Pontificia Universidad Católica de Chile
Facultad de Ciencias Biológicas
Programa de Doctorado en Ciencias Biológicas
Mención Genética Molecular y Microbiología

TESIS DOCTORAL:

EFFECTS OF TEMPERATURE ON CARBON AND NITROGEN METABOLISMS OF PORCELANA MICROBIAL MAT COMMUNITY: RATES, STRATEGIES AND GENE EXPRESSION

Por

MARÍA ESTRELLA ALCAMÁN ARIAS

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Por

MARÍA ESTRELLA ALCAMÁN ARIAS

Director de Tesis: Dra. Beatriz Díez M.

Comisión de Tesis: Dra. Susan Bueno R.
Dra. Margarita Carú M.
Dr. Rodrigo De la Iglesia

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SRTA. MARÍA ESTRELLA ALCAMÁN ARIAS

Ha sido aprobada por el Tribunal Examinador, constituido por los profesores abajo firmantes, calificándose el trabajo realizado, el manuscrito sometido y la defensa oral con nota _____ (_____).

DRA. SUSAN BUENO R.
Coordinadora Comité de Tesis
Facultad de Ciencias Biológicas-UC

DR. JUAN A. CORREA M.
Decano
Facultad de Ciencias Biológicas-UC

DRA. MARGARITA CARÚ M.
Profesor Invitado
Universidad de Chile

DRA. BEATRIZ DÍEZ M.
Directora de Tesis
Facultad de Ciencias Biológicas-UC

DR. RODRIGO DE LA IGLESIA C.
Miembro Comité de Tesis
Facultad de Ciencias Biológicas-UC

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A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales.

-Marie Curie.

DEDICATORIA

A mis padres, quienes sin su incondicional apoyo ninguna de mis metas serian realidad.
Por hacer de mí una persona perseverante y sensible.
Y por sobre todo…siempre confiar en mí, comprender mis ideales y el tiempo que no he estado con ellos.

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ABBREVIATIONS

AHB: Aerobic and Anaerobic Heterotrophic Bacteria
AOA: Amonia Oxidizer Archaea
AOB: Ammonia Oxidizer Bacteria
ARA: Acetylene Reduction Assay
BLASTN: Basic local Alignment Search Tool Nucleotide C: Carbon
C:N: organic matter composition
DGGE: Denaturing Gradient Gel Electrophoresis
DIC: Dissolved Inorganic Carbon
DNRA*:* Dissimilatory Nitrate Reduction to Ammonium
FAP: Filamentous Anoxygenic Phototroph
HK: Housekeeping
HH: Hydroxypropionate-Hydroxybutyrate
KEGG: Kyoto Encyclopedia of Genes and Genomes
LCA: Last Common Ancestor
LGT: Lateral Gene Transfer
LL: constant light
LM: Light Microscopy
N: Nitrogen
NA: Nitrogenase Activity
NR: NCBI non-redundant database
OTU: Operational Taxonomic Unit
PP: Primary Production
SRB: Sulfate-Reducing Bacteria
TEM: Transmission Electron Microscopy
YNP: Yellowstone National Park
3-HP: 3-Hydroxypropionate bicycle

SUMMARY

Microbial mats associated to hot springs are characterized as dynamic and complex ecosystems exhibiting spatial and temporal heterogeneity, being studied for decades as they represent a good extreme model system to discover the principles of microbial community ecology.

The microbial mat community along the temperature gradient (66, 58 and 48°C) on the non-acidic hot spring of Porcelana (Northern Chilean Patagonia) were investigated by integrative approaches such as DNA/RNA sequencing, isotopic labeled carbon ($H^{13}CO_3$) and nitrogen ($^{15}N_2$, $^{15}NH_4Cl$ and $K^{15}NO_3$), and culture controlled conditions assays.

The Porcelana taxonomic microbial mat composition reveals that phyla Chloroflexi and Cyanobacteria were prevalent and dominant (>80% of total community) along the temperature gradient and co-occurring at intermediate temperatures, with contribution to the most representative active metabolisms such as photosynthesis, CO_2 and nitrogen fixation, as well as the sulfur, phosphorous and iron cycles. Particularly, Chloroflexi members (*Roseiflexus* sp. and *Chloroflexu*s sp.) were >90% responsible for all the active energetic and nutrients metabolisms found at 66°C, while Cyanobacteria (*Mastigocladus* spp.) contributed most at 58°C with a maximum at lowest temperatures (48°C). Three pathways of carbon fixation; Calvin-Benson (Cyanobacteria members), 3-Hydroxypropionate bicycle (Chloroflexi members) and Hydroxypropionate-hydroxybutyrate (Archaea members) were represented along the temperature gradient. Similar taxonomical profile was obtained for the nitrogen cycle routes, where Cyanobacteria have an important role in the incorporation of new nitrogen into the microbial mat.

Concerning with the high importance of Cyanobacteria in the Porcelana microbial mat, the distribution and diversity of their diazotrophic members were analyzed along the temperature gradient in an inter-annual (2009, 2011-2013) period. The dominance of the filamentous heterocystous genus *Mastigocladus* (Order Stigonematales) belonging to subsection V along the entire temperature gradient (69-38ºC) of Porcelana was revealed. According to that, *in situ* nitrogenase activity, cellular uptake of $^{15}N_2$, and *nif*H mRNA expression demonstrate that nitrogen fixation was light dependent and detected at temperatures ranging from 46 to 58°C but not at 66ºC. This nitrogenase activity pattern strongly suggests a heterocystous cyanobacterial origin and reveals a correlation with the *nif*H gene expression obtained during diurnal cycles in this thermal microbial mat. The contribution of N and C fixation was approximately 3 g N m^{-2} y^{-1} and 27 g C m^{-2} y^{-1}, respectively, suggesting that these vital demands are fully met by the diazotrophic and photoautotrophic capacities of the Stigonematal cyanobacteria in the Porcelana hot spring.

A Stigonematal cyanobacterium isolated from Porcelana hot spring (strain CHP1) was characterized morphological and genetically being identified as a *Mastigocladus* sp., with a moderately thermophilic character (grows at 50°C but not at 60ºC). *Mastigocladus* sp. strain CHP1 fixes maximum atmospheric dinitrogen (nitrogenase activity) in light independent of temperature. The *nif*H gene expression was rhythmic at 50ºC, while at 45ºC the *nif*H gene expression remaining constant during the light/dark period, suggesting arrhythmicity. A high affinity for nitrate and a low tolerance to high ammonium concentrations were apparent for the strain CHP1; hence, certain plasticity in regards to the use of different nitrogen sources was suggested.

It is proposed that *Mastigocladus* sp. strain CHP1, and probably other close relatives, may be of a general importance in hot springs colonized by representatives within the Order Stigonematales, and to represent major players in hot springs like Porcelana by contributing new nitrogen through the biological nitrogen fixation.

RESUMEN

Los tapetes microbianos asociados a sistemas termales se caracterizan por ser ecosistemas dinámicos y complejos, exhibiendo heterogeneidad espacial y temporal. Estos sistemas han sido estudiados por décadas ya que representan modelos de estudio ideales para entender los principios de la ecología microbiana.

La comunidad del tapete microbiano en el gradiente de temperatura (66, 58 y 48°C) del sistema termal no ácido de Porcelana (Patagonia Norte de Chile), se investigó mediante aproximaciones integrativas como; secuenciación masiva de ADN/ARN, carbono ($H^{13}CO_3$) y nitrógeno ($^{15}N_2$,$^{15}NH_4Cl$ y $K^{15}NO_3$) isotópicamente marcado, y ensayos en cultivos bajo condiciones controladas de laboratorio.

La composición taxonómica del tapete microbiano reveló que los taxones pertencientes a Chloroflexi y Cianobacteria fueron frecuentes y dominantes (>80% de la comunidad total) a lo largo del gradiente de temperatura, co-ocurriendo a temperaturas intermedias, y contribuyendo con los metabolismos activos más representativos tales como; fotosíntesis, fijación de CO_2 y nitrógeno, así como los ciclos de azufre, fósforo y hierro. En particular, los miembros de Chloroflexi (*Roseiflexus* sp. y *Chloroflexus* sp.) fueron >90% responsables de todos los metabolismos energéticos activos y de nutrientes encontrados a 66°C, mientras que las Cianobacterias (*Mastigocladus* sp.) contribuyeron mayoritariamente a 58°C, con un máximo a temperaturas menores (48°C). Tres vías de fijación de carbono: Calvin-Benson (usado por miembros de las Cianobacterias), biciclo 3-Hidroxipropionato (usado por miembros de los Chloroflexi) y ciclo Hidroxipropionato-hidroxibutirato (usado por miembros de las Archaeas) fueron representados a lo largo del gradiente de temperatura en el tapete de Porcelana. Perfiles taxonómicos similares fueron obtenidos para las rutas del ciclo del nitrógeno, donde las cianobacterias tuvieron un papel primordial en la incorporación de nitrógeno nuevo en el tapete microbiano.

Dada la gran importancia de las cianobacterias en el tapete microbiano de Porcelana, la distribución y diversidad de sus miembros diazotróficos se analizó a lo largo del gradiente de temperatura en un período interanual (2009, 2011-2013). Este estudio reveló un predominio del género filamentoso con heterocistos *Mastigocladus* (Orden Stigonematales), que pertenece a la subsección V, a lo largo de todo el gradiente de temperatura (69-38ºC). De acuerdo con esto, la actividad *in situ* de la enzima nitrogenasa, la captación celular de $^{15}N_2$, y la expresión del gen *nif*H, demostraron que la fijación de nitrógeno es dependiente de luz, detectándose a temperaturas entre los 46 a 58°C pero no a 66ºC. Este patrón de actividad sugiere fuertemente la presencia y actividad de cianobacterias con heterocistos, revelando una correlación positiva entre la actividad de la enzima nitrogenasa y la expresión del gen *nif*H durante ciclos diurnos en el tapete

microbiano. La contribución de la fijación de N y C fue de aproximadamente 3 g de N m^{-2} $año^{-1}$ y 27 g C m^{-2} $año^{-1}$, respectivamente, sugiriendo que las demandas de estos compuestos son alcanzadas completamente por la actividad diazotrófica y autótrofa de las cianobacterias Stigonematales presentes en el sistema termal de Porcelana.

La cianobacteria Stigonematal cepa CHP1, aislada desde el sistema termal de Porcelana fue caracterizada morfológica y genéticamente, siendo identificada como *Mastigocladus* sp., presentando un carácter moderadamente termófilo (crece a 50°C pero no a 60°C). *Mastigocladus* sp. cepa CHP1 fija nitrógeno (actividad nitrogenasa) en periodos de luz independientemente de la temperatura. El patrón de expresión del gen *nif*H mostró fluctuación a 50ºC, mientras que a 45°C la expresión del gen permanecio constante durante el período de luz/oscuridad, sugiriendo aritmicidad. Una alta afinidad para el nitrato y una baja tolerancia a altas concentraciones de amonio fueron evidenciadas para la cepa CHP1, por lo tanto, sugiriéndose cierta plasticidad respecto a la utilización de diferentes fuentes de nitrógeno para esta cepa.

Se propone que *Mastigocladus* sp. cepa CHP1, y probablemente otros miembros cercanos, son relevantes en tapetes microbianos colonizados por estos representantes del Orden Stigonematal, siendo uno de los principales actores en sistemas termales como Porcelana contribuyendo a la incorporación de nitrógeno nuevo a través de la fijación biológica de nitrógeno.

GENERAL INTRODUCCTION

Microbial life began about 3 billion years ago, and the first evolutionary steps began in a world without oxygen, with a different atmosphere unable to provide protection from ultraviolet radiation, and probably in water bodies, which were hot and acidic. It was a physiologically challenging scenario, but one in which the microbes survived and succeeded, and through their success they began to change the world, adding oxygen to the atmosphere and changing the microenvironments into less stressed places (Brock, 1978).

1. Extremophiles

At the dawning of the twenty-first century we know that the Solar System, and even Earth, contains environmental extremes unimaginable to the 'ancients' of the nineteenth century. Equally marvellous is the detection of organisms that thrive in these extreme environments. Macelroy, (1974) named these lovers ("philos") of extreme environments as "*Extremophiles*".

Extremophiles are organisms found living in extreme harsh environments that we might think should be uninhabitable, and most of its members come from all three domains, being Bacteria and Archaea (Mondigan and Marrs 1997; Fredrickson and Onstott 1996; Brock, 1978) the most abundant and diverse (Figure 1) (Canganella and Wiegel, 2011). In particular, Archaea are presumably the first version of life on our planet when its atmosphere was devoid of oxygen, and comprised largely of ammonia, methane, water vapor and carbon dioxide (Brock, 1978).

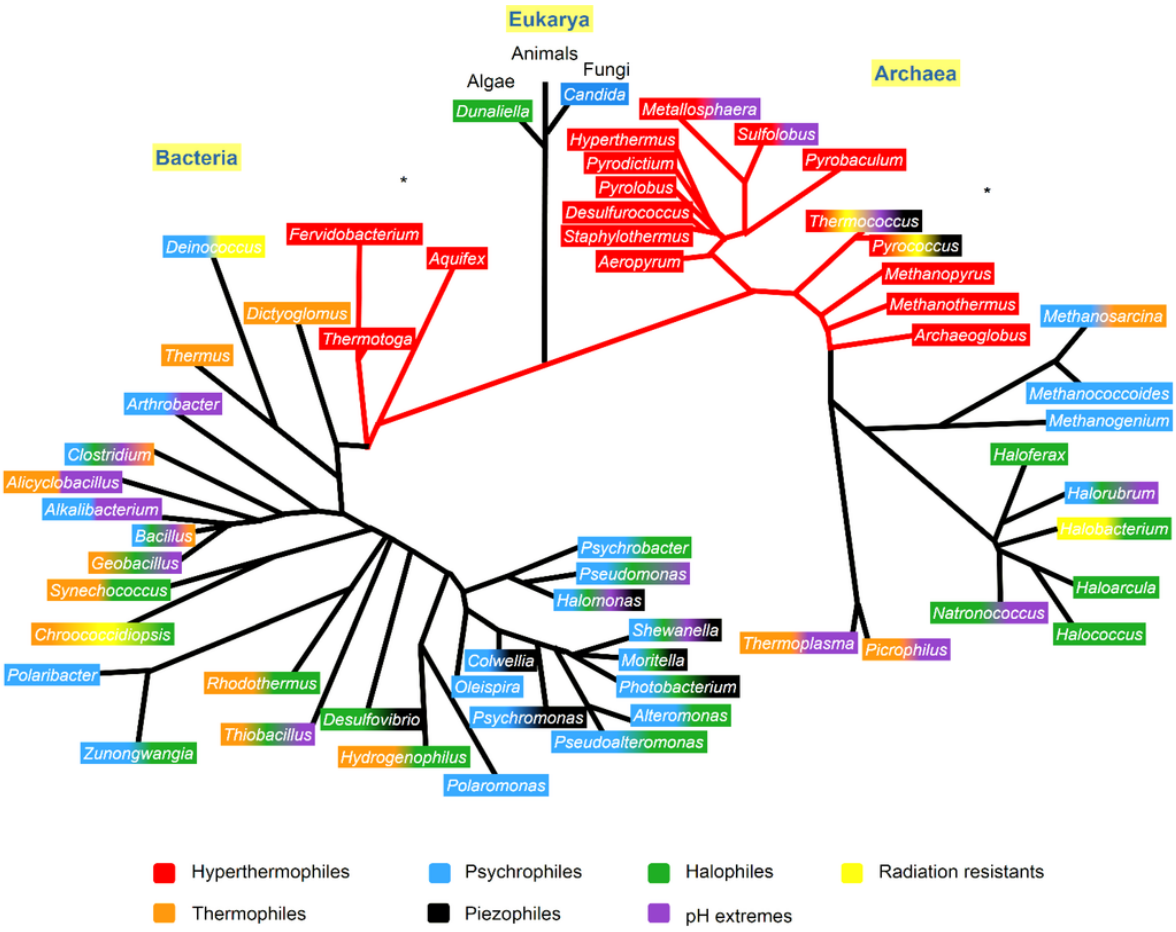


Figure 1. Phylogenetic tree that classified the different extremophiles on life domains. The tree shows the extremophiles and the resistant characteristics that appear in at least one species of each genera, identified with the color code (Woese et al, 1990; Lang et al. 2013; Dereeper et al. 2008). Extracted from Leal et al. (2015)

Extreme environments can be alkaline (>pH 9; soda lakes), acidic (<pH 5; solfataras and mine drainage systems), extremely cold (<10ºC; glaciers, high mountain, polar sites, and deep ocean) or hot (>45ºC; terrestrial hot springs, geysers, deep-sea vents), hypersaline (>3.5%; solar salters), under pressure (>400 atm; deep oceans and lakes), with

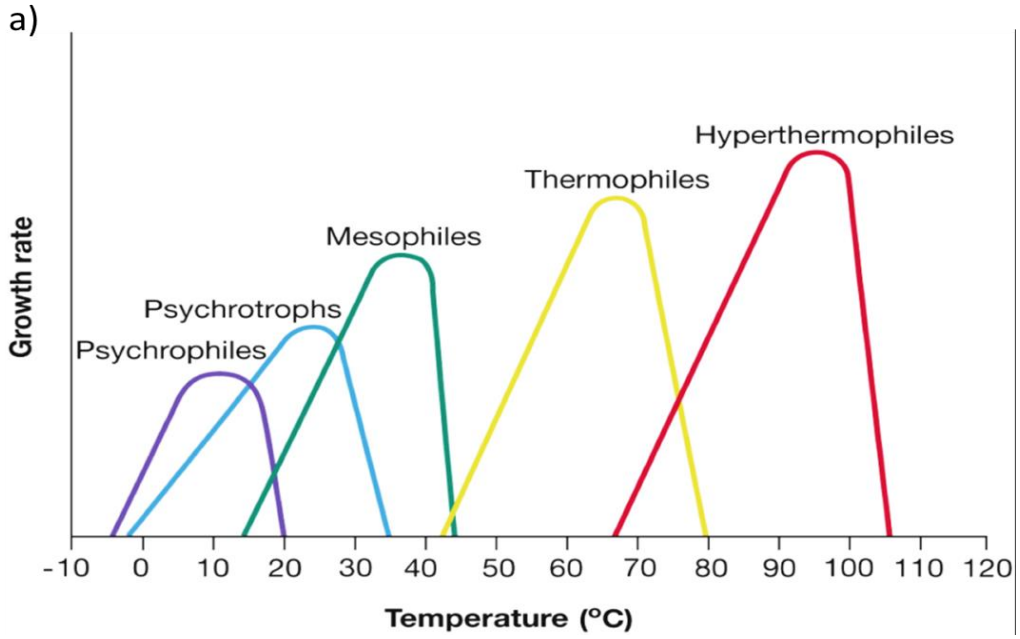
high radiation (UV and IR), extremely dry (hot and cold deserts, some endolithic habitats), suboxic (< $0.5 ml O_2 L^{-1}$; deep sediments, eutrophic lakes), etc. In this sense, the extremophiles have been classified as thermophiles (40-80ºC), hyperthermophiles (>80ºC), acidophiles (<pH 5), alkaliphiles (>pH 7), psychrophiles (<10ºC), halophiles (15-36% salinity), barophiles (>400 atm), etc, according with the extreme systems where they live (Figure 1).

The study of these extremophiles challenges our concept of the limits of life, and informs our quest for the comprehensive tree of life, helping us to understand how evolution has taken place. In particular, at the world most of the visible organisms live in a relatively limited range of temperatures (from 5°C to 40°C), that is defines as "normal". However, there are many microorganisms that live far away of this temperature range (Figure 2). In accordance to that, the present study is focused in "*Thermophiles*": microorganisms that inhabit extreme environments that are modulated by high temperature (between 40 to 80ºC), such as those found in hot spring systems.

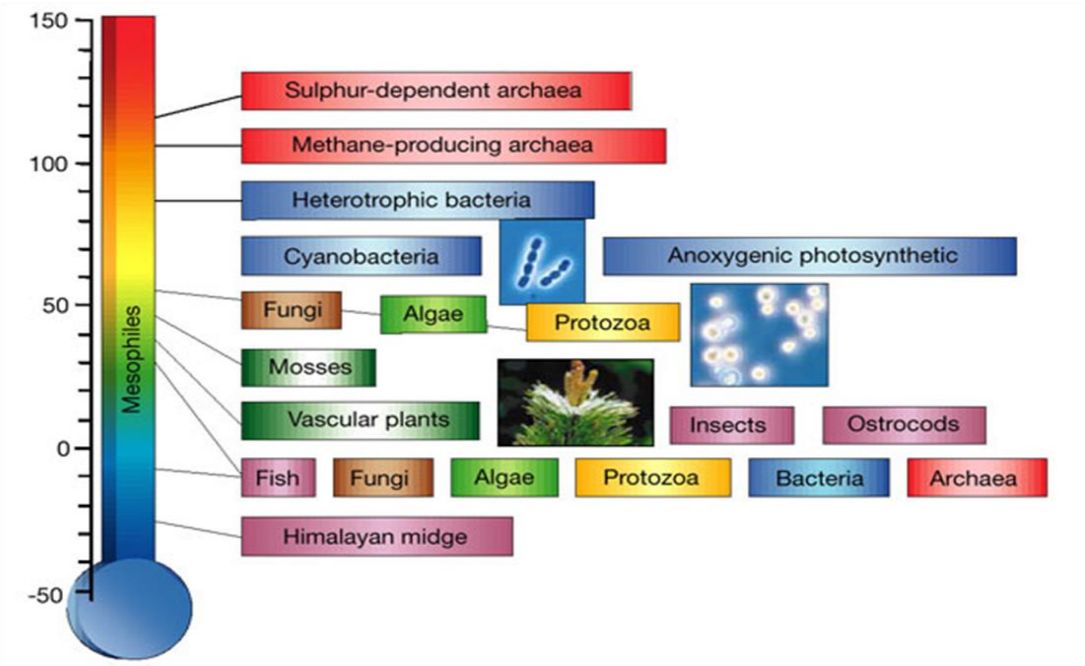
2. Life at high temperature

Temperature greatly influences physical and biological processes. According to temperature, it is possible to distinguish organisms from low to high temperature tolerance as: psychophiles (<10ºC), mesophiles (15-37ºC), thermophiles (40-80ºC) and hyperthermophiles (>80ºC) (Figure 2a). The thermophilic microorganisms are more widespread than hyperthermophiles, and they are nearest to the basis of the phylogenic tree of the origin of life (Figure 2c).

a)


Growth rate
Psychrophiles
Psychrotrophs
Mesophiles
Thermophiles
Hyperthermophiles
-10 0 10 20 30 40 50 60 70 80 90 100 110 120
Temperature (°C)

b)


150
100
50
0
-50
Mesophiles
Sulphur-dependent archaea
Methane-producing archaea
Heterotrophic bacteria
Cyanobacteria
Anoxygenic photosynthetic
Fungi
Algae
Protozoa
Mosses
Vascular plants
Insects
Ostrocods
Fish
Fungi
Algae
Protozoa
Bacteria
Archaea
Himalayan midge

c)

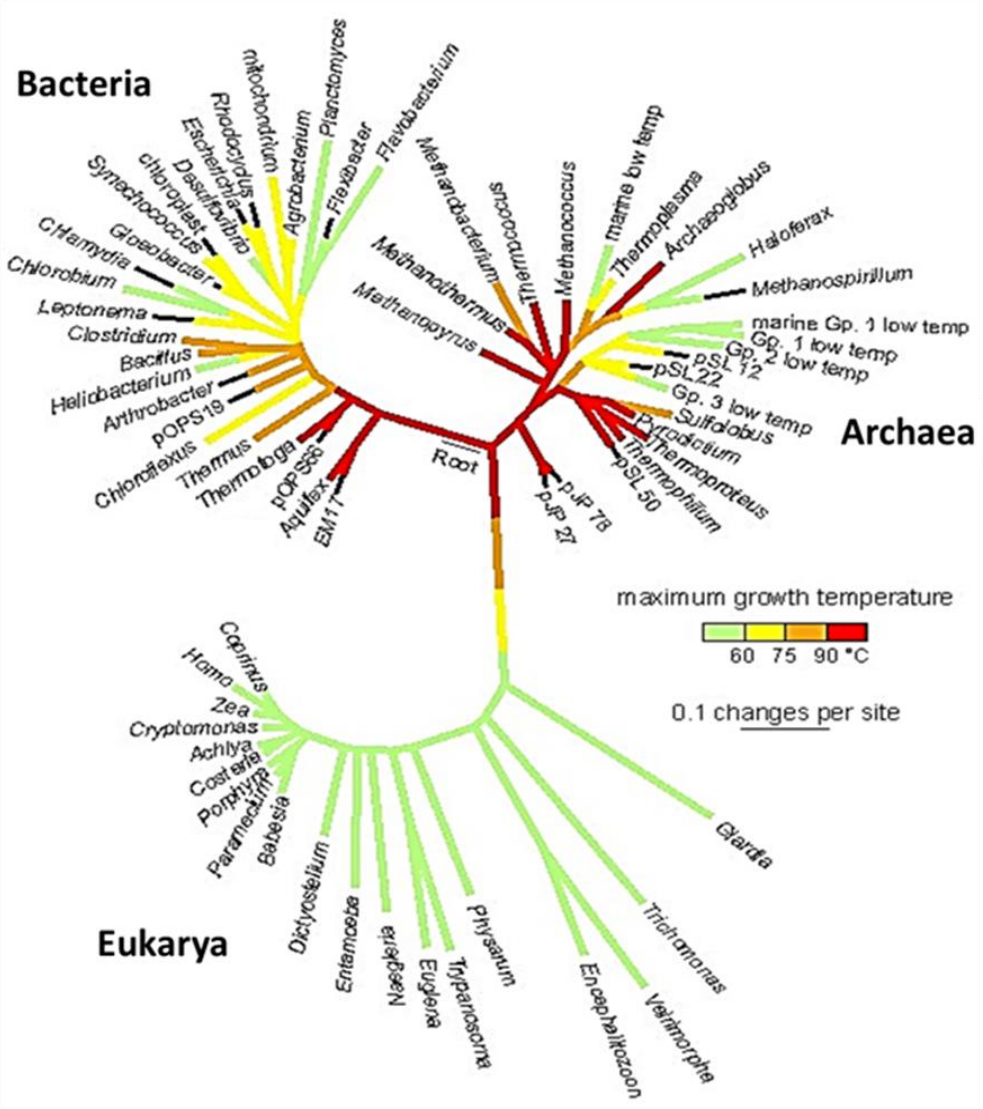


Figure 2. Classification of Thermophiles and its maximum growth temperature. a and b) Categories of microbes based on temperature ranges growth. Any organism with an optimum temperature above 45°C is classified as a thermophile, but this range is extremely broad and extends 80°C up to the upper limit of life. Therefore, thermophiles have been further subdivided into moderate thermophiles that grow optimally between 45 and 70°C, extreme thermophiles that grow optimally at 70°C and above, and hyperthermophiles that grow optimally at 80°C and above (Modified of Rothschild and Mancinelli, 2001). c) Phylogenetic Tree based on rRNA sequences. The scale bar corresponds to 0.1 changes per nucleotide. The last common ancestor (LCA) is located on the bacterial line between the node to *Aquifex* and the Y intersection of the three domains. Maximal growth temperatures have been used to colour-code the branches (Extracted from Schwartzman and Lineweaver, 2004).

Biologically, organisms at high temperature have to reduce the fluidity of membranes to maintain optimal conditions for the cell. This adjust comprise the composition of the membrane including the amount and type of saturated versus unsaturated lipids (Driessen *et al*., 1996). Temperature also affects the structure and function of proteins, including increase of ion-pair content, formation of higher-order oligomers and decreasing flexibility at room temperature (Jaenicke, 1996). In the same way, DNA at high temperature (>70°C) is subject normally to denaturation and chemical modification, yet the DNA of hyperthermophiles such as *Pyrococcus furiosus* is known to be more stable *in vivo* than that of a mesophile such as *Escherichia coli* (Peak *et al*., 1995). Monovalent and divalent salts enhance the stability of nucleic acids, as these salts screen the negative charges of the phosphate groups, and because KCl and $MgCl_2$ protect the DNA from depurination and hydrolysis (Marguet and Forterre, 1998). The G-C pair of nucleic acids is more thermostable than the A-T or A-U pairs due to the additional hydrogen bond (Galtier *et al*., 1997).

The most hyperthermophilic organisms belong to the Archaea Domain, as *Pyrolobus fumarii* (Crenarchaeota), a nitrate reducing chemolithoautotroph capable of growing at the highest temperatures of up to 113°C (Blöchl *et al*., 1997). However, at the thermophilic range there are representatives among the Bacteria Domain as phototrophic bacteria (Cyanobacteria, purple and green bacteria) and Eubacteria (*Bacillus*, *Clostridium*, *Thiobacillus*, *Desulfotomaculum*, *Thermus*, lactic acid bacteria, Actinomycetes, Spirochetes and numerous other genera), as well as among the Archaea (*Pyrococcus*, *Thermococcus*, *Thermoplasma*, *Sulfolobus* and many methanogens). Additionally to temperature tolerance, many organisms can thrive under more than one factor of stress at a time, and so they are named polyextremophiles. As an example, the archaea *Sulfolobus*

acidocaldarius, that flourishes in hot springs and sulfataras at pH 3 at 80°C, so it is an acid-thermophile. In contrast to Prokaryotes, the upper limit for Eukaryotes is normally ~60°C, a temperature suitable for some protozoa, algae and fungi (Figure 2a).

3. Hot spring systems

Hot springs are formed when cool groundwater (from rain, snowmelt, rivers or lakes) near the surface percolates down into the earth, approaches a heat source such as a hot magma chamber, and it is steadily heated towards its boiling point rising to the surface driven by pressure or density (Lahsen, 1988). When the water arrives to the surface, it can explode into steam in an enormous expansion of volume confining water out of the vent as a geyser, fumaroles and hot springs (Figure 3). This surface water is characterized by high temperature (>100°C), high concentration of minerals (mainly Fe, Cl, S and HCO_3), and a variation of pH from acid to alkaline (Heasler *et al*., 2009).

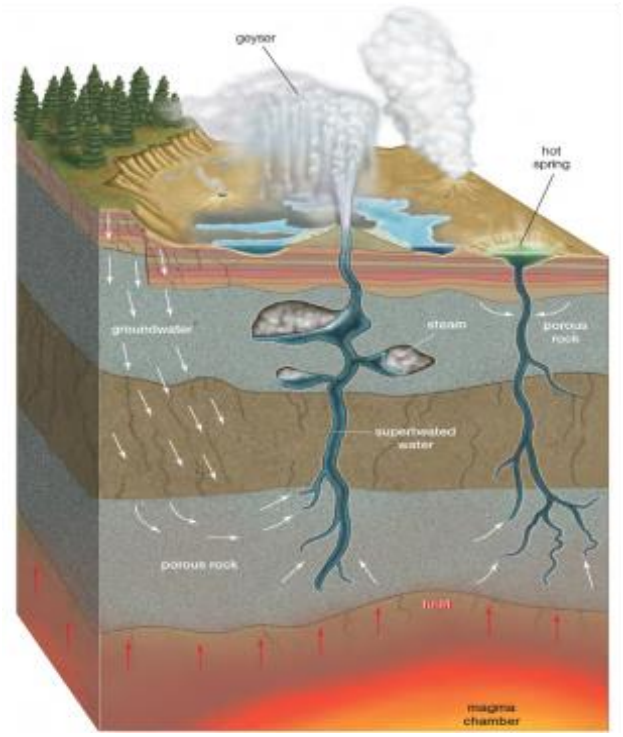


Figure 3. Cross section of hot spring origin; geyser and hot spring. In geysers and fumaroles the superheated water collects in underground pockets. In hot springs the rising superheated water is cooled below the boiling point by groundwater before reaching the surface. When the supply of steam and hot water is exhausted, the spouting stops and the cycle begin again (Encyclopedia Britanica, Inc 2006).

Hot spring systems have its origin in volcano regions, and are distributed in regions associated with fire hot spots worldwide (Garcia *et al*., 2009). According to that, Chile (i.e. El Tatio geysers field) is characterized as the third-most geothermal active country in the world, after USA (i.e. Yellowstone National Park) and Russia (i.e. Dolina, Geizerov, Kronotsky National Park) (Figure 4). Also, these thermal systems are geographically isolated and therefore represent dispersal barriers that resulted in the genetic isolation and diversification of their living organisms (Miller *et al.*, 2007; Takacs-Vesbach *et al.*, 2008; Lau *et al.*, 2009).

Figure 4. Global representations map of geyser fields. The map shows the location of world countries with most active geyser fields. *http://geology.com/articles/geyser.shtml*

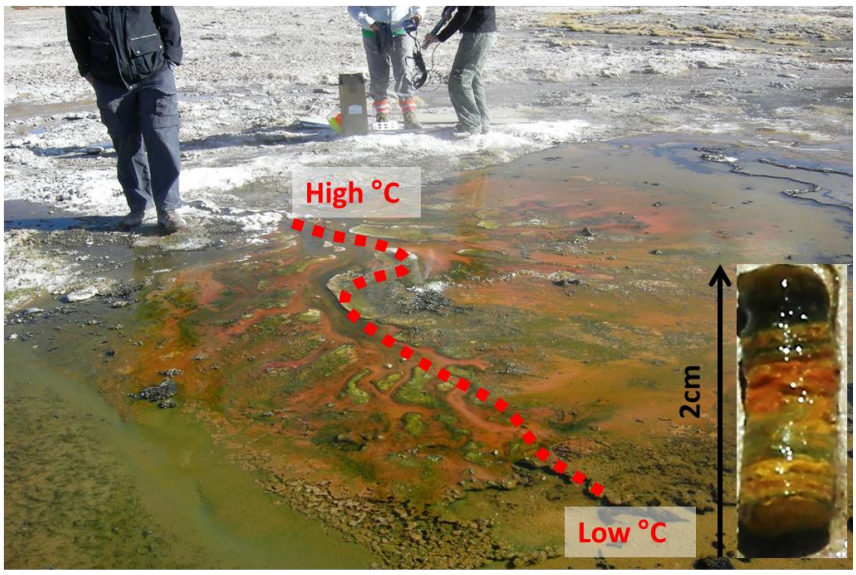
In hot springs as in other environments, organisms respond to the stress variable (in this case temperature) forming compact structures so-called microbial mats. In these complex structures these organisms are adapted at high temperatures, and also they can obtain light, oxygen, nutrients forming a redox potential between the different populations that live there.

4. Microbial mats in hot springs

Microbial mats are dense communities of benthic organisms distributed in a stratified manner that develop at the interface between water and soil substrates (Stal *et al*., 1994) (Figure 5). These are relatively stable, so they are a good natural model to investigate the ecology of their microbial communities (Ward *et al*., 1998). Several microbial mats from alkaline (Loiacono *et al*., 2012), acidic (Hamilton *et al*., 2011) and neutral (Cole *et al*., 2013; Mackenzie *et al*., 2013) hot springs have been described in the last decade, showing the diversity and importance of microorganisms in these extreme systems.

In hot springs, as similar to the other environments, the size of microbial mats varied from millimeters (mm) to centimeters (cm) thick, and can be several meters long as the temperature gradient is formed (Figure 5a). In the vertical distribution of these mats, oxygenic processes occur in the first few millimeters exposed to light, and at deeper areas anoxic processes take place generating a redox flux of nutrients that support the metabolic requirements of the present microbial communities (Pentecost, 1996) (Figure 5b).

a)



b)

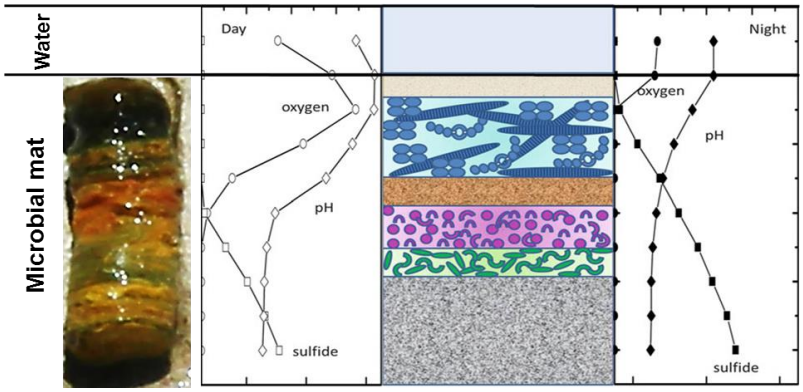


Figure 5. Microbial mat representation. a) Pigmented microbial mat developed along the temperature gradient, and vertical picture of the stratified microbial community, b) Vertical representation that shows the principal taxonomical actors along oxygen and sulfide evolution layers, during day and night period. Imagen of microbial mat was taken in El Tatio Geysers, Chile. The schematic representation was extracted from Stal, 2014.

In the layered microbial mat (Figure 5b), the light penetrates only a few millimeters and the oxygen produced by photosynthesis rapidly diminishes with depth (Jorgensen, 1992). Commonly, the oxygenic layer of the mat is composed mainly by photosynthetic bacteria, whose function is to be primary producers. Among them, members of the Cyanobacteria phylum have been reported as a major active and constitutive component

(Miller *et al*., 2006; Jing *et al*., 2006; McGregor and Rasmussen, 2007; Klatt *et al*., 2013; Alcamán *et al*., 2015). Metabolic versatility of this group of bacteria confers them high competitiveness in extreme environments (Stal, 1991b). Cyanobacteria perform photosynthesis, aerobic respiration, fermentation and some are able to fix dinitrogen under light/darkness or aerobic/anaerobic conditions (Stal, 2007). Furthermore, other phyla such as Chloroflexi, Proteobacteria, Bacteroidetes, Firmicutes and Deinococcus-Thermus are also conspicuous in the upper and intermediate portion of these mats (Harris *et al*., 2013; Klatt *et al*., 2013). Sulfate-reducing bacteria (SRB) commonly are present in the bottom layers as they are restricted by the presence of oxygen (Jørgensen, 1982). However, highest rates of sulfate reduction have been observed in the highly oxic near-surface region of phototrophic microbial mats (Canfield and Marais, 1991; Fründ and Cohen, 1992). Also, members of the Archaea Domain belonging to Crenarchaeota, Euryarchaeota and Thaumarchaeota orders, have been characterized as typical from acidic and alkaline thermal microbial mats (Huang *et al*., 2013; Quaiser *et al*., 2014).

5. Biogeochemical cycles in hot spring microbial mats

The thermophilic microbial community contributes by its functional metabolisms to support live in hot springs mats (Marais, 2003). Temperature is a major environmental factor determining the diversity and metabolic activities of the organisms in hot springs, crucial to understand biological adaptations that have evolved to live in these particular systems. However, very little is still known about contribution of the community to the C and N biogeochemical cycles through their metabolic activities along the temperature gradient in these systems (Paerl *et al*., 2000; Inskeep *et al*., 2010; Alcamán *et al*., 2015).

Generally, in microbial mats, the organic matter resulting from productivity of phototrophs is the energy source for other aerobic and anaerobic heterotrophic microorganisms. In the other hand, aerobic heterotrophic organisms are functionally important as their activity leads to oxygen depletion, and fermentative organisms provide with growth substrates for sulfate-reducing bacteria. Other groups numerically less important are nitrifying and denitrifying bacteria, and methanogenic bacteria (Van Gemerden, 1993) (Figure 6). Cyanobacteria as primary producers are particularly relevant in many microbial mats (Steward, 1967; Miller *et al*., 2006; Steunou *et al*., 2006), and they can combine CO_2 and N_2 fixation, the two most important biogeochemical processes on the Earth (Steward, 1970; Steunou *et al*., 2008; Klatt *et al*., 2011).

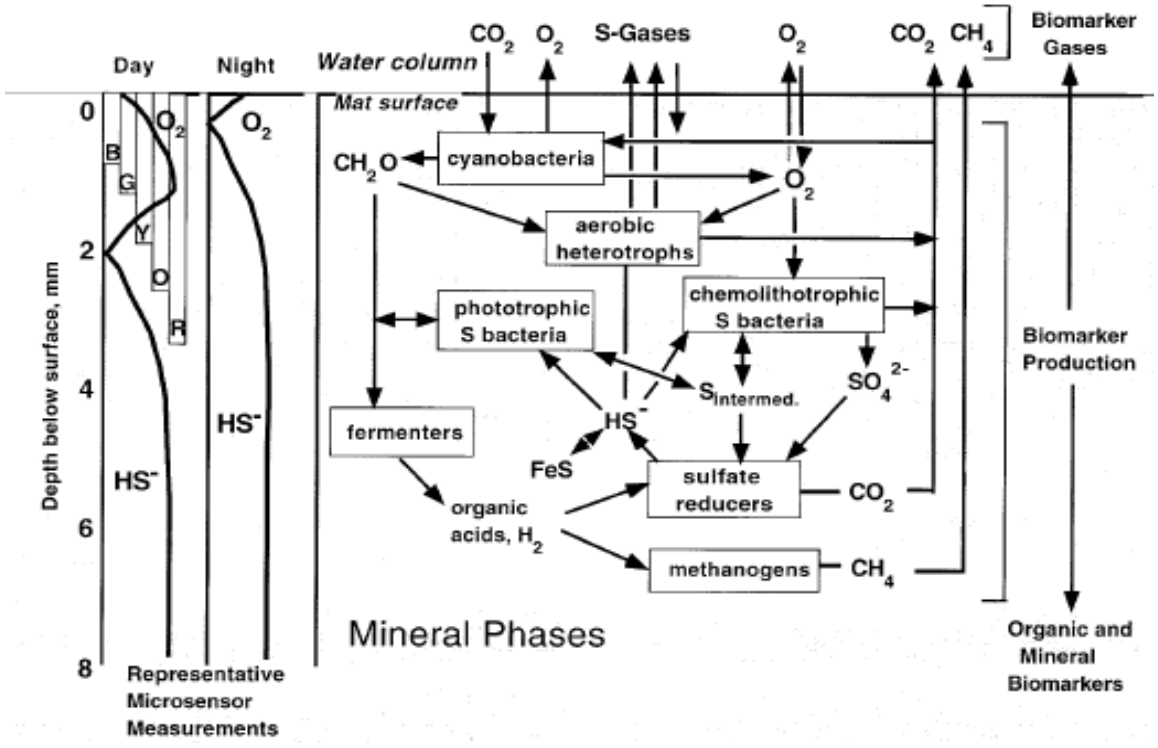


Figure 6. Schematic of a phototrophic microbial mat with associated depth-related light and chemical gradients. Flow diagram at center is modeled after Fenchel and Finlay (1995). Boxes denote functional groups of microorganisms, and arrows denote flows of chemical species into or out of microorganisms. $S_{intermed}$ indicates sulfur in intermediate oxidation states. Schematic at left depicts vertical gradients of O_2 and sulfide during the day and at night. Oxygen concentrations are shown decreasing to zero at a depth of 2 mm during the day and just below the mat surface at night. The vertical bars at upper left represent the relative depths of penetration of blue (B), green (G), yellow (Y), orange (O), and red (R) light. Such chemical gradients and light penetration profiles of both filamentous and unicellular microbial mats are qualitatively similar, although the depth scale (mm) of such profiles tends to be greater for unicellular mats (Extracted from Marais, 2003).

5.1 Photosynthesis and CO_2 fixation in thermophilic microbial mats

Photosynthesis is the process that plants, algae and some types of bacteria use to transform light energy into chemical energy. During the photosynthesis process, light energy is captured and used to convert water, carbon dioxide, and minerals into oxygen and energy-rich organic compounds. The photosynthetic process depends on a set of complex protein molecules that are located in and around a highly organized membrane. The oxygenic photosynthesis comprises two separate phases; the light dependent reaction (phosphorilation) (Frenkel, 1995) and the Calvin cycle (light independent reactions) (Ivanovsky, 1999). During the light dependent reactions, the energy of light is captured by photosystems II (PSII) and I (PSI) and converted into the chemical energy (ATP) needed to run the Calvin cycle, who builds the glucose from carbon dioxide (Baker, 1996). Furthermore, there are organisms that perform anoxygenic photosynthesis in which these species only have one type of reaction center or photosystem (PSI) (Blankenship *et al*., 1995). Each year more than 10% of the total atmospheric CO_2 is reduced to carbohydrate by photosynthetic organisms (Barber, 1992).

Furthermore, although many photosynthetic bacteria depend on Rubisco and the Calvin cycle for the reduction of CO_2, some microorganisms are able to fix atmospheric CO_2 by other biochemical pathways. In nature, there are other five mechanisms than Calvin cycle to assimilate CO_2 into cell material (Stadtman, 1957): the reductive citric acid (Arnon-Buchanan) cycle performed by Proteobacteria, Aquificae, Green Sulfur Bacteria among others (Albert *et al*., 2008), the reductive acetyl-CoA (Wood-Ljungdahl) pathway performed by for instance some members of Proteobacteria, Planctomycetes, Spirochaetes, and Euryarchaeota (Holo and Sirevåg, 1986), the 3-hydroxypropionate bicycle so far just performed by the Chloroflexaceae family, the Hydroxypropionate-hydroxybutyrate pathway performed by Crenarchaeota (Berg *et al*., 2010), and finally the Dicarboxylate-hydroxybutirate cycle that is performed by the anaerobic Crenarchaeal from Orders Thermoproteales and Desulfurococcales (van der Meer *et al*., 2000; House *et al*., 2003).

In hot springs, due to the lack of phototrophic eukaryotes CO_2 fixation is been attributed to Cyanobacteria (oxygenic process using Calvin Benson) and Chloroflexi (anoxygenic process using 3-hydroxypropionate bicycle) (van der Meer *et al*., 2007; Zarzycki *et al*., 2009; Liu *et al*., 2011; Zarzicki *et al*., 2009-2011). In these extreme systems, oxic (Steunou *et al*., 2006, 2008) and anoxygenic (Klatt *et al*., 2013) photosynthesis have been reported through the quantification of specific target genes, but how the temperature could affect these processes has never been described. Moreover, other possible ways of CO_2 fixation, especially those that involve members of the Archaea Domain, have been totally neglected in hot spring mat studies.

5.2 Nitrogen cycle in thermophilic microbial mats

Nitrogen is an essential element for organisms, because it is component of proteins and nucleic acids. This element is available at 80% in the atmosphere in gaseous form (N_2). The remaining percentage is found as part of the living organisms, and cycling in soils and aquatic environments.

The combined inorganic nitrogen sources such as ammonium (NH_4^+) and nitrate (NO_3^-), generally are limiting in natural environments, including hot springs (Hamilton *et al*., 2011; Wang *et al*., 2013). However, when they are present, NH_4^+ is the inorganic nitrogen form preferred by microorganisms to assimilate into the cell, because less energy expenditure is required to break its structure. To assimilate these two nitrogen combined compounds, serial active genes are needed. To NO_3^- uptake, *nir*A-*nrt*ABCD-*nar*B operon is activated (Frías *et al*., 1997; Muro-Pastor *et al*., 2005) and genes that encode GS-GOGAT pathway are necessary to ammonium assimilation (Luque *et al*., 1993; Levitan *et al*., 2010) (Figure 7).

On the other hand, when the concentrations of these nutrients are minimal, the primary production and growth of microbial mat communities is limited (Paerl *et al*., 2000; Pinckney *et al*., 1995). Under these conditions, the biological fixation of atmospheric molecular nitrogen (N_2) plays an important role as it is the only input of new nitrogen into these natural systems. In this sense, available molecular N_2 in the atmosphere can only be biologically fixed through the activity of prokaryotic microorganisms called diazotrophs (Stewart, 1970; Wickstrom, 1984). Until now, none eukaryote microorganism has this capacity (Stal, 1995). In aquatic systems, many species of Cyanobacteria are capable of fixing atmospheric molecular nitrogen (N_2), as well of assimilate combined inorganic

nitrogen sources (NH_4^+ and NO_3^-, mainly), contributing to important pathways whithin the nitrogen cycle (Figure 7).

In hot springs, the importance of biological nitrogen fixation not only derives from its contribution to the incorporation of "new" nitrogen combined into the system, but also counteract/ offsetting the combined nitrogen losses through denitrification (Otaki *et al*., 2012). Therefore, in hot springs the nitrogen fixation process is listed as crucial (Villbrandt *et al*., 1990), and in neutral hot spring, Cyanobacteria have been suggested to be key microorganisms in this process (Steunou *et al*., 2006). The combined analysis of the molecular marker *nif*H gene (encoding one subunit in the nitrogenase enzyme), together with direct measurements of nitrogenase activity (using the acetylene reduction assay) has been widely studied in hot springs from Yellowstone National Park (YNP, USA) (Miller *et al.*, 2009; Hamilton *et al.*, 2011; Loiacono *et al.*, 2012). Nitrogenase activity attributed to the filamentous heterocystous cyanobacterium *Mastigocladus laminosus* has been recorded in several alkaline hot springs at temperatures of ~50°C (Stewart, 1970; Miller *et al.*, 2006), while at higher temperatures, this activity was associated to the unicellular cyanobacterium *Synechococcus* (Steunou *et al.*, 2006, 2008). However, other potential diazotrophs from thermophilic and hyperthermophilic heterotrophic bacteria and Archaea are also described as active nitrogen fixers at temperatures up to 82°C in YNP acidic hot springs (Hamilton *et al.*, 2011). Moreover, *nif*H genes have also been detected in widely range of temperatures between 16 to 89°C in hot springs with varied pH values (1.9 to 9.8) and associated to *Roseiflexus* sp., *Synechococcus* sp. and α-Proteobacteria (Hall *et al.*, 2008; Loiacono *et al.*, 2012).

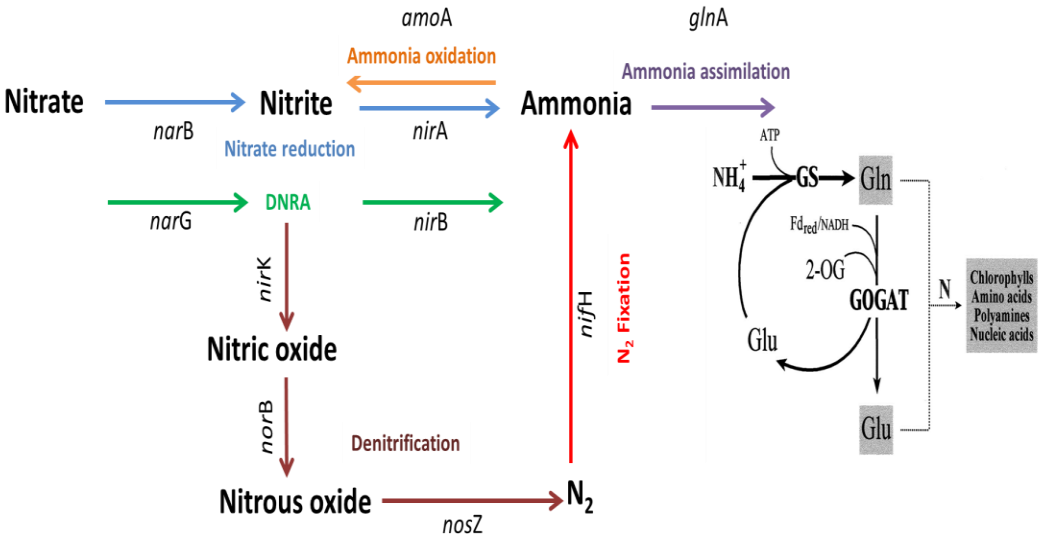


Figure 7. Nitrogen cycle representation**.** The diagram shows the principal pathways in the nitrogen cycle including assimilative, dissimilative (DNRA) pathways and genes involved in the key process.

6. Thermophilic Cyanobacteria from hot spring systems

For any understanding of the evolution of life and the biogeochemical cycles on Earth, knowledge about the ecology and evolution of the Cyanobacterial phylum is a prerequisite.

Cyanobacteria and, hence, oxygenic photosynthesis evolved 2.7–2.2 billion years ago and had therefore ample time to diversify and adapt to newly evolving niches that emerged on Earth (Schopf *et al.*, 2002). Moreover, Cyanobacteria are a morphologically highly diverse, yet phylogenetically coherent group of oxygenic phototrophs, with their oldest known fossils indicating the presence of filamentous forms, growing in microbial assemblages similar to those still found in stromatolites now days (Schopf and Packer,

1987). Several different members of the Cyanobacteria fix atmospheric nitrogen, and they are the only eubacteria so far with a true circadian clock (Golden *et al*., 1997).

Cyanobacteria now days are abundant in aquatic environments but also occur in terrestrial ecosystems including deserts, polar soils in Antarctica and other extreme environments as hot springs where they play a prominent role (Stal, 1995).

Cyanobacterial diversity in hot spring systems is represented by all subsections (I, II, III, IV and V) (Rippka *et al*., 1979). Among them, unicellular cyanobacteria appear more frequently represented by *Synechococcus* and *Cyanothece* genera (subsection I) (Steunou *et al*., 2006, 2008; Ward *et al*., 1998), while within filamentous cyanobacteria, *Phormidium* sp. (subsection III), *Calothrix* sp., *Nodularia sp.*, *Anabaena* sp., *Aphanizomenon* sp. and *Scytonema* sp. (subsection IV), as well as *Fischerella* sp. and *Mastigocladus* sp. (subsection V) are present (Miller *et al*., 2006; Sompong *et al*., 2006; Khumanthem *et al*., 2007; Finsinger *et al*, 2008) (Figure 8). It has been seen that their diversity changes according with temperature variations. For example, at temperatures >60°C unicellular cyanobacteria such as *Synechococcu*s sp. have been found dominant (Ward *et al*, 1998; Steunou *et al*., 2006, 2008), while at lower temperatures (between 60 to 40°C) filamentous cyanobacteria are described as dominant (Ward and Castenholzs, 2000; Lau *et al*., 2005) (Figure 8). Thus, Cyanobacteria seem to be distributed on the mat depending primarily on their temperature tolerance and also by the specific metabolic requirements under such conditions.

Based on the interest to understand in more detail the specific adaptations of these thermophilic bacteria, is that several representatives of Cyanobacteria inhabiting hot springs have been isolated and characterized (Steunou *et al*., 2008; Miller *et al*., 2006; Finsinger *et al*., 2008). Some of the most studied cyanobacterial isolates with high

representation in hot spring microbial mats belong to the Stigonematales Order, being filamentous heterocystous *Fischerella* sp. and *Mastigocladus* sp. the most common genera (Miller *et al*., 2006; Finsinger *et al*., 2008).

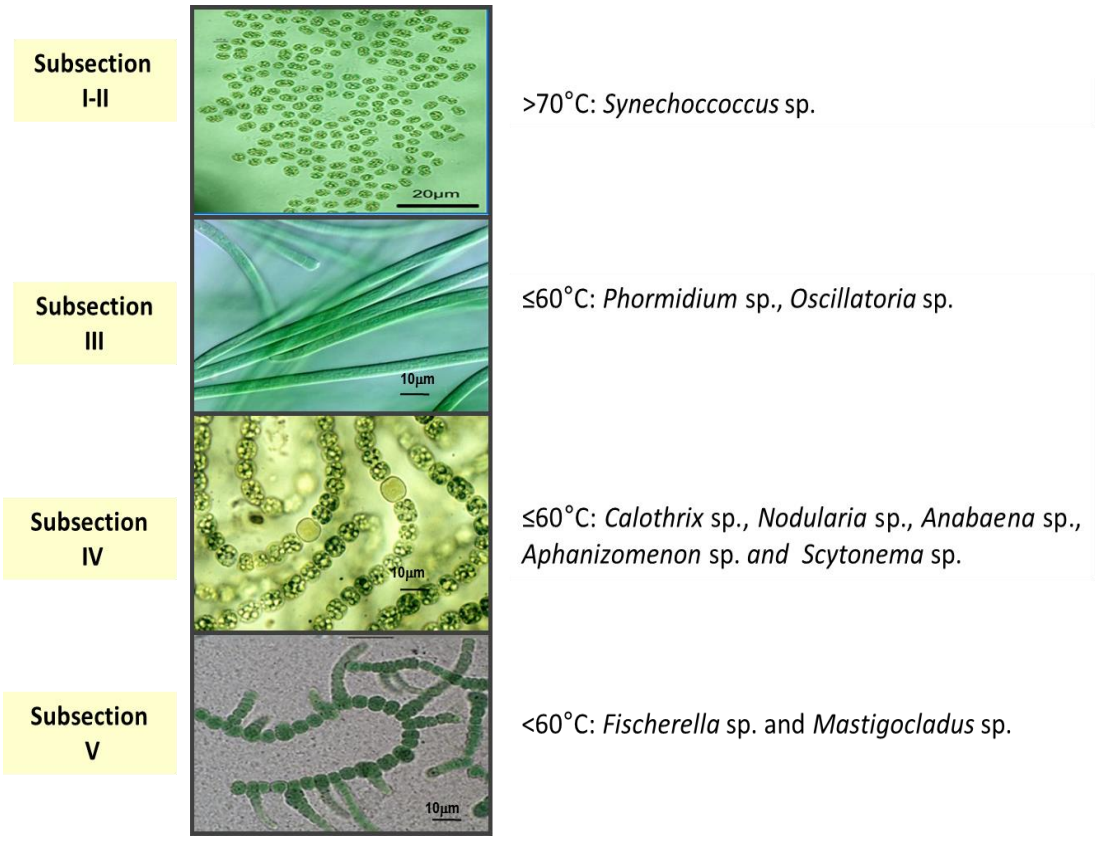


Figure 8. Cyanobacteria subsections reported in microbial mats of hot springs. Unicellular (*Synechococcus* sp.; Steunou *et al*., 2006-2008), filamentous without heterocystous (*Phormidium* sp. *Oscillatoria* sp.; Lau *et al.*, 2005) and filamentous with heterocystous (*Calothrix* sp., *Nodularia* sp., *Anabaena* sp., *Aphanizomenon* sp. and *Scytonema* sp.; Ward and Castenholzs, 2000; *Fischerella* sp., *Mastigocladus* sp.; Steward *et al.*, 1967; Miller *et al.*, 2006; Finsinger *et al.*, 2008).

6.1 Stigonematal Cyanobacteria

The Stigonematales order (section V) comprises the morphologically most complex species of Cyanobacteria (Figure 8), with up to four distinct cell types in their filaments: vegetative cells, performing oxygenic photosynthesis; heterocysts, in which nitrogen fixation takes place; hormogonia, motile short filaments; and akinetes, a type of resting cell (Finsinger *et al*., 2008) (Figure 8-9). Also multiseriate and true-branching trichomes are a common feature found in this group; however, the developmental biology of these organisms is still poorly understood.

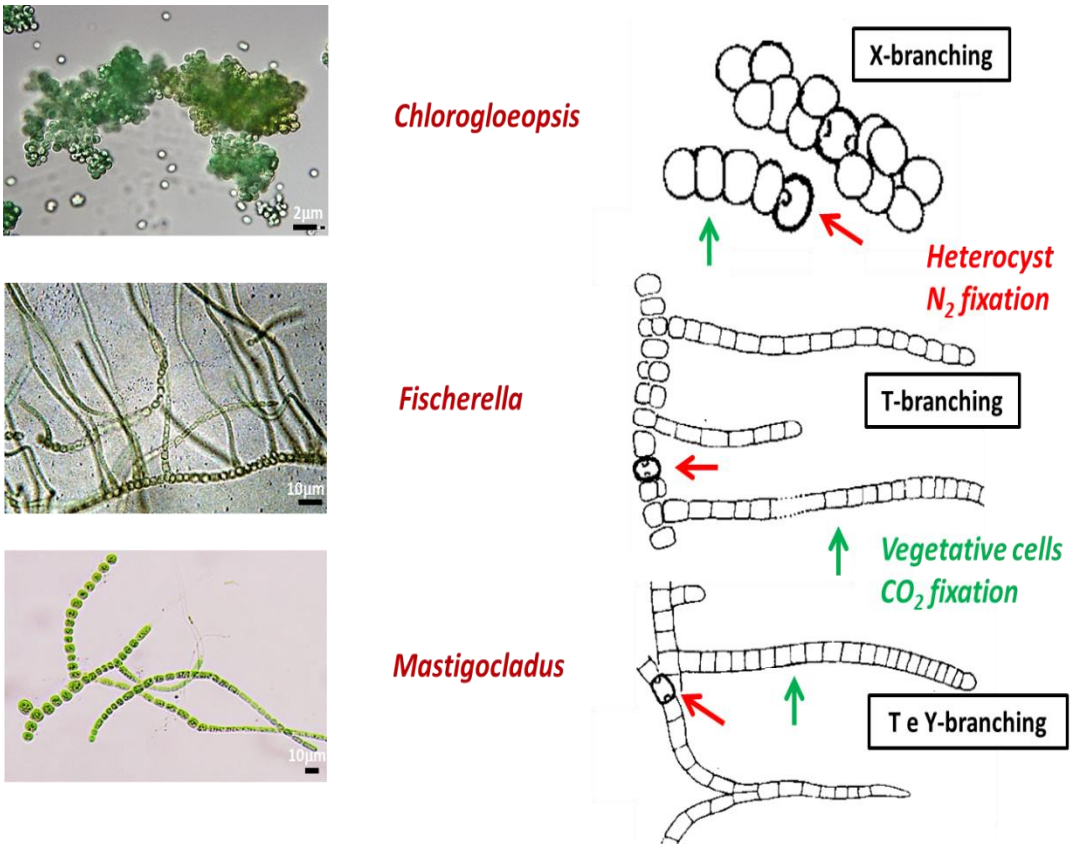


Figure 9. Morphological characterization of Stigonematal order**.** Different division plane of principal filament, width of vegetative cells, type of branching and heterocysts are the principal differences between them according with Anagnostidis and Komarek (1990).

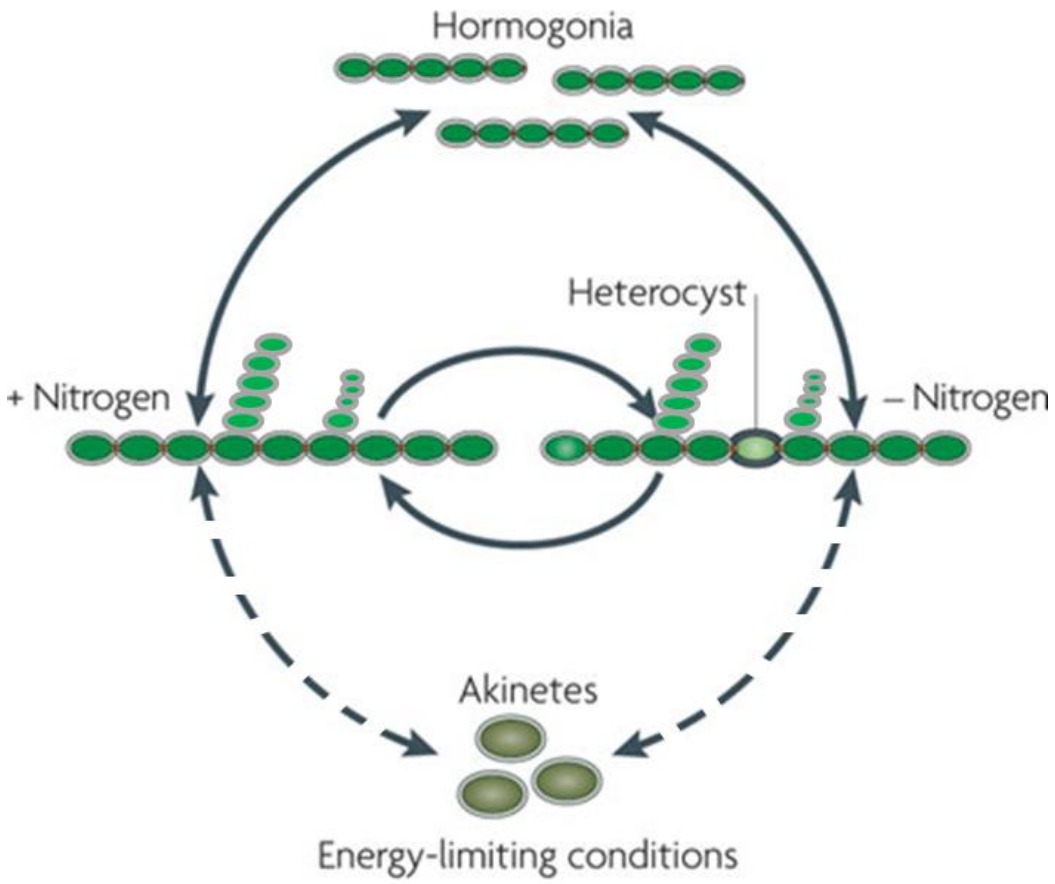


Figure 10. Diagram representing the complex life cycle of some heterocyst forming cyanobacteria from Order Stigonematal. The different phases of life cycle comprise heterocysts forming in response to deprivation of combined nitrogen, some vegetative cells differentiate into akinetes in response to energy-limiting conditions; dotted line is represented were the cellular differentiation is no clear in this order. Hormogonia represent the start of the life cycle, and are dispersal forms that can also function as infection units for the establishment of symbiotic associations with plants. The differentiation of hormogonia can be stimulated by some environmental conditions, such as nitrogen deprivation, and by a plant hormogonium-inducing factor60; when hormogonia resume growth, they produce filaments with or without heterocysts, depending on the availability of combined nitrogen. The diagram was adapted to the Stigonematal order from the Nostocales order describe by Flores and Herrero (2010).

Stigonematal cyanobacteria are typically found in the flowing waters of hot springs up to 58ºC (Khumanthen *et al*., 2007; Miller *et al*., 2007, 2009; Alcamán *et al*., 2015). Close to those temperatures, several isolates of thermophilic *Fischerella* and *Mastigocladus* spp. have been used as strain models to describe morphological characters and physiological performance as well as temperature tolerance, optimal growth and nitrogen fixation/assimilation (Nierzwicki-Bauer *et al*., 1984; Stevens *et al*., 1985; Miller and Bebout, 2004; Miller *et al*., 2006; 2007). However, there is a need to gain information from more isolates in culture to better understand their requirements and adaptations to particular environmental conditions, as this is difficult to be determined just by studying its environmental distributions (Stal, 2007).

According to major biological processes that occur in the first layers of the thermal mats, and the metabolic importance that Cyanobacteria have as primary producers and nitrogen fixers, is that unveiling their diversity and metabolic capacities compared to other organisms along the temperature gradient in hot spring microbial mats, was the focus of this study.

In particular, a previous study on Chilean hot springs mats, demonstrated that Stigonematal Cyanobacteria conform an important part of the community in the mat, therefore this specific order of Cyanobacteria was used as model to perform this thesis.

7.- Field characteristics and questions

Chile is a country with a volcanic history, with more than 200 active volcanos at the moment. That activity generates a large number of hot springs all over the Chilean geography. Among them, Porcelana hot spring located in the northern Patagonia (42º 27’

29.1’’S - 72º 27’ 39.3’’W) (Figure 9) can be defined as an extreme system according to its volcano origin (Lahsen, 2000). This thermal system presents a temperature gradient from >70 to 40°C, from the origin to the farthest point; distance that does not exceed the 10 meters of extension. The temperature has been suggest as a major factor determining the distribution and abundance of species that are living in the microbial mat of Porcelana, since it is the only parameter that changes drastically in the few meters long (Mackenzie *et al.*, 2013). Porcelana is also a pristine spring that provides with a decent accessibility to conduct research on field, which as permitted in this doctoral thesis to gain information on the diversity and metabolic activity contributions that the thermophilic microbial mat community has along the temperature gradient.

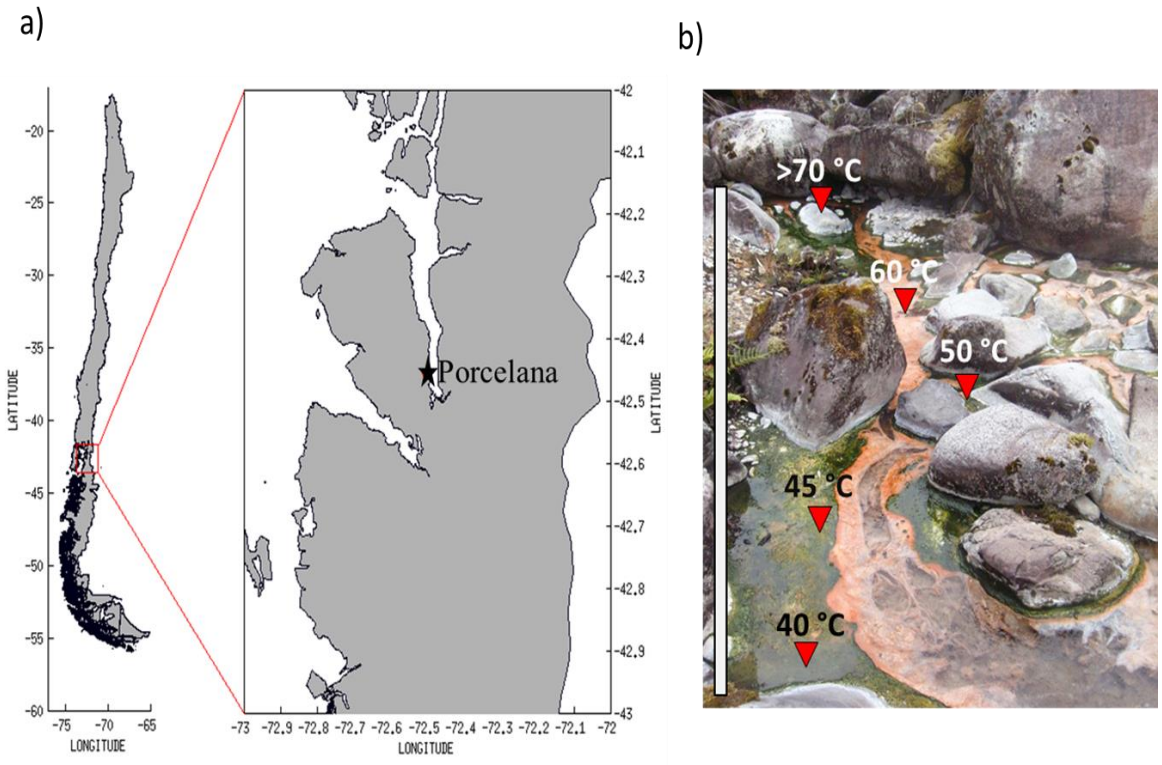


Figure 11. Field sampling study**:** a) Porcelana hot spring geographical location; and b) Pigmented microbial mat along the thermophilic gradient in Porcelana.

In a previous study carry out by our group at Porcelana microbial mat, the 16S rRNA gene was analyzed by denaturing gradient gel electrophoresis (DGGE), showing Cyanobacteria members of the Order Stigonematales (subsection V) as well represented (MacKenzie *et al*., 2013). According to that, an effort was done to isolate a representative of this group of Stigonematales (Figure 12), that was characterized during the present doctoral thesis, and which behavior and capabilities in culture under controlled conditions was compared to our (*in situ*) results obtained at the field studies.

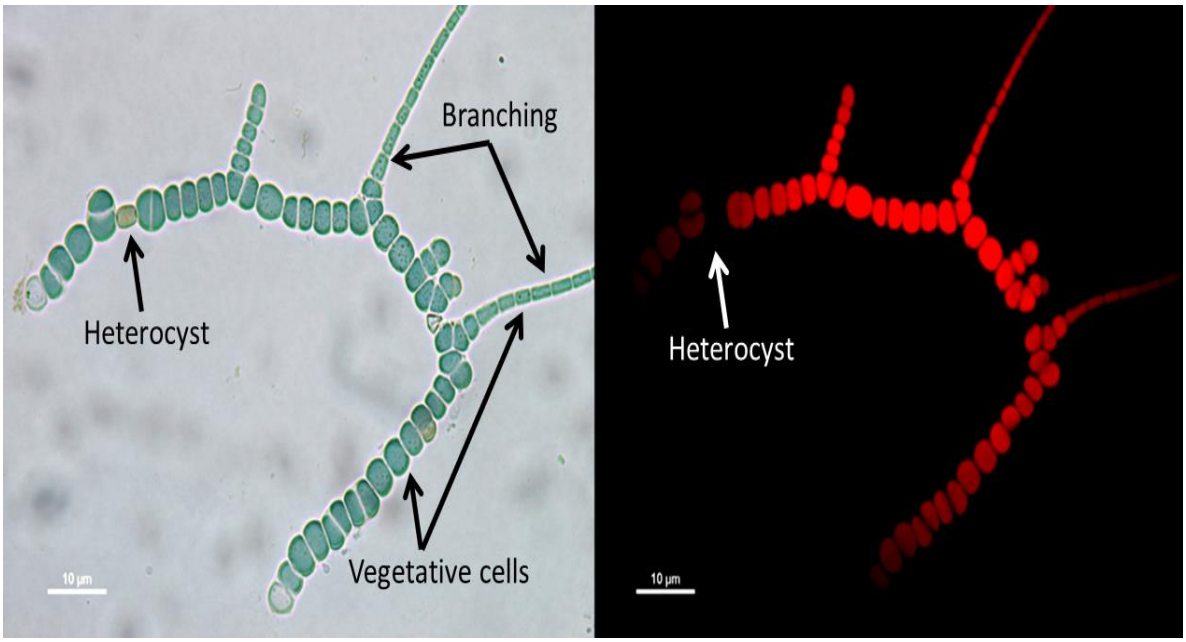


Figure 12. Stigonematal cyanobacteria "strain CHP1" isolated from Porcelana hot spring**.** a) Light, and b) Epifluorescence microscopy showing vegetative cells, branching of principal filamentous and heterocyst nitrogen fixation specialized cells.

In total, this doctoral thesis comprises three years of field work in Porcelana thermophilic microbial mat, and a physiological characterization of the Stigonematal isolate strain, called "CHP1". Both approximations have permitted us to unveil not only the diversity, biogeochemistry and metabolic activity contributions of thermophilic microorganisms associated to the C and N cycles at Porcelana hot spring but also to understand better the metabolic strategies and optimal growth conditions at high temperatures of main actors in these mats such as Cyanobacteria.

The field studies permitted us to answer: "*How temperature affects the diversity and metabolic processes in the microbial mat communities?; What are the main actors and strategies for nitrogen and carbon compounds assimilations?; What is the gene expression pattern associated with the carbon and nitrogen cycle pathways at different temperatures?; What is the contribution of the thermophilic cyanobacterial members in the microbial community?.*

The cultures under controlled conditions in the laboratory permitted us to answer: *What is the identity and optimal temperature growth of the isolate cyanobacteria strain CHP1?; Is CHP1 a thermophilic nitrogen fixer?; Has CHP1 preference for any especial nitrogen source?; What is the contribution of CHP1 cyanobacterium on the Porcelana microbial mat community?"*

HYPOTHESIS

"The temperature modulates the composition and carbon/nitrogen metabolisms of the microbial mat community, particularly Stigonematal diazotrophic cyanobacteria, present in Porcelana hot spring (X Region de los Lagos)".

GENERAL AIM

Determine how the temperature influences in bacterial composition, the carbon and nitrogen fixation (CO_2 and N_2), assimilation (NH_4^+ and NO_3^-) and expression of metabolic related genes (*psb*A, *rbc*L; *nif*H, *gln*A and *nar*B), in the microbial mat community, particularly Stigonematal diazotrophic Cyanobacteria, present in Porcelana hot spring (X Region de los Lagos).

SPECIFIC AIMS AND THESIS STRUCTURE

To describe and determine how the temperature affects the microbial mat diversity and specifically the molecular gene expressions and adapted physiological behavior of microorganisms involved in the carbon and nitrogen cycles, four specific aims were planed and conducted along three connected chapters:

Aim *1. Determine the composition of the microbial mat community present at different temperatures in Porcelana hot spring, by metagenomic and metatranscriptomic analysis* (Illumina Hi-seq). All microbial mat samples where obtained from the temperature gradient established in year 2013, and the results are exposed in **Chapter I**. Omics data set, permitted to reveal the complete microbial mat composition in the temperature gradient.

Aim *2. Determine the presence and activity (gene transcripts and rates) of main metabolic pathways associated to carbon (e.g. photosynthesis, CO_2 fixation) and nitrogen (e.g. N_2 fixation, NH_4^+ and NO_3^-assimilations) along the temperature gradient at Porcelana hot spring, by metatranscriptomic analysis (Illumina Hi-seq) and* in situ *stable isotopic ($H^{13}CO_3$/$^{15}NH_4Cl$/$K^{15}NO_3$) uptake incubations.* Omics data demonstrated that Chloroflexi and Cyanobacteria were the higher active phyla along the temperature gradient in Porcelana mat, and were involved in the majorly of the pathways of the carbon fixation and nitrogen cycle. Few totals reads for CO_2 fixation pathways at the highest temperature were attributed to Chloroflexi and Thaumarchaeota, but most autotrophic CO_2 fixation

could just be attributed to Cyanobacteria at the lower temperatures. Also, nitrogen fixation was the major process in the contribution of new nitrogen at the lower temperatures and, more specifically attributed to the genus *Mastigocladus* sp.

In accordance, the C (CO_2) and N (N_2) fixation rates were detected during the light period; however, ammonium and nitrate assimilation rates demonstrated very low incorporations. The expression recorded for genes involved at each metabolic C and N route suggested a temperature-dependency pattern. Therefore, Porcelana microbial mat is a photosynthetic and diazotrophic system that is modulates taxonomically by temperature. All these results are exposed in **Chapter I.**

Aim 3. *Determine the d and activity of nitrogen-fixing bacteria (specifically Cyanobacteria) along the temperature gradient in Porcelana hot spring*, by *in situ* studies during four years (2009, 2011, 2012 and 2013). The diazotrophic cianobacterial diversity along the temperature gradient for each year was determined by the analysis of the 16S rRNA and *nif*H genes using denaturing gradient gel electrophoresis (DGGE), while their potential nitrogen fixation activity was investigated through acetylene reduction assays and isotopic $^{15}N_2$ rates. Main results are displayed in **Chapter II**. Analyses of the 16S rRNA and *nif*H genes demonstrate that Stigonematal diazotrophic members are most abundant among Cyanobacteria at all years studied, similarly to the results obtained for year 2013 by our omic analyses. Our results also demonstrated that high nitrogenase activities and ^{15}N cell incorporation rates were light dependent with maximum activity and *nif*H gene expression at midday, and at temperatures from 46 to 58ºC. This insight suggests that Porcelana thermal microbial mat behave as an important atmospheric N scavenger, contributing with up to 90% of the new nitrogen to the total microbial community.

Aim 4. *Characterization of the strain CHP1 (Stigonematal) isolated from Porcelana hot spring, under differential (temperatures and combined nitrogen sources) culture controlled conditions.* To understand the potential environmental performance of Stigonematal cyanobacteria in Porcelana, the strain CHP1 isolated from Porcelana was characterized and its performance under controlled conditions in the laboratory was then compared with the field work results. Strain CHP1 was identified as belonging to the genus *Mastigocladus* (branched, heterocystous filaments) and represents a major player in Porcelana spring by contributing to the new nitrogen through its nitrogen fixation activity. CHP1 growth curves demonstrated that this strain is a moderately thermophilic cyanobacterium that grows at 45 and 50°C but not at 60ºC. The strain CHP1 fixes maximum atmospheric dinitrogen in light independent of temperature. However, the *nif*H gene expression was arrhythmic at 45ºC remaining constant during the day/night period, while at 50ºC *nif*H gene showed a high expression during light period. High affinity for nitrate but low tolerance to high ammonium concentrations, showing an apparent plasticity in regards to the use of nitrogen sources. Also, the sequenced genome of strain CHP1 together with metatranscriptomic data from Porcelana, the cyanobacteria "*Mastigocladus* sp. strain CHP1" was confirmed as one of the most important nitrogen fixer in the Porcelana microbial community. All these results are presented at **Chapter III.**

CHAPTER I

Temperature modulated microbial diversity involved in carbon and nitrogen metabolisms in Porcelana hot spring mat.

ORIGINAL ARTICLE

Temperature modulates microbial diversity involved in carbon and nitrogen metabolisms in Porcelana hot spring mats.

M. Estrella Alcamán[1], Carlos Pedrós-Alió[2], Javier Tamames[2], Gonzalo Sepúlveda-Hermosilla[1], Germán Marchandon[1], Camila Fernández[3,4,5], Danilo Pérez-Pantoja[6] and Beatriz Díez[1,7*]

[1]*Department of Molecular Genetics and Microbiology, Pontificia Universidad Católica de Chile, Libertador Bernardo O´Higgins 340, Casilla 144-D, C.P. 651 3677, Santiago, Chile.*

[2]*Programa de Biología de Sistemas, Centro Nacional de Biotecnología (CSIC), Darwin 3, 28049 Madrid, Spain.*

[3]*Sorbonne Universités, UPMC Univ Paris 06, CNRS, Laboratoire d'Océanographie Microbienne (LOMIC), Observatoire Océanologique, F-66650, Banyuls/mer, France*

[4]*Department of Oceanography, Interdisciplinary center for Aquaculture Research (INCAR) and COPAS SURAUSTRAL program, Universidad de Concepción, Chile.*

5 Fondap IDEAL, Universidad Austral de Chile, Chile

[6]*Department of Biochemistry and Molecular Biology, Universidad de of Concepción, Chile*

[7]*Center for Climate and Resilience Research (CR)2, Chile*

Running title

Temperature modulates microbial diversity and metabolisms

*Correspondence: Beatriz Díez, Department of Molecular Genetics and Microbiology, Faculty of Biological Sciences, Pontificia Universidad Católica de Chile, Libertador Bernardo O´Higgins 340, Casilla 144-D, C.P. 651 3677, Santiago, Chile.
E-mail: bdiez@bio.puc.cl

Abstract

The phototrophic microbial mat communities collected along the temperature gradient (66, 58 and 48 °C) at noon in the neutral pH Porcelana hot spring (Northern Chilean Patagonia) was investigated by metagenomic, metatranscriptomic and isotopically labeled carbon ($H^{13}CO_3$) and nitrogen ($^{15}NH_4Cl$ and $K^{15}NO_3$) assimilation assays. The taxonomic microbial mat composition was represented by 31 taxa, and confirmed that phyla Chloroflexi and Cyanobacteria were prevalent and dominant (>80% of total community relative abundance). Minor members at all temperatures were Bacteroidetes, Proteobacteria and Deinococcus-Thermus that together accounted for less than 15%, while rare members of the community (i.e. Chlorobi, Firmicutes, etc) represented only <1%. Metatranscriptomic analysis revealed that energy metabolism (Photosynthesis and CO_2 fixation) and nitrogen, sulfur, phosphorous and iron cycles were active at noon preferentially at 58 and 48 °C. These activities were mostly carried out by Chloroflexi and Cyanobacteria phyla. At 58 °C both phyla co-occurred, with similar contribution to the most representative active metabolisms. However at 66 °C, filamentous anoxygenic phototrophic Chloroflexi members such as *Roseiflexus* sp. and *Chloroflexu*s sp. were >90% responsible for all active energetic and nutrients metabolisms, being CO_2 fixation by the 3-hydroxypropionate autotrophic pathway present in this phyla preferably at this temperature

as well as at 58 °C. In the other hand, filamentous heterocystous Cyanobacteria from the genus *Mastigocladus* sp., contributed most at 58 and 48 °C in photosynthesis and CO_2 fixation by Calvin cycle, with a maximum at lowest temperatures. The molecular data suggest that the high *in situ* isotopically labeled C fixation rates (up to 362 nmoles C $cm^{-2}d^{-1}$ between 58 and 48 °C), were associated to Chloroflexi and Cyanobacteria activities. Low ammonium and nitrate assimilation rates with fast and low turnover were recorded, respectively. Ammonia oxidation, DNRA and denitrification were the most N active routes at 66 ºC, and were associated to minority and rare taxa such as genera *Nitrososphaera* sp. (Thaumarqueota), *Burkholderia* sp. (Proteobacteria) and *Rhodothermus* (Bacteroidetes), respectively. At 58 and 48 °C nitrogen fixation and nitrate assimilation were the most relevant N active process and were totally carried out by Cyanobacteria from *Mastigocladus* spp. Our findings not only conclude that Porcelana microbial mat diversity and C/N metabolisms are modulated taxonomically by temperature but also that not only a few genera with well known dominance in neutral hot springs globally, but also some minoritary and rare taxa might contribute with their metabolisms to maintain these systems functioning.

Keywords: carbon and nitrogen uptake / metagenomics / metatranscriptomics / microbial mat / nitrogen cycle / photosynthesis.

Introduction

Microbial mats have been studied for decades and represent model systems for discovering the principles of microbial community ecology (Stal, 1995; Ward, 2006; Hou *et al*., 2013; Inskeep *et al*., 2013). Microbial mats associated with hot spring are dynamic and complex ecosystems exhibiting spatial and temporal heterogeneity (Ward *et al*., 1998, 2006; Bhaya *et al*., 2007; Klatt *et al*., 2011; Alcamán *et al*., 2015). These microenvironments support a diversity of species carrying out a wide range of metabolic processes. The upper few millimeters in alkaline and neutral microbial mats are dominated by oxygenic phototrophs, such as the unicellular cyanobacterium *Synechococcus* sp. (Steunou *et al*., 2006, 2008; Bhaya *et al*., 2007; Klatt *et al*., 2011), the filamentous non-heterocystous *Oscillatoria* sp. and heterocystous *Mastigocladus* sp. cyanobacteria (Stewart, 1970; Miller *et al*, 2006; Alcamán *et al*., 2015), as well as the Chloroflexi filamentous anoxygenic phototrophs (FAPs) *Roseiflexus* sp. and *Chloroflexus* sp. (van der Meer *et al*., 2010; Liu *et al.*, 2011; Klatt *et al*., 2011). Several diversity studies in hot spring microbial mats have demonstrated that members of the phototrophic Cyanobacteria and Chloroflexi phyla can co-occur in a collaborative manner (Liu *et al.*, 2011). For instance, *Synechococcus* sp. produces low-molecular weight organic compounds as byproducts of its metabolism (as primary producers), that are assimilated photoheterotrophically by FAPs (Sandbeck & Ward 1981; Anderson *et al*., 1987; Bateson & Ward, 1988). A similar co-occurrence has been reported between photosynthetic Cyanobacteria, sulfur oxidizing Chlorobi (green sulfur bacteria) or filamentous anoxygenic phototrophic Chloroflexi in sulfidic hot springs mats (Bryant *et al*., 2012; Klatt *et al*., 2013). These phyla also co-occur in neutral and alkaline hot springs, however, less is

known about how these communities contribute to biogeochemical cycles at different thermophilic temperatures that define the temperature gradient in the mat.

It is known that the first millimeters of the microbial mat are the most metabolically active layer with respect to carbon cycling. During daytime, this upper part of the mat is supersaturated with photosynthetically produced oxygen (Canfield & Marais, 1994), whereas at night, anoxic conditions prevail due to continued respiration activities of aerobic and anaerobic heterotrophic bacteria (AHB) such as sulfate reducing bacteria (SRB) that reduce sulfate to sulfide (Wieland & Kühl, 2000; Baumgartner *et al*., 2006). Photo-oxygenic microorganisms, like Cyanobacteria, perform photosynthesis in two stages: light-dependent reactions and the Calvin cycle (light-independent reactions) to synthesize organic molecules, and consequently biomass. On the other hand, anoxigenic photosynthetic microorganisms, such as Chloroflexi, use sulfide (Madigan & Brock, 1977a) or hydrogen (Holo & Siverag, 1986) as electron donors to fix carbon dioxide (CO_2) through the 3-hydroxypropionate (3-HP) bicycle (Strauss & Fuchs, 1993; Klatt *et al*., 2007; Zarzycki *et al*., 2009, 2011). Mixotrophy has been also described in several Chloroflexi. These organisms can simultaneously incorporate inorganic (CO_2) and organic carbon, such as acetate (generated under anoxic conditions at night) and glycolate (generated of photorespiration under O_2 supersaturation during the day) produced by Cyanobacteria (van der Meer *et al*., 2005, 2007; Bateson & Ward 1988; Zarzycki and Fuchs, 2011; Bryant *et al*., 2011). The availability and abundance of these organic compounds, combined with the availability of inorganic carbon, light energy and hydrogen or sulfide as electron sources, are factors that shape the relative degree to which FAPs behave as heterotrophs, mixotrophs or autotrophs (van der Meer *et al*., 2005; Zarzycki & Fuchs, 2011; Klatt *et al*., 2013). Therefore, the different forms of C uptake in the mat, can

reveal whether Cyanobacteria and Chloroflexi are collaborating or competing by substrates.

Moreover, the study of other essential macronutrients in the biogeochemistry of the mat in hot springs like those related to the nitrogen cycle, has been focused on the assimilation pathways such as N_2-fixation attributed to Cyanobacteria (*Synechococcus* sp. and *Mastigocladus* sp.) (Steunou *et al*., 2006, 2008; Miller *et al*., 2006; Alcamán *et al*., 2015), ammonia oxidation attributed to Archaea (*Candidatus* Nitrosocaldus yellowstonii) (de la Torre *et al*., 2008; Reigstad *et al*., 2008; Hamilton *et al*., 2014), and denitrification and dissimilatory nitrate reduction to ammonia (DNRA) associated with different members of Aquificales and Thermales (*Hydrogenobacter* sp., *Sulfurihydrogenibium* sp., *Anoxybacillus* sp. and *Thermus* sp.) (Dodsworth *et al*., 2011). Generally, hot springs are N-limited systems due to the fast assimilation and turnover of inorganic nitrogen forms (Lin *et al*., 2015; Alcamán *et al*., 2015). According to this, N_2-fixation carried out by Cyanobacteria has been reported as the most relevant biological process for the input of exogenous nitrogen in the microbial mat community of the Chilean neutral hot spring of Porcelana (Alcamán *et al*., 2015). Additionally, evidence to maintain the balance between input and output of N was demonstrated by Hamilton *et al*., (2014) in the YNP hot spring of Perpetual Spouter (pH 7.03, 86.4 °C), where a rapid consumption of NH_4^+ by putative oxidizing archaea (affiliated to the phylum Thaumarchaeota) forced the activity of the putative nitrogen-fixing (diazotrophic) bacteria *Thermocrinis albus* from phyla Aquificae, in the absence of other nitrogen sources. It has also been reported that Archaea are able to perform different N reductive pathways, including nitrate assimilation, N_2 fixation, and dissimilatory reactions (Cabello *et al*., 2004), reveling a strong co-distribution pattern in the populations at >70 °C in hot springs (Hamilton *et al*., 2014). However, little is known

about the pathways and main actors that carry out the N-Cycle in neutral hot springs with moderated temperatures (<70 °C). Insights into the expression of protein-coding genes involved in the N cycle pathways and/or quantification of assimilation rates of reduced inorganic (ammonium and ammonia) and oxidized inorganic forms (e.g., nitrate, nitrite, nitric acid and nitrogen oxides) are completely unknown in these extreme environments.

In the last decades the development of high-throughput sequencing techniques has expanded our knowledge of taxonomical diversity as well as protein-coding genes in hot springs (Bhaya *et al*., 2007; Liu *et al*., 2011; Klatt *et al*., 2011). Metagenomics and metatranscriptomics are effective approaches to target the community structure and the expressed genes that reveal community functions carried out at the time of sampling (Urich *et al*., 2008; Bhaya *et al*., 2007). Some metagenomic studies have revealed new aspects of microbial diversity distribution with temperature in hot springs (Bhaya *et al*., 2007), as well as the characterization of dominant chlorophototrophic populations and previously unidentified members of the Chloroflexi phylum (Klatt *et al*., 2011). In addition, metatranscriptomic analyses have revealed important information about temporal patterns of expression of key genes involved in processes such as N_2-fixation (*nif*H) (Alcamán *et al*., 2015), aerobic (*pca*A) and anaerobic (*puf*M) photosynthesis (Liu *et al*., 2011), as well as survival strategies of relevant populations (Quaiser *et al*., 2014).

In the present study we used metagenomic and metatranscriptomic analyses to determine the structure and activity of the microbial mat communities that develop along a moderately thermophilic gradient (66 to 48 °C) at noon in the neutral hot spring of Porcelana (Northern Patagonia, Chile). Previous studies of partial 16S rRNA gene conducted in the microbial mat of Porcelana (MacKenzie *et al*., 2013), revealed that Chloroflexi, Cyanobacteria and Proteobacteria were the most abundant phyla, and studies

of nitrogen fixation activities (*nif*H gene) showed that Cyanobacteria made the most important contribution of new nitrogen to that system (Alcamán *et al*., 2015). Hence, we quantify assimilatory-N ($^{15}NH_4Cl$ and $K^{15}NO_3$) and C-fixation ($H^{13}CO_3$) rates at the different temperatures along Porcelana gradient, together with the taxonomy distribution and the active metabolisms related to the C and N cycles, to determine the role of main members of the microbial community in the different carbon and nitrogen processes.

Materials and Methods

Sampling

Porcelana hot spring is located in Chilean Patagonia (42º 27’ 29.1’’S - 72º 27’ 39.3’’W). It is a neutral pH system with a maximal temperature of 70 °C when sampled on March 2013. Microbial mats (up to 3 cm thick) grow downstream down to 46 ˚C (Mackenzie *et al*., 2013, Alcamán *et al*., 2015). Microbial mats growing at 66, 58 and 48 °C were sampled using a cork borer with a diameter of 7 mm. Cores 1 cm thick were collected in triplicate at noon (12:00 h PM) for DNA and RNA (metagenomic/metatranscriptomic) analyses. Samples were transported in liquid nitrogen and kept at -80ºC until extraction.

For isotopic nitrogen ($^{15}NH_4Cl$, and $K^{15}NO_3$) and carbon ($H^{13}CO_3$) uptake experiments cores from microbial mats growing at 58 and 48 ºC were collected in the same way. All experiments were done in triplicate.

Nitrogen and carbon uptake experiments

Three biological replicates composed of three microbial mat cores each (7 mm in diameter and 1 cm thick), were transferred to pre-sterilized 12 ml vials with 1 ml of pre-filtered spring water (0.2 μm filter pore) at their *in situ* temperature. Dual $^{15}N/^{13}C$ uptake experiments were started by adding 50 μL of $^{15}NH_4Cl$ (500 μM) plus 500 μl of $H^{13}CO_3$ (500 μM) or 30 μl $K^{15}NO_3$ (500 μM stock solution) plus 500 μL of $H^{13}CO_3$ (500 μM) to each vial. The ^{15}N tracer additions were generally kept close to 10% of ambient concentration; 3.2 μmol L^{-1} NO_3^- and 0.04 μmol L^{-1} NH_4^+, respectively. Vials were incubated at 58 and 48 °C *in situ* temperatures, and triplicates were sacrificed after 2, 6, 12 and 24 h. In addition, two replicates (for each experiment) without the isotope (^{15}N and ^{13}C) were also incubated in order to determine the natural isotopic composition and to be used as negative controls. After incubations, microbial mat samples were dried at 70 °C for 48 h. Isotopic analyses of ^{15}N and ^{13}C and the C:N ratio (organic matter composition in the sample) were carried out using a IRMS delta plus Thermo FinniganH mass spectrometer (Stable Isotope Laboratory, Granada, Spain). Rates of carbon fixation and nitrogen assimilation were expressed as carbon or nitrogen assimilation rates (nmoles cm^{-2} h^{-1}), and calculate as:

$$\%R_{exc} = \frac{\left(\frac{V_{add} * T_{conc}}{V_{inc}}\right) + Nat_{conc} * Nat_{abundance}}{Nat_{conc} + \frac{V_{add} * T_{conc}}{V_{inc}}} * 100 - (\%)Nat_{abundance} \quad (1)$$

$$\rho = \left[\frac{(\%AT_f - Nat_{abundance})}{\%R_{exc}}\right] * [PON\ or\ POC] \ / \text{mg} \ / \text{Time}_{inc} \quad (2)$$

where $\%R_{exc}$; the excess enrichment of the tracer after inoculation; is calculated using Eq. (1): V_{add} indicates the volume of isotope added to the sample during the inoculation; T_{conc} is the tracer concentration added to the sample; V_{inc} is the volume of incubation; Nat_{conc} represents the initial amount C or N in the sample; $Nat_{abundance}$ corresponds to natural abundance of C or N. For this study, a constant DIC value of 0.8 μM, NH_4^+ equal to 0.04 μM and 3.2 μM to NO_3^-, were used based on previous measurements in Porcelana mat. To calculate the rate of assimilation (ρ) Eq. (2): $\%AT_f$ represent the percentage of total atoms after incubation; PON or POC are the amounts of particulate organic nitrogen or carbon recovered after incubation and measured by mass spectrometry; mg is the microbial mat mass analyzed and $Time_{inc}$ is the final time of incubation (hrs).

Also, considering that the incubation time was six hours, an extrapolation to 12 light hours per day was used to obtain daily assimilation rates. Daily rates of ammonia and nitrate contribution to primary production (PP) were determined considering the particulate C:N ratio obtained in each experiment and the corresponding daily rates of ammonium or nitrate ($\rho^{15}N$) and $\rho^{13}C$ at each temperature (Eq. 3)

$$\%\text{Contribution (PP)} = \frac{(CN * \rho 15N)}{\rho^{13}C} * 100 \quad (3)$$

Nucleic acid extraction and high throughput sequencing

Nucleic acids were extracted as previously described (Alcamán *et al*., 2015). Briefly, glass beads were added to the DNA samples that were then homogenized by bead beating three times for 20 seconds. In the case of RNA samples the Trizol-mat mixture was

subjected to beating only two times. Quality and quantity of the extracted nucleic acids were checked and kept at -80 °C. DNA (20 ng mL^{-1}) and RNA (1µg) from all temperatures were sequenced by Illumina Hi-seq technology (Research and Testing Laboratory, Texas, USA). Briefly, enzymatic fragmentation was done prior to DNA library construction using NEBNext dsFragmentase, then fragmented DNA was cleaned up by column purification. The construction of libraries (Ultra DNA Library Prep Kit for Illumina) by: End prep., adaptor ligation, size selection of Adaptor-ligated DNA, PCR amplification and cleanup of PCR amplification. Finally, fragment size was checked by Fragment Analyzer. For metatranscriptomes gel-free or low input small RNA library prep kit with reduced bias for Illumina sequencing was conducted. The specific kit (NEXTflex™ Illumina Small RNA Sequencing Kit v3) and protocol is available at: http://www.biooscientific.com/next-gen-sequencing/nextflex-illumina-small-rna-seq-library-prep-kits.

Taxonomic and functional assignment of reads

To ensure downstream analysis feasibility, FastQC (www.bioinformatics.bbsrc.ac.uk/projects/fastqc/) was used to assess the quality of the sequences data. To correct the quality issues found the following low quality filters were applied using Cutadapt (http://journal.embnet.org/index.php/embnetjournal/article/view/200), leaving only mappable sequences longer than 30 bp (-m 30), with a 3' end trimming for bases with quality below 28 (-q 28), a hard clipping of the first 5 leftmost bases (-u 5), and finally a perfect match of at least 10 bp (-O 10) against the standard Illumina adaptors. This procedure reduced the total number of sequences from 452.7 to 394.7 million (Table 1).

For the classification of 16S rRNA gene sequences, the rRNA reads in DNA and RNA high quality samples were identified and separated using Ribopicker (Schmieder *et al*., 2012) with the non-redundant rRNA database that combines Silva, Greengenes, RDP-II, NCBI archaeal/bacterial, HMP and Rfam databases. Ribosomal RNA sequences amounted to 4.2% of the total, leaving 378.2 million reads of functional genes (Table 1).

The next step was to carry out a taxonomical and functional assignment of the non rRNA high quality reads. For this purpose sequences were searched against the NCBI non-redundant (NR) database, previously filtered by bacterial sequences (ids obtained from gbbct database ftp://ftp.ncbi.nlm.nih.gov/genbank/) and added new bacterial genomes not incorporated yet from JGI (http://genome.jgi.doe.gov/). Diamond (Buchfink *et al*., 2015) with default parameters was used for this purpose. The NCBI taxonomic identifiers (taxid) for each match were manually added as a new column in Diamond's output (CSV file), using an in-house bash script based on Unix command line programs that take each GI code and match it with the corresponding taxid from the NCBI taxonomy tree (Sayers *et al*., 2009). Subsequently, the results were parsed using the lowest common ancestor algorithm trough MEGAN 5 (Huson *et al*., 2007) under default parameters (score =50), to display a graphical representation of abundance for each taxonomic group identified. All the sequences that matched Bacterial and Archaeal phyla were extracted for further analysis. The total number of non-redundant hits was 105.9 millions. On most samples around 80% of such hits could be taxonomically assigned. Only in sample 66 RNA the percentage was near 20% (Table 1). Another approach to assess taxonomic composition was using the KRAKEN classifier system (Wood & Salzberg, 2014), that was used to infer taxonomic origins and compare both tools (Supplementary data, Figure S1).

Functional classification was obtained using the SEED database (Overbeek *et al.*, 2005) within MEGAN 5, with up-to-date mapping files. The total reads assigned to each metabolism were extracted and normalized to the total amount of reads assigned. To analyze specific metabolisms (both in DNA and cDNA) only the reads associated with photosynthesis, respiration, fermentation, CO_2 fixation, and acquisition of nitrogen, phosphorous, sulfur, and iron were extracted and matched with the previously Bacterial and Archaeal assigned reads, using an in-house bash script that took the read name as a common key. This allowed identification of the community members involved in each metabolism.

To identify the specific pathways of photosynthesis, CO_2 fixation and nitrogen metabolisms, the two metagenomes (48 °C and 58 °C) were co-assembled using the Spades software (Bankevich *et al*., 2012). Key genes from pathways of interest were used as indicators of the importance of each pathway (Table 2). For taxonomic and functional annotation of the genes Prodigal software (Hyatt *et al*., 2010) was used to predict genes. A homology search on the amino acid translations of the genes was done using RapSearch2 (Zhao *et al*., 2012) against the GenBank non-redundant protein database. Functional annotation in KEGG and COG codes was done using the fun3 software, as described in Guazzaroni *et al*., (2013). The abundance of genes and transcripts in each sample was determined by mapping the corresponding reads to the co-assembled metagenomic contigs. We used Bowtie2 (Langmead & Salzberg, 2012) for that purpose, and quantified the number of mapped reads to each gene using HTSeq (Anders *et al*., 2015). The raw counts were normalized to a FPKM (Fragments per Kilobase and Million reads) measure using a custom script. Many of the transcripts do not map to the reference contigs of the metagenome. These correspond to highly expressed genes of minority species that could

not be assembled because of their low abundance. To include these genes in the analysis, we extracted the unmapped transcripts from the three metatranscriptomes, and assembled them. The resulting contigs from the metatranscriptomes (composed often by a single gene, but also of polycistrons corresponding to operons) were added to the metagenomics contig set, and treated as described above (taxonomic and functional assignment, mapping and quantification of reads).

Results

Temperature ranged between 69 and 48 °C along the gradient. pH~6.8 was comparatively constant over time and temperatures. Macronutrient concentrations were on overage 3.2 μmol L^{-1} NO_3^-, 0.1 μmol L^{-1} NO_2^-, 0.04 μmol L^{-1} NH_4^+ and 62.5 μmol L^{-1} PO_4^{3-}. Thus, nitrogen was the limiting nutrient.

Taxonomic assignment of DNA and cDNA reads

In Porcelana the total richness was represented by a total of 31 taxa (including dominant, minority and rare), independently of the temperature along the gradient.

Bacterial phyla Chloroflexi and Cyanobacteria were the most abundant and active microorganisms in this hot spring (Figure 1a). Chloroflexi showed higher relative abundance (DNA) and activity (cDNA) at the highest temperature (66 °C), decreasing considerably at 48 °C. On the contrary, Cyanobacteria increased their relative abundance at lower temperatures, reaching their maximal abundance and activity (up to 80% of all reads) at 48 °C. At the intermediate temperature of 58 ºC, both phyla were represented in similar amounts both in metagenomic and metatranscriptomic libraries. Members

belonging to phyla Deinococcus-Thermus, Proteobacteria and Bacteroidetes contributed with less than 15% each to the total assigned reads (Figure 1a). The only exception was that of Proteobacteria abundance, that increased as temperature decreased, reaching 30% relative abundance at 48 °C, but their activity remained constant (representing less than 10%) at all temperatures.

The remaining 26 bacterial taxa detected (e.g. Chlorobi, Firmicutes, Planctomycetes, Actinobacteria, etc.) accounted for less than 1% to the total abundance and activity in the mat at all temperatures (Figure S2). However, it comprises a large proportion of the microbial community richness reaching ~80% of total microbial community. There were substantial differences in the representation of these phyla in the DNA and the RNA libraries. For example, Planctomycetes were the most active at 66 ˚C but Chlorobi were more abundant. On the contrary, Chlorobi were the most active at 48 ˚C while Planctomycetes were the most abundant (Supplementary data, Figure S2).

On the other hand, Archaea also was represented in the microbial mat reach only 0.7% to 0.1% from 66 to 48°C. The main phyla were referenced to Thaumarchaeota as dominant in abundance and activity at the three temperatures studied (Figure 1b). However, at 58 °C the dominant relative abundance was shared with the Euryarchaeota, but most of the activity was due to Thaumarchaeota. Finally, <10% of reads were assigned to Crenarchaeota with similar amounts both in metagenomic and metatranscriptomic analyses (Figure 1b).

Most filamentous anoxygenic bacteria (FAPs) of the Phylum Chloroflexi belonged to the Order Chloroflexales. Anaerolineales and Caldilineales were also present but accounted for less that 2% of the reads (Figure 2a). Members of Order Chloroflexales dominated the assemblage at all temperatures studied, contributed more than 90% of the

activity. These Chloroflexales were primarily represented by two genera: *Chloroflexus* sp. and *Roseiflexus* sp., while *Oscillochloris* sp. was present in very low abundance (Figure 2b). *Chloroflexus* sp. increased its abundance from 66 to 58 ˚C while its activity decreased at the lower temperature. *Roseiflexus* sp., in turn, had larger abundance and activity at 48 °C. Surprisingly, *Roseiflexus* sp was not well represented at 58 °C.

The Phylum Cyanobacteria was represented mostly by filamentous non-heterocystous members of subsection III (Order Oscillatoriales), and filamentous heterocystous members of subsections IV (Order Nostocales) and V (Order Stigonematales) (Figure 3a). A higher activity of the Order Stigonematales was found at 48 °C, while at higher temperatures the Order Oscillatoriales was the most active. Stigonematales were largely (>80%) represented by members of the genus *Mastigocladus* sp. (Figure 3b). Considering the total contribution to the communities at the different temperatures, Oscillatoriales were abundant and most active at 66 ˚C (Figure 3a) but the total contribution of Cyanobacteria was relatively small at that temperature (Figure 1a). At the lowest temperatures, in turn, the Cyanobacteria contributed most of the activity (>90%) (Figure 1a) and this was mostly due to Stigonematales and *Mastigocladus* sp. in particular (Figure 3b), even though Nostocales were also abundant (<40%) but with minor activity (<5%) (Figure 3a).

Functional assignment of DNA and cDNA reads

Reads were analyzed with the SEED classification database (Meyer *et al*., 2008). The analysis was followed by normalization (to 100%) of all assigned reads to obtain relative abundances of all metabolisms (Figure 4). Metabolisms that were represented by

366 less than 1%, were grouped together in the category “Others <1%”. The DNA samples
367 from 58 and 66 °C were identical and very similar in cDNA. The 48 °C samples differed
368 more from the other two, especially in the cDNA samples. The most represented
369 metabolisms at all temperatures were carbohydrates (20%), amino acids (13%), cofactors
370 (8%) and proteins (8%). The same metabolisms were also well represented in cDNA
371 samples. A high fraction of the non-rRNA cDNA reads were related to photosynthesis
372 activity. This process was more active at 48 °C, and decreased gradually as temperature
373 increased, reaching an activity of <1% at 66 °C. Similarly, active CO_2 fixation was
374 increasing from 8, 17 and 24% of total reads as the temperature decreased. Other well
375 represented metabolisms were respiration (5%), cell wall (5%) and DNA-RNA
376 metabolisms (5 and 3%, respectively), which showed no significant differences between
377 metagenomes and metatranscriptomes. Nitrogen (2%), phosphorus (1%), iron (1%),
378 fermentation (5%) and sulfur (<1%), were found in similar proportions as those related to
379 stress response (2%), membrane transport (2%) and cell division (2%) at all temperatures
380 (Figure 4).
381
382 ***Attribution of metabolisms to dominant taxa***
383 Energetic metabolisms and principal nutrient cycles are represented in Figure 5,
384 which shows the proportion of reads attributed to the main phyla at the different
385 temperatures, both with DNA and cDNA. The general picture was similar to that shown for
386 the global data in Figure 1: Chloroflexi dominated abundance and activity at the highest
387 temperature, while Cyanobacteria did so at the lowest temperature. But the details of the
388 different metabolism departed from the general pattern in many cases. Just as in Figure 1,
389 Proteobacterial genes became abundant in most metabolisms examined at the lowest

temperature, and there were small contributions from Bacteroidetes and Deinococcus-Thermus to abundance and from the former to activity.

Photosynthesis was mostly due to Cyanobacteria at all temperatures. It must be recalled, however, that the expression of photosynthesis genes at 66 ˚C was very low. As indicated in Table 2, some specific genes were used as indicators of particular metabolisms. Relative abundances of these genes are shown in context in Figure 6. Both PSI and PSII indicator genes, and chlorophyll a/b binding light-harvesting protein PcbD, were basically inactive at 66˚C (grey bars) and significantly expressed at lower temperatures (red and green bars; Figure 6). The abundance of photosystems I (PSI) and II (PSII) total transcripts also showed that the process was essentially not existent at 66 ˚C (Supplementary data, Figure S3), so that, actually most photosynthesis took place at the lower temperatures. *Mastigocladus* sp. was the genus responsible for most of the activity, with a minor contribution of Oscillatoriales members affiliated to the LPP group (*Leptolyngbya* sp., *Plectonema* sp. and *Phormidium* sp.) at 66 °C. Also some reads assigned to anoxygenic photosynthesis represented by *puf*M (photosynthetic reaction center M subunit) gene and *bch*C (bacteriochlorophyll a dehydrogenase) gene were preferentially more actives at 66 °C and 58 °C, with Chloroflexi genera *Chloroflexus* sp. and *Roseiflexus* sp. as the major representatives (see horizontal gray and red bars in Figure 6). As could be expected, the oxygenic photosynthesis was the major light capturing process at low temperatures, being the anoxygenic more important at 66 °C (Figure 6; Supplementary data, Figure S3). At any rate, the contribution of Chloroflexi to photosynthesis at noon was minor compared with the high transcripts of Cyanobacteria at low temperatures (Figure 5).

Alternative energy gathering metabolisms such as respiration and fermentation were mostly due to Chloroflexi at 66 °C and to Cyanobacteria at 48 °C, and both at 58 °C. Proteobacteria and Bacteroidetes genes were present at all temperatures, particularly at the lowest one, but their contribution to activity was very small (Figure 5).

Carbon fixation (CO_2) was found at all temperatures and was associated mainly with Chloroflexi and Cyanobacteria phyla (Figure 5). Moreover, three CO_2 fixation pathways were detected (Figure 7). Calvin-Benson cycle was attributed mostly to Cyanobacteria (*Mastigocladus* sp. and a small proportion of LPP) at all temperatures, correlating with the high *rbc*L (ribulose-bisphosphate carboxylase large chain) gene transcripts at 58 and 48 °C (Figure 7a). Proteobacteria (*Bradyrhizobium* sp. and *Nitrospirilum* sp.) were more active at 66 °C, but this metabolism was minor at this temperature. Second, the 3-hydroxypropionate bicycle characteristic of Chloroflexi was quantitatively important at 66 and 58 °C. Transcripts were mostly assigned to *Chloroflexus* sp. and *Roseiflexus* sp., and to a lesser extent to Orders Flavobacteria and Rhodospirillales at the lowest temperature (Figure 7b). The relatively high transcription levels of *acc*A (acetyl-CoA carboxylase) and *pcc*A (propionyl-CoA carboxylase), demonstrate that CO_2 was actively fixed through this cycle. Several steps involved in this cycle (showen by green arrows in Figure 7b) are shared, with the aerobic carbon fixation by Archaea through the Hydroxypropionate-hydroxybutyrate (HH) cycle. In this way, transcript levels of *paa*F (3-hydroxypropionyl-coenzyme A dehydratase), *pcc*A and *epi* ([*S*] Methylmalonyl-CoA) were associated also with Archaea at all temperatures. The second cycle, however, has several steps that are exclusively archaeal (yellow arrows in Figure 7b). Genes such as *suc*D (succinyl-CoA), *abf*D (4-hydroxybutyryl-CoA dehydratase), *crt* (crotonyl-CoA reductase), *hbd* ([*S*] 3-hydroxybutyryl-CoA) and *ato*B (Acetoacetyl-CoA), were

assigned to Thaumarchaeota at all temperatures (Figure 7b; Supplementary data, Figure S4).

Once more, due to the few total reads for CO_2 fixation pathways at the highest temperature attributed to Chloroflexi and Thaumarchaeota, most autotrophic CO_2 fixation could just be attributed to Cyanobacteria at the lower temperatures and, more specifically, to the genus *Mastigocladus* sp.

Pathways for acquisition of nitrogen, phosphorus, sulfur and iron followed the same general pattern (Figure 5), with Chloroflexi dominating abundance and activity at 66 ˚C and Cyanobacteria dominating activity (but not so much abundance) at 48 °C. An intriguing departure from this pattern was the relatively large contribution to iron uptake activity by Bacteroidetes at 66 °C. Since nitrogen is the most common limiting nutrient in natural systems as microbial mats (Alcamán *et al*., 2015), we dedicated special attention to the genes involved in nitrogen transformations (Figure 8).

As with most metabolic pathways, ammonia and nitrate assimilation were due to *Chloroflexus* sp. and *Roseiflexus* sp. at the highest temperature and to *Mastigocladus* sp. at the lower ones (Fig 8b). Ammonia assimilation was active at all temperatures but decreased with the temperature, with the Order Rhodospirillales (Proteobacteria) and the genus *Rhodothermus* sp. (Bacteroidetes) as minor contributers. Nitrate assimilation was active at all temperatures, but more so at 58 and 48 °C (Supplementary data, Figure S5) according to the high account for *nar*B and *nir*A gene transcripts and with low contribution associated to *Nitrosospira* sp. (Proteobacteria) and *Sphingobacterium* sp. (Bacteroidetes) (Figure 8b). The other nitrogen acquisition pathway, nitrogen fixation, was entirely due to Cyanobacteria, particularly *Mastigocladus* sp. and, accordingly, the largest activity of the

460 *nif*H gene was registered at the lowest temperature, and there was no activity at 66 °C
461 (Figure 8; Supplementary data, Figure S4).

462 In effect, the *amo*A gene (indicative of ammonia oxidation) was present at the three
463 temperatures, although it was active mostly at 66 °C (Figure 8a), and all the transcripts of
464 this gene were taxonomically assigned to Thaumarchaeota, particularly to *Nitrososphaera*
465 sp. relatives (Figure 8b). In effect, the genus *Nitrososphaera* was the dominant
466 Thaumarchaeota at all temperatures (Supplementary data, Figure S6). Interestingly, the
467 dominance and activity of *Nitrososphaera* sp. decreased with decreasing temperature, but
468 other Thaumarchaeota such as *Nitrosopumilus* sp. and *Candidatus* Nitrosoarchaeum
469 increased their relative abundances and activities along the gradient (Supplementary data,
470 Figure S6).

471 The two dissimilatory processes, denitrification (represented by *nir*K, *nor*B and
472 *nos*Z gene transcripts) and DNRA (represented by *nar*G and *nir*B gene transcripts) were
473 found at all temperatures, with *nos*Z gene (denitrification) more active at 66 °C compared
474 to the other genes and temperatures (Figure 8a, b), and most of the activity could be
475 attributed to genera *Rhodothermus* sp., *Flavobacterium* sp. (Bacteroidetes) and
476 *Burkholderia* sp. (Proteobacteria). At the lower temperatures activity was also lower (~1%)
477 and Cyanobacteria (*Leptolyngbya* sp. and *Mastigocladus* sp.; associated to *nor*B gene)
478 were responsible in <45% of the total denitrification, while Proteobacteria (*Burkholderia*
479 sp.) contributed significantly to DNRA (>90%) at high temperatures (Figure 8a, b).

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481

482

Carbon and nitrogen assimilation rates

In situ $H^{13}CO_3$ assimilation rates were 26.9±6.08 nmoles C cm^{-2} h^{-1} and 33.5±7.62 nmoles C cm^{-2} h^{-1} at 58 and 48 °C, respectively (Table 3). Ammonia assimilation rates varied from 0.015±0.004 nmoles N cm^{-2} h^{-1} at 58 °C to 0.031±0.004 nmoles N cm^{-2} h^{-1} at 48 °C, whereas nitrate uptake (ρNO_3) was generally similar to ammonium uptake (Table 3). Daily carbon assimilation rates were 323±73.0 nmoles C cm^{-2} d^{-1} at 58 °C and 402±91.5 nmoles C cm^{-2} d^{-1} at 48 °C. In turn, daily rates for ammonium and nitrate were 0.180±0.043 and 0.142±0.109 nmoles N cm^{-2} d^{-1} at 58 °C, and at 48 °C were 0.371±0.048 and 0.107±0.009 nmoles N cm^{-2} d^{-1}, respectively.

Considering the natural concentrations of ammonia and nitrate (0.04 and 3.2 μM, respectively) reported in Porcelana by Alcamán *et al*. (2015) and the potential rates of ρNH_4 and ρNO_3 mentioned above, the turnover of each nutrient was calculated at each temperature (Table 3; 0.69 h at 58 °C and of 1.69 h at 48 °C). The turnover of NO_3^- was slower than that for ammonium, with values of 34.3 h at 58 °C and 11.5 h at 48 °C. The contribution of ρNH4 to daily primary production (on the basis of C), was 0.71% at 58 °C and 0.91% at 48 °C, and the contribution of nitrate reached 0.50 % and 0.36% at 58 and 48 °C, respectively.

Discussion

Metagenomic and metatranscriptomic approaches have been useful tools to analyze in depth the microbial composition of hot springs, revealing even less abundant microorganisms and their *in situ* functions in hot springs (van der Meer *et al*., 2005; Bhaya *et al*., 2007; Inskeep *et al*., 2010; Klatt *et al*., 2011, 2013; López-López *et al*., 2013). The

most extensive metagenomic studies of hot spring microbial mats have been carried out in Yellowstone National Park (Wyoming, USA). There are a variety of spring types, including those dominated by chemotrophs such as Aquificales or Archaea (Inskeep *et al*., 2013), and phototrophic mats (Klatt *et al*., 2011, 2013). The latter, in particular, have been studied for many years, becoming the gold standard of phototrophic hot microbial mats. However, there is still poor and many cases contradictory knowledge related to taxa composition and *in situ* activities on specific metabolisms such as photosynthesis, carbon and nitrogen metabolisms in thermal mats (Klatt *et al*., 2013; Liu *et al*., 2011; Steunou *et al*., 2008; Klatt *et al*., 2013).

The Porcelana mats are similar in pH (slightly alkaline) and temperature range (70 to 40 °C) to those from phototrophic mats in Yellowstone. However, they are in another hemisphere at thousands of kilometers of distance. Thus, it was of interest to compare the communities and activities associated between mats in the two areas.

Microbial community mat composition in Porcelana

Temperature is one of the most important environmental factors affecting microbial community composition in hot springs (Miller *et al*., 2009; Everroad *et al*., 2012, Cole *et al*., 2013, Wang *et al*., 2013), where the complexity of the microbial mat community increases with decreases in the temperature, as already described in Porcelana hot spring (Mackenzie *et al*., 2013). Such previous studies, however, lacked information regarding the complete taxonomic community structure and function.

In the present study we confirmed the dominance and higher activity of the phyla Cyanobacteria and Chloroflexi with smaller representation/contribution of phyla Deinococcus-Thermus, Proteobacteria and Bacteroidetes. This composition was similar to

that found at other hot springs such as the alkaline Mushroom hot spring in Yellowstone National Park (USA) (Miller *et al*., 2009; Klatt *et al*., 2013, Liu *et al*., 2011). Cyanobacteria and Chloroflexi showed opposing trends in abundance and activity along the temperature gradient. As was the case in Yellowstone, *Roseiflexus* was more important than *Chloroflexus*, both in abundance and transcripts, and its activity were maximal at the lower temperature of 48 °C. Cyanobacteria (Stigonematales) were responsible for a large fraction of the metabolic activity, especially at 58 and 48 °C. These temperatures have been commonly accepted as optimal temperature for growth of thermotolerant cyanobacteria, both *in situ* and in culture (Miller *et al*., 2006; Alcamán *et al*., 2015; Finsinger *et al*., 2008). For instance, the high temperature tolerance of Oscillatoriales (that were abundant at 66 and 58 °C in Porcelana) has been previously demonstrated in other hot springs (Sompong *et al*., 2005)..

In many extreme environments, it is known that diversity tends to be lower at high temperatures (Brock, 1978). In Porcelana the detection and subsequent analysis of the organisms agruped in the <1% (26 of 31 total taxa; ~80%) have demonstrated high richness of these taxa with potential importance for the system. Therefore, some members of Bacteria and Archaea neglected potential relevance in hot springs are now revealed by these methodologies. For instance, Firmicutes, reported here to reach <1% of the total community as in Mushroom hot spring (Liu *et al*., 2011), point them as one of the decomposers into the mat. Archaea which are key players in acidic hot spring increasing at higher temperature and pH (Huang *et al*., 2013), are usually less represented in neutral hot springs (Huang *et al*., 2011; Wang *et al*., 2013), however in Porcelana their abundance reached <10% of total community. In particular, Thaumarchaeota reached ~0.7% and 0.1%

553 of relative abundance at lower and higher temperatures, respectively, revealing major
554 activity at high temperature.
555
556 ***Microbial phototrophy in Porcelana***
557 In slightly alkaline springs such as Octopus and Mushroom in YNP mats show a
558 succession of dominant phototrophs from high to low temperatures. The unicellular
559 cyanobacterium *Synechococcus* accompanied by Chloroflexi filaments predominate at
560 temperatures between 73 and 60 °C forming green-orange mats (van der Meer *et al*.,
561 2005), whereas between 60 and 30 °C phototrophy is due to filamentous cyanobacteria, in
562 particular *Mastigocladus* and *Phormidium*-like, as well as some eukaryotic algae.
563 Photosynthesis was detected by transcript levels of *psa*B (PSI reaction center protein) and
564 *psb*B (PSII chlorophyll binding protein) genes, and was attributed to *Synechococcus* sp.
565 (Steunou *et al*., 2008; Liu *et al*., 2011). One first and obvious difference between Porcelana
566 and the YNP and other hot spring studies (Sompong *et al*., 2005) was that *Synechococcus*
567 was absent and only filamentous and particularly the Stigonematal *Mastigocladus* are the
568 cyanobacteria responsible for oxygenic photosynthesis in the mats (Mackenzie *et al*., 2013;
569 Alcamán *et al*., 2015). Although *puf*M (photosynthetic reaction center M subunit) gene
570 transcripts involved in anoxygenic photosynthesis were detected and attributed to
571 *Chloroflexus* sp. and *Roseiflexus* sp. at all temperatures (mostly at 66 and 58°C), they were
572 very low in comparison to those related to oxygenic photosynthesis. This was probably due
573 to the fact that the DNA/cDNA material was obtained from high illuminated and oxygen
574 saturated microbial mats at noon. In this sense, Chloroflexales (both *Chloroflexus* and
575 *Roseiflexus*) must have been essentially photoheterotrophic in Porcelana as also found in
576 some Yellowstone studies (van der Meer et al., 2005). In some sulfide rich (Mammoth

Springs, YNP) (Giovannoni *et al*., 1987) and alkaline (van der Meer *et al*., 2003; Strauss & Fuchs, 1993; Zarzycki *et al*., 2009) hot springs, *Chloroflexus* sp. has been described as mixotrophic, as opposed to *Roseiflexus* sp. who cannot grow photoautotrophically (Hanada *et al*., 2002; Madigan *et al*., 2005). This could explain the distribution of abundance and activity of this bacterium in the Porcelana: *Roseiflexus* sp. might need the cyanobacterial excretion products to grow (van der Meer *et al*., 2005). Thus, in Porcelana the dominance of *Roseiflexus* sp. at the lower temperature of 48 ºC can be correlated with the higher abundance of Cyanobacteria. Moreover, metatranscriptome analysis in Mushroom hot spring mat (obtained at hourly intervals during 24 hours) revealed that chlorophototrophic members of the Chloroflexi phylum transcribed several photosynthesis related genes (*puf*M) during the night (Klatt *et al*., 2013). So, an additional analysis of the night activity of the Porcelana microbial mat is needed now to determine the real contribution of this group to the process.

Another difference with YNP was the absence of *Candidatus* Chloracidobacterium thermophilum and phototrophic Anaerolineae sequences. The case of the Anaerolineae is particularly interesting, since the members of this group in pure culture are not phototrophs but phototrophic genes were found in YNP. We only found a few reads of Chlorobiaceae corresponding to Bchl c synthesis genes, and some *puf*M genes related to the alpha proteobacterium *Elioraea tepidiphila*. The original strain of this bacterium was isolated from a microbial mat in the Azores with optimal temperature for growth between 45 and 50 ˚C, and a pH optimum between 8.0 and 8.5. These parameters fit every well with those of Porcelana, where the *puf*M gene was most abundant and expressed at 48 ˚C. The original strain, however, did not have the Bchl a or *puf* genes.

In summary, phototrophy in Porcelana was essentially due to oxygenic photosynthesis by *Mastigocladus* cyanobacteria at the lower temperatures. Only marginal activity of anoxygenic photorophy by Chloroflexi and *Elioraea tepidiphila* was detected at all temperatures.

Microbial contribution to CO_2 fixation in Porcelana

Inorganic carbon fixation rates amounted to 16 g C m^{-2} y^{-1} on average. These rates are similar to those measured in previous years (Alcamán et al. 2015). This CO_2 fixation could be attributed to three different carbon fixation pathways. Most of it was done through the Calvin cycle by the cyanobacterium *Mastigocladus* and most of this activity occurred at 58 and 48 °C. Interestingly, there was some contribution of alpha-Proteobacteria at 66 °C but total activity was very low at this temperature. Another portion of carbon fixation was through the 3-Hydroxypropionate bicycle. This was most active at 66 and 58 ˚C, just the opposite of the Calvin cycle. And it was almost entirely due to Chloroflexi. Only at 48 °C, when this pathway was less active, there was a contribution from Flavobacteria and Rhodospirillaceae. Finally, the Hydroxypropionate-hydroxybutyrate cycle was also detected and could be attributed entirely to Thaumarchaeota. *Nitrososphaera* was the most important both in abundance and activity at all temperatures, while *Nitrosopumilius* and *Candidatus* Nitrosoarchaeum had very small contributions. This pathway had similar levels of activity at the three temperatures analyzed. Thaumarchaeota have been previously found in hot springs (Berg *et al*., 2010) and, for instance the thermophilic Thaumarchaeota *Nitrosophaera* sp. has been reported to have potential to carry out CO_2 fixation under experimental conditions (Hatzenpichler *et al*., 2008).

At noon most autotrophy could be attributed to the Cyanobacteria, while FAPs and Archaea might be responsible for the activity at sunrise (Meer *et al*. 2005; Stetter, 1988). van der Meer *et al*. (2005), suggested that FAPs have potential for photoautotrophic metabolism during low-light periods, in contrast to Cyanobacteria that incorporate inorganic carbon during high-light periods. In addition, it has also been described that some Archaea are known anaerobic sulfate-reducing carbon fixers during the night or under low oxygen saturation (Stetter, 1988). Therefore, it would be of interest in the future to investigate the contribution of FAPs and Archaea when the mat is completely depleted of oxygen at night in order to fully understand carbon metabolism in this system.

It has been suggested that Cyanobacteria can provide organic carbon sources to heterotrophic bacteria and archaea in the mat (van der Meer *et al*., 2005, 2007; Bateson & Ward 1988; Zarzycki & Fuchs, 2011; Bryant *et al*., 2011). Such compounds can be used under heterotrophic conditions by some Chloroflexi members (Ward *et al*., 1987; Strauss & Fuchs, 1993; Zarzycki *et al*., 2009). In this case, Cyanobacteria and Chloroflexi would avoid competition for light. The two groups can also avoid competition by carrying our photosynthesis at different times of the day.

Other heterotrophic bacteria in the mat were Proteobacteria and Bacteroidetes, both of which have been recorded in other hot springs (Cole et al. 2013, Harris *et al*., 2013). The co-occurrence of Cyanobacteria and Proteobacteria has also been shown in hypersaline microbial mats (Jonkers & Abed, 2003), where aerobic heterotrophic Proteobacteria can mineralize photosynthetic excretion products (such as glycolate) derived from Cyanobacteria, to grow.

Microbial contribution to N biogeochemical cycle

Ammonia and nitrate assimilation genes were actively transcribed, and the main components of the mats, Cyanobacteria and Chloroflexi, expressed genes for both processes. Obviously, Chloroflexi contributed more at 66 ˚C and Cyanobacteria at the lower temperatures. Ammonia assimilation was more important than nitrate assimilation at the highest temperature and this was the other way around at the lower temperature. However, both ammonia and nitrate assimilation rates as determined by stable isotopes were relatively low. In the case of Cyanobacteria, this low uptake of ammonia and nitrate could be compensated by nitrogen fixation. In effect, the large numbers of transcript of *nif*H gene recorded at 58 and 48 ºC were attributed to the cyanobacterium *Mastigocladus* sp., and was consistent with high nitrogen fixation reported previously in this hot spring (Alcamán *et al*., 2015). However, no alternative is apparent for Chloroflexi.

Besides assimilation, ammonia oxidation also contributed to decrease the ammonia concentrations in situ, which were relatively low. The *amo*A gene was expressed especially at 66 ˚C and could be assigned to the Taumarchaeota genus *Nitrososphaera*. This thermophilic microorganism was reported to grow under low ammonia concentrations (Hatzenpichler *et al*., 2008), such as those found in Porcelana.

The widespread distribution of putative archaeal ammonia monooxygenase (*amo*) genes and their numerical dominance over their bacterial counterparts in most marine and terrestrial environments suggest that ammonia oxidizing archaea (AOA) play a major role in global nitrification (Francis *et al*., 2005; Zhang *et al*., 2008). Dodsworth *et al*. (2011) in Great Boiling Spring (GBS) and Sandy's Spring West (SSW) (US Great Basin), found the autotrophic ammonia-oxidizing archaeon *Candidatus* Nitrosocaldus yellowstonii to be abundant, postulating ammonia oxidation as a major source of energy fuelling primary

production in these two environments. The high numbers of transcripts of *Nitrososphaera* sp. *amo*A genes registered at all temperatures in Porcelana strongly suggests that ammonia can be rapidly oxidized by these organisms.

The two known denitrification mechanisms were active active at all temperatures, but denitrification was particularly active at 66 °C and primarily attributed to heterotrophic organisms whose function occurs under low oxygen conditions (Zumft, 1997). For the DNRA process some ammonia-oxidizing bacteria (AOB) such as Nitrospira sp., have been reported to carry out both nitrification and denitrification under aerobic conditions (Shaw et al., 2006), as we found in Porcelana. As could be expected, several different microorganisms were involved in these processes, including the Cyanobacteria.

Anammox could be expected to be present in Porcelana according to the few gene transcripts of nitrite reductase enzyme associated to Planctomycetes. However, the absence of the enzyme hydrazine synthase (hzsA gene) in our data and the low activity of this phylum (only reached <1% of activity) at high temperatures, prevent determining the relevance of this process in Porcelana. Again, further studies in Porcelana mats at night when oxygen is depleted, are needed to understand the relevance of this and other anoxygenic processes.

Conclusions

Through metagenomic and metatranscriptomic analysis and *in situ* isotopic experiments, diversity and activities of Porcelana microbial mat at noon were unveiled. Chloroflexi and Cyanobacteria dominated at all temperatures, suggesting potential competition of these two groups of organisms for physical space, nutrient limitation or difference in their temperature for optimal growth. Metabolisms such as photosynthesis

and CO_2 fixation were mainly due to Cyanobacteria, Chloroflexi and Thaumarchaeota activities, revealing the presence of different ways of autotrophic carbon fixation. Thaumarchaeota was the principal player in ammonia oxidation at high temperatures. Also, ammonia and nitrate were actively assimilated at all temperatures mainly by Cyanobacteria and Chloroflexi. Nitrogen fixation by *Mastigocladus* spp. was the major process in the contribution of new nitrogen at the lower temperatures. Active genes involved in denitrification pathways were important nitrogen loss routes at higher temperatures. Finally, we can conclude that the Porcelana microbial mat is a photosynthetic and diazotrophic system that is modulated taxonomically by temperature.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary information is available at ISME J's website

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Titles and legends to Tales and figures

Table 1. Results of high throughput sequencing: raw reads, quality reads, percent of reads corresponding to ribosomal RNA, Non ribosomal genes, non-redundant hits and percent of the latter that could be taxonomically assigned. Numbers of sequences are shown in millions.

Sample	Raw Reads	High quality Reads	rRNA Reads (%)	Non-rRNA Reads	NR hits	Taxonomically-assigned (%)
48-DNA	168.4	141.0	0.2	140.7	23.7	72.8
48-RNA	15.5	13.4	5.7	12.7	4.7	80.7
58-DNA	70.6	62.0	0.2	61.8	21.3	82.6
58-RNA	38.5	33.6	4.5	32.1	15.2	89.4
66-DNA	10.5	10.4	2.2	10.2	4.5	84.5
66-RNA	149.2	134.3	10.1	120.6	36.6	19.8
Total	452.7	394.7	4.2	378.2	105.9	59.7

Table 2. Main pathways analyzed and genes used for photosynthesis, CO_2 fixation and nitrogen cycle transformations.

Process and pathways	genes
Oxygenic photosynthesis	
PII	*psb*A; *psb*D
PI	*psa*A; *psa*B
Chlorophyll	*pcb*D
Anoxygenic photosynthesis	
RC	*puf*M
Bacteriochlorophyll	*bch*C
Calvin Cycle	*rbc*L
3-Hydroxypropionate autotrophic pathway	*acc*A; *paa*F;*acd*; *pcc*A; *epi*; *mut*; *smt*A; *sdh*A
Hydroxypropionate-hydroxybutyrate	*ato*B*; hbd; crt; abfD; suc*D
Nitrogen cycle	
Nitrate reduction	*nar*B; *nir*A
Nitrite reduction/DNRA	*nar*G; *nir*B
Nitrogen fixation	*nif*H
Ammonia oxidation	*amo*A
Ammonia assimilation	*gln*A
Denitrification	*nir*K*; nor*B; *nos*Z

Table 3. Carbon ($\rho DI^{13}C$), ammonia (ρNH_4) and nitrate (ρNO_3) assimilation rates at noon in Porcelana Hot Spring.

	Mean values $\rho^{15}NH_4$	Mean values $\rho^{15}NO_3$	Mean values $\rho DI^{13}C$	Mean values $\rho^{15}NH_4$	Mean values $\rho^{15}NO_3$	Mean values $\rho DI^{13}C$	Turnover time NH_4^+	Turnover time NO_3^-
T °C	nmoles cm^{-2} h^{-1}	nmoles cm^{-2} h^{-1}	nmoles cm^{-2} h^{-1}	nmoles cm^{-2} d^{-1}	nmoles $cm^{-2}d^{-1}$	nmoles $cm^{-2}d^{-1}$	hours	
58	0.015 ±0.004	0.012 ±0.009	26.9 ±6.08	0.180 ±0.043	0.142 ±0.109	323 ±73.0	0.69	34.3
48	0.031 ±0.004	0.009 ±0.0008	33.5 ±7.62	0.371 ±0.048	0.107 ±0.009	402 ±91.5	1.69	11.5

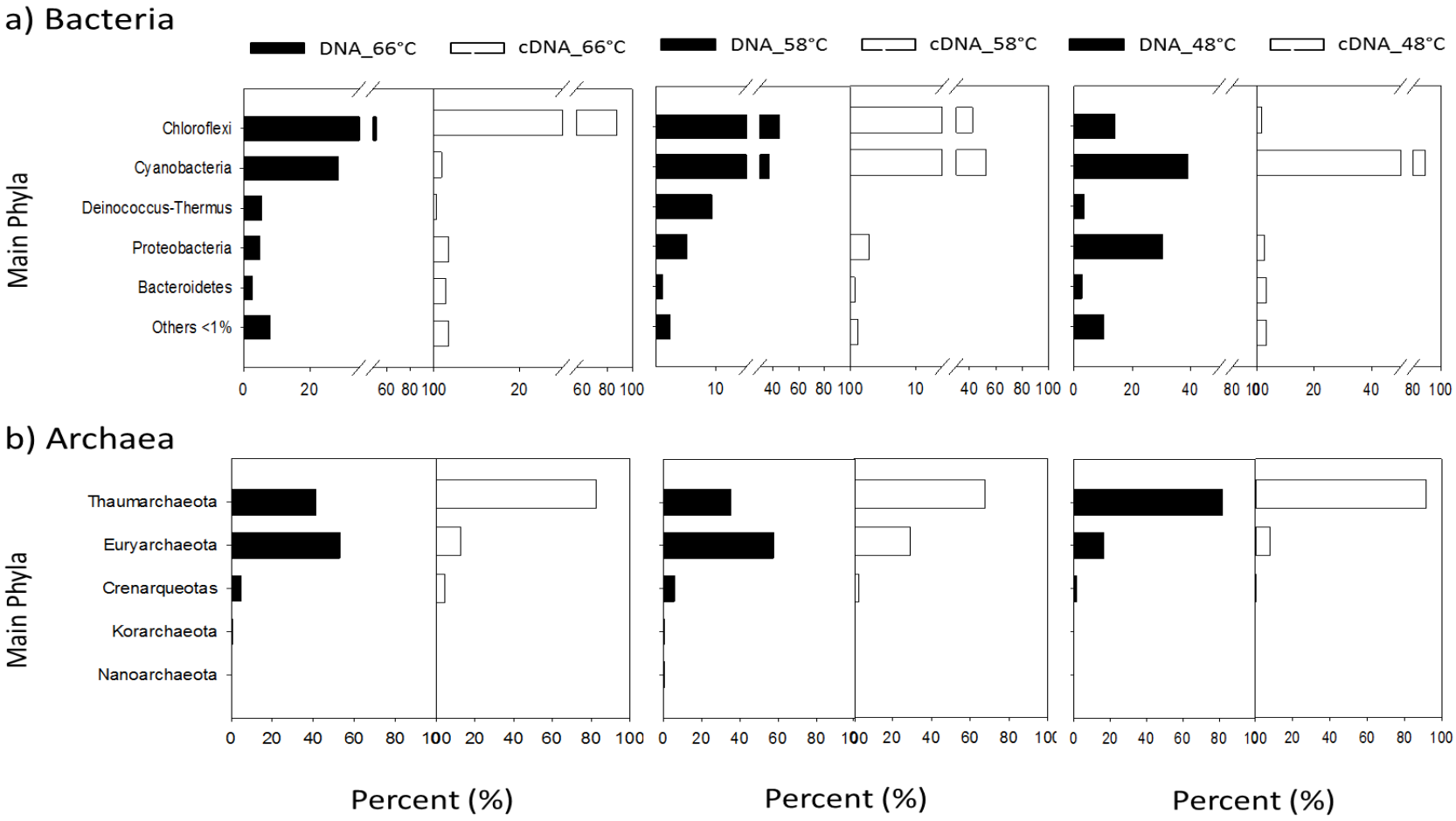


Figure 1. Taxonomic assignment at the Phylum level of metagenomic (DNA; black bars) and metatranscriptomic (cDNA; white bars) to Bacteria and Archaea reads at the three temperatures studied.

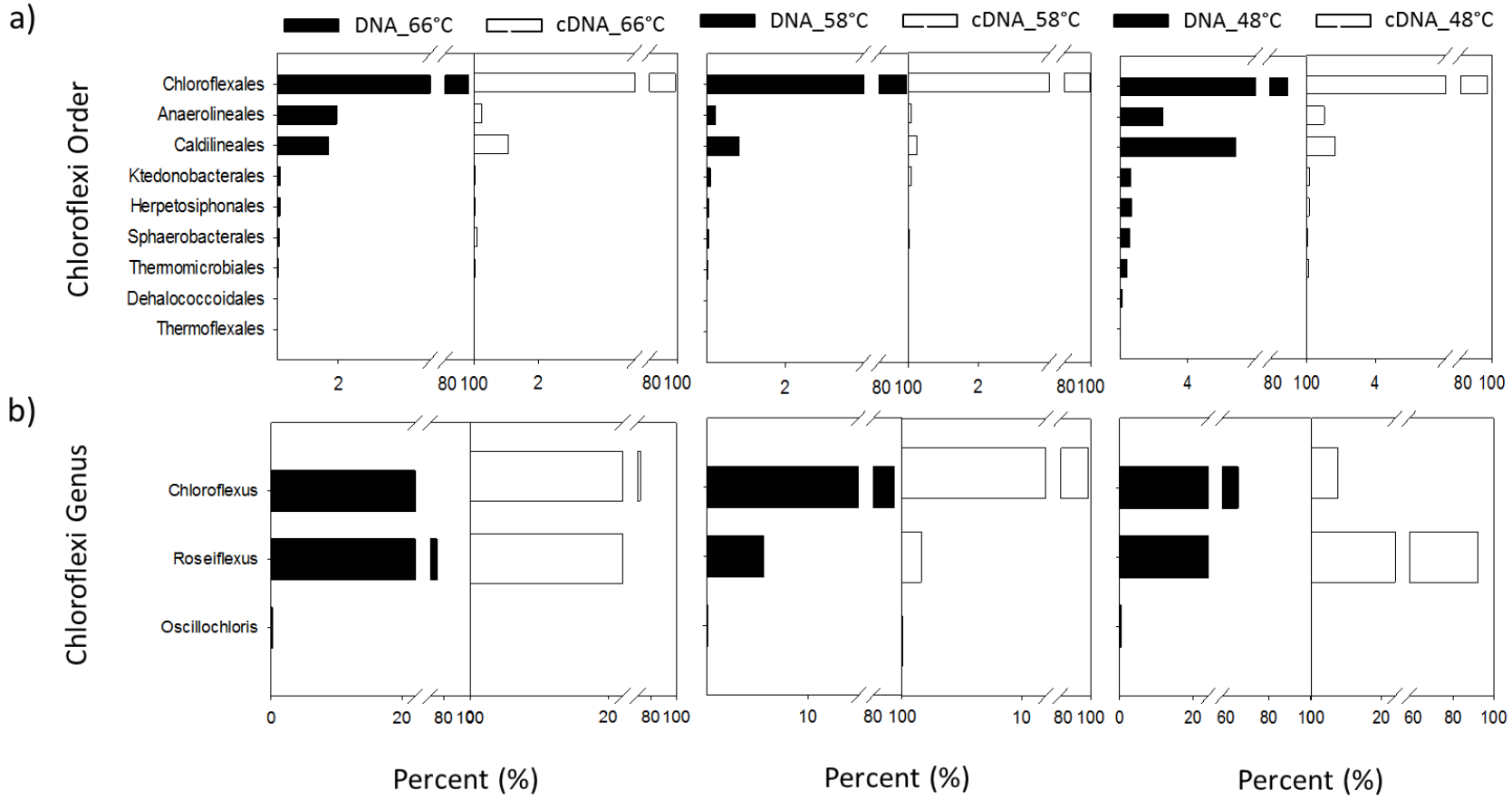


Figure 2. Taxonomic assignment of Chloroflexi at the Order (a) and genus (b) levels of metagenomic (DNA) and metatranscriptomic (cDNA) reads at the three temperatures studied.

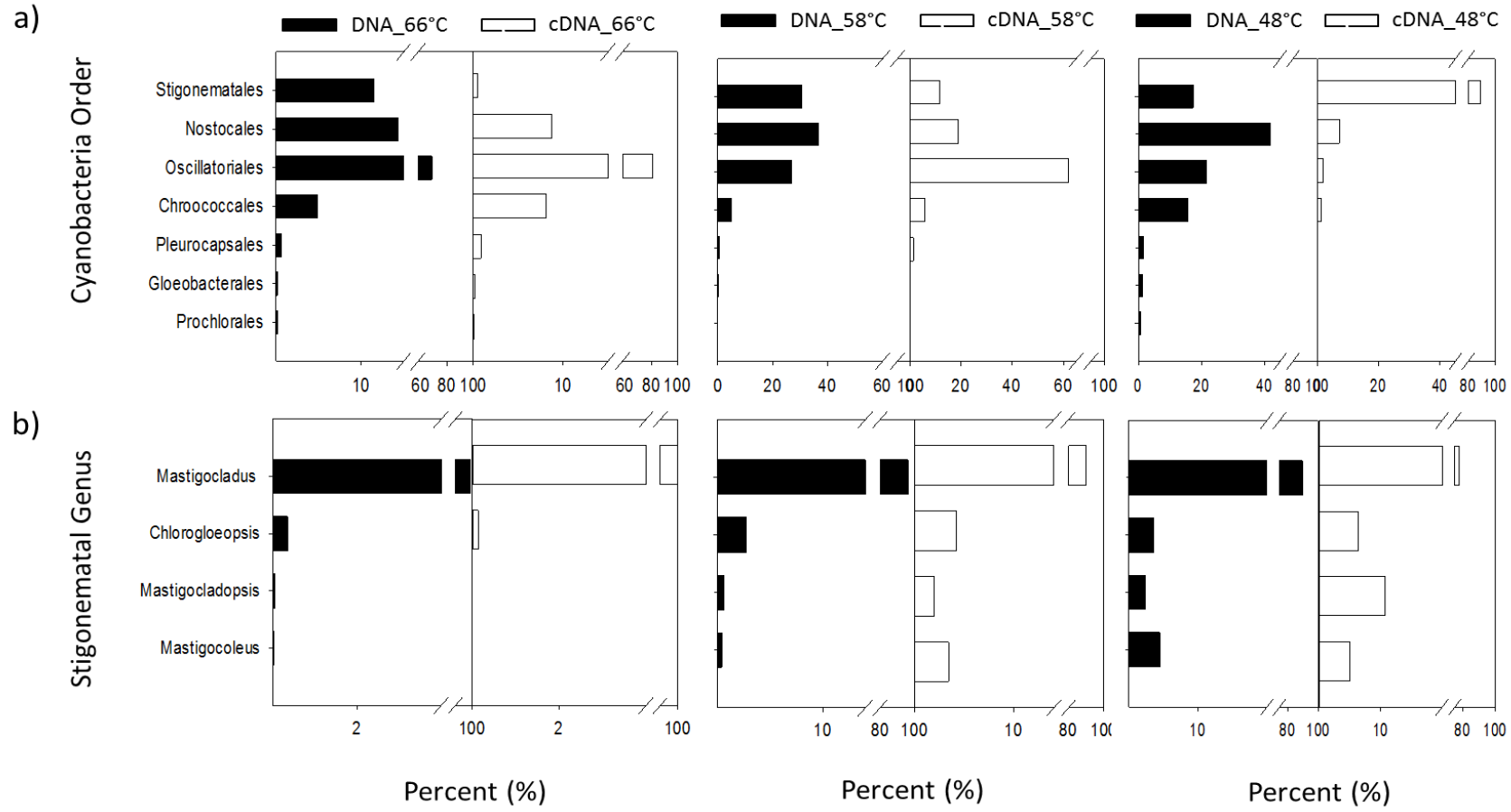


Figure 3. Taxonomic assignment of Cyanobacteria at the Order (a) and genus (b) levels of metagenomic (DNA) and metatranscriptomic (cDNA) reads at the three temperatures studied.

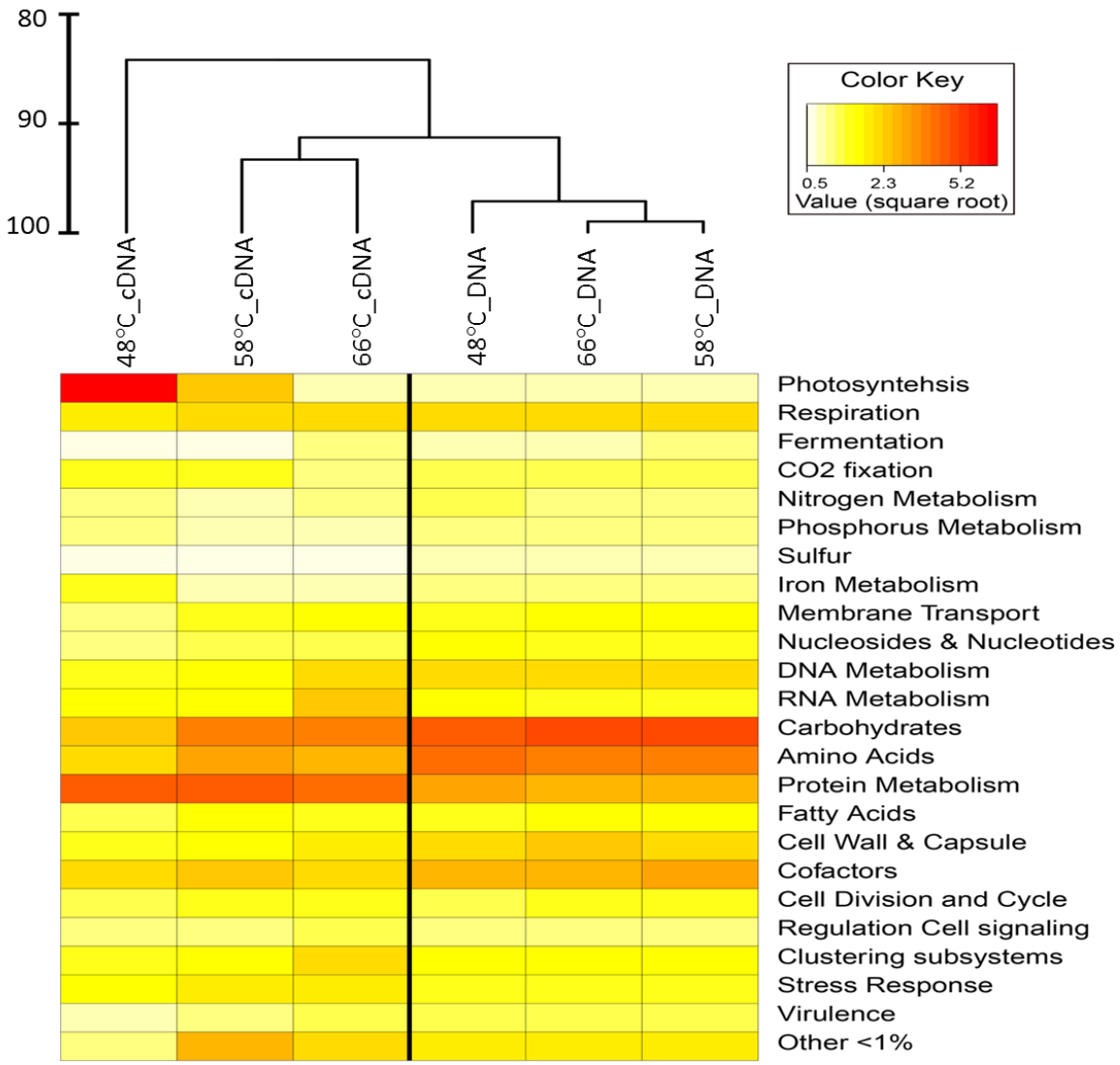


Figure 4. Clustering of DNA and cDNA samples representing the global metabolisms recorded and actives across the temperature gradient in Porcelana hot spring.

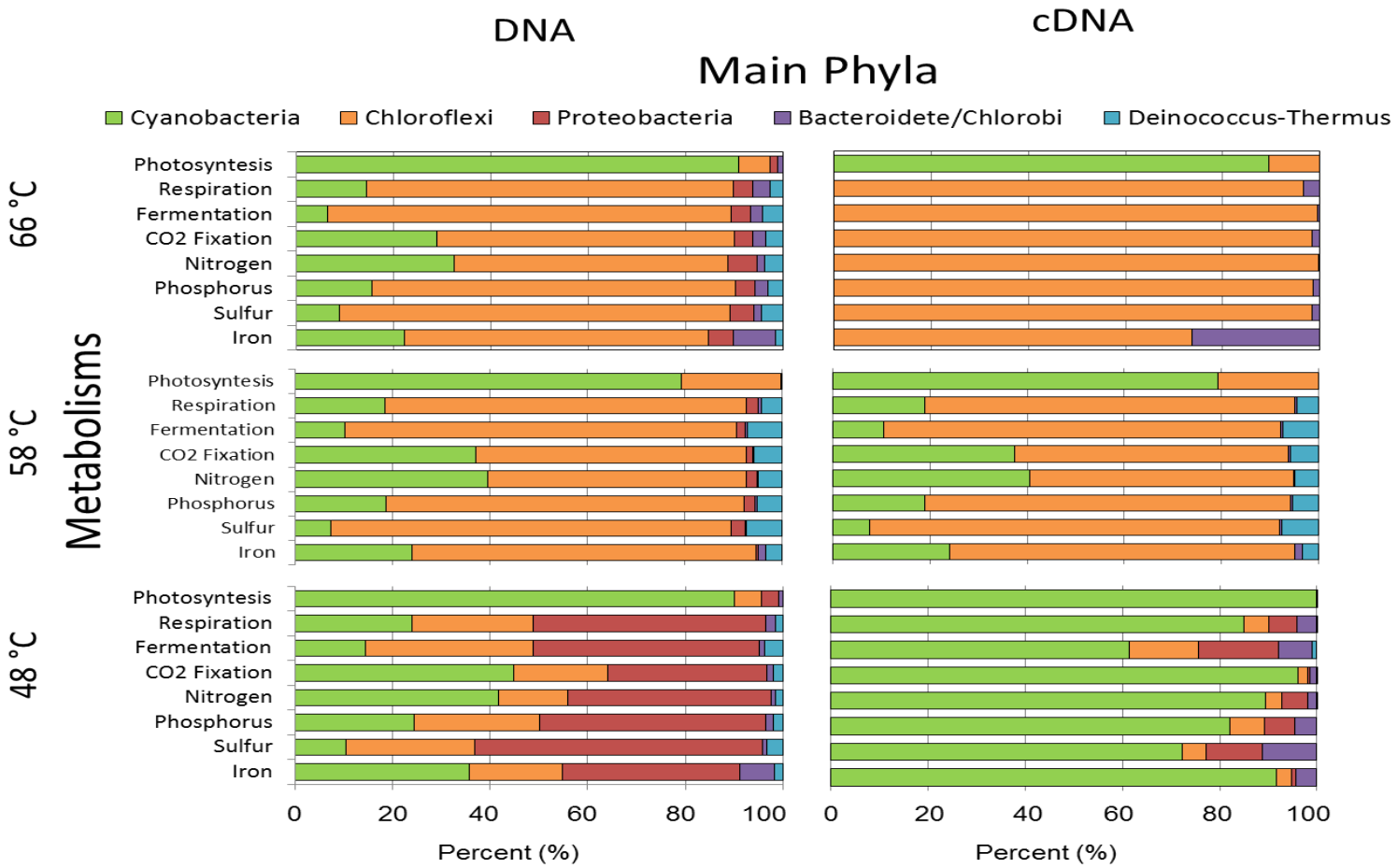


Figure 5. Functional assignment of metagenomic (DNA) and metatranscriptomic (cDNA) reads, for each main Phylum, at the three temperatures studied. Energy conservation (photosynthesis, respiration, and fermentation), CO_2 fixation, and nutrient acquisition pathways have been selected.

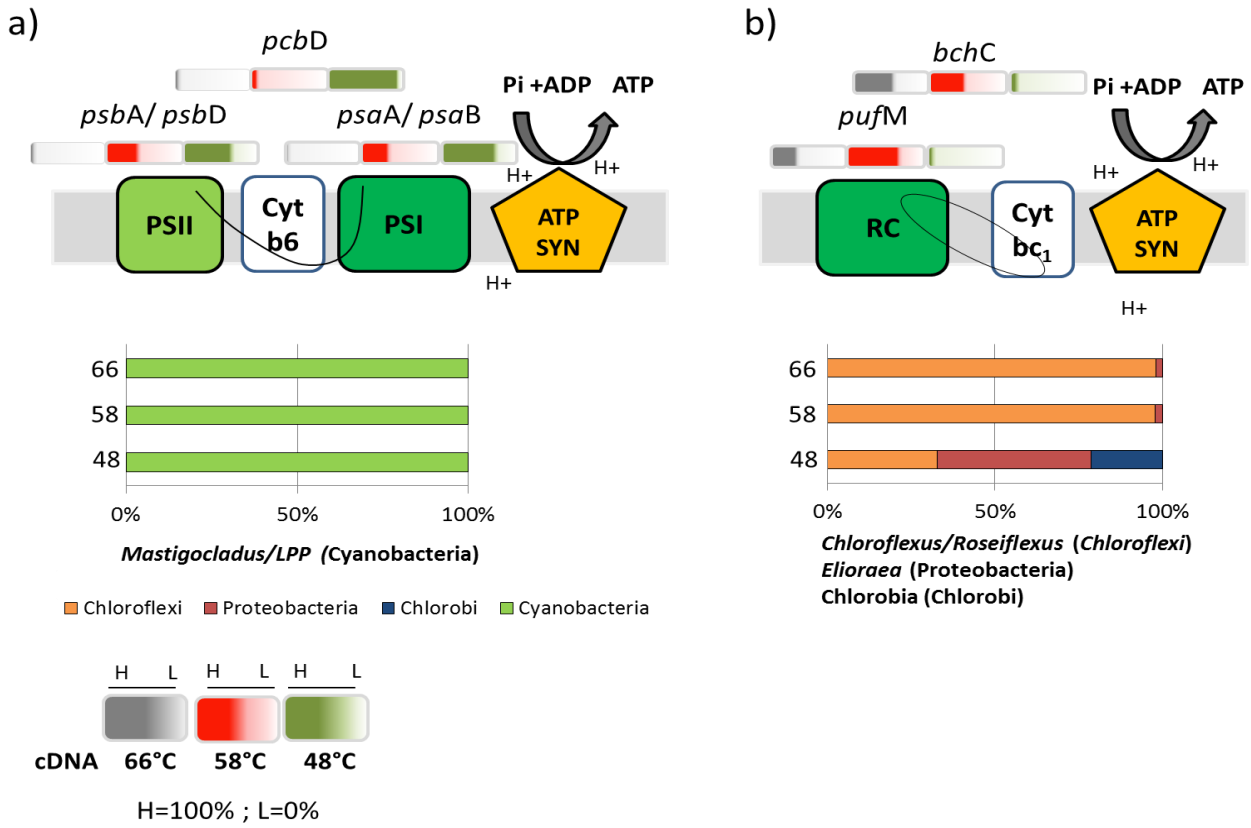


Figure 6. Main photosynthesis components and ATPase enzyme. In all cases, the percent of relative abundance of cDNA reads is shown for each gene by small rectangles at 66 (gray), 58 (red) and 48 ˚C (green). The percent contribution of the three main Phyla to expression for each gene is shown by the horizontal bars. The most important genera in each case are noted to the right.

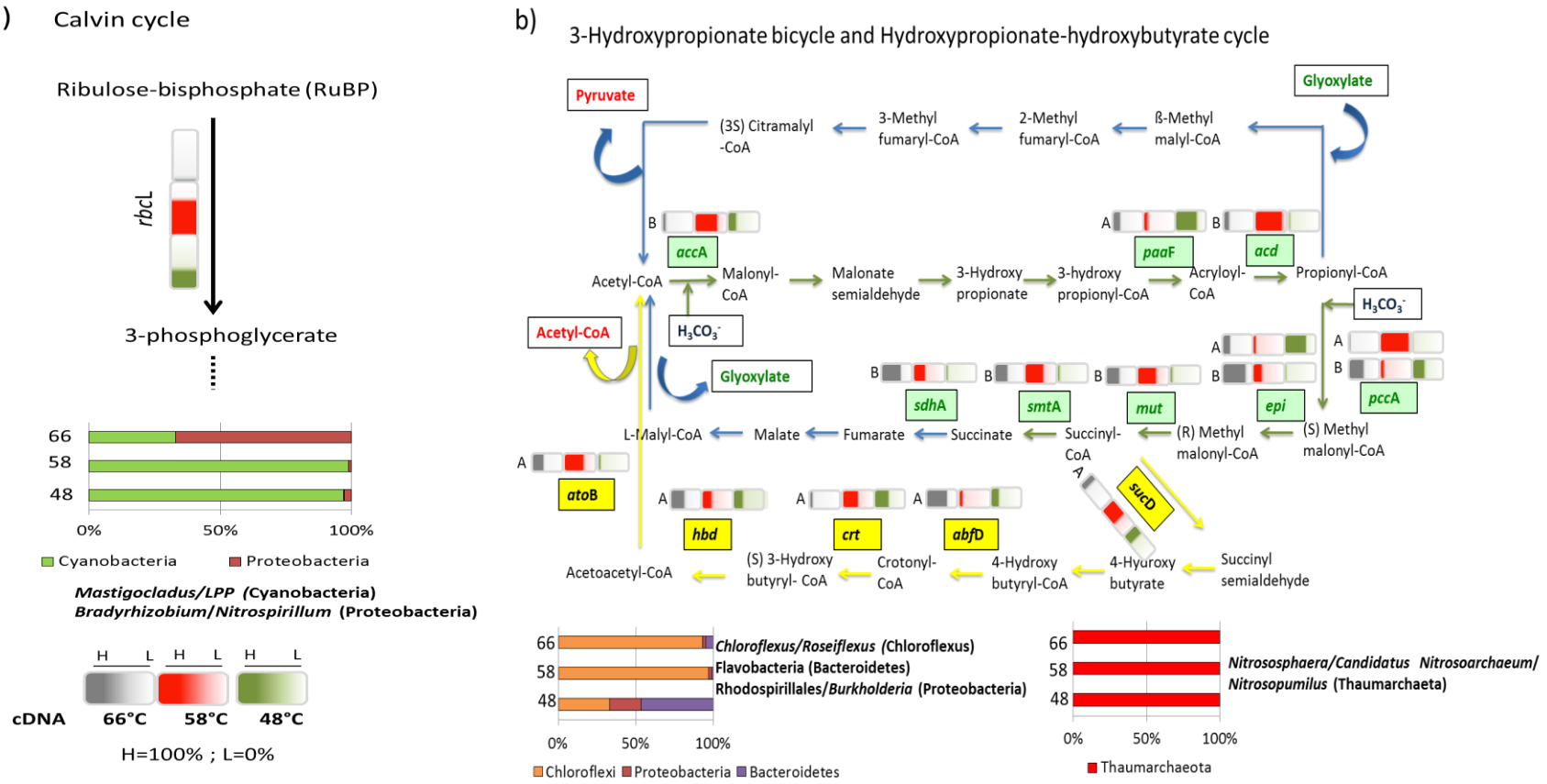


Figure 7. Autotrophic carbon fixation: a) Calvin cycle; b) 3-hydroxypropionate bicycle representated by blue arrows, and Hydroxypropionate-hydroxybutyrate cycle represented by yellow arrows. Green arrows represent the shared route between Bacteria and Archaea. The horizontal histograms represent the gene transcripts associated to taxonomical phyla at the different temperatures. The grey (66 °C), red (58 °C) and green (48 °C) bars represented the percent (%) of transcripts number of specific gene at each temperature; the letter A (in front bars) represents Archaea, and B to Bacteria.

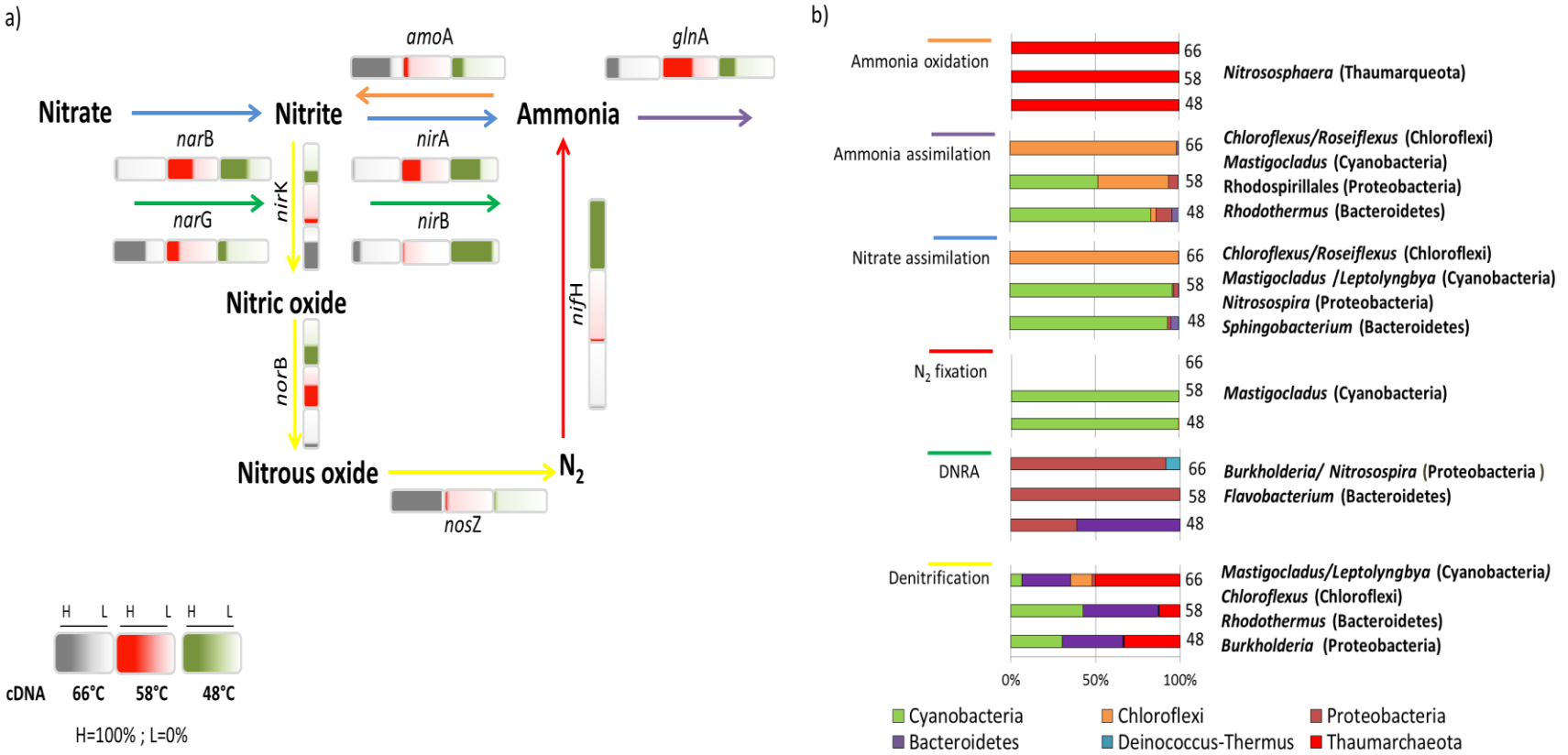


Figure 8. Nitrogen cycle transformations. a) Scheme of the reactions, representative genes and relative abundance of cDNA reads for each gene shown by small rectangles at 66 (gray), 58 (red) and 48 ˚C (green) that represent the percent (%) of transcripts number of specific gene at each temperature. b) Percent contribution of the six main Phyla to expression for each gene is shown by the horizontal bars. The most important genera in each case are noted to the right.

Supplementary data

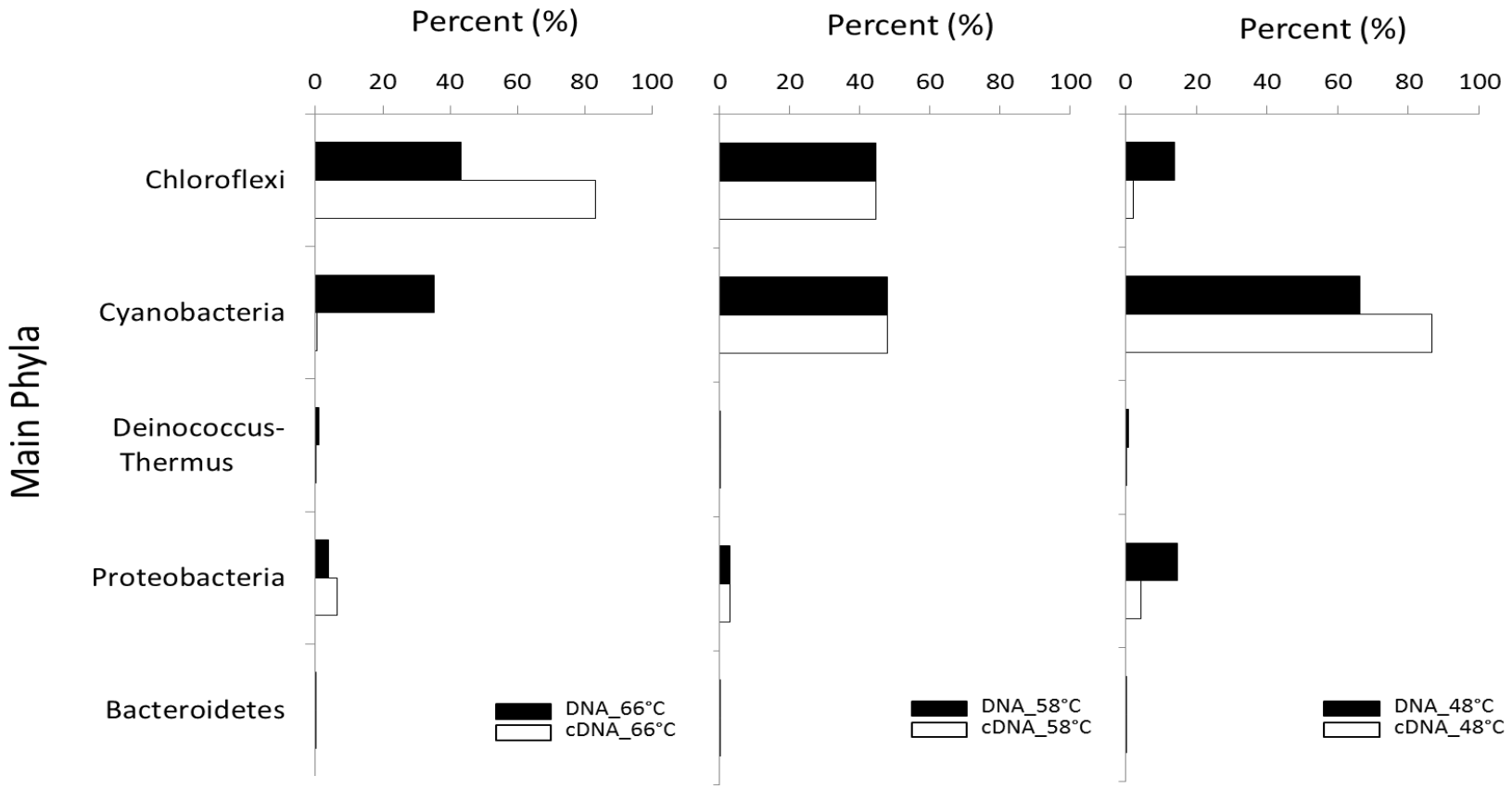


Figure S1. Taxonomic assignment at the Phylum level of metagenomic (DNA) and metatranscriptomic (cDNA) reads of the most abundant phyla using the KRAKEN classifier system, at the three temperatures studied

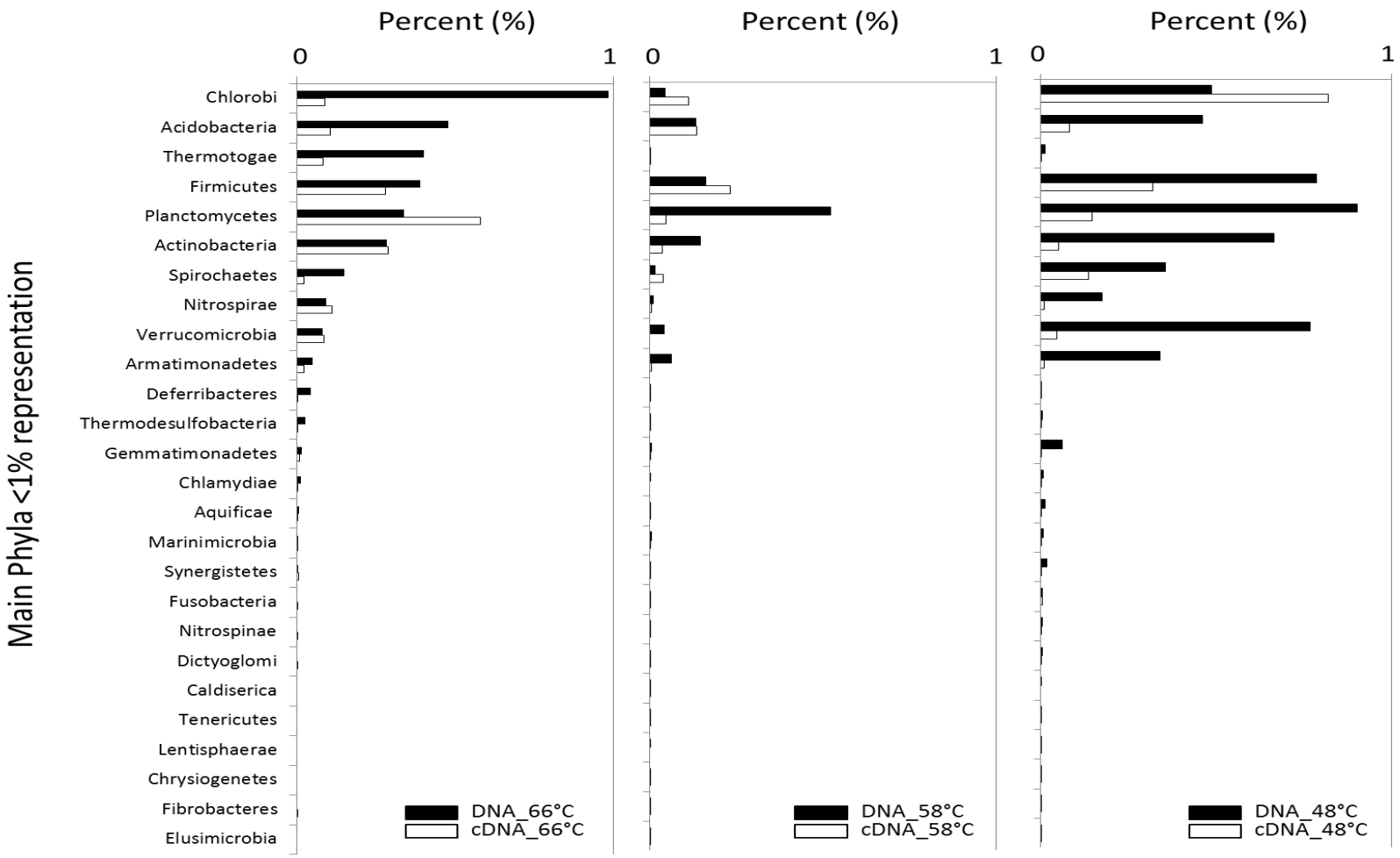


Figure S2. Taxonomic assignment at the Phylum level of metagenomic (DNA) and metatranscriptomic (cDNA) reads of the phyla represented by less than 1% of the reads, at the three temperatures studied..

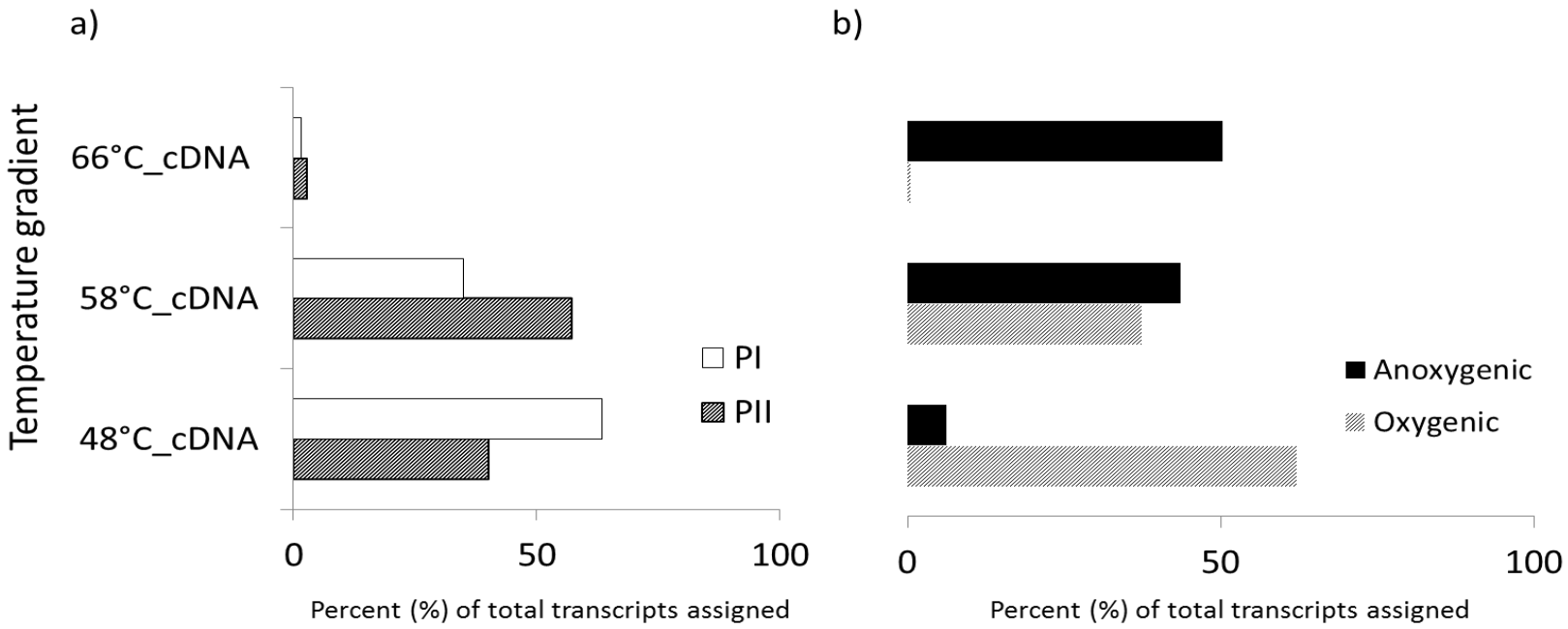


Figure S3. Total transcripts (cDNA) assigned to photosynthesis components. a) Oxygenic photosynthesis; photosystems I (PI) and II (PII), b) Proportion between oxygenic and anoxygenic photosynthesis, the relation between both is 1000:1.

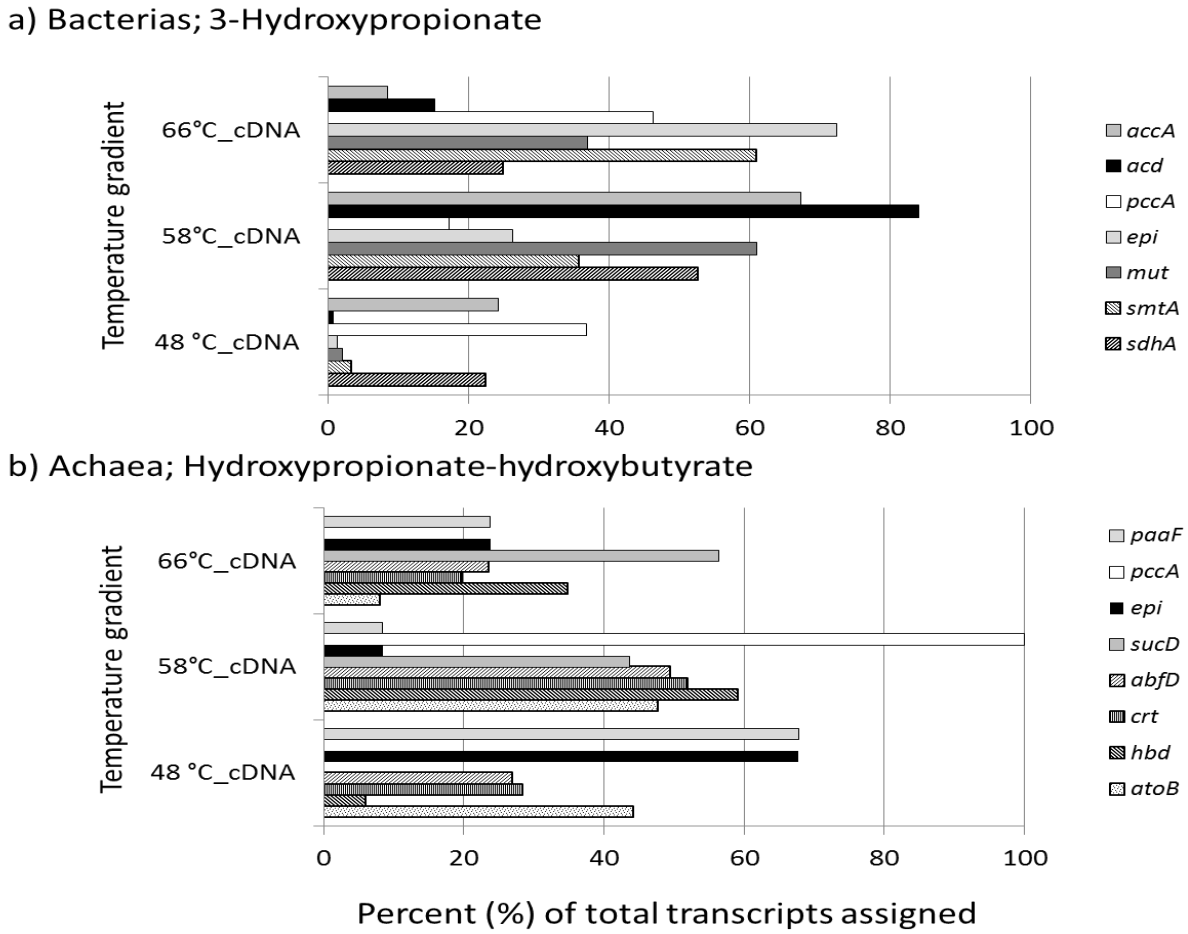


Figure S4. Total transcripts (cDNA) assigned to the carbon fixation cycles. a) 3-hydroxypropionate bicycle and b) Hydroxypropionate-hydroxybutyrate cycle.

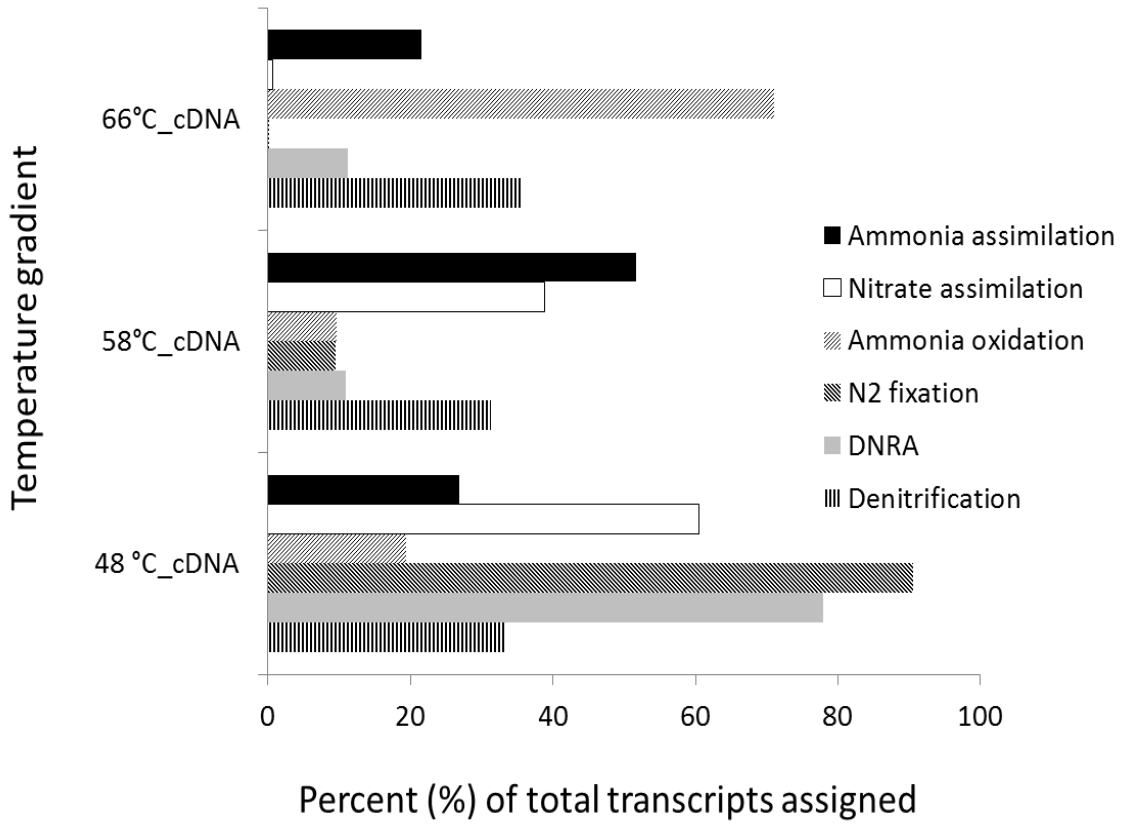


Figure S5. Total transcripts (cDNA) assigned to nitrogen cycle pathways.

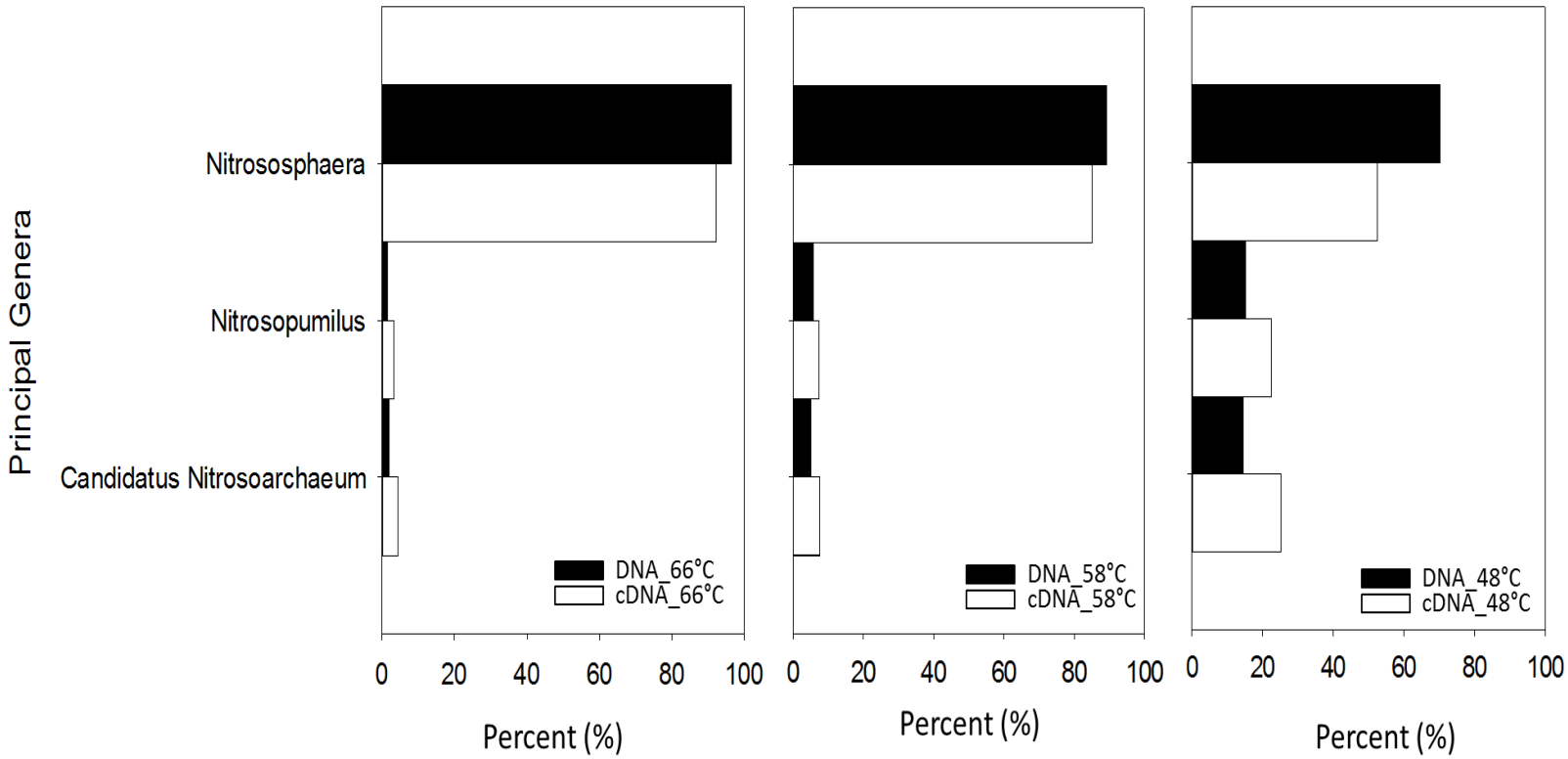
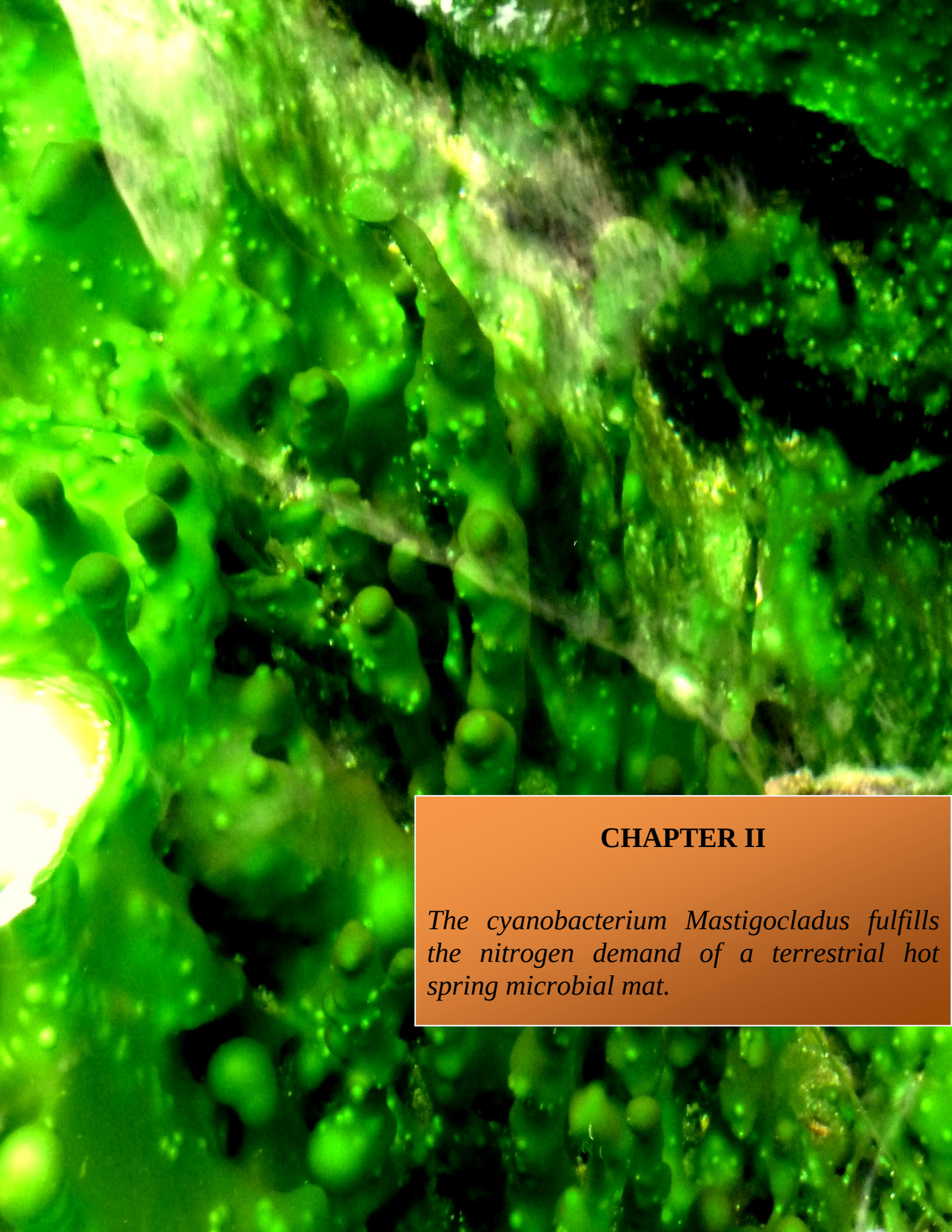


Figure S6. Principal Archaeal genera of metagenomic (DNA) and metatranscriptomic (cDNA) reads at the three temperatures studied.



CHAPTER II

The cyanobacterium Mastigocladus fulfills the nitrogen demand of a terrestrial hot spring microbial mat.

ORIGINAL ARTICLE

The cyanobacterium *Mastigocladus* fulfills the nitrogen demand of a terrestrial hot spring microbial mat

M. Estrella Alcamán[1], Camila Fernández[2, 3, 4], Antonio Delgado[5], Birgitta Bergman[6], Beatriz Díez[1*]

[1]*Department of Molecular Genetics and Microbiology and Center for Climate and Resilience Research (CR)2, Pontificia Universidad Católica de Chile, Alameda 340, Casilla 144-D, C.P. 651 3677, Santiago, Chile.*

[2]*Sorbonne Universités, UPMC Univ Paris 06, UMR 7621, Laboratoire d'Océanographie Microbienne, Observatoire Océanologique, F-66650 Banyuls/mer, France.*

[3]*CNRS, UMR 7621, Laboratoire d'Océanographie Microbienne, Observatoire Océanologique, F-66650 Banyuls/mer, France.*

[4]*Department of Oceanography, Interdisciplinary center for Aquaculture Research (INCAR) and COPAS SURAUSTRAL program, University of Concepción, Chile.*

[5]*Instituto Andaluz de Ciencias de la Tierra (CSIC-Univ. Granada), Armilla, Granada, Spain.*

[6]*Department of Ecology, Environment and Plant Sciences and Science for Life Laboratory, Stockholm University, S-10691 Stockholm, Sweden.*

Running title

Diazotrophy in a hot spring microbial mat

*Correspondence: Beatriz Díez, Department of Molecular Genetics and Microbiology, Faculty of Biological Sciences, Pontificia Universidad Católica de Chile, Alameda 340, Casilla 144-D, C.P. 651 3677, Santiago, Chile.

E-mail: bdiez@bio.puc.cl

Abstract

Cyanobacteria from Subsection V (Stigonematales) are important components of microbial mats in non-acidic terrestrial hot springs. Despite their diazotrophic nature (N_2-fixers), their impact on the nitrogen cycle in such extreme ecosystems remains unknown. Here, we surveyed the identity and activity of diazotrophic cyanobacteria in the neutral hot spring of Porcelana (Northern Patagonia, Chile) during 2009 and 2011-2013. We used 16S rRNA and the *nif*H gene to analyze the distribution and diversity of diazotrophic cyanobacteria. Our results demonstrate the dominance of the heterocystous genus *Mastigocladus* (Stigonematales) along the entire temperature gradient of the hot spring (69-38 ºC). In *situ* nitrogenase activity (acetylene reduction), nitrogen fixation rates (cellular uptake of $^{15}N_2$), and *nif*H transcription levels in the microbial mats showed that nitrogen fixation and *nif*H mRNA expression were light dependent. Nitrogen fixation activities were detected at temperatures ranging from 58 to 46 °C, with maximum daily rates of 600 nmol C_2H_4 cm^{-2} d^{-1} and 94.1 nmol N cm^{-2} d^{-1}. These activity patterns strongly suggest a heterocystous cyanobacterial origin and reveal a correlation between nitrogenase activity and *nif*H gene expression during diurnal cycles in thermal microbial mats. N and C fixation in the mats contributed approximately 3 g N m^{-2} y^{-1} and 27 g C m^{-2} y^{-1}, suggesting that these vital demands are fully met by the diazotrophic and photoautotrophic capacities of the cyanobacteria in the Porcelana hot spring.

Keywords: diazotroph / microbial mat / nitrogenase activity, ARA / nitrogen fixation, $^{15}N_2$ / thermophilic cyanobacteria**.**

Introduction

Hot springs represent extreme environments for life. They are typically dominated by a range of microorganisms that form well-defined 'mats' that are constantly being over-run by hot spring water. A variety of physical and chemical features, such as the pH (Hamilton *et al.*, 2011; Huang *et al.*, 2013; Loiacono *et al.*, 2012), sulfide concentration (Purcell *et al.*, 2007), and temperature (Miller *et al.*, 2009; Wang *et al.*, 2013) shape the microbial presence and life cycle in these ecosystems. Temperature is considered the most important variable associated with changes and metabolic adaptations in microbial mat communities in hot springs with a neutral pH (Cole *et al.*, 2013; Mackenzie *et al*., 2013).

Recently, the diversity of microbial thermophiles in many hot springs has been characterized (Nakagawa & Fukui, 2002; Meyer-Dombard *et al.*, 2005; Hou *et al.*, 2013; Inskeep *et al.*, 2013). A range of thermophilic microorganisms (~75-40 °C) has been identified. Representatives of the bacterial phyla Cyanobacteria, Chloroflexi and Proteobacteria are the most commonly found microbes in neutral to alkaline hot springs (Otaki *et al.*, 2012; Cole *et al.*, 2013; Mackenzie *et al.*, 2013; Wang *et al.*, 2013). Within the cyanobacteria, unicellular members such as *Synechococcus* and *Cyanothece* typically dominate at temperatures above 60 °C (Ward *et al.*, 1998, 2000; Papke *et al.*, 2003; Steunou *et al*., 2006, 2008). At lower temperatures (~60-40 °C), filamentous, non-heterocystous genera such as *Phormidium* and *Oscillatoria* and heterocystous genera such as *Calothrix, Fischerella*, and *Mastigocladus* are common (Sompong *et al.*, 2005; Miller *et al.*, 2006; Finsinger *et al.*, 2008; Coman *et al.*, 2013). Although heterocystous cyanobacteria are richly represented in many hot springs with a neutral pH, their role and capacity as providers of fixed nitrogen is still unknown.

Nitrogen fixation is the process by which selected diazotrophs from Archaea and Bacteria consume atmospheric N_2 gas as a substrate for growth (Stewart *et al*., 1967). This process may represent an important source of 'new' nitrogen in the often nitrogen-limited hot spring waters. This process also counteracts the loss of combined nitrogen caused by denitrification in the poorly ventilated substrates of terrestrial hot springs (Otaki *et al.*, 2012). N_2 fixation has been assessed by screening for specific *nif* genes such as *nif*H (encoding the α-subunit of the nitrogenase enzyme complex), which is the most widely used molecular marker in the search for diazotrophs. Hence, the analysis of the presence of the *nif*H gene combined with measurements of nitrogenase activity (using the acetylene reduction assay) has been widely used to identify diazotrophs and diazotrophy in microbial mats from diverse environments (Stal *et al*., 1984; Bergman *et al*., 1997; Díez *et al*., 2007; Steunou *et al*., 2006; Severin & Stal, 2009; Desai *et al*., 2013).

Currently, the most thoroughly studied hot springs are those in Yellowstone National Park (YNP, USA), in which both nitrogenase activity and *nif*H gene transcription patterns have been examined (Miller *et al.*, 2009; Hamilton *et al.*, 2011; Loiacono *et al.*, 2012). For example, nitrogenase activity was recorded in alkaline hot springs at temperatures of ~50 °C and was attributed to the heterocystous cyanobacterium *Mastigocladus laminosus* (Stewart, 1970; Miller *et al.*, 2006), while at higher temperatures in two other hot springs, the activity was credited to the unicellular cyanobacterium *Synechococcus* (Steunou *et al.*, 2006, 2008). However, heterotrophic bacteria and archaea are also highly represented as thermophiles in YNP acidic hot springs, including the presence of some active nitrogen fixers at temperatures up to 82 °C (Hamilton *et al.*, 2011). Moreover, *nif*H genes have been detected at 89 °C in hot springs with varying pH values (1.9 to 9.8) (Hall *et al.*, 2008; Loiacono *et al.*, 2012).

Due to the more 'indirect' character of the 'nitrogen fixation' activity provided by the acetylene reduction technique (which measures nitrogenase enzyme activity), verification of the results through measurements of the nitrogen fixation activity (*i.e.,* N_2 gas uptake and cellular N incorporation using the $^{15}N_2$ stable isotope assay) is highly recommended (Peterson & Burris, 1976; Montoya *et al.*, 1996). However, $^{15}N_2$ gas uptake has rarely been used to study nitrogen fixation by microorganisms in thermal hot springs. The only exception is the study of Stewart (1970) in an alkaline hot spring in YNP. Furthermore, measurements of nitrogenase activity by ARA, $^{15}N_2$ uptake and *nif*H gene expression have not been evaluated together in a thermal microbial mat.

The aim of our study was to evaluate the role of diazotrophs in the nitrogen economy of the pristine, neutral terrestrial hot spring of Porcelana (Chile) with a focus on cyanobacteria. To achieve this goal, we examined the molecular identity (16S rRNA and *nif*H genes) of diazotrophic cyanobacteria and estimated their daily *in situ* nitrogenase activity and $^{15}N_2$ uptake in combination with *nif*H gene expression in a series of inter-annual analyses (2009, 2011-2013). Our data show that cyanobacteria are capable of fulfilling the nitrogen demands of hot spring microbial mats through their nitrogen fixation activity.

Materials and Methods

Study site and sampling strategies

The study was conducted in the hot spring of Porcelana located ~100 m above sea level at 42º 27' 29.1''S - 72º 27' 39.3''W in northern Patagonia, Chile (Figure 1a). A similar thermophilic temperature range (>69 to 38 °C) was registered during the sampling and experimentation during late summer (March) of the four years 2009 and 2011-2013.

Temperature, pH and dissolved O_2 percentages were monitored using a multiparameter instrument (Oakton, model 35607-85). Microbial mat samples (1 cm thick) used for *in situ* ARA and $^{15}N_2$ uptake experiments and DNA/RNA analysis were obtained using a cork borer with a diameter of 7 mm. An extra 3 cores not used in the *in situ* analysis were included to generate enough material for the DNA/RNA analyses. Spring water (5 ml) and microbial mat samples were collected in triplicate for nutrient (NH_4^+, NO_3^-, NO_2^-, and PO_4^{3-}) and total chlorophyll determinations. Dissolved Fe concentrations were determined in the same samples using ICP-Mass spectrometry X series 2 (Thermo Fisher Scientific, Inc.) after pre-concentration with ammonium1-pyrrolidinedithiocarbamate/diethylammonium diethyldithiocarbamate (APDC/DDDC) organic extraction (Bruland *et al*., 1985). All samples were stored in liquid nitrogen during transportation to the laboratory and at -80 °C until processing.

DNA extraction, PCR amplification, and denaturing gradient gel electrophoresis (DGGE)

DNA was extracted as previously described (Bauer *et al.*, 2008). Prior to DNA extraction, the samples were placed in a Lysing Matrix E tube (QBIO gene, Carlsbad, CA, USA) containing lysis buffer and solid-glass beads (1 mm) to homogenize the microbial cells by bead beating (4.0 ms^{-1} for 20 sec). The quality and quantity of the extracted DNA were determined using a spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and by inspection after separation in a 1% agarose gel. Then, the total DNA was used as the template for PCR amplifications of cyanobacterial 16S rRNA genes using the cyanobacteria-specific primers CYA106F with a GC-clamp (5’ 40 nt GC tail) and CYA781R(a) - CYA781R(b) (Nübel *et al.*, 1997) to generate amplicons 600 nucleotides in

length. The DNA was also used as a template for amplification of the *nif*H genes using the diazotrophic cyanobacteria-specific primers CNF with a GC-clamp and CNR (Olson *et al.*, 1998) to generate amplicons 350 nucleotides in length. The amplicons were resolved using a denaturing gradient gel electrophoresis (DGGE) approach with a D-code system (BioRad) according to the protocol of Díez *et al*. (2007). The DNA-denaturant agent gradients used in the gels were 45 to 75% for the 16S rRNA gene and 45 to 65% for the *nif*H gene. DGGE bands located in the same position in the gel were assigned to the same microbial population. Several of the bands with the same position were excised from the gel, re-amplified and sequenced, as were all bands located at different positions along the gel. The excised DGGE bands were eluted in 20 μL DNAse/RNAse-free dH_2O (ultraPURE, Gibco) and stored at 4 °C overnight. An aliquot of the eluted DNA was subjected to an additional PCR amplification using the corresponding primers (without GC-clamp) prior to sequencing (Macrogen Inc., Korea). Each specific DGGE band retrieved was assigned to one sequence representing a specific phylotype. The sequences were edited using the BioEdit software (Sequence Alignment Editor Software V 7.0.5.3.), followed by a basic local alignment and the use of a search tool (BLASTN) (Altschul *et al*., 1997).

Bacterial* nif*H gene clone library

The diversity of diazotrophic prokaryotes present in the microbial mat throughout the thermal gradient was determined using *nif*H gene clone libraries. PCR amplifications of the *nif*H gene were performed using the universal primers PolF/PolR (Poly *et al.*, 2001) that cover most of the known diazotrophic organisms (Bacteria and Archaea), including

cyanobacteria (Mårtensson *et al*., 2009; Díez *et al*., 2012). These primers amplify fragments 360 bp in length. The PCR products were purified (Wizard clean-up system, Promega) and cloned using the commercial pJET1.2/blunt cloning kit (Thermo Scientific) according to the manufacturer`s instructions. Clones with the proper insert sequence were validated using the primer vector set pJetF/pJetR (amplicon length ~550-600 bp). Fifty to one hundred clones obtained from each library (12 clone libraries in total) were selected for cyanobacterial-specific *nif*H gene amplifications using the primers CNF and CNR (Olson *et al*., 1998). These primers amplify a fragment within the insert generated by the universal primers PolF/PolR (Poly *et al.*, 2001). Several of the amplified PCR products were sequenced to check for cyanobacterial genetic identities. Clones that did not amplify with the cyanobacterial primers CNF and CNR were assumed to correspond to other types of bacteria and were also sequenced. All sequences obtained were edited using the BioEdit software (Sequence Alignment Editor Software V 7.0.5.3.). The OTUs with 98% similarity were assigned using BLASTCLUST-BLAST score-based single-linkage clustering (Schloss & Westcott, 2011). The closest relatives to all OTUs were assigned using the BLASTN tool (NCBI database).

Phylogenetic reconstruction and statistical analysis

The 16S rRNA phylotypes retrieved from the DGGE band sequences**,** the reference taxa and the closest relatives from GenBank (only from published studies or cultures) were aligned using Bioedit with the ClustalW tool (Tom Hall, Ibis Therapeutics, Carlsbad, CA, USA). The same procedure was used for the *nif*H-DGGE band sequences and the *nif*H OTUs from the constructed clone libraries. The subsequent phylogenetic reconstruction using the maximum likelihood search strategy with 10,000 bootstrap replicates was

191 performed for each gene dataset. The sequences of *Gloeobacter violaceus* and
192 *Desulfovibrio salexigens* were used as outgroups for the 16S rRNA and *nif*H gene
193 phylogenetic reconstructions, respectively.

194 The obtained 16S rRNA and *nif*H sequences (16S rRNA-DGGE band and *nif*H OTUs)
195 were subjected to cluster analysis and BEST tests using PRIMER 6. The dendrograms
196 generated for both genes were constructed to elucidate the similarity between the samples
197 collected during different years and along the temperature gradient. The BEST test was
198 performed to estimate the environmental factors that best explained the microbial species
199 distributions. Additionally, CCA and RDA analyses (Clarke, 1993) were performed based
200 on the relative abundances of 16S rRNA-DGGE bands and *nif*H OTUs and the
201 environmental variables recorded each year to pinpoint the environmental variable/s that
202 most strongly influenced the microbial mat community.

203

204 ***RNA extraction and real-time qPCR measurements***

205 Biological replicates from the acetylene reduction assay (3 cores each) plus some
206 additional non-assayed samples were used for the subsequent RNA analysis. These
207 samples were collected throughout the day-night cycle (at 12:00, 13:00, 14:00, 16:00,
208 18:00, 20:00, 23:00 and 03:00 h) and at three different temperatures (58, 48 and 47 °C) in
209 two years (2012 and 2013). RNA from the samples was extracted using Trizol and the
210 RNeasy Plant mini kit according to manufacturer's specifications (Qiagen, Germany). The
211 quality and quantity of the RNA were determined using a spectrophotometer (NanoDrop
212 Technologies, Inc., Wilmington, DE, USA) and by electrophoresis in an RNase-free 1%
213 agarose gel. DNase treatment (TURBO, Applied Biosystems, USA) was performed, and 1
214 μg of RNA from each sample (in duplicate) was used for qPCR standardization. Then, the

cDNA was synthesized using a selective cDNA kit (Bio-Rad, USA) according to the manufacturer's specifications with the universal *nif*H gene primers PolF/PolR (Poly *et al.*, 2001). For qPCR, the *nif*H gene was cloned into the TOPO vector plasmid to obtain the plasmid stock concentration (10^{10} copies) and the plasmid curve (10^{2} to 10^{8} copies). The SensiMix kit (Bioline, USA) was used for the fluorescence signal, and the real-time qPCR (Roche LC 480) program was run as follows: 40 cycles at 95 °C for 10 s, 59 °C for 20 s, and 72 °C for 30 s. To avoid non-specific fluorescence, only fluorescence within the CP range given by the plasmid standard curve was considered and melting curves were only considered if they showed a unique product.

Measurement of nitrogenase activity (acetylene reduction assay, ARA)

The acetylene reduction assay (ARA) was used to assess nitrogenase activity (NA) in the microbial mats throughout the temperature gradient of the hot spring. This assay was performed according to the procedure described by Capone (1993). At each temperature, four biological replicates composed of three microbial mat cores each (7 mm in diameter and 1 cm thick) were placed in pre-sterilized 10 ml glass incubation vials containing 1 ml of pre-filtered (0.2 μm filter pore) spring water and sealed using Mininert valves STD (Sigma-Aldrich). The samples were incubated for 2 hours following replacement of 1 ml of air with 1 ml of acetylene gas (10-20% of the gas phase) generated from calcium carbide ($CaC_2 + H_2O = Ca(OH)_2 + C_2H_2$). The four replicates plus two controls (one with microbial mat cores but no acetylene gas and one containing only acetylene gas but no cores) were incubated at their original *in situ* temperature in the field. The first control was used to estimate any natural 'background' ethylene generated by the microbial community, and the

second control was used to estimate any ethylene generated in the calcium carbide reaction. After incubations during diel cycles (13:00, 14:00, 17:00, 23:00 and 03:00 h), 5 ml of the gas phase was withdrawn from each vial using a hypodermic syringe and transferred to a 5 ml BD vacutainer (no additive Z plus tube, REF367624). After transporting the vacutainers to the laboratory, the ethylene produced was analyzed by injecting 1 ml of the gas using a gas-tight syringe (Hamilton) into a GC-8A gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with an 80/100 Porapak Q (Supelco, St. Louis, MO, USA) 1 m x 1/4" column and a flame ionization detector using helium as the carrier gas. A commercial ethylene standard of 100 ppm (Scotty Analyzed Gases, USA) in air was used to estimate the ethylene produced. Acetylene (20% in air) was used as an internal standard (Stal, 1988). The nitrogenase activity calculated from the ethylene produced was corrected using the two controls and expressed per surface area of microbial mat cores and time.

Isotopic nitrogen assimilation ($^{15}N_2$) and carbon ($H^{13}CO_3^-$) uptake

In parallel to the ARA measurements performed in 2012 and 2013, samples from the microbial mats were collected for ^{15}N and ^{13}C uptake experiments. The experiments ($^{15}N_2$ and $H^{13}CO_3^-$) were performed using three biological replicates composed of three microbial mat cores each (7 mm in diameter and 1 cm thick). The cores were placed in pre-sterilized 12 ml vials with 1 ml of pre-filtered (0.2 μm filter pore) spring water and incubated at the corresponding *in situ* temperatures. The ^{15}N uptake experiments were initiated through the addition of 1 ml of $^{15}N_2$ gas (98% atom $^{15}N_2$ gas, Sigma-Aldrich) through a gas-tight syringe into the headspace of each vial. To estimate the carbon

($H^{13}CO_3^-$) uptake, 500 µl of $H^{13}CO_3^-$ (500 µM) was added to the vials. Additionally, two replicate vials without the isotope ($^{15}N_2$ and ^{13}C) were incubated to determine the natural isotopic composition (control). The vials were incubated *in situ* for 2 or 6 hours and then the cores were dried at 70 °C for 48 h. Measurement of ^{15}N and ^{13}C atom incorporation (AT ^{15}N and ^{13}C) were performed using a mass spectrometer (IRMS delta plus, Thermo FinniganH, Stable Isotope Laboratory, Granada, Spain), and the C:N ratio (organic matter composition of the sample) was determined. Calculations of the ^{15}N and ^{13}C assimilation rates were performed as described by Montoya *et al.* (1996) and Fernández *et al*. (2009), including corrections by dilutions of $^{15}N_2$ gas and controls.

Results

Geochemistry of the Porcelana hot spring

The Porcelana hot spring (Figure 1) shows a continuous outflow of hot water, thereby forming a decreasing temperature gradient away from the well. The water temperature ranged from 69 to 38 °C over the gradient investigated, with some variation in maximum temperatures between years (Table 1). A brightly pigmented microbial mat (~3 cm deep) extended 7-10 meters away from the well (Figure 1b) at the bottom of the water stream. The decreasing temperature gradient resulted in increasing water oxygen solubility. The physicochemical features of the mat were comparatively constant over time (Table 1). The pH was close to neutral (approximately 6.5), and the macronutrient concentrations were on average 1.9 µmol L^{-1} NO_3^-, 0.6 µmol L^{-1} NO_2^-, 0.03 µmol L^{-1} NH_4^+ and 51.4 µmol L^{-1} PO_4^{3-} over the 2011-2013 period (Table 1). The nitrate concentration was examined during the day and night periods and at two temperatures (52 and 47 ºC) in 2012. No variations

284 were apparent between day and night, although the nitrate levels were almost 3-fold higher
285 at 52 ºC (Supplementary data, Figure S1). The dissolved Fe concentrations were
286 approximately 0.07 μmol L^{-1} in 2012 and 2013 (Table 1).
287
288 ***Inter-annual cyanobacterial diversity***
289 The cyanobacterial diversity in the microbial mat growing along the temperature
290 gradient was examined during the years 2009 and 2011-2013. The analyses were
291 performed by DGGE using the cyanobacterial-specific 16S rRNA gene as the diversity
292 marker (Supplementary data, Figure S3a). The resulting DGGE bands (five in total)
293 revealed the existence of differently distributed sub-populations along the temperature
294 gradient. The bands corresponded with members of the phylum Cyanobacteria and
295 specifically with members within the heterocystous order Stigonematales (DGGE band
296 CYA5; GenBank accession numbers for nucleotide sequences: KJ696694) and the non-
297 heterocystous order Oscillatoriales (DGGE band CYA1-4; GenBank accession numbers
298 for nucleotide sequences: KJ696687 to KJ696690) (Supplementary data, Table S1).
299 Cluster analysis of the 16S rRNA gene marker was performed using PRIMER 6 (Bray-
300 Curtis dissimilarity index dendrogram) assuming the presence-absence of the DGGE bands
301 together with their relative abundance throughout the temperature gradient in the four years
302 (Figure 2). Up to 70% similarity was apparent for all samples denoted as cyanobacteria in
303 the dendrogram (Figure 2a). However, samples collected from similar temperatures within
304 the same year grouped as pairs showed >90% similarity. This result may be explained by
305 the similar relative abundances of the cyanobacteria (analyzed by 16S rRNA genes)
306 exhibited by the pairs (Figure 2b). Additionally, most pairs showed >80% similarity with a

third sample collected during the same year or at a similar temperature. This result illustrates the strong influence of temperatures and inter-annual variations on the cyanobacterial community.

Phylogenetic reconstructions of the sequences retrieved from the DGGE bands using the 16S rRNA gene confirmed the placement of the hot spring cyanobacteria within the filamentous non-heterocystous order Oscillatoriales (Section III) and the heterocystous order Stigonematales (Section V) (Supplementary data, Figure S2). The four 16S rRNA-DGGE bands CYA1 to CYA4 (Supplementary data, Figure S3a) formed clusters with members of the genera *Leptolyngbya* and *Oscillatoria* (Oscillatoriales) with a 99% similarity according to the BLASTN analyses (Supplementary data; Table S1). Moreover, the even more prevalent 16S rRNA-DGGE band CYA5 (Supplementary data, Figure S3a) was closely related to members of the *Mastigocladus* and *Fischerella* genera (Supplementary data; Table S1). The tentatively identified *Mastigocladus* phylotype (CYA5) was the only phylotype present along the entire temperature gradient (*i.e.*, from 69 to 38 °C); CYA5 also exhibited the highest relative abundance in 16S rRNA gene sequences at higher temperatures (57-46 °C) (Figure 2b). Within this temperature range, the *Mastigocladus* phylotype represented an average of 66% of the total cyanobacterial community; the remaining 34% was represented by Oscillatoriales phylotypes.

BEST analysis relating the 16S rRNA phylotypes identified by DGGE to the recorded *in situ* environmental variables including temperature (°C), dissolved oxygen (%), pH, and nitrogen compounds (NO_3^-, NO_2^-, NH_4^+) (Table 1) showed that variations in temperature and pH explained 77% of the similarity between the phylotypes (Rho value 0.109; significance level 91%). These results were corroborated using canonical correspondence analysis (CCA), which showed that temperature, pH, and NO_2^- represented the major

ecological drivers of the phylotype distribution in the Porcelana hot spring (Supplementary data, Figure S5).

Inter-annual diversity of cyanobacterial diazotrophs

The diversity of diazotrophs in the hot spring was investigated by constructing clone libraries targeting the *nif*H gene using universal primers (Poly *et al.*, 2001). Fifty to one hundred clones were obtained from the 12 libraries constructed (Supplementary data, Table S1). To identify clones related to cyanobacteria, the clones were re-amplified using the cyanobacterial specific *nif*H gene primers (Olson *et al*., 1998). Fifteen to fifty clones in each library were found to represent cyanobacterial phylotypes. All of the retrieved sequences (GenBank accession numbers for nucleotide sequences: KM507492 to KM507497) were analyzed using BLASTCLUST-BLAST (Schloss & Westcott, 2011) to identify the OTUs present in each clone library (Supplementary data, Table S1). A total of six cyanobacterial *nif*H OTUs were apparent, three of which were determined to be closely affiliated (>98% nucleotide sequence identity) to the heterocystous genus *Mastigocladus* (Stigonematales) by BLASTN analysis (Figure 3b; Supplementary data, Table S1). The other three OTUs were affiliated with the Oscillatoriales (>88% nucleotide sequence identity) and more specifically with the genera *Leptolyngbya* and *Oscillatoria* (Supplementary data, Table S1). A phylogenetic reconstruction of the six *nif*H gene OTUs and the closest related sequences from the database confirmed the identities obtained by BLASTN (Supplementary data; Figure S4). As shown in Figure 3a, similarity cluster analysis of the *nif*H OTUs demonstrated that all of the microbial mat samples collected in the spring were highly stable and exhibited >95% similarity in the community that was independent of the temperature and the year investigated. The dominance of the

355 *Mastigocladus* OTUs identified by *nif*H gene analysis was confirmed (93% on average) at
356 all temperatures, whereas the Oscillatoriales OTUs were comparatively rare (7% average)
357 (Figure 3b).

358 Redundancy analysis (RDA) of the *nif*H gene OTUs and the *in situ* recorded
359 environmental variables including temperature (°C), dissolved oxygen (%), pH, and
360 nitrogen compounds (NO_3^-, NO_2^-, and NH_4^+) (Table 1) showed that the temperature and
361 nutrients (NH_4^+ and NO_2^-) explained the distribution and high relative abundance of the
362 *Mastigocladus nif*H gene OTUs in the spring (Supplementary data, Figure S6).

363 The identity of the cyanobacterial OTUs obtained using the *nif*H clone libraries were
364 verified via the DGGE approach using the same cyanobacterial-specific *nif*H primers
365 (Olson *et al.*, 1998). Three *nif*H-DGGE bands (Supplementary data, Figure S3b) were
366 retrieved and affiliated with *Mastigocladus laminosus* with 99% sequence similarity
367 (BLASTN tool; GenBank accession numbers for nucleotide sequences: KJ696698 to
368 KJ696700) (Supplementary data, Table S1). None of the *nif*H-DGGE bands were affiliated
369 with members of the Oscillatoriales.

370 A phylogenetic reconstruction combining the sequenced *nif*H gene OTUs and *nif*H
371 DGGE bands with their closest matches in the database (Supplementary data, Figure S4)
372 showed that all of the genes clustered to Stigonematales with sequences related to the
373 thermophilic *Mastigocladus laminosus*. The Oscillatoriales OTUs clustered with the
374 'Filamentous thermophilic cyanobacterium sp.' (accession number: KM507495 and
375 KM507496) and *Leptolyngbya* sp. (accession number: KM507497).

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Biological nitrogen fixation

Due to the high presence of potential diazotrophic cyanobacteria in the microbial mat of the Porcelana hot spring, the nitrogen fixation process was recorded using two approaches: the sensitive acetylene reduction assay (ARA-GC) to estimate the nitrogenase enzyme activity (years 2009 and 2011-2013) and the $^{15}N_2$ stable isotope uptake to estimate the biological incorporation of nitrogen into the biomass (years 2012 and 2013) using mass spectroscopy. As shown in Figure 4 and Table 2, the total nitrogenase activity recorded along the temperature gradient at midday (12:00-14:00) varied from 0.2 (± SD 0.01) to 50.0 (± SD 7.0) nmol C_2H_4 cm^{-2} h^{-1}. The highest rates were recorded at 46-48 °C, while higher temperatures (Figure 4) and darkness (Figure 5) gave a lower activity.

Analysis of the cellular incorporation of nitrogen (Table 2) after 2-hour (12:00-14:00) and 6-hour (12:00-18:00) incubations showed incorporation of ^{15}N (Table 2, Figure 4). The highest nitrogen incorporation recorded was 7.8 nmol N cm^{-2} h^{-1} (± SD 0.6) at 48 °C in 2013, coinciding with the highest nitrogenase activity at the same temperature and year (Table 2). No difference in activity was observed following incubations for 2 or 6 hours (Table 2). The theoretical ratio between the acetylene reduction (ARA) and the isotopic N_2 fixation method (C_2H_4:N_2) is 4:1. The ratio for the Porcelana hot spring microbial mat was close to this theoretical ratio, ranging from 2.2:1 to 6.8:1 (Table 2).

Based on the ^{15}N uptake quantities, the 'new' yearly nitrogen inputs into the Porcelana hot spring were extrapolated to represent up to 2.9 g N m^{-2} y^{-1} in 2012 and 4.8 g N m^{-2} y^{-1} in 2013 (Table 2).

Diel cycles of nitrogenase activity and* nif*H gene expression

Based on the fact that the optimum temperature for nitrogenase activity in the Porcelana hot spring was between 58 and 46 °C (Figure 4), this temperature interval were selected to determine the nitrogenase activity and *nif*H gene expression in greater detail throughout the day during two consecutive days in 2012 and 2013. As shown in Figure 5, the nitrogenase activity peaked at mid-day (at approximately 13:00-14:00 h) irrespective of the temperature and approached zero at night. Similar diel nitrogenase activity patterns were observed in both years, peaking at 22.9 nmol C_2H_4 cm^{-2} h^{-1} at 47 °C in 2012 and 32.4 nmol C_2H_4 cm^{-2} h^{-1} at 48 °C in 2013 (Table 2). The nitrogenase activity was consistently higher at lower temperatures (47-48 °C). Next, the biological sample replicates used for the nitrogenase assays (3 cores in each vial) were combined with extra microbial mat material to examine the diel cycles of *nif*H gene expression (Figure 5). In 2012, the *nif*H gene expression was measured only at 47 °C. Maximum transcript levels occurred around midday (day 2) with 2.1 x 10^7 *nif*H gene transcripts identified. In 2012, two lower expression peaks were noted at 16:00 h (5.2 x 10^5) and 20:00 h (6.6 x 10^5); this pattern was also observed in 2013. The highest transcription level (2.4 x 10^4) was found at approximately 16:00 h and 58 °C, while no *nif*H expression took place in the dark/night time when examined in 2012 and 2013.

Carbon fixation

Because the data showed that the Porcelana microbial mat was dominated by cyanobacteria, the *in situ* incorporation of ^{13}C-labeled bicarbonate ($H^{13}CO_3^-$) was followed in 2012 and 2013. The incubations with $H^{13}CO_3^-$ lasted 2 hours (12:00 to 14:00) under the same conditions described for the nitrogen fixation assays (*i.e.*, at 52 and 58 °C) (Table 2).

The highest carbon incorporation recorded was 53.0 (± SD 4.1) and 45.8 (± SD 8.7) nmol C cm^{-2} h^{-1} at 52 °C and 58 ºC, respectively, during the two consecutive years (Table 2). Extrapolation to a yearly incorporation showed an average C uptake of approx. 27 g C fixed m^{-2} y^{-1} in the Porcelana hot spring.

Contribution of combined nitrogen to the Porcelana microbial community

Taking into account the daily rates of $^{15}N_2$ uptake, $H^{13}CO_3^-$ assimilation and the C:N ratio (Table 2), it was apparent that the photoautotrophic nitrogen fixers present in the Porcelana microbial mat sustained these key nutrient demands to a large extent. Even when the daily rates found for nitrate assimilation ($^{15}NO_3^-$) (data not shown) were considered, the total 'new' production of nitrogen fixation (^{15}N) contributed up to 99% of the 'new' N input into the microbial mat of the Porcelana hot spring (Table 2). The analyses were performed according to the protocol of Raimbault & Garcia (2008), although the data were not corrected for nitrification.

Discussion

Although thermal systems around the world have attracted considerable interest and their overall biology and organisms have been characterized (Stewart, 1970; Miller *et al.*, 2006; Steunou *et al*., 2008; Hamilton *et al.*, 2011; Loiacono *et al.*, 2012; Huang *et al.*, 2013), our knowledge on the identity and relevance of diazotrophs in such systems has remained surprisingly rudimentary. The distinct microbial mats or biofilms formed in hot springs typically harbor phototrophic microorganisms that often belong to the phyla Cyanobacteria and Chloroflexi (Liu *et al*., 2011; Klatt *et al*., 2013). Because certain

members of these phyla (together with archaea) may fix atmospheric dinitrogen gas (N_2), this organismal segment may serve an important key nutrient (N) role in these ecosystems, as was recently suggested (Steunou *et al*., 2006, 2008; Hamilton *et al.*, 2011; Loiacono *et al.*, 2012).

To extend our knowledge concerning the significance of thermal diazotrophs, we performed the first detailed examination by combining analyses of the genetic diversity of microbes, their diazotrophic capacity and estimates of their contribution to 'new' nitrogen in the neutral hot spring Porcelana (Patagonia, Chile). The high volcanic activity in Chile has generated a large number of largely unexplored terrestrial hot springs with distinct physicochemical parameters; some of the hot springs exhibit characteristics resembling those of other well-studied hot spring areas (*e.g.,* Yellowstone National Park) (Hauser, 1989; Hamilton *et al.*, 2011; Loiacono *et al.*, 2012; Wang *et al*., 2013). The pristine hot spring of Porcelana was selected because this spring represents a stable ecosystem appropriate for identifying microbes and factors that control their behavior in the community. The lush microbial mats of the Porcelana thermal gradient (~69-38 ºC) are likely supported by the nitrogen, phosphate and iron levels typical for the Porcelana water system and contain microbes belonging to Cyanobacteria, Proteobacteria and Chloroflexi (Mackenzie *et al*., 2013). Hence, we hypothesized the existence of a rich diazotrophic community in the Porcelana spring, making it an ideal model system for exploration.

The polyphasic approach used in our study of the Porcelana hot spring in combination with several methodological approaches such as molecular markers (16S rRNA and *nif*H genes), molecular techniques (clone libraries, DGGE and RT-qPCR), *in situ* enzyme activities (ARA) and isotope uptake ($^{15}N_2$ and $H^{13}CO_3^-$) established that the Porcelana hot spring is dominated by cyanobacteria, particularly the diazotrophic genus *Mastigocladus*

(Stigonematales). Cyanobacteria have been identified in other thermal microbial mats included members of the unicellular Synechococcales (mainly the genus *Synechococcus*) (Sompong *et al*., 2005; Steunou *et al*., 2006, 2008) and the filamentous Stigonematales (genera *Fischerella* and *Mastigocladus*) (Schwabe, 1960; Lacap *et al*., 2007; Miller *et al*., 2006, 2007; Finsinger *et al*., 2008). The dominating cyanobacterial phylotypes discovered in the microbial mats of the Porcelana hot spring corroborated these data, with the exception of the unicellular cyanobacteria. The presence of the Stigonematales phylotypes was also verified by morphological analysis (microscopy; data not shown).

Using the *nif*H genes as a marker allowed a more accurate determination of the affiliation of the dominating cyanobacteria OTUs and revealed the dominance of the heterocystous genus *Mastigocladus*; however, the affiliations were less apparent using the 16S rRNA marker gene. The latter is likely due to the low number of sequences and sequenced genomes from the order Stigonematales in the databases. The *Mastigocladus* phylotypes were present throughout the temperature gradient (69 ºC to near 38 °C), thereby expanding their upper temperature limit compared to the results of other thermal or laboratory systems (Finsinger *et al*., 2008; Miller *et al*., 2009). The 16S rRNA and *nif*H gene approach also identified members of the non-heterocystous Oscillatoriales (including both non-diazotrophs and diazotrophs), although they were present at a lower abundance; this group was not detected using the DGGE approach. Taken together, the data show that the Porcelana spring has a unique microbial composition devoid of unicellular cyanobacteria and other diazotrophic bacteria.

To broaden our knowledge of the importance of nitrogen fixation in the Porcelana spring, diel activities were examined using both the nitrogenase activity and $^{15}N_2$ isotope uptake approaches; the use of these complementary techniques reflect different aspects of

498 the fixation process (Peterson & Burris, 1976; Montoya *et al.*, 1996). To date,
499 measurements of cyanobacterial-associated nitrogenase activity (acetylene reduction assay)
500 have dominated hot spring analyses (Steunou *et al.,* 2006, 2008; Miller *et al.,* 2009).
501 Recent studies showed that heterotrophic bacteria and archaea may serve as significant
502 nitrogen-fixers in hot springs (Hamilton *et al.*, 2011; Loiacono *et al.*, 2012). However, the
503 only study following $^{15}N_2$ isotope uptake was conducted in 1970 in thermal microbial mats
504 (YNP) dominated by the cyanobacterial genera *Calothrix* and *Mastigocladus* (Stewart,
505 1970). Nitrogen fixation assessed using nitrogenase activity in combination with $^{15}N_2$ gas
506 uptake provided different but complementary information; therefore, we used these
507 techniques in the present study of the Porcelana hot spring. The data show that diazotrophy
508 is the norm in this hot spring in all four years examined. Furthermore, the activity was only
509 apparent during the day time (13:00-14:00 h) and was highest at 58 to 46 °C but was not
510 detected above 60 °C. The nitrogenase activity recorded was on a similar order of
511 magnitude (50.0 nmol C_2H_4 cm^{-2} h^{-1}) to that reported for the Mushroom Spring (YNP; 40
512 to 180 nmol C_2H_4 cm^{-2} h^{-1}; Steunou *et al*., 2008), although there were differences in the
513 retrieval of the diazotrophic biomass. Similarly, nitrogen fixation rates in the Porcelana hot
514 spring (ranging from 2 to 8 nmol N cm^{-2} h^{-1}) were in agreement with the activities reported
515 for other non-thermal aquatic ecosystems (Fernández *et al*., 2011). The data further
516 demonstrated that the nitrogen fixation rates in the Porcelana microbial mat fell within the
517 theoretical ratio for C_2H_4:N_2 of 4:1 (if hydrogen production was taken into account;
518 Crawford *et al.*, 2000).

519 The use of the *nif*H gene as a potent molecular marker for diazotrophs in natural
520 ecosystems has been extensive in recent years (Díez *et al*., 2007; Severin & Stal, 2009,
521 2010; Fernandez *et al*., 2011). However, the presence of *nif* genes or transcripts is not

necessarily coupled to activity, as shown for *Synechococcus*-dominated hot spring mats (Steunou *et al*., 2006, 2008) where *nif*H gene expression peaked in the evening and nitrogenase activity peaked in the morning. A similar phenomenon was also observed in cyanobacterial microbial mats from temperate regions (Stal *et al*., 1984; Severin & Stal, 2009, 2010). In contrast, the nitrogen fixation activity (nitrogenase activity and N_2 uptake) in the Porcelana hot spring showed a positive correlation with *nif*H gene expression. Moreover, because nitrogen fixation during the daytime is typical for ecosystems dominated by heterocystous cyanobacteria (Stal, 1995; Evans *et al*., 2000; Charpy *et al*., 2007; Bauer *et al*., 2008), our data infer the predominance, if not the exclusive role, of the heterocystous *Mastigocladus*-type cyanobacteria in nitrogen fixation in the Porcelana hot spring.

It cannot be excluded that the low concentrations of combined inorganic nitrogen (*e.g.*, ammonium and nitrate) in the Porcelana hot spring may be the result of a rapid turnover of these compounds (Herbert, 1999). However, the distinct nitrogen fixation activities recorded (on average 3 g N m^{-2} y^{-1}) in the Porcelana hot spring suggest that this process is not diminished by other sources of combined nitrogen. Rather, we can conclude that the entry of 'new' nitrogen by diazotrophic cyanobacteria supports most of the total daily nitrogen demand (up to 99%) of the microbial mat. Comparing this nitrogen input with that of rain water (ca. 0.1 g N m^{-2} y^{-1}) for the geographical region related to Porcelana (Weathers & Likens, 1997), we suggest that the biological nitrogen fixation found in our study may constitute the major source of "new" nitrogen into this ecosystem.

The fact that both the nitrogen and CO_2-fixation coincided at midday in the Porcelana cyanobacterial mat may explain the substantial nitrogen fixation activity recorded.

Photosynthesis would not only cover the high energy demand (ATP) of the nitrogen fixation process but also provide the required reducing power and carbon skeletons.

Conclusions

Our data demonstrate that the microbial mats covering the thermal gradient of the Porcelana hot spring out-flow represent a well-organized and functioning ecosystem dominated by diazotrophic cyanobacteria of the *Mastigocladus*-type and may represent a typical scenario for neutral hot springs. Our results further emphasize the pivotal role of such diazotrophic cyanobacteria in maintaining this microbial-dominated ecosystem by delivering most of its nitrogen demand through nitrogen fixation. These findings may also have important implications for other thermal or extreme environments dominated by cyanobacterial microbial mats.

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569 **Conflict of Interest**

570 The authors declare no conflict of interest.

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572 **Supplementary information is available at ISME J's website**

573

574 **References**

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Titles and legends to Tables and figures

Table 1. Physical and chemical variables registered in the Porcelana hot spring at different locations along the microbial mat during the years 2009 and 2011-2013.

Year	T°C	O_2 % Sat.	pH	NO_3^- (mmol L^{-1})	NO_2^- (mmol L^{-1})	NH_4^+ (mmol L^{-1})	PO_4^- (mmol L^{-1})	Fe (mmol L^{-1})
2009	46	42	5.2	ND*	ND*	ND*	ND*	ND*
2009	42	46	6.4	ND*	ND*	ND*	ND*	ND*
2009	40	43	6.1	ND*	ND*	ND*	ND*	ND*
2009	38	48	5.1	ND*	ND*	ND*	ND*	ND*
2011	69	54	6.9	ND*	ND*	ND*	ND*	ND*
2011	64	59	6.7	ND*	ND*	ND*	ND*	ND*
2011	61	80	6.9	0.8	1.3	ND*	ND*	ND*
2011	57	82	6.8	ND*	ND*	ND*	ND*	ND*
2011	51	90	6.7	ND*	ND*	ND*	ND*	ND*
2012	52	104	6.7	1.7	0.2	0.01	29.7	ND*
2012	47	108	7.1	1.2	0.2	0.01	43.5	0.02
2013	66	72	6.8	6.5	0.01	0.02	115	0.05
2013	65	73	6.8	0.9	0.2	0.01	47.4	0.07
2013	58	86	6.8	1.9	0.1	0.01	38.4	0.14
2013	48	94	7.1	1.2	0.2	0.1	34.1	0.06

ND*, Data not determined

Table 2. The contribution of nitrogenase activity, nitrogen fixation and carbon assimilation rates, the C_2H_4:N_2 ratio and the percentage of nitrogen fixation to the total primary production [PP (nmoles C cm^{-2} d^{-1})] and new nitrogen production [Pnew (nmoles N cm^{-2} d^{-1})] in the Porcelana hot spring. The values were calculated from those obtained during daytime (12:00 to 14:00 h).

		Hourly rates				Daily rates			Ratios		% Nitrogen fixation contribution to		Input of daily nitrogen fixation to microbial mat
Year	T °C	Nitrogenase activity (nmol cm^{-2} h^{-1})	Nitrogen fixation (nmol N cm^{-2} h^{-1})	Nitrogen fixation[a] (nmol N cm^{-2} h^{-1})	Carbon assimilation (nmol C cm^{-2} h^{-1})	Nitrogenase activity (nmol cm^{-2} d^{-1})	Nitrogen fixation (nmol N cm^{-2} d^{-1})	Carbon assimilation (nmol C cm^{-2} d^{-1})	C_2H_4:N_2	C:N[b]	Total daily primary production PP (C)	Total new production Pnew (N)	g N m^{-2} y^{-1}
2009	46	50.0 ± 7.0	ND*	ND*	ND*	600 ± 84.1	ND*	ND*	ND*	ND*	ND*	ND*	ND*
2009	42	14.6 ± 1.8	ND*	ND*	ND*	175 ± 21.8	ND*	ND*	ND*	ND*	ND*	ND*	ND*
2009	40	11.3 ± 3.1	ND*	ND*	ND*	136 ± 36.6	ND*	ND*	ND*	ND*	ND*	ND*	ND*
2011	61	0.3 ± 0.1	ND*	ND*	ND*	3.6 ± 1.6	ND*	ND*	ND*	ND*	ND*	ND*	ND*
2011	57	0.5 ± 0.3	ND*	ND*	ND*	6.1 ± 3.0	ND*	ND*	ND*	ND*	ND*	ND*	ND*
2011	51	26.5 ± 6.2	ND*	ND*	ND*	318 ± 74.4	ND*	ND*	ND*	ND*	ND*	ND*	ND*
2012	52	16.5 ± 2.7	2.6 ± 0.3	1.4 ± 0.1	53.0±4.1	198 ± 32.4	31.4 ± 3.24	636±49	6.3	18.7	92.2	99.1	1.6
2012	47	22.9 ± 3.5	3.4 ± 1.8	4.6 ± 1.8	ND*	275 ± 42.5	40.2 ± 21.6	ND*	6.8	ND*	ND*	ND*	2.9
2013	66	0.2 ± 0.01	ND*	ND*	ND*	2.8 ± 0.1	ND*	ND*	ND*	ND*	ND*	ND*	ND*
2013	58	14.5 ± 1.6	6.7 ± 1.2	1.6 ± 0.3	45.8±8.7	174 ± 19.7	80.0 ± 14.3	550±105	2.2	9.1	132	99.8	4.1
2013	48	32.4 ± 3.6	7.8 ± 0.6	6.3 ± 1.8	ND*	388 ± 42.9	94.1 ± 6.9	ND*	4.1	ND*	ND*	ND*	4.8

ND*: Data not determined

[a]: Nitrogen fixation rates for 6 hours in situ incubation

[b]: C:N based on organic matter calculated by mass spectrometer instrument

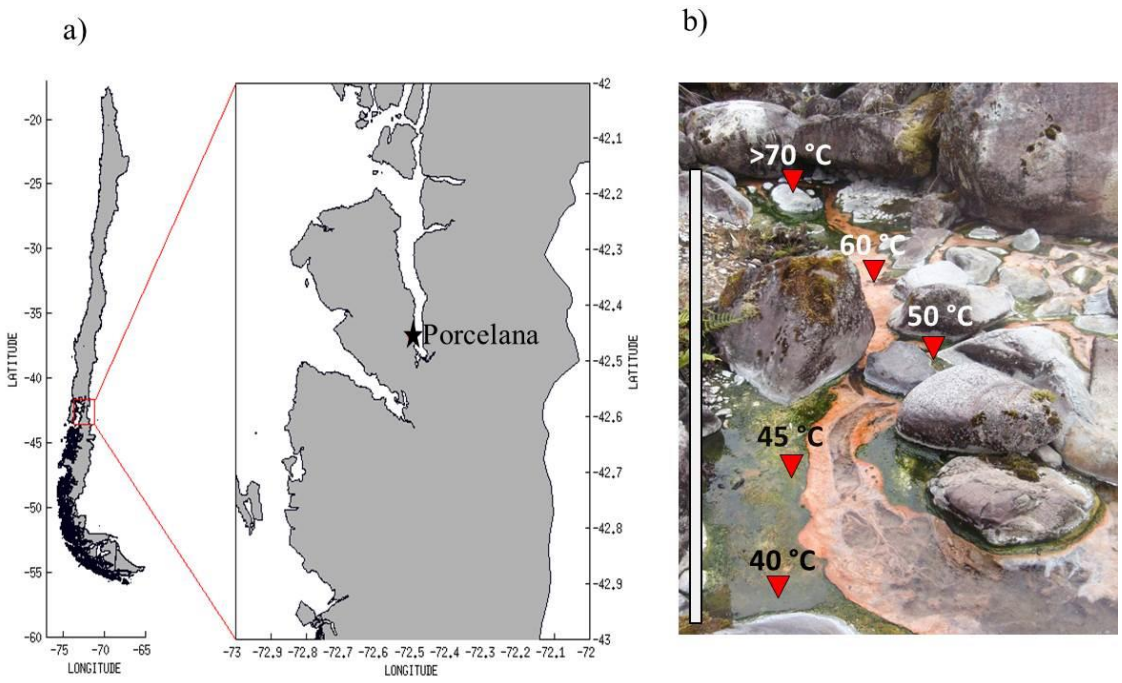


Figure 1. a) Location of the Porcelana hot spring in northern Patagonia, Chile (X Region, Comau fjord). b) The pigmented microbial mat was formed throughout the temperature gradient; the sampling sites are indicated by red triangles. The gray bar represents the mat extension (~10 m) within the thermophilic temperature gradient.

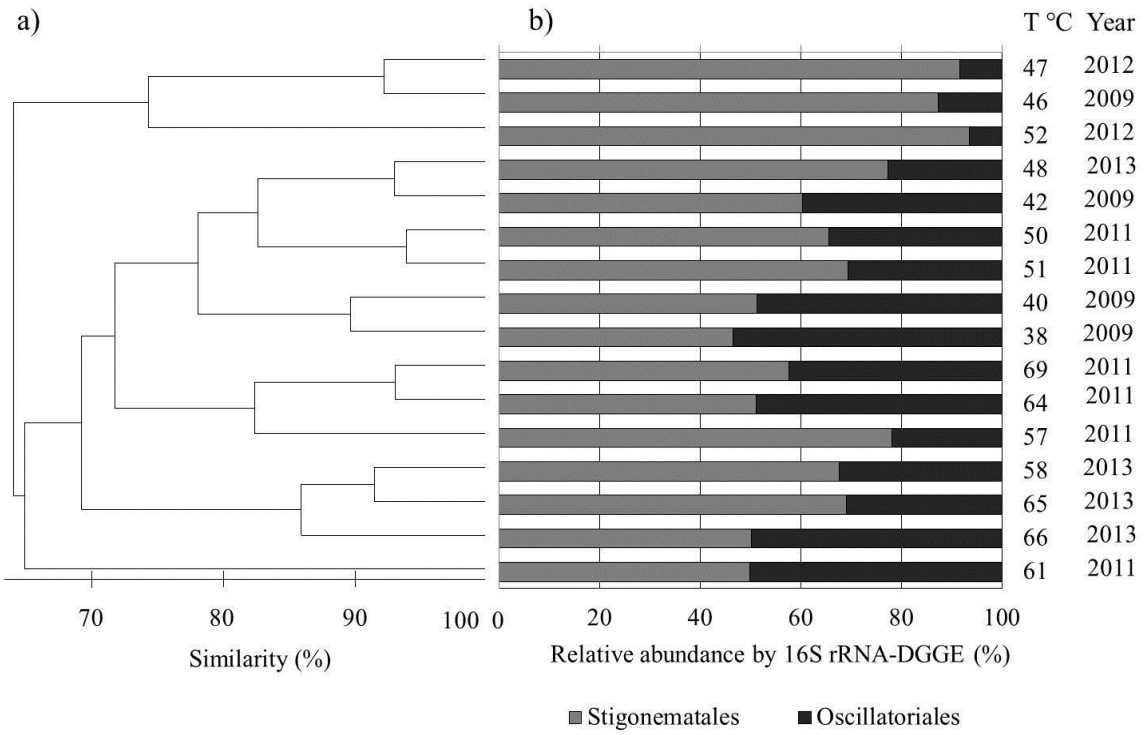


Figure 2. Comparison of the inter-annual cyanobacterial diversity at different temperatures in the Porcelana hot spring based on the 16S rRNA gene and denaturing gradient gel electrophoresis (DGGE). a) Bray-Curtis dissimilarity index dendrogram. b) Relative abundance of 16S rRNA-DGGE bands (phylotypes) for each temperature and year investigated.

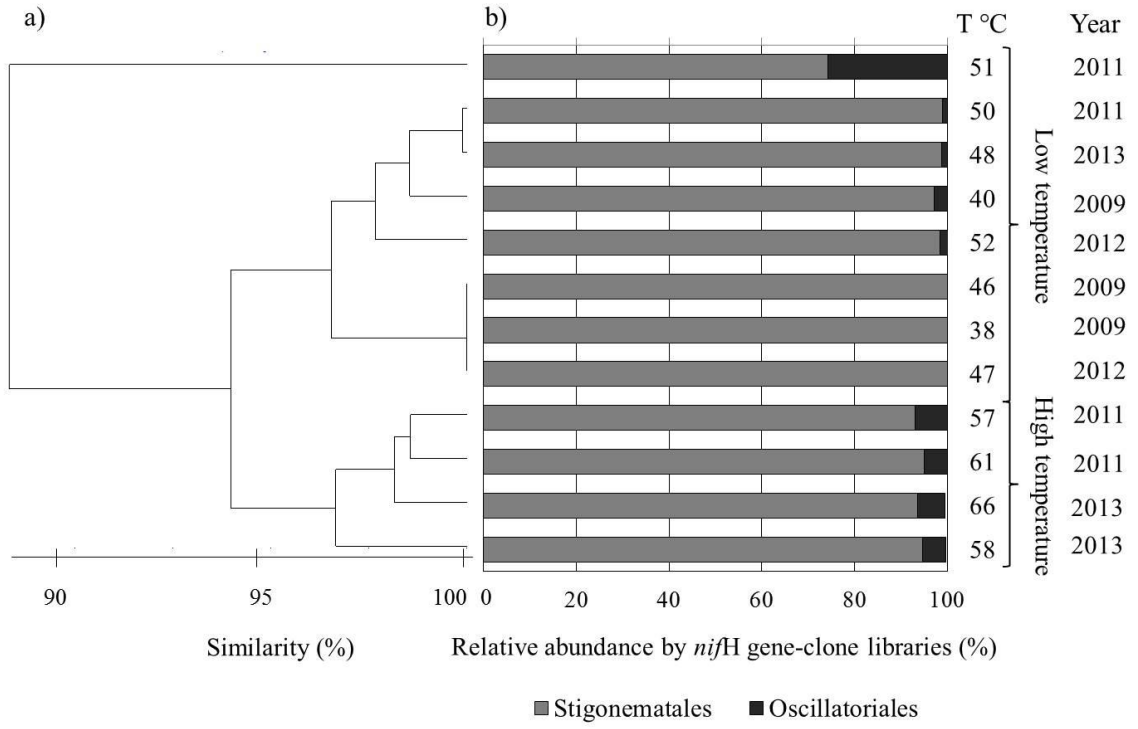


Figure 3. Relative abundance and inter-annual diazotrophic bacterial diversity in the Porcelana hot spring based on the *nif*H marker gene and clone libraries. a) Bray-Curtis dissimilarity index dendrogram. b) Relative abundance of the *nif*H gene (OTUs) determined using clone libraries obtained for each temperature and year investigated.

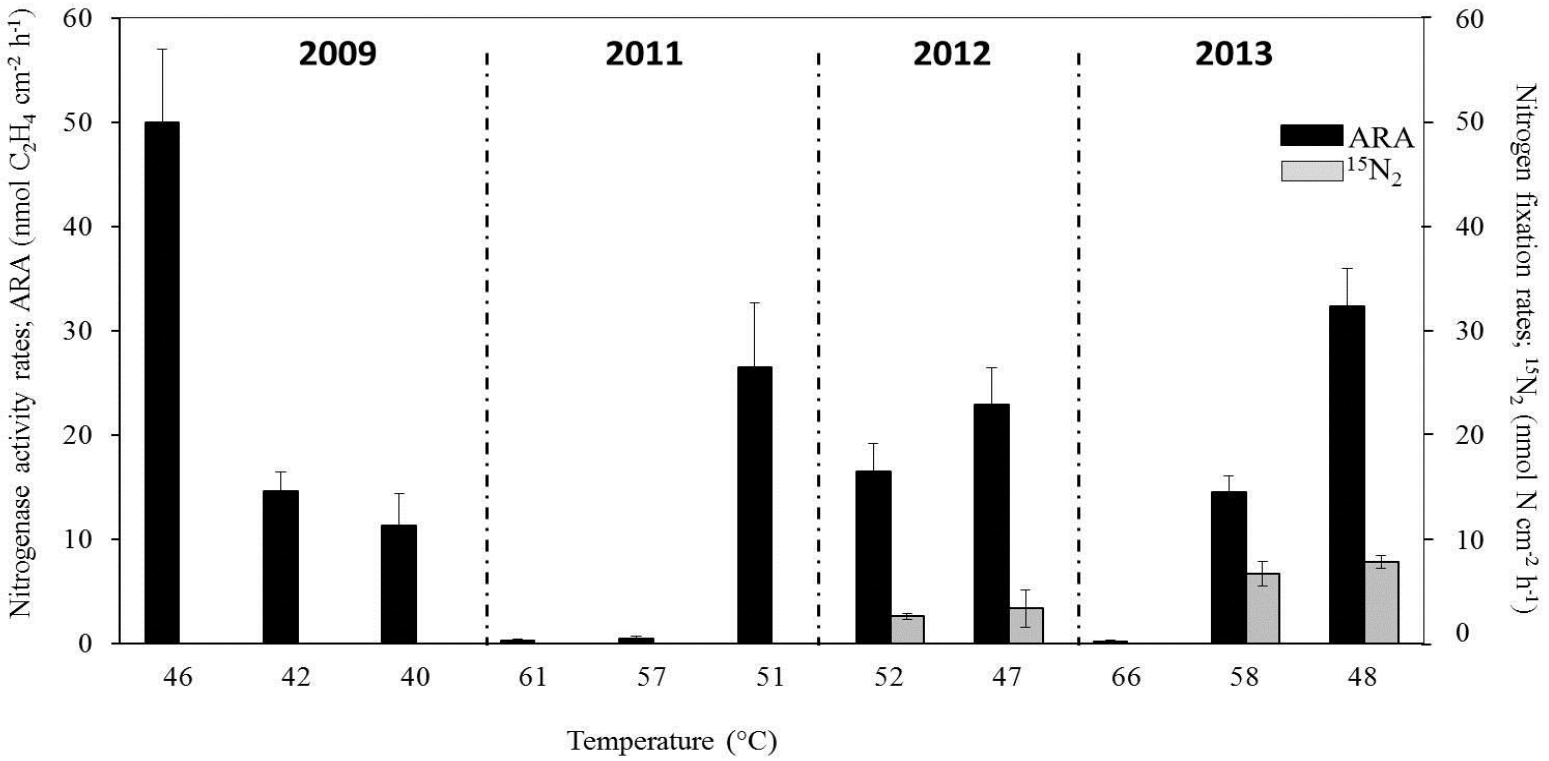


Figure 4. Nitrogen fixation assessed by the acetylene reduction assay (ARA) and $^{15}N_2$ uptake analysis for the different temperatures and years investigated. ARA measurements (black bars) were conducted during the four years, while $^{15}N_2$ uptake measurements (gray bars) were performed in 2012 and 2013.

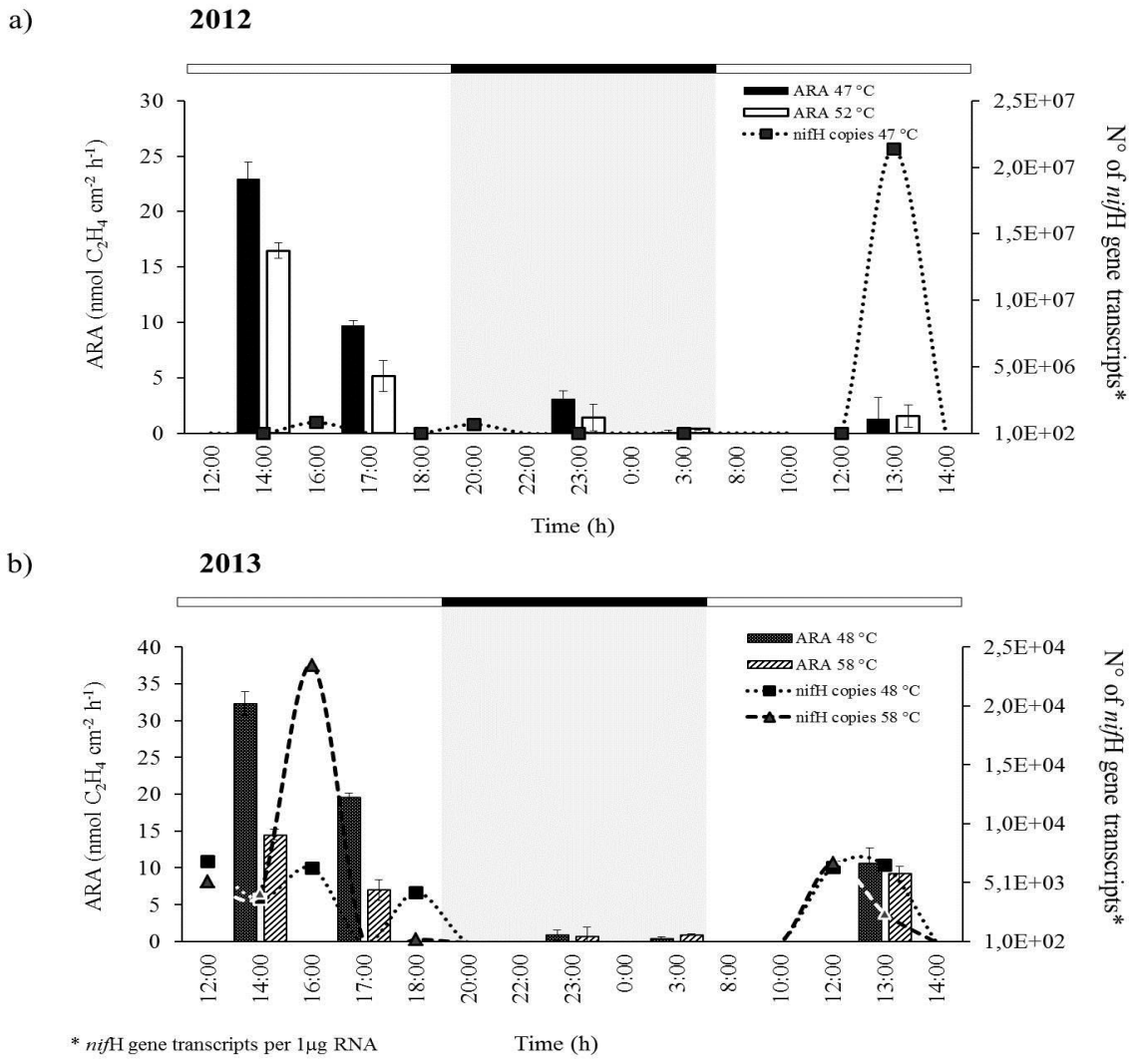


Figure 5. Diel cycles in nitrogenase activity (NA) and n*if*H gene expression in the Porcelana hot spring. a) Diel cycles at different temperatures in 2012. The bars represent ARA and the dotted line represents the number of *nif*H gene transcripts at 47 °C. b) Diel cycles at different temperatures in 2013. The bars and the dashed line represent activities at 58 °C and 48 °C. Error bars indicate the standard deviations. The top bar represents the light (white) and night (black) periods; the latter is also illustrated by gray shading.

Supplementary data

Table S1. Affiliation and accession number to OTUs from *nif*H gene clone libraries, 16S rRNA and *nif*H gene DGGE bands.

*nif*H gene clone libraries								
Year	T°C	Nº of nifH gene clones	OTUs and nº of clones sequenced per OTU	Order	Closest match	Query cover (%)	Identity (%)	Accession number
2009	46	61	OTU 1 (12)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507492
			OTU 2 (8)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507493
	40	53	OTU 1 (11)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507492
			OTU 2 (11)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507493
	38	55	OTU 1 (12)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507492
			OTU 2 (3)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507493
2011	61	74	OTU 1 (24)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507492
			OTU 2 (9)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507493
	57	85	OTU 1 (41)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507492
			OTU 2 (7)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507493
	51	83	OTU 1 (25)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507492
			OTU 4 (15)	Oscillatoriales	*Filamentous thermophilic cyanobacterium* sp.	96	89	KM507495
			OTU 5 (7)	Oscillatoriales	*Filamentous thermophilic cyanobacterium* sp.	96	89	KM507496
2012	52	80	OTU 1 (17)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507492
			OTU 2 (10)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507493
	47	89	OTU 1 (16)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507492
			OTU 2 (4)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507493
			OTU 3 (3)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507494
	66	90	OTU 1 (24)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507492
			OTU 2 (10)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507493
			OTU 4 (6)	Oscillatoriales	*Filamentous thermophilic cyanobacterium* sp.	96	89	KM507495
			OTU 5 (4)	Oscillatoriales	*Filamentous thermophilic cyanobacterium* sp.	96	89	KM507496
			OTU 6 (4)	Oscillatoriales	*Leptolyngbya* sp.	94	87	KM507497
2013	65	82	OTU 1 (18)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507492
			OTU 2 (10)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507493
	58	86	OTU 1 (26)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507492
			OTU 2 (10)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507493
			OTU 6 (8)	Oscillatoriales	*Leptolyngbya* sp.	94	87	KM507497
	48	82	OTU 1 (16)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507492
			OTU 2 (6)	Stigonemtales	*Mastigocladus laminosus*	95	99	KM507493

16S rRNA gene DGGE bands

Year	T°C	DGGE bands	Phylotypes	Order	Closest match	Query cover (%)	Identity (%)	Accession number
2009	46	2	CYA 1	Oscillatoriales	*Leptolyngbya* sp.	100	99	KJ696687
			CYA 5	Stigonemtales	*Mastigocladus laminosus/Fischerella* sp. JSC-11	100	100	KJ696694
	42	2	CYA 3	Oscillatoriales	*Leptolyngbya* sp.	100	99	KJ696689
			CYA 5	Stigonemtales	*Mastigocladus laminosus/Fischerella* sp. JSC-11	100	100	KJ696694
	40	2	CYA 2	Oscillatoriales	*Leptolyngbya* sp.	100	99	KJ696688
			CYA 5	Stigonemtales	*Mastigocladus laminosus/Fischerella* sp. JSC-11	100	100	KJ696694
	38	2	CYA 1	Oscillatoriales	*Leptolyngbya* sp.	100	99	KJ696687
			CYA 5	Stigonemtales	*Mastigocladus laminosus/Fischerella* sp. JSC-11	100	100	KJ696694
	69	4	CYA 3	Oscillatoriales	*Leptolyngbya* sp.	100	99	KJ696689
			CYA 4	Oscillatoriales	*Leptolyngbya* sp.	100	99	KJ696690
			CYA 5	Stigonemtales	*Mastigocladus laminosus/Fischerella* sp. JSC-11	100	100	KJ696694
2011	64	2	CYA 3	Oscillatoriales	*Leptolyngbya* sp.	100	99	KJ696689
			CYA 5	Stigonemtales	*Mastigocladus laminosus/Fischerella* sp. JSC-11	100	100	KJ696694
	61	2	CYA 5	Stigonemtales	*Mastigocladus laminosus/Fischerella* sp. JSC-11	100	100	KJ696694
			CYA 3	Oscillatoriales	*Leptolyngbya* sp.	100	99	KJ696689
	57	2	CYA 3	Oscillatoriales	*Leptolyngbya* sp.	100	99	KJ696689
			CYA 5	Stigonemtales	*Mastigocladus laminosus/Fischerella* sp. JSC-11	100	100	KJ696694
	51	2	CYA 3	Oscillatoriales	*Leptolyngbya* sp.	100	99	KJ696689
			CYA 5	Stigonemtales	*Mastigocladus laminosus/Fischerella* sp. JSC-11	100	100	KJ696694
2012	52	2	CYA 3	Oscillatoriales	*Leptolyngbya* sp.	100	99	KJ696689
			CYA 5	Stigonemtales	Mastigocladus laminosus/Fischerella sp. JSC-11	100	100	KJ696694
	47	2	CYA 3	Oscillatoriales	*Leptolyngbya* sp.	100	99	KJ696689
			CYA 5	Stigonemtales	*Mastigocladus laminosus/Fischerella* sp. JSC-11	100	100	KJ696694
2013	66	2	CYA 4	Oscillatoriales	*Leptolyngbya* sp.	100	99	KJ696690
			CYA 5	Stigonemtales	*Mastigocladus laminosus/Fischerella* sp. JSC-11	100	100	KJ696694
	65	2	CYA 4	Oscillatoriales	*Leptolyngbya* sp.	100	99	KJ696690
			CYA 5	Stigonemtales	*Mastigocladus laminosus/Fischerella* sp. JSC-11	100	100	KJ696694
	58	2	CYA 3	Oscillatoriales	*Leptolyngbya* sp.	100	99	KJ696689
			CYA 5	Stigonemtales	*Mastigocladus laminosus/Fischerella* sp. JSC-11	100	100	KJ696694
	48	2	CYA 4	Oscillatoriales	*Leptolyngbya* sp.	100	99	KJ696690
			CYA 5	Stigonemtales	*Mastigocladus laminosus/Fischerella* sp. JSC-11	100	100	KJ696694

*nif*H gene DGGE bands

Year	T°C	DGGE bands	Phylotypes	Order	Closest match	Query cover (%)	Identity (%)	Accession number
2009	46	1	CN 1	Stigonemtales	*Mastigocladus laminosus*	86	99	KJ696698
	42	1	CN 1	Stigonemtales	*Mastigocladus laminosus*	86	99	KJ696698
	40	2	CN 1	Stigonemtales	*Mastigocladus laminosus*	86	99	KJ696698
			CN 2	Stigonemtales	*Mastigocladus laminosus*	89	99	KJ696699
	38	3	CN 1	Stigonemtales	*Mastigocladus laminosus*	86	99	KJ696698
			CN 2	Stigonemtales	*Mastigocladus laminosus*	89	99	KJ696699
			CN 3	Stigonemtales	*Mastigocladus laminosus*	92	98	KJ696670
2011	69	3	CN 1	Stigonemtales	*Mastigocladus laminosus*	86	99	KJ696698
			CN 2	Stigonemtales	*Mastigocladus laminosus*	89	99	KJ696699
			CN 3	Stigonemtales	*Mastigocladus laminosus*	92	98	KJ696670
	64	3	CN 1	Stigonemtales	*Mastigocladus laminosus*	86	99	KJ696698
			CN 2	Stigonemtales	*Mastigocladus laminosus*	89	99	KJ696699
			CN 3	Stigonemtales	*Mastigocladus laminosus*	92	98	KJ696670
	61	3	CN 1	Stigonemtales	*Mastigocladus laminosus*	86	99	KJ696698
			CN 2	Stigonemtales	*Mastigocladus laminosus*	89	99	KJ696699
			CN 3	Stigonemtales	*Mastigocladus laminosus*	92	98	KJ696670
	57	3	CN 1	Stigonemtales	*Mastigocladus laminosus*	86	99	KJ696698
			CN 2	Stigonemtales	*Mastigocladus laminosus*	89	99	KJ696699
			CN 3	Stigonemtales	*Mastigocladus laminosus*	92	98	KJ696670
	50	3	CN 1	Stigonemtales	*Mastigocladus laminosus*	86	99	KJ696698
			CN 2	Stigonemtales	*Mastigocladus laminosus*	89	99	KJ696699
			CN 3	Stigonemtales	*Mastigocladus laminosus*	92	98	KJ696670
2012	52	3	CN 1	Stigonemtales	*Mastigocladus laminosus*	86	99	KJ696698
			CN 2	Stigonemtales	*Mastigocladus laminosus*	89	99	KJ696699
			CN 3	Stigonemtales	*Mastigocladus laminosus*	92	98	KJ696670
	47	2	CN 1	Stigonemtales	*Mastigocladus laminosus*	86	99	KJ696698
			CN 2	Stigonemtales	*Mastigocladus laminosus*	89	99	KJ696699
	66	2	CN 1	Stigonemtales	*Mastigocladus laminosus*	86	99	KJ696698
			CN 2	Stigonemtales	*Mastigocladus laminosus*	89	99	KJ696699
	65	3	CN 1	Stigonemtales	*Mastigocladus laminosus*	86	99	KJ696698
			CN 2	Stigonemtales	*Mastigocladus laminosus*	89	99	KJ696699
			CN 3	Stigonemtales	*Mastigocladus laminosus*	92	98	KJ696670
2013	58	3	CN 1	Stigonemtales	*Mastigocladus laminosus*	86	99	KJ696698
			CN 2	Stigonemtales	*Mastigocladus laminosus*	89	99	KJ696699
			CN 3	Stigonemtales	*Mastigocladus laminosus*	92	98	KJ696670
	48	3	CN 1	Stigonemtales	*Mastigocladus laminosus*	86	99	KJ696698
			CN 2	Stigonemtales	*Mastigocladus laminosus*	89	99	KJ696699
			CN 3	Stigonemtales	*Mastigocladus laminosus*	92	98	KJ696670

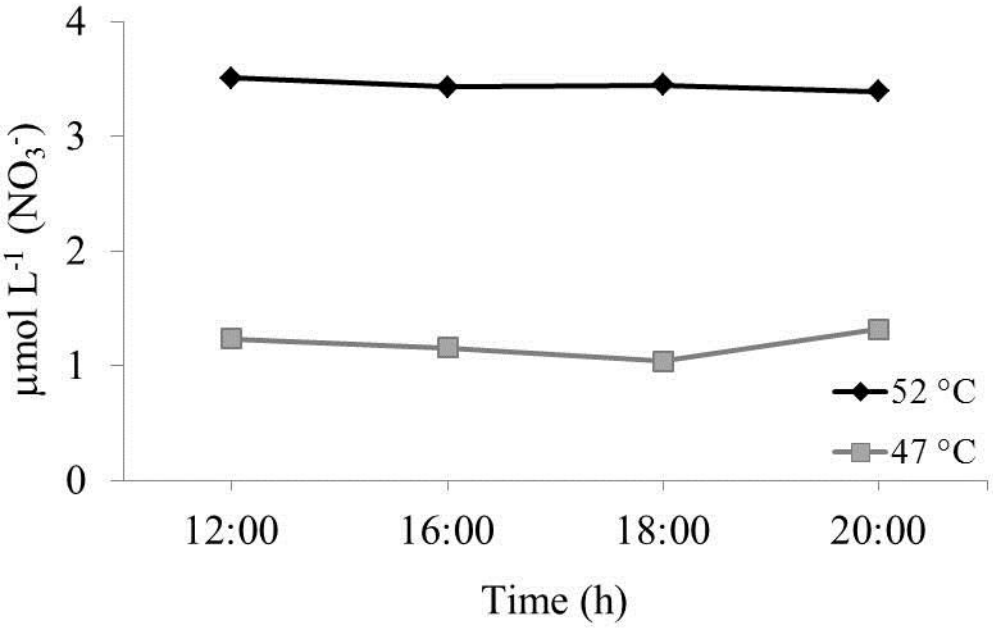


Figure S1. Nitrate concentration along the light period for temperatures of 52 and 47 ºC during year 2012 at Porcelana hot spring.

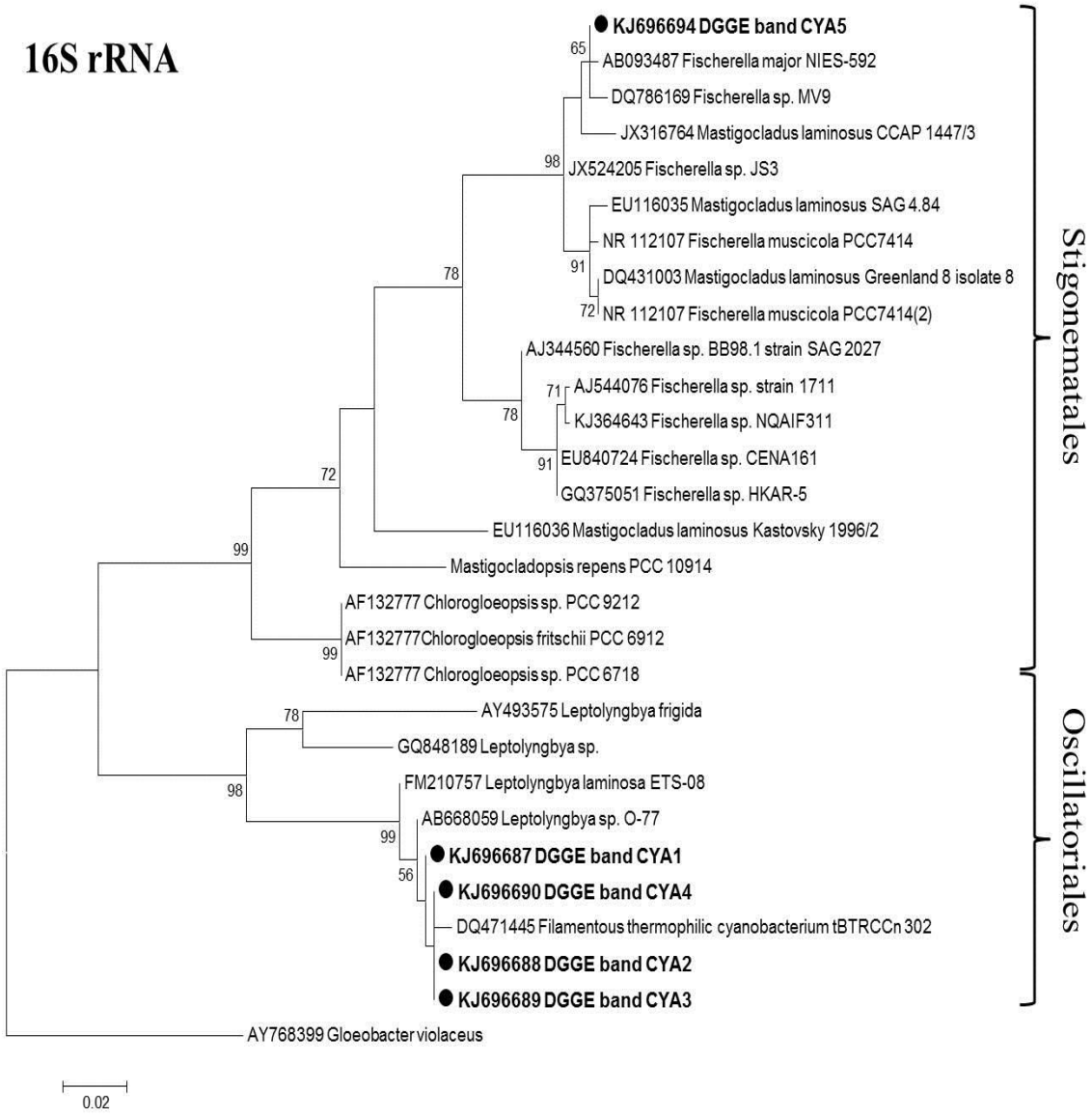


Figure S2. Phylogenetic affiliation of sequences retrieved using cyanobacterial specific 16S rRNA genes as targets combined with DGGE analyses of microbial mat associated DNA samples from Porcelana hot spring (Chile). The phylogram was constructed using the maximum likelihood distance method. Sequences obtained are given in bold, and each DGGE phylotype is designated by their accession number. The numberings are the same as those given in the 16S rRNA-DGGE profiles in Fig. S3a. The numbers given at the nodes represent bootstrap values of 10.000 replicates.

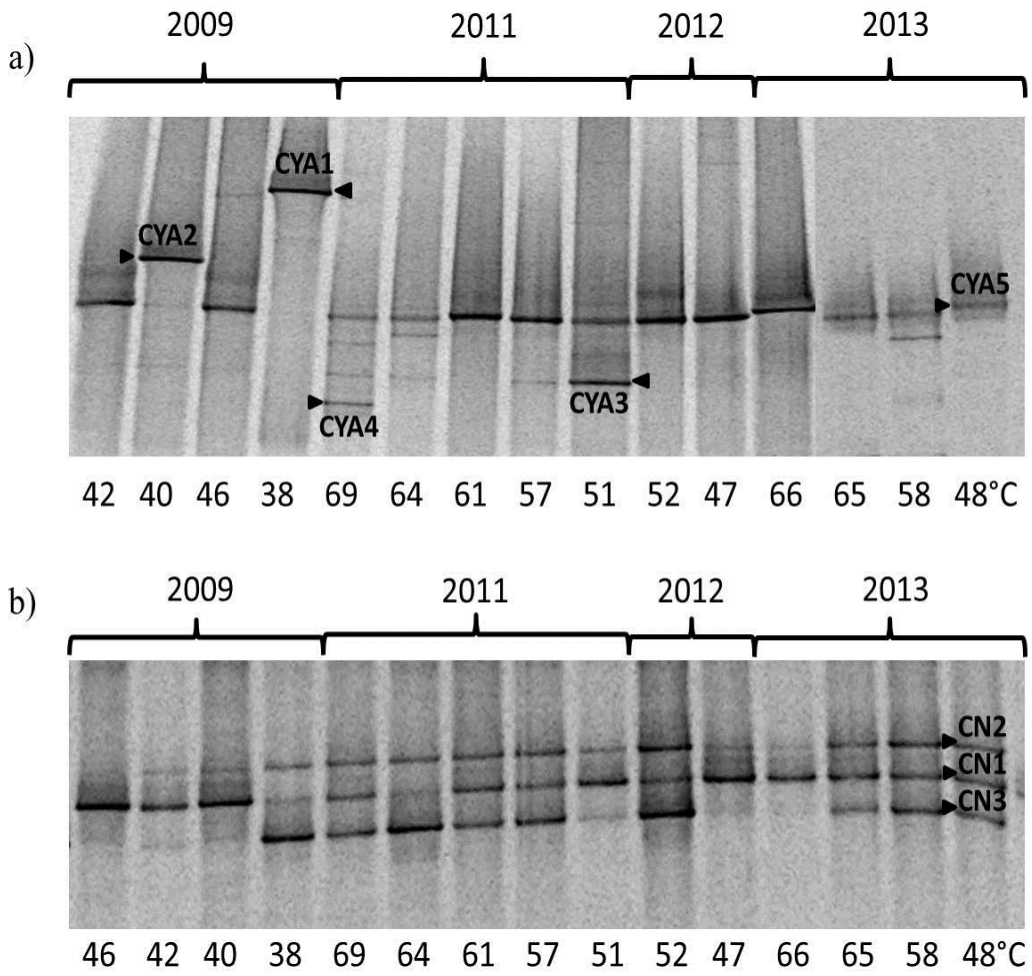


Figure S3. DGGE fingerprint images showing microbial mat associated DNA samples along the temperature gradient and the four years investigated at Porcelana hot spring (Chile). For identity results (phylogenetic reconstructions), see Supplementary data, Figure S2 and S4. a) Image of the 16S rRNA gene analysis with cyanobacterial specific 16S rRNA primers, indicating the sequenced bands CYA1-CYA5. b) Image of the *nif*H gene analysis with cyanobacterial specific *nif*H primers, indicating the sequenced bands CN1-CN3. Abbreviations used in a): CYA1, CYA2, CYA3, CYA4 = Oscillatoriales and CYA5 = Stigonematales. Abbreviations used in b) CN1, CN2 y CN3= Stigonematales.

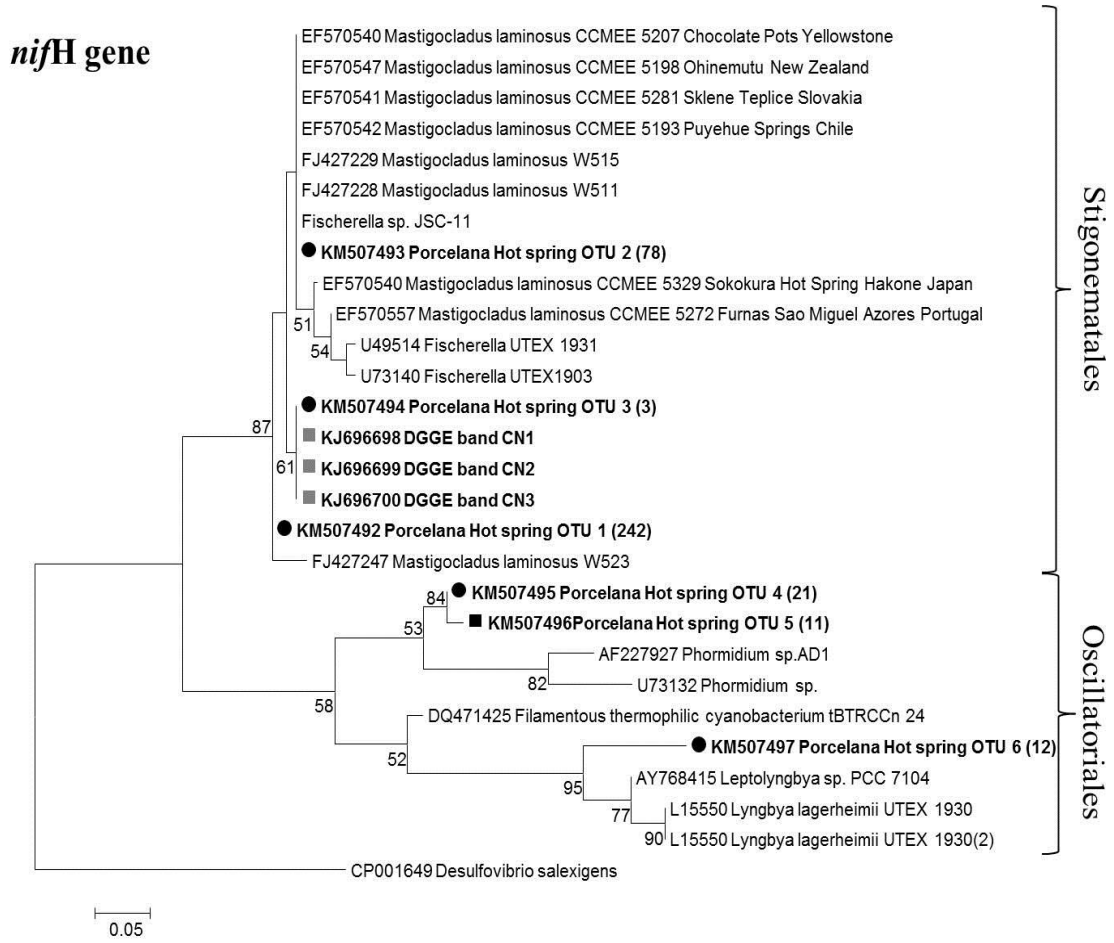


Figure S4. Phylogenetic affiliation of sequences retrieved using universal and cyanobacterial specific *nif*H genes as targets combined with clone libraries (OTUs) and DGGE (phylotypes) analyses of microbial mat associated DNA samples from Porcelana hot spring (Chile). The phylogram was constructed using the maximum likelihood distance method. Sequences obtained are given in bold, and each OTU and DGGE phylotype is designated by their accession number. The numberings are the same as those given in the Supplementary data, Table S1 and *nif*H-DGGE profiles in the Fig. S3b. The numbers given at the nodes represent bootstrap values of 10.000 replicates.

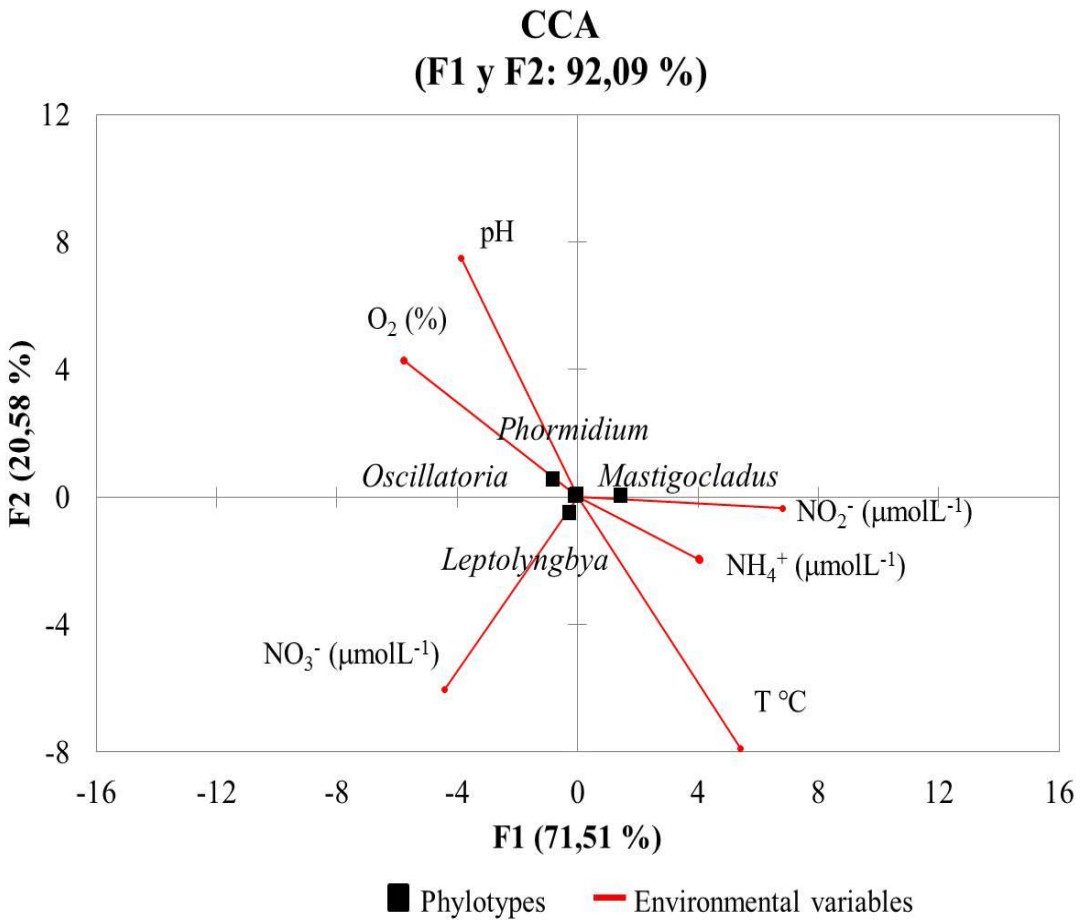


Figure S5. Canonical Correspondence Analysis (CCA) for the total environmental and biological variables at the four years of study (2009, 2011-2013).

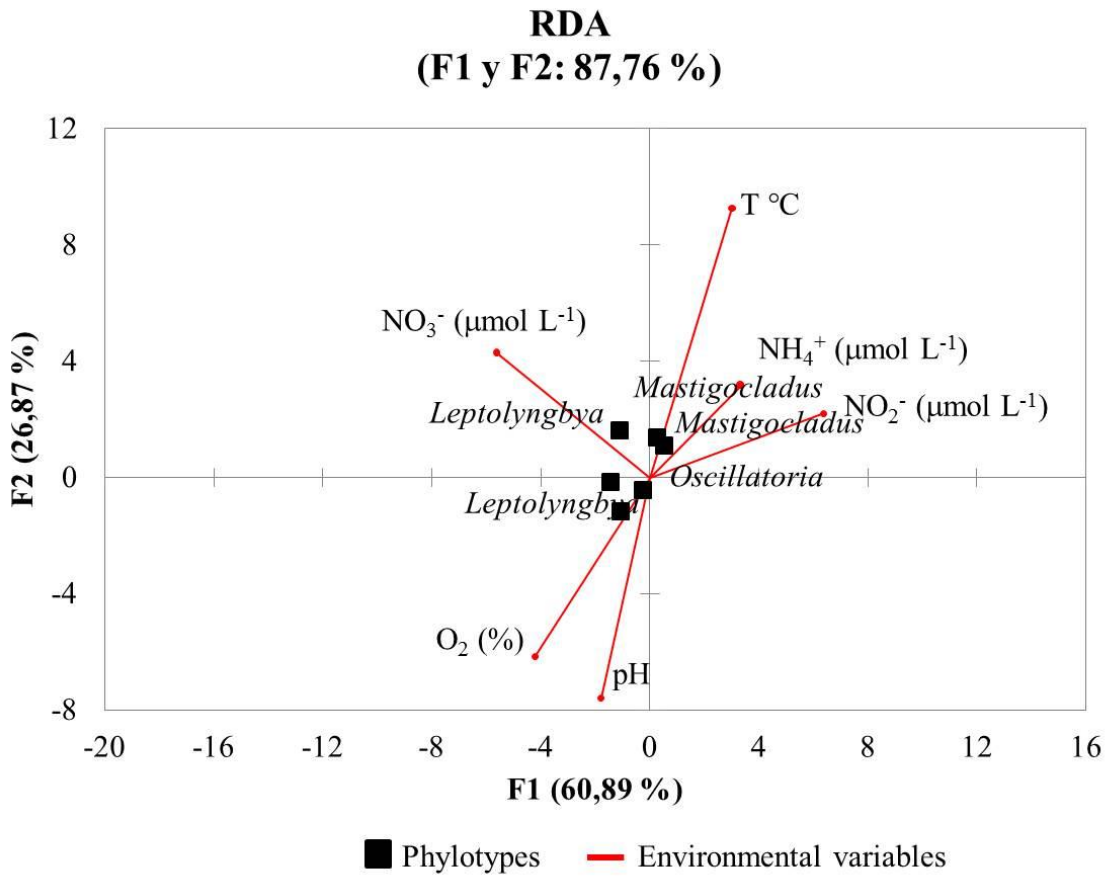
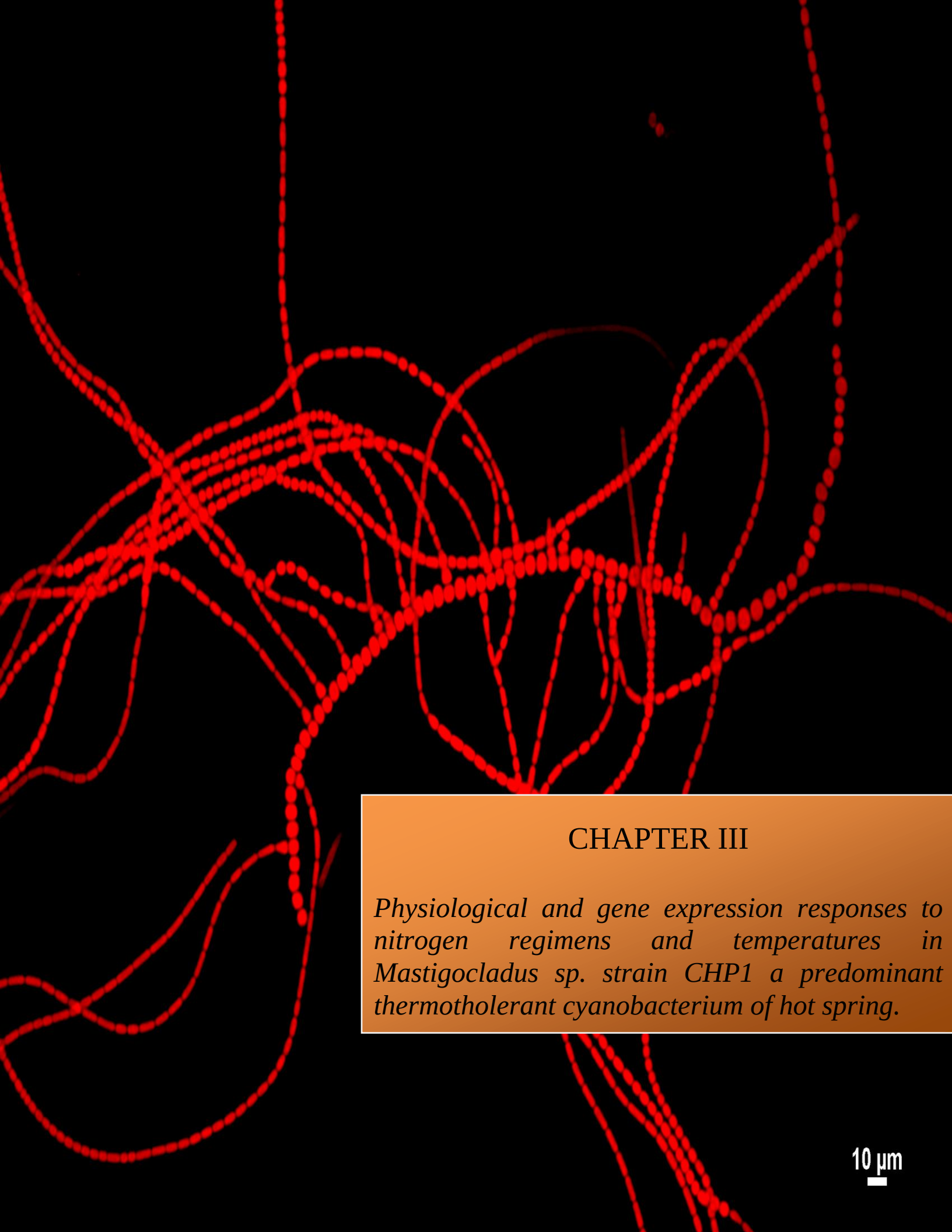


Figure S6. Redundancy analysis (RDA) for total environmental and biological variables obtained at the four years of study (2009, 2011-2013).



CHAPTER III

Physiological and gene expression responses to nitrogen regimens and temperatures in Mastigocladus sp. strain CHP1 a predominant thermotholerant cyanobacterium of hot spring.

Physiological and gene expression responses to nitrogen regimes and temperatures in *Mastigocladus* sp. strain CHP1, a predominant thermotholerant cyanobacterium of hot springs

M. Estrella Alcamán[1], Jaime Alcorta[1], Birgitta Bergman[2], Mónica Vásquez[1], Martin Polz[3], Beatriz Díez[1, 4*]

[1]*Department of Molecular Genetics and Microbiology, Pontificia Universidad Católica de Chile, Libertador Bernardo O´Higgins 340, Casilla 144-D, C.P. 651 3677, Santiago, Chile.*

[2]*Department of Ecology, Environment and Plant Sciences and Science for Life Laboratory, Stockholm University, S-10691 Stockholm, Sweden.*

[3]*Environmental Science and Engineering, Department of Civil and Environmental Engineering, Massachusetts Institute of Technology (MIT), 15 Vassar Street, Cambridge, MA 02139, USA.*

[4]*Center for Climate and Resilience Research (CR)2, Chile*

Running title

Gene expression, *Mastigocladus* sp., nitrate/ammonium preference, thermotolerance

*Correspondence: Beatriz Díez, Department of Molecular Genetics and Microbiology, Faculty of Biological Sciences, Pontificia Universidad Católica de Chile, Libertador Bernardo O´Higgins 340, Casilla 144-D, C.P. 651 3677, Santiago, Chile.

E-mail: bdiez@bio.puc.cl

Abstract

Cyanobacteria are widely distributed primary producers with significant implications for the global biogeochemical cycles of carbon and nitrogen. Diazotrophic cyanobacteria from subsection V (Order Stigonematales) are particularly ubiquitous in hot springs photoautotrophic microbial mats. A Stigonematal cyanobacterium isolated from the Porcelana hot spring (Chile) was recently identified as *Mastigocladus* (branched, heterocystous filaments) and to represent a major player in this spring by contributing with most of the new nitrogen through nitrogen fixation. A further morphological and genetic characterization of this key-player (strain CHP1), using epifluorescence and transmission electron microscopy (TEM) as well as 16S rRNA and *nif*H gene based phylogenies, verified the placement of the strain CHP1 within the Stigonematales, and that it forms a separate clade together with other thermophiles of the genera *Fischerella* and *Mastigocladus*, while being moderately thermophilic (grows at 50°C but not at 60ºC). The strain CHP1 fixes maximum atmospheric dinitrogen (nitrogenase activity) in light, independent of the thermophilic temperature range. However, the gene expression represented by *nif*H transcripts levels was arrhythmic at 45ºC remaining constant during the light/dark period, while at 50ºC *nif*H transcripts showed a high expression during the light period. A high affinity for nitrate and a low tolerance to high ammonium concentrations was apparent; and the *gln*A and *nar*B genes, related to ammonium and nitrate assimilation, showed higher expression levels in light and early into the dark phase; hence, certain plasticity in regards to the use of these two different nitrogen sources was evidenced. It is proposed that the diazotrophic cyanobacterium *Mastigocladus* sp. strain CHP1 is a good model to study thermophilic and other possible adaptations of photoautrophic organisms inhabiting microbial mats in hot springs globally.

Introduction

Cyanobacteria are microorganism responsible for the oxygenation of the Earth's atmosphere (Buick [1]), thereby structuring the biosphere for the evolution of plants and animals. Today, cyanobacteria colonize successfully almost any illuminated environment and numerous plant hosts (Whitton and Potts [2] and Usher et al. [3]). Furthermore, being important primary producers in many environments they are also responsible for a substantial proportion of the new nitrogen fixed into the oceans (Capone et al. [4]), soils (Belnap [5] and DeLuca et al. [6]), lakes (Stewart et al. [7]), and hot springs (Castenholz [8]). Also, cyanobacteria exhibit a remarkable morphological diversity with their members being subdivided into subsections I - V (Rippka et al. [9]), with the last two subsections, being largely supported by recent 16S rRNA gene phylogeny (Wilmotte and Herdman [10]). Recently, Dagan et al. [11] showed that members of subsections IV and V cyanobacteria have a pronounced genetic similarity with eukaryotic nuclear genes depending on their cyanobacterial origin (plastid ancestors). While our understanding of multicellularity in subsection IV has deepened considerably by studying *Anabaena* sp. PCC7120 as a model organisms (Flores and Herrero [12]), there is little known about cyanobacteria of subsection V.

Members of the Order Stigonematales (subsection V) are among the most complex cyanobacterial types, divided into eight families that show thermophilic, branching and diazotrophic features. Till today, the genomes of six genera from this order have been sequenced, but only one is been completed (*Fischerella* sp. NIES-3754; Hirose et al. [13]). Among these genomes, only members within families Fischerellaceae, Mastigocladaceae and Chlorogloeopsaceae have thermal representatives, and are characterized by true lateral

branching filaments (Anagnostidis and Komarek [14]). The Stigonematales are commonly found in a variety of terrestrial environments (Srivastava et al. [15], Roldán and Hernández-Mariné [16], Singh et al. [17] and [6]), including endolithic cavities (Chacón et al. [18] and Gaylarde et al. [19]), and microbial mats of polar (Lacap et al. [20], Roeselers et al. [21], Boomer et al. [22] and Soe et al. [23]) and hot springs ecosystems ([7], [Alcamán et al., [24], Stewart [25], Miller et al. [26], Mackenzie et al. [27]), considering these as cosmopolitan organisms of relevant importance as phototrophs and diazotrophic cyanobacteria [8]. The best understood an organism within this section is probably *Mastigocladus laminosus*, which is a major component of epilithic microbial mats in Yellowstone National Park (YNP, USA) [26].

In filamentous cyanobacteria, growth under nitrogen depletion, show changes in photosynthetic vegetative cells ultrastructure with distinct reorganization patterns of subcellular components (*e.g.* thylakoid membranes, cell walls) (Stevens et al. [28] and Kumar et al. [29]), eventually leading to the differentiation of heterocysts, specialized nitrogen-fixing cells (Malder and Muro-Pastor [30]). Most N_2-fixing cyanobacteria are mesophilic and show optimum N_2-fixing ability in the moderate temperature range of 20-25°C (Issa et al. [31]). At high temperature various metabolic processes including N_2-fixing ability might be adversely affected, but information about N_2-fixing and assimilation is scanty in the case of thermophilic cyanobacteria.

Members of the Stigonematales in hot springs, specifically *Mastigocladus* sp. have been found to be dominant and responsible for the nitrogen fixed at temperatures between 28 to 60°C (Wickstrom [32], Stewart [33], [24] and [25]). In accordance, 60ºC is the highest temperature recorded so far for the presence of N_2-fixing filamentous cyanobacteria with a proposed upper temperature limit for *in situ* nitrogen fixation. This temperature is also near

the upper limit for photosynthesis, suggesting that the major biological mechanisms for capturing gaseous C and N are restricted by temperature (Belay et al. [34]). The features of *Mastigocladus* spp. make them ecologically important as a component of microbial mats in neutral to alkaline thermal springs (Casztenholz [35]). In the Chilean hot spring of Porcelana, *in situ* assays have showed that maximum *nif*H gene transcript levels were associated with the genus *Mastigocladus*, which correlated with the higher nitrogenase activity observed during the light period [24].

In cyanobacteria the assimilation of other N sources as ammonium is usually the preferred inorganic nitrogen source of cyanobacteria, due to its lower energy requirement. Ammonium is generated from reduction of nitrate, through nitrogen fixation or directly through incorporation via the permease transport system (Amt) and subsequently incorporated through the GS;*gln*A–GOGAT;*glt*S route into amino acids and proteins (Muro-Pastor et al. [39]). In the other hand, nitrate assimilation involves the transport system encoded by *nrt*ABCD genes, and the subsequent reduction into ammonium is catalyzed by the nitrate reductase (NR; *nar*B) and nitrite reductase (NiR; *nir*A) enzymes (Flores et al. [36], Rubio et al. [37], Frías et al. [38]).

Under controlled conditions the thermophilic Stigonematal *Mastigocladus laminosus*, shows a typical phenotypic (differentiation of heterocysts) and genetic (*nif*H gene expression; encoding one of the subunits in the nitrogenase enzyme) responses to the lack of combined nitrogen ([28], Miller et al. [40] and Khumanthem et al. [41]). Further, a thermotolerant *Mastigocladus laminosus* species isolate from the Jakrem hot spring in Meghalaya (India), showed that nitrogenase activity is inhibited in presence of combined nitrogen sources, such as nitrate (NO_3^-), ammonium (NH_4^+) and amino acids [41], similar to that also established for other non-thermal heterocyst-forming cyanobacteria (*e.g.*

Anabaena sp. PCC7120; subsection IV) (Meeks et al. [42], Vintila et al. [43, 44], Martin-Nieto et al. [45] and [36]). This thermophilic *Mastigocladus laminosus* species was able to efficiently assimilate NO_3^- and NH_4^+ at 45°C under laboratory conditions [41]; however, its behavior under different thermophilic temperatures and levels of gene transcripts of the principal enzymes involved in the substrate incorporation, as well as the genomic context of genes involved in the nitrogen assimilation are unknown as the temperature dependency. Recently, it was shown that Stigonematal members and specially *Mastigocladus* spp. are the most active and relevant nitrogen producers at high temperatures in the Chilean Porcelana hot spring [24]. According to that a representative of the Stigonematales was isolated from the microbial mat of Porcelana, denoted CHP1 (CHilean Porcelana 1). This strain was tentatively identified as belonging to the genus *Mastigocladus*, based on morphological features, such as type of branching [14]. The aim of the present study was corroborate the affiliation and genetic adaptation of this prominent and highly relevant phototrophic and diazotrophic thermophilic strain CHP1 under controlled conditions, in regards to the regulation of its nitrogen fixation and combined nitrogen assimilation at high temperatures. This was evaluated morphologically (morphology and subcellular structures) and genetically (using ribosomal and functional marker genes) under controlled conditions by following growth, nitrogen fixation (nitrogenase enzyme activity), nitrogen assimilation (isotopic ^{15}N uptake) and the expression of related key genes (*nif*H, *nar*B, *gln*A) at different temperatures (45-60°C). Our data demonstrated that strain CHP1 is affiliated with the Stigonematal thermophilic clade formed by *Mastigocladus* spp., and performs optimally (growth and nitrogenase activity) at 45-50ºC, further stressing this strain as a major nitrogen provider in the Porcelana hot spring [24]. Also, its metabolic temperature dependency was consistent with the previous *in situ* study [24], therefore *Mastigocladus*

sp. strain CHP1 become an optimal thermophilic model to future inquiries into the cellular and genetic adaptations at high temperatures for phototrophic organisms inhabiting microbial mats.

Material and Methods

CHP1 strain isolation in culture and different temperature growth curves

The CHP1 strain was collected at 46°C from Porcelana hot spring located in Northern Patagonia Chile (42º 27’ 29.1’’S - 72º 27’ 39.3’’W). This isolate was obtained through the serial dilution technique under microscope observation until obtaining one unique filament. The filament was plated on sterile BG11 medium [9], and grown up at three different temperatures; 45, 50 and 60°C. Each plate was maintained in batch culture under 12light-12dark photoperiod with 30 µmol photons m^{-1} s^{-1} of white-light intensity. After three weeks of growth the biomass formed was transferred to a borosilicate glass flask (250 ml) and maintained for one month at the condition described above until obtained enough biomass to perform the different temperature growth curves.

Growth curves were conducted in batch cultures (500 ml flask) using BG11 free-nitrogen ($BG11_0$), and BG11 medium supplemented separately with $NaNO_3$ 9mM ($BG11_{NO3}$) and NH_4Cl 200µM ($BG11_{NH4}$) at 45, 50 and 60°C. Growth was measured as increase in concentration of Chlorophyll *a* (Chl *a*) content and dry weight (mg L^{-1}). The culture curves were initiated with an inoculum of 2 mg L^{-1} of Chl *a* at each temperature and N supplement condition: two replicates of 200 ml were used for Chl *a* determinations, and six replicates of 200 ml each for dry weight measurements. Samples for Chl *a* determinations were obtained every three days and processed according to Chorus and Bartram [46], but modified with an additional step to homogenize the cells by bead beating (4.0 ms^{-1} for 2

min) using solid-glass beads (1 mm). In the course of 42 days, every 7 days one replicate (200 ml flask) was filtered using filter isopore™ 10 μm (Merck Milipore, USA), and the pellet was dried at 60°C for dry biomass determinations.

DNA extraction to genome sequencing

The culture biomass of CHP1 strain grown up at 50°C was selected to DNA extraction. Primarily 250 ml of biomass was washed using BG11-Triton, and then incubated with 5 mg ml^{-1} of lysozyme, and over night (ON) incubation under stirring with Kanamycin (100 $\mu g ml^{-1}$) and Spectinomycin (20 $\mu g ml^{-1}$) antibiotics, to eliminate the accompanying bacteria. DNA extraction and purification was performed as described by Alcamán et al. [24]. The genomic DNA obtained was then sequenced according to Rodrigue et al. [47]. Briefly, DNA molecules were end-repaired and phosphorylated according to manufacturer's recommendations (End-repair kit, Enzymatics or New England Biolabs). The amplified libraries were subjected to an additional round of AMPure XP SPRI beads purification to remove residual primers and adapter dimers. The samples were next quantified by real-time PCR on a LightCycler II 480 (Roche). Illumina libraries were loaded on to an Illumina GAIIx sequencer, and data was analyzed using the Illumina pipeline 1.4.0 to generate fastq files. The raw sequences were cleaned of barcode and the quality was checked with fastqc software and filtering with trimmomatic (Bolger et al. [48]). Finally, the CHP1 genome was assembled with SPAdes assembler (Bankevich et al. [49]), and annotated with PROKKA software (Seemann [50]).

Phylogenetic analysis of Porcelana strain CHP1

RNAmmer software (Lagesen *et al*. [51]) was used to search complete 16S rRNA gene sequence (>1300 pb) for all available Stigonematales in the GenBank public database (only sequences from published studies or cultures were included), and at five genomes sequenced by own laboratory, including strain CHP1 (CHP1 complete 16S rRNA gene was deposited in the public database with the accession number KXO35101). Also, partial 16S rRNA gene sequences (~1300 pb) from SILVA SSU database were used for comparisons between subsections. All sequences were aligned using MEGA5.2 software (Tamura et al. [52]) with the ClustalW algorithm (Tom Hall, Ibis Therapeutics, Carlsbad, CA, USA). Substitution models used for the phylogenetic analysis were cheeked by Model Selection (ML) option of MEGA5.2 software by the lowest BIC score obtained for each dataset [51]. The subsequent 16S rRNA gene phylogenetic reconstruction was performed using the maximum likelihood search strategy, Kimura 2-parameters substitution model with G+I rates between sites, and 10.000 bootstrap replicates. Additionally, a phylogenetic reconstruction of the *nif*H gene from all available Stigonematales including CHP1 was performed using the GTR substitution model and the same procedure mentioned above for the 16S rRNA gene sequences. The sequences of *Trichodesmium erythaeum* IMS101 and *Gloeobacter violaceus* PCC7421 were used as outgroups for the 16S rRNA, and *Trichodesmium erythaeum* IMS101 for the *nif*H gene phylogenetic reconstructions.

Morphological characterization by light microscopy and TEM

From each culture growth curve condition, two independent samples of 1 ml were fixed with 2.5% glutaraldehyde for morphological characterizations. One sample was used for light microscopy morphological characterization using a microscope Labophot-2 (Nikon,

Japan) coupled with mercury lamp of 100W (LH-M100C-1 Model), and the other 1 ml sample was used for transmission electron microscopy (TEM) analysis. The morphological characterization was done according to Anagnostidis and Kómarek [14], and dimensions of vegetative cells in the branching and leader filament according to Kastovsky and Johansen [53]. For TEM, 500 µL of fixed cells with 2.5% glutaraldehyde in cacodylate buffer (0.1M y pH 7.2) were maintained for 16 h at room temperature. The cells were washed with cacodylate buffer (0.1M y pH 7.2) for 2 h at 4ºC, and post-fixed with 1% (w/v) osmium tetroxide during 90 min, rinsing with distilled water, and stained with uranyl acetate at 1% for 60 minutes. Then, cells were dehydrated in acetone (50%, 70%, twice 95% y three times 100%) and polymerized in Epon:Acetone (1:1) overnight, and finally polymerized in Epon at 60°C for 24 h. Ultra-thin sections (70 nm) were obtained in ultramicrotome Sorval MT-5000 (MTS, USA) mounted on Formvar-coated copper grids and stained with uranyl acetate 4%, followed by lead citrate. The samples were examined and photographed in a Philips Tecnai (Philips, USA) electron microscope operating at 12 a 80 kV with integrated BioScan camera model ccd Megaview G2 (Olympus-Sis, Japan). The heterocyst characterization was done according to Nierzwicki-Bauer et al. [54]).

Nitrogen assimilation assays in growth culture curves

Based on the results of growth culture curves, biomass in exponential phase was used to start the daily cycle of nitrogen fixation ($^{15}N_2$: ARA) and nitrogen assimilation ($K^{15}NO_3$ and $^{15}NH_4Cl$) assays. The nitrogen fixation measures were performed by activity of the nitrogenase enzyme (acetylene reduction assays: ARA) and the isotopic technique ($^{15}N_2$ isotopic uptake). ARA assay was performed at 45 and 50°C, with three biological replicates (3 culture flasks of 250 ml). 5 ml of CHP1 culture from each replicate was

placed in quadruplicate into sterilized 10 ml glass incubation vials. The assays started when 1 ml of air in each vial is replaced with 1 ml of acetylene gas (10-20% of the gas phase), and then incubation for 3 hours. Additionally to the four replicates, two controls (one with CHP1 culture but no acetylene gas and one containing only acetylene gas) were conducted. The samples were incubated in six periods of 3 hours each (08:00-11:00; 11:00-14:00: 15:00-18:00, 19:00-22:00; 23:00-02:00; 03:00-06:00) over a dial cycle. From each vial 4 ml of the gas phase was withdrawn using a hypodermic syringe and transferred to a 5 ml BD vacutainer (no additive Z plus tube, REF367624). The ethylene produced was analyzed by injecting 1 ml of the gas using a gas-tight syringe (Hamilton) into a GC-8A gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with an 80/100 Porapak Q (Supelco, St. Louis, MO, USA) 1 m x 1/4" column and a flame ionization detector using helium as the carrier gas. The nitrogenase activity was calculated from the ethylene produced, corrected by controls and expressed per dry weight and incubation time.

In parallel to the ARA assays, $^{15}N_2$ uptake experiments were performed in 150 ml polycarbonate bottles. The ^{15}N assimilation experiments (N_2) were initiated through the addition of 1 ml of $^{15}N_2$ gas (98% atom $^{15}N_2$ gas, Sigma-Aldrich) through a gas-tight syringe into the bottles. Additionally, two replicates of 20 ml each (without isotope $^{15}N_2$) at each temperature (45 and 50ºC) were collected to determine the natural isotopic composition (control). Six bottles were inoculated at the same time (08:00 h) and harvested after 3, 6, 10, 14, 18 and 24 hours. After each incubation period the content of each bottle was filtered (filter isopore™ 10 μm; Merck Millipore, USA), and the pellets obtained were dried at 70°C for 48 h. Measurements of ^{15}N atom incorporation (AT ^{15}N) and particulate organic nitrogen (PON) were performed using a mass spectrometer (IRMS Delta V

Advantage, Thermo Finnigan, Laboratory of Biogeochemistry and Applied Stable Isotopes: LABASI). Calculations of the ^{15}N rates were performed as described by Alcamán et al. [24] and Fernandez et al. [55], including corrections by dilutions of $^{15}N_2$ gas and controls.

Isotopic ammonium ($^{15}NH_4$) and nitrate ($^{15}NO_3$) assimilation experiments with isotopic solutions ($^{15}NH_4Cl$ and $K^{15}NO_3$, respectively) were performed independently using cultures previously grown at 45°C in $BG11_{NO3}$ and $BG11_{NH4}$. The experiments began washing the cultures with distilled water to eliminate the debris and nitrogen sources, and then each culture was re-suspended in $BG11_0$, and maintained in darkness during six hours for synchronization in order to shifts the phase of circadian rhythms in cyanobacteria (Kiyohara et al. [56]). The washed-pellet from $BG11_{NO3}$ was divided in two; one of this was inoculated with BG11 medium contained KNO_3 9mM + $K^{15}NO_3$ 1mM, and the other was inoculated with BG11 $^{15}NH_4Cl$ 200μM, in triplicate. The same procedure was done for washed-pellet from $BG11_{NH4}$. For each assay, six times over 48 h cycles were conducted in triplicate. Each 3, 6, 12, 18, 24 and 48 h subsamples for RNA, nutrients (NO_3^- and NH_4^+) and isotopic analyses were also obtained. Nutrients analyses were performed using an autoanalyzer (Seal Analytical AA3; Biogeochemistry laboratory, Universidad of Concepción, Chile), and isotopic analyses were carried out as describe above.

Nitrogen assimilation related cluster genes in strain CHP1

Reference sequences from *Anabaena* sp. PCC7120 (Kaneko et al. [57]) were used to search for: *nif*H, *nar*B, *nir*A and *gln*A genes in the CHP1 strain genome. The accession numbers of corresponding strain CHP1 genes were: KXO35102, KXO35103, KXO35104 and KXO35105 for *gln*A, *nar*B, *nif*H and *nir*A genes, respectively. Moreover, data

obtained from the US DOE JGI IMG (https://img.jgi.doe.gov/cgi-bin/m/main.cgi) and PATRIC (https://www.patricbrc.org/portal/portal/patric/Home) databases for *Fischerella* sp. PCC 9431 (ID 2512875027), *Mastigocladus laminosus* UU774 (ID 1594576), *Fischerella* sp. JSC-11 (ID 2505679024) and *Fischerella* sp. NIES-3754 (ID 1752063) representatives of mesophilic and thermophilic cyanobacteria, were used to compare nitrogen genes synteny between them. For all genomes, once the region in the genome was located, surrounding region sequences were extracted and examined in the ORF Finder program (http://www.bioinformatics.org/sms2/orf_find.html) to define the open reading frames (ORFs) in the DNA sequence. This program returns the range of each ORF, along with its protein translation. Each ORF found was then blasted in the NCBI database using BLASTP and BLASTN tools to assign potential protein encoding segments. This search allowed finding neighboring genes, and therefore the genomic cluster context for each gene.

RNA extraction from N assimilation experiments and RT-qPCR for gene expression analysis

Three biological replicates from the isotopic assays were used for gene expression by RNA analysis. These samples were collected at the same incubation times where the bottles for $K^{15}NO_3$ and $^{15}NH_4Cl$ at 45°C were harvested (at 3, 6, 12, 18, 24, 48h), and also for nitrogen fixation at the two temperatures investigated (45 and 50°C). RNA from samples was extracted using Trizol and kit RNA Clean & Concentrator™ (Zymo Research, USA). The quality and quantity of the RNA were determined using a spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and by electrophoresis in an RNase-free 1% agarose gel. DNase treatment (TURBO, Applied Biosystems, USA) was

311 performed, and 1.5 μg of RNA from each sample (in triplicate) was used for qPCR
312 standardization. Then, the cDNA was synthesized using ImProm-II™ Reverse
313 Transcription System (Promega, USA) according to the manufacturer's specifications. For
314 *nif*H, *nar*B and *gln*A genes qPCR measurements, specific primers for strain CHP1 were
315 designed (Supplementary data, Table S1). Each gene PCR product obtained was cloned
316 into the pGEM-T® (Promega, USA) vector plasmid to obtain the plasmid stock
317 concentration (10^{10} copies) and the plasmid curve (10^2 to 10^8 copies). The SensiMix kit
318 (Bioline, USA) was used for the fluorescence signal, and the real-time qPCR (Roche LC
319 480) program was run as follows: 40 cycles at 95 °C for 10 s, 55 °C for 20 s, and 72 °C for
320 30 s. All primers were designed to have a similar annealing temperature in order to run
321 under similar conditions. To normalize the quantification of transcripts, three different
322 housekeeping genes were tested. Stability analyses for *pet*B, 16S rRNA and *ilv*D genes in
323 the different media ($BG11_0$, $BG11_{NO3}$ and BG_{NH4}) were conducted (Pinto et al. [58]). The
324 most stable gen tested by the geNorm algorithm for all conditions was *ilv*D gene
325 (Supplementary data, Fig. S1). Finally, all fluorescence genes quantifications where
326 extrapolated using the respective plasmid standard curve, and normalized with the absolute
327 quantification of housekeeping (HK) *ilv*D gene.
328
329 **Results**
330 *Phylogenetic affiliation of the Porcelana strain CHP1*
331 A phylogenetic reconstruction based on complete 16S rRNA and *nif*H gene sequences
332 from all Stigonematales cyanobacteria sequenced genomes (subsection V), and some other
333 selected sequences from SILVA SSU database, was first performed. Reconstruction using
334 the 16S rRNA gene (Fig. 1) demonstrated that the Porcelana strain CHP1 clustered

(bootstrap 97%) exclusively with members of the genera *Mastigocladus* and *Fischerella*. Moreover, all members within this clade are characterized as thermophilic, while the rest of the sequences from Stigonematal members were non-thermal and clustered separately (*Fischerella*, *Hapalosiphon*, *Westiellopsis*, *Nostochopsis*, *Westiella* and *Mastigocladus*; bootstrap 95%) from the thermal clade. All being diazotrophs, a second phylogenetic reconstruction based on the complete *nif*H gene was performed (Supplementary data; Fig. S2). This reconstruction verified the affiliation of strain CHP1 within the thermophilic Stigonematales, forming a tight clade (99% sequence identity) with *Mastigocladus* and *Fischerella* spp. isolated from hot springs.

To widen the affiliation basis further, strain CHP1 was examined morphologically under light (LM; Fig. 2) and transmission electron microscopy (TEM; Figs. 3 and 4). LM examinations showed that the principal filaments were mostly uniseriated with true lateral branches due to cell division in more than one plane. The vegetative cells were on average 7.6 µm SD±1.3 long and 6.0 µm SD±1.8 wide, while branched cells were 8.0 µm SD±2.1 long and of 4.8 µm SD±1.4 wide (Fig. 2). As seen in Fig. 2, branching of the T-Bd (T-bd) and Y-Di (Y-di) types were observed (Fig. 2b, e), with V and X-branching types occasionally being present (Fig. 2b).

Subjecting the cells to growth on different nitrogen sources (atmospheric nitrogen gas-$BG11_0$; and the combined nitrogen sources nitrate-$BG11_{NO3}$ and ammonium-$BG11_{NH4}$), all vegetative cells were characterized by a strong red chlorophyll *a* fluorescence (Figs. 3h, i), in contrast to the non-fluorescent heterocysts, cells developing in the absence of a combined nitrogen source (Fig. 3g). The heterocyst/vegetative cell frequency was 2-4%, irrespective of temperature. Heterocysts in primary trichomes were predominantly intermediate and less frequently terminal (Figure 2a, d, g). Ultrastructural (TEM) analyses

of strain CHP1 exposed no major differences between cells grown at the various temperatures and nitrogen-regimes examined (Figs. 3-4). Both proheterocysts and mature heterocysts showed the sub-cellular reorganizations expected for vegetative cells, such as the development of extra wall layers, a narrow neck leading to adjacent vegetative cells, and an extensive reorganization of the thylakoid membranes.

As all these features are found in and typical for members the genus *Mastigocladus*, specifically the uniseriate filaments, true lateral branching and a vegetative cell size between 8-10 µm, an affiliation of the strain CHP1 to *Mastigocladus* sp. is concluded.

Thermo-tolerance under different nitrogen regimes

To test the genetic adaptation through thermophilic characters and nitrogen requirements of strain CHP1, growth was followed under combination of different temperatures and nitrogen regimes: $BG11_0$ at 45, 50 and 60°C; $BG11_{NO3}$ and $BG11_{NH4}$ at 45 and 50°C. When cultivated at 60°C (Fig. 5a), strain CHP1 did not survive beyond 6 days in $BG11_0$, nor under $BG11_{NO3}$ and $BG11_{NH4}$ (data not shown). In contrast, at the lower temperatures (45 and 50°C) and under both $BG11_0$ and $BG11_{NO3}$ regimes, the biomass of the strain CHP1 increased substantially over time (Fig. 5a, b). Initially, the increase in biomass was low in $BG11_{0,}$ which was followed by a more significant increase in biomass (dry weight) at 50°C, reaching up to 66.1 mg after 38 days. However, biomass under $BG11_{NO3}$ (9mM $NaNO_3$) was consistently highest, reaching 166.3 mg at 45°C and 122.8 mg at 50°C, respectively. The increase in biomass measured as chlorophyll a showed a similar pattern (Fig. 5a, b).

Previous studies in the literature suggested that optimal ammonium concentrations for cyanobacterial growth are in the range of 2 to 10 mM NH_4Cl. However, 2 mM of

NH_4Cl caused severe bleaching of strain CHP1 cells (after day 6; data not shown), therefore 0.02, 20 and 200 μM ammonium concentrations in $BG11_{NH4}$ were tested. The concentration of 0.02 μM NH_4Cl, close to that found in the Porcelain hot spring [24], was depleted after 4 days as evidenced by the appearance of heterocysts in the filaments of strain CHP1. As heterocysts also developed under 20 μM and 200 μM after 6 and 21 days, respectively, a concentration of 200 μM NH_4Cl was selected for comparative examinations (Fig. 2c). At both temperatures growth on NH_4Cl was considerably lower measured as dry weight or chlorophyll *a*, reaching 20.8 mg at 45°C and 17.2 mg at 50°C, being approx. 20 folds lower than when grown in $BG11_{NO3}$.

Altogether, this comparison illustrates that strain CHP1 was clearly thermo-tolerant up to about 50°C, and that the tolerance was influenced by the prevailing nitrogen regime, with nitrogen fixation ($BG11_0$) consistently supporting highest growth at 50°C (dry weight and chlorophyll a), while nitrate ($BG11_{NO3}$) at 45°C, although growth under $BG11_{NO3}$ was 2-3 times higher. Growth under $BG11_{NH4}$ showed no clear thermo-preference and was many folds lower than under the other nitrogen regimes.

Nitrogen assimilation capacity and rates

The nitrogen assimilation capacity of strain CHP1 was next examined. Nitrogen fixation rates during light-dark regimes were examined by both $^{15}N_2$ assimilation assay and acetylene reduction assay (the nitrogenase activity). As seen in Fig. 6, exposing cultures to $^{15}N_2$ gas at 45 and 50°C, showed that $^{15}N_2$ gas uptake of 7.5 (at 50°C) and 8.3 (at 45°C) nmoles PON mg^{-1} throughout the light/dark cycle; with an activity 2 folds higher at midday and with a marginal effect of temperature (Fig. 6a). Nitrogenase activity (acetylene to

406 ethylene) was recorded throughout the light/dark period but as for the isotopic uptake with
407 a distinct peak in light at midday, reaching 54.3 (SD±3.9) nmoles ethylene produced mg^{-1}
408 at 45°C, and up to 62.4 (SD±10.8) nmoles ethylene mg^{-1} at 50°C.
409 At 45°C, the cellular uptake of nitrate as $K^{15}NO_3$ in cultures previously grown in $BG11_{NO3}$
410 was light dependent, ranging from 342 (SD ±14.7) to 412 (SD ± 9.5) nmoles N mg^{-1} h-1,
411 while if pre-grown in $BG11_{NH4}$ the uptake was up to 10 folds lower 28 nmoles (SD ± 6.2)
412 and 54 nmoles (SD ± 2.1) N mg^{-1} h^{-1}; Fig. 6b. The concentrations of nitrate in the $BG11_{NO3}$
413 medium remained high during the 48 h time course of the experiment.
414 Likewise, cellular uptake of ammonium as $^{15}NH_4Cl$ was higher in cultures previously
415 grown in $BG11_{NH4}$ than in $BG11_{NO3}$, although this uptake was at a considerably lower level
416 than the $K^{15}NO_3$ uptake (Fig. 6c). A maximum incorporation of 48 nmoles (SD ± 3.7) N
417 mg^{-1} h^{-1} was found after 18 h. Ammonium approached zero after a 48 h of incubation (Fig.
418 6c), while high nitrate levels still remained in the medium after that period (Fig. 6b).
419
420 *Gene expression of key genes in the nitrogen fixation and assimilation pathways*
421 Fluctuations in the transcripts of *nif*H (encoding the Fe subunit of the nitrogenase
422 complex), *gln*A (encoding the ammonium assimilating enzyme, glutamine synthetase) and
423 *nar*B (encoding nitrate reductase involved in nitrate assimilation) in strain CHP1 was
424 followed during light/dark cycles using RT-qPCR, combined with normalization to the
425 standard plasmid curve of the housekeeping *ilv*D gene [57]. The *nif*H gene in the strain
426 CHP1 is only expressed in the presence of mature heterocysts, and constituted from 2 to
427 4% in CHP1 grown in $BG11_0$ medium. The *nif*H transcript levels remained fairly constant
428 throughout the experimental period at 45ºC, while at 50ºC the *nif*H levels were high under

429 the light, decreasing radically (1500 to 100 *nif*H gene copies) during the light to dark
430 transition (Fig. 7a).
431 The *gln*A gene that encodes the primary ammonium assimilation protein glutamine
432 synthetase, is typically reported to be present in the nitrogen-fixing heterocysts but also in
433 all vegetative cells, therefore a high expression of this gene was expected. Transcription
434 levels of *gln*A gene (normalized to the HK *ilv*D gene) in strain CHP1 cells under nitrogen-
435 fixing condition were apparent at both 45 and 50ºC, peaking in light (7928 gene copies) as
436 seen in Fig. 7b, although a pronounced *gln*A activity was also recorded in the dark period
437 (5800 gene copies). As expected, when strain CHP1 was grown under combined nitrogen
438 sources, the *nif*H gene was not expressed under any circumstances (Fig. 8a, b).
439 In the $BG11_{NO3/15NO3}$ experiment at 45°C, *gln*A and *nar*B gene expressions were high,
440 reaching 1853 (SD±263) and 1645 (SD±150) transcripts, respectively (Fig. 8a; left).
441 Expression in the $BG11_{NO3/15NO3}$ experiment was one order of magnitude higher than in the
442 $BG11_{NH4/15NO3}$ experiment (Fig. 8a). In the $BG11_{NH4/15NO3}$ experiment (45°C), the
443 maximum expression of *gln*A and *nar*B genes reached similar levels of 443 (SD ± 110)
444 and 361 (SD ± 17) in the light, respectively (Fig. 8a; right), coincident with nitrate
445 incorporation (Fig. 6b). However, in contrast to in the results obtained for the $BG11_0$
446 medium, the *gln*A transcript levels were low in the dark period.
447 Similar trends were observed in the cultures supplemented with $^{15}NH_4Cl$ ($BG11_{NO3/15NH4}$
448 and $BG11_{NH4/15NH4}$) at 45°C (Fig. 8b). The *gln*A and *nar*B gene transcript levels in
449 $BG11_{NO3/15NH4}$ reached 636 (SD ± 75) and 490 (SD ± 97), and under $BG11_{NH4/15NH4}$ 870
450 (SD ± 498) and 344 (SD ± 78) for *gln*A and *nar*B genes, respectively (Fig. 8b).
451
452

Nitrogen cluster gene organization

Next, the genic context in regards to the *nif*HDK cluster genes in the genome of strain CHP1 was investigated and compared with: *Mastigocladus laminosus* UU774, *Fischerella* sp. JSC-11, *Fischerella* sp. NIES-3754 and finally with *Fischerella* sp. 9431 a mesophilic representative of the Stigonematales. The *nif*HDK cluster of strain CHP1 has synteny with those recovered at the reference genomes used for comparison (Fig. S3), except for *Mastigocladus laminosus* UU774 and *Fischerella* sp. 9431, the latter interrupted by hypothetical proteins, a phage integrase and a methylase protein. In addition, extra copies of *nif*D and *nif*K were also found near the CHP1 *nif*HDK cluster. The *nar*B and *nir*A genes, related to nitrate and nitrite assimilation respectively, formed a complete *nir*A-*nrt*ABCD cluster in the genome of strain CHP1 (Fig. S4), but located in the complementary strand. The *nar*B gene, although located in another region of the CHP1 genome, was in synteny with the *nar*B gene of *Mastigocladus laminosus* UU774 and *Fischerella* sp. JSC-11. In contrast to most of the other freshwater strains of cyanobacteria, the bi-specific (nitrate and nitrite) *nrt*P transporter was not detected in the strain CHP1 genome. Finally, the *gln*A gene in CHP1 showed no synteny with the *gln*A context of adjacent genes within the other genomes analyzed (Fig. S5).

Discussion

Filamentous cyanobacteria isolated from various thermal ecosystems worldwide have often been characterized as members of the subsection V Order Stigonematales, and particularly as *Mastigocladus laminosus* Cohn ex Kirchner 1898, in accordance with the morphotype classified as *M. laminosus* ([14], [41] and [52]). The geographically wide distribution of *Mastigocladus* genotypes, is likely explained by a high tolerance against elevated

477 temperatures as well as desiccation. In addition, *Mastigocladus* spp. grow at non-thermal
478 temperatures (25-30°C), albeit slowly, and may even tolerate temperatures approaching
479 freezing while developing akinetes (Miller et al. [59]; Ionescu et al. [60]).
480 From our phylogenetic (16R rRNA and *nif*H genes) and morphological analyses it is
481 apparent that the strain CHP1 isolated from the neutral pH Porcelana hot spring (Chile) is a
482 member of the Order Stigonematales (subsection V), family Mastigocladaceae Geitl. 1925,
483 subfamily Mastigocladoiceae and the genus *Mastigocladus* Cohn 1862, to which a >98-
484 99% genetic identity was found. Furthermore, the morphological features identified in
485 strain CHP1 (*e.g.* uniseriate leading strand, true branching of type T and Y) including also
486 its subcellular organization (*e.g.* a central distribution of thylakoid membranes), suggest a
487 taxonomic affiliation specifically to *Mastigocladus laminosus* (Nierzwicki et al. [61]). The
488 fact that this strain was unable to survive at 60°C (under any of the nitrogen regimes tested,
489 see below), but can grow rapidly at 45 and 50°C classifies the *Mastigocladus* strain CHP1
490 as a moderately thermophilic cyanobacterium. In general, 50°C promotes higher growth
491 than at 45°C, which may reflect a potential genetic adaptation to 50ºC. Similar thermo-
492 tolerance is shared with other *Mastigocladus* spp. isolated from other geographically
493 separated hot springs in for instance Yellowstone National Park (YNP), also showing a
494 greater fitness at 40-55ºC [40]. Hence, the previous tentative affiliation of strain CHP1 to
495 *Mastigocladus* [24], is confirmed, as is the fact that strain CHP1 belongs to a clade
496 exclusive for thermophilic or thermotolerant representatives from within the genera
497 *Mastigocladus* and *Fischerella*. The latter, further stress the close relationship between
498 thermotolerant members irrespective of genus and geographic origin. However, to fully
499 explore the biogeography of thermal Stigomenatales additional strains from other hot

springs in other continents are needed to test Bass-Beckings theory [62] that "everything is everywhere but the environment selects".

Our data also demonstrate that *Mastigocladus* sp. strain CHP1 has the capacity to grow on different nitrogen regimes, besides on atmospheric dinitrogen gas as under natural conditions in the Porcelana hot spring [24], although at dissimilar rates. Besides the appreciable growth sustained on nitrogen gas ($BG11_0$), the most prolific growth of *Mastigocladus* sp. strain CHP1 took place in presence of nitrate ($BG11_{NO3}$) at both 45 and 50°C, while growth was minimal under a medium supplemented with ammonium. This is in accordance with data of a thermophilic *Mastigocladus laminosus* strain from Jakrem hot spring (Meghalaya, India) [41], while not with two *Mastigocladus* populations from White Creek and Boiling River (YNP), showing similar growth yields under both $BG11_0$ and $BG11_{NO3}$ regimes [26]. These differences may suggest physiological diversifications and adaptations of strains within the thermal *M. laminosus* clade due to the various physical and geochemical conditions offered [59].

Expectedly, nitrogen fixation peaked in light in the heterocystous *Mastigocladus* sp. strain CHP1 under controlled conditions irrespective of temperature (45 and 50°C) and was abolished under combined nitrogen regimes. Maximum activity coincided with that found *in situ* in the Porcelana hot spring [24] as well as with that of one thermophilic *Mastigocladus laminosus* isolate from YNP [26]. Also, *nif*H gene expression in *Mastigocladus* sp. strain CHP1 was higher and apparent in light at 50ºC similar to recorded previously *in situ* in the 48-58°C temperature range [24]. However, *nif*H transcript levels were generally low and fluctuated moderately in the light/dark cycles at 45ºC. There is also the possibility that *nif*H gene transcription may not be affected by a circadian control at 45ºC. This is supported by the finding that *nif*H gene expression was constant under

524 constant low light (30 μmol m^{-2} s^{-1}) in *Mastigocladus* sp. strain CHP1 (45-50°C)
525 (Arancibia-Loewe *et al*. unpublished). A similar behavior has been observed in the
526 heterocystous cyanobacterium *Anabaena* sp. PCC 7120 (subsection IV), *i.e.* showing no
527 rhythmicity in the *nif*HDK gene expression under low light (30 μmol m^{-2} s^{-1}), and therefore
528 classified as arrhythmic under low light condition (Kushige et al. [63]). A deeper
529 understanding of the over-all molecular regulation of nitrogen fixation in *Mastigocladus*
530 sp. strain CHP1 at different temperature and light regimes is now warranted.
531 The organization of the *nif*HDK gene cluster in the genome of *Mastigocladus* sp. strain
532 CHP1, showed that it holds a close synteny with that of *Fischerella* sp. JSC-11
533 (Supplementary data, Fig. S3). A synteny of the *nif*HDK cluster has previously been
534 demonstrated between Stigonematal cyanobacteria, such as *Fischerella* sp. and
535 *Mastigocladus laminosus* (Saville et al. [64]; Singh and Stevens [65]), suggesting that the
536 *nif*HDK gene cluster tightly resemble that of the *Mastigocladus* sp. strain CHP1 genome
537 information available.
538 Under nitrogen-fixing conditions at 50ºC, the *gln*A gene transcription, underpinning
539 primary ammonia assimilation in all cell types, was high in *Mastigocladus* sp. strain CHP1,
540 and a positive correlation between *nif*H and *gln*A gene expression patterns was observed.
541 The two distinct *gln*A transcription peaks found in light and dark, respectively, is well-
542 known for cyanobacteria, *e.g.* the heterocystous *Anabaena* sp. PCC7120 [60] and the
543 unicellular *Crocosphaera watsonii* WH8501 (Shi et al. [66]). Moreover, the high *nar*B
544 gene transcript levels (related to ferredoxin dependent nitrate reductase) found in light and
545 nitrate amended ($BG11_{NO3\text{-}15NO3}$) cultures contrasted to the low transcript levels retrieved
546 under pre-adaption in ammonium, which suggests a severe inhibition of the *nrt*ABCD

547 transporter under ammonium ([36] and Aichi et al. [67]). The low ammonium tolerance (≤
548 200 μM) found in *Mastigocladus* sp. strain CHP1 may potentially trigger the minor
549 changes seen in the *gln*A transcription. A quick drop in the glutamine synthetase activity
550 on ammonium upshift has been shown for the unicellular *Synechocystis* sp. PCC6803
551 (Mérida et al. [69]). Differences in NH_4^+ tolerance among cyanobacteria is well-known
552 (Dai et al. [68]) and due to pH shifts (Stewart [70]), triggering a disruption of the
553 manganese cluster in the photosystem II oxygen-evolving complex (Belkin and Boussiba
554 [71] and Drath et al. [72]).
555 On the other hand, the data shows similar efficiencies in ammonium and nitrate uptake in
556 *Mastigocladus* sp. strain CHP1, even though the cells are not able to switch quickly from
557 one to the other. For instance, nitrate uptake was reduced by 89% in cultures previously
558 grown on ammonium ($BG11_{NH4}$), potentially due to the lack of an active NRT substrate-
559 binding protein (NrtA), needed to assimilate nitrate (Omata et al. [73]). Likewise, the
560 ammonium uptake was reduced by 87% in cultures acclimated to nitrate ($BG11_{NO3}$). This
561 preference to incorporate particular nitrogenous substances is also known for other thermal
562 *Mastigocladus* spp. [41].
563 From the data obtained it may be concluded that *Mastigocladus* sp. strain CHP1 has a great
564 metabolic plasticity when it comes to the usage of nitrogen regimes offered, and this
565 genetic "versatility" may confer great competition under natural conditions. Indeed, the
566 importance of the members of Stigonematales as contributors of fixed nitrogen to the
567 nitrogen cycle in Porcelana hot spring was recently demonstrated by Alcamán et al. (2015).
568 In addition, recent metatranscriptomic data from Porcelana microbial mats (at 48 and
569 58°C) demonstrate that the *nif*H gene transcripts of *Mastigocladus* sp. strain CHP1

represent 87% of the total *nif*H gene transcripts in this hot spring. This further stresses the pivotal role of this specific strain in Porcelana, and potentially in other globally distributed hot springs where members of *Mastigocladus* dominate.

Our findings thus highlights the use of *Mastigocladus* sp. strain CHP1 as an excellent model to answer still open questions about for instance which evolutionary strategies that enable the extremophiles to evolve and functionally adapt in such extreme environments. Specifically, such data may provide valuable insights into the potential use of thermophilic microorganisms in biotechnology, equipped with unique macromolecular properties and high metabolic rates. In addition, *Mastigocladus* sp. strain CHP1 has excellent biotechnological potential given its competitive ability to fulfill its carbon and nitrogen requirements using light energy only.

Conclusions

Collectively our data demonstrate that the CHP1 cyanobacterium strain isolated from Porcelana hot spring phylogenetically belongs to an exclusive thermal clade within the Stigonematales. The strain is taxonomically identified as *Mastigocladus* sp., in combination with morphological characters (uniseriate filaments and its true branching types, T and Y). Its capacity to grow at 50°C but not at 60°C demonstrates that *Mastigocladus* sp. strain CHP1 represents a moderately thermophilic cyanobacterium. Daily *nif*H gene expression patterns suggest different rhythmicity controls depending on temperature. High expression was found for the *gln*A and *nar*B genes in light, being differently regulated by the nitrogen regime available. A clear preference for nitrate and a low tolerance to ammonium was noticeable. Being a major player in the nitrogen economy of the microbial mats in Pocelana hot spring (Alcamán et al. 2015), we here provide

insights into nitrogen acquisition behavior of *Mastigocladus* sp. strain CHP1, revealing its physiological adaptations at high temperatures. Further analyses are now warranted to better understand the unique genomic features and adaptation abilities that gave members of this clade capacity to conquer these extremely hot environments.

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Conflict of Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

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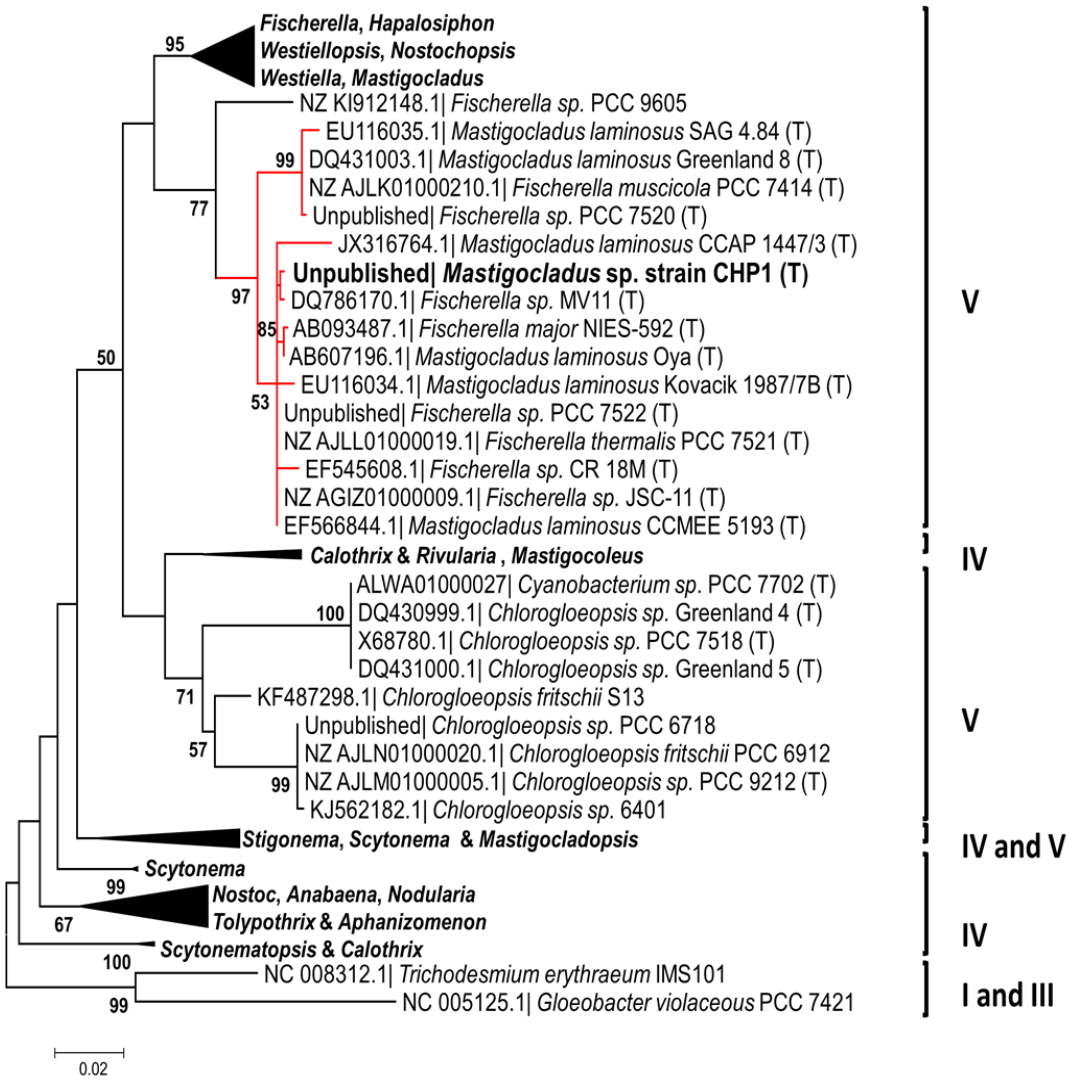

Figure 1. Phylogenetic reconstruction built on the 16S rRNA gene from major representatives of filamentous cyanobacteria. The affiliation of sequences classified as *Mastigocladus* sp. strain CHP1 is clearly located within the order Stigonematales (the heterocystous branching Subsection V). The red lines show clades with representatives most closely related to *Mastigocladus* sp. strain CHP1. All represent strains of thermal origin (T) which stresses the close phylogenetic relatedness between thermophiles. The cyanobacteria *Trichodesmium erythaeum* IMS101 (Subsection III) and the unicellular *Gloeobacter violaceus* PCC7421 (Subsection I) were used as outgroups. The numbers given at the nodes represent bootstrap values of 10.000 replicates.

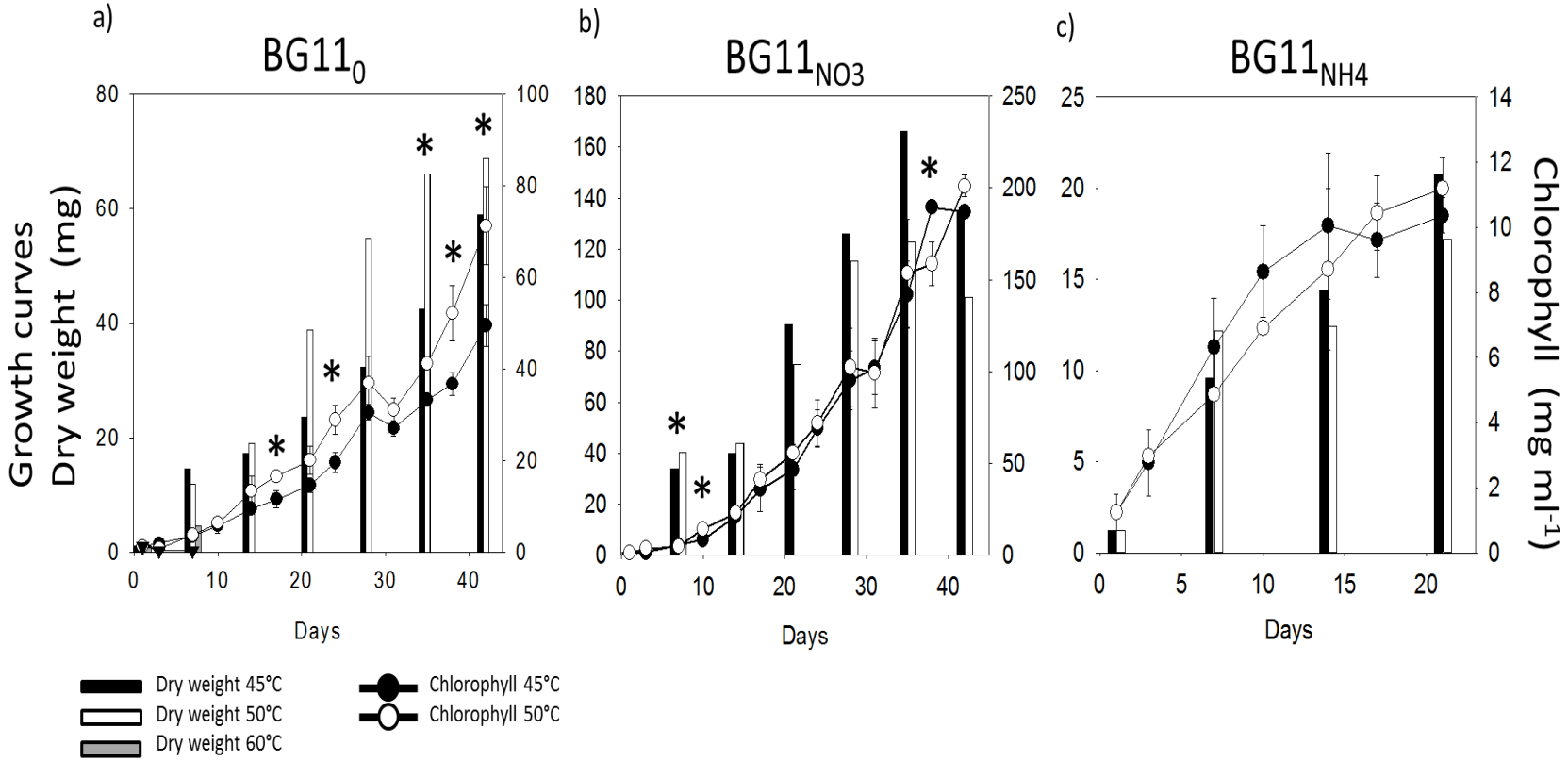

Figure 2. Growth of *Mastigocladus* sp. strain CHP1 incubated under different nitrogen regimes and temperatures. (a) Under nitrogen-fixing conditions, *i.e.* in absence of a combined nitrogen source: $BG11_0$; (b) in a medium supplemented with KNO_3 (9 mM): $BG11_{NO3}$; and (c) supplement with NH_4Cl (200 μM): $BG11_{NH4}$. Each culture initially held 2 mg chlorophyll L^{-1} and were incubated at 45, 50 and 60°C in $BG11_0$, and at 45 and 50°C in $BG11_{NO3}$ and $BG11_{NH4}$. Biomass is given as dry weight (mg) and chlorophyll *a* (mg ml^{-1}) during a growth period of six weeks, except for in c. Note differences in scales at the Y-axis. Growth in $BG11_0$ at 60°C ceased after 6 days (grey bars). Asterisks denote significant differences (valor $p<0.05$) using t-student tests for chlorophyll *a* at the two temperatures.

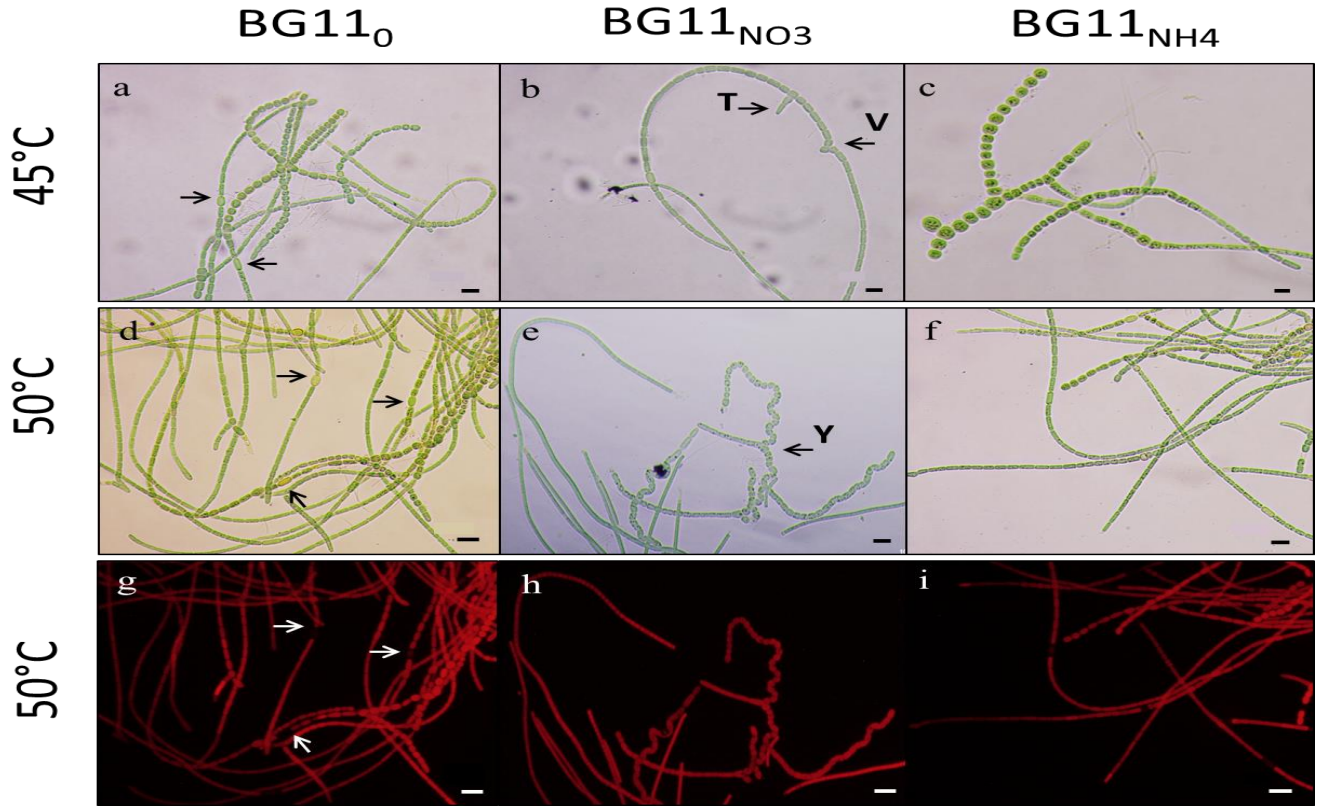

Figure 3. Morphological appearance of *Mastigocladus* sp. strain CHP1 in light and epifluorescence microscopy when grown under different nitrogen sources and temperatures. (a, d) Growth under nitrogen-fixing conditions ($BG11_0$). Heterocysts were apparent as more transparent and larger cells being mostly intercalary (black arrows). (b, e) Growth under $BG11_{NO3}$ and (c, f) under $BG11_{NH4}$. Non-heterocystous uniseriate filaments and filaments with type T, V and Y branching points predominated under all nitrogen regimes. (g, h, i) Epifluorecence micrographs show the red fluorescence of chlorophyll in vegetative cells under all growth conditions. (g) The lack of epifluorescence in heterocysts is illustrated (white arrows). Bar represent 10 μm.

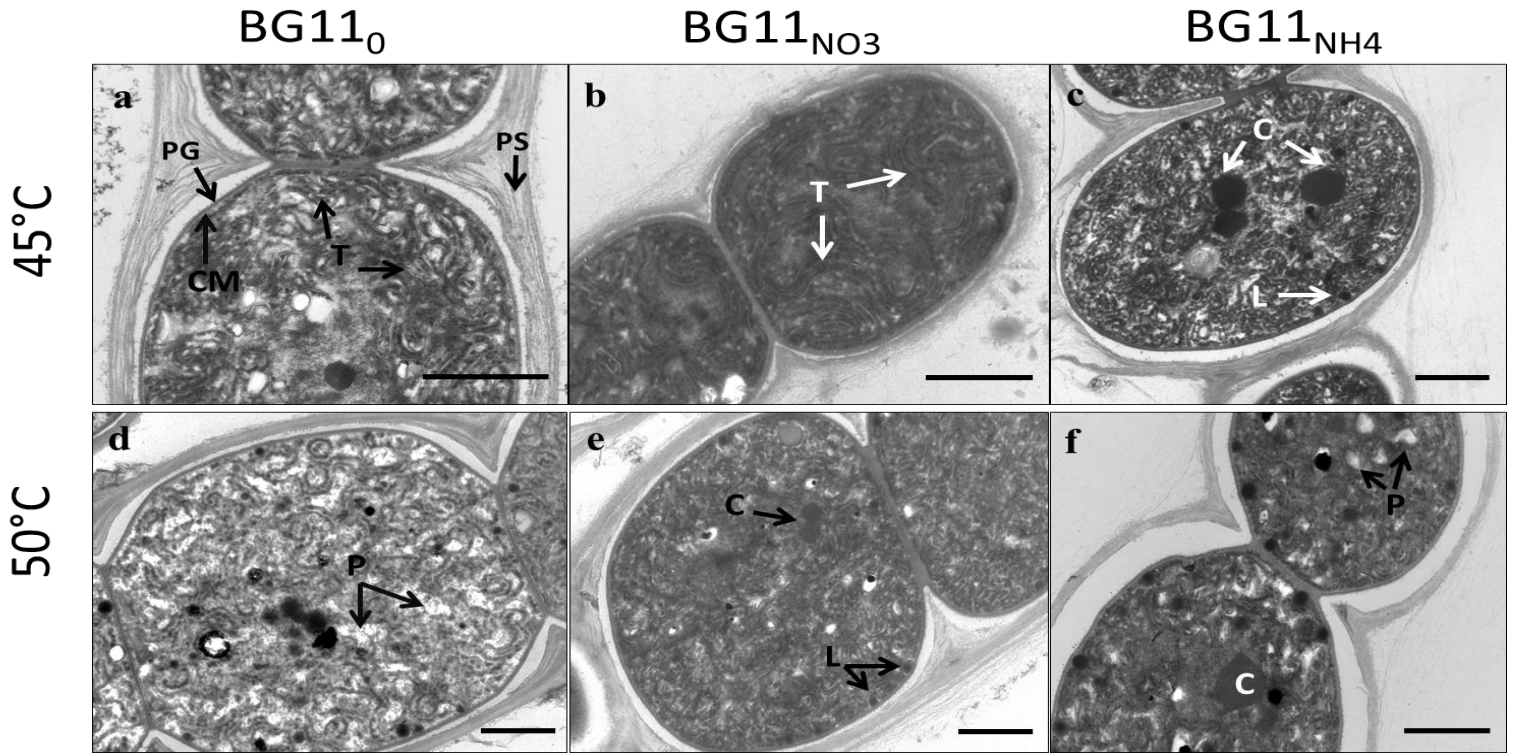

Figure 4. Electron micrographs of ultrathin sections of vegetative cells in *Mastigocladus* sp. strain CHP1 when grown under different nitrogen sources and temperatures. (a, d) Cells grown under nitrogen-fixing conditions ($BG11_0$), *i.e.* in absence of a combined nitrogen source. (b, e) cells grown on $BG11_{NO3}$; and (c, f) on $BG11_{NH4}$ and at the different temperatures indicated. Abbreviations: PS, external polysaccharide layers; PG, peptidoglycan layer and CM, cytoplasmic membrane; and T, thylakoids; C, Carboxysomes; L, lipid bodies; PS, clusters of polysaccharides (glycogen) granules; P, polyphosphate granules. Bar correspond to1μm.

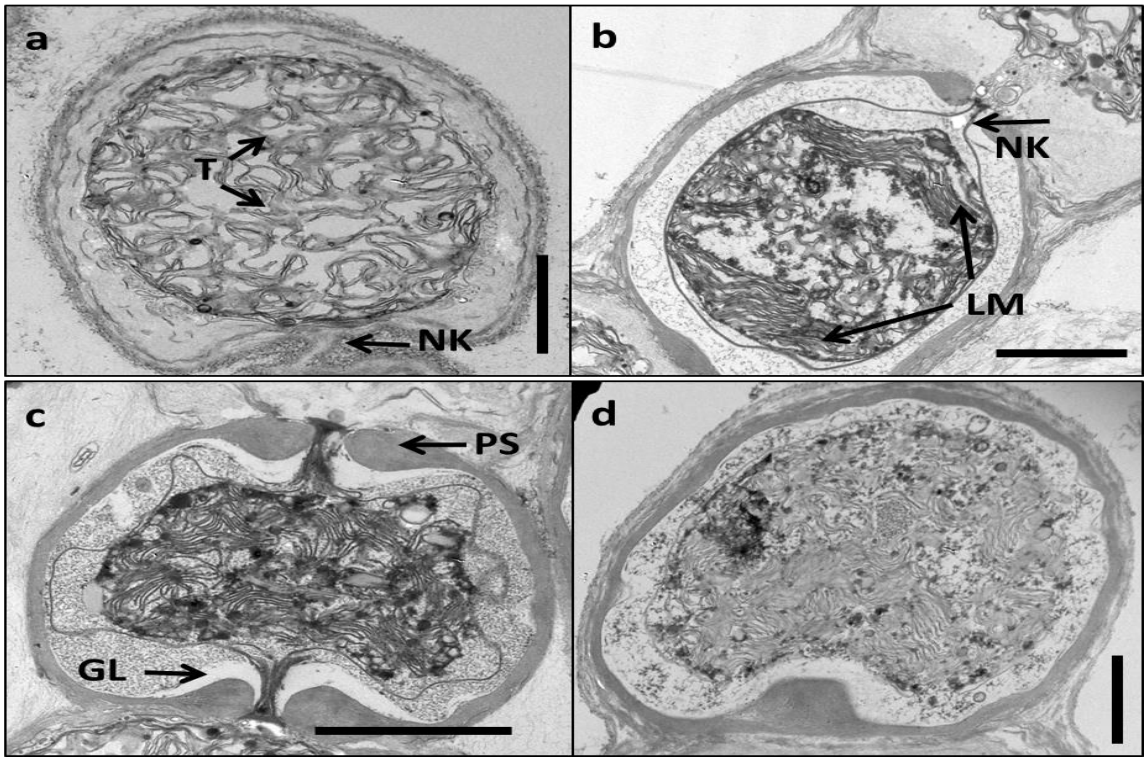

Figure 5. Ultrastructural characteristics of developmental stages in heterocysts of *Mastigocladus* sp. strain CHP1 grown under nitrogen-fixing conditions ($BG11_0$). (a) Proheterocyst at an early stage of the development characterized by loose external cell wall layers, rearrangement of thylakoids (T), and narrowing of the neck (NK) leading to the adjacent vegetative cell. (b) Intermediate developmental stage with the heterocyst characterized by a narrow neck but with still not fully developed external cell wall layers. (c, d) Mature heterocysts with distinct bundles of stacked, lamellar membranes (LM) filling up the entire cytoplasm, well defined narrow necks,external polysaccharide layers (PS) and glycolipid layers (GL). Note the absence of other subcellular structures of vegetative cells at all stages (compare to Figure 3). Bar 2 μm.

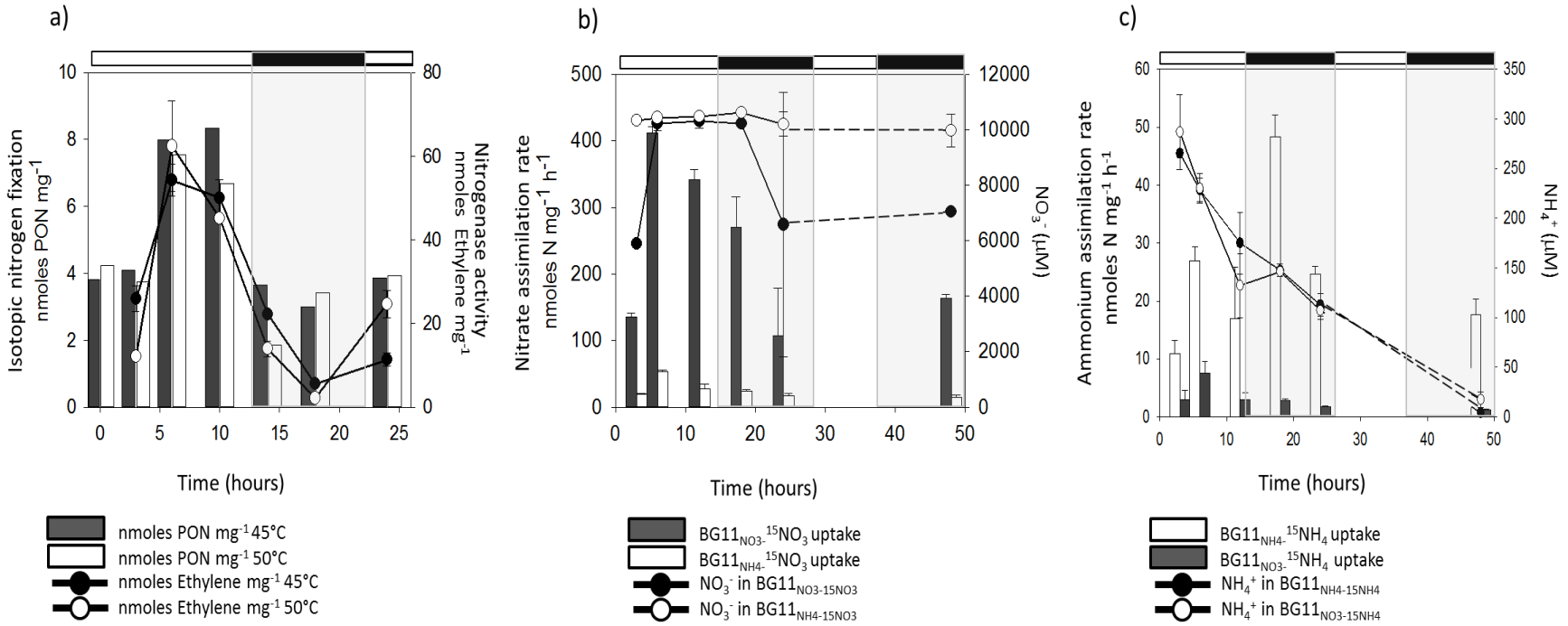

Figure 6. Nitrogen assimilation in *Mastigocladus* sp. strain CHP1 during light/dark cycles at different temperatures. (a) Assimilation under nitrogen-fixing conditions using i) $^{15}N_2$ incorporation, given as particulate organic nitrogen (PON) formed (grey and white vertical bars) and ii) the acetylene reduction assay (ARA; nitrogenase activity), given as the ethylene produced (black and white circles). (b) Nitrate assimilation: growth in $BG11_{NO3}$ (grey bars) and $BG11_{NH4}$ (white bars) was followed by growth on isotopic $K^{15}NO_3$ added at time zero. The black and white circular symbols show nitrate concentrations during the experiment. (c) Ammonium assimilation: growth in $BG11_{NH4}$ (grey bars) and $BG11_{NO3}$ (white bars) was followed by growth on $^{15}NH_4Cl$ at time zero. The black and white circular symbols show ammonium concentrations during experiment. Note the differences in scales at the Y-axis. Horizontal bars above the graphs represent durations and pattern in light/dark cycles during the 24 or 48 hour cycles.

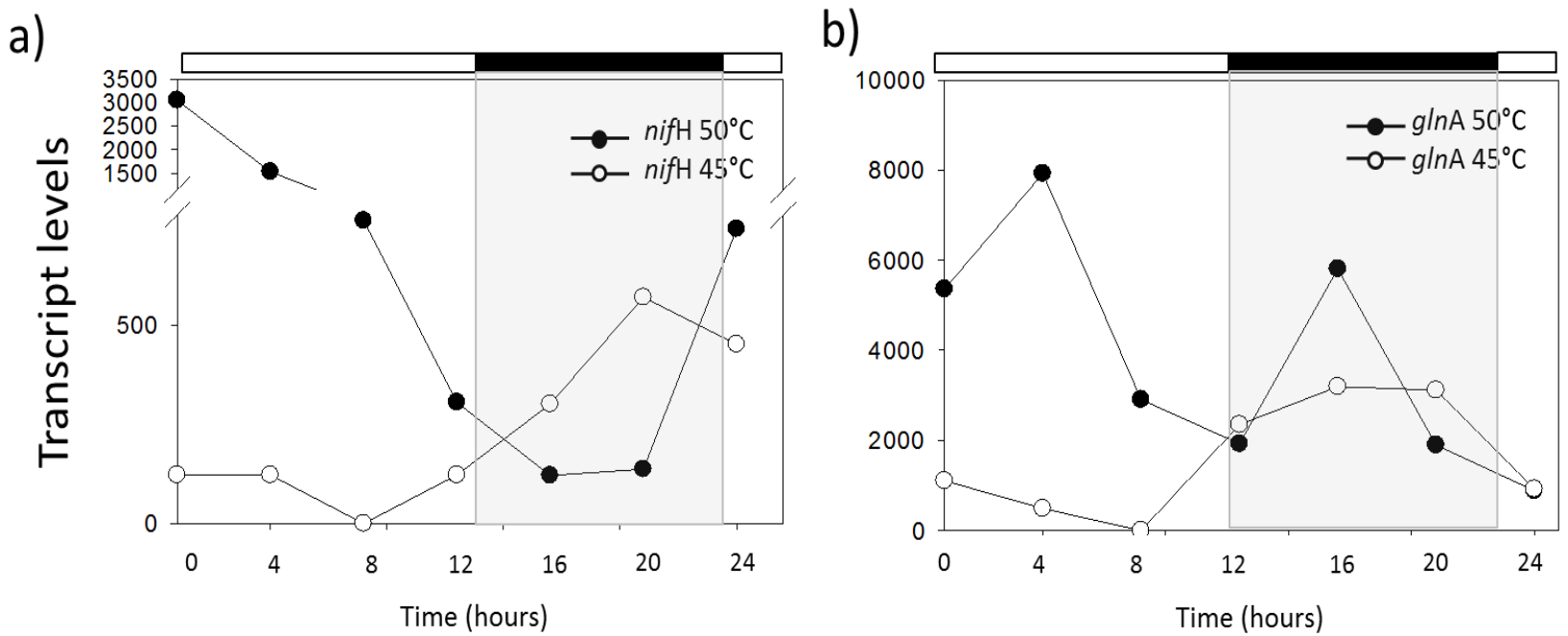

Figure 7. Transcript levels of genes involved in atmospheric nitrogen fixation in *Mastigocladus* sp. strain CHP1. Transcript levels of a) the *nif*H and b) the *gln*A genes at 45 and 50°C under nitrogen-fixing conditions ($BG11_0$) during light-dark transitions. The horizontal bars above the graph indicate the durations of the light and dark period of the experiment.

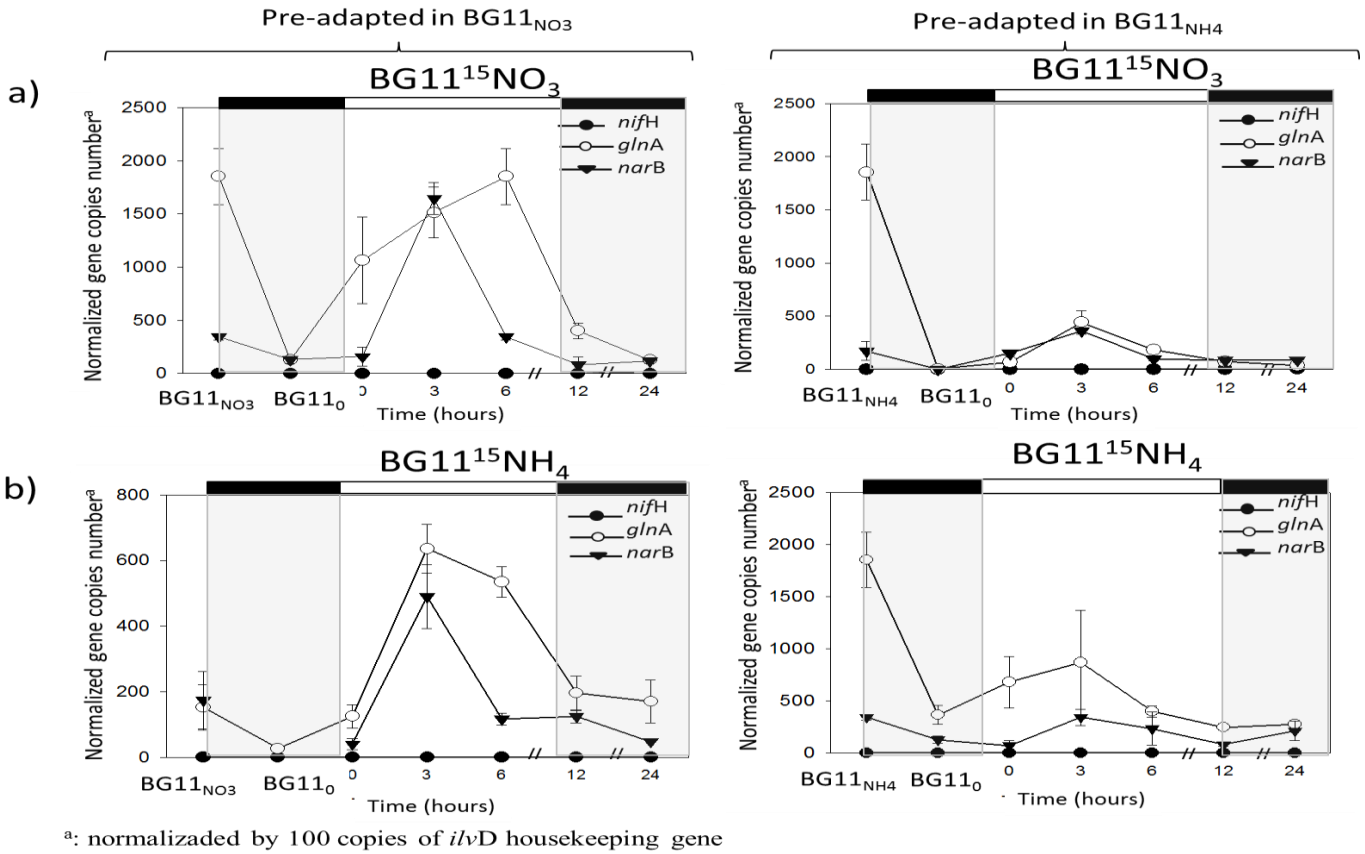

Figure 8. Transcript levels of genes involved in nitrogen assimilation in *Mastigocladus* sp. strain CHP1 at 45°C. Transcript patterns of *nif*H, *gln*A and *nar*B genes in cultures growth pre-adapted under $BG11_{NO3}$ and $BG11_{NH4}$ prior to the start of the experiment, and at time 0 inoculated with (a) $K^{15}NO_3$ and (b) $^{15}NH_4Cl$. The grey area represent dark periods, the first represent a 6 hour synchronization time in darkness prior to the start of the 24 h cycle. The horizontal bars above the graph indicate the durations of the light and dark period of the experiment.

Supplementary data

Figure S1. geNorm algorithm analysis for housekeeping gene stability under different nitrogen regimes. The genes *ilv*D, *pet*B and 16S rRNA were examined as potential housekeeping in each light/dark of *Mastigocladus* strain CHP1 grown on various nitrogen sources ($BG11_0$, $BG11_{NO3}$ and $BG11_{NH4}$). Numbers at the top indicate the stability of the genes. The *ilv*D gene was most stable of the genes tested, therefore used to normalize all transcripts gene levels studied.

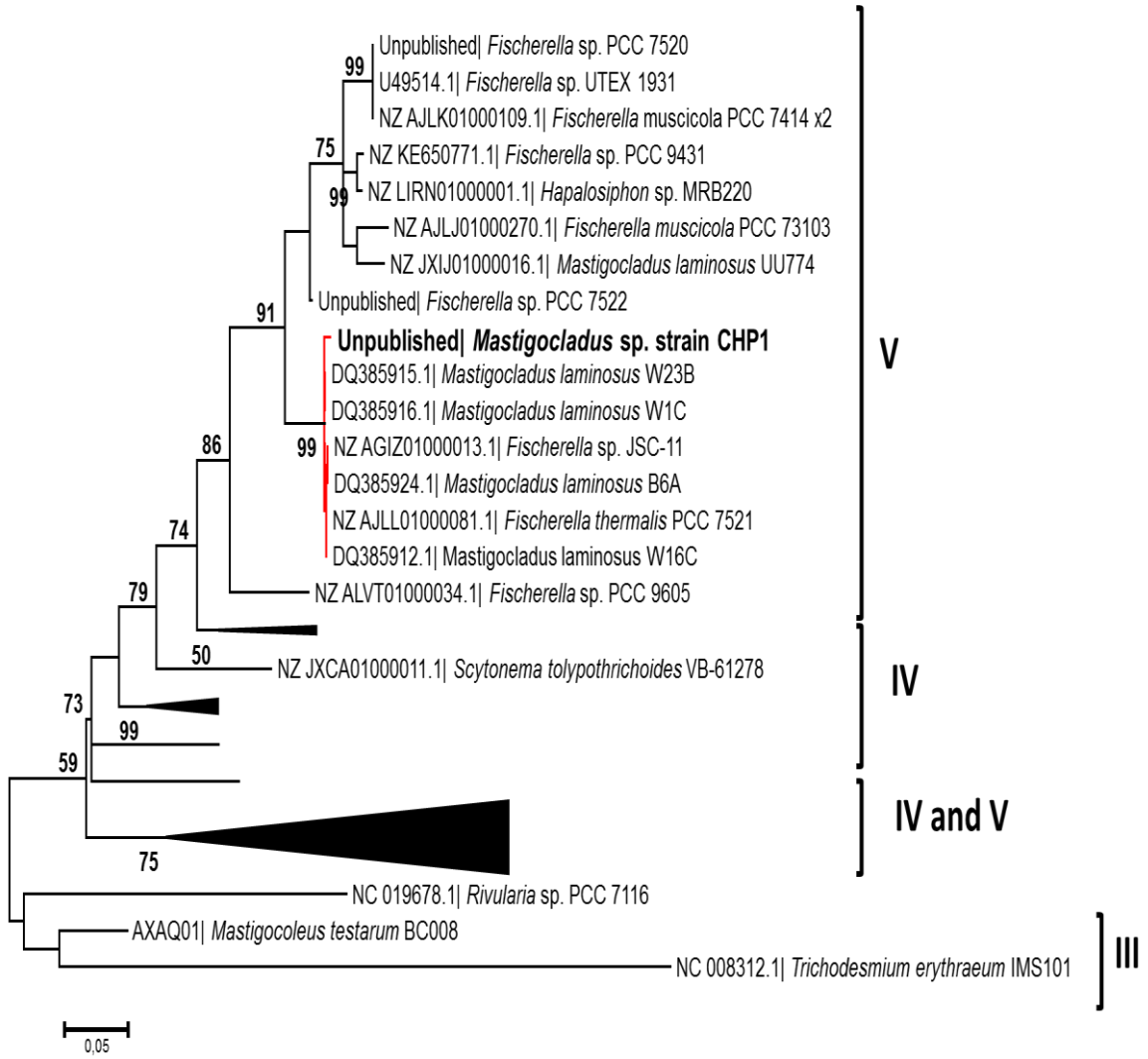

Figure S2. Phylogenetic *nif*H gene reconstruction from major representatives of diazotrophic cyanobacteria. The affiliation of sequences classified to *Mastigocladus* sp. strain CHP1 as diazotrophic with closest relation with *Mastigocladus* spp. represented by red lines clades. The cyanobacteria *Trichodesmium erythaeum* IMS101 (subsection III) was used as outgroups. The numbers given at the nodes represent bootstrap values of 10.000 replicates.

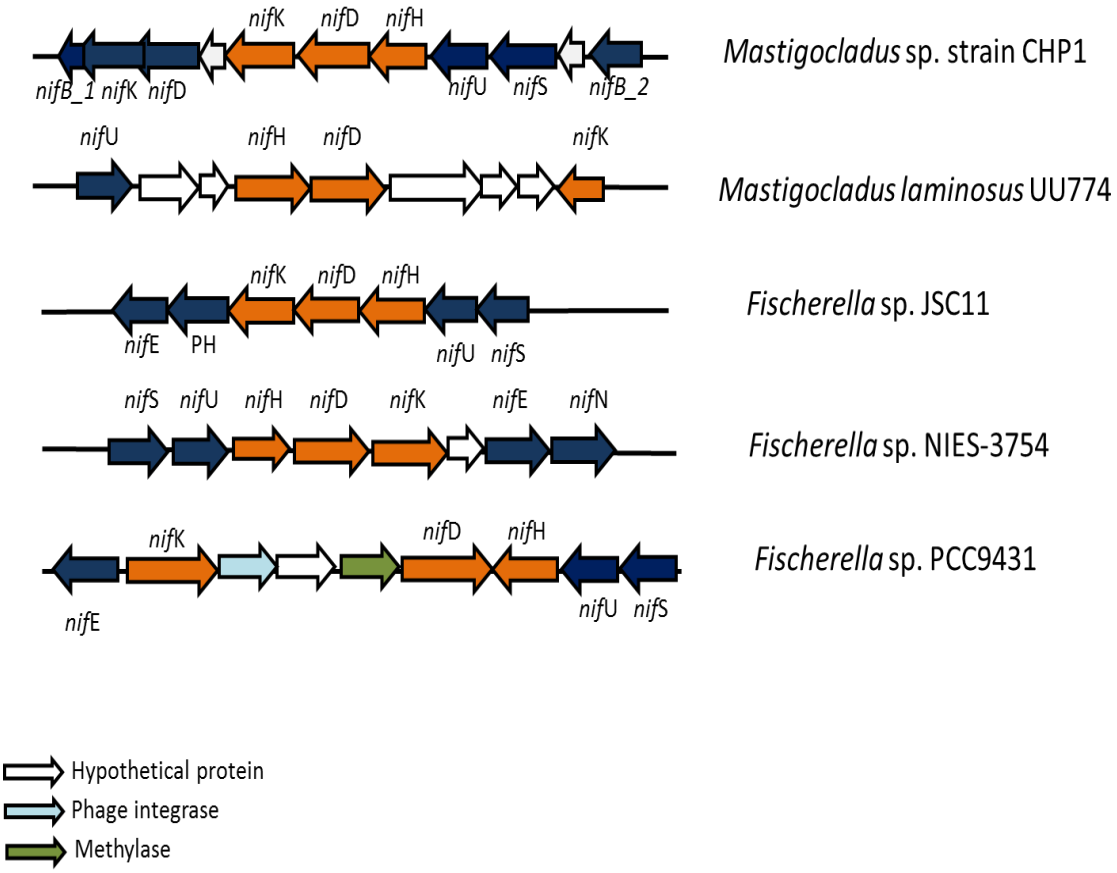

Figure S3. *nif* gene clusters in *Mastigocladus* sp. strain CHP1 and surrounding gene context. For comparison the thermal *Mastigocladus laminosus* UU774, *Fischerella* sp. JSC-11, *Fischerella* sp. NIES-3754 and mesophilic *Fischerella* sp. PCC9431 (subsection V) are included.

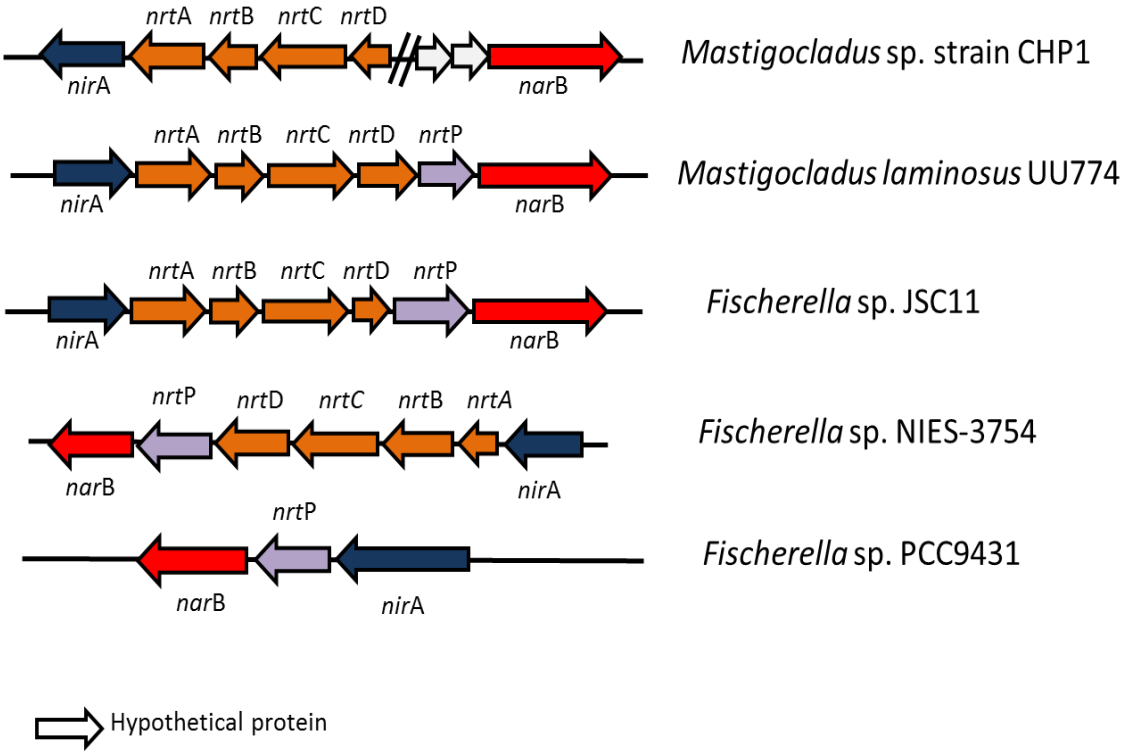

Figure S4. Gene clusters of ABC-NRT nitrate transporter and NR/NiR enzymes in the assimilation of nitrate/nitrite in *Mastigocladus* sp. strain CHP1. For comparison the thermal *Mastigocladus laminosus* UU774, *Fischerella* sp. JSC-11, *Fischerella* sp. NIES-3754 and mesophilic *Fischerella* sp. PCC9431 (subsection V) are included.

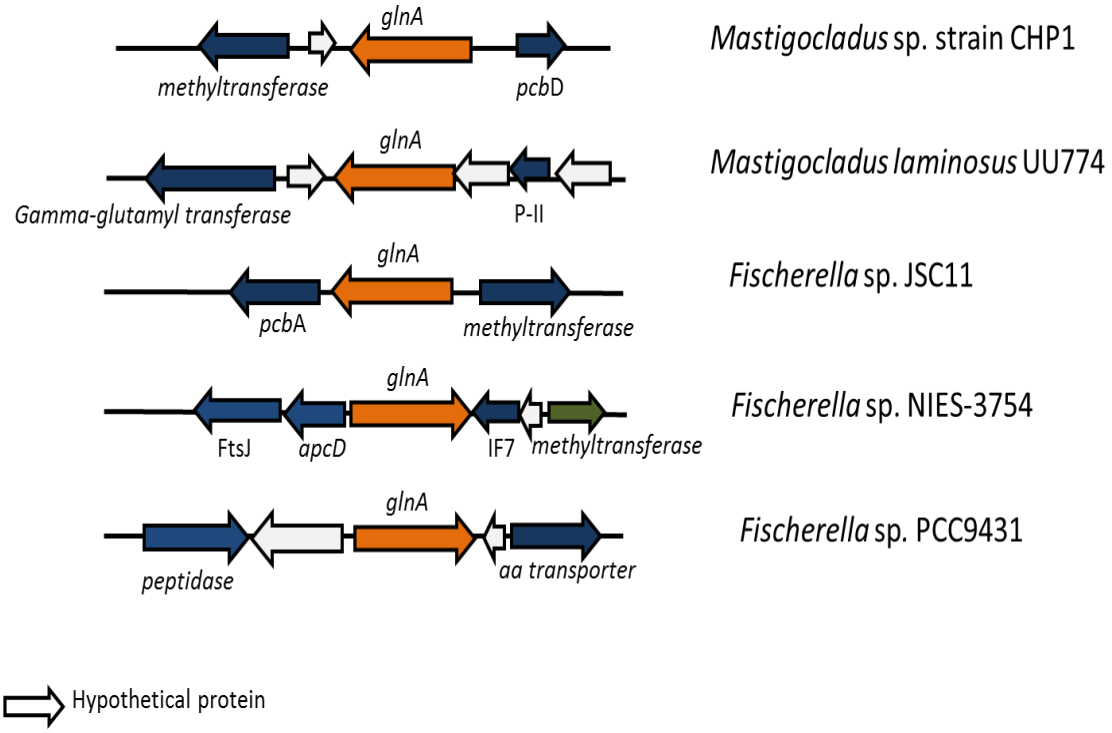

Figure S5. Genetic context of glutamine synthetase enzyme involved in the ammonium assimilation in *Mastigocladus* sp. strain CHP1. For comparison the thermal *Mastigocladus laminosus* UU774, *Fischerella* sp. JSC-11, *Fischerella* sp. NIES-3754 and mesophilic *Fischerella* sp. PCC9431 (subsection V) are included.

GENERAL DISCUSSION

Microbiology of hot springs around the world had attracted considerable interest for decades (Stewart, 1967; Miller *et al*., 2006; Steunou *et al*., 2008; Hamilton *et al*., 2011; Cole *et al*., 2013). There are many studies characterizing the biology and microorganisms inhabiting these extreme systems (Stewart, 1970; Miller *et al.*, 2006; Steunou *et al*., 2008; Hamilton *et al.*, 2011; Loiacono *et al.*, 2012; Huang *et al.*, 2013). However, our knowledge on the biogeochemical contributions to the carbon and nitrogen cycles in non-acidic phototrophic hot spring microbial mats is still surprisingly rudimentary (Alcamán *et al*., 2015; Li *et al*., 2016). In particular, the characterization and contribution of thermophilic filamentous heterocystous Cyanobacteria, a globally common group of organisms that dominate in these systems, has been mostly neglected.

In Chile, the high volcanic activity along its geography has generated a large number of unexplored terrestrial hot springs with distinct physicochemical conditions. Many of these hot springs exhibit characteristics resembling others well-studied such as those in Yellowstone National Park (Hauser, 1989; Hamilton *et al.*, 2011; Loiacono *et al.*, 2012; Wang *et al*., 2013). In particular, the pristine neutral- slightly acidic hot spring of Porcelana located in the Chilean Patagonia (X Region de los Lagos) was selected for our studies given its constant thermophilic gradient (70-40ºC), presence of phototrophic microbial mats, and accessibility for field work.

In Porcelana hot spring (Mackenzie *et al*., 2013) as in many other hot springs (Miller *et al*., 2009; Everroad *et al*., 2012, Cole *et al*., 2013, Wang *et al*., 2013, 2014) has been reported that temperature is one of the most important environmental factors affecting

microbial community composition, where the complexity of the microbial mat community increases with decline in the temperature. A previous study to this thesis in the lush microbial mats of the Porcelana hot spring, revealed through 16S rRNA gene analysis that Cyanobacteria and Chloroflexi were two of the principal community phyla present along the temperature gradient (Mackenzie *et al*., 2013).

As in many other environmental systems, metagenomic and metatranscriptomic approaches have become very useful tools to deepen in the understanding of the microbial mat composition and their activities. The results from Porcelana hot spring by using such techniques revealed that Cyanobacteria and Chloroflexi together with some other minoritary phyla such as Deinococcus-Thermus, Proteobacteria and Bacteroidetes and rare biosphere are constantly represented and active in the microbial mat like those at the alkaline Mushroom hot spring in Yellowstone National Park (USA) (Miller *et al*., 2009; Klatt *et al*., 2013, Liu *et al*., 2011) **Chapter I**.

The results also demonstrate an apparent transition on abundance and activity between Chloroflexi and Cyanobacteria observed from high to lower temperatures, with a co-habitation in the intermediate temperatures (58ºC). The later could be explain as a matter of collaboration by the exchange of for instance organic carbon sources provided by the Cyanobacterial members, which can be used under heterotrophic conditions by the Chloroflexi (Ward *et al*., 1987; Strauss & Fuchs, 1993; Zarzycki *et al*., 2009). Other studies have also suggested that these two groups of taxa might also avoid competition for resources by temporally partitioning nutrient uptake during the day (van der Meer *et al*., 2005; Klatt *et al*., 2013).

In Porcelana, Chloroflexi member of the genera *Chloroflexus* sp. and *Roseiflexus* sp., were major active representatives at 66°C and 48°C, respectively. Similarly, in other

alkaline thermal systems such as that of Mushroom spring (YNP) and Alla mats in Russia, it was reported that the water temperature decrease, resulted in the preferential development of *Roseiflexus* sp. over *Chloroflexus* sp. (Klatt *et al*., 2013; Gaisin *et al*., 2015). All these studies are in disagreement with the study of Ward *et al*. (2012) performed in a hot spring also at YNP showed that at temperatures close to 68°C both FAPBs were present in approximately equal proportion. In the other hand, Cyanobacteria and particularly the diazotrophic genus *Mastigocladus* (Stigonematales), reported widely both in neutral and alkaline thermal systems (Steward *et al*., 1967; Steward, 1970; Miller *et al*., 2006, 2009), was in Pocelana potentially responsible for all the metabolic activity at 48°C at noon, sharing activity with Chloroflexi relatives at 58°C. The presence of *Chloroflexi* and *Mastigocladus* OTUs inhabit in the range of 40 to 58°C along the thermal gradients of White Creek (YNP) has also been reported by Miller *et al*. (2009). Other cyanobacteria than Stigonematales belonging to subsection IV, Nostocales order (e.g. *Nostoc* sp.) and subsection III, Oscillatoriales order related to the LPP group (*Leptolingbya* sp., *Plectonema* sp. and *Phormidium* sp.) were also revealed, mostly at higher temperatures and always representing minor contribution that members of the *Mastigocladus*. High temperature tolerance of Oscillatoriales (at 66 and 58°C) has been previously demonstrate in other hot springs such as those in Thailand (Sompong *et al*., 2005), were its gliding motility into the microbial mat by a combination of phototaxis and photokinesis, permit the onset of their activity as anoxygenic photosynthetic organisms by consuming sulfide (Richardson *et al*., 1987).

According with the high relative abundance and activity of Cyanobacteria recorded by metagenomic and metatranscriptomic results presented in the **Chapter I**, as well as their specific diversity and diazotrophic capability studied respectively by 16S rRNA and

*nif*H genes in the **Chapter II**, allowed us to perform a more accurate affiliation of the dominant cyanobacteria present in Porcelana, revealing again the dominance of these filamentous heterocystous members of the genus *Mastigocladus*.

In the other hand the *nif*H gene, has been the more extensively molecular marker used to target diazotrophs in natural ecosystems (Zehr *et al*., 1997; Díez *et al*., 2007; Severin and Stal, 2009, 2010; Fernandez *et al*., 2011), and in hot springs (Steunou *et al*., 2006, 2008; Miller *et al*., 2006; Liu *et al*., 2011; Hamilton *et al*., 2011). The results for the diazotrophic diversity by *nif*H gene marker by direct amplification and also through metagenomics in Porcelana hot spring demonstrate a unique dizotrophic microbial composition formed by Stigonematal cyanobacteria devoid of diazotrophic unicellular cyanobacteria and bacteria (see **Chapter II**). That is different to other studies were unicellular cyanobacteria have been suggested as most important diazothops (Steunou *et al*., 2006, 2008). In order to broaden our knowledge of nitrogen fixation in Porcelana microbial mat, diel *in situ* activities were also performed. Nitrogenase activity measurements obtained by acetylene reduction assay, have previously been associated to cyanobacteria in several hot springs (Steunou *et al.*, 2006, 2008; Miller *et al.*, 2009). However, more recently some studies also suggest that heterotrophic bacteria and archaea may be significant nitrogen-fixers in hot springs (Hamilton *et al*., 2011; Loiacono *et al*., 2012). In Porcelana hot spring, the results demonstrated that the nitrogen fixation process is totally associated to the activity of cyanobacterial members of the order Stignematales, and that other potential bacteria does not have any significant contribution to this process (see **Chapter I**). Furthermore, *in situ* biological nitrogen fixation examined using both the nitrogenase activity and $^{15}N_2$ isotope uptake techniques presented at **Chapter II**, shows apparent nitrogenase activity values only during the daytime and at temperatures from 58

to 46°C (were the heterocystous Stigonematal are actively present; **Chapter I**), being not detected above 60°C. Similar range values have been reported in other hot springs such as Mushroom Spring (Steunou *et al*., 2008), and were in agreement with the activities reported in other non-thermal aquatic ecosystems (Fernandez *et al*., 2011) (see **Chapter II**). The maximum nitrogen fixation recorded at daytime in Porcelana mat pointed out once more the presence and activity of heterocystous cyanobacteria such as Stigonematales that typically perform this process during the light period (Stal, 1995; Evans *et al*., 2000; Charpy *et al*., 2007; Bauer *et al*., 2008). In that sense, the data infer the predominance, if not the exclusive role, that the heterocystous *Mastigocladus* cyanobacteria have as nitrogen fixers in Porcelana hot spring and possible in many other hot springs over the world.

Additionally, maxima nitrogen fixation activities recorded in Porcelana at 48ºC and attributed to *Mastigocladus* sp., showed a positive correlation with *nif*H gene expressions (see **Chapter II**), and *nif*H gene transcripts retrieved also in the metatranscriptome at this lower temperature (48°C) (see **Chapter I**). Similar pattern has also been found in thermal mats dominated by *Synechococcus* (Octopus Spring; YNP), where the *nif*HDK transcripts increased in accordance with the maximum nitrogenase activity during the dark period (Steunou *et al*., 2006). However, a correlation between the *nif*H transcripts and the nitrogenase activity during the light/dark period in the *Mastigocladus*-mat along temperatures and years has only been until now reported for Porcelana hot spring (Alcamán *et al*., 2015). Additionally, our results demonstrated that the nitrogen fixation activity reported in Porcelana, is not diminished by the assimilation of other sources of combined nitrogen (**Chapter I and II**). *In situ* measurements of ammonium concentration in Porcelana indicate its low availability, which can be explained by the rapid assimilation of this compound by Chloroflexi and Cyanobacteria. In the same way, assimilatory rates of

isotopic labeled ammonium ($^{15}NH_4Cl$) were also very low, probably due to high ammonia oxidation by Thaumarchaeota, resulting in high ammonia turnover rates into the mat. A similar pattern was also associated to the transcript genes (*nar*B, *nir*A) related to nitrate assimilation ($K^{15}NO_3$) and the lower rates of cellular incorporation recovered.

Rather, nitrogen fixation contributes with up to 99% of the 'new' nitrogen to the microbial mat community, supporting the total daily nitrogen demand in this extreme system (Alcamán *et al*., 2015; **Chapter II**). In our knowledge this is the first time than identity, activity and gene expression by diazotrophs, and contribution rates of nitrogen fixation are investigated together in a temperature gradient in a hot spring.

Regarding nitrogen cycle, also other important pathways than nitrogen fixation were present in the Porcelana hot spring (see **Chapter I**). The data reveals the high levels of *gln*A gene transcripts associated with the ammonia assimilation. Chloroflexi and Cyanobacteria members were associated to this process at high (66 and 58°C) and low (48°C) temperatures, respectively. Also, the putative archaeal ammonia monooxygenase (*amo*) genes involved in the ammonia oxidation pathways were identified along the temperature gradient similar to that at the Perpetual Spouter hot spring (YNP) (Hamilton *et al*., 2014). High numbers of *Nitrososphaera* sp. *amo*A genes transcripts, were registered at all temperatures in the Porcelana mat, suggesting that ammonia can be rapidly oxidized by these organisms reflected by the low $^{15}NH_4CL$ assimilation rates obtained at 58 and 48°C (**Chapter I**). Similarly, Hatzenpichler *et al*. (2008) reported the genus *Candidatus* Nitrososphaera gargensis as a thermophilic ammonia oxidizer in the microbial mats of the Siberian Garga hot spring, with temperature optimum for ammonia oxidation at 46°C. In the other hand, denitrification and dissimilatory nitrate reduction to ammonium (DNRA) processes, also were active and widely found at all temperatures, with denitrification

particularly active at 66°C. These pathways were primarily attributed to the heterotrophic bacteria such as *Nitrospira* sp. (Proteobacteria) and *Rhodothermus* sp. and *Flavobacterium* sp. (Bacteroidetes). These results confirm an active nitrogen cycle in Porcelana hot spring, suggesting similar insight found in other two ~80°C springs in the US Great Basin, where ammonia oxidation, denitrification and DNRA were actives in water and sediments, directing that ammonia oxidation may be a major source of energy fuelling primary production (Dodsworth *et al*., 2011).

In Porcelana hot spring, both the nitrogen and CO_2-fixation coincided at midday in the cyanobacterial mat, which explains the substantial nitrogen fixation activity recorded at that time of the day (see **Chapter II**). In this mat, photosynthesis would not only cover the high energy demand (ATP) of the nitrogen fixation process, but also provides with the required reducing power and carbon skeletons. As shows in the **Chapter I**, oxygenic photosynthesis was totally attributed to Cyanobacteria from genus *Mastigocladus* sp. and no transcripts of anoxygenic photosynthesis by *Chloroflexus* sp. and *Roseiflexus* sp. were detected at noon. Given that at the present study all DNA/cDNA material was obtained from high illuminated and oxygen saturated microbial mats, is that transcripts associated to anoxygenic photosynthesis are not expected as they might decrease as the light intensity increases (Liu *et al*., 2011). Also, at the Mushroom hot spring microbial mat is being suggested that Chloroflexi members transcribe photosynthesis related genes during the night (Klatt *et al*., 2013). In that sense, analysis of the night activity of the microbial mat of Porcelana is now needed in order to fully understand the real contributions and in particular that of the Chloroflexi members related to this processes.

In particular CO_2 fixation by Calvin cycle in Cyanobacteria, and the presence of transcripts associated with the 3-hydroxypropionate bicycle associated to members of

Chloroflexi, demonstrates that these two phototrophs are active CO_2 fixers along the temperature gradient in Porcelana (**Chapter I**). Interestingly, besides Cyanobacteria and Chloroflexi, in Porcelana, Archaea are also fixing CO_2 by the hydroxypropionate-hydroxybutyrate cycle at high temperatures, revealing a major activity of the Thaumarchaeota members related to *Nitrosophaera* sp. This thermophilic ammonia oxidizer Thaumarchaeota has been reported as adapted to grow under low ammonium concentrations, with potential to carry out CO_2 fixation under experimental conditions (Hatzenpichler *et al*., 2008). In agreement with the retrieved CO_2 fixation pathways (see **Chapter I**), *in situ* rates of $H^{13}CO_3$ assimilation obtained in Porcelana, suggest a high activity of autotrophs at noon, and specially attributed to Cyanobacteria according with the their associated high number of gene transcripts, being FAPs and archaeal members much less represented (**Chapter I**).

As *Mastigocladus* sp. cyanobacteria, was found as the dominant oxygenic photoautotroph and most active nitrogen fixer along the temperature gradient (69ºC to near 38°C) recorded in the Porcelana mat (**Chapter II**), physiological and genetic characterization of this Stigonematal was of much interest. In that sense, an isolate from Porcelana hot spring; CHP1 (CHilean Porcelana 1) tentatively identified as Stigonematal was characterized and investigated under differential culture growth conditions, and further comparisons were done to the *in situ* results in the field.

The characterization of strain CHP1 (see **Chapter III**), revealed its tentative affiliation to the *Mastigocladus* genus according to some of their morphological characteristics (e.i type of branching) previously described for that genus by Anagnostidis and Komárek, (1990). The strain also presents similarities to ramification features of *Fischerella* spp. but with the T-type branching as common as the V-type. However, cells

leading strand and secondary filament long were consistent with *Mastigocladus laminosus* features. Additionally, ultrastructure organization such as central distribution of thylakoid membranes, also suggested that morphologically the strain CHP1 is a member of the Order Stigonematales (Subsection V). Moreover, closest phylogenetic affiliation of the 16S rRNA gene encloses at the strain CHP1 with other thermal *Mastigocladus* spp.

Additionally, the physiological performance of CHP1 at high temperatures, as well as its preferential nitrogen sources, demonstrates that *Mastigocladus* sp. strain CHP1 has the capacity to grow at high temperatures under culture medium without combined nitrogen ($BG11_0$), as well as under the presence of ammonium ($BG11_{NH4}$) and nitrate ($BG11_{NO3}$) in the media. The optimal growth conditions for CHP1 were 45-50°C, while it was not able to survive at 60°C. The later correlates with the temperature range for other thermal Stigonematales isolates (Finsinger *et al*., 2008; Miller *et al.*, 2009). Interestingly, an apparent low NH_4^+-concentration dependency was found for the strain CHP1 under laboratory conditions, therefore their prevalence in the Porcelana mat would be according to the low natural NH_4^+ concentrations (**Chapter II**). However, deeper studies are now needed in the subsection V of cyanobacteria and in particular the thermal *Mastigocladus* genus to fully understand their nitrogen acquisition metabolisms.

The capacity of the strain CHP1 as nitrogen fixer during the light period was validated at both 45 and 50°C, and was similar to that previously reported for another thermophilic cyanobacterium population of *Mastigocladus laminosus* (Miller *et al*., 2006). This nitrogen fixation capacity in culture of strain CHP1 was correlated to the nitrogen fixation rates related *in situ* to *Mastigocladus* sp. as exposed in the **Chapter II**. Additionally, the metatranscriptomic data (see **Chapter I**) analysis revealed that *Mastigocladus* sp. strain CHP1 might reach in average 87% of the total *nif*H gene

transcripts at 48 and 58°C, suggesting that this strain could be the most relevant diazotroph contributing to new nitrogen into this hot spring. Fluctuations of CHP1-*nif*H gene transcripts at 50°C (along the light/dark cycle), were in accordance with the high *nif*H gene transcripts pattern observed during the light period in Porcelana microbial mat (**Chapter II**). However, at 45°C the results showed not rhythmicity of CHP1-*nif*H gene. Therefore, greater emphasis on studies related to transcription factors that could be controlling the gene expression, and/or post-translational modifications in the nitrogenase enzyme are now necessary.

Additionally, results of combined nitrogen compounds assimilation in the strain CHP1 demonstrated that although ammonium assimilation requires less energy to be incorporated, if the strain was grown (pre-adapted) in $BG11_{NO3}$, it shows a preference to incorporate nitrate instead of ammonium (see **Chapter III**). This behavior was previously reported for other *Mastigocladus* spp. (Khumanthem *et al*., 2007), suggesting that the uptake was NO_3-induced. A similar behavior was also observed for nitrate assimilation in cultures pre-adapted in $BG11_{NH4}$. In this case, an explanation for that behavior is that the *nrt*ABCD transporter might be inhibited under the presence of ammonium and therefore all the machinery remains inactivated when the culture is maintained under $BG11_{NH4}$ condition. Furthermore, nitrate uptake declined up to 89% on cultures previously grown in $BG11_{NH4}$, possibly due to the no-active NRT substrate-binding protein anchored to the plasma membrane (NrtA) needed to assimilate nitrate (Omata *et al*., 1993). In the other hand, ammonium uptake declined up to 87% in cultures acclimated nutritionally with $BG11_{NO3}$. Under this condition, the *gln*A gene transcripts were slightly induced. In general, *gln*A and *nar*B genes those are related to ammonium and nitrate assimilation, showed higher expression levels in the light and early into the dark phases. The minor change on

induction of *gln*A gene expression observed could be directly related to the low ammonium tolerance (≤ 200 μM) of *Mastigocladus* sp. strain CHP1. The effect of ammonium toxicity is well-known to promote the disruption of the manganese cluster in the PSII oxygen-evolving complex under light condition in cyanobacteria (Belkin & Boussiba, 1991; Drath *et al*., 2008).

Finally, both ammonium and nitrate uptake behavior are very similar and might represent a cell adaptation in the *Mastigocladus* sp. strain CHP1 to utilize one or another nitrogen source, not allowing the cell to switch quickly enough from one to another, independently of how easy can it be assimilated; hence, certain plasticity in regards to the use these different nitrogen sources was apparent. These patterns can also be directed to the ecophysiological adaptations and energy savings of the strain CHP1, once nitrate/nitrite or ammonia is bioavailable in the medium.

According with all the above results obtained in this doctoral thesis, now we have a wider perspective of how taxonomic composition and metabolic activities in terms of C and N cycle contribution are associated to the temperature gradient in Porcelana hot spring. Also, the new important insights obtained by the comparison at the different temperatures along the gradient will permit us to extrapolate our results to other known thermal systems around the world with similar physico-chemical features. Also, the genomic adaptation at high temperatures for *Mastigocladus* sp. strain CHP1, provide future challenges in to understand its macromolecular properties and chemically stable enzymes, because their unique ability to function at high temperature enables development of improved or new biotechnology.

GENERAL CONCLUSIONS

The phototrophic mat of Porcelana hot spring represent a well-organized and functional ecosystem dominated at temperature <60ºC by the oxygenic phothosynhetic and diazotrophic cyanobacteria *Mastigocladus* spp., and at higher temperatures by the anoxygenic photosynthetic Chloroflexi members from genera *Roseiflexus* and *Chloroflexus* spp., which may represent a typical scenario for many neutral hot springs. The temperature transition pattern observed in Porcelana suggests potential competition of these two groups of organisms for physical space, nutrients limitation or difference in their temperature optimal growth conditions.

Metabolisms such as photosynthesis and CO_2 fixation were mainly linked with Cyanobacteria, Chloroflexi and Thaumarchaeota activities, revealing the presence of several different ways to autotrophic carbon fixation in Porcelana depending of temperature. Ammonia and nitrate were actively assimilated at all temperatures, principally by Cyanobacteria and Chloroflexi. Thaumarchaeota was present in all temperatures, and was the principal ammonia oxidizer at the most higher (66°C), while nitrogen fixation by Cyanobacteria was the major process in the contribution of new nitrogen at the lower temperatures in the Porcelana mat. Active genes involved in denitrification pathways can reflect important nitrogen loss routes at higher temperatures.

The isolate strain CHP1 was taxonomically identified as *Mastigocladus* sp. based on its morphological and genetic characterization and might represent one of the most dominant cyanobacteria (Stigonematales) in Porcelana. *Mastigocladus* sp. strain CHP1 represents a moderately thermophilic cyanobacterium able to grow at 45 and 50°C but not

at 60°C. This strain has the capacity to generated heterocysts, the specialized cells for nitrogen fixation, and it was demonstrated its capacity to perform such a process with a maximum of nitrogenase activity at noon during the light period, independently of the temperature. Daily *nif*H gene expression observed at 50°C in strain CHP1 was consistent with the *in situ* patterns reported in Porcelana mat; however, *nif*H transcripts at 45°C together with nitrogenase enzyme presence were constant during light/dark period. The results further emphasize the pivotal role of such diazotrophic cyanobacterial strain in maintaining the microbial ecosystem by delivering most of the nitrogen demands through its activity by the biological nitrogen fixation process. Also, the strain CHP1 has an apparent affinity to specific nitrogen sources according with a potential previous nutrient adaptation, reflected by *gln*A and *nar*B genes expression levels and rates of incorporation. Also, low tolerance to high concentrations of ammonium with potential toxicity for the cells was detected in this strain, however further investigations are now necessary to fully understand this response.

Altogether, we demonstrate that the temperature seems to modulate the distribution and consequent activities of the microbial mat community in Porcelana hot spring, revealing a close relationship between Bacteria and Archaea, which have high implications to the C and N cycle in this extreme ecosystem. *Mastigocladus* sp. strain CHP1 represents a substantial part of the Stigonematales that possible together with other closer relatives dominates the microbial mat of Porcelana, being responsible for the total biological nitrogen fixation in this hot spring. These findings may have global important implications for other thermal microbial mats or extreme environments dominated by Cyanobacteria. And in that sense the strain CHP1 shows genomic adaptation at high temperatures, become a good model to study thermophilic diazotrophic cyanobacteria living in hot springs.

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ORIGINAL ARTICLE

The cyanobacterium *Mastigocladus* fulfills the nitrogen demand of a terrestrial hot spring microbial mat

María Estrella Alcamán[1], Camila Fernandez[2,3,4], Antonio Delgado[5], Birgitta Bergman[6] and Beatriz Díez[1,7]

[1]*Department of Molecular Genetics and Microbiology, Faculty of Biological Sciences, Pontificia Universidad Católica de Chile, Santiago, Chile;* [2]*Laboratoire d'Océanographie Microbienne, Observatoire Océanologique, Sorbonne Universités, UPMC Univ Paris 06, UMR 7621, Banyuls/mer, France;* [3]*CNRS, UMR 7621, Laboratoire d'Océanographie Microbienne, Observatoire Océanologique, Banyuls/mer, France;* [4]*Department of Oceanography, Interdisciplinary Center for Aquaculture Research (INCAR) and COPAS SURAUSTRAL Program, University of Concepción, Concepción, Chile ;* [5]*Instituto Andaluz de Ciencias de la Tierra (CSIC-Univ. Granada), Armilla, Granada, Spain;* [6]*Department of Ecology, Environment and Plant Sciences and Science for Life Laboratory, Stockholm University, Stockholm, Sweden and* [7]*Center for Climate and Resilience Research (CR)2, Santiago, Chile*

Cyanobacteria from Subsection V (Stigonematales) are important components of microbial mats in non-acidic terrestrial hot springs. Despite their diazotrophic nature (N_2 fixers), their impact on the nitrogen cycle in such extreme ecosystems remains unknown. Here, we surveyed the identity and activity of diazotrophic cyanobacteria in the neutral hot spring of Porcelana (Northern Patagonia, Chile) during 2009 and 2011–2013. We used 16S rRNA and the *nif*H gene to analyze the distribution and diversity of diazotrophic cyanobacteria. Our results demonstrate the dominance of the heterocystous genus *Mastigocladus* (Stigonematales) along the entire temperature gradient of the hot spring (69–38 °C). *In situ* nitrogenase activity (acetylene reduction), nitrogen fixation rates (cellular uptake of $^{15}N_2$) and *nif*H transcription levels in the microbial mats showed that nitrogen fixation and *nif*H mRNA expression were light-dependent. Nitrogen fixation activities were detected at temperatures ranging from 58 °C to 46 °C, with maximum daily rates of 600 nmol C_2H_4 cm^{-2} per day and 94.1 nmol N cm^{-2} per day. These activity patterns strongly suggest a heterocystous cyanobacterial origin and reveal a correlation between nitrogenase activity and *nif*H gene expression during diurnal cycles in thermal microbial mats. N and C fixation in the mats contributed ~3 g N m^{-2} per year and 27 g C m^{-2} per year, suggesting that these vital demands are fully met by the diazotrophic and photoautotrophic capacities of the cyanobacteria in the Porcelana hot spring.
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Introduction

Hot springs represent extreme environments for life. They are typically dominated by a range of microorganisms that form well-defined 'mats' that are constantly being over run by hot spring water. A variety of physical and chemical features, such as the pH (Hamilton *et al.*, 2011; Loiacono *et al.*, 2012; Huang *et al.*, 2013), sulfide concentration (Purcell *et al.*, 2007) and temperature (Miller *et al.*, 2009; Wang *et al.*, 2013) shape the microbial presence and life cycle in these ecosystems. Temperature is considered the most important variable associated with changes and metabolic adaptations in microbial mat communities in hot springs with a neutral pH (Cole *et al.*, 2013; Mackenzie *et al.*, 2013).

Recently, the diversity of microbial thermophiles in many hot springs has been characterized (Nakagawa and Fukui, 2002; Meyer-Dombard *et al.*, 2005; Hou *et al.*, 2013; Inskeep *et al.*, 2013). A range of thermophilic microorganisms (~75–40 °C) has been identified. Representatives of the bacterial phyla Cyanobacteria, Chloroflexi and Proteobacteria are the most commonly found microbes in neutral to alkaline hot springs (Otaki *et al.*, 2012; Cole *et al.*, 2013; Mackenzie *et al.*, 2013; Wang *et al.*, 2013). Within the cyanobacteria, unicellular members such as *Synechococcus* and *Cyanothece* typically dominate at temperatures above 60 °C (Ward *et al.*, 1998;

Correspondence: B Díez, Department of Molecular Genetics and Microbiology, Faculty of Biological Sciences, Pontificia Universidad Católica de Chile, Alameda 340, Casilla 144-D, CP 651 3677 Santiago, Chile.
E-mail: bdiez@bio.puc.cl
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Ward and Castenholz, 2000; Papke *et al*., 2003; Steunou *et al*., 2006, 2008). At lower temperatures (~60–40 °C), filamentous, non-heterocystous genera such as *Phormidium* and *Oscillatoria* and heterocystous genera such as *Calothrix*, *Fischerella* and *Mastigocladus* are common (Sompong *et al*., 2005; Miller *et al*., 2006; Finsinger *et al*., 2008; Coman *et al*., 2013). Although heterocystous cyanobacteria are richly represented in many hot springs with a neutral pH, their role and capacity as providers of fixed nitrogen is still unknown.

Nitrogen fixation is the process by which selected diazotrophs from Archaea and Bacteria consume atmospheric N_2 gas as a substrate for growth (Stewart *et al*., 1967). This process may represent an important source of 'new' nitrogen in the often nitrogen-limited hot spring waters. This process also counter acts the loss of combined nitrogen caused by denitrification in the poorly ventilated substrates of terrestrial hot springs (Otaki *et al*., 2012). N_2 fixation has been assessed by screening for specific *nif* genes such as *nif*H (encoding the α-subunit of the nitrogenase enzyme complex), which is the most widely used molecular marker in the search for diazotrophs. Hence, the analysis of the presence of the *nif*H gene combined with measurements of nitrogenase activity (using the acetylene reduction assay) has been widely used to identify diazotrophs and diazotrophy in microbial mats from diverse environments (Stal *et al*., 1984; Bergman *et al*., 1997; Steunou *et al*., 2006; Díez *et al*., 2007; Severin and Stal, 2009; Desai *et al*., 2013).

Currently, the most thoroughly studied hot springs are those in Yellowstone National Park (YNP, Wyoming, USA), in which both nitrogenase activity and *nif*H gene transcription patterns have been examined (Miller *et al*., 2009; Hamilton *et al*., 2011; Loiacono *et al*., 2012). For example, nitrogenase activity was recorded in alkaline hot springs at temperatures of ~50 °C and was attributed to the heterocystous cyanobacterium *Mastigocladus laminosus* (Stewart, 1970; Miller *et al*., 2006), whereas at higher temperatures in two other hot springs, the activity was credited to the unicellular cyanobacterium *Synechococcus* (Steunou *et al*., 2006, 2008). However, heterotrophic bacteria and archaea are also highly represented as thermophiles in YNP acidic hot springs, including the presence of some active nitrogen fixers at temperatures up to 82 °C (Hamilton *et al*., 2011). Moreover, *nif*H genes have been detected at 89 °C in hot springs with varying pH values (1.9–9.8) (Hall *et al*., 2008; Loiacono *et al*., 2012).

Owing to the more 'indirect' character of the 'nitrogen fixation' activity provided by the acetylene reduction technique (which measures nitrogenase enzyme activity), verification of the results through measurements of the nitrogen fixation activity (that is, N_2 gas uptake and cellular N incorporation using the $^{15}N_2$ stable isotope assay) is highly recommended (Peterson and Burris, 1976; Montoya *et al*., 1996). However, $^{15}N_2$ gas uptake has rarely been used to study nitrogen fixation by microorganisms in thermal hot springs. The only exception is the study of Stewart (1970) in an alkaline hot spring in YNP. Furthermore, measurements of nitrogenase activity by acetylene reduction assay (ARA), $^{15}N_2$ uptake and *nif*H gene expression have not been evaluated together in a thermal microbial mat.

The aim of our study was to evaluate the role of diazotrophs in the nitrogen economy of the pristine, neutral terrestrial hot spring of Porcelana (Chile) with a focus on cyanobacteria. To achieve this goal, we examined the molecular identity (16S rRNA and *nif*H genes) of diazotrophic cyanobacteria and estimated their daily *in situ* nitrogenase activity and $^{15}N_2$ uptake in combination with *nif*H gene expression in a series of interannual analyses (2009, 2011–2013). Our data show that cyanobacteria are capable of fulfilling the nitrogen demands of hot spring microbial mats through their nitrogen fixation activity.

Materials and methods

Study site and sampling strategies

The study was conducted in the hot spring of Porcelana located ~100 m above sea level at 42°27′29.1″S–72°27′39.3″W in northern Patagonia, Chile (Figure 1a). A similar thermophilic temperature range (>69–38 °C) was registered during the sampling and experimentation during late summer (March) of the 4 years, 2009 and 2011–2013. Temperature, pH and dissolved O_2 percentages were monitored using a multiparameter instrument (Oakton, Des Plaines, IL, USA; model 35607-85). Microbial mat samples (1 cm thick) used for *in situ* ARA and $^{15}N_2$ uptake experiments and DNA/RNA analysis were obtained using a cork borer with a diameter of 7 mm. An extra three cores not used in the *in situ* analysis were included to generate enough material for the DNA/RNA analyses. Spring water (5 ml) and microbial mat samples were collected in triplicate for nutrient (NH_4^+, NO_3^-, NO_2^- and PO_4^{3-}) and total chlorophyll determinations. Dissolved Fe concentrations were determined in the same samples using ICP-Mass spectrometry X series 2 (Thermo Fisher Scientific Inc.) after preconcentration with ammonium 1-pyrrolidinedithiocarbamate/diethylammonium die thyldithiocarbamate organic extraction (Bruland and Coale, 1985). All samples were stored in liquid nitrogen during transportation to the laboratory and at −80 °C until processing.

DNA extraction, PCR amplification and denaturing gradient gel electrophoresis

DNA was extracted as described previously (Bauer *et al*., 2008). Before DNA extraction, the samples were placed in a Lysing Matrix E tube (Qbiogene, Carlsbad, CA, USA) containing lysis buffer and solid-glass beads (1 mm) to homogenize the microbial cells by bead beating (4.0 ms^{-1} for 20 s). The quality and

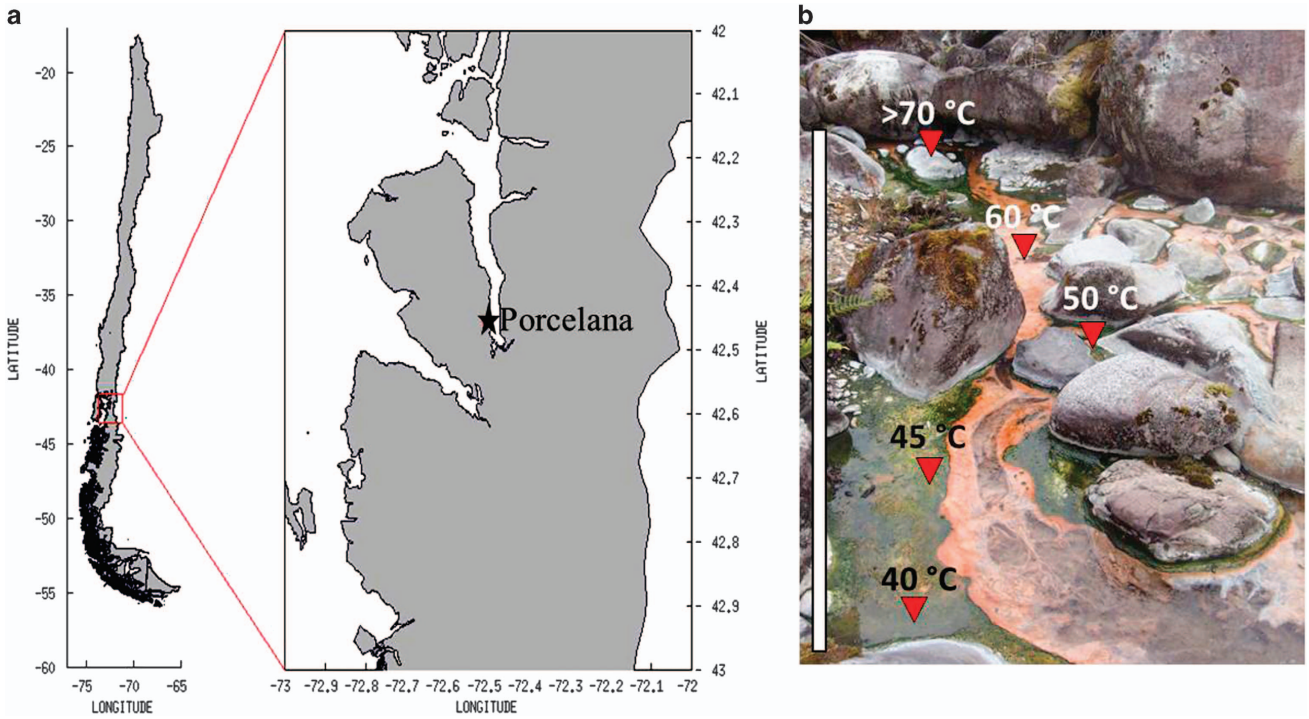

Figure 1 (**a**) Location of the Porcelana hot spring in northern Patagonia, Chile (X Region, Comau fjord). (**b**) The pigmented microbial mat was formed throughout the temperature gradient; the sampling sites are indicated by red triangles. The gray bar represents the mat extension (~10 m) within the thermophilic temperature gradient.

quantity of the extracted DNA were determined using a spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and by inspection after separation in a 1% agarose gel. Then, the total DNA was used as the template for PCR amplifications of cyanobacterial 16S rRNA genes using the cyanobacteria-specific primers CYA106F with a GC clamp (5′ 40 nucleotide GC tail) and CYA781R(a)–CYA781R(b) (Nübel *et al.*, 1997) to generate amplicons 600 nucleotides in length. The DNA was also used as a template for amplification of the *nif*H genes using the diazotrophic cyanobacteria-specific primers CN Forward (CNF) with a GC clamp and CN Reverse (CNR) (Olson *et al.*, 1998) to generate amplicons 350 nucleotides in length. The amplicons were resolved using a denaturing gradient gel electrophoresis (DGGE) approach with a D-code system (Bio-Rad Laboratories, Hercules, CA, USA) according to the protocol of Díez *et al.* (2007). The gradients of DNA denaturant agents used in the gels were 45–75% for the 16S rRNA gene and 45–65% for the *nif*H gene. DGGE bands located in the same position in the gel were assigned to the same microbial population. Several of the bands with the same position were excised from the gel, reamplified and sequenced, as were all bands located at different positions along the gel. The excised DGGE bands were eluted in 20 µl DNAse/RNAse-free dH_2O (ultraPURE; Apiroflex, Santiago, Chile) and stored at 4 °C overnight. An aliquot of the eluted DNA was subjected to an additional PCR amplification using the corresponding primers (without GC clamp) before sequencing (Macrogen Inc., Seoul, Korea). Each specific DGGE band retrieved was assigned to one sequence representing a specific phylotype. The sequences were edited using the BioEdit software (Sequence Alignment Editor Software V.7.0.5.3., Carlsbad, CA, USA), followed by a basic local alignment and the use of a search tool (BLASTN) (Altschul *et al.*, 1997).

Bacterial nifH gene clone library

The diversity of diazotrophic prokaryotes present in the microbial mat throughout the thermal gradient was determined using *nif*H gene clone libraries. PCR amplifications of the *nif*H gene were performed using the universal primers PolF/PolR (Poly *et al.*, 2001) that cover most of the known diazotrophic organisms (Bacteria and Archaea), including cyanobacteria (Mårtensson *et al.*, 2009; Díez *et al.*, 2012). These primers amplify fragments 360 bp in length. The PCR products were purified (Wizard Clean-Up System; Promega, Madison, WI, USA) and cloned using the commercial pJET1.2/blunt Cloning Kit (Thermo Scientific) according to the manufacturer's instructions. Clones with the proper insert sequence were validated using the primer vector set pJetF/pJetR (amplicon length ~550–600 bp). Fifty to one hundred clones obtained from each library (12 clone libraries in total) were selected for cyanobacterial-specific *nif*H gene amplifications using the primers CNF and CNR (Olson *et al.*, 1998). These primers amplify a fragment within the insert generated by the

universal primers PolF/PolR (Poly *et al*., 2001). Several of the amplified PCR products were sequenced to check for cyanobacterial genetic identities. Clones that did not amplify with the cyanobacterial primers CNF and CNR were assumed to correspond to other types of bacteria and were also sequenced. All sequences obtained were edited using the BioEdit software (Sequence Alignment Editor Software V.7.0.5.3.). The operational taxonomic units (OTUs) with 98% similarity were assigned using BLASTCLUST-BLAST score-based single-linkage clustering (Schloss and Westcott, 2011). The closest relatives to all OTUs were assigned using the BLASTN tool (National Center for Biotechnology Information database).

Phylogenetic reconstruction and statistical analysis
The 16S rRNA phylotypes retrieved from the DGGE band sequences, the reference taxa and the closest relatives from GenBank (only from published studies or cultures) were aligned using BioEdit with the ClustalW tool (Tom Hall; Ibis Therapeutics, Carlsbad, CA, USA). The same procedure was used for the *nif*H-DGGE band sequences and the *nif*H OTUs from the constructed clone libraries. The subsequent phylogenetic reconstruction using the maximum-likelihood search strategy with 10 000 bootstrap replicates was performed for each gene data set. The sequences of *Gloeobacter violaceus* and *Desulfovibrio salexigens* were used as outgroups for the 16S rRNA and *nif*H gene phylogenetic reconstructions, respectively.

The obtained 16S rRNA and *nif*H sequences (16S rRNA-DGGE band and *nif*H OTUs) were subjected to cluster analysis and BEST tests using PRIMER 6. The dendrograms generated for both genes were constructed to elucidate the similarity between the samples collected during different years and along the temperature gradient. The BEST test was performed to estimate the environmental factors that best explained the microbial species distributions. Additionally, correspondence analysis and redundancy analysis analyses (Clarke, 1993) were performed based on the relative abundances of 16S rRNA-DGGE bands and *nif*H OTUs and the environmental variables recorded each year to pinpoint the environmental variable (s) that most strongly influenced the microbial mat community.

RNA extraction and real-time qPCR measurements
Biological replicates from the acetylene reduction assay (three cores each) plus some additional non-assayed samples were used for the subsequent RNA analysis. These samples were collected throughout the day–night cycle (at 1200, 1300, 1400, 1600, 1800, 2000, 2300 and 0300 hours) and at three different temperatures (58 °C, 48 °C and 47 °C) in 2 years (2012 and 2013). RNA from the samples was extracted using Trizol and the RNeasy Plant Mini Kit according to manufacturer's specifications (Qiagen, Hilden, Germany). The quality and quantity of the RNA were determined using a spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and by electrophoresis in an RNase-free 1% agarose gel. DNase treatment (TURBO DNA-free kit; Applied Biosystems, Carlsbad, CA, USA) was performed, and 1 μg of RNA from each sample (in duplicate) was used for quantitative PCR (qPCR) standardization. Then, the cDNA was synthesized using a selective cDNA Kit (Bio-Rad Laboratories) according to the manufacturer's specifications with the universal *nif*H gene primers PolF/PolR (Poly *et al*., 2001). For qPCR, the *nif*H gene was cloned into the TOPO vector plasmid to obtain the plasmid stock concentration (10^{10} copies) and the plasmid curve (10^{2}–10^{8} copies). The SensiMix kit (Bioline, Taunton, MA, USA) was used for the fluorescence signal, and the real-time qPCR (Roche LC 480 Roche diagnostics Ltd., Mannheim, Germany) program was run as follows: 40 cycles at 95 °C for 10 s, 59 °C for 20 s and 72 °C for 30 s. To avoid nonspecific fluorescence, only fluorescence within the CP (crossing point) range given by the plasmid standard curve was considered and melting curves were only considered if they showed a unique product.

Measurement of nitrogenase activity ARA
The ARA was used to assess nitrogenase activity in the microbial mats throughout the temperature gradient of the hot spring. This assay was performed according to the procedure described by Capone (1993). At each temperature, four biological replicates composed of three microbial mat cores each (7 mm in diameter and 1 cm thick) were placed in presterilized 10 ml glass incubation vials containing 1 ml of prefiltered (0.2 μm filter pore) spring water and sealed using Mininert valves STD (Sigma-Aldrich, St Louis, MO, USA). The samples were incubated for 2 h following replacement of 1 ml of air with 1 ml of acetylene gas (10–20% of the gas phase) generated from calcium carbide ($CaC_2+H_2O=Ca(OH)_2+C_2H_2$). The four replicates plus two controls (one with microbial mat cores but no acetylene gas and one containing only acetylene gas but no cores) were incubated at their original *in situ* temperature in the field. The first control was used to estimate any natural 'background' ethylene generated by the microbial community, and the second control was used to estimate any ethylene generated in the calcium carbide reaction. After incubations during diel cycles (1300, 1400, 1700, 2300 and 0300 hours), 5 ml of the gas phase was withdrawn from each vial using a hypodermic syringe and transferred to a 5 ml BD vacutainer (no additive Z plus tube, REF367624). After transporting the vacutainers to the laboratory, the ethylene produced was analyzed by injecting 1 ml of the gas using a gas-tight syringe (Hamilton) into a GC-8A gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with an 80/100 Porapak Q (Supelco, St Louis, MO, USA) 1 m × 1/4 in

column and a flame ionization detector using helium as the carrier gas. A commercial ethylene standard of 100 p.p.m. (Scotty Analyzed Gases, Sigma-Aldrich) in air was used to estimate the ethylene produced. Acetylene (20% in air) was used as an internal standard (Stal, 1988). The nitrogenase activity calculated from the ethylene produced was corrected using the two controls and expressed per surface area of microbial mat cores and time.

Isotopic nitrogen assimilation ($^{15}N_2$) and carbon ($H^{13}CO_3^-$) uptake

In parallel to the ARA measurements performed in 2012 and 2013, samples from the microbial mats were collected for ^{15}N and ^{13}C uptake experiments. The experiments ($^{15}N_2$ and $H^{13}CO_3^-$) were performed using three biological replicates composed of three microbial mat cores each (7 mm in diameter and 1 cm thick). The cores were placed in presterilized 12 ml vials with 1 ml of prefiltered (0.2 μm filter pore) spring water and incubated at the corresponding *in situ* temperatures. The ^{15}N uptake experiments were initiated through the addition of 1 ml of $^{15}N_2$ gas (98% atom $^{15}N_2$ gas; Sigma-Aldrich) through a gas-tight syringe into the headspace of each vial. To estimate the carbon ($H^{13}CO_3^-$) uptake, 500 μl of $H^{13}CO_3^-$ (500 μM) was added to the vials. Additionally, two replicate vials without the isotope ($^{15}N_2$ and ^{13}C) were incubated to determine the natural isotopic composition (control). The vials were incubated *in situ* for 2 or 6 h and then the cores were dried at 70 °C for 48 h. Measurement of ^{15}N and ^{13}C atom incorporation (AT ^{15}N and ^{13}C) were performed using a mass spectrometer (IRMS delta plus, Thermo FinniganH; Stable Isotope Laboratory, Granada, Spain), and the C:N ratio (organic matter composition of the sample) was determined. Calculations of the ^{15}N and ^{13}C assimilation rates were performed as described by Montoya *et al.* (1996) and Fernandez *et al.* (2009), including corrections by dilutions of $^{15}N_2$ gas and controls.

Results

Geochemistry of the Porcelana hot spring

The Porcelana hot spring (Figure 1) shows a continuous outflow of hot water, thereby forming a decreasing temperature gradient away from the well. The water temperature ranged from 69 °C to 38 °C over the gradient investigated, with some variation in maximum temperatures between years (Table 1). A brightly pigmented microbial mat (~3 cm deep) extended 7–10 m away from the well (Figure 1b) at the bottom of the water stream. The decreasing temperature gradient resulted in increasing water oxygen solubility. The physicochemical features of the mat were comparatively constant over time (Table 1). The pH was close to neutral (~6.5), and the macronutrient concentrations were on average 1.9 μmol l^{-1} NO_3^-, 0.6 μmol l^{-1} NO_2^-, 0.03 μmol l^{-1} NH_4^+ and 51.4 μmol l^{-1} PO_4^{3-} over the 2011–2013 period (Table 1). The nitrate concentration was examined during the day and night periods and at two temperatures (52 °C and 47 °C) in 2012. No variations were apparent between day and night, although the nitrate levels were almost threefold higher at 52 °C (Supplementary Data and Supplementary Figure S1). The dissolved Fe concentrations were ~0.07 μmol l^{-1} in 2012 and 2013 (Table 1).

Table 1 Physical and chemical variables registered in the Porcelana hot spring at different locations along the microbial mat during the years 2009 and 2011–2013

Year	T *(°C)*	O_2 *% Sat.*	*pH*	NO_3^- *(μmol l^{-1})*	NO_2^- *(μmol l^{-1})*	NH_4^+ *(μmol l^{-1})*	PO_4^{3-} *(μmol l^{-1})*	*Fe (μmol l^{-1})*
2009	46	42	5.2	ND*	ND*	ND*	ND*	ND*
2009	42	46	6.4	ND*	ND*	ND*	ND*	ND*
2009	40	43	6.1	ND*	ND*	ND*	ND*	ND*
2009	38	48	5.1	ND*	ND*	ND*	ND*	ND*
2011	69	54	6.9	ND*	ND*	ND*	ND*	ND*
2011	64	59	6.7	ND*	ND*	ND*	ND*	ND*
2011	61	80	6.9	0.8	1.3	ND*	ND*	ND*
2011	57	82	6.8	ND*	ND*	ND*	ND*	ND*
2011	51	90	6.7	ND*	ND*	ND*	ND*	ND*
2012	52	104	6.7	1.7	0.2	0.01	29.7	ND*
2012	47	108	7.1	1.2	0.2	0.01	43.5	0.02
2013	66	72	6.8	6.5	0.01	0.02	115	0.05
2013	65	73	6.8	0.9	0.2	0.01	47.4	0.07
2013	58	86	6.8	1.9	0.1	0.01	38.4	0.14
2013	48	94	7.1	1.2	0.2	0.1	34.1	0.06

Abbreviation: ND*, data not determined.

Interannual cyanobacterial diversity

The cyanobacterial diversity in the microbial mat growing along the temperature gradient was examined during the years 2009 and 2011–2013. The analyses were performed by DGGE using the cyanobacterial-specific 16S rRNA gene as the diversity marker (Supplementary Data and Supplementary Figure S3a). The resulting DGGE bands (five in total) revealed the existence of differently distributed sub-populations along the temperature gradient. The bands corresponded with members of the phylum Cyanobacteria and specifically with members within the heterocystous order Stigonematales (DGGE band CYA5; GenBank accession numbers for nucleotide sequences: KJ696694) and the non-heterocystous order Oscillatoriales (DGGE band CYA1-4; GenBank accession numbers for nucleotide sequences: KJ696687–KJ696690) (Supplementary Data and Supplementary Table S1).

Cluster analysis of the 16S rRNA gene marker was performed using PRIMER 6 (Bray–Curtis dissimilarity index dendrogram) assuming the presence or absence of the DGGE bands together with their relative abundance throughout the temperature gradient in the 4 years (Figure 2). Up to 70% similarity was apparent for all samples denoted as cyanobacteria in the dendrogram (Figure 2a). However, samples collected from similar temperatures within the same year grouped as pairs showed >90% similarity. This result may be explained by the similar relative abundances of the cyanobacteria (analyzed by 16S rRNA genes) exhibited by the

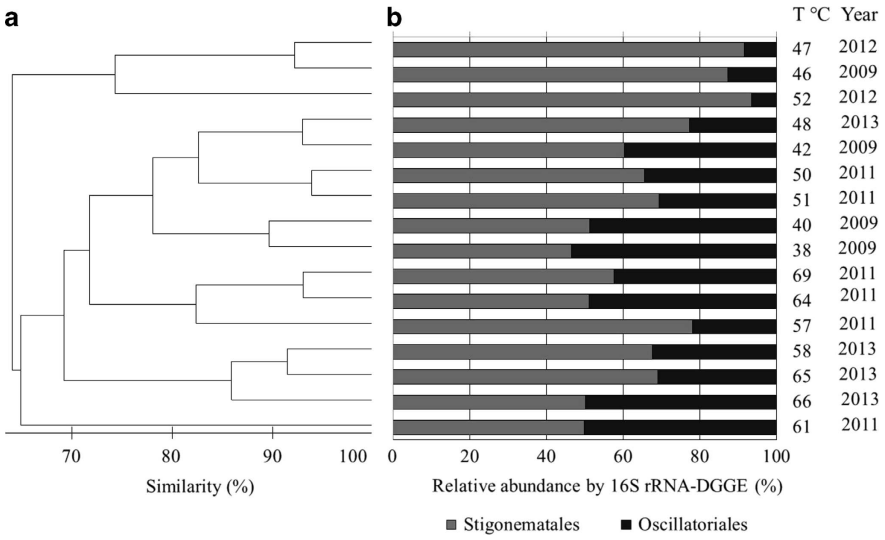

Figure 2 Comparison of the interannual cyanobacterial diversity at different temperatures in the Porcelana hot spring based on the 16S rRNA gene and DGGE. (**a**) Bray–Curtis dissimilarity index dendrogram. (**b**) Relative abundance of 16S rRNA-DGGE bands (phylotypes) for each temperature and year investigated.

pairs (Figure 2b). Additionally, most pairs showed >80% similarity with a third sample collected during the same year or at a similar temperature. This result illustrates the strong influence of temperatures and interannual variations on the cyanobacterial community.

Phylogenetic reconstructions of the sequences retrieved from the DGGE bands using the 16S rRNA gene confirmed the placement of the hot spring cyanobacteria within the filamentous non-heterocystous order Oscillatoriales (Section III) and the heterocystous order Stigonematales (Section V) (Supplementary Data and Supplementary Figure S2). The four 16S rRNA-DGGE bands CYA1–CYA4 (Supplementary Data and Supplementary Figure S3a) formed clusters with members of the genera *Leptolyngbya* and *Oscillatoria* (Oscillatoriales) with a 99% similarity according to the BLASTN analyses (Supplementary Data and Supplementary Table S1). Moreover, the even more prevalent 16S rRNA-DGGE band CYA5 (Supplementary Data and Supplementary Figure S3a) was closely related to members of the *Mastigocladus* and *Fischerella* genera (Supplementary Data and Supplementary Table S1). The tentatively identified *Mastigocladus* phylotype (CYA5) was the only phylotype present along the entire temperature gradient (i.e., from 69 °C to 38 °C); CYA5 also exhibited the highest relative abundance in 16S rRNA gene sequences at higher temperatures (57–46 °C) (Figure 2b). Within this temperature range, the *Mastigocladus* phylotype represented an average of 66% of the total cyanobacterial community; the remaining 34% was represented by Oscillatoriales phylotypes.

BEST analysis relating the 16S rRNA phylotypes identified by DGGE to the recorded *in situ* environmental variables including temperature (°C), dissolved oxygen (%), pH and nitrogen compounds (NO_3^-, NO_2^- and NH_4^+) (Table 1) showed that variations in temperature and pH explained 77% of the similarity between the phylotypes (ρ-value 0.109; significance level 91%). These results were corroborated using canonical correspondence analysis, which showed that temperature, pH and NO_2^- represented the major ecological drivers of the phylotype distribution in the Porcelana hot spring (Supplementary Data and Supplementary Figure S5).

Interannual diversity of cyanobacterial diazotrophs

The diversity of diazotrophs in the hot spring was investigated by constructing clone libraries targeting the *nif*H gene using universal primers (Poly *et al*., 2001). Fifty to one hundred clones were obtained from the 12 libraries constructed (Supplementary Data and Supplementary Table S1). To identify clones related to cyanobacteria, the clones were reamplified using the cyanobacterial-specific *nif*H gene primers (Olson *et al*., 1998). Fifteen to fifty clones in each library were found to represent cyanobacterial phylotypes. All of the retrieved sequences (GenBank accession numbers for nucleotide sequences: KM507492–KM507497) were analyzed using BLASTCLUST-BLAST (Schloss and Westcott, 2011) to identify the OTUs present in each clone library (Supplementary Data and Supplementary Table S1). A total of six cyanobacterial *nif*H OTUs were apparent, three of which were determined to be closely affiliated (>98% nucleotide sequence identity) to the heterocystous genus *Mastigocladus* (Stigonematales) by BLASTN analysis (Figure 3b, Supplementary Data and Supplementary Table S1). The other three OTUs were affiliated with the Oscillatoriales (>88% nucleotide sequence identity) and more specifically with the genera *Leptolyngbya* and *Oscillatoria* (Supplementary Data

and Supplementary Table S1). A phylogenetic reconstruction of the six *nif*H gene OTUs and the closest related sequences from the database confirmed the identities obtained by BLASTN (Supplementary Data and Supplementary Figure S4). As shown in Figure 3a, similarity cluster analysis of the *nif*H OTUs demonstrated that all of the microbial mat samples collected in the spring were highly stable and exhibited >95% similarity in the community that was independent of the temperature and the year investigated. The dominance of the *Mastigocladus* OTUs identified by *nif*H gene analysis was confirmed (93% on average) at all temperatures, whereas the Oscillatoriales OTUs were comparatively rare (7% average) (Figure 3b).

Redundancy analysis of the *nif*H gene OTUs and the *in situ* recorded environmental variables including temperature (°C), dissolved oxygen (%), pH and nitrogen compounds (NO_3^-, NO_2^- and NH_4^+) (Table 1) showed that the temperature and nutrients (NH_4^+ and NO_2^-) explained the distribution and high relative abundance of the *Mastigocladus nif*H gene OTUs in the spring (Supplementary Data and Supplementary Figure S6).

The identity of the cyanobacterial OTUs obtained using the *nif*H clone libraries were verified via the DGGE approach using the same cyanobacterial-specific *nif*H primers (Olson *et al*., 1998). Three *nif*H-DGGE bands (Supplementary Data and Supplementary Figure S3b) were retrieved and affiliated with *M. laminosus* with 99% sequence similarity (BLASTN tool; GenBank accession numbers for nucleotide sequences: KJ696698–KJ696700) (Supplementary Data and Supplementary Table S1). None of the *nif*H-DGGE bands were affiliated with members of the Oscillatoriales.

A phylogenetic reconstruction combining the sequenced *nif*H gene OTUs and *nif*H-DGGE bands with their closest matches in the database (Supplementary Data and Supplementary Figure S4) showed that all of the genes clustered to Stigonematales with sequences related to the thermophilic *M. laminosus*. The Oscillatoriales OTUs clustered with the '*Filamentous thermophilic cyanobacterium* sp.' (accession number: KM507495 and KM507496) and *Leptolyngbya* sp. (accession number: KM507497).

Biological nitrogen fixation

Owing to the high presence of potential diazotrophic cyanobacteria in the microbial mat of the Porcelana hot spring, the nitrogen fixation process was recorded using two approaches: the sensitive acetylene reduction assay (ARA-GC) to estimate the nitrogenase enzyme activity (years 2009 and 2011–2013) and the $^{15}N_2$ stable isotope uptake to estimate the biological incorporation of nitrogen into the biomass (years 2012 and 2013) using mass spectroscopy. As shown in Figure 4 and Table 2, the total nitrogenase activity recorded along the temperature gradient at mid-day (1200–1400 hours) varied from 0.2 (± s.d. 0.01) to 50.0 (± s.d. 7.0) nmol C_2H_4 cm^{-2} h^{-1}. The highest rates were recorded at 46–48 °C, whereas higher temperatures (Figure 4) and darkness (Figure 5) gave a lower activity.

Analysis of the cellular incorporation of nitrogen (Table 2) after 2-h (1200–1400 hours) and 6-h (1200–1800 hours) incubations showed incorporation of ^{15}N (Table 2 and Figure 4). The highest nitrogen incorporation recorded was 7.8 nmol N cm^{-2} h^{-1} (± s.d. 0.6) at 48 °C in 2013, coinciding with the highest nitrogenase activity at the same temperature and year (Table 2). No difference in activity was observed following incubations for 2 or 6 h (Table 2). The theoretical ratio between the acetylene reduction (ARA) and the isotopic N_2 fixation method

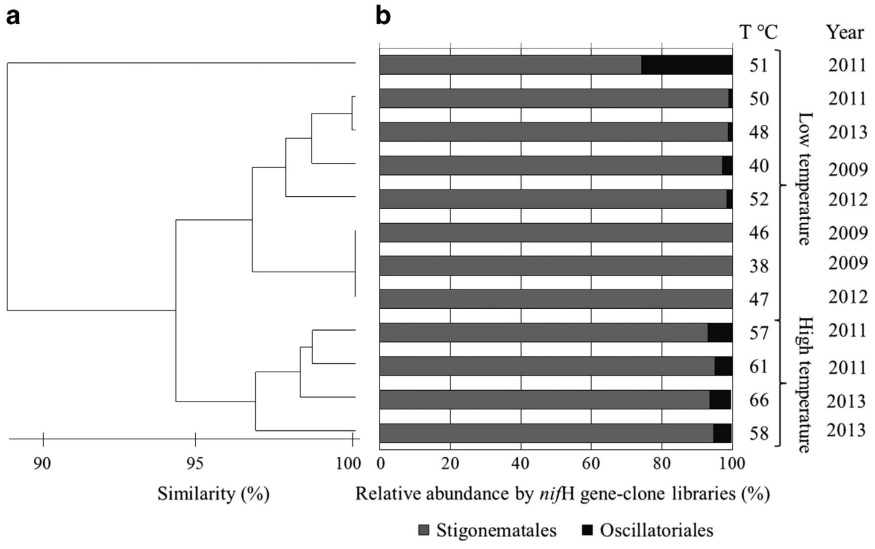

Figure 3 Relative abundance and interannual diazotrophic bacterial diversity in the Porcelana hot spring based on the *nif*H marker gene and clone libraries. (**a**) Bray–Curtis dissimilarity index dendrogram. (**b**) Relative abundance of the *nif*H gene (OTUs) determined using clone libraries obtained for each temperature and year investigated.

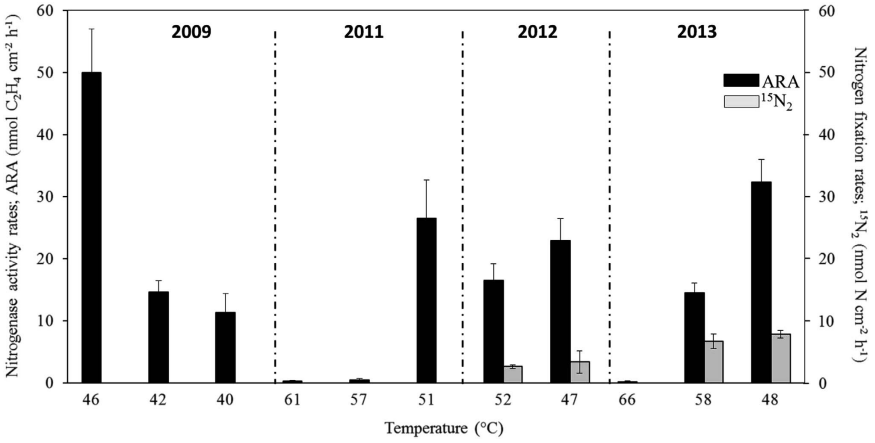

Figure 4 Nitrogen fixation assessed by the ARA and $^{15}N_2$ uptake analysis for the different temperatures and years investigated. ARA measurements (black bars) were conducted during the 4 years, whereas $^{15}N_2$ uptake measurements (gray bars) were performed in 2012 and 2013.

(C_2H_4:N_2) is 4:1. The ratio for the Porcelana hot spring microbial mat was close to this theoretical ratio, ranging from 2.2:1 to 6.8:1 (Table 2).

Based on the ^{15}N uptake quantities, the 'new' yearly nitrogen inputs into the Porcelana hot spring were extrapolated to represent up to 2.9 g N m^{-2} per year in 2012 and 4.8 g N m^{-2} per year in 2013 (Table 2).

Diel cycles of nitrogenase activity and nifH gene expression

Based on the fact that the optimum temperature for nitrogenase activity in the Porcelana hot spring was between 58 °C and 46 °C (Figure 4), this temperature interval were selected to determine the nitrogenase activity and *nif*H gene expression in greater detail throughout the day during two consecutive days in 2012 and 2013. As shown in Figure 5, the nitrogenase activity peaked at mid-day (at ~1300–1400 hours) irrespective of the temperature and approached zero at night. Similar diel nitrogenase activity patterns were observed in both years, peaking at 22.9 nmol C_2H_4 cm^{-2} h^{-1} at 47 °C in 2012 and 32.4 nmol C_2H_4 cm^{-2} h^{-1} at 48 °C in 2013 (Table 2). The nitrogenase activity was consistently higher at lower temperatures (47–48 °C). Next, the biological sample replicates used for the nitrogenase assays (three cores in each vial) were combined with extra microbial mat material to examine the diel cycles of *nif*H gene expression (Figure 5). In 2012, the *nif*H gene expression was measured only at 47 °C. Maximum transcript levels occurred around mid-day (day 2) with 2.1×10^7 *nif*H gene transcripts identified. In 2012, two lower expression peaks were noted at 1600 hours (5.2×10^5) and 2000 hours (6.6×10^5); this pattern was also observed in 2013. The highest transcription level (2.4×10^4) was found at ~1600 hours and 58 °C, whereas no *nif*H expression took place in the dark–night time when examined in 2012 and 2013.

Carbon fixation

Because the data showed that the Porcelana microbial mat was dominated by cyanobacteria, the *in situ* incorporation of ^{13}C-labeled bicarbonate ($H^{13}CO_3^-$) was followed in 2012 and 2013. The incubations with $H^{13}CO_3^-$ lasted 2 h (1200–1400 hours) under the same conditions described for the nitrogen fixation assays (i.e., at 52 °C and 58 °C) (Table 2). The highest carbon incorporation recorded was 53.0 (± s.d. 4.1) and 45.8 (± s.d. 8.7) nmol C cm^{-2} h^{-1} at 52 °C and 58 °C, respectively, during the two consecutive years (Table 2). Extrapolation to a yearly incorporation showed an average C uptake of ~27 g C fixed m^{-2} per year in the Porcelana hot spring.

Contribution of combined nitrogen to the Porcelana microbial community

Taking into account the daily rates of $^{15}N_2$ uptake, $H^{13}CO_3^-$ assimilation and the C:N ratio (Table 2), it was apparent that the photoautotrophic nitrogen fixers present in the Porcelana microbial mat sustained these key nutrient demands to a large extent. Even when the daily rates found for nitrate assimilation ($^{15}NO_3^-$) (data not shown) were considered, the total 'new' production of nitrogen fixation (^{15}N) contributed up to 99% of the 'new' N input into the microbial mat of the Porcelana hot spring (Table 2). The analyses were performed according to the protocol of Raimbault and Garcia (2008), although the data were not corrected for nitrification.

Discussion

Although thermal systems around the world have attracted considerable interest and their overall biology and organisms have been characterized (Stewart, 1970; Miller *et al*., 2006; Steunou *et al*., 2008; Hamilton *et al*., 2011; Loiacono *et al*., 2012; Huang *et al*., 2013), our knowledge on the identity and relevance of diazotrophs in such systems has

Table 2 The contribution of nitrogenase activity, nitrogen fixation and carbon assimilation rates, the C_2H_4:N_2 ratio and the percentage of nitrogen fixation to the total PP[a] and Pnew[b] in the Porcelana hot spring

Year	T *(°C)*	*Hourly rates*				*Daily rates*			*Ratios*		*% Nitrogen fixation contribution to*		*Input of daily nitrogen fixation to microbial mat*
		Nitrogenase activity (nmol cm^{-2} h^{-1})	*Nitrogen fixation (nmol N cm^{-2} h^{-1})*	*Nitrogen fixation[c] (nmol N cm^{-2} h^{-1})*	*Carbon assimilation (nmol C cm^{-2} h^{-1})*	*Nitrogenase activity (nmol cm^{-2} per day)*	*Nitrogen fixation (nmol N m^{-2} per day)*	*Carbon assimilation (nmol C cm^{-2} per day)*	*C_2H_4: N_2*	*C:N[d]*	*Total daily primary production PP (C)*	*Total new production Pnew (N)*	*g N m^{-2}* per *year*
2009	46	50.0 ± 7.0	ND*	ND*	ND*	600 ± 84.1	ND*	ND*	ND*	ND*	ND*	ND*	ND*
2009	42	14.6 ± 1.8	ND*	ND*	ND*	175 ± 21.8	ND*	ND*	ND*	ND*	ND*	ND*	ND*
2009	40	11.3 ± 3.1	ND*	ND*	ND*	136 ± 36.6	ND*	ND*	ND*	ND*	ND*	ND*	ND*
2011	61	0.3 ± 0.1	ND*	ND*	ND*	3.6 ± 1.6	ND*	ND*	ND*	ND*	ND*	ND*	ND*
2011	57	0.5 ± 0.3	ND*	ND*	ND*	6.1 ± 3.0	ND*	ND*	ND*	ND*	ND*	ND*	ND*
2011	51	26.5 ± 6.2	ND*	ND*	ND*	318 ± 74.4	ND*	ND*	ND*	ND*	ND*	ND*	ND*
2012	52	16.5 ± 2.7	2.6 ± 0.3	1.4 ± 0.1	53.0 ± 4.1	198 ± 32.4	31.4 ± 3.24	636 ± 49	6.3	18.7	92.2	99.1	1.6
2012	47	22.9 ± 3.5	3.4 ± 1.8	4.6 ± 1.8	ND*	275 ± 42.5	40.2 ± 21.6	ND*	6.8	ND*	ND*	ND*	2.9
2013	66	0.2 ± 0.01	ND*	ND*	ND*	2.8 ± 0.1	ND*	ND*	ND*	ND*	ND*	ND*	ND*
2013	58	14.5 ± 1.6	6.7 ± 1.2	1.6 ± 0.3	45.8 ± 8.7	174 ± 19.7	80.0 ± 14.3	550 ± 105	2.2	9.1	132	99.8	4.1
2013	48	32.4 ± 3.6	7.8 ± 0.6	6.3 ± 1.8	ND*	388 ± 42.9	94.1 ± 6.9	ND*	4.1	ND*	ND*	ND*	4.8

Abbreviations: ND*, data not determined; Pnew, new nitrogen production; PP, primary production.
The values were calculated from those obtained during daytime (1200–1400 hours).
[a]Nmol C cm^{-2} per day.
[b]Nmol N cm^{-2} per day.
[c]Nitrogen fixation rates for 6 h *in situ i*ncubation.
[d]C:N based on organic matter calculated by mass spectrometer instrument.

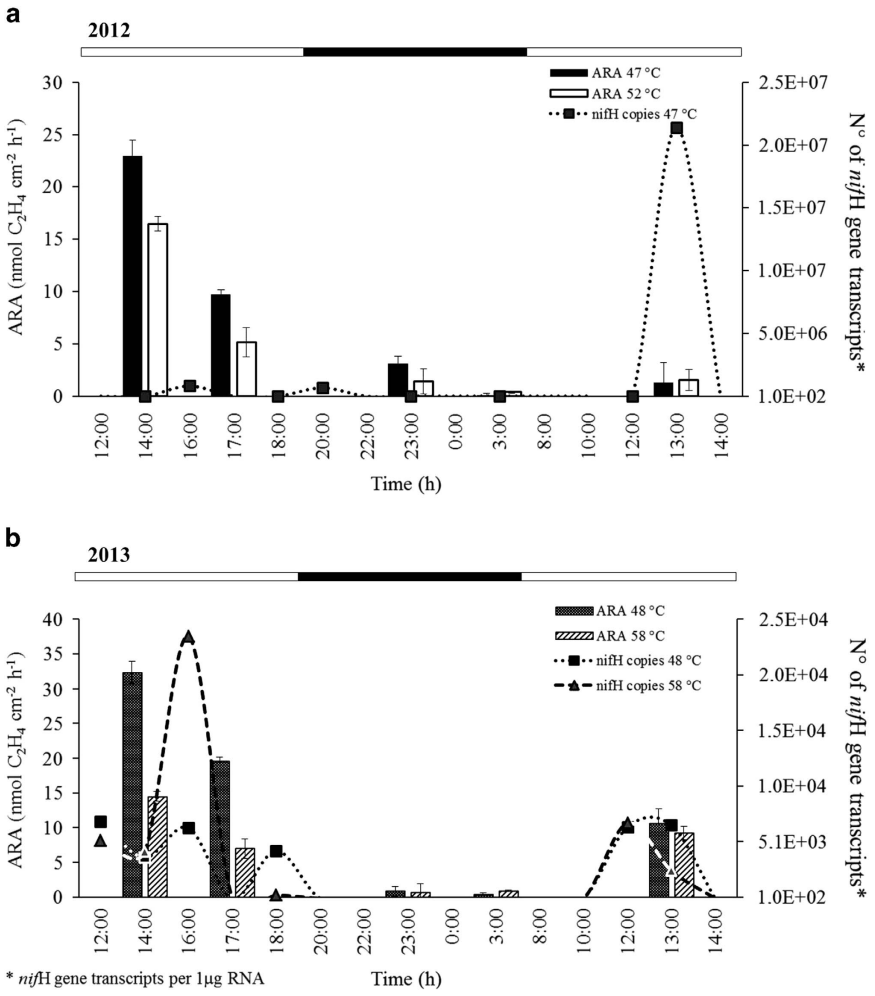

Figure 5 Diel cycles in nitrogenase activity (NA) and n*if*H gene expression in the Porcelana hot spring. (**a**) Diel cycles at different temperatures in 2012. The bars represent ARA and the dotted line represents the number of *nif*H gene transcripts at 47 °C. (**b**) Diel cycles at different temperatures in 2013. The bars and the dashed line represent activities at 58 °C and 48 °C. Error bars indicate the s.d. The top bar represents the light (white) and night (black) periods; the latter is also illustrated by gray shading.

remained surprisingly rudimentary. The distinct microbial mats or biofilms formed in hot springs typically harbor phototrophic microorganisms that often belong to the phyla Cyanobacteria and Chloroflexi (Liu *et al.*, 2011; Klatt *et al.*, 2013). Because certain members of these phyla (together with archaea) may fix atmospheric dinitrogen gas (N_2), this organismal segment may serve an important key nutrient (N) role in these ecosystems, as was recently suggested (Steunou *et al.*, 2006, 2008; Hamilton *et al.*, 2011; Loiacono *et al.*, 2012).

To extend our knowledge concerning the significance of thermal diazotrophs, we performed the first detailed examination by combining analyses of the genetic diversity of microbes, their diazotrophic capacity and estimates of their contribution to 'new' nitrogen in the neutral hot spring Porcelana (Patagonia, Chile). The high volcanic activity in Chile has generated a large number of largely unexplored terrestrial hot springs with distinct physicochemical parameters; some of the hot springs exhibit characteristics resembling those of other well-studied hot spring areas (for example, YNP) (Hauser, 1989; Hamilton *et al.*, 2011; Loiacono *et al.*, 2012; Wang *et al.*, 2013). The pristine hot spring of Porcelana was selected because this spring represents a stable ecosystem appropriate for identifying microbes and factors that control their behavior in the community. The lush microbial mats of the Porcelana thermal gradient (~69–38 °C) are likely supported by the nitrogen, phosphate and iron levels typical for the Porcelana water system and contain microbes belonging to Cyanobacteria, Proteobacteria and Chloroflexi (Mackenzie *et al.*, 2013). Hence, we hypothesized the existence of a rich diazotrophic community in the Porcelana spring, making it an ideal model system for exploration.

The polyphasic approach used in our study of the Porcelana hot spring in combination with several methodological approaches such as molecular markers (16S rRNA and *nif*H genes), molecular techniques (clone libraries, DGGE and RT-qPCR), *in situ* enzyme activities (ARA) and isotope uptake ($^{15}N_2$ and $H^{13}CO_3^-$) established that the Porcelana

hot spring is dominated by cyanobacteria, particularly the diazotrophic genus *Mastigocladus* (Stigonematales). Cyanobacteria have been identified in other thermal microbial mats included members of the unicellular Synechococcales (mainly the genus *Synechococcus*) (Sompong *et al.*, 2005; Steunou *et al.*, 2006, 2008) and the filamentous Stigonematales (genera *Fischerella* and *Mastigocladus*) (Miller *et al.*, 2006; Lacap *et al.*, 2007, 2007; Finsinger *et al.*, 2008). The dominating cyanobacterial phylotypes discovered in the microbial mats of the Porcelana hot spring corroborated these data, with the exception of the unicellular cyanobacteria. The presence of the Stigonematales phylotypes was also verified by morphological analysis (microscopy; data not shown).

Using the *nif*H genes as a marker allowed a more accurate determination of the affiliation of the dominating cyanobacteria OTUs and revealed the dominance of the heterocystous genus *Mastigocladus*; however, the affiliations were less apparent using the 16S rRNA marker gene. The latter is likely due to the low number of sequences and sequenced genomes from the order Stigonematales in the databases. The *Mastigocladus* phylotypes were present throughout the temperature gradient (69 °C to near 38 °C), thereby expanding their upper temperature limit compared with the results of other thermal or laboratory systems (Finsinger *et al.*, 2008; Miller *et al.*, 2009). The 16S rRNA and *nif*H gene approach also identified members of the non-heterocystous Oscillatoriales (including both non-diazotrophs and diazotrophs), although they were present at a lower abundance; this group was not detected using the DGGE approach. Taken together, the data show that the Porcelana spring has a unique microbial composition devoid of unicellular cyanobacteria and other diazotrophic bacteria.

To broaden our knowledge of the importance of nitrogen fixation in the Porcelana spring, diel activities were examined using both the nitrogenase activity and $^{15}N_2$ isotope uptake approaches; the use of these complementary techniques reflect different aspects of the fixation process (Peterson and Burris, 1976; Montoya *et al.*, 1996). To date, measurements of cyanobacterial-associated nitrogenase activity (acetylene reduction assay) have dominated hot spring analyses (Steunou *et al.*, 2006, 2008; Miller *et al.*, 2009). Recent studies showed that heterotrophic bacteria and archaea may serve as significant nitrogen fixers in hot springs (Hamilton *et al.*, 2011; Loiacono *et al.*, 2012). However, the only study following $^{15}N_2$ isotope uptake was conducted in 1970 in thermal microbial mats (YNP) dominated by the cyanobacterial genera *Calothrix* and *Mastigocladus* (Stewart, 1970). Nitrogen fixation assessed using nitrogenase activity in combination with $^{15}N_2$ gas uptake provided different but complementary information; therefore, we used these techniques in the present study of the Porcelana hot spring. The data show that diazotrophy is the norm in this hot spring in all 4 years examined. Furthermore, the activity was only apparent during the day time (1300–1400 hours) and was highest at 58 °C to 46 °C but was not detected above 60 °C. The nitrogenase activity recorded was on a similar order of magnitude (50.0 nmol C_2H_4 cm^{-2} h^{-1}) to that reported for the Mushroom Spring (YNP; 40–180 nmol C_2H_4 cm^{-2} h^{-1}; Steunou *et al.*, 2008), although there were differences in the retrieval of the diazotrophic biomass. Similarly, nitrogen fixation rates in the Porcelana hot spring (ranging from 2 to 8 nmol N cm^{-2} h^{-1}) were in agreement with the activities reported for other non-thermal aquatic ecosystems (Fernandez *et al.*, 2011). The data further demonstrated that the nitrogen fixation rates in the Porcelana microbial mat fell within the theoretical ratio for C_2H_4:N_2 of 4:1 (if hydrogen production was taken into account; Postgate, 1982).

The use of the *nif*H gene as a potent molecular marker for diazotrophs in natural ecosystems has been extensive in recent years (Díez *et al.*, 2007; Severin and Stal, 2009, 2010; Fernandez *et al.*, 2011). However, the presence of *nif* genes or transcripts is not necessarily coupled to activity, as shown for *Synechococcus*-dominated hot spring mats (Steunou *et al.*, 2006, 2008) where *nif*H gene expression peaked in the evening and nitrogenase activity peaked in the morning. A similar phenomenon was also observed in cyanobacterial microbial mats from temperate regions (Stal *et al.*, 1984; Severin and Stal, 2009,2010). In contrast, the nitrogen fixation activity (nitrogenase activity and N_2 uptake) in the Porcelana hot spring showed a positive correlation with *nif*H gene expression. Moreover, because nitrogen fixation during the daytime is typical for eco systems dominated by heterocystous cyanobacteria (Stal, 1995; Evans *et al.*, 2000; Charpy *et al.*, 2007; Bauer *et al.*, 2008), our data infer the predominance, if not the exclusive role, of the heterocystous *Mastigocladus*-type cyanobacteria in nitrogen fixation in the Porcelana hot spring.

It cannot be excluded that the low concentrations of combined inorganic nitrogen (for example, ammonium and nitrate) in the Porcelana hot spring may be the result of a rapid turnover of these compounds (Herbert, 1999). However, the distinct nitrogen fixation activities recorded (on average 3 g N m^{-2} year) in the Porcelana hot spring suggest that this process is not diminished by other sources of combined nitrogen. Rather, we can conclude that the entry of 'new' nitrogen by diazotrophic cyanobacteria supports most of the total daily nitrogen demand (up to 99%) of the microbial mat. Comparing this nitrogen input with that of rain water (ca. 0.1 g N m^{-2} per year) for the geographical region related to Porcelana (Weathers and Likens, 1997), we suggest that the biological nitrogen fixation found in our study may constitute the major source of 'new' nitrogen into this ecosystem.

The fact that both the nitrogen and CO_2 fixation coincided at mid-day in the Porcelana cyanobacterial

mat may explain the substantial nitrogen fixation activity recorded. Photosynthesis would not only cover the high energy demand (ATP) of the nitrogen fixation process but also provide the required reducing power and carbon skeletons.

Conclusions

Our data demonstrate that the microbial mats covering the thermal gradient of the Porcelana hot spring outflow represent a well-organized and functioning ecosystem dominated by diazotrophic cyanobacteria of the *Mastigocladus*-type and may represent a typical scenario for neutral hot springs. Our results further emphasize the pivotal role of such diazotrophic cyanobacteria in maintaining this microbial dominated ecosystem by delivering most of its nitrogen demand through nitrogen fixation. These findings may also have important implications for other thermal or extreme environments dominated by cyanobacterial microbial mats.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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