The ISME Journal (2015) 9, 2290–2303

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ORIGINAL ARTICLE

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

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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.
The ISME Journal (2015) **9,** 2290–2303; doi:10.1038/ismej.2015.63; published online 31 July 2015

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;

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Received 25 May 2014; revised 23 March 2015; accepted 25 March 2015; published online 31 July 2015

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 diethyldithiocarbamate 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

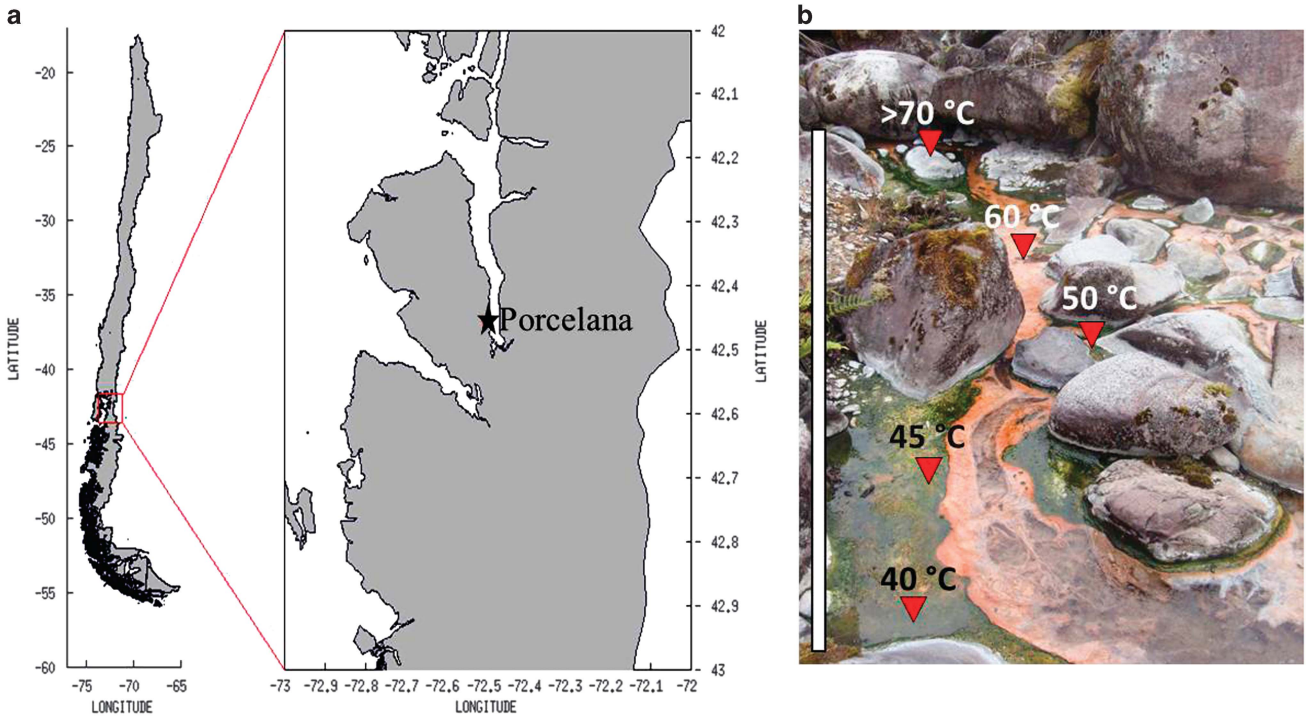

Figure 1 (**a**) Location of the Porcelana hot spring in northern Patagonia, Chile (X Region, Comau fjord). (**b**) The pigmented microbial mat was formed throughout the temperature gradient; the sampling sites are indicated by red triangles. The gray bar represents the mat extension (~10 m) within the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

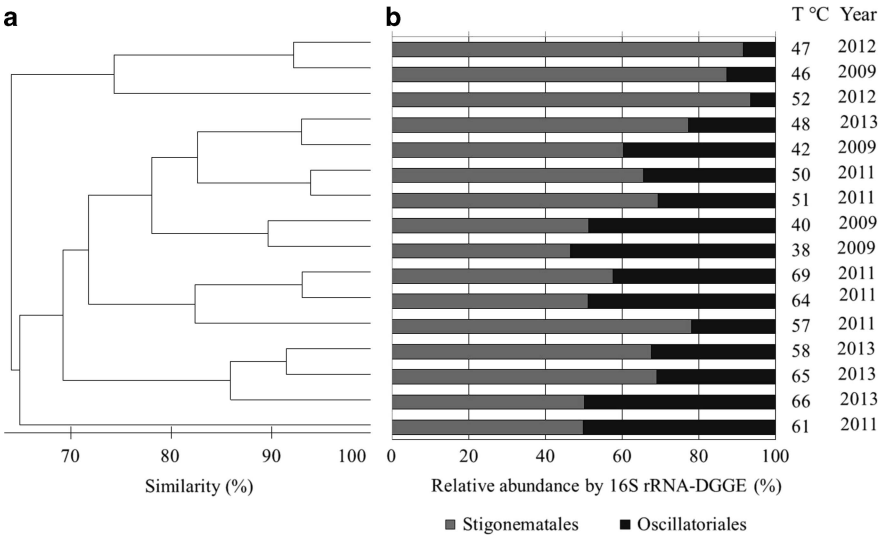

Figure 2 Comparison of the interannual cyanobacterial diversity at different temperatures in the Porcelana hot spring based on the 16S rRNA gene and DGGE. (**a**) Bray–Curtis dissimilarity index dendrogram. (**b**) Relative abundance of 16S rRNA-DGGE bands (phylotypes) for each temperature and year investigated.

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

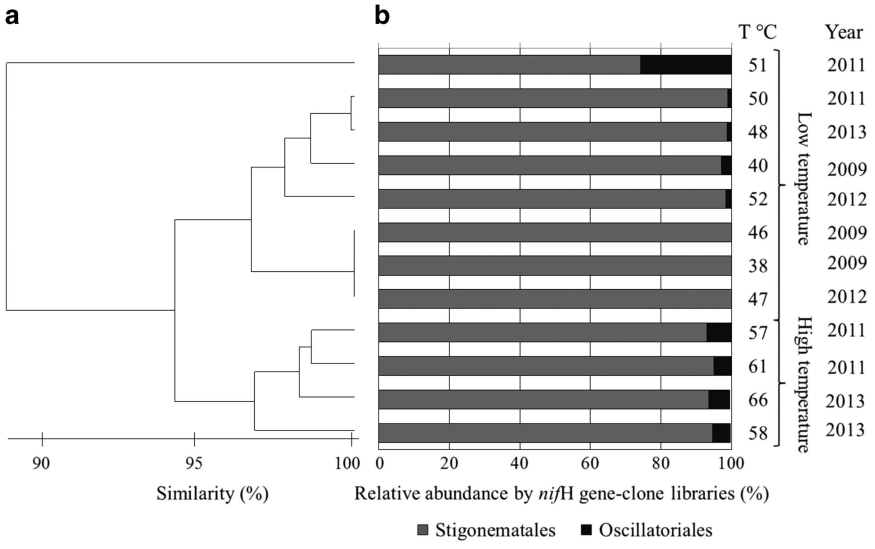

Figure 3 Relative abundance and interannual diazotrophic bacterial diversity in the Porcelana hot spring based on the *nif*H marker gene and clone libraries. (**a**) Bray–Curtis dissimilarity index dendrogram. (**b**) Relative abundance of the *nif*H gene (OTUs) determined using clone libraries obtained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

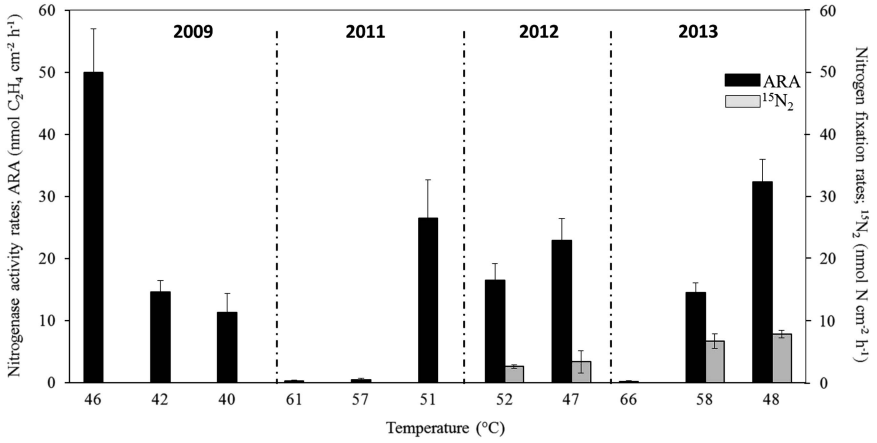

Figure 4 Nitrogen fixation assessed by the ARA and $^{15}N_2$ uptake analysis for the different temperatures and years investigated. ARA measurements (black bars) were conducted during the 4 years, whereas $^{15}N_2$ uptake measurements (gray bars) were performed in 2012 and 2013.

(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.

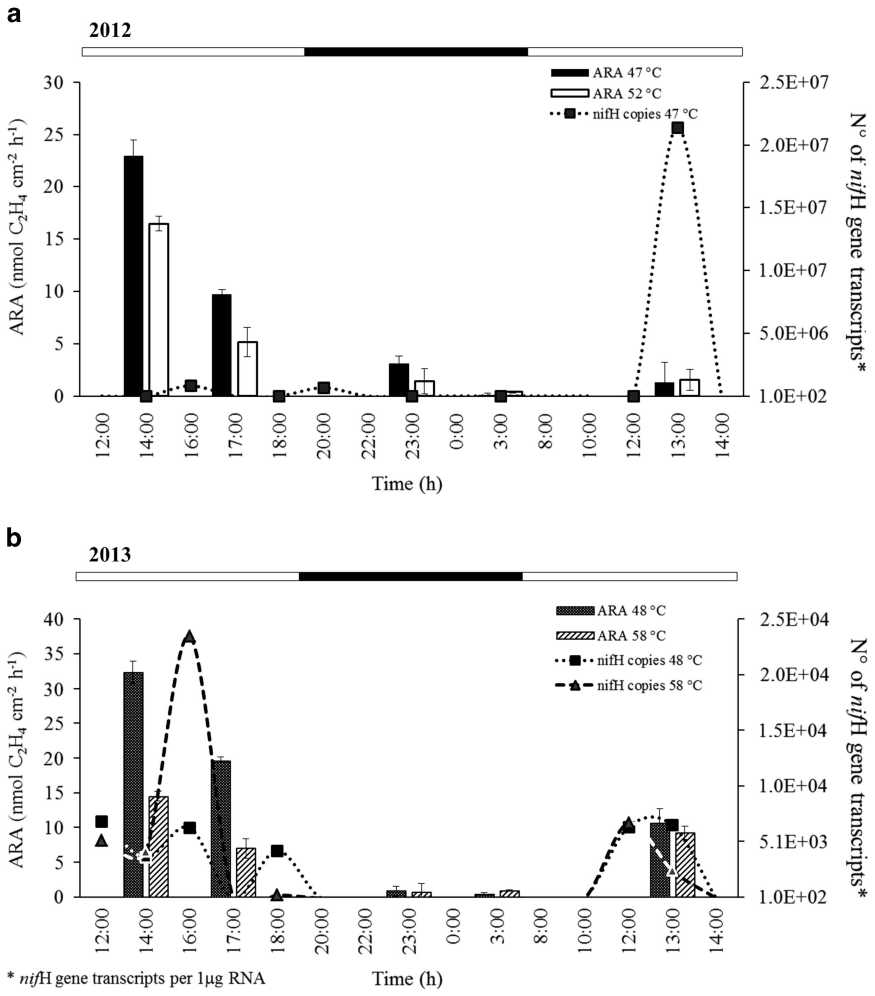

Figure 5 Diel cycles in nitrogenase activity (NA) and n*if*H gene expression in the Porcelana hot spring. (**a**) Diel cycles at different temperatures in 2012. The bars represent ARA and the dotted line represents the number of *nif*H gene transcripts at 47 °C. (**b**) Diel cycles at different temperatures 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

We are grateful to the staff of the Huinay Scientific Field Station for making our visits to the Porcelana hot spring possible. We also thank Dr C Pedrós-Alió, and colleagues R MacKenzie, T Quiroz, S Guajardo and S Espinoza for their assistance with sample collection and analysis, and Dr S Andrade for his help with the analysis of the metal samples. This work was financially supported by PhD scholarship CONICYT No. 21110900, the French Embassy for PhD mobility (Chile) and LIA MORFUN (LIA 1035), and the following grants funded by CONICYT: FONDECYT No. 1110696 and FONDAP No. 15110009.

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