Universitat Autònoma de Barcelona

Facultad de Biociències

Departament de Genètica i de Microbiologìa

Doctoral Thesis

Ecology of hot spring microbial mats: Diversity, microheterogeneity, and biogeography

Roy Mackenzie Calderón

Directors: Prof. Beatríz Díez Moreno and Prof. Carles Pedrós-Alió

Tutor: Prof. Jordi Mas Gordi

October 2014

Ecology of hot spring microbial mats: Diversity, microheterogeneity, and biogeography

Thesis submitted by Roy Mackenzie Calderón to obtain the degree of Doctor in Microbiology by the Universitat Autònoma de Barcelona

Roy Mackenzie Calderón,

Barcelona, October 2014

With the approval of the Thesis Directors and Tutor,

Prof. Beatriz Díez Moreno
Research Professor
P. Universidad Católica de Chile

Prof. Carles Pedrós-Alió
Research Professor
Institut de Ciències del Mar, CSIC

Prof. Jordi Mas Gordi
Research Professor
Universitat Autònoma de Barcelona

"Ecology of hot spring microbial mats: Diversity, microheterogeneity, and biogeography"

The author was funded by the Chilean Government through the National Commission for Scientific and Technological Research (CONICYT) – BECASCHILE Scholarship progam (PFCHA/Doctorado al Extranjero 2° Convocatoria, 72100502)

This work has been carried out in the framework of the following research projects:

Work in Chile was funded by grants FONDECYT 1110696 and FONDAP 15110009 (CONICYT), and in Barcelona by grant MarineGems CTM2010-20361 from the Spanish Ministry of Economy and Competitiveness.

Sampling was funded by grants TERMOCHILE by the Spanish Ministry of Science and Innovation, INACH15-10 (Instituto Nacional Antártico Chileno), Fundación San Ignacio del Huinay including three cooperation projects with CSIC, 2012CL, 2013CL0017, and 2014CL18 (Southern Chile), FONDECYT 1110696 (El Tatio Geyser Field, Atacama Desert), and cooperation project CSIC-CRUSA (Costa Rica). We thank Maria Estrella Alcamán, Sebastián Espinoza, Martin Polz, Mónica Vásquez and Lorena Uribe for help in the field.

A mi adorada Madre que me motivó y a mi amada hija que me inspiró…

¿O era al revés?

Y a ti, por aguantar-me, y por invertir tus presentes en nuestros mañanas

… as we know, there are known knowns;

there are things we know we know.

We also know there are known unknowns;

that is to say we know there are some things we do not know.

But there are also unknown unknowns

-- the ones we don't know we don't know.

Nassim Nicholas Taleb

Summary

The results presented in this PhD thesis include a description on the thermophilic populations thriving in biodiversity hot spots such as the Valdivian Rain Forest in Patagonia, and Cloud Forests in Costa Rica, as well as the Atacama Desert and Deception Island in the South Shetland Archipelago in Antarctica. The bacterial community structure of several hot spring microbial mats was characterized by means of molecular microbial ecology methods, and the relationship between diversity and temperature was measured at different spatial scales.

Microbial mat samples from three hot springs in Northern Patagonia showed large cell counts, compared with microbial mats elsewhere, as well as a high percentage of thermophilic culturable bacteria. SEM images revealed that filaments with and without sheaths dominated in different temperature ranges.

Phylum Cyanobacteria was identified as the major component of the microbial community by at least three different sequencing methods with different taxonomic resolution, and a deeper diversity analysis on this group showed no substantial differences between samples taken in winter and summer, although significant similarities were observed among hot springs with common surrounding environments.

The horizontal microheterogeneity of microbial mats at a centímeter scale also revealed Chloroflexi as dominant phylum together with Cyanobacteria, and a temperature differentiation was observed between both potential primary producers. Significant diversity shifts were detected among closely situates samples in single mats, but at the same time, samples within mat patches showed a high community structure similarity.

A deeper characterization of the bacterial community structure revealed the presence of several heterotrophic groups such as Cytophagia and Sphingobacteria; Thermales; and Alpha, Beta, and Gammaproteobacteria; among others. When compared with other thermophilic microbial mats along a latitudinal gradient, no relationship was detected between richness and latitude, as samples closer to the equator were not more diverse or harbored more richness than

samples closer to the pole. Temperature, on the other hand, influenced significantly the bacterial richness. Finally, samples at local, regional and global scales (up to 1000 m^2) showed a decrease in similitude as geographic distance, but not at the continental scale.

Resumen

Los resultados que se presentan en esta Tesis doctoral comprenden una revisión de las poblaciones termófilas que habitan en algunos Hot Spots de diversidad de América, como lo son el bosque lluvioso Valdiviano en la Patagonia, o los Bosques Nubosos de Costa Rica. También se incluyen ecosistemas extremos como el desierto de Atacama y las Isla Decepción en el Archipiélago Shetland del Sur, en la Antártica. En este trabajo de tesis se caracterizó la estructura de las comunidades bacterianas en tapetes microbianos de varias termas terrestres diferentes mediante métodos de ecología molecular microbiana, y se midió la relación de los cambios de diversidad con respecto a la temperatura a diferentes escalas espaciales..

Los recuentos celulares en muestras de tres tapetes microbianos pertenecientes a tres termas en la Patagonia Norte de Chile mostraron una gran abundancia de individuos comparado con tapetes microbianos en termas de otros lugares del mundo; así también se determinó un alto porcentaje de bacterias termófilas cultivables. Las imágenes de microscopía electrónica de barrido (SEM) revelaron que dos tipos diferentes de bacterias filamentosas dominaban a diferentes rangos de temperatura.

El filo Cyanobacteria fue identificado como uno de los mayores componentes de la comunidad bacteriana por al menos tres métodos moleculares de huella dactilar a diferentes niveles taxonómicos, y al analizar este grupo en detalle no se observaron diferencias sustanciales entre muestras obtenidas en invierno y verano del mismo año en las mismas termas. Por otra parte, se detectó una similitud significativa entre las termas que comparten ambientes semejantes a su alrededor.

El estudio de la microheterogeneidad horizontal de los tapetes microbianos a pequeña escala (centímetros) reveló que también miembros del filo Chloroflexi dominaban alternativamente junto con Cyanobacteria en ciertas zonas del tapete, y se observó una diferenciación por temperatura entre ambos productores primarios. En este sentido, se detectaron cambios significativos de diversidad entre muestras en un mismo tapete microbiano, y al mismo tiempo, se detectó una alta similitud en la estructura de las comunidades dentro de algunos “parches” en los tapetes.

Una caracterización más profunda de la estructura de las comunidades bacterianas reveló la presencia de varios grupos heterotróficos como Cytophagia y Sphingobacteria; Thermales; y Alpha, Beta y Gammaproteobacteria, entre otros menos abundantes. Cuando se comparó la estructura de estas comunidades con otras también fototróficas-termófilas de tapetes microbianos a miles de kilómetros de distancia en un gradiente latitudinal, no se detectó una relación entre riqueza bacteriana y latitud, ya que las muestras más cercanas a la línea del Ecuador no mostraron mayor diversidad o riqueza que las más cercanas al Polo Sur. Por otra parte, la temperatura exhibió una relación negativa significativa con respecto a la riqueza bacteriana independientemente de la latitud geográfica: a mayor temperatura de la muestra, menor riqueza bacteriana. Finalmente, las muestras dentro de una escala local, regional y global (hasta 1000 m^2) mostraron una disminución significativa de la diversidad a medida que la distancia geográfica y la temperatura se incrementaban. A nivel continental, estas relaciones no fueron significativas.

Resum

Els resultats presentats en aquesta tesi inclouen una descripció de les poblacions termòfiles que prosperen en alguns punts calents de biodiversitat com és ara la selva Valdiviana a la Patagònia, o els boscos de boira a Costa Rica, així com al Desert d'Atacama i a l'Illa Decepció, a l'arxipèlag de les Shetland del Sud, Antàrtida. L'estructura de la comunitat bacteriana de diversos tapets microbians d'aigües termals es van caracteritzar mitjançant tècniques moleculars de l'ecologia microbiana i es va determinar la relació entre la diversitat i la temperatura a diferents escales espacials.

Els tapets microbians de tres fonts termals al nord de la Patagònia van mostrar recomptes de cèl·lules alts, en comparació amb els tapets microbians d'altres llocs, així com un alt percentatge de bacteris cultivables termòfils. Imatges de SEM van revelar que els filaments amb i sense beines dominaven a diferents rangs de temperatura.

El Phylum Cyanobacteria va ser identificat com el principal component de la comunitat microbiana per almenys tres mètodes de seqüenciació diferents amb diferent resolució taxonòmica. Una anàlisi més detallada sobre la diversitat d'aquest grup no va mostrar diferències substancials entre les mostres preses a l'hivern i a l'estiu, però sí que es van observar similituds significatives entre aigües termals amb característiques ambientals comunes.

La microheterogeneitat horitzontal dels tapets microbians a una escala de cm també va revelar Chloroflexi com phylum dominant juntament amb els cianobacteris, i es va observar una diferenciació de temperatura entre els dos potencials productors primaris. També es van detectar canvis significatius de diversitat entre mostres localitzades a pocs cm dins d'un mateix tapet, encara que en general les mostres del mateix tapet eren molt similars entre si.

Una caracterització més detallada de l'estructura de la comunitat bacteriana va revelar la presència de diversos grups heterotròfics com és ara Cytophagia i Sphingobacteri, Thermales, i Alpha, Beta, i Gammaproteobacteria, entre d'altres. Quan es van comparar amb altres tapets microbians termòfiles al llarg d'un gradient latitudinal, no es va detectar cap relació entre la

riquesa i la latitud, ja que mostres més properes a l'equador no eren més diverses o tenien més riquesa que les mostres més a prop del pols. D'altra banda, la temperatura va influir significativament en la riquesa bacteriana. Finalment, les mostres van mostrar una disminució de la similitud amb l'increment de la distància geogràfica a escales local, regional i global (fins a 1.000 m2) però no a escala continental.

INDEX

GENERAL INTRODUCTION

General Introduction

All living beings have certain tolerance limits to one or many environmental factors, and they cannot survive above or under these limits. This is what we call the "limits of life" (Pikuta et al. 2007). Nevertheless, there are specially adapted microorganisms, which are able to colonize, and moreover, to develop successfully in ecophysiological severe conditions. We call these microorganisms "Extremophiles". From our anthropocentric point of view, habitats where our surviving is not possible are considered "extreme", although by the microorganisms themselves these places are basically oasis (Javaux, 2006).

Extremophiles prosper on the edge of temperature, hypersalinity, pH, pressure, dryness and dessication. Although all three domains have extremophilic representatives, the Bacteria and Archaea domains probed to be (so far) the most abundant and diverse (Canganella and Wiegel, 2011). In words of Horikoshi (1998), as conditions become more demanding, extreme environments become exclusively populated by prokaryotes. In the last few decades, extremophilic microorganisms have been found in a wide range of environments, wherever there is liquid water, even briefly over a year such as in the Dry Valleys of Antarctica (De los Ríos et al., 2004) or in the Atacama Desert of Chile (De los Ríos et al., 2010). Extremophiles also thrive at acid mine drainages and rivers (such as the Rio Tinto in Spain) (Amils et al., 2007), hot springs (Brock, 1967), and many other environments summarized by Bell and Callaghan (2011). Table 1 shows the environmental ranges of most studied extremophilic microorganisms.

The discovery of extremophiles has put vitality into the biotechnology industry, since several reviews and books have been published on this topic, together with an increasing number of meetings held, genomes sequenced and patents filed in the past decade (Rothschild and Mancinelli, 2001). A classic example of the value of basic research on extremophiles is the discovery and use of Taq polymerase in the polymerase chain reaction (PCR) from *Thermus aquaticus* (Brock and Freeze, 1969), and the substantial industry built upon it, not to mention

the research improvements to molecular biologists ever since (Brock, 1997). *Thermus aquaticus* is a thermophilic and heterotrophic microorganism isolated by Thomas D. Brock (Brock and Freeze, 1969) in hot springs from Yellowstone National Park, USA. Other thermozymes has also been subject of intense studies for biotechnological applications (Zeikus et al., 1998; Bruins et al., 2001). In 1997, Brock stated he could not have made such discovering without studies directly on field, illuminating the importance of investigations such as the present thesis.

Table 1: Extreme definitions and some examples of extremophilic microorganisms

Environmental Parameter	Extremophile	Definition	Example
Temperature	Hyperthermophile	Growth >80º C	*Pyrolobus fumarii*, 113º C
	Thermophyle	Growth 60-80º C	*Synechococcus lividus*
	Psychrophile	<15º C	*Psychrobacter sp.*
Pressure	Piezophile	Pressure loving	*Shewanella violacea*
pH	Acidophile	Low pH loving	*Ferroplasma sp.*
	Alkaliphile	High pH loving (pH >9)	*Spirulina sp.*
Salinity	Halophile	Salt loving (2-5 M NaCl)	Halobacteriacea
Oxygen	Anaerobe	Cannot tolerate O2	*Methanococcus jannaschii*
	Microaerophil	Tolerates some O2	*Clostridium spp.*
Radiation	Radioresistant	Infra-red Ionizing radiation tolerant	*Deinococcus radiodurans*

Modified from Stojanovic et al. 2008

Life at high temperatures

High temperature accelerates the denaturation of biomolecules, and increases the solubility of gases in hot water, causing a problem in the exchange of O_2 and CO_2. To overcome these conditions, microorganisms have made physiological adaptations that allow them to colonize and develop at high temperatures, basically by adding several ionic bonds and other

internal forces that help to stabilize all enzymes (Madigan and Marrs, 1997). These adaptations work at high temperature, but rarely do the same job at lower temperature, circumscribing heat-loving microbes to hot environments. Figure 1 shows the most representative taxa at different temperatures. Archaea (red) and Bacteria (blue) are the most thermophilic and hyperthermophilic major taxa. The temperature in hot springs is usually over the limit of eukaryotic life (near to 60 °C), which limits the microbial life to Bacteria and Archaea (and their viruses) (López-López et al., 2013).

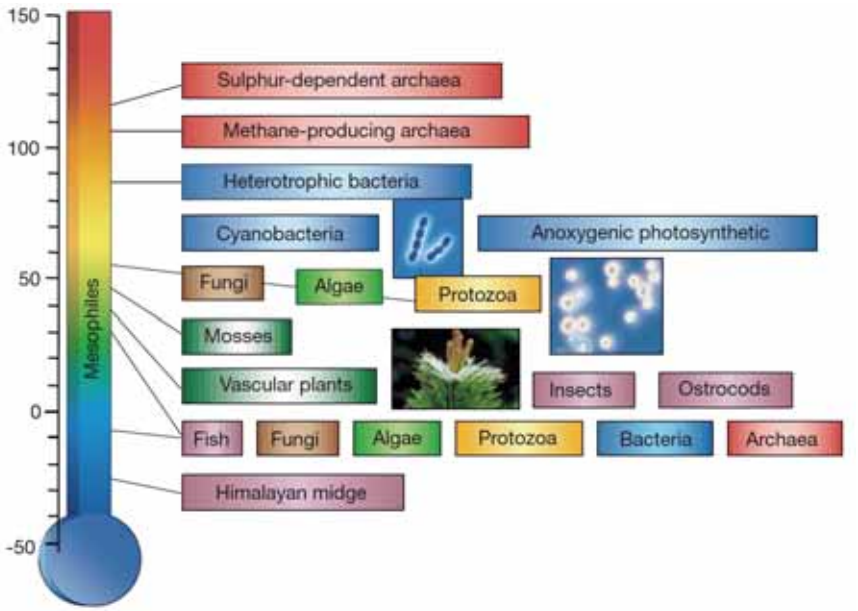


Figure 1: Temperature limits for life. The highest and lowest temperature for each major taxon is given. Taken from Rothschild and Mancinelli (2001).

Thermophilic microorganisms

The physiology of thermophilic microorganisms has a wide diversity. They could be primary producers of organic matter, as well as primary and secondary decomposers within the community. Chemolithoautotrophs and organotrophs with mixotrophic and heterotrophic anabolism can be found. Some of them are dependent upon external electron acceptors (sulfur, iron, sulfate, nitrate, oxygen), or have fermentative metabolism (Pikuta et al., 2007). Moreover, the last common ancestor of all live forms could have metabolized hydrogen for energy at high

temperatures (Pace et al., 1997), and as can be seen in Figure 2, deeply branched bacterial lineages (such as *Thermotoga* and *Aquifex*) are also thermophilic chemolithotrophs, suggesting that adaptations of thermophiles and hyperthermophiles to temperature are important features in the early history of life on earth.

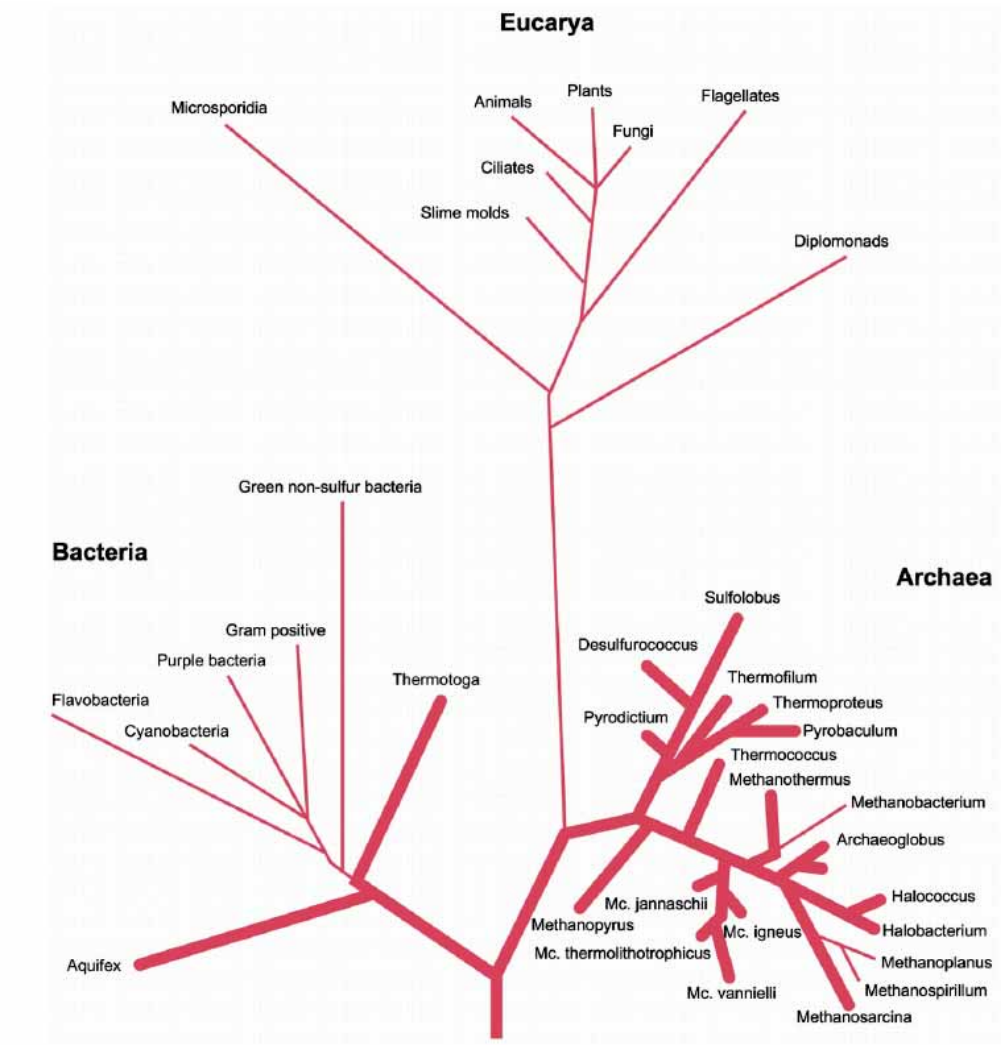


Figure 2: Classical tree of life based on SSU rRNA, rooted in the bacterial branch. Bold lines represent hyperthermophilic lineages. Taken from Xu and Glansdorff (2002).

Hot springs anatomy

Superficial geothermal events, commonly known as hot springs and geysers, are frequently found all over the globe. Strictly defined, any spring or well water whose average temperature is noticeably above the mean annual temperature of the air at the same locality may be classed as thermal (Waring, 1969). The most notable feature of the distribution of thermal springs is their close association with the main belts and areas of volcanoes of present or geologically recent activity. Nearly all thermal springs are associated with volcanic rocks, so the origin of heat has always been assumed as from volcanic origin, in a cycle schematized in Figure 3.

Each hot spring differs from others in temperature, chemical composition and its gradients of temperature or light. Hot springs comprise several habitats, such as thermal water, microbial mats and sediments. This diversity of habitats provides a vast number of sites to sample, all with potential interest for metagenomic analysis. 16S ribosomal RNA-based studies revealed that microbial diversity was much broader than suggested by culture-dependent techniques (Pedrós-Alió, 2007). In combination with the construction of metagenomic libraries, research on total environmental DNA produced a vast amount of information, providing detailed pictures of the microbial communities present in diverse thermal environments (López-López et al., 2013). The increasing number of reports makes it easier to understand how physicochemical conditions and biological interactions have shaped these microbial communities within their specific environments (López-López et al., 2013).

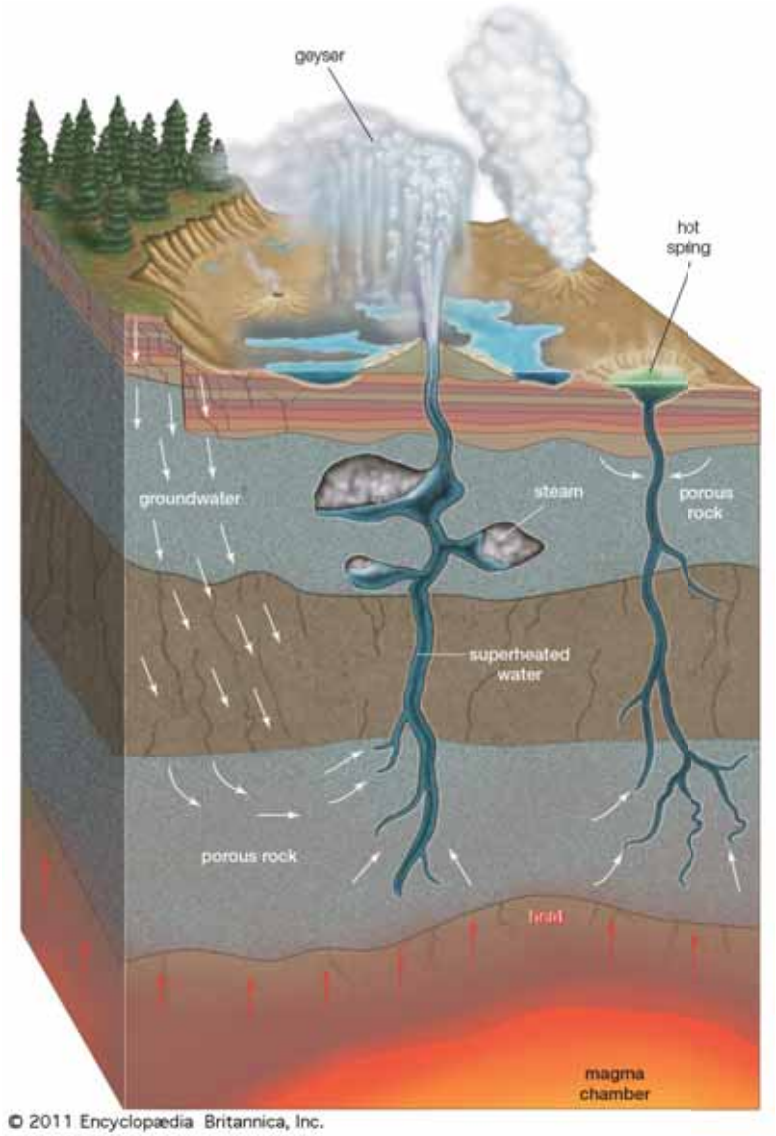


Figure 3: Groundwater percolates through porous rock into fractures deep underground, where heat from a nearby magma chamber superheats the pressurized water to a temperature above the boiling point of water at surface pressure. In hot springs the rising superheated water is cooled below the boiling point by groundwater before reaching the surface. In geysers the superheated water collects in underground pockets. When the supply of steam and hot water is exhausted, the spouting stops and the cycle begin again. Taken from Art. Encyclopædia Britannica Online. Web. 20 Sep. 2014. (http://global.britannica.com/EBchecked/media/91382/Cross-section-of-a-geyser-and-hot-spring-Groundwater-percolates).

Thermophilic Microbial Mats

In contrast to the multicellular structure of eukaryotes (tissues, organs, systems of organs, whole organism), the highest organized form of prokaryotic life in nature is presented by the benthic colonization in biofilms and microbial mats (Van Gemerden, 1993). In these complex structures, all microbial cells of different species are distributed in space and time according to their functions and to physicochemical gradients that allow more effective system support, self-protection, and energy distribution (Van Gemerden, 1993; Pikuta et al. 2007).

The presence and abundance of bacterial species in a microbial mat is strongly influenced by physical properties such as temperature, besides other key chemical parameters such as oxygen, pH, redox potential, salinity, and the availability of electron acceptors and donors (Franks and Stolz 2009). Brock (1978) described that hot spring microbial mats are typically zoned downstream according to temperature. Hot spring microbial mats usually present steep gradients of pH, oxygen, and sulfates (Van Gemerden 1993), as thermal water is quickly mixed with sediments and surrounding environment when emerging from the subsurface (Reysenbach and Cady 2001).

Usually, photoautotrophs (mainly Cyanobacteria and Chloroflexi) are dominant and overlie heterotrophic prokaryotes that form the base of a mat (Van Gemerden 1993, Ward et al., 1998). Thus, it is evident that the driving force of most microbial mats is photosynthesis by phototrophs. Figure 4 schematizes the microbial organization according to functional metabolisms and ecological roles usually found in a thermophilic microbial mat. The organic matter, resulting from phototrophic productivity, is the energy source for other aerobic and anaerobic microorganisms. Aerobic heterotrophic organisms are functionally important as their activity leads to oxygen depletion, and fermentative organisms provide growth substrates for sulfate-reducing bacteria. Other groups, numerically less important, are nitrifying and denitrifying bacteria, and methanogenic bacteria (Van Gemerden, 1993).

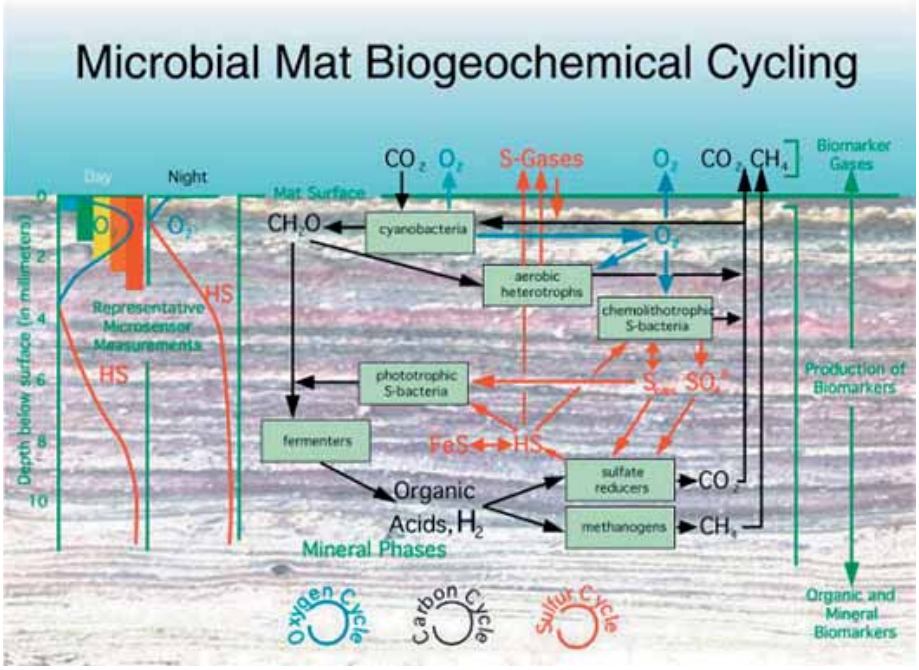


Figure 4: A few representative pathways in the cycling of carbon, oxygen and sulfur in a microbial mat. Figure modified after a figure by Fenchel, T., and B. J. Findlay. 1995. Ecology and Evolution in Anoxic Worlds. Oxford University Press.

Approaches to bacterial diversity

Many hot spring microbial mat studies do not reflect the centimeter-scale shifts of microbes, and replicate samples from a single microbial mat might differ drastically from one to another. The millimetric vertical stratification of mats from different natural sources has been described by several authors (Franks and Stolz, 2009; De los Ríos et al., 2004; Demergasso et al., 2003; Brock, 1969). However, studies on microbial community patch size are scarce, and little is known about the horizontal scales at which microbial interactions and associations become important (Franklin et al., 2002).

There are many tools to measure spatial or temporal variations in microbial community structure associated to environmental gradients. Most of these diversity measurements rely mainly on species richness and relative abundance, and were well revised by Magurran (1988, 2004) and Oren (2004). As diversity measurement is a comparative discipline (Magurran,

2004), it is possible to compare different environments by the biological datasets found in them (Hughes et al., 2001). However, most of studies on microbial diversity have focused on the most abundant populations, dismissing the rare species in microbial communities, which remain largely unexplored (Watve and Gangal, 1996).

Anderson et al. (2011) distinguished two types of diversity shifts among sites or time (also known as β diversity): variation and turnover (Figure 5). The difference mostly relies on the rare species. In variation, the compared samples are discrete and are not connected to each other. Turnover, on the other hand, requires the investigator to define a specific gradient or directionality. Studies in this thesis include both types of diversity analyses, but at different spatial scales, in order to better describe the shift patterns of bacterial communities. Turnover at a micro-scale within centimeters and meters, according to a narrow range temperature gradient; and variation, comparing diversity differences among different hot springs separated by several kilometers from each other, with clear environmental distinctions.

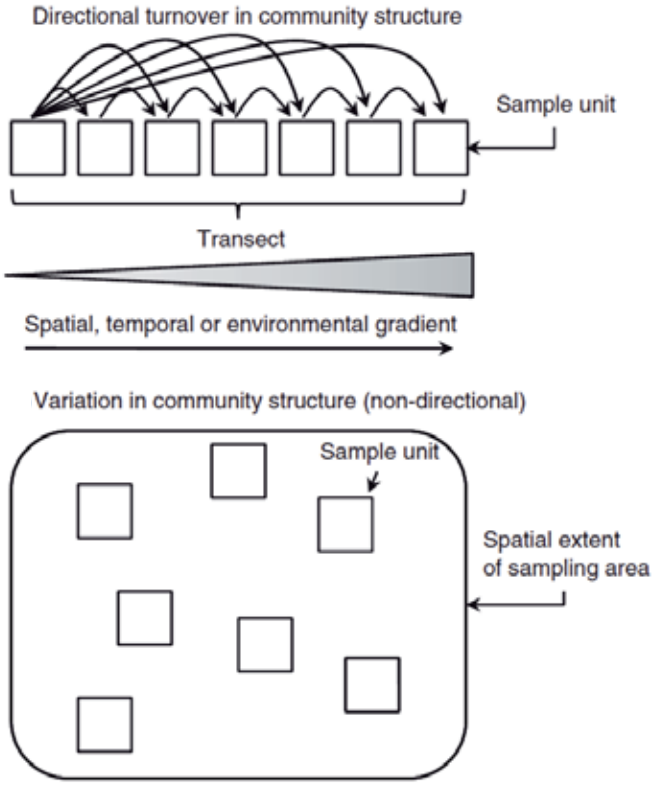


Figure 5: Schematic diagram of two conceptual types of diversity for ecology: On top, turnover in community structure along a gradient. Below, variation in community structure among sample units within a given area. Modified from Anderson et al., 2011.

Microbial Biogeography

As stated earlier, microbial diversity changes across space and time, and for an extremophilic organism, extreme environments may be seen as island-like habitats dispersed in a hostile environment, leading to geographic isolation and eventually to divergence (Ramette and Tiedje, 2007). Hot springs naturally occurring in geographically distant areas have been used as "island" models for biogeography studies, since a wide variety of microbial taxa exhibit biogeographical patterns (Whitaker et al., 2003; Martiny et al., 2006). Additionally, hot springs are well-isolated habitats occurring as clusters in globally distant regions. The microorganisms that thrive in them are extremophiles adapted to drastically different conditions from the ambient milieu through which they would have to disperse. As such, it would be expected that geographical isolation might be an important component in the diversification of hot spring microorganisms (Papke et al., 2003).

It is remarkable that in macro-biogeography, one of the fundamental patterns is that diversity tends to increase when latitude decreases, meaning that there is more diversity close to the equator than to the poles (Lomolino et al., 2006). Resurgence in microbial biogeography studies has been motivated mainly by the advancements in molecular techniques that allow us to survey the extent of microbial diversity (Martiny et al., 2006; Ramette and Tiedje, 2007), but despite these advances, Ramette and Tiedje (2007) stated that although the island-like nature of extreme environments was essential to reveal endemic patterns, predictions based on island biogeographic theories have yet not been extensively tested using prokaryotic diversity data.

Thermophilic phototrophic microbial mats are widely distributed in hot springs around the world (Ward et al., 1998; Papke et al., 2003; Miller et al., 2007), and cyanobacterial and *Chloroflexus*-like populations have been subject of biogeographic or beta-diversity variation studies elsewhere (Miller et al., 2007 and Liu et al., 2011, respectively). Possibly, the most extended study is that of Whitaker et al. (2003): by means of *Sulfolobus* strains isolated from hot springs world wide, the authors concluded that geographic distance between hot springs

explained the strain differences. The cited papers had based their research on single groups or populations of prokaryotes, but little is known about the biogeographic patterns of the complete bacterial community diversity at different spatial scales.

Hot springs assessed in this thesis were located in protected areas belonging to Costa Rica (Miravalles, Rocas Calientes, Bajo las Peñas, and Rio Negro), Chile (El Tatio Geyser Field in Atacama Desert; and Porcelana hot spring, Cahuelmó hot spring, and Porcelana Geyser, in Northern Patagonia) and Antarctica (Deception Island in Southern Shetland Islands).

Most of the hot springs in this study are still safe from human intervention and pollution. Some have probably remained in the same native condition since the last glaciation. The complexity of sampling in these remote hot springs adds an invaluable merit to this work. A perfect example is Porcelana geyser; a three hours walk paving the way through the rainforest, wading through the river, swimming against the current together with Pacific Salmons and climbing ropes just to get to this unknown geothermal event. Considering the equipment necessary for a proper sampling, and the few daylight hours during winter, it is necessary and fair to recognize the effort of the samplers and logistic support of the scientific team of Fundación Huinay, a scientific field station who made the project possible.

The history of Porcelana geyser is curious, and goes back to the beginning of XX century, when Boris Hernández's grandfather, both settlers in the Patagonian fjord (only 12 families are settled there), went deep into the forest looking for *Fitzroya cupressoides* (Alerce), an ancient tree highly coveted by its timber, and found an impressive scene: a 4 meter white column blurred in vapor, standing in a multicolored altar. He run off back terrified, and told his family the holy virgin appeared to him in the middle of the forest. Apparently, no one else attempted to go inside the forest again for years, until the late 90's, when his grandson, Boris, took the old trail into the depth of the woods to find the hidden virgin. And without knowing it then, this thesis began to unfold…

Given the limited scientific data available on these areas, the importance of this thesis was to characterize and compare the prokaryotic communities in microbial mats from hot springs in Central and South America. The results obtained represent a comprehensive review on the thermophilic microorganisms that thrive in these biodiversity hot spots such as the Valdivian Rain Forest in Patagonia, and cloud forests in Costa Rica, as well as the Atacama Desert and Southern Shetland Island in Antarctica. The improvement of knowledge around the microbial diversity of these fragile ecosystems reinforces the need for their protection, moreover this biodiversity could be the source for further biotechnological applications, embodying a key cornerstone for a developing region.

Objectives and structure of the following doctoral thesis

Using the previous background, the main objectives of this thesis were to characterize and compare the bacterial communities in hot springs on which no previous data was available, and to identify the most relevant environmental factors influencing community-level diversity shifts at different spatial scales, by means of molecular microbial ecology methods.

The objectives of this Thesis were:

- Describe and add value to hot springs in Patagonia which were unknown until now (Chapters I and II), and
- To seize the advantages that hot springs offer for studying bacterial diversity insights: biogeography, latitude relationships, and spatial distribution at different scales (Chapters III and IV).

Below, a brief overview of each chapter is given:

In Chapter I, we determined the environmental parameters and compared thermophilic prokaryotic communities from microbial mats along the thermal gradients of three unexplored hot springs from Chilean Northern Patagonia (Porcelana and Cahuelmó hot springs, and Porcelana geyser). Samples were used for fingerprinting analysis by means of the ribosomal RNA small subunit, total cell counts, and isolation of thermophilic heterotrophic strains. Additionally, scanning electron microscopy images were used to observe the complexity and obvious differences in microbial mat structures along the thermal gradients of the three hot springs. The data presented in this chapter constituted a first step in the analysis of these pristine hot springs, and was essential to better plan the following expeditions, as the complexity of traveling in and out of each hot spring represented a challenge of its own. Unfortunately, all samples obtained in this expedition were lost in the 2010 earthquake in Concepción, and no

further experiments were possible. Nevertheless, a framework for the thesis was achieved by the quick microbiological overview and the field experience.

In Chapter II, the study included the same points sampled in Chapter I, which were obtained in two different expeditions. Dominant populations were characterized by fingerprinting of the SSU rRNA, and a seasonal and spatial analysis was made to better understand the diversity shifts of dominant populations. Phylum Cyanobacteria was identified as the major component of the microbial community, and a deeper analysis was made on this group in parallel to other less dominant Phyla such as Bacteroidetes and Deinococcus-Thermus. No substantial diversity differences were detected between samples in winter and summer, but significant similarities were observed among hot springs in similar surrounding environments, mainly in Cyanobacteria.

In Chapter III, the horizontal microheterogeneity of microbial mats at a cm scale was analyzed by using high-throughput sequencing of rRNA SSU of samples in the same hot springs analyzed previously. Dominant and major phyla were consistent with previous observations, plus other very abundant phyla undetected before. Additionally, a wide diversity of minor and rare bacterial groups was characterized. A temperature-niche transition was observed among primary producers Cyanobacteria and Chloroflexi, and significant diversity shifts were detected between nearby samples. Similarity among hot springs maintained, as previously reported.

In Chapter IV, biogeography of thermophilic microbial mat samples was analyzed. Samples were collected from hot springs in Costa Rica, Atacama Desert, Northern Patagonia (from the same hot springs above), and Antarctica. High-throughput sequencing of the rRNA SSU was used for bacterial community structure characterization. Cyanobacteria and Chloroflexi were dominant in almost all samples. No latitudinal relationship was detected, as samples closer to the equator were not more diverse or harbored more richness than samples closer to the pole. Nevertheless, samples showed a negative relationship between diversity and geographic distance, and temperature.

REFERENCES

Amils R., González-Toril E., Fernández-Remolar D., Gómez F., Aguilera Á., Rodríguez N., Malki M., García-Moyano A., Fairén A. G., de la Fuente V. and Sanz J. L. 2007. Extreme environments as Mars terrestrial analogs: The Rio Tinto case. Planet. Space Sci. 55:370–381.

Anderson M. J., Crist T. O., Chase J. M., et al. 2011. Navigating the multiple meanings of β diversity: a roadmap for the practicing ecologist. Ecology letters, 14:19–28.

Bell E. M. and Callaghan T. V. 2011. What are extreme environments and what lives in them?. In: Bell E. M. (ed) Life at extremes: environments, organisms and strategies for survival. Chapter I, pp 1-12. CABI, Australia.

Brock T. D. 1967. Life at high temperatures. Science. 158:1012–1019.

Brock T. D., and Freeze H. 1969. *Thermus aquaticus* gen. n. and sp. n., a Non-sporulating extreme thermophile. J. Bacteriol. 98:289–297.

Brock T. D. 1969. Vertical zonation in hot spring algal mats. Phycologia 8:201–205.

Brock T. D. 1978. Thermophilic microorganisms and life at high temperatures. Springer-Verlag, New York, Heidelberg, Berlin.

Brock T. D. 1997. The value of basic research: Discovery of *Thermus aquaticus* and other extreme thermophiles. Genetics 146:1207–1210.

Bruins M. E., Janssen A. E. M. and Boom R. M. 2001. Thermozymes and their applications. Appl. Biochem. Biotechnol. 90:155–186.

Canganella F. and Wiegel J. 2011. Extremophiles: from abyssal to terrestrial ecosystems and possibly beyond. Naturwissenschaften 98:253–279.

Da Costa M. S., Rainey F. A. and Nobre M. F. 2006. The genus *Thermus* and relatives, p 7:797-812. In Dworkin M, S Falkow, E Rosenberg, KH Schleifer, E Stackebrandt (ed.), The Prokaryotes, Third Edition, Springer. Singapore.

Demergasso C., Chong G., Galleguillos P., Escudero L., Martínez-Alonso M. and Esteve I. 2003. Microbial mats from the Llamará salt flat, northern Chile. Rev. Chil. Hist. Nat. 76:485–499.

De los Ríos A., Ascaso C., Wierzchos J., Fernández-Valiente E. and Quesada A. 2004. Microstructural characterization of cyanobacterial mats from the McMurdo Ice Shelf, Antarctica. Appl. Environ. Microbiol. 70:569–580.

De los Ríos A., Valea S., Ascaso C., Davila A., Kastovsky J., Mckay C. P., Gómez-Silva B. and Wierzchos J. 2010. Comparative analysis of the microbial communities inhabiting halite evaporites of the Atacama Desert. Int. Microbiol. 13:79–89.

Fierer N. and Jackson R. B. 2006. The diversity and biogeography of soil bacterial communities. PNAS. 103:626–631.

Franklin R. B., Blum L. K., McComb A. C. and Mills A. L. 2002. A geostatistical analysis of small-scale spatial variability in bacterial abundance and community structure in salt marsh creek bank sediments. FEMS Microbiol. Ecol. 42:71–80.

Franks J. and Stolz J. F. 2009. Flat laminated microbial mat communities. Earth-Science Rev. 96:163–172.

Glennon J. A. and Pfaff R. M. 2003. The extraordinary thermal activity of El Tatio Geyser Field, Antofagasta region, Chile. GOSA Trans Vol VIII: 31-78.

Horikoshi K. 1998. Introduction. In: Extremophiles: microbial life in extreme environments. Horikoshi K., Grant W. D. (eds). Wiley-Liss, New York.

Hughes J. B., Hellmann J. J., Ricketts T. H. and Bohannan B. J. M. 2001. Counting the uncountable: Statistical approaches to estimating microbial diversity. Appl Environ Microbiol, 67:4399–4406.

Javaux E. J. 2006. Extreme life on Earth-- past, present and possibly beyond. Res. Microbiol. 157:37–48.

Jones B. and Renaut R. W. 1997. Formation of silica oncoids around geysers and hot springs at El Tatio, northern Chile. Sedimentol. 44:287-304.

Kassen R. and Rainey P. B. 2004. The ecology and genetics of microbial diversity. Annu. Rev. Microbiol. 58:207–231.

Kristjansson J. K. and Alfredsson G. A. 1983. Distribution of *Thermus spp*. in Icelandic hot springs and a thermal gradient. Appl. Environ. Microbiol. 45:1785-1789.

Liu Z., Klatt C. G., Wood J. M., Rusch D. B., Ludwig M., Wittekindt N., Tomsho L. P., Schuster S. C., Ward D. M. and Bryant D. A. 2011. Metatranscriptomic analyses of chlorophototrophs of a hot spring microbial mat. ISMEJ. 5:1279–1290.

Lomolino M. V, Riddle B. R. and Brown J. H. 2006. The science of biogeography, p. 3–12. In Biogeography. Sinauer Associates, Inc, Sunderland, Massachussets.

López-López O., Cerdán M. E. and González-Siso M. I. 2013. Hot spring metagenomics. Life. 2:308–320.

Madigan M. T. and Marrs B. L. 1997. Extremophiles. Sci. Am. 82–87.

Magurran A. E. 1988. Ecological Diversity and Its Measurement. 179 pp. Princeton University Press, New Jersey, USA.

Magurran A. E. 2004. Measuring Biological Diversity. Chapter 5, pp. 131-160. Blackwell Publishing, Cornwall, UK.

Martiny J. B. H., Bohannan B. J. M., Brown J. H., Colwell R. K., Fuhrman J. A., Green J. L., Horner-Devine M. C., Kane M., Krumins J. A., Kuske C. R., Morin P. J., Naeem S., Øvreås L., Reysenbach A., Smith V. H. and Staley J. T. 2006. Microbial biogeography: Putting microorganisms on the map. Nature 4:102–112.

Martiny J. B. H., Eisen J. A., Penn K., Allison S. D. and Horner-Devine M. C. 2011. Drivers of bacterial β -diversity depend on spatial scale. PNAS 108:7850–7854.

Miller S. R., Castenholz R. W. and Pedersen D. 2007. Phylogeography of the thermophilic cyanobacterium *Mastigocladus laminosus*. Appl. Environ. Microbiol. 73:4751–4759.

Oren A. 2004. Prokaryote diversity and taxonomy : current status and future challenges. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 359:623–638.

Pace N. R. 1997. A molecular view of microbial diversity and the biosphere. Science. 276:734–740.

Papke R. T., Ramsing N. B., Bateson M. M. and Ward D. M. 2003. Geographical isolation in hot spring cyanobacteria. Environ. Microbiol. 5:650–659.

Pedrós-Alió C. 2007. Ecology. Dipping into the rare biosphere. Science. 315:192–193.

Phoenix V. R., Bennett P. C., Engel A. S., Tyler S. W. and Ferris F. G. 2006. Chilean high-altitude hot-spring sinters: a model system for UV screening mechanisms by early Precambrian cyanobacteria. Geobiology. 4:15-28.

Pikuta E. V, Hoover R. B. and Tang J. 2007. Microbial extremophiles at the limits of life. Crit. Rev. Microbiol. 33:183–209.

Ramette A. and Tiedje J. M. 2007. Biogeography. An emerging cornerstone for understanding prokaryotic diversity, ecology and evolution. Microb. Ecol. 53:197–207.

Reysenbach A. L. and Cady S. L. 2001. Microbiology of ancient and modern hydrothermal systems. Trends Microbiol. 9:79–86.

Rothschild L. J. and Mancinelli R. L. 2001. Life in extreme environments. Nature. 409:1092–1101.

Stojanovic D., O. Fojkar, A. Drobac-Cik, K. Cajko, T. Dulic, and Z. Svircev. 2008. Extremophiles - Link between Earth and Astrobiology. Proc. Natl. Acad. Sci. Matica Srp. Novi Sad 114:5–16.

Tassi F., Aguilera F., Darrah T., Vaselli O., Capaccioni B., Poreda R. J. and Delgado-Huertas A. 2010. Fluid geochemistry of hydrothermal systems in the Arica-Parinacota, Tarapacá and Antofagasta regions (northern Chile). J. Volcanol. Geotherm. Res. 192 (1-2):1-15

Van Gemerden H. 1993. Microbial mats: A joint venture. Mar. Geol. 113:3–25.

Ward D. M., Ferris M. J., Nold S. C. and Bateson M. M. 1998. A natural view of microbial biodiversity within hot spring cyanobacterial mat communities. Microbiol. Mol. Biol. Rev. 62:1353–1370.

Watve M. G. and Gangal R. M. 1996. Problems in measuring bacterial diversity and a possible solution. Appl. Environ. Microbiol. 62, 4299–4301.

Whitaker R. J., Grogan D. W. and Taylor J. W. 2003. Geographic barriers isolate endemic populations of hyperthermophilic Archaea. Science. 301:976–978.

Xu Y. and Glansdorff N. 2002. Was our ancestor a hyperthermophilic procaryote? Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 133:677–688.

Zeikus J. G., Vieille C. and Savchenko A. 1998. Thermozymes: Biotechnology and structure-function relationships. Extremophiles. 2:179–183.

CHAPTER I

The thermophilic microbial mats from hot springs in Chilean Northern Patagonia – A first overview

Roy Mackenzie, Beatriz Diez, Leslie E. Abarzúa, Homero E. Urrutia, Carlos Pedrós-Alió

ABSTRACT

The aim of this work was to determine environmental parameters and compare prokaryotic communities in microbial mats along the thermal gradients of three unexplored hot springs from Chilean North Patagonia (Porcelana and Cahuelmó hot springs, and Porcelana geyser), as part of an exploratory expedition. Physico-chemical data were obtained *in situ* (temperature, pH, conductivity and dissolved oxygen) and in the laboratory (sulfate concentration). Total and live bacterial counts were performed on fresh samples using DAPI staining and BacLight viability kit, respectively. Colony forming units of thermophilic heterotrophic aerobic bacteria were also determined. Results revealed total cell counts higher than in other hot springs (3.8×10^{12} cells per dry gram of mat, Cahuelmó hot spring), and a high percentage of thermophilic (55º C) culturable bacteria (20% of live cell counts in Porcelana geyser point F2). Morphological analyses by SEM demonstrated the complexity and obvious differences in microbial mat structures along the thermal gradients of the three sites surveyed. Filaments with and without sheaths (2 and 0.5 µm diameter sizes in average) dominated in different temperature ranges. Larger biovolume of 0.5 µm diameter filaments was found at higher temperatures. 16S rRNA gene fingerprinting analysis by denaturing gradient gel electrophoresis (DGGE) demonstrated changes in bacterial community composition along transects in each hot spring. A multidimensional scaling (MDS) analysis showed that bacterial communities at high temperature had low resemblance to the corresponding mesophilic communities in each system; on the contrary, mesophilic communities were similar among them in each hot spring. The data presented in this study are a first step to the analysis of these pristine hot springs.

INTRODUCTION

In hydrothermal systems the pressurized-boiling water will be eventually forced up to the surface as a hot hydrothermal fluid, rich in dissolved minerals and reduced gases. The chemical composition of the hydrothermal fluid differs as a function of the underground rock composition and the residence time of the fluid below surface (Reysenbach and Cady, 2001). In these environments, microbial communities are usually organized in thick matrices of polymers and sediment, named microbial mats (Ward et al., 1998). The presence and abundance of species in a microbial mat is strongly influenced by physical properties such as temperature, besides other key chemical parameters such as oxygen, pH, redox potential, and the availability of electron acceptors and donors (Franks and Stolz, 2009). Hot spring microbial mats usually present steep gradients of pH, oxygen, and sulfides (Van Gemerden, 1993), as thermal water is quickly mixed with sediments and surrounding environment when emerging from the subsurface (Reysenbach and Cady, 2001).

Brock (1978) described that hot spring microbial mats are typically zoned downstream according to temperature. Typically, photoautotrophs (mainly Cyanobacteria) are dominant and overlie heterotrophic prokaryotes that form the base of a mat (Van Gemerden, 1993). Members of the *Thermus* genus, a ubiquitous heterotrophic thermophilic bacterium, are commonly found in non-acidic thermal areas all over the world in a temperature range of 40º-70º C (da Costa et al., 2006), and may well be the most important heterotrophs in the hot spring ecosystem (Kristjansson and Alfredsson, 1983).

Where visible, cyanobacteria form streamers of different macroscopical characteristics attached to substrates. Cyanobacteria can be found in water up to 73-75 ºC (Brock, 1967), usually associated to photosynthetic bacteria such as *Chloroflexus* (Ward et al., 1998), forming large streamers. *Synechococcus* is frequently the dominant genus at temperatures ranging from ~74-65 ºC (Ferris et al., 1996a; Ramsing et al., 2000). In waters cooler than 65ºC, filamentous cyanobacteria such as *Oscillatoria*, *Phormidium*, *Fischerella* and *Calothrix* (Ferris and Ward, 1997; Roeselers et al., 2007; Boomer et al., 2009; Portillo et al., 2009), with eukaryotic algae (<60ºC) (Foster et al., 2009) can be found, forming extensive mats over the sediment or floating

on the water. Elements such as the shape of the cells, and structure of microbial mats have been studied without disrupting the sample using scanning electron micrography (SEM), in ecosystems as different as stromatolites (Konhauser et al., 2001), hot springs (Jones and Renaut, 1997), or hypersaline lakes (Jørgensen et al., 1983).

Denaturing gradient gel electrophoresis, a culture-independent, has been used for determining the composition and diversity of microbial communities in hot springs (Ferris et al., 1996b; Nocker et al., 2007). Some of these authors have focused on the dominant bacterial groups such as phototrophs (Roeselers et al., 2007), Bacteroidetes (Portillo et al., 2009), Proteobacteria (Nold et al., 1996), Archaea (Barns et al., 1994) and Cyanobacteria (Ramsing et al., 2000), in hot springs from Yellowstone (USA), Kamchatka (Russia), Indonesia, and Thailand, among other places. The use of the 16S rRNA gene as molecular markers together with the DGGE fingerprinting methodology also allows obtaining ecological indexes to compare communities, such as similarity and diversity coefficients, species richness, and dominance, among others (Vallaeys et al., 1997; Fromin et al., 2002; Nocker et al., 2007).

In the south-central and austral Andes (between latitudes 17º30’ S - 56º32’ S), hydrothermal systems are widespread due to several quaternary active volcanoes in the region, resulting from subduction of the Nazca and Antarctic oceanic plates below South America (Stern, 2004). Some geological and geochemical studies of hydrothermal events have been conducted in the Atacama Desert and Altiplano (Northern Chile) (Jones and Renaut, 1997; Glennon and Pfaff, 2003; Phoenix et al., 2006; Tassi et al., 2010). These studies mention the presence and potential importance relation to geological processes of microorganisms. Therefore, no microbiological studies have been conducted in any hot spring in the area.

In the Andes from Northern Patagonia, in remote locations inside the rainforest, several hot springs remain undescribed; some of them are still safe from human intervention and pollution. Some of these hot springs have probably remained in the same native condition since the last glaciation. Hot springs Porcelana, Cahuelmó and Porcelana geyser are natural ecosystems located in Chilean Northern Patagonia. Hauser (1989), in his study of the thermal springs of the area, mentioned hot springs Porcelana and Cahuelmó as constant water discharge

sources with temperatures of 60º and 84º C, respectively. Hauser also determined some hydro-chemical properties of the water, such as pH, conductivity, and metal content. This author emphasized the problems and errors of such estimations, due to logistic problems and difficulties to reach the hot springs. Waring, in his monumental work on Thermal Springs of the World (1965), mentioned Cahuelmó and provided a measured temperature of 55 ˚C. Porcelana geyser (from now on, and to avoid confusion, we will refer to this system as geyser) was discovered by a local resident 40 years ago, and has not been described in the literature before. Given the limited scientific data available on these areas, the aim of this paper was to characterize and compare the prokaryotic communities in microbial mats from hot springs Porcelana and Cahuelmó, and geyser Porcelana. The data presented here were collected in an exploratory expedition to locate the hot springs in the Southern Andes.

MATERIALS AND METHODS

Sampling sites and sampling collection

Microbial mat samples were collected from hot springs Cahuelmó (42º 15’ 11.8’’S - 72º 22’ 4.4’’W), Porcelana (42º 27’ 29.1’’S - 72º 27’ 39.3’’W), and Porcelana geyser (42º 24’ 51’’S / 72º 29’ 02.2’’ W) in March, 2008. The three hot springs are located in the surroundings of fjords Comau and Cahuelmó, Los Lagos region, North Patagonia of Chile (Fig. 1). Linear distances between hot springs are 5.1 km from Porcelana to the geyser, 25.4 km from Porcelana to Cahuelmó, and, 20.6 km from Cahuelmó to the geyser.

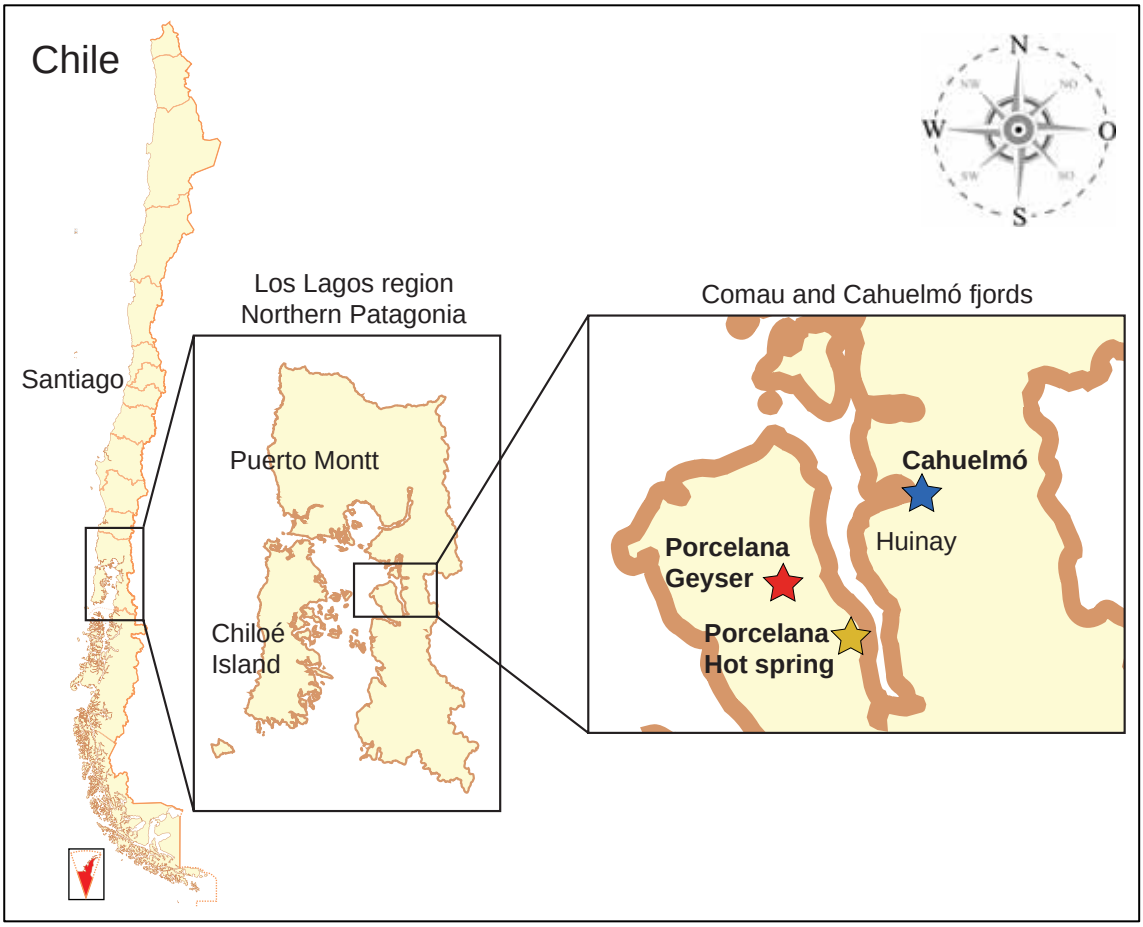


Figure 1: Map of Los Lagos region (Chile). Stars represents sampled hot springs. Cahuelmó hot spring (Blue), Porcelana geyser (Red), and Porcelana hot spring (Yellow).

In each hot spring, an approximately 6-8 m long transect was analyzed following a temperature gradient, downstream from the water source, with four sampling points in each transect (Fig. 2). At each sampling point, temperature was determined using a thermometer (Oakton, model 35607-85). The pH was approximated to the nearest integer using pH-indicator paper (Merck, Germany). A 40-50 ml sample was collected from each sampling point using a 2 cm diameter cork borer when the substrate was consistent, or directly with 50 mL plastic tubes (4 cm in diameter, Falcon tubes, BD Biosciences, NJ USA) when it was soft. Ethanol rinsed forceps were used to accommodate the sample in sterile 50 ml tubes.

Bacterial counts and SEM

Samples for electron microscopy were collected in triplicate directly from mats into 1.7 mL Eppendorf tubes. Aliquots were fixed in situ with glutaraldehyde (2.5% final volume) for SEM imaging in a scanning electron microscope model ETEC Autoscan U-1 (Perkin-Elmer, Electron Beam Technology, CA USA). Fresh samples were used for culturable aerobic heterotrophic counts (55º C).

In order to homogenize the mats, 1 g of material was diluted in 1 ml of physiological saline solution, and 300 µm diameter glass beads were added. The mat slurry was subjected to two pulses of shaking (5 seconds) in a mini-beadbeater cell disrupter, alternated with two sonication intervals of 30 seconds in a sonicator model 8890E-MT (Cole-Parmer instruments Co, IL USA). Three aliquots of several dilutions of each sample were plated on R2A agar (Difco BD Biosciences, NJ USA) made with filter-sterilized water from each hot spring. Plates were incubated at 55 ˚C in the dark, until no new colony growth was observed (5-6 days). Aliquotes were used for total, as well as live-and-dead cell counts (five replicates each) through epifluorescence microscopy with a Zeiss model Axiostar microscope. Total counts were done using DAPI (1 mg/ml), and filtering through black polycarbonate filters (25 mm diameter, 0.22 µm pore size) (Porter and Feig, 1980), using 300 µl of DAPI and 300 µl of diluted sample. Cell counts were made by counting ten fields in an epifluorescence microscope. In each case, the standard deviation was <10% of the mean. For the live-and-dead counts, aliquots were diluted and stained with Baclight Viability kit (Molecular Probes, OR USA) according to the manufacturer's instructions.

Biovolumes of filamentous bacteria were estimated as described by Olson (1950). Briefly, filaments were considered to be randomly distributed over the observed field, in a theoretical grid with squares of 100 µm. Based on the distribution of sizes of filaments in the samples, two average diameters of filaments were considered; 0.5 µm diameter (thin) and 2 µm diameter (thick), determined in SEM microphotographs. Both types of filaments were counted separately. The length of filaments was estimated by counting the intersections of filaments

with the horizontal and vertical lines of the grid, starting from the first horizontal line below the top line, and including the bottom line. Vertical lines were counted next, starting from the first vertical line to the right of the left hand boundary, and including the right hand vertical line. The number of intersections counted was multiplied times 0.786 ($\pi/4$) to obtain the total length of filaments. Total length of filaments was used to calculate the volume of theoretical cylinders of 0.5 and 2 μm diameters, resulting in final biovolume. The standard deviation was usually below 12% of the mean. In one case it was up to 35% of total biovolume count in both filaments. Biovolumes of cocci were calculated considering an average size of 0.5 μm radius, and those of bacilli by considering 0.2 μm radius by 1 μm length after measurement of a variable number of cells in SEM images.

Nucleic acid extraction, amplification, and DGGE

Genetic material was extracted from 0.3 g of microbial mat, using the MO BIO Soil DNA Isolation kit (Invitrogen, USA). Universal bacteria primers 341F – 907R (Muyzer et al., 1995) and 341FGC – 534R (Muyzer et al., 1993) were used to amplify by nested PCR the 16S rRNA gene, as described by Boon et al. (2002).

A DGGE (made in a DCode System, BioRad CA, USA) was made with the amplification products, in a urea/formamide denaturing gradient between 45-70% denaturant. Operational conditions for DGGE were as reported elsewhere (Boon et al., 2002). Gels were stained with ethidium bromide (0.5 mg ml^{-1}) and recorded. Image analysis was done using the Quantity One 1D Analysis software (Bio-Rad, CA, USA). Bray-Curtis distances were calculated from absence-presence data, and a multidimensional scaling (MDS) analysis was carried out within and among hot springs, using the Primer 6 Software (V 6.1.2).

RESULTS AND DISCUSION

The geothermal systems explored in the present work (Cahuelmó, Porcelana, and Geyser) are surrounded by mountains and fjords, with no apparent direct contact among them. These hot springs exhibited physico-chemical and biological differences, summarized as follows.

Cahuelmó hot spring.

This hot spring is located at the shoreline of Cahuelmó Fjord (Fig. 1), in a metamorphic complex of the main mountain chain, originated in the Paleozoic period. Temperature decreased from 56.5º to 40.5º C, and pH decreased two units (from 7 to 5) (Table 1).

Table 1: Physico-chemical characteristics of interstitial water in sampled microbial mats

Hot spring	Cahuelmó				Porcelana				Geyser			
Sample	C1	C2	C3	C4	P1	P2	P3	P4	F1	F2	F3	F4
Temperature (ºC)	56.5	55	46	40.5	53	47.5	45	38.5	66	57	51	41
pH	7	6	6	5	6	6	6	5	5	5	6	6

The microbial mat sampled in C1 was formed by white long filaments attached to sediments or small tree branches submerged in water (Fig. 2A). Sample C2 showed shorter orange filaments; sample C3 exhibited pale green gelatinous mat intimately adhered to the sediment; and sample C4 was formed by brilliant green mushroom-like structures.

Figure 2: Appearance of transects in (A) Cahuelmó hot spring, (B) Porcelana hot spring, and (C) Porcelana geyser, showing studied mats and temperature of the water.

SEM images showed evident differences in filamentous morphology (Fig. 3A and B). Sample C1 showed a complex net of thin filaments (Fig. 3A). This net corresponds to the macroscopic aspect of the microbial mat observed in C1, as white filamentous streamers (Fig. 2A). Sample C4 (Fig 3B) revealed thick filaments, which resembled cyanobacterial sheaths.

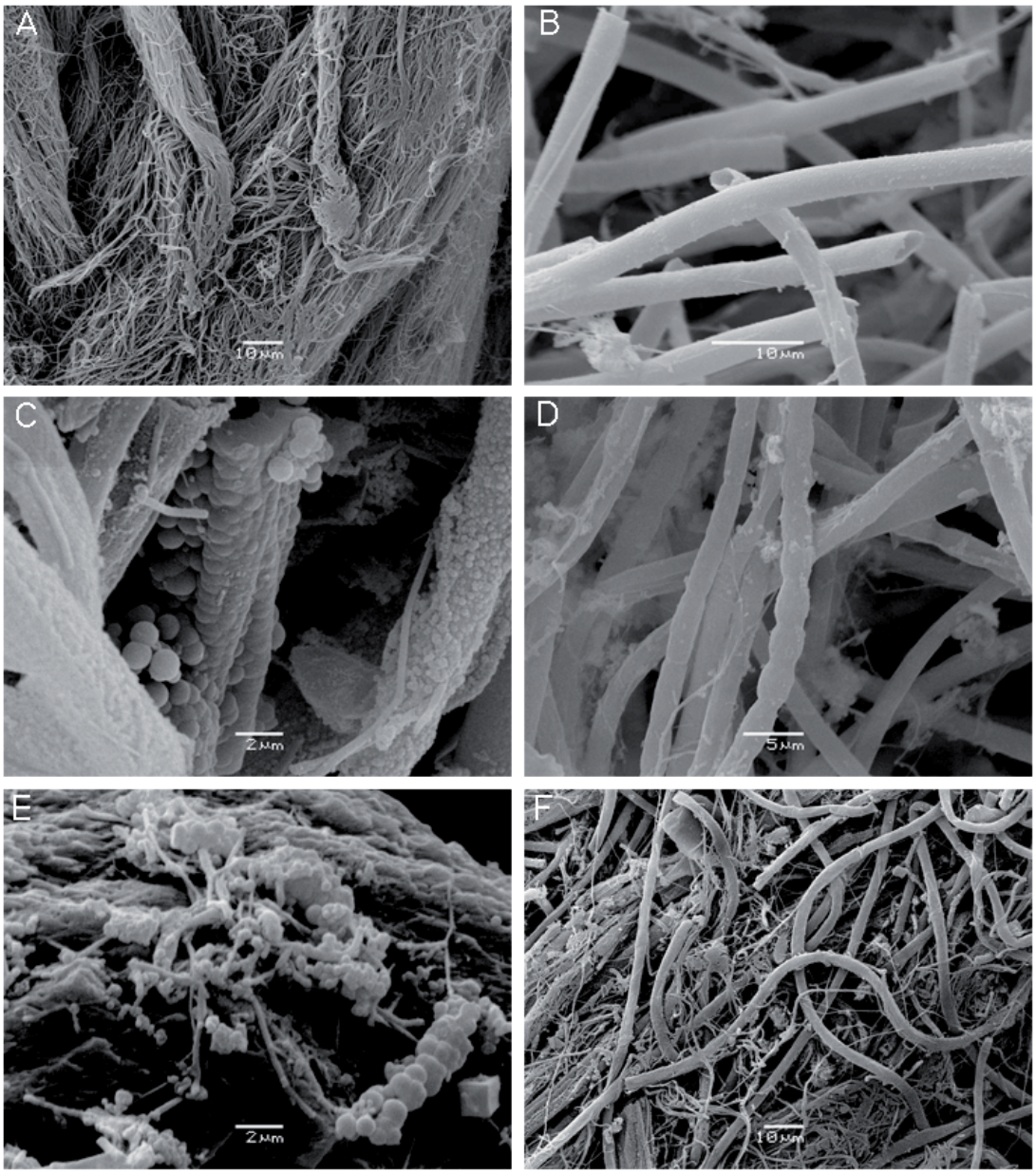


Figure 3: SEM micrographs of two sampled points in each hot spring. A and B: Cahuelmó hot spring, samples C1 and C4 respectively. C and D: Porcelana hot spring, samples P1 and P4, respectively. E and F: Porcelana geyser, samples F1 and F4, respectively.

Biovolume of bacilli was lower than that of cocci, and the highest relative biovolume of bacilli in Cahuelmó was 26% (sample C2, Fig. 4). Cocci biovolume was as high as 60% in this hot spring (sample C2, Fig. 4). Biovolume of 0.5 μm diameter filaments dominated the high temperature mat (92% in sample C1, Fig 4), which could explain the observations by SEM, but decreased drastically downstream (Fig. 4). On the contrary, biovolume of 2 μm diameter filaments increased downstream up to 72% in sample C3, but it was not detected in sample C4 (Fig. 4). This could be due to unstained empty sheaths of filamentous cyanobacteria, which cannot be detected by epifluoresence microscopy.

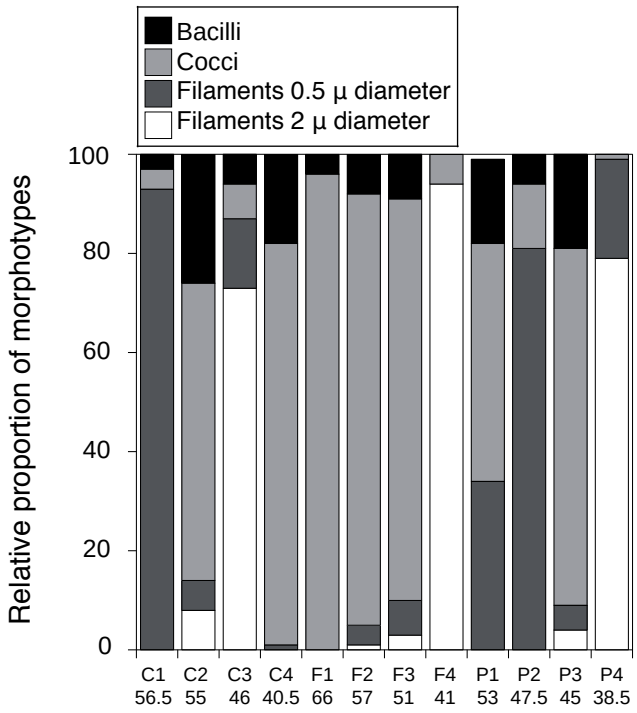


Figure 4: Relative biovolume of different morphotypes in samples, determined by epifluorescence microscopy. Temperature of each sample is included.

The abundance of unicellular bacteria in Cahuelmó diminished downstream, ranging from 10^{12} to 10^{9} cells g^{-1} (Fig. 5). Sample C1 showed the highest relative abundance of live cells (17% of total cell counts), with an average of 10% living cells in the remaining samples.

Percentage of culturable cells at 55º C, as expected, was higher at high temperature (up to 8.8% of live cells), and diminished downstream (less than 1% in points C3 and C4, Fig. 5).

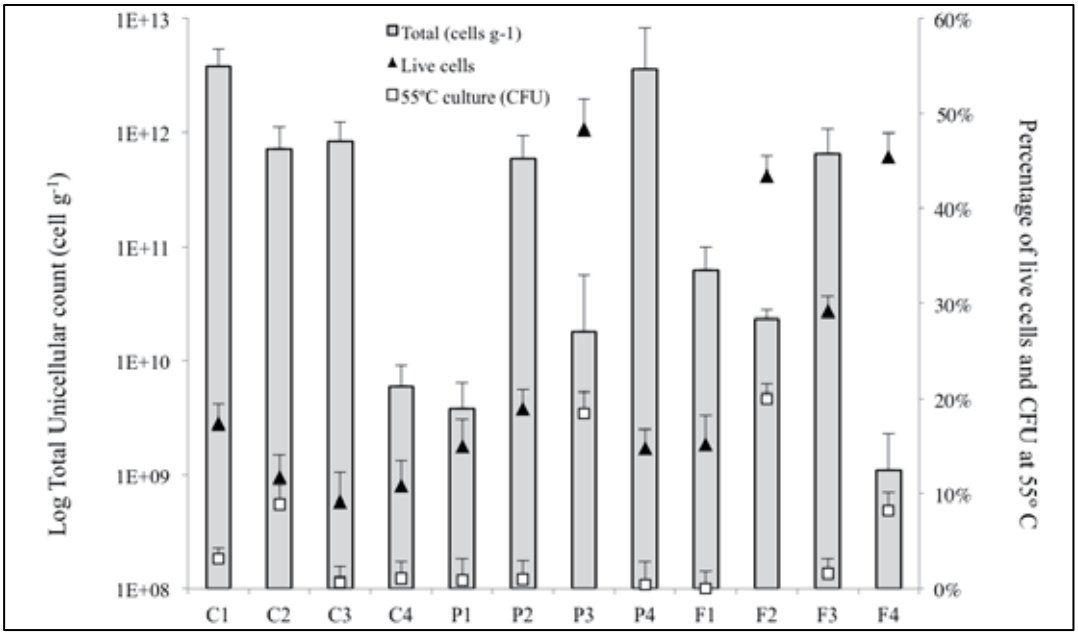


Figure 5: Total cell count, and relative abundance of live and cultivable cells in samples.

MDS analyses showed two clear clusters of over 60% similarity, one composed of samples C1 56.5º and C2 55º C, and a second composed of C3 46º and C4 40.5º C (Fig. 6A). This type of clustering suggests a temperature defined ecosystem with an ecotone around 50º C.

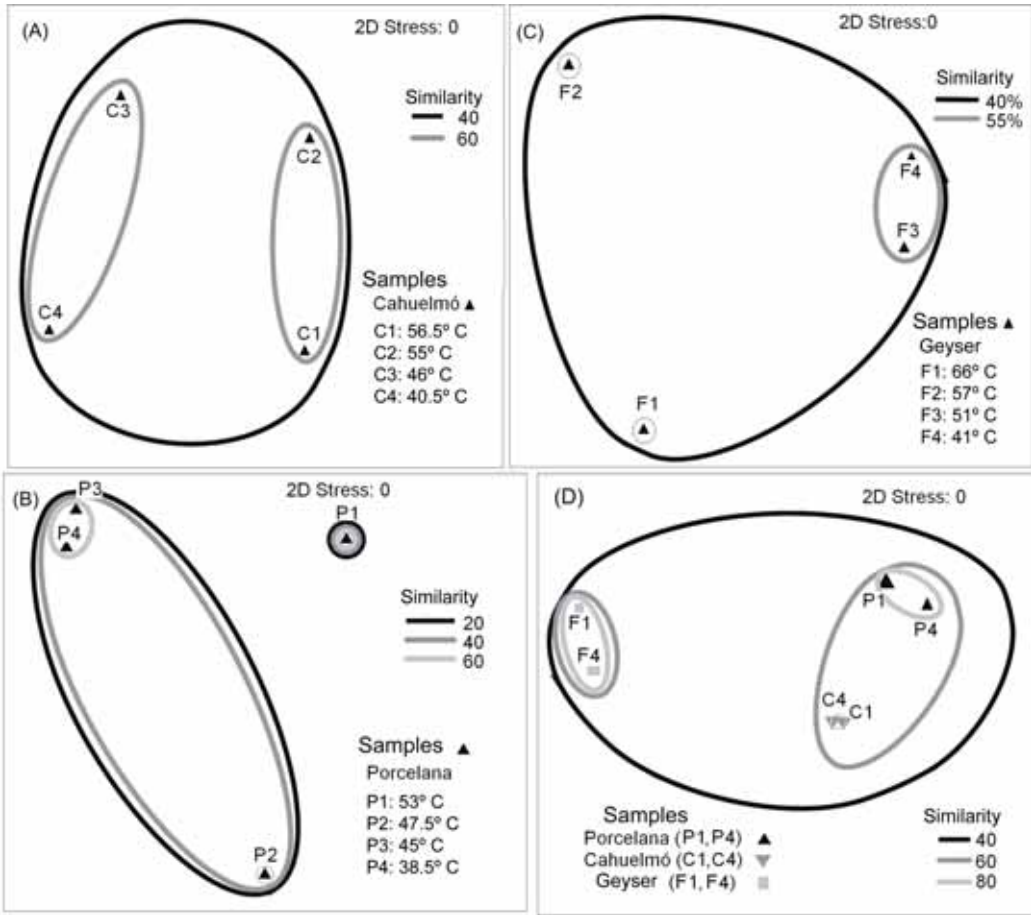


Figure 6: MDS analysis by Bray Curtis distance index of 16S rRNA-DGGE fingerprinting of all samples. A: Cahuelmó hot spring. B: Porcelana hot spring C: Porcelana geyser. D: Porcelana geyser (F1 and F4), Cahuelmó (C1 and C4), and Porcelana (P1 and P4).

Porcelana hot spring.

This hot spring is located in the rain forest, away from the fjord influence (Fig. 1). The site is in volcanic rocks originated in the quaternary period (Duhart et al. 2000). The volcanic rock in this region is formed mainly by silicates and carbonates, and is rich in minerals such as pyrite, chalcopyrite, arsenopyrite and antimony (Fortey et al. 1992). Temperature decreased from 53º to 38.5º C, and pH remained constant around 6 along the transect (Table 1).

The microbial mat in sample P1 of Porcelana was formed by white, thick filaments (5-7 cm), anchored to the rocks surrounding the water stream (Fig. 2B). The white mat had orange

(inside) and green (outside) edges. Sample P2 was formed by a white, thick mat (10-12 cm), with an orange (inside) and black or dark green (outside) edge, anchored to rocks surrounding the water stream. Several insect larvae were observed all over the mats. Sample P3 showed a brilliant green mat with big and small oxygen bubbles, anchored to sediments or rocks, and forming branches flowing with the water stream. Sample P4 mat was dark green with filaments flowing down stream, rooted on a bigger non-filamentous brilliant pale-green mat (Fig. 2B). SEM images of these mats revealed a dominance of cocci intermixed with bacilli and thin filaments in sample P1 (Fig. 3C). Sample P4 revealed a dominance of thick filaments of approximately 2 µm diameter, and a very low presence of cocci (Fig. 3D). In the microbial mat from sample P4, no sheaths were detected.

Biovolume of bacilli in Porcelana was three times lower than that of cocci. Cocci biovolume in this hot spring was as high as 72% (sample P3, Fig. 4). No 2 µm diameter filaments were detected at points P1 and P2, but 0.5 µm diameter filaments dominated these mats (34% and 81% relative biovolume, respectively). 2 µm diameter filaments were detected at points P3 and P4, widely dominating sample P4 (78.8% relative biovolume, Fig. 4).

The abundance of unicellular bacteria in this hot spring increased downstream, unlike in Cahuelmó. The sample with the highest total abundance of unicellular bacteria (sample P4 $3.6x10^{12}$ cells g^{-1}) did not correlate with the highest proportion of live cells (Table 2). Sample P3 exhibited the highest proportion of live cells (48.3% relative abundance), also showed the highest culturability of heterotrophic CFUs at 55ºC (18.4%), the highest assessed, as expected (Fig 5). All other sampled points in Porcelana presented over 14% of live cells, but less than 1% of culturable heterotrophic CFUs at 55ºC (Fig. 5).

MDS analyses revealed that microbial mats from points P3 and P4 were closely related (over 60% Bray Curtis similarity index), but sample P2 had a similarity of 40% (Fig. 6B). Sample P1 had a similarity index lower than 20% with the other samples in Porcelana, even though the difference in temperature between P1 and P2 was only 5.5º C.

TABLE 2: Total and live unicellular counts by epifluorescence microscopy, and culturable bacterial count.

	Cahuelmó				Porcelana				Geyser			
Cell counts	C1	C2	C3	C4	P1	P2	P3	P4	F1	F2	F3	F4
Total cells (10^9 cells g^{-1})	3800	720	830	5.90	3.8	580	18	3600	62	23	650	1.1
Live (10^9 cells g^{-1})	660	85	76	0.6	0.57	110	8.7	530	9.4	10	190	0.5
Live (% of total)	17.4%	11.8%	9.2%	10.8%	15.0%	19.0%	48.3%	14.7%	15.2%	43.5%	29.2%	45.5%
55º C culture (10^9 CFU g^{-1})	21	7.5	0.52	0.0007	0.0005	1	1.6	2.3	0.0001	2	3	0.004
55ºC culture (% of live cells)	3.18%	8.82%	0.68%	0.12%	0.88%	0.91%	18.39%	0.43%	0.01%	20%	1.58%	8.2%

Porcelana geyser.

The distance between Porcelana hot spring and geyser is only 5.1 km (Fig. 1), meaning that the same geological description can be applied to both geothermal events. Nevertheless, a different chemical interaction within the rock must be occurring at the geyser, as calcium carbonate structures dominated the area. Temperature decreased from 66º to 41º C (Table 1). The pH at the source was 5, but slightly increased to downstream.

Porcelana geyser exhibited large active (spraying hot water) and inactive (neither water nor heat) calcium carbonate structures from 1.5 and 5 to 7 meters, respectively. The active structure had several siphons that were permanently spraying water out, and it was considered as the thermal water source. The mat sampled at the source of the geyser (F1) was taken in the upper 2 mm. F1 microbial mat was brilliant orange or green, being part of a calcium carbonate structure (Fig. 2C). The mat from sample F2 was dominated by white filaments, surrounded by a thick bright green mat, and intimately adhered to the rocky surface. Sample F3 presented a mix of dark green, bright blue-green, pale yellow, pink and brown mats, of approximately 6 cm thick. A complex sample was obtained, comprising all mats observed at that temperature (50-51 ºC). Mats from sample F4 presented several bright blue-green bumps, with an orange lower layer adhered to the calcium carbonate surface (Fig. 2C).

Intermixed populations of 2 μm diameter filaments and cocci can be seen in SEM images (Figs. 3E and F, respectively). Biovolume of bacilli was one of the lowest of all morphotypes found, together with 0.5 μm diameter filaments (together they accounted for less than 10% of the total biovolume). Cocci were dominant in points F1, F2 and F3 with over 80% relative biovolume, but were replaced by 2 μm diameter filaments in sample F4 (Fig. 4).

Relative abundance of total unicellular bacteria did not show any trend downstream (Table 2), but geyser exhibited the highest percentage of living cells, up to 45.5% in sample F4. Similarly, geyser presented the highest percentage of culturability of heterotrophic CFUs at 55º C, up to 20% in sample F2 (Fig. 5).

DGGE based MDS analyses showed that low temperature microbial mats F3 and F4 were more similar (over 55% Bray Curtis similarity index) than high temperature microbial mats. Points F1 and F2 showed a community similarity index of 55%, and both with F3 and F4, a similarity index of 40% between themselves (Fig. 6C).

Comparison among the three hydrothermal systems

Porcelana Hot Spring and geyser are in the same peninsula and only about 5 km from each other. On the other hand, Cahuelmó is located in a different area separated by the Comau Fjord (Figure 1). A priori we could expect more similar hot water sources for the two former systems than for the latter. Twenty-two years ago, Cahuelmó hot spring was described by Hauser (1989) as a slightly basic (pH 8.25) and hyperthermic (84º C at the source) hydrothermal system. In this study, physico-chemical properties of water in Cahuelmó were different: temperature did not reach 60º C, and pH was neutral or slightly acidic.

On the other hand, the same author described Porcelana as a neutral, thermal hot spring (60º C), but in this study, Porcelana hot spring showed a temperature of 53º C, and pH 6.

Hauser (1989) attributed differences among hot spring waters to temperature and pressure in the underground cortex caused by volcanic activity, which could have change through years because of volcanic eruptions and earthquakes, common in the region. These events could also explain why Waring (1965) reported a temperature of 55º C in Cahuelmó (more similar to this study) in his World Thermal Springs Summary, 46 years ago. If the methods used several decades ago can be trusted, these data would indicate that the conditions of these thermal springs are quite variable in time.

The physical appearance was similar between Cahuelmó and Porcelana, where water emerged forming relatively large pools and flowed slowly downstream. In geyser, on the

contrary, water vapor was expelled at pressure and condensed water quickly drained downstream over hard, calcium carbonate edifices (Figure 2C).

In effect, the macroscopic appearance of the microbial mats was also more similar in Cahuelmó and Porcelana than in geyser (Figure 2). In both Cahuelmó and Porcelana (Figure 2A, B) large white filaments dominated at the highest temperatures, orange-pink filaments were present at intermediate temperatures, and green filaments dominated the low temperature samples. Although we do not have molecular data to identify these organisms, they are probably related to similar filaments described from other thermal springs. Thus, the white filaments could belong to *Thermus*-like bacteria, as described by Kristjansson and Alfredsson (1983) in a thermal gradient from Yellowstone, and the orange-pink ones to similar filaments described from Yellowstone (Reysenbach et al. 1994, Huber et al. 1998). With respect to the green filaments, macroscopic and microscopic appearance and color indicate they were cyanobacteria.

The mats at geyser were apparently more heterogeneous spatially (Figure 2C) and they were more tightly bound to the hard substrate. Besides, temperature was 10 degrees higher than at the two hot springs. We do not have enough information to speculate on the identity of the orange and white mats in geyser, although the green mats were most likely formed by cyanobacteria. These differences between the hot spring on the one hand and geyser on the other were confirmed by the MDS of the molecular fingerprints (Figure 6D). Perhaps the microbial assemblage at geyser participates in the calcium carbonate precipitation, as has been shown in other places (Kandianis et al. 2008), and this would contribute to explain the differences in the microbial communities from those at the two hot springs, where this precipitation does not take place.

Considerable heterogeneity was observed microscopically in the morphology of the filaments forming the matrix of the mats (Figure 3). Filamentous bacteria changed with temperature within each mat and among the three different thermal systems. The unicellular components of mats are usually ignored in favor of the more apparent, mat-building filaments.

However, in the present work, unicellular prokaryotes formed a significant portion of the total biovolume in all mats (Figure 4). In one case, cocci actually accounted for most of the biovolume (Figure 3E, corresponding to sample F1 in Figure 4). Furthermore, the relative proportions of the different morphotypes varied substantially from one sampling point to another in all three systems. These differences could be observed even between samples where the macroscopic appearance was similar, such as the green mats or the white filaments in both hot springs. Table 3 summarizes results from different studies of hot springs that have considered the unicellular prokaryotes. The total concentration ranged three orders of magnitude among the three systems in the present study (from 10^9 to 10^{12} cells per g). The three values, however, were higher than the two systems from the literature (10^7 and 10^8 cells per g respectively). This stresses once more the large variability in bacterial abundance and composition despite the apparent similarity of the mats in hot springs.

The percent of live cells, according to the method used, was very constant: between 15 and 17%. As a comparison, Janssen et al. (2002) found 1.33 x 10^9 cells per g and 9% active cells with the same method. Thus, most of the biomass of the unicellular prokaryotes is inactive in these systems. When CFU at 55 ˚C were determined, the percentages were very different in each system. The percentage of the total count retrieved in these plates in geyser was only 0.01%. The two hot springs had 0.88 and 3.18% respectively, which show a fourfold difference only. For comparison, an Australian soil had 1.9% CFUs at 26 ˚C (Janssen et al. 2002). The extremely low percent at geyser suggest that the heterotrophic assemblage is very different from the others and the medium used was not appropriate to retrieve them. This is again consistent with the MDS analysis.

TABLE 3: Total, live and culturable cell counts in this and other studies.

Environment	Temperature (ºC)	Total cell count (cells per g of dry soil)	Live cell count (cells per g of dry soil)	% Cultivable (of total cells)	Reference
Cahuelmó hot spring *(mat C1, Patagonia)*	57.8	3.8×10^{12}	6.6×10^{11} (17.4%)	3,18	This study
Porcelana hot spring *(mat P1, Patagonia)*	52.4	3.8×10^{9}	5.7×10^{8} (15%)	0.88	This study
Geyser *(mat F1, Patagonia)*	68.3	6.2×10^{10}	9.4×10^{9} (15.2%)	0.01	This study
Steep Cone hot spring *(sinter, Yellowstone park)*	75.8	1.3×10^{7}	ND	ND	Inagaki et al. 2001
Macao hot spring *(mat, Yangshang park)*	84-94	2.6×10^{8}	ND	ND	Ng et al. 2005

Considering the noticeable differences in macroscopic and microscopic appearances of the mats along the temperature gradients, we were expecting that communities from similar temperature environments would be more similar among them than communities from different temperatures in the same hot spring. However, the opposite was true (Figure 6D). The samples from the highest and lowest temperature in each hydrothermal system grouped together to the exclusion of the other systems. Next, the two hot springs were closer to each other and geyser was the most distinct. One possible explanation is that the microorganisms responsible for the mat structure and appearance (that is, the main filaments) belong to only one single taxon in each mat and are, thus, reflected in the DGGE by just one band. On the other hand, perhaps the numerous cocci and bacilli belong to many different species that are more closely related within hot springs rather than across the three systems. If this were true, they would weigh heavily in the comparison of fingerprints by constituting many bands. Further molecular studies will be necessary to discern this question. At any rate, the three systems described are clearly of interest for further studies of microbial ecology of high temperature systems.

Acknowledgments

The authors are thankful for the sponsorship given by Fundación Huinay (Huinay Scientific Field Station) and their staff, who made possible the visits to the hot springs. We thank Boris Hernández for bringing Porcelana geyser to our attention and for guiding us to the place. This is the publication number 56 of Fundación Huinay. Special thanks to Rosa Soto, Gerardo Cárcamo, Victor Campos and Vannesa Balagué. This work was supported by the Biofilms and Environmental Microbiology Laboratory (Universidad de Concepción, Chile) and project TERMOCHILE (CGL2009-06449-E/BOS), Spanish Ministry of Science.

REFERENCES

Apha, 2005. Standard methods for the examination of water and wastewater, 20th ed. Washington, DC: American Public Health Association.

Barns S. M., Fundyga R. M., Jeffries M. W. and Pace N. R. 1994. Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. Proc Natl Acad Sci USA 91: 1609-13.

Belkova N. L., Tazaki K., Zakharova J. R., Parfenova V. V. 2007. Activity of bacteria in water of hot springs from Southern and Central Kamchatskaya geothermal provinces, Kamchatka Peninsula, Russia. Microbiol Res 162:99-107.

Blanc M., Marilley L., Beffa T. and Aragno M. 1999. Thermophilic bacterial communities in hot composts as revealed by most probable number counts and molecular (16S rDNA) methods. FEMS Microbiol Ecol 28: 141-149.

Boomer S. M., Noll K. L., Geesey G. G. and Dutton B. E. 2009. Formation of multilayered photosynthetic biofilms in an alkaline thermal spring in Yellowstone National Park, Wyoming. Appl Environ Microbiol 75: 2464-75.

Boon N., Windt W., Verstraete W., and Top E. M. 2002. Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. FEMS Microbiol Ecol 39: 101-12.

Brock T. D. 1967. Life at high temperatures. Science 158:1012-1019.

Brock T. D. 1978. Thermophilic microorganisms and life at high temperatures. Springer-Verlag, New York, Heidelberg, Berlin.

Da Costa M. S., Rainey F. A. and Nobre M. F. 2006. The genus *Thermus* and relatives, p 7:797-812. In Dworkin M., S. Falkow, E. Rosenberg, K. H. Schleifer, E. Stackebrandt (ed.), The Prokaryotes, Third Edition, Springer. Singapore.

De los Ríos A., Ascaso C., Wierzchos J., Ferna E. and Quesada A. 2004. Microstructural Characterization of Cyanobacterial Mats from the McMurdo Ice Shelf, Antarctica. Appl Environ Microbiol 70: 569-580.

Ferris M. J., Ruff-Roberts A. L., Kopczynski E. D., Bateson M. M. and Ward D. M. 1996a. Enrichment culture and microscopy conceal diverse thermophilic Synechococcus populations in a single hot spring microbial mat habitat. Appl Environ Microbiol 62: 1045-50.

Ferris M. J., Muyzer G. and Ward D. M. 1996b. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Appl Environ Microbiol 62: 340-6.

Ferris M. J. and Ward D. M. 1997. Seasonal distributions of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. Appl Environ Microbiol 63: 1375-81.

Fortey R., Pankhurst R. J. and Hervé F. 1992. Devonian Trilobites at Buill, Chile (42ºS). Rev Geol Chile, Vol. 19, No. 2, p. 133-144.

Foster J. S., Green S. J., Ahrendt S. R., Golubic S., Reid R. P., Hetherington K. L. and Bebout L. 2009. Molecular and morphological characterization of cyanobacterial diversity in the stromatolites of Highborne Cay, Bahamas. ISME J 3: 573-87.

Franks J. and Stolz J. F. 2009. Flat laminated microbial mat communities. Earth-Sci Rev 96: 163-172.

Fromin N., Hamelin J., Tarnawski S., Roesti D., Jourdain-Miserez K. et al. 2002. Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. Environ Microbiol 4: 634-643.

Glennon J. A. and Pfaff R. M. 2003. The Extraordinary Thermal Activity of El Tatio geyser Field, Antofagasta Region, Chile. GOSA Trans Vol VIII: 31-78.

Hauser A. 1989. Fuentes Termales y Minerales en torno a la carretera Austral, regiones X-XI, Chile. Rev Geol Chile 16(2): 229-239.

Huber R., Eder W., Heldwein S., Wanner G., Huber H., Rachel R. and Stetter K. O. 1998. *Thermocrinis ruber* gen. nov., sp. nov., a Pink-Filament-Forming Hyperthermophilic Bacterium Isolated from Yellowstone National Park. Appl Environ Microbiol 64: 3576-3583.

Inagaki F., Motomura Y., Doi K., Taguchi S., Izawa E., Lowe D., Ogata S. 2001. Silicified microbial community at steep cone hot spring, Yellowstone National Park. Microbes Environ. 16(2):125-130.

Janssen P. H., Yates P. S., Grinton B. E., Taylor P. M. and Sait M. 2002. Improved Culturability of Soil Bacteria and Isolation in Pure Culture of Novel Members of the Divisions Acidobacteria, Actinobacteria, Proteobacteria and Verrucomicrobia. Appl Environ Microbiol 68:2391-2396.

Jones B. and Renaut R. W. 1997. Formation of silica oncoids around geysers and hot springs at El Tatio, northern Chile. Sedimentol 44: 287-304.

Jørgensen B. B., Revsbech N. P. and Cohen Y. 1983. Photosintesis and structure of benthic microbial mats: Microelectrode and SEM studies of four cyanobacterial communities. Limnol Oceanogr 28(6): 1075-1093.

Kandianis M. T., Fouke B. W., Johnson R. W., Veysey J. and Inskeep W. P. 2008. Microbial biomass: A catalyst for CaCO3 precipitation in advection-dominated transport regimes. Geol Soc Am Bull 120: 442.

Konhauser K. O., Phoenix V. R., Bottrell S. H., Adams D. G. and Head I. M. 2001. Microbial-silica interactions in Icelandic hot spring sinter: possible analogues for some Precambrian siliceous stromatolites. Sedimentol 48: 415-433.

Kristjansson J. K. and Alfredsson G. A. 1983. Distribution of Thermus spp. in Icelandic hot springs and a thermal gradient. Appl Environ Microbiol 45:1785-1789.

Muyzer G., De Waal E. C. and Uitterlinden A. G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59: 695-700.

Muyzer G., Teske A., Wirsen C., Jannasch H. W. 1995. Phylogenetic relationships of Thiomicrospira species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch Microbiol 164:165-172.

Nannipieri P. J., Ascher M. T., Ceccherini L., Landi G., Pietramellara G., Renella G. 2003. Microbial diversity and soil functions. Eur J Soil Sci 54:655-670.

Ng C., Huang W., Chang C., Tzeng W., Chen T., Liu Y., Shyu Y. 2005. Tufa microbial diversity revealed by 16S rRNA cloning in Taroko National Park, Taiwan. Soil Biol Biochem 38:342-348.

Nocker A., Burr M. and Camper A. K. 2007. Genotypic microbial community profiling: a critical technical review. Microb ecol 54: 276-89.

Nold S. C., Kopczynski E. D. and Ward D. M. 1996. Cultivation of aerobic chemoorganotrophic proteobacteria and gram-positive bacteria from a hot spring microbial mat. Appl Environ Microbiol 62: 3917-21.

Olson F. C. W. 1950. Quantitative estimates of filamentous Algae. T Am Microsc Soc 69(3): 272-279.

Phoenix V. R., Bennett P. C., Engel A. S., Tyler S. W. and Ferris F. G. 2006. Chilean high-altitude hot-spring sinters: a model system for UV screening mechanisms by early Precambrian cyanobacteria. Geobiology 4:15-28.

Porter K. G. and Feig Y. S. 1980. The Use of DAPI for Identifying and Counting Aquatic Microflora. Limnol Oceanogr 25(5):943-948.

Portillo M. C., Sririn V., Kanoksilapatham W. and Gonzalez J. M. 2009. Differential microbial communities in hot spring mats from Western Thailand. Extremophiles 13: 321-31.

Ramsing N. B., Ferris M. J. and Ward D. M. 2000. Highly ordered vertical structure of Synechococcus populations within the one-millimeter-thick photic zone of a hot spring cyanobacterial mat. Appl Environ Microbiol 66: 1038-49.

Reysenbach A. L., Wickham G. S. and Pace N. R. 1994. Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus Spring, Yellowstone National Park. Appl Environ Microbiol 60: 2113-9.

Reysenbach A. L. and Cady S. L. 2001. Microbiology of ancient and modern hydrothermal systems. Trends Microbiol 9: 79-86.

Roeselers G., Norris T. B., Castenholz R. W., Rysgaard S., Glud R. N., Kühl M. and Muyzer G. 2007. Diversity of phototrophic bacteria in microbial mats from Arctic hot springs (Greenland). Environ Microbiol 9: 26-38.

Skirnisdottir S., Hreggvidsson G. O., Hjörleifsdottir S., Marteinsson V. T., Petursdottir S. K., Holst O. and Kristjansson J. K. 2000. Influence of sulfide and temperature on species composition and community structure of hot spring microbial mats. Appl Environ Microbiol 66: 2835-41.

Stern C. R. 2004. Active Andean volcanism: its geologic and tectonic setting. Rev Geol Chile, 31(2): 161-206.

Tassi F., Aguilera F., Darrah T., Vaselli O., Capaccioni B., Poreda R. J. and Delgado-Huertas A. 2010. Fluid geochemistry of hydrothermal systems in the Arica-Parinacota, Tarapacá and Antofagasta regions (northern Chile). J Volcanol Geotherm Res 192 (1-2):1-15

Vallaeys T., Topp E., Muyzer G., Macheret V., Laguerre G., Rigaud A. and Soulas G. 1997. Evaluation of denaturing gradient gel electrophoresis in the detection of 16s rDNA sequence variation in rhizobia and methanotrophs. FEMS Microbiol Ecol 24: 279-285.

Van Gemerden H. 1993. Microbial mats: A joint venture. Marine Geol 113: 3-25.

Ward D. M., Ferris M. J., Nold S. C. and Bateson M. M. 1998. A natural view of microbial biodiversity within hot spring cyanobacterial mat communities. Microbiol Mol Biol R 62: 1353-70.

Waring G. A. 1965. Thermal springs of the United States and other countries in the world - A summary. United States Government Printing Office, Washington, US.

CHAPTER II

Bacterial composition of microbial mats in hot springs in Northern Patagonia: Variations with seasons and temperature

Roy Mackenzie, Carlos Pedrós-Alió, Beatriz Díez

Published in *Extremophiles: life under extreme conditions*. Impact Factor (2013): +2.174

DOI: 10.1007/s00792-012-0499-z

ABSTRACT

Seasonal shifts in bacterial diversity of microbial mats were analyzed in three hot springs (39 ºC to 68 ºC) of Patagonia, using culture-independent methods. Three major bacterial groups were detected in all springs: Phyla Cyanobacteria and Bacteroidetes, and Order Thermales. Proteobacteria, Acidobacteria and Green Non-Sulfur Bacteria were also detected in small amounts and only in some samples. Thermophilic filamentous heterocyst-containing *Mastigocladus* were dominant Cyanobacteria in Porcelana Hot Spring and Geyser, and *Calothrix* in Cahuelmó, followed by the filamentous non-heterocyst *Leptolyngbya* and *Oscillatoria*. Bacteroidetes were detected in a wide temperature range and their relative abundance increased with decreasing temperature in almost all samples. Two *Meiothermus* populations with different temperature optima were found. Overall, fingerprinting analysis with universal bacterial primers showed high similarities within each hot spring despite differences in temperature. On the other hand, Cahuelmó Hot Spring showed a lower resemblance among samples. Porcelana Hot Spring and Porcelana Geyser were rather similar to each other, possibly due to a common geological substrate given their geographic proximity. This was even more evident with specific cyanobacterial primers. The different geological substrate and the seawater influence in Cahuelmó might have caused the differences in the microbial community structure with the other two hot springs.

INTRODUCTION

Hot springs with source water temperatures above 40 °C can be found all along the Southern Andes (Lahsen 1988). Most of the previously studied hot springs and geysers are located in the Atacama Desert and Altiplano (18º - 27º S, Northern Chile). Most of these studies focused on geological and geochemical features (Jones and Renaut 1997; Glennon and Pfaff 2003; Fernandez-Turiel *et al*. 2005; Tassi *et al*. 2010), on the resistance to ultraviolet radiation (Phoenix *et al*. 2006) or on development of an infrared sensing system to map temperature in the mats (Dunckel *et al*. 2009).

The Patagonian Andes (39 – 47º South latitude) are also volcanically active and, as a consequence, many hot springs are scattered throughout the fjords and forests. Many of these have remained unknown until recently and most have not been studied at all (Hauser 1989). The region is covered by Valdivian temperate rainforest, a unique ecosystem considered to be a hotspot for biodiversity of plants and vertebrates (Arroyo *et al*. 2004). Likewise, hot springs harbor a diversity of thermophilic microbial lineages, with potential interest for evolution, biogeography, microbial ecology studies (Whitaker *et al*. 2003; Papke *et al*. 2003; Miller *et al*. 2007), as well as for biotechnology (Brock 1997; Klatt *et al*. 2011). Moreover, a better knowledge of these systems will allow better sustainable management of the ecosystems (Smith-Ramírez 2004).

One aspect that has received little attention is the seasonal variations in the hot spring communities, maybe assuming that the supposedly stable physico-chemical condition of the source water would attenuate seasonal changes. The few studies carried out, however, did show significant seasonal changes (Ferris and Ward 1997; Lacap *et al*. 2007). Here, we compared the composition of the mat communities at two different seasons.

Finally, hot springs can be considered as biogeographic islands (Papke *et al*. 2003), and useful to explore the potential existence of biogeography in microorganisms (Martiny *et al*. 2006). Here, We analyzed three different hot springs at different distances from each other.

Different molecular methods have been used to analyze the composition of microbial mats in hot springs, for example, in Yellowstone (USA, Ward *et al*. 1998), Kamchatka (Russia, Perevalova *et al*. 2008), Seltun and Hveradalir (Iceland, Aguilera *et al*. 2010), Boekleung (Thailand, Portillo *et al*. 2009), and El Coquito (Colombia, Bohorquez *et al*. 2012). DGGE has been effective in allowing efficient comparison of different communities (Nocker *et al*. 2007) including some studies of hot springs (Perevalova *et al*. 2008, Portillo *et al*. 2009). Here we used DGGE with two different sets of primers: general for bacteria and specific for cyanobacteria and chloroplasts.

Cahuelmó and Porcelana Hot Springs have only received a few sporadic visits determining a few physico-chemical parameters (Hauser 1989, Waring 1965), but no microbiological studies have been carried out. The Porcelana Geyser, in turn, has not been described at all. The purpose of the present study was to obtain information on the bacterial community composition and its changes in space and time.

MATERIALS AND METHODS

Sampling sites

Microbial mat samples were obtained from hot springs Porcelana (42º 27’ 29.1’’S - 72º 27’ 39.3’’W), and Cahuelmó (42º 15’ 11.8’’S - 72º 22’ 4.4’’W) in June (austral winter) and December (austral summer) 2009. Porcelana Geyser (42º 24’ 51’’S / 72º 29’ 02.2’’ W) was sampled only in June 2009 due to the trail being impassable in December. These hot springs are located in the surroundings of Comau and Cahuelmó Fjords, North Chilean Patagonia. The linear distance between hot springs is 5.1 km between Porcelana Hot Spring and Porcelana Geyser, 25.4 km between Porcelana and Cahuelmó, and 20.6 km between Cahuelmó and Porcelana Geyser (Figure 1).

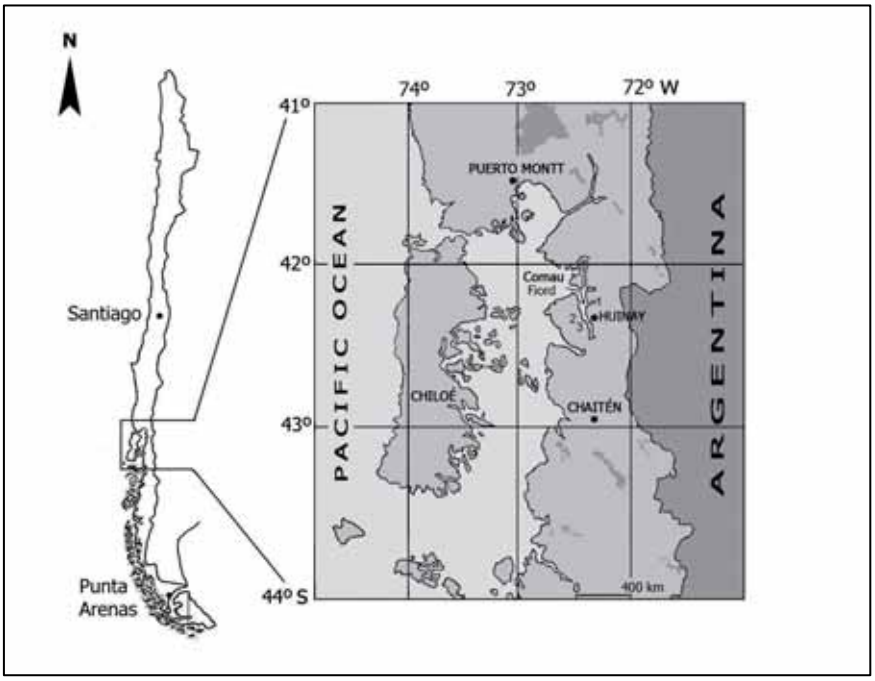


Figure 1: Map of Los Lagos District, Chile. Hot springs included in the present study are indicated by numbers 1 = Cahuelmó Hot Spring; 2 = Porcelana Geyser; 3 = Porcelana Hot Spring.

Cahuelmó Hot Spring is in the coast of Cahuelmó Fjord (at sea level), exposed to brackish water influence (salinity 3%) and wind. This system is located in a metamorphic rock complex, closer to the Andes mountain chain, so it is rich in metallic minerals and elements such as pyrite, polonium, magnetite, and chalcopyrite (Duhart *et al*. 2000).

Porcelana Hot Spring and Porcelana Geyser are located in Huequi Peninsula. They are in an area of shallow depth geothermic events whose outflow pours over volcanic rocks originated in the Quaternary Period, and are encircled by active Quaternary volcanoes such as the Huequi Volcano (Duhart *et al*. 2000). The volcanic rocks in this region are formed mainly by silicates and carbonates, and are rich in metallic minerals and elements such as pyrite, chalcopyrite, arsenopyrite, and Antimony (Fortey *et al*. 1992).

Four points were sampled per transect. The different points along the transects were defined by the differences in water temperature encountered along the gradient. Thus, some sampling points were slightly different in the June and December visits.

Environmental characteristics of hot springs and sample collection

Temperature and conductivity were determined using a conductivity meter (Oakton, model 35607-85); dissolved oxygen was measured using a dissolved oxygen probe (YSI, model 550I); and pH using pH-indicator paper (Merck, Germany). Salinity of Cahuelmó Fjord was measured using a refractometer (ATAGO S/Mill-E, Japan). Two-milliliter samples of microbial mats at each selected temperature point (in triplicate biological replicates) were collected in sterile plastic cryovials, and frozen in liquid nitrogen *in situ* for DNA analyses.

Nucleic acid extraction, PCR and DGGE

Samples were thawed and approximately 200 µl of microbial mat were added to a bead beating tube with different glass beads for rapid and thorough homogenization. Nucleic acids were extracted using a modified phenol : chloroform : IAA protocol, and purified using Amicon Ultra-15 (Millipore, MA, USA). Resulting total DNA was measured in a Nanodrop (Thermo Scientific, DE, USA). The 16S rRNA gene was amplified using universal bacterial primers (358Fgc – 907R) (Muyzer *et al*. 1995), as described by Schauer *et al*. (2003); cyanobacterial-specific primers (CYA106Fgc – CYA781R(a) and CYA781R(b)), as described by Díez *et al*. (2007). Each PCR reaction contained 1.25 µl of 10 mM primer (each), 0.5 µl of deoxynucleoside triphosphates (dNTP Mix 10 mM, Applied Biosystems), 2.5 µl of PCR Flexi colourless buffer 1X, 0.75 µl of $MgCl_2$ solution 25 mM, 1.25 µl of BSA 3 mg/ml, 0.125 U polymerase (HotStar Taq polymerase, Qiagen), and a 2-microliter aliquot of the DNA template

for each PCR reaction. All reactions were made up to 25 μL with DNAse/RNAse free H_2O (ultraPURE, Gibco).

DGGE was carried out using a DGGE-2000 system (CBS Scientific Company) as described by Muyzer *et al*. (1995). All DGGEs were carried out casting a 6% polyacrylamide gel, with gradients of DNA-denaturant agent of 50 – 75% for universal bacterial primers, and 45 – 75% for cyanobacterial specific primers. Approximately 800 ng of PCR product was loaded for each sample and the gel was run at 100 V for 16 h at 60 °C in 1 × TAE buffer (40 mM Tris [pH 7.4], 20 mM sodium acetate, 1 mM EDTA). Gels were stained with SybrGold (Molecular Probes) for 30 min, rinsed with MilliQ water, removed from the glass plate to a UV transparent gel scoop, and visualized with UV in a Fluor-S MultiImager (Bio-Rad) with the Multi-Analyst software (Bio-Rad) and recorded. Image analysis was performed using the Quantity One 1D Analysis software (Bio-Rad, CA, USA). DGGE gels were used for Bray Curtis similarity index calculation in each and between hot springs, using the Primer 6 Software (V 6.1.2).

The 43 most intense bands from both bacterial and cyanobacterial DGGE gels (25 and 18 bands, respectively) were excised, reamplified and sequenced. Closest relatives were determined with BLAST. The 16S rDNA gene sequences from this study have been deposited in the European Nucleotide Archive under accession numbers HE979738 to HE979757. Sequence analysis was carried out using the Chromas Software V 2.33 and the BioEdit Sequence Alignment Editor software V 7.0.5.3. Phylogenetic trees were constructed using the MEGA Software V 5.01. Diversity graphics were constructed using the OriginPro 8 SR0 V. 8.0724 (MA, USA), summing up the intensity of bands belonging to the same bacterial phylum, and calculating the proportion of each phylum in the total bacterial community.

RESULTS AND DISCUSSION

Physico-chemical characteristics

The three hydrothermal systems studied showed a continuous water discharge with temperature ranging from 45 to 68 ºC. In all cases temperature decreased and oxygen increased downstream, as expected (Table 1). Both temperature and oxygen values at the sources were very similar in winter and summer in Cahuelmó and Porcelana Geyser. In Porcelana hot spring there was a difference of seven degrees between the two seasons. This difference in temperature was due to the presence of several sources at this place that are not always active and, thus, the source sampled in June could not be sampled in December. Porcelana Geyser showed the highest temperature (68.3 ºC) followed by Cahuelmó (~ 58 ºC) and Porcelana (45.6 - 52.4 ºC). In fact, temperature at the source in Porcelana Geyser was 99 ºC, but it cooled down quickly before reaching the first microbial mats that was possible to sample. Temperature in Porcelana Hot Spring was higher during winter than in summer, almost 7ºC difference in the hottest point sampled. Dissolved oxygen was lower in Cahuelmó (11.2 – 13.3% saturation) than at the two other springs (around 40% saturation).

Table 1: Physicochemical characteristics of the three hot springs. Measurements of Porcelana and Cahuelmó were made in winter (w) and summer (s) in year 2009. Measurements of Porcelana Geyser were made only in June 2009. The available data from the literature are also shown.

Hot spring	Sample	Tº (ºC)	Dissolved oxygen (%)	Conductivity (µs/cm)	pH	Publication
Cahuelmó	C1w	57.8	13.3	2150	7	This study
	C2w	54.8	15.4	2120	6	
	C3w	48	35	2080	5	
	C4w	41.7	38.4	2090	5	
	C1s	58.7	11.2	2621	6	This study
	C5s	46.6	17.8	2652	6	
	C6s	45.1	33.5	-	6	
	C2s	-	64.4	2631	6	
	*	84	-	256	8.25	Hauser, 1989
	*	58	-	-	-	Waring, 1965
Porcelana	P1w	52.4	39.4	1086	6	This study
	P2w	48.6	51	972	6	
	P3w	43.7	55.4	952	6	
	P4w	39.5	61.2	905	6	
	P7s	45.6	41.9	1092	5	This study
	P6s	41.5	46.1	1071	6	
	P5s	40.7	47.7	1082	5	
	P2s	39.7	42.9	1113	6	
	*	60	-	-	7	Hauser, 1989
Porcelana Geyser	F1w	68.3	40.2	1224	5	This study
	F2w	55	26.5	1491	6	
	F3w	50.8	38.3	2030	6	
	F4w	40.8	44.3	2300	6	

* The authors reported no information on the exact point measured. It was assumed they measured the source of the springs

Hauser (1989) provided data such as temperature, conductivity or pH, for 20 different hot springs in the same area, with temperatures ranging from 18 to 84˚C, while Waring (1965) reported some data from 9 hot springs in the area, covering a temperature range between 22 and 60˚C. Table 1 summarizes the information from those studies, together with the data obtained in this study. In the case of Cahuelmó, Waring (1965) reported a temperature essentially identical

to that found in our study. However, in a study published two decades later, Hauser (1989) reported 84 ºC in Cahuelmó. The temperature difference in these two decades is approximately 26 ºC. Hauser (1989) also reported that the Porcelana Hot Spring was at 60 ºC, 8 ºC higher than reported in our study. As mentioned above, the Porcelana Geyser had not been studied before.

Conductivity and pH only showed minor differences between seasons and among sampling points. The pH was slightly acidic (around 5-6) or neutral. The pH reported by Hauser (1989) was also higher than the pH detected in this study in Cahuelmó and Porcelana (Table 1), and was measured by electrometric determination. On the contrary, conductivity was ten times higher in Cahuelmó in the present study than in Hauser (1989). Hauser reported no conductivity data for Porcelana. Conductivity was higher in Cahuelmó (above 2000 µS cm^{-1}) than in the other two springs (around 1000 µS cm^{-1}). Sulfate ranged between 0.43 and 7.89 mg L^{-1} in Cahuelmó, between 40.6 and 66.9 mg L^{-1} in Porcelana and between 46.1 and 61.7 mg L^{-1} in Porcelana Geyser. Clearly, the two Porcelana springs were around 10 times richer in sulfate than Cahuelmó.

These results indicate that these geothermal systems experience substantial environmental shifts, probably related to the variable volcanic activities in the area.

Bacterial assemblages

Comparison among bacterial communities at the three hot springs

A dendrogram comparing DGGE fingerprints from all samples showed differences among all three hot springs (<50% Bray-Curtis similarity). All samples from Porcelana Geyser formed a separate cluster, higher than 55% Bray Curtis similarity. Porcelana Hot Spring winter samples also formed a separate cluster (Bray-Curtis index larger than 55%). However, the Porcelana Hot Spring samples collected in summer were more different from each other, in particular points P2s and P5s, within this spring and season (Fig 2A). Cahuelmó samples

showed the largest differences with temperature and season, resulting in a complex pattern of clusters when analyzing its DGGE fingerprints. This is probably due to human influence, since Cahuelmó is the only hot spring of the three used by tourists. Even though the human presence is very low, and only in summer, tourists change the water courses to take hot baths in pools nearby, disrupting the stability and development of microbial communities in the mats.

Bacterial community composition at the three hot springs

All three hot springs showed a dominance of Phyla Cyanobacteria (23.5% on average), Bacteroidetes (21.5%), and the Order Thermales (19.6%) (Fig. 2B). Combined they accounted for 71% of total band intensity in Porcelana Geyser, 58% in Porcelana, and 65% in Cahuelmó. The remaining groups detected, Acidobacteria, Green Non-sulfur Bacteria, and Beta-, Gamma-, and Epsilon Proteobacteria, were present in small amounts and not in all samples. In general, the retrieved sequences had a higher phylogenetic resemblance with uncultured members of these phyla rather than with cultured species. As expected, most of the closest relatives in GenBank had originated in high temperature environments. In Porcelana Geyser, only 3.7% of the total band intensity could not be assigned to any group. The percentage of undetermined band intensity in Porcelana was 32.5% in winter and 22.3% in summer. In Cahuelmó percentages were 16.1% in winter and 8.7% in summer (Fig. 2B).

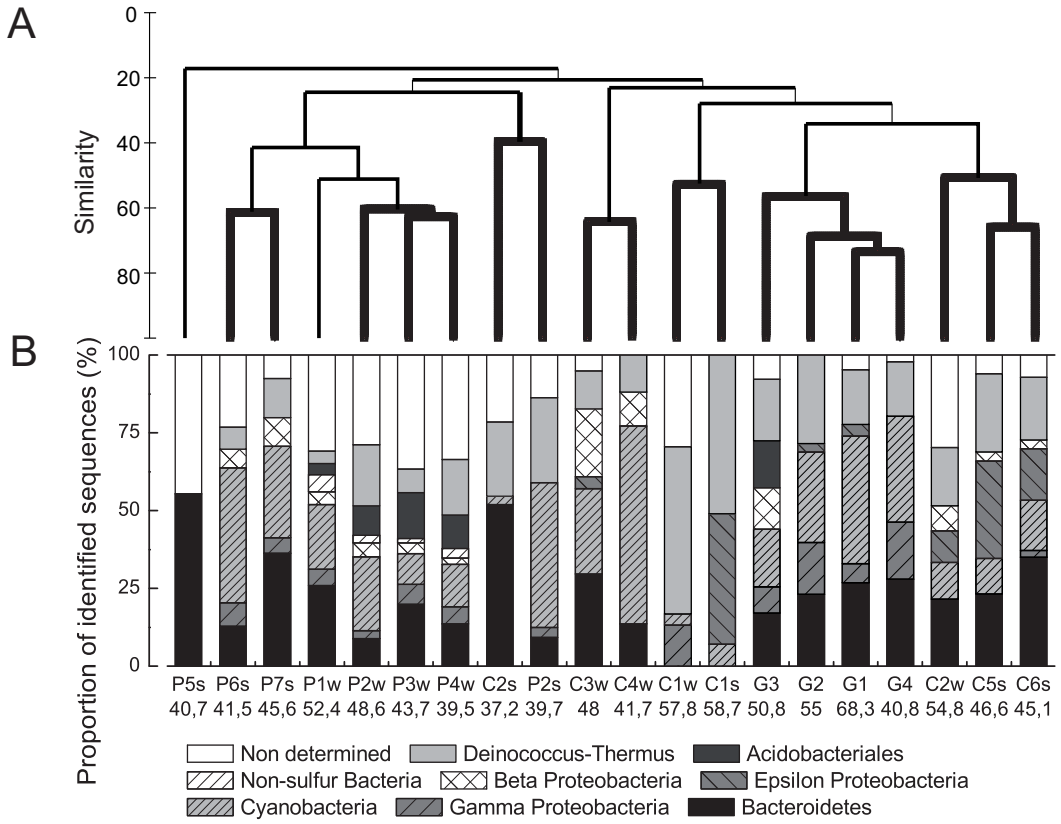


Figure 2: (A) Dendrogram comparing all samples according to the relative band intensities of the different bacterial groups. The vertical axis is shown as the Bray-Curtis similarity distances. (B) Relative composition of each bacterial assemblage based on 16S ribosomal RNA DGGE band intensities. The letters w and s in sample code indicate winter and summer respectively. Temperature (ºC) appears below sampled points.

Phylum Cyanobacteria

Cyanobacteria are usually the dominant phototrophic organisms in non-acidic hot environments (Ferris *et al*. 1996; Ward *et al*. 1998; Roeselers *et al*. 2007; Klatt *et al*. 2011; Miller *et al* 2007). In our study, the presence or dominance of Cyanobacteria was confirmed in all samples except for P5s at Porcelana Hot Spring. This sample had the largest percent of unaccounted band intensity (46%) and only Bacteroidetes sequences could be retrieved (see below). Perhaps the absence of Cyanobacteria sequences was related to this large percent of unidentified DGGE bands (Fig. 2B). On average, Cyanobacteria accounted for 29% of total

band intensity in Porcelana Geyser. In Cahuelmó their contribution decreased from 26.6% in winter to 9.3% in summer. On the contrary, in Porcelana, it increased from 16.9% in winter to 29.8% in summer, similar to Porcelana Geyser.

Our analysis of 16S rRNA genes with general bacterial primers detected several bands affiliated to Cyanobacteria, which were separated in two major groups: filamentous heterocystous (HCYA) and filamentous non-heterocystous cyanobacteria (NHCYA) (Fig. 3A). NHCYA were present in all samples, while HCYA were absent from six samples, five from Cahuelmó and one from Porcelana Hot Spring.

Cyanobacterial specific primers were used to analyze these important organisms in more detail. Unicellular cyanobacteria not retrieved with the general bacterial primers were found using the specific primers. These unicellular *Synechococcus*-type sequences only appeared in samples C1w and C5s from Cahuelmó Hot Spring (Fig. 3C).

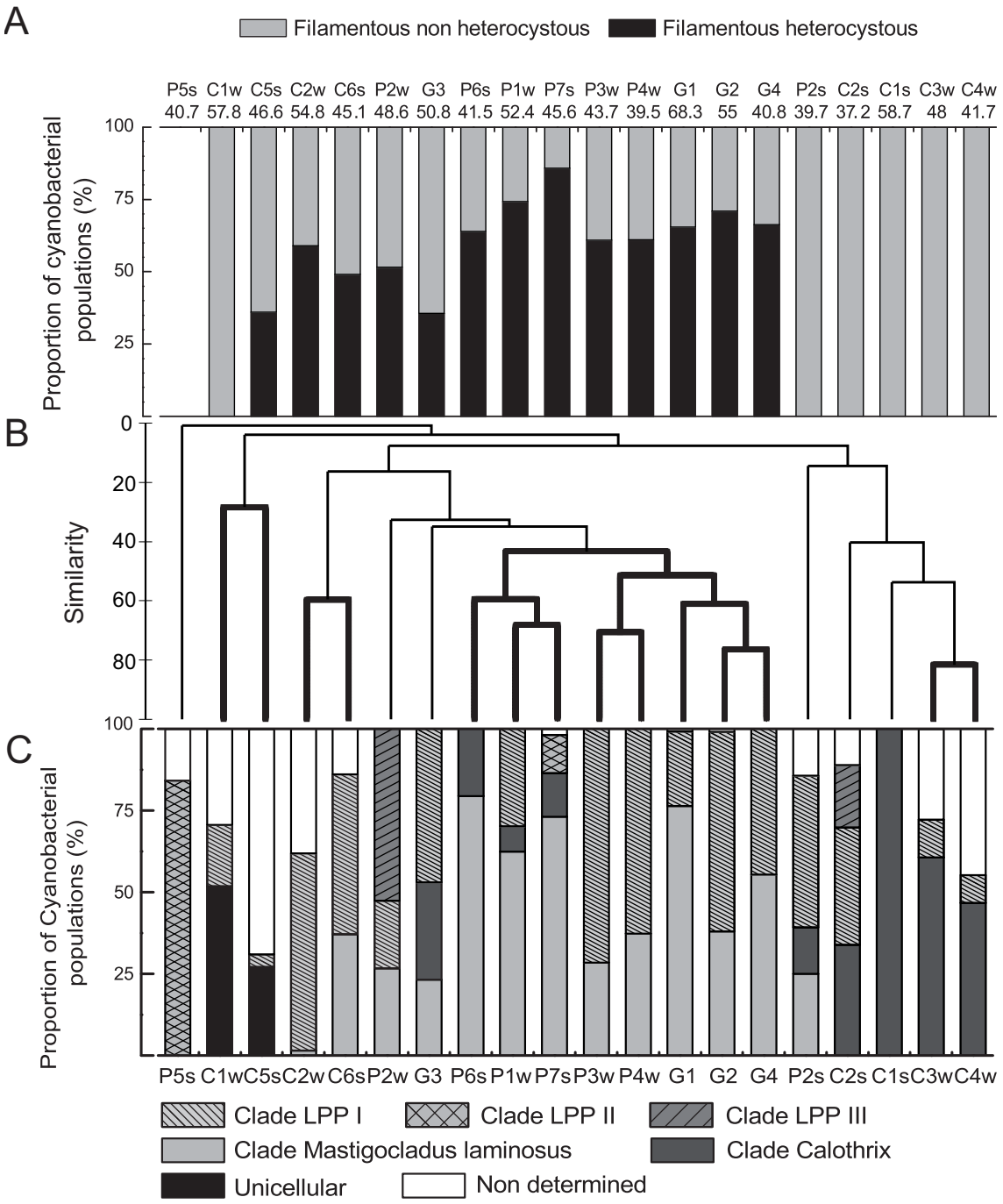


Figure 3: (A) Proportion of filamentous heterocystous and non heterocystous cyanobacteria in each sample according to their relative band intensity in 16S rRNA -DGGE fingerprints obtained with general bacterial primers. Temperature (ºC) appears below the sampled points. (B) Dendrogram comparing all samples according to the relative band intensities of the different cyanobacterial groups. The vertical axis is shown as the Bray-Curtis similarity distances. (C) Relative composition of each cyanobacterial assemblage based on DGGE band intensities with specific cyanobacterial primers. The letters w and s in sample code indicate winter and summer respectively.

In general, when using the specific primers, we were rather successful in identifying most of the important DGGE bands present at the Porcelana Geyser and Hot Spring fingerprints, with an average of only 0.4% and 4% of the total bands not identified, respectively. In Cahuelmó, however, a substantial percent (29% approx.) could not be identified, mainly due to sample C5s (Fig. 3C) that showed difficulties in amplification and further sequencing of the extracted bands.

As expected, most bands retrieved belonged to the filamentous cyanobacteria, either HCYA (four bands) or NHCYA (nine bands). The former fit into two different clusters: *Mastigocladus* and *Calothrix* (Fig. 4A). In turn, the NHCYA could be placed in three clusters that we have named LPP-I, LPP-II and LPP-III (Fig. 4B).

One of the most important bands in all hot springs, CYA22, together with other two bands (CYA27 and CYA46), were affiliated with the genus *Mastigocladus*, a typical thermophylic, branching, heterocyst-containing cyanobacterium of the order Stigonematales. Band CYA22 accounted for the highest band intensity, particularly in Porcelana Geyser and Hot Spring in winter. It was present in low abundance in Cahuelmó, and showed intermediate values in Porcelana Hot Spring in summer. Band CYA15, was closely affiliated with *Calothrix* spp., another HCYA (Fig. 4A).

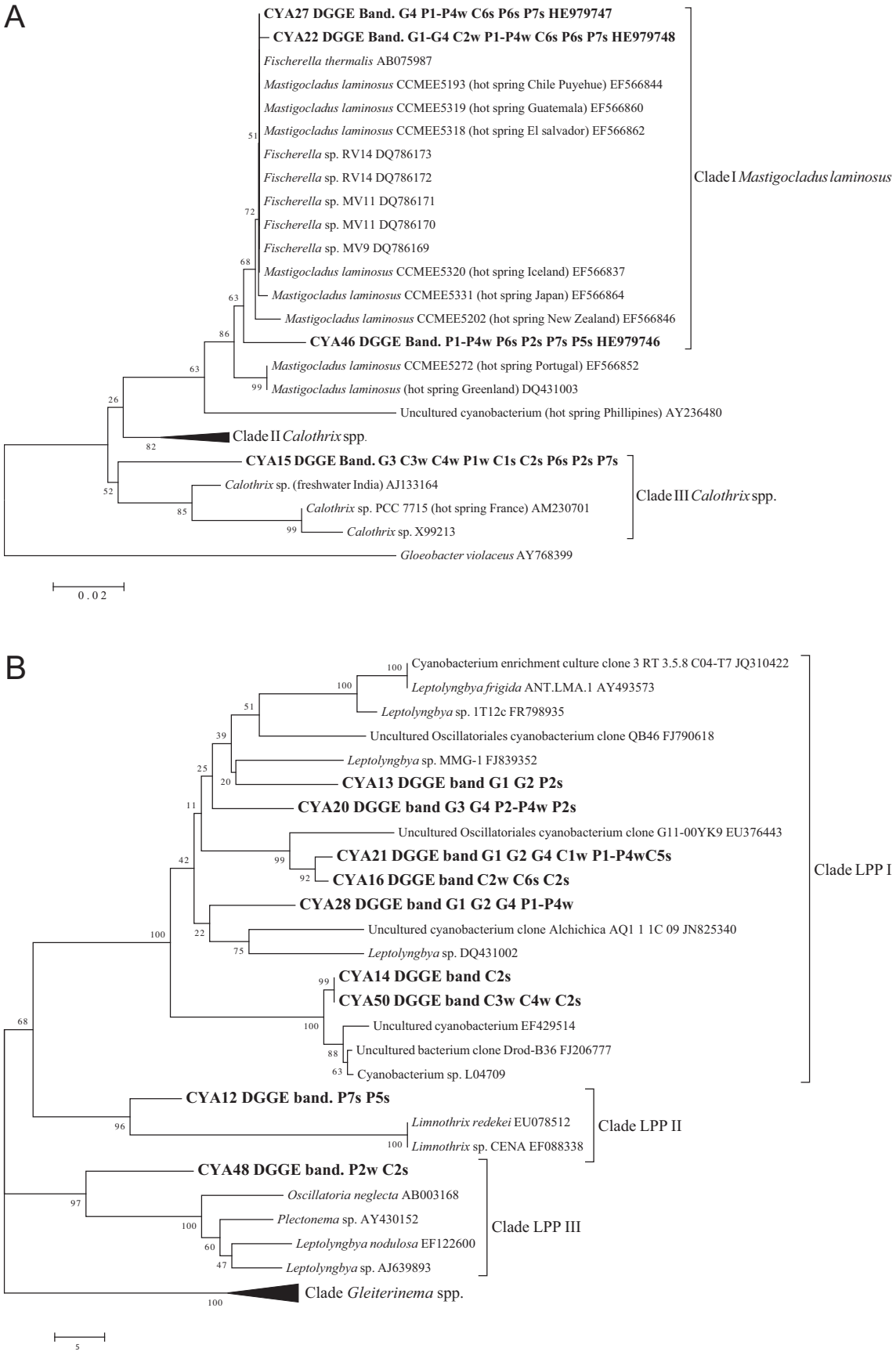


Figure 4: Phylogenetic reconstruction including the partial 16S rRNA gene sequences obtained by DGGE bands of (A) Cyanobacterial populations with heterocysts; (B) Cyanobacterial populations without heterocysts, denominated *Leptolyngbya*, *Phormidium* and *Plectonema*

(LPP) group. The 16S rRNA gene sequences used to generate the reconstructions were obtained with specific cyanobacterial primers. Samples where every band was present are indicated after the name of the band.

Nine bands were related to cyanobacteria of the filamentous non-heterocystous Order Oscillatoriales. Many of these were members of the thin filamentous genera *Leptolyngbya* and *Oscillatoria*, which lack distinctive morphology and have a poorly resolved taxonomy (Litvaitis 2002). Bands CYA21, CYA20 and CYA28 were widespread among all three hot springs and accounted for large percentages of the total band intensity (over 40%) in Porcelana Hot Spring in winter and Porcelana Geyser. However, none of them were present in all samples. The other bands corresponded to ill-defined environmental clones from diverse hot spring mats all over the world, and always occurred in low percentages (Fig. 3C and 4B).

At the highest temperature of 68.3 ºC at Porcelana Geyser mats, the *Mastigocladus*-like band accounted for 20.6% of the total band intensity, and two Oscillatoriales-related bands accounted together for 13%. The temperature at Porcelana Geyser is above the currently accepted upper limit for filamentous cyanobacteria, which is around 55-62 ºC (Seckbach, 2007). *Mastigocladus* is a genus commonly found in hot environments such as Yellowstone, USA (Miller *et al*. 2007, 2009), Costa Rica (Finsinger *et al.* 2008), Italy, Iceland, New Zealand, Russia and Chile (Miller *et al.* 2007), among others. As described by Miller *et al.* (2007), the presence of *Mastigocladus* in such distant places, and the high resemblance of sequences could be related to a recent dispersal event, and with time, new strains or even species might arise in each of these biogeographical islands.

A cluster analysis with all DGGE bands retrieved (including the non taxonomically identified ones) separated Cahuelmó samples from those of Porcelana and Porcelana Geyser with only a few exceptions (Fig. 3B). It appears, therefore, that the cyanobacterial assemblages do not follow the same geographical distribution as the total bacterial assemblages (Fig 2A). In this case, geographical proximity might be the reason for the closer clustering of Porcelana

Geyser and Porcelana Hot Spring. When the dendrogram is compared to the percent composition of each sample in Fig. 3C, it seems that the significant presence of members of the *Mastigocladus* clade determines the clustering together of most Porcelana Hot Spring and all Porcelana Geyser samples. This clade was absent from most Cahuelmó samples. In fact, when this group was removed from the analysis, the similarity index between all samples decreased almost 20% between Porcelana Hot Spring and geyser, and 6% between both springs and Cahuelmó. This was already the case with our general bacterial primers analysis. The cluster formed by four Cahuelmó samples and sample P2s from Porcelana Hot Spring seemed to be determined by the massive presence of members of other cyanobacteria, the *Calothrix* clade. Therefore, among the HCYA, the *Mastigocladus* clade predominated in Porcelana Geyser and Hot Spring, while the *Calothrix* clade did so in Cahuelmó, although not in all samples. Thus, different heterocystous cyanobacteria were dominant in different springs. A possible explanation for this finding could be the origin of both genera. While *Calothrix* has both freshwater and marine species and is not reported as a thermophylic genus, *Mastigocladus* is a typical thermophylic genus that has only been reported in freshwater hot springs. As mentioned, Cahuelmó Hot Spring is in close proximity to the fjord waters, and the mats are partially submerged in marine or brackish waters during high tides.

Independently of the primer sets used, *Mastigocladus*-related sequences were detected in 13 samples and were absent in five samples (all from Cahuelmó, three in winter and two in summer). The only discrepancies between both sets of primers were found in samples P2s and C5s. In sample C2w, the relative representation of this cluster was close to 50% with general bacterial primers but only a very small percent with the cyanobacterial primers. Both samples C5s and C2w had a large proportion of the band intensity unidentified with the cyanobacterial primers, and this could be the reason for the discrepancies. We do not have an explanation for the case of sample P2s.

Order Thermales

The Order Thermales is commonly found in hot environments (Brock, 1997). A *Thermus* species was detected for the first time in a hot spring in Yellowstone, USA by Brock and Freeze (1969). The heterotrophic nature of this group places them as consumers of the organic matter produced by Cyanobacteria and other phototrophic, as well as lithotrophic, primary producers in these extreme ecosystems.

This order was well represented in most of our samples, but its contribution varied significantly among sites. Its average contribution to the total band intensity was 20.6 %, ranging between 4 and 53.7% with one exception: no Thermales sequences could be retrieved from sample P5s. This sample was already mentioned in the section on Cyanobacteria as it had a large percent of undetermined bands (Fig. 2B). The presence of Thermales decreased from 24.1 and 30.1% in Cahuelmó (in winter and summer, respectively) to 19.8% in Porcelana Geyser, and down to 12% in Porcelana in both winter and summer.

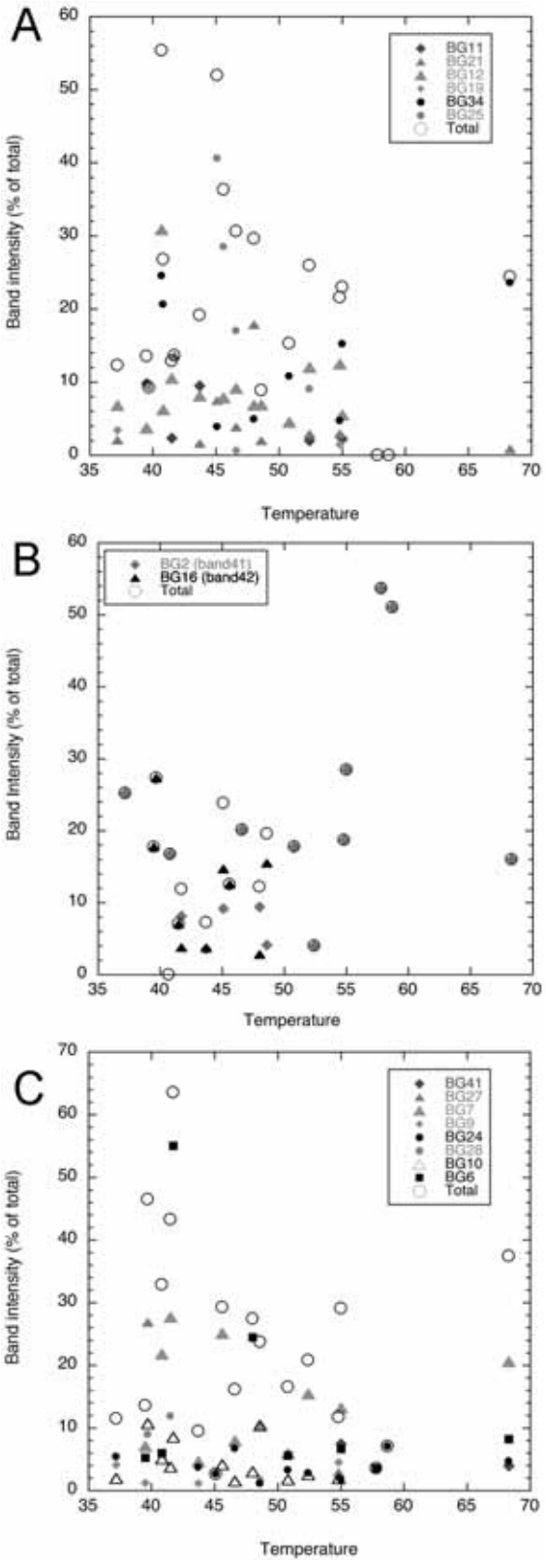


Figure 5: (A) Relative abundance of the cyanobacterial affiliated bands with respect to temperature. (B) Relative abundance of the order Thermales affiliated bands with respect to temperature. (C) Relative abundance of the Bacteroidetes affiliated bands with respect to temperature.

Considering each sample individually, the percent contribution of Thermales ranged between 4 and 26% throughout the temperature gradient (except the above mentioned sample P5s). Two points with temperatures between 57 and 59 ºC showed the highest contributions around 52% (Fig. 5B). If this can be taken as an indication of the temperature optima of the Thermales present in these springs, it would coincide with the optimum found for members of the genus *Meiothermus* (50 to 65 ºC in different species, Nobre *et al*. 1996). The genus *Meiothermus* was formerly included in the genus *Thermus* (Nobre *et al*. 1996), but the former has lower temperature optima, and the two genera use different niches (Chen *et al*. 2002).

The lower percentage of order Thermales found at the highest temperature (16% at 68.3 ºC) suggests that no representatives of the genus *Thermus* were present (with optimal temperatures around 70-75 ºC, Nobre *et al*. 1996). In fact, the two DGGE bands that could be assigned to the Thermales (bands BG2 and BG16) clustered with the genus *Meiothermus* (Fig. 6A). Their closest relatives were *Meiothermus ruber* and an environmental sequence recovered from a hot spring in Thailand (Portillo *et al*. 2009). Band BG16 was absent from samples with temperatures above 50 ºC (Fig 5B). This band was absent from Porcelana Geyser, increased with decreasing temperature in Cahuelmó (up to 30% in winter, and to almost 60% in summer), and was more represented in Porcelana at temperatures around 40º C, but also at 45º C in sample P7s (Fig. 6B). Band BG2, on the other hand, thrived throughout the temperature gradient, but reached its highest relative abundance above 55 ºC. This was absent from Porcelana in summer and was less important than BG16 in winter, and its importance decreased with decreasing temperature in Cahuelmó. Thus, it appears that some other environmental parameter besides temperature was influencing the distribution of these two possible ecotypes.

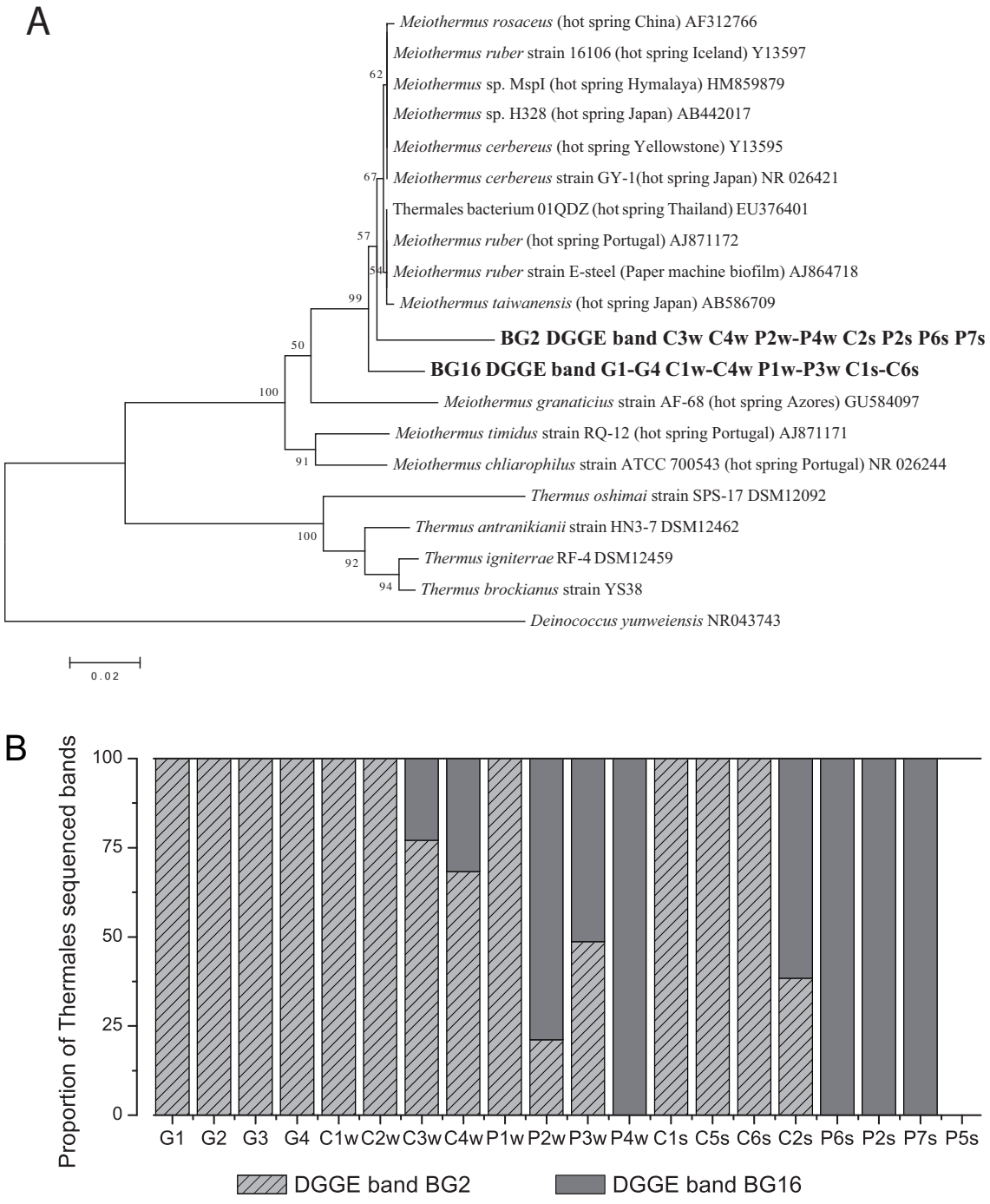


Figure 6: (A) Thermales phylogenetic reconstruction including sequences retrieved from partial 16S rRNA sequences obtained from DGGE bands using universal bacterial primers. Each sequence includes the sites where they were found. (B) Percent of relative contributions in each sample of the two Thermales bands identified in the DGGE analysis using the universal bacterial primers.

Phylum Bacteroidetes

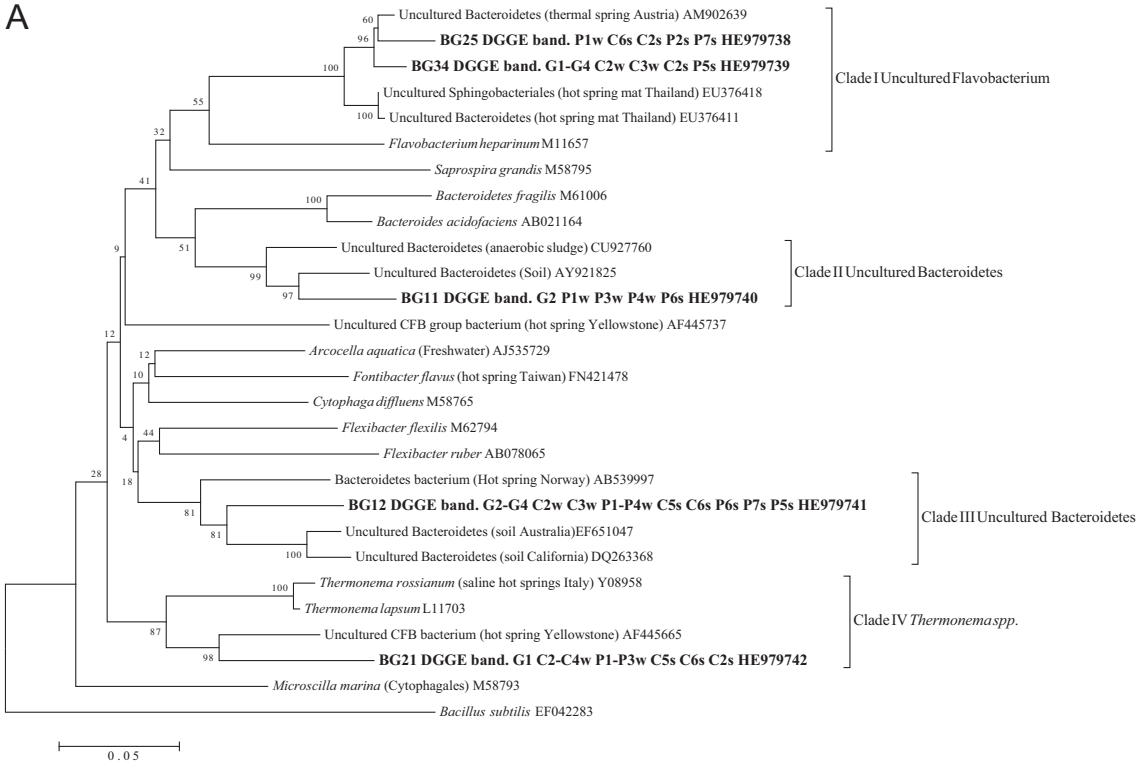
The class Bacteroidetes is a heterotrophic bacterial group not associated specifically with hot springs. Nevertheless, in the last decade, some studies have reported Bacteroidetes as an important group in hot springs from Thailand (Kanokratana *et al*. 2004; Portillo *et al*. 2009). The high diversity of species in this group also includes a wide variety of heterotrophic metabolisms, representing potentially important consumers of the organic matter produced by Cyanobacteria and other autotrophic organisms such as the green non-sulfur bacteria from the genus *Chloroflexus,* also detected in the study.

Class Bacteroidetes was a very well represented group in most of our samples, accounting for 22.4% of the total band intensity in Porcelana Geyser, 15.8 to 22.7% in Cahuelmó (winter and summer, respectively), and 16.9 to 28.5% in Porcelana (winter and summer, respectively). No relationship with temperature was apparent for any of the clades retrieved (Fig. 5C). This group of bacteria was absent only from the two hottest points, around 58 ºC, in Cahuelmó (Fig. 2B). Since Bacteroidetes were present at higher temperatures in Porcelana Geyser, clearly high temperature was not the reason for this absence in Cahuelmó. In the other Cahuelmó samples the contribution of Bacteroidetes ranged between 9 and 55%. In both Porcelana and Cahuelmó, their contribution seemed to increase with decreasing temperature in summer, but not in winter. It is worth noting that the temperature range was very similar in both seasons (Table 1).

Five discrete bands provided good sequences related to Bacteroidetes and were incorporated into the phylogenetic reconstruction of this class (Fig. 7A). They appeared in four different clusters, named I to IV for the present discussion. The relative band intensity contributions to the total Bacteroidetes can be seen in Fig. 7B. Clade I was the most abundant in Porcelana Geyser and in the summer samples from both Cahuelmó and Porcelana Hot Spring (Fig. 7A). The two bands in this cluster were related to sequences from hot springs in Thailand as their closest relatives (Portillo *et al*. 2009). Clade II was related to Bacteroidetes sequences

from soil, and was detected in low temperature samples from Porcelana Hot Spring, and in one sample from Porcelana Geyser. Clade III, on the other hand, was significant in almost all samples. It was related to the Bacteroidetes bacterium sequence AB539997, with over 95% of similarity. Clade IV was represented by only one band associated with the genus *Thermonema* and its closest relative was a sequence from a saline hot spring in Naples, Italy (Tenreiro *et al*. 1997) (Fig. 7A). This population was well represented in Cahuelmó, especially in winter, and it was present also in Porcelana (winter only). The *Thermonema* isolate sequence reported by Tenreiro *et al*. (1997) was slightly halophilic, which could explain its presence in Cahuelmó, as it is the only hot spring studied subjected to seawater influence.

A



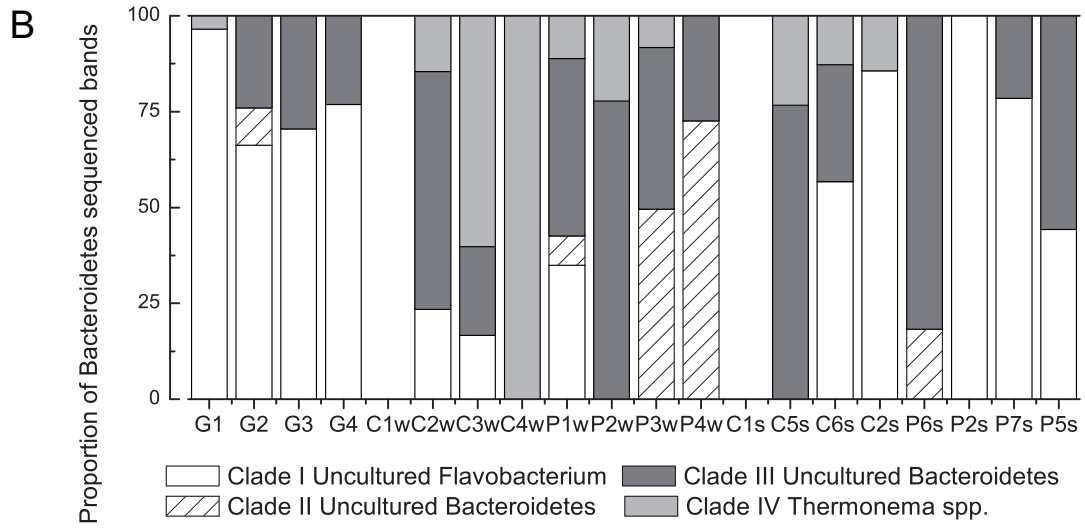


Figure 7: (A) Bacteroidetes phylogenetic reconstruction including partial 16S rRNA sequences retrieved from DGGE bands using universal bacterial primers. Each sequence includes the sites where they were found. (B) Percent of relative contributions of the four clades of Bacteroidetes retrieved in the different samples.

Other bacterial groups

Acidobacteria, Green Non-sulfur Bacteria, and Beta-, Gamma-, and Epsilon Proteobacteria, were detected in small relative abundances and not in all the samples investigated in this study. A high diversity of Proteobacteria and Acidobacteria members have been also reported in hot environments from volcanic areas in the Canary Islands (Portillo and González 2008), and in western Thailand (Portillo *et al*. 2009). Phototrophic green non-sulfur bacteria *Chloroflexi* were also well represented in tropical hot spring from the island of Luzon, in the Philippines (Lacap *et al*. 2007). The metabolisms found among these bacterial groups in hot springs are diverse, from autotrophic photosynthetic *Chloroflexi* (Lacap *et al*. 2007) and chemolithotrophic epsilon-proteobacteria (Takai *et al*. 2005), to anaerobic heterotrophs, which have been shown to be abundant in hot springs well above the temperature limit for photosynthesis (Kristjansson *et al*. 1985). The three hot springs in this study have different surrounding environmental conditions, and the diversity of organic substrates must be causing

differences in the development of the heterotrophic fraction of microbial communities, as shown in our fingerprinting analyses (Figs. 2B and 8).

Altogether, the overall similarity found in bacterial communities from Porcelana Hot Spring and Porcelana Geyser microbial mats, and the differences of both with Cahuelmó (Fig. 8) suggest the influence of geographical distance as well as differences in the geological substrate in these hot spring environments. Additionally, the influence of seawater in Cahuelmó hot sprint, although with low salt concentration (3%), might also affect bacterial diversity, for example, in the case of the slightly halophilic *Thermonema* spp.

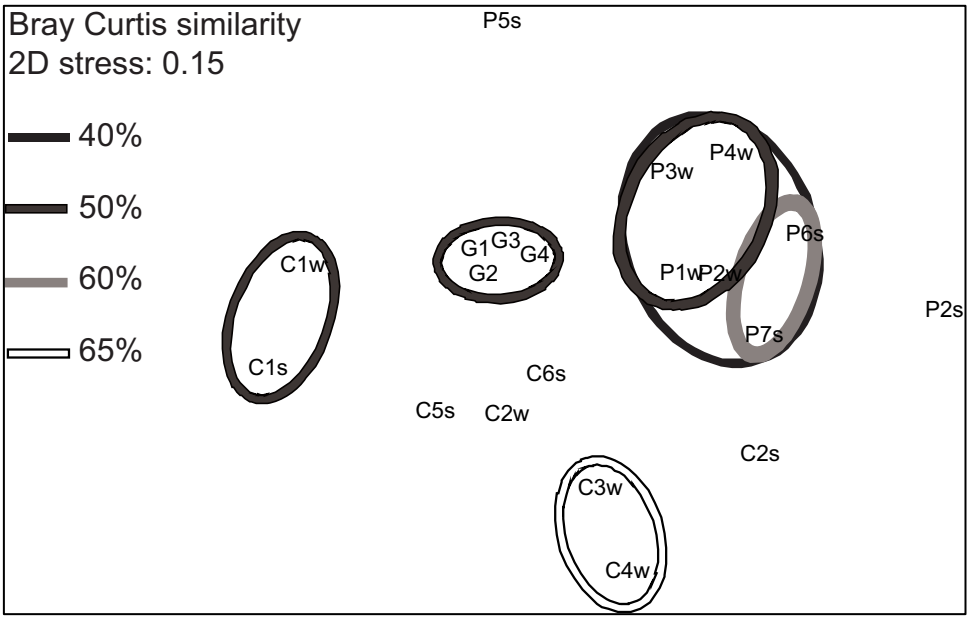


Figure 8: Multidimensional scale analysis of all samples by Bray Curtis similarity distances. Similarity distances between samples are shown in circles.

Small seasonal variations in bacterial communities were detected in Porcelana Hot Spring (Fig. 8). Porcelana Hot Spring samples were clustered with over 40% similarity in both seasons except for samples P5s and P2s, both from summer, although this hot spring showed a variation of 7 ºC in the hottest points (P1w and P7s) between winter and summer. On the other

hand, Cahuelmó Hot Spring did not show a temperature variation between winter and summer. No clear similarities were observed among samples in this hot spring, except for samples C1s and C1w, with the same temperature, which showed over 50% similarity. As mentioned before, Cahuelmó Hot Spring is the only hot spring of the three that experiences to some extent human intervention, except for points C1s and C1w (source of the spring) where the temperature is so high that remains free of attention by tourists. Our results suggest that the temperature at these hot springs is differently affected by seasons. According to the results retrieved, the temperature would be the main –but maybe not the only- environmental factor influencing the bacterial community structure and composition in the microbial mats present in these ecosystems.

Also, the fingerprint similarity between Porcelana Hot Spring and Geyser, and the dissimilarity of both with Cahuelmó Hot Spring may be due to geographical distance and rock-substrate differences.

As the economical value of these hot springs is being assessed in terms of ability to attract tourists, it is now very important to document the biodiversity of these biologically unknown environments and to recognize other potential values such as their genetic resources for the developing field of biotechnology. Thus, a more sustainable management can be achieved in the coming years for these hot springs in Northern Patagonia.

Acknowledgments

The authors are thankful for the sponsorship given by Fundación Huinay (Huinay Scientific Field Station) and their staff, who made possible the visits to the hot springs. We thank Boris Hernández for guiding us to Porcelana Geyser. This is the publication number 63 of Fundación Huinay. Special thanks to Rosa Soto and Vannesa Balagué. This work was supported by the project TERMOCHILE (CGL2009-06449-E/BOS), Spanish Ministry of Science and Innovation, and project FONDECYT nº 1110696 funded by CONICYT, Chile.

REFERENCES

Aguilera Á., Souza-Egipsy V., González-Toril E., Rendueles O., Amils R. 2010. Eukaryotic microbial diversity of phototrophic microbial mats in two Icelandic geothermal hot springs. Int Microbiol 13:21–32

Arroyo M. T. K., Marquet P. A., Marticorena C., Simonetti J. A., Cavieres L., Squeo F., Rozzi R. 2004. Chilean winter rainfall-Valdivian forests. In: Mittermeier RA, Gil PR, Hoffmann M, Pilgrim J, Brooks T, Mittermeier CG, Lamoreux J da Fonseca GAB (eds) Hotspots Revisited: Earth's Biologically Wealthiest and most Threatened Ecosystems. CEMEX, México DF, pp 99-103

Bohorquez L. C., Delgado-Serrano L., López G., Osorio-Forero C., Klepac-Ceraj V., Kolter R., Junca H., Baena S., Zambrano M. M. 2012. In-depth characterization via complementing culture-independent approaches of the microbial community in an acidic hot spring of the Colombian Andes. Microbial Ecol 63:103–15

Brock T. D., Freeze H. 1969. *Thermus aquaticus gen. n. and sp. n.*, a non-sporulating extreme thermophile. J Bacteriol 98:289-297

Brock T. D. 1997. The value of basic research: Discovery of *Thermus aquaticus* and other extreme thermophiles. Genetics 146:1207-1210

Chen M., Lin G., Lin Y., Tsay S. 2002. *Meiothermus taiwanensis* sp. nov., a novel filamentous, thermophilic species isolated in Taiwan. Int J Syst Evol Microbiol 52:1647-1654.

Díez B., Bauer K., Bergman B. 2007. Epilithic cyanobacterial communities of a marine tropical beach rock (Heron Island, Great Barrier Reef): diversity and diazotrophy. Appl Environ Microbiol 73(11):3656–3668.

Duhart P., Crignola P., Ordoñez A., Muñoz J. 2000. Franjas metalogénicas en Chiloé Continental (41º - 44ºS), pp 1–5. In: IX Congreso Geológico Chileno.

Dunckel A. E., Cardenas M. B., Sawyer A. H., Bennett P. C. 2009. High-resolution *in-situ* thermal imaging of microbial mats at El Tatio Geyser, Chile shows coupling between community color and temperature. Geophys Res Lett 36:1-5

Fernandez-Turiel J. L., Garcia-Valles M., Gimeno-Torrente D., Saavedra-Alonso J., Martinez-Manent S. 2005. The hot spring and geyser sinters of El Tatio, Northern Chile. Sediment Geol 180:125-147

Ferris M. J., Muyzer G., Ward D. M. 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Appl Environ Microbiol 62:340-6

Ferris M. J., Ward D. M. 1997. Seasonal distributions of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. Appl Environ Microbiol 63:1375–1381

Ferris M. J., Nold S. C., Revsbech N. P., Ward D. M. 1997. Population structure and physiological changes within a hot spring microbial mat community following disturbance. Appl Environ Microbiol 63:1367–1374

Ferris M. J., Palenik B. 1998. Niche adaptation in ocean cyanobacteria. Nature 396:226-228

Finsinger K., Scholz I., Serrano A., Morales S., Uribe-Lorio L., Mora M., Sittenfeld A., Weckesser J., Hess W. R. 2008. Characterization of true-branching cyanobacteria from geothermal sites and hot springs of Costa Rica. Environ Microbiol 10:460-473

Fortey R., Pankhurts R., Hervé F. 1992. Devonian trilobites at buill, Chile (42ºS). Rev Geol Chile 19:133–139

Glennon J. A., Pfaff R. M. 2003. The extraordinary thermal activity of El Tatio Geyser Field, Antofagasta region, Chile. GOSA Transactions 8:31–77.

Hauser A. 1989. Fuentes termales y minerales en torno a la carretera Austral, Regiones X-XI, Chile. Rev Geol Chile 16(2):229-239

Jones B., Renaut R. W. 1997. Formation of silica oncoids around geysers and hot springs at El Tatio, Northern Chile. Sedimentology 44:287–304

Kanokratana P., Chanapan S., Pootanakit K., Eurwilaichitr L. 2004. Diversity and abundance of Bacteria and Archaea in the Bor Khlueng Hot Spring in Thailand. J Basic Microbiol 44(6):430-444

Klatt C. G., Wood J. M., Rusch D. B., Bateson M. M., Hamamura N., Heidelberg J. F., Grossman A. R., Bhaya D., Cohan F. M., Kühl M., Bryant D. A., Ward D. M. 2011. Community ecology of hot spring cyanobacterial mats: predominant populations and their functional potential. ISME J 5:1262-1278

Kristjansson J. K., Ingason A., Alfredsson G. A. 1985. Isolation of thermophilic obligately autotrophic hydrogen-oxidizing bacteria, similar to *Hydrogenobacter thermophilus*, from Icelandic hot springs. Arch Microbiol 140:321–325

Lacap D. C., Barraquio W., Pointing S. B. 2007. Thermophilic microbial mats in a tropical geothermal location display pronounced seasonal changes but appear resilient to stochastic disturbance. Environ Microbiol 9:3065–3076

Lahsen A. 1988. Chilean geothermal resources and their possible utilization. Geothermics 17:401–410

Lahsen A., Muñoz N., Parada M. A. 2010. Geothermal development in Chile. Proceedings World Geothermal Congress 2010 (pp. 1-7)

Litvaitis M. K. 2002. A molecular test of cyanobacterial phylogeny: inferences from constraint analyses. Hydrobiologia 468:135-145

Martiny J. B. H., Bohannan B. J. M., Brown J. H., Colwell R. K., Fuhrman J. A., Green J. L., Horner-Devine M. C., *et al*. 2006. Microbial biogeography: putting microorganisms on the map. Nature 4:102-112

Miller S. R., Castenholz R. W., Pedersen D. 2007. Phylogeography of the thermophilic cyanobacterium *Mastigocladus laminosus*. Appl Environ Microbiol 73:4751-4759

Miller S. R., Williams C., Strong A. L., Carvey D. 2009. Ecological specialization in a spatially structured population of the thermophilic cyanobacterium *Mastigocladus laminosus*. Appl Environ Microbiol 75:729-34

Muyzer G., Teske A., Wirsen C. O., Jannasch H. W. 1995. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch Microbiol 164:165-72

Muyzer G., de Waal E. C., Uitterlinden A. G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695-700

Nobre M. F., Trüper H. G., Da Costa M. S. 1996. Transfer of *Thermus ruber* (Loginova *et al*. 1984), *Thermus silvanus* (Tenreiro *et al*. 1995), and *Thermus chiliarophilus* (Tenreiro *et al*. 1995) to *Meiothermus* gen. nov. as *Meiothermus ruber* comb. nov., *Meiothermus silvanus*

comb. nov., and *Meiothermus chilarophilus* comb. nov., respectively, and emendation of the genus *Thermus*. Int J Syst Bacteriol 56:604-606

Nocker A., Burr M., Camper A. K. 2007. Genotypic microbial community profiling: a critical technical review. Microb Ecol 54:276–89

Nold S. C., Ward D. M. 1996. Photosynthate partitioning and fermentation in hot spring microbial mat communities. Appl Environ Microbiol 62:4598-607

Nübel U., Garcia-Pichel F., Muyzer G. 1997. PCR primers to amplify 16S rRNA genes from cyanobacteria. Appl Environ Microbiol 63:3327-3332

Papke R. T., Ramsing N. B., Bateson M. M., Ward D. M. 2003. Geographical isolation in hot spring cyanobacteria. Environ Microbiol 5(8):650-659

Perevalova A. A., Kolganova T. V., Birkeland N. K., Schleper C., Bonch-Osmolovskaya E. A., and Lebedinsky A. V. 2008. Distribution of *Crenarchaeota* representatives in terrestrial hot springs of Russia and Iceland. Appl Environ Microbiol 74:7620–7628

Phoenix V. R., Bennett P. C., Engel A. S., Tyler S. W., Ferris F. G. 2006. Chilean high-altitude hot-spring sinters: a model system for UV screening mechanisms by early Precambrian cyanobacteria. Geobiology 4:15–28

Portillo M. C., Sririn V., Kanoksilapatham W., Gonzalez J. M. 2009. Differential microbial communities in hot spring mats from Western Thailand. Extremophiles 13:321-331

Portillo M. C., Gonzalez J. M. 2008. Microbial communities and immigration in volcanic environments of Canary Islands (Spain). Naturwissenschaften 95:307-315

Roeselers G., Norris T. B., Castenholz R. W., Rysgaard S., Glud R. N., Kühl M., Muyzer G. 2007. Diversity of phototrophic bacteria in microbial mats from Arctic hot springs (Greenland). Environ Microbiol 9:26-38

Schauer M., Balagué V., Pedrós-Alió C., Massana R. 2003. Seasonal changes in the taxonomic composition of bacterioplankton in a coastal oligotrophic system. Aquat Microb Ecol 31:163-174

Seckbach J. (ed) 2007. Algae and cyanobacteria in extreme environments. Springer, Secaucus.

Smith-Ramírez C. 2004. The Chilean coastal range: a vanishing center of biodiversity and endemism in South American temperate rainforests. Biodiversity and Conservation 13:373-393

Sompong U., Hawkins P. R., Besley C., Peerapornpisal Y. 2005. The distribution of cyanobacteria across physical and chemical gradients in hot springs in Northern Thailand. FEMS Microb Ecol 52:365-376

Stal L. J., Caumette P. 1994. Microbial mats: structure, development and environmental significance. Springer-Verlag, Berlin

Takai K., Campbell B. J., Cary S. C., Suzuki M., Oida H., Nunoura T., Hirayama H., Nakagawa S., Suzuki Y., Inagaki F., Horikoshi K. 2005. Enzymatic and genetic characterization of carbon and energy metabolisms by deep-sea hydrothermal chemolithoautotrophic isolates of *Epsilonproteobacteria*. Appl Environ Microbiol 71:7310–7320

Tassi F., Aguilera F., Darrah T., Vaselli O., Capaccioni B., Poreda R. J., Delgado-Huertas A. 2010. Fluid geochemistry of hydrothermal systems in the Arica-Parinacota, Tarapacá and Antofagasta regions (Northern Chile). J Volcanol Geotherm Res 192:1–15

Taton A., Grubisic S., Brambilla E., De Wit R., Wilmotte A. 2003. Cyanobacterial diversity in natural and artificial microbial mats of Lake Fryxell (McMurdo Dry Valleys, Antarctica): a morphological and molecular approach. Appl Environ Microbiol 69:5157-5169

Tenreiro S., Nobre M. F., Rainey F. A., Miguel C., da Costa M. S. 1997. *Thermonema rossianum* sp. nov., a new thermophilic and slightly halophilic species from saline hot springs in Naples, Italy. Int J Syst Bacteriol 47:122–126

Ward D. M., Ferris M. J., Nold S. C., Bateson M. M. 1998. A natural view of microbial biodiversity within hot spring cyanobacterial mat communities. Microbiol Mol Biol Rev 62:1353-1370

Waring G. A. 1965. Thermal springs of the United States and other countries of the world; a summary. Blankeship RR, Bentall R (eds). US Govt. Print. Office, Washington

West N. J., Schönhuber W. A., Fuller N. J., Amann R. I., Rippka R., Post A. F., Scanlan D. J. 2001. Closely related *Prochlorococcus* genotypes show remarkably different depth distributions in two oceanic regions as revealed by in situ hybridization using 16S rRNA-targeted oligonucleotides. Microbiology 147:1731-1744

Whitaker R. J., Grogan D. W., Taylor J. W. 2003. Geographic barriers isolate endemic populations of hyperthermophilic Archaea. Science, 301:976–978

Chapter III.

Spatial microheterogeneity of microbial mat communities in hot springs

Roy Mackenzie, Antonio Martin-Platero, Sarah P Preheim, Pau Corral, Carles Pedrós-Alió,

Martin F. Polz, Beatriz Díez

Abstract

The non-acidic Cahuelmó, Porcelana, and Porcelana Geyser hot spring microbial mats from Northern Patagonia (Chile) were sampled during 2011 to analyze horizontal patchiness in terms of abundance and diversity of the thermophilic bacterial communities in response to small-scale temperature gradients. Thirty samples were collected at 2 cm intervals in microbial mat rectangles of approximately 150 cm^2 for horizontal microheterogeneity analysis in the three hot springs. Temperature within such quadrats changed by 8, 14 and 21 ºC respectively at the three spring mats. By means of Illumina sequences of the 16S rRNA gene (V4 hypervariable region), we observed that the top five most abundant Operational Taxonomic Units (OTU) in the samples belonged to phyla Cyanobacteria, Chloroflexi, Proteobacteria, Bacteroidetes, and Deinococcus-Termus in all the springs. 80 OTUs —almost one-fifth of the total— were common to all hot springs, mostly belonging to phyla Cyanobacteria and Bacteroidetes. However, multidimensional scale (MDS) analysis at the community level based on Bray-Curtis similarities revealed that Porcelana and Geyser were more similar at high temperatures (over 45º C), probably due to the spatial proximity and the similar geological setting of both hot springs. Cyanobacteria and Chloroflexi clearly dominated the microbial mats in Geyser and Porcelana, but not in Cahuelmó, probably due to the anthropogenic and the marine environmental influence in the latter. In Cahuelmó, a heterotrophic Gammaproteobacteria dominated the mat. Diversity indices such as Shannon (H'), or Evenness (J') showed no correlation with temperature in any hot spring mat. However, patchiness in Porcelana hot spring mat revealed a niche partitioning related to temperature, and a turnover of primary producers from Cyanobacteria to Chloroflexi was observed when a threshold temperature of approximately 58º C was reached. Some other heterotrophic dominant phyla such as Proteobacteria, Bacteroidetes, and Deinococcus-Thermus, showed relative abundance peaks correlated with temperature, mostly at 45º C. Microheterogeneity and spatial dynamics of bacterial communities were different among hot springs, and were strongly affected by the surrounding environment. Porcelana geyser did not show the patchiness transition observed in

Porcelana hot spring, and slight diversity shifts were observed among samples in that hot spring, illustrating the complexity of sampling design in microbial mats. Finally, a considerable number of retrieved bacterial sequences were not found in databases, suggesting a high degree of novel diversity in these hot springs.

INTRODUCTION

Thermophilic prokaryotic communities from terrestrial hot springs have been a target for several studies by microbial ecologists and biotechnologists due to the amazing adaptations these microbes show to the environment (Adams and Kelly, 1995; Madigan et al., 1997; Madigan and Marrs, 1997). Bacterial populations in these harsh systems are commonly organized in microbial mats, defined by van Gemerden (1993) as vertically stratified organo-sedimentary structures, usually several millimeters thick that can be adhered to the bottom sediment or float downstream of the thermal water source.

In natural environments, microorganisms are not distributed uniformly, as their abundance and activity change along environmental gradients (Franklin and Mills, 2003). The heterogeneity in microbial communities arises as a response to interrelated variables that fluctuate at many different temporal and spatial scales, which can be both biotic (e.g. competition) and abiotic (pH, temperature, substrate availability, among others) (Becker et al., 2006). In general, the scientific literature is more in agreement with temperature being the main factor shaping microbial communities in terrestrial hot springs (Brock 1967; Miller et al., 2009b; Mackenzie et al., 2013). Therefore, the aquatic environments downstream of hot springs presents well defined physico-chemical gradients with opportunities for niche colonization that might allow interpretation of species diversity and community structure in terms of environmental gradients (Sompong et al., 2005), such as temperature.

Most bulk microbial mats studies have described the vertical stratification of mats within millimeters (i.e. Ramsing et al., 2000; Brock, 1969). However, studies on microbial community patch size are rare, and little is known about the horizontal scales at which microbial interactions and associations become important. In hot springs, the distribution of thermophilic phototrophic or heterotrophic populations has been described on the horizontal plane of microbial mats in single samples (Cuecas et al., 2014), by identification of phototrophs

according to a temperature gradient (Jørgensen and Nelson, 1988), or by molecular fingerprint (Roeselers et al., 2007; Mackenzie et al 2013).

In general, acidic hot springs have been explored more than alkaline hot springs, the former being dominated by thermoacidophilic methanotrophs and sulfur-oxidizing microorganisms that use inorganic substrates of volcanic origin (Mardanov et al., 2011). Recent metagenomic analysis showed that only a small proportion of the sequences retrieved in acidic systems had matches against databases, suggesting a high proportion of novel taxa (Jiménez et al., 2012). Research on alkaline hot springs is much more recent and has already provided some fascinating findings related to the oxygenic and anoxygenic phototrophic microorganisms inhabiting the microbial mats that typically develop in these type of springs, including the discovery of novel functions such as the potential ability of thermophilic *Synechococcus* spp. to fix nitrogen (Steunou et al., 2006, 2008). In these microbial mats, the currently uncultured members of the phyla Chloroflexi and Chlorobi appear to be the most abundant primary producers responsible for most inorganic carbon fixation together with Cyanobacterial members (Bhaya et al., 2007; Miller et al., 2009 a/b; Portillo et al., 2009; van der Meer et al., 2010; Klatt et al., 2011; Liu et al., 2011; Swingley et al., 2012; De León et al., 2013; Mackenzie et al., 2013).

In alkaline springs, a general tendency to an alternation in dominance between Chloroflexi and Cyanobacteria dependent on temperature has been suggested, as a result of competition for physical space and/or limited nutrients (Miller et al., 2009a), and with different strategies for maximizing solar-energy capture, usage and growth (Liu et al., 2011). The later explanation has been proposed as more reliable than the longstanding hypothesis that co-adapted lineages of these bacteria maintain tightly co-occurring distributions along the gradient as a result of a producer-consumer relationship.

However, most of studies of microbial diversity have focused on the most abundant populations, dismissing the rare species in microbial communities, which remain largely

unexplored (Watve and Gangal, 1996). Additionally, the microbial mat studies in hot springs do not adequately reflect what microbes are experiencing on a centimeter-scale level, and replicate samples from the same mat might differ drastically from one another. The objective of this study was to elucidate whether temperature influences the shape of microbial community structure at fine-scale and in different steep thermic gradients.

In recent years, one of the main questions that the study of microbial diversity and ecology has addressed is whether communities are organized by specific assembly rules, leading to nonrandom distribution of species or not (Weltzer and Miller, 2013). Jørgensen and Nelson (1988) stated that temperature is one of the most important environmental factors affecting microbial community composition, and which species of mat-building organisms becomes dominant depends mostly on the temperature and geographical location. Also, Weltzer and Miller (2013) stated that phototrophic taxa (such as Chloroflexi and Cyanobacteria) might be highly structured due to competition for light. By contrast, heterotrophic groups (e.g., Proteobacteria, Bacteroidetes, etc.) may exhibit less structure due to the diversity of potential energy sources. Hot springs, thus, represent a fine model for microbial diversity studies.

Hot springs are frequently found along the Andes mountain range, and in the last decade two main eruptions in the central-south frontier of Chile and Argentina (currently the most active zone of all the Andes), has caused great damage to environmental and human structures in the surroundings (Chaitén volcano, 2008; and Puyehue-Cordón Caulle volcanic complex, in 2011) (Lara, 2009; Collini et al., 2013). The southern volcanic zone is covered by Valdivian rainforest, which is a biodiversity hot spot (Myers et al., 2000), increasing the interest of describing the hidden biological resources lying inside. We have previously described bacterial populations in microbial mats in three hot springs from Northern Patagonia and found that they have a well-defined distribution that correlates with the temperature gradient (Mackenzie et al., 2013. In that study, we showed that Chloroflexi and Cyanobacteria from Subsection V, co-dominate, and that the cyanobacterial members might supply and sustain the total N demands of these systems (Alcamán et al., submitted).

In the present study, by using high-throughput sequencing of the 16S ribosomal RNA V4 region, we detected temperature-related patterns of diversity distribution at the fine scale, especially in the phototrophic fraction dominated by Cyanobacteria and Chloroflexi, which showed a turnover phenomenon in the mat as temperature changed. Additionally, all major groups revealed a richness peak close to 45º C, but overall diversity did not show any correlation with temperature. The relevance and potential of common and rare taxa in these extreme systems will help us to achieve a better estimation of the local and global genetic resources in hot springs and to foster the development of new strategies to obtain new uses of thermophilic organisms inhabiting these still poorly explored systems.

MATERIALS AND METHODS

Sampling sites and collection of samples

Microbial mat samples were collected in the 2011 austral summer (March) at three hot springs: Porcelana (42º 27’ 29.1’’ S – 72º 27’ 39.3’’ W), Cahuelmó (42º 15’ 11.8’’S / 72º 22’ 4.4’’W) and Porcelana geyser (42º 24’ 51’’S / 72º 29’ 02.2’’ W). Map in Figure 1A shows the region where the samples were taken. Approximately two-cubic centimeter samples of microbial mat were collected along a temperature gradient (five samples in total at each location) and also within a grid at a given point in the gradient using a cork borer.

A grid of approximately 15 x 10 cm in the horizontal plane of the microbial mat, with 30 samples of 1 cm diameter each (six rows per five columns) and approximately 2 cm thick was sampled in Porcelana. In Cahuelmó, a grid of approximately 20 x 8 cm (three rows per ten columns), also with 30 samples of 1 cm diameter and 0.5 to 1 cm thick were collected. Finally, at the microbial mat of Porcelana Geyser, a grid of approximately 20 x 8 cm (three rows per ten columns), with 30 samples of 1 cm diameter and approximately 2 cm thick were collected. Temperature was measured at each point within the grid at all three hot springs before the extraction of samples using a digital thermometer (Oakton, model 35607-85). All samples from

the tree locations were stored in cryovials and kept at natural temperature during the way back, and were frozen in liquid nitrogen before nucleic acid extractions. Temperature ambiente

DNA extraction, 16S rDNA library preparation and sequencing

Extraction of nucleic acids was done as described by Mackenzie et al. (2013). Briefly, samples were thawed and approximately 200 µl of microbial mat was bead-beaten for rapid and thorough homogenization. Nucleic acids were extracted using a modified phenol: chloroform: IAA protocol, and purified using Amicon Ultra-15 (Millipore, MA, USA). 16S Ribosomal DNA libraries were prepared following the procedure described by Preheim et al. (2013). In summary, the 16S rRNA gene was amplified using as few cycles as possible (Polz and Cavanaugh 1998). This was achieved by carrying out qPCR to identify the amplification cycle threshold (Ct) of each sample, and modifying the thermocycle program to the minimal number of cycles possible. This qPCR was carried out in a final volume of 25 µl containing 5 µl of 5x HF buffer, 250 µM of dNTPs, 0.3 µM of the primers PE_16S_V4_U515_F (5' - ACA CGA CGC TCT TCC GAT CTY RYR GTG CCA GCM GCC GCG GTA A – 3') and PE_16S_V4_E786_R (5' – CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC TGG ACT ACH VGG GTW TCT AAT), 0.5x SYBR Green I nucleic acid stain (Invitrogen^{TM}), 2.5 U of Phusion® High-Fidelity DNA Polymerase (New England BioLabs Inc.), and 10 ng of template DNA. The amplification program for these initial qPCR reactions consisted of an initial denaturing step at 98 ºC for 3 min followed by an amplification step of 45 cycles of 30 s at 98 ºC, 30 s at 52 ºC, and 30 s at 72 ºC, and a final extension of 5 min at 72 ºC.

All samples were normalized to the least concentrated sample according to the QPCR results. Samples were diluted according to $Df = 2^n$, where Df is the dilution factor and n is the difference in Ct between the Ct of the sample to dilute and lowest Ct (15 cycles in this experiment). Normalized templates were processed for 15 cycles (based on QPCR results) with the same conditions as the qPCR reaction without SYBR Green with 5 µl of normalized

template DNA. Due to the low number of cycles used, this PCR was done in quadruplicate and products were pooled after amplification.

Pooled PCR products were purified using magnetic beads (AgenCourt® AMPure® XP, Beckman Coulter). Each 100 µl of PCR was mixed with 85.5 µl of beads and left 13 min to let the DNA bind to the beads. Then, samples were incubated during 15 min on a magnet (SPRIplate® 96-Ring) and washed three times with 100 µl of ethanol 70%. After ethanol evaporation, the DNA was eluted by 40 µl of EB buffer (QIAGEN) by incubating 7 min for elution and 15 min on magnet to separate DNA from the beads.

Specific barcodes were added to each sample during the 9th cycle of PCR, with primers that overlapped with the former primers amplified region. This PCR was carried out in a final volume of 25 µl containing 5 µl of 5x HF buffer, 250 µM of dNTPs, 0.3 µM of the primers PE_III_F (5' – AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC T – 3') and PE_III_001-096 (5' – CAA GCA GAA GAC GGC ATA CGA GAT NNN NNN N CGG TCT CGG CAT TCC TGC TGA ACC GCT – 3'; NNNNNNN represents the specific barcode), 2.5 U of Phusion® High-Fidelity DNA Polymerase (New England BioLabs Inc.), and four microliters of the previous purified PCR product as template. The amplification program consisted of an initial denaturing step at 98 ºC for 2 min followed by an amplification step of 9 cycles of 30 s at 98 ºC, 9 s at 70 ºC, and 30 s at 72 ºC, and a final extension of 2 min at 72 ºC. This PCR was also done in quadruplicate, pooled after amplification, and purified by AgenCourt® AMPure® XP magnetic beads as described above.

Finally, the libraries were multiplexed for Illumina sequencing. The multiplexing ratios were estimated by qPCR with the Illumina sequencing primers. This qPCR was carried out in a final volume of 25 µl containing 12.5 µl of 2x QuantiTec mastermix, 0.2 µM of the primers PE_seq_F (5' – ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT – 3') and PE_seq_R (5' – CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC T – 3'), and

5 µl of the libraries as template. The amplification programme consisted of an initial denaturing step at 95 ºC for 15 min followed by an amplification step of 45 cycles of 10 s at 95 ºC, 20 s at 60 ºC, and 30 s at 72 ºC, and a final extension of 5 min at 72 ºC.

The multiplexed samples were submitted for MiSeq Illumina sequencing at the BioMicro Center (MIT, Cambridge, MA). The obtained 16S iTag sequences were 100 bp long for the grid samples.

Processing and analysis of 16S reads

The analysis of Illumina (MiSeq Sequencing System) sequences was performed first with Qiime (Caporaso et al., 2010) using its split_libraries_fastq.py function to demultiplex and quality filter sequences with the following parameters: the length of the barcode --barcode_type 7; maximal number of consecutive low quality base -r 0; Q was used for quality --phred_quality_threshold 17. Next, demultiplexed and filtered reads were trimmed using MOTHUR (Schloss et al, 2009) to remove primers using the trim.seqs function and all reads were truncated at 77 and 81 bp for the forward and reverse read, respectively. This corresponded to positions 533-609 (forward) and 705-785 (reverse) of the *Escherichia coli* 16S rRNA sequence (Baker et al., 2003). After carrying out the previous steps on both forward and the reverse reads independently, paired surviving reads were concatenated. The distribution-based clustering (DBC, Preheim et al., 2013) method was used to create OTUs from the concatenated sequences, which uses genetic distance and ecological information (i.e. the distribution of sequences across sampled environments) to inform the clustering algorithm. The goal of DBC is to accommodate differences in the level of genetic differentiation across taxa, reducing the number of redundant OTUs from sequences within the same population or created by sequencing error. The DBC algorithm uses read distribution information, the relative abundances of sequences within all samples, and genetic distance to inform clustering. Concatenated sequences were aligned to a modified SILVA bacterial alignment, which was

trimmed to the same positions of the *E. coli* 16S rRNA sequence and concatenated in the same manner as the sequence data. Jukes-Cantor corrected distances were created using FastTree with the -makematrix option (Price et al., 2010) for both the aligned and unaligned sequences, and used as input to the DBC algorithm. After creating OTUs with DBC, OTU representatives were defined as the most abundant sequence in the OTU. Non-16S rRNA sequences were removed from the final OTU list if the representative sequence alignment did not begin and end at the same positions as the reference alignment. Chimeric OTU representatives were identified with UCHIME 4.0 (Edgar et al., 2011) and removed. OTUs were assigned taxonomic identity based on the representative sequence with the RDP classifier (Wang et al., 2007) using a bootstrap cut-off of 50% as recommended for sequences shorter than 250 bp.

Bacterial community diversity analysis

OTUs were analyzed at the community level using the Vegan v2.0-5 package (Oksanen et al., 2012) for R v2.15.2. Shannon diversity index (H'), Bray Curtis similarity index, Chao1 index, and Richness (S) were calculated with R. Maximal Shannon index (Hmax=ln(S)) and Evenness index (H'/Hmax) were calculated afterwards.

Multidimensional scale analysis (MDS) was performed using Primer-E software v6.1.2. Data was transformed to square root before Bray-Curtis similarity calculation.

A phylogenetic reconstruction using all OTUs obtained, with the exception of those showing only one read (singletons), or two reads (doubletons), was conducted using Maximum likelihood (ML). Tree construction was carried out on full-length of 16S rRNA gene sequences, using the SILVA database. The sequences were aligned using BioEdit Sequence Alignment Editor v. 7.2.5 with the ClustalW algorithm. An additional more stringent alignment was made by removing and adjusting ambiguously aligned sites by visual examination using GeneDoc Multiple Sequence Alignment tool. Phylogenies were constructed using Maximum Likelihood as implemented in RAxML v.7.2.8 (Stamatakis et al., 2008), with GTR nucleotide substitution

model. The tree generated was visualized and edited in Interactive Tree Of Life v 2.2.2 (Ciccarelli et al., 2006). In order to find whether OTUs had any habitat associations, the abundance-weighted mean temperature (AWMT) was calculated for each OTU. The AWMT was obtained by adding up the resulting product of the temperature of each sample by the number of reads of each OTU at that sample, and dividing the result by the total number of reads of each OTU. This is the OTU's 'niche value' for temperature (Stegen et al., 2012).

The tree generated was visualized and edited in Interactive Tree Of Life (http://itol.embl.de/, visited on August 2014. Letunic and Bork, 2007).

RESULTS AND DISCUSSION

The geographic distance of the three studied hot springs of Cahuelmó, Porcelana and Geyser can be seen in Figure 1. Distance between Porcelana hot spring and Porcelana geyser is 5.1 km lineal (approx.). Cahuelmó hot spring is located across the fjord, at 19.3 km from Porcelana Geyser, and 23.1 km from Porcelana hot spring.

Figure 1: Map of Los Lagos region (Chilean Northern Patagonia) showing the location of the hot springs studied: (1) Cahuelmó hot spring (2) Porcelana Geyser, and (3) Porcelana hot spring.

Porcelana hot spring and Geyser are located in a pristine temperate rainforest environment. Due to their proximity, it is likely that both thermal events share a common underground water source. Also, both hot springs are deep inside the rain forest, and close to rivers, where they discharge. On the other hand, Cahuelmó spring is located in Cahuelmó fjord, exposed to the wind, the fjord brackish water, and tidal fluctuations that flood part of the microbial mats. It is also subjected to human interference, as tourists do recreational baths constantly changing the water flow to fill artificial pools (Figure 2).

Figure 2: Cahuelmó hot spring. Water channels, covered with orange thermophilic microorganisms, end in artificial pools (left and bottom left) on which tourists take baths by blocking or releasing the water course to fill them with hot water.

Each hot spring harbored microbial mats of different characteristics that could be easily observed by naked eye. Figure 3 shows the microbial mats sampled in each hot spring. In Porcelana, the microbial mat exhibited two different colors separated by a clearly marked the limit between two types of microbial communities; an orange one and a dark green one (Figure 3A). The microbial mat in Cahuelmó was dark green and showed a black spotted-like pattern all across (Fig 3B). Microbial mat sampled in Porcelana Geyser was dark green and pink with a hard, flat coverage of white calcium carbonate (Figure 3C). Thermophilic microbial mat images obtained by Portillo et al. (2009) at western Thailand were very similar at naked eye to the microbial mats in our study, as mats sampled by them were both orange and dark green.

Figure 3: Microbial mats sampled. A: Porcelana hot spring. B: Cahuelmó hot spring. C: Porcelana geyser (the green areas were sampled).

General physicochemical setting

In Cahuelmó spring, temperature in the grid ranged between 31.1º and 44.7 ºC (13.6º C difference). In Porcelana, 30 samples were sequenced. A difference of 7.8 ºC was detected among samples in the grid (from 52.4º to 60.2º C). Samples at Porcelana Geyser were also obtained as a grid where temperature ranged from 32.8º to 53.7 ºC (a 20.9º C difference). A total of 30 samples were also obtained forming a grid. Table S1 shows the temperature of each sample in the three hot springs. Figure 4 shows the grid configuration of sampling in Porcelana hot spring microbial mat.

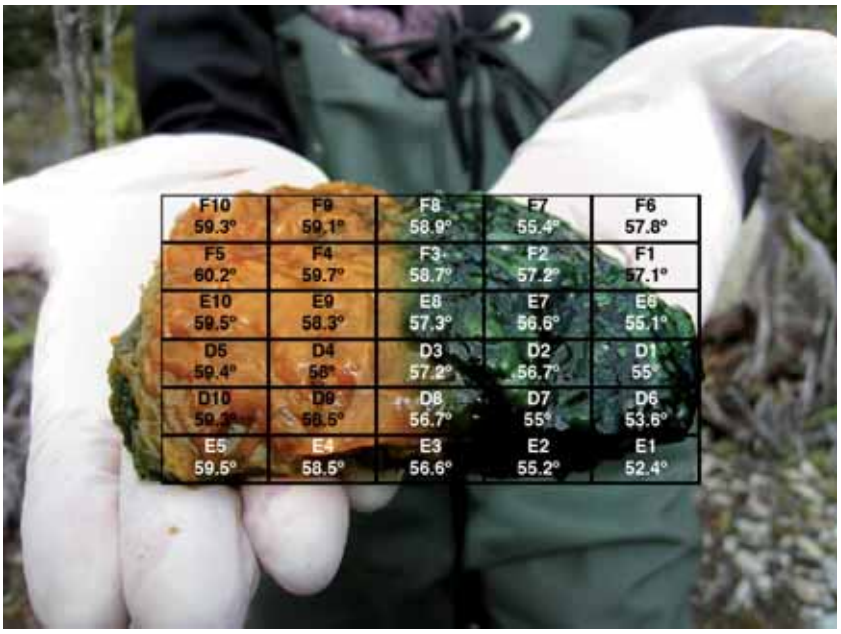


Figure 4: A piece of microbial mat (10 x 15 cm approx.) taken from Porcelana hot spring, showing the distribution of samples (30) obtained for microheterogeneity analysis (mat in the image is leaned). Similar pieces of microbial mats were taken from Cahuelmó and Geyser (20 x 8 cm approx. each, also 30 samples).

Figure 5: Calcium carbonate columnar structures in Porcelana Geyser before (A) and after (B) self-collapse (1.5 years difference). The orange-green structure in (A) brakes due to its own weight down to the river course, and microbial colonization starts again (B), favoring the mineral precipitation within the biofilm and allowing the calcium carbonate column-like formation again.

Diversity of bacterial communities

The total number of reads obtained after the quality filtering and cleaning was 151771, considering all 86 samples for the three hot spring sampled. The abundance of reads is summarized in Table S1, and varied considerably among samples, ranging between 271 (Cahuelmó) and 5591 (Geyser). Values were normalized by rarefaction before further analysis. A total of 549 OTUs were detected considering the three hot springs. A total of 374 OTUs were detected in Geyser, the highest richness estimated, as expected due to the large dataset of sequenced retrieved in this hot spring. Followed by Cahuelmó (308 OTUs), and finally Porcelana (185 OTUs). A detailed summary of OTU richness per sample can be found in Table S1. A large fraction of the OTUs (80 OTUs) were shared among the three hot springs (Figure 3). Cahuelmó and Geyser had more OTUs in common than with Porcelana, probably because both hot springs had the largest datasets. Porcelana Geyser showed 149 unique OTUs, the largest amount of the three hot springs, followed by Cahuelmó (124 unique OTUs), and Porcelana (36 unique OTUs), as expected by the size of datasets.

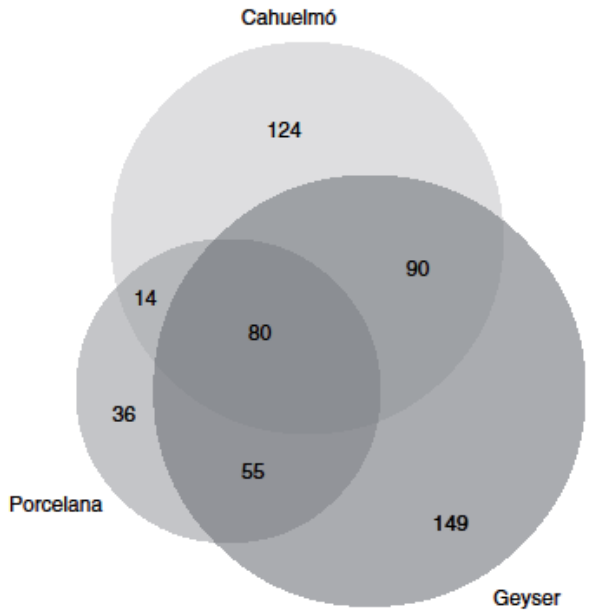


Figure 6: Venn diagram showing operational taxonomic unit (OTU) distribution between hot springs. The size of the circles is proportional to the number of OTUs. Plots were obtained in http://www.cmbi.ru.nl/cdd/biovenn.

Over 60% of retrieved Illumina reads could be determined correctly to genus level, but none could be assigned to a species level (Figure 7). Liu et al. (2007) discussed the feasibility to apply phylogenetic diversity measures to microbial community data, relying on the capability to build phylogenetic trees from fragments of the 16S rRNA sequence. Because the accuracy of phylogenetic reconstruction depends sensitively on the number of informative sites, and tends to be much worse below a few hundred base pairs, the short sequences used in this study do not allowed to assign species-level taxonomy to OTUs. The proportion of correctly identified sequences is consistent with that reported by Degnan and Ochman (2012), who stated that the RDP Classifier accurately identified all of the 19 previously known bacterial strains in an artificial community to taxonomic Class by means of Illumina reads, but its success rate progressively decreased for lower taxonomic divisions, with only about 60–90% assigned correctly.

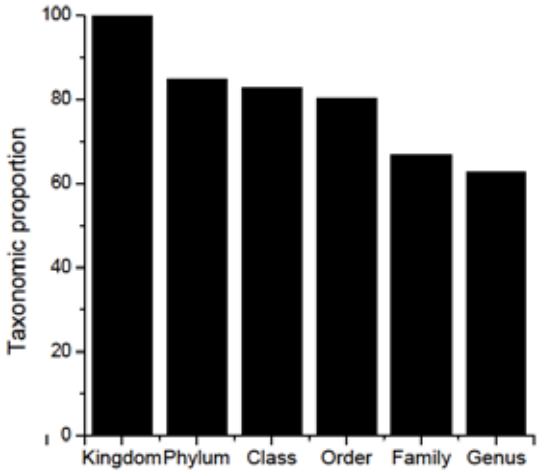


Figure 7: Taxonomic level that could be assigned to reads from the three springs. A 0.97 identity threshold was used to classify the iTags against the Greengenes database.

Taxonomic assignment revealed a total of 17 phyla: Acidobacteria, Actinobacteria, Bacteroidetes, Chlamydiae, Chloroflexi, Chlorobi, Cyanobacteria, Deinococcus-Thermus, Firmicutes, Fusobacteria, Nitrospira, OP10, Planctomycetes, Proteobacteria, Spirochaetes, Thermodesulfobacteria, and Verrucomicrobia. Phylum Tenericutes showed one read only. All phyla were detected in Cahuelmó, with exception of Chlamydiae. In Porcelana hot spring, all phyla but Chlorobi, Fusobacteria and Thermodesulfobacteria were detected. In Geyser, only pylum Thermodesulfobacteria was not detected. Therefore, phylum Thermodesulfobacteria was found in Cahuelmó hot spring only. Thermodesulfobacteria is a phylum of thermophilic sulphur-reducing bacteria, described first in hot springs in Japan (Garrity et al., 2001), but found in hot springs elsewhere, such as Yellowstone National Park (Vick et al., 2010), Tibet (Lau et al., 2009), and in Argentinian Northern Patagonia (Urbieta et al., 2014). Major phyla, specifically Cyanobacteria, Bacteroidetes, Proteobacteria, Deinococcus-Thermus, and Acidobacteria detected in this study were also previously described in Mackenzie et al. (2013). Additionally, in that article, unidentified non-sulfur bacteria were reported, which probably corresponded in this study to the largely abundant phylum Chloroflexi. Other authors have reported similar presence and relative abundance of these phyla in hot springs from Yellowstone National Park (De León et al., 2013; Hugenholtz et al., 1998), Thailand (Sompong et al., 2005;

Portillo et al., 2009; Cuecas et al., 2014), Kamchatka Geyser Field in Russia (Gumerov et al., 2011), Tibet (Lau et al., 2009; Huang et al., 2011), Greenland (Roeselers et al., 2007), Japan (Nakagawa and Fukui, 2002; Nakagawa and Fukui, 2003), the Colombian Andes (Bohorquez et al., 2012), and the Copahue region in Argentinian Northern Patagonia (Urbieta et al., 2014), among others.

Despite the small size of fragments (78 bp forward and reverse, paired ends assembled into a 156 bp fragment with a gap in between), less than 20% remained unclassified. The unclassified proportion in this study was less than in other hot springs studies using pyrotags like Bohorquez et al. (2012) from high mountain hot spring water, where 45% of unclassified sequences were reported. In our study, most of the major taxa were consistent with our own previous study (Mackenzie et al., 2013) as stated earlier; nevertheless, in this study we obtained more taxonomic diversity depth of the total bacterial community, and detailed information on rare biosphere of these thermophilic mats.

As stated by Magurran in her book *Measuring Biological Diversity* (2004), diversity measurement is a comparative discipline. A single value is not informative, and only when we compare two or more communities, or ask about temporal or spatial diversity shifts, values acquire a meaning. Therefore, to assess variations in community structure associated to any environmental gradient, we used diversity measurements, which rely mostly on species richness and relative abundance (Oren, 2004), such as Shannon (diversity), Evenness (equitability), and Inverse Simpson (dominance) indices (Magurran, 1988).

Additionally, there are estimators that assess how well the microbial communities where sampled, i.e. rank abundance and rarefaction curves. These models, besides representing a quality-proof exercise, allow comparing different environments by the biological datasets found in them (Hughes et al., 2001).

With regard to alpha diversity, S and Chao 1 estimators showed the same trend but the latter provided higher estimates than the former, as could be expected. The difference between

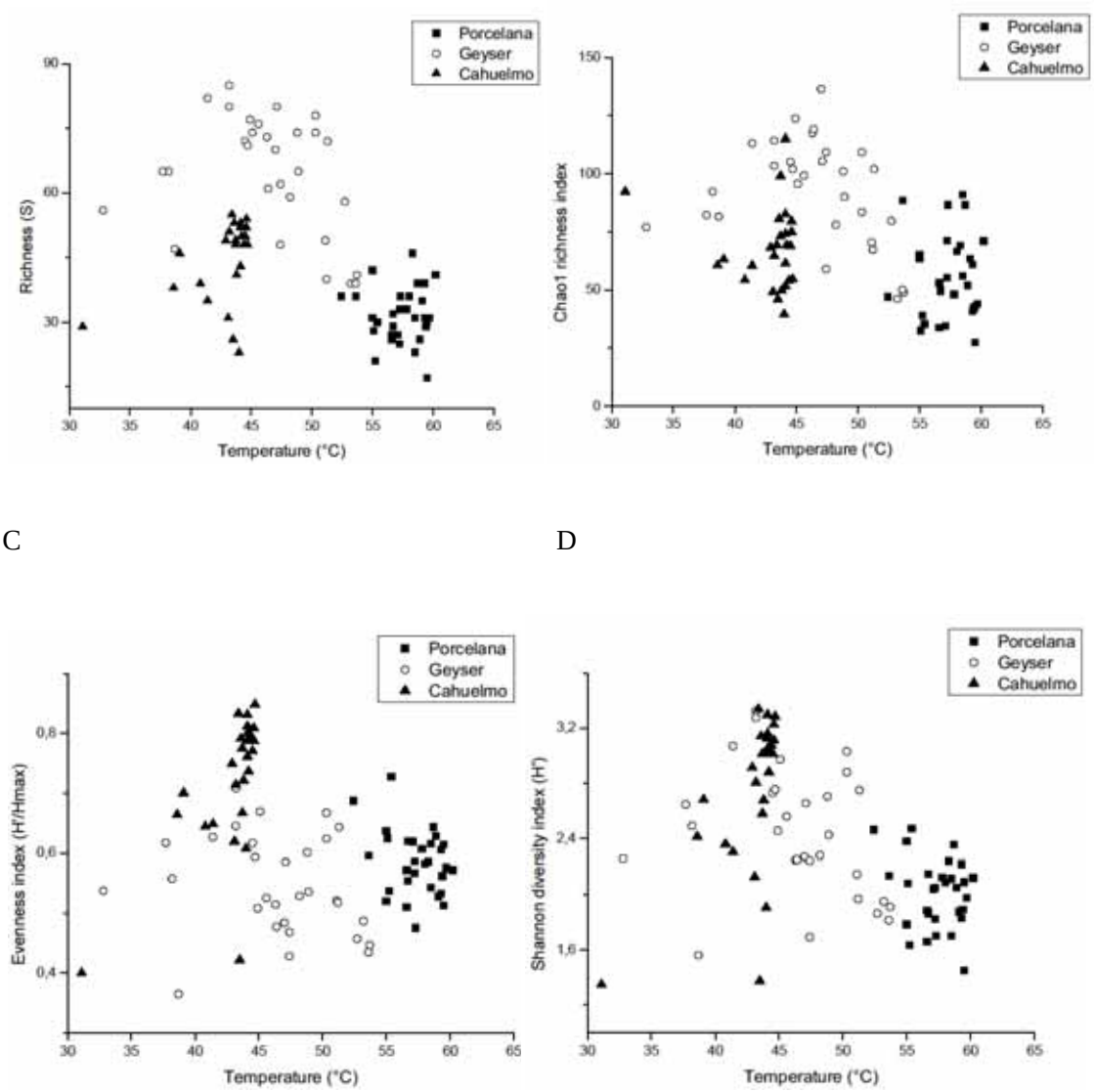
both estimators was less than two times, which suggests the effect of the high proportion of singletons and doubletons, and therefore, many bacteria remained undetected in samples (Chao, 2005). Similar differences between S and Chao1 estimators were measured by Miller et al. (2009a) in bacterial communities from two hot springs in Yellowstone National Park, and in mats from high-mountain thermophilic mats in the Colombian Andes (Bohorquez et al. 2012). Samples from Porcelana hot spring and Geyser followed the same trend: a decrease in richness and no change in Evenness when increasing temperature (Figure 4A and C, respectively). This proportionally inverse trend resulted in a slight decrease in Shannon index. This decrease in diversity estimates with increasing temperature was what could be expected from general ecological theory. For instance, Everroad et al. (2012) found a similar decrease by using T-RFLP along a temperature gradient ranging from 52º to 75º C in Nakabusa spring, Japan. Other studies did not find a relationship between bacterial community diversity shifts and temperature, such as described by Lau et al. (2009), although the study used a narrower temperature range of 60º to 65º C. In this particular case, the 5º C difference was probably not enough to show any relevant difference in the community diversity.

Evenness in Cahuelmó samples, however, showed a different pattern. They showed lower richness and higher evenness than Porcelana Geyser samples in a similar temperature range (close to 45º C, Figures 7A and C). In all cases, evenness index in samples below 42 ˚C were rather variable and did not show any relationship to temperature. Most Cahuelmó samples corresponded to this lower range of temperature. In this case we cannot assure whether the differences between Cahuelmó and the other two hot springs were due to a lower temperature range, or to other factors.

As a general overview on diversity shifts according to temperature, all samples (86) from the three hot springs together, sorted in a temperature gradient from 30º to 65º C, were negatively related with increasing temperature (Figure 7D). The negative relationship between Shannon index and temperature is consistent with that shown by Sharp et al. (2014), who found similar results in microbial communities from hot springs in Canada and New Zealand,

concluding that temperature exerts a strong control on microbial diversity when considered over most of the temperature range within which life is possible.

A B



C D

Figure 7: Diversity estimators (A) Richness (S) as number of OTUs; (B) Chao1 Richness estimator; (C) Evenness (J'); and (D) Shannon's diversity index (H') of all samples versus temperature.

A multidimensional scaling analysis was made using the 86 samples in the three hot springs by means of Bray-Curtis similarity index to detect similarities between samples considering their source, temperature and community structure (Figure 8).

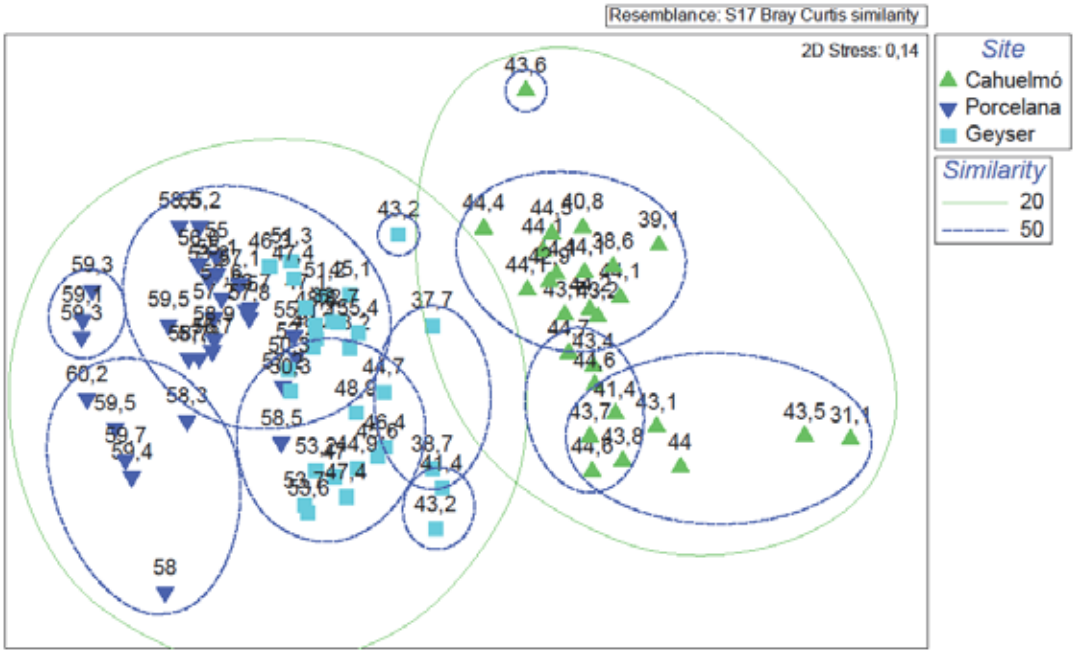


Figure 8: Multidimensional-Scale analysis (MDS) of all samples from Porcelana, Geyser and Cahuelmó by means of Bray-Curtis similarity index.

Regarding the MDS in Figure 8, a similar spatial distribution by means of Bray-Curtis based MDS was previously reported by Mackenzie et al. (2013) at the same three hot springs, using DGGE-16S rRNA fingerprint patterns of most represented bacterial groups. Again in this study, MDS showed that Cahuelmó samples clustered separately from the other two hot spring samples (less than 20% similarity with them), revealing differences in bacterial community structure compared to the other two springs. On the other hand, by including the rare bacterial groups, this result confirmed that both Geyser and Porcelana hot springs share environmental conditions which affect and shape the community structure of microbial mats especially at the temperature range from 45º to 55 ºC.

Taxonomic composition of bacterial community

A phylogenetic reconstruction was made of the bacterial communities in microbial mats, using 170 representative sequences of the most abundant OTUs retrieved (5% cutoff), which provided an overall picture of the diversity of the five main dominating phyla in these three hot springs (Figure 9).

In the reconstruction, colored leaves and external rings are based on the classification of sequences using Distribution-Based Clustering (Preheim et al., 2013), and BLAST. Both independent approaches were mostly concordant with the exception of 8 unclassified sequences identified as possible Bacteroidetes or Proteobacteria, that were removed from the tree due to taxonomic incongruence. Singletons and doubletons were also removed from the phylogenetic analysis to facilitate visualization, and because they represented less than 5% of the total. From the five more diverse phyla (regarding the number of OTUs), Proteobacteria (purple) was the richest phylum, followed by Bacteroidetes (red), Cyanobacteria (green), Chloroflexi (yellow), and Deinococcus-Thermus (light blue).

In Figure 9, the outer ring (with shades of red) shows the abundance-weighted mean temperature (AWMT), used to estimate the temperature at which each OTU was more abundant, thereby characterizing each OTU niche in terms of the habitats where it occurs (Stegen et al., 2012). Black squares indicate reference 16S rRNA sequences extracted from complete genome sequenced organisms. The average of the AWMT was 45º C, temperature that will be discussed later on, as peaks of abundance can be observed in several OTUs from a diverse number of taxa that appeared at this temperature. The external colored heatmap rings in the phylogenetic tree (Figure 9) represent the abundance of reads in each sample within hot springs. Samples from Cahuelmó (inner ring) were colored blue; samples from Porcelana (middle ring) were colored green, and samples from Porcelana Geyser (outer ring) were colored magenta.

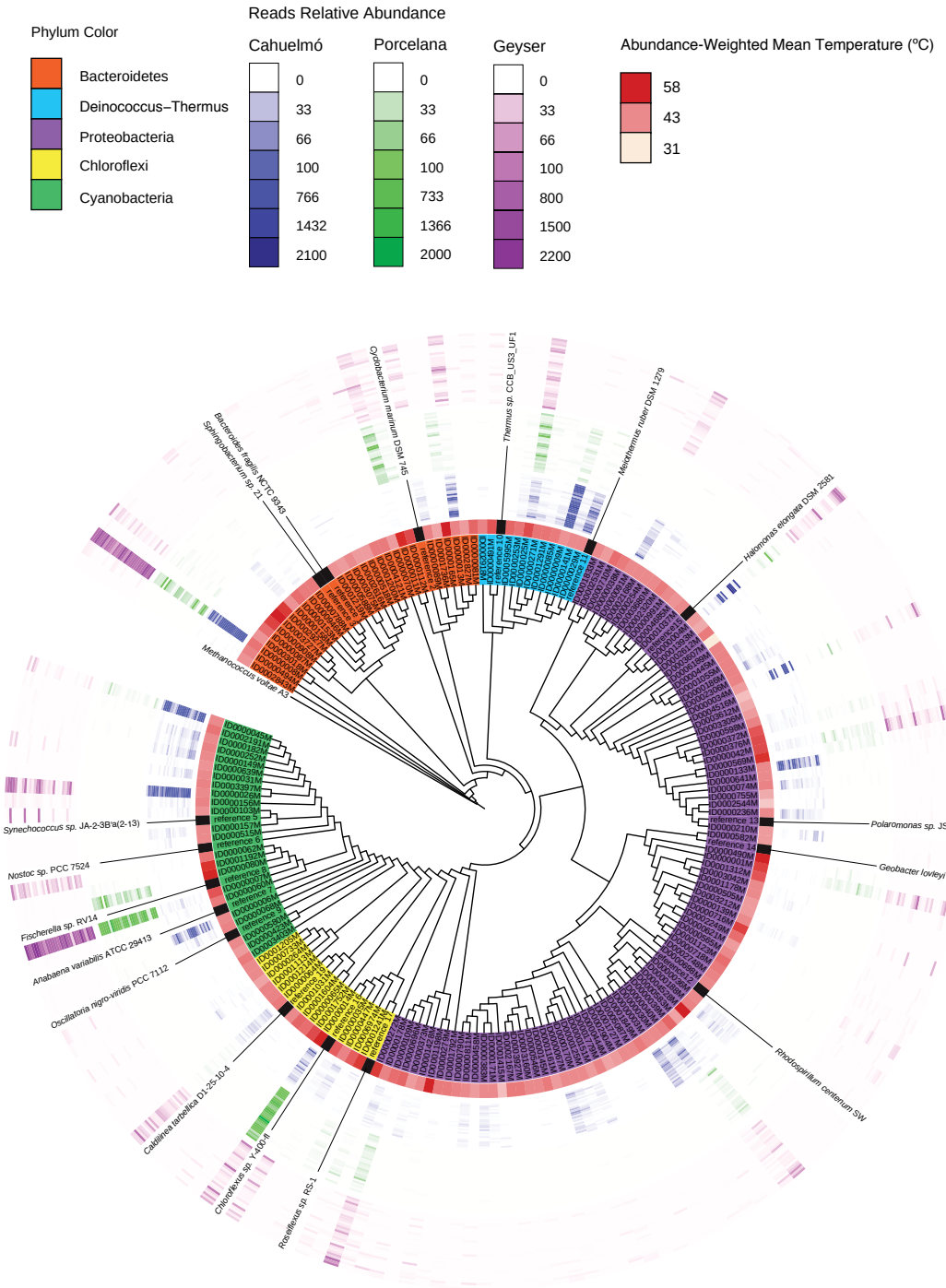


Figure 9: Maximum likelihood phylogenetic tree with 16S rDNA sequences of prokaryotes retrieved from the hot springs. The tree was constructed using 170 illumina Tags (V4 hypervariable region, positions 515 to 786), each representing an OTU. The tree ignores branch lengths. The inner colored ring shows the taxonomic assignments of sequences to prokaryotic phyla. The red-gradient ring outside the taxonomic ring represents the Abundance-weighted mean temperature, indicating the temperature preference of each OTU. Black squares represent reference sequences. Bars in the outer ring show as heatmaps the read relative abundance in each sample from each hot spring.

93 OTUs belonged to Proteobacteria, the most abundant phylum . 25 of these OTUs were shared among the three hot springs, while 23 were found exclusively in Porcelana Geyser, 17 in Cahuelmó, and 4 in Porcelana hot spring. The OTUs affiliated to the Gammaproteobacteria *Halomonas sp*. (ID0000004M) and *Shewanella sp.* (ID0000034M) were abundant throughout the gradient especially in Cahuelmó and Porcelana Geyser at temperatures lower than 46º C, with maxima at 32º and 45º, and were almost absent at higher temperatures (Figure 9). In Porcelana hot spring, both *Halomonas sp*. (ID0000004M) and *Shewanella sp.* (ID0000034M) showed a significant abundance peak in a sample at 58 °C.

Halomonas is as a heterotrophic, moderately halophilic Gammaproteobacteria. The cells have a well-known exopolysaccharide production activity in natural environments (Arahal and Ventosa, 2006). Kaye et al. (2004) isolated six *Halomonas* strains from a low temperature deep-sea hydrothermal vent fluid, and measured a wide temperature range (-1 to 35° C with a growth optimum at 30° C). Therefore, the *Halomonas* found at 58 °C in the present study suggest that strains adapted to higher temperatures exist. According to Ghosh et al. (2003), thermophilic relatives of the genus *Shewanella* can be isolated from hot environments at temperatures as high as 66 °C, and are well known iron-reducers that might be obtaining energy from iron forms in the thermal water (data not shown).

The second largest group of sequences in the three hot springs was affiliated to phylum Bacteroidetes and included 30 different OTUs (Figure 9). The highest relative abundances in this group were achieved by two unknown Sphingobacteriales (OTUs ID0000013M and ID00000008M), that were detected at a wide temperature range (especially at 42º to 46º C) in the three hot springs, particularly in Cahuelmó and Porcelana Geyser. Above 55º C, another unknown Bacteroidetes (ID0000011M) was detected in high abundance at the three hot springs, being more represented in Porcelana hot spring and Geyser than in Cahuelmó. Above this temperature (>55º C) at Porcelana hot spring exclusively, other Bacteroidetes OTUs related to unknown Sphingobacteriales (ID00003727M), and to the genus *Thermonema* (ID00000165M) were detected in small relative abundances. Sphingobacteriales-like OTUs are commonly found

in many hot springs elsewhere such as in Yellowstone (Meyer-Dombard et al., 2011), Iceland (Tobler and Benning, 2011), or South Africa (Tekere et al., 2011), and were also detected by using the DGGE technique in Porcelana hot spring (Mackenzie et al., 2013). In particular, the genus *Thermonema* is a heterotrophic, thermophilic (60º C growth optimum), long and thin filament-forming bacterium isolated initially from hot springs in New Zealand, but has been also found later on in Italy in saline hot springs and hydrtothermal vents in the submarine seafloor in Iceland, suggesting its worldwide dispersion (Oren, 2006).

23 OTUs were identified as belonging to the phylum Cyanobacteria (Figure 9). Several very abundant OTUs were closely related to the Order Stigonematales (heterocystous forming Cyanobacteria) and in particular to the genus *Mastigocladus*. It can be seen in Figure 9 that *Fischerella*-like OTU ID0000007M was the most abundant of Cyanobacteria, and was widely distributed along samples in Porcelana hot spring and geyser. Additionally, *Fischerella*-like OTUs ID0000080M and ID0000062M were very abundant in samples from Porcelana hot spring, and Porcelana geyser, respectively. The filamentous heterocystous Cyanobacteria from order Stigonematales have been commonly found in hot springs elsewhere (Miller et al. 2006, 2009), mostly at temperature ranges below 60º C, dominating at the range between 40º and 50º C (Sompong et al., 2005). Different OTUs were also found affiliated to other Cyanobacteria groups, such as the previously reported thermophilic unicellular species of *Synechococcus spp.* OTUs closely related to this unicellular cyanobacteria were OTUs ID0000157M, detected in Cahuelmó; and ID0000103M, very abundant in some geyser samples at 41-43º C). Other *Synechococcus*-like affiliated OTUs such as ID0000026M, ID0000045M were mostly detected in Cahuelmó, and the first was abundant in Geyser. Filamentous non-heterocystous Cyanobacteria from the order Oscillatoriales were also found, such as *Leptolyngbya*, OTUs ID0000026M; present in Cahuelmó and Porcelana Geyser, and ID0000045M, present in all hot springs at temperatures as low as 35 ºC (Figure 9).

OTUs belonging to phylum Chloroflexi (15 in total) were very abundant above 58º C in the three hot springs. Particularly OTU ID0000014M, affiliated to the genus *Chloroflexus*, was

the most abundant in the three hot springs, but especially in Porcelana hot spring. In addition, OTUs related to the genus *Caldilinea* and another *Chloroflexus* (OTUs ID0000064M and ID0000035M, respectively) were detected in less abundance at a lower temperature range (40-45º C) especially in Cahuelmó hot spring and Porcelana Geyser. The family Chloroflexaceae was first discovered in thermal environments, and its members described as filamentous anoxygenic phototrophic bacteria (Hanada and Pierson, 2006). According to Wang et al. (2013), organisms belonging to the order Chloroflexales are usually found dominating the phototrophic microbial mats at a temperature range of 55º to 75º C, coinciding closely with the maximum of tolerance of most Cyanobacteria. Klatt et al. (2013) also described filamentous anoxygenic phototrophs (FAPs), commonly associated to phylum Chloroflexi, as facultative phototrophs in absence of oxygen. In aerobic environments, their metabolic metabolism turns to heterotrophy.

Finally, OTUs affiliated to the phylum Deinococcus-Thermus formed two groups: at 52º C, OTU ID000025M tentatively identified as *Thermus sp.* CCB_US3_UF1 was present in the three hot springs; while approximately at 43º C the OTUs ID0000029M and ID0000009M, identified as *Meiothermus ruber* DSM 1279 was present in xxxxxxx (Figure 9). Despite of being detected in the three hot springs, these Deinococcus-Thermus were highly abundant in Cahuelmó only. Genera *Thermus* and *Meiothermus* are both thermophilic and heterotrophic, the first isolated in hot springs from Yellowstone (Brock and Freeze, 1969), and the second reported in hot springs from Portugal by Tenreiro et al. (1995). Both genera are widely distributed in natural or artificial thermal sources (Kristjansson and Alfredsson, 1983), and due to their distribution along thermal gradients, a close relationship between Cyanobacterial primary producers and *Meiothermus* heterotrophic consumers could be inferred at temperatures below 55 ºC, as well as between *Chloroflexus* primary producers and *Thermus* heterotrophic consumers above 55º C.

Bacterial community structure

The community structure and spatial distribution of samples in the three microbial mats is shown in Figure 10. Out of the 549 OTUs found in all three hot springs, 193 were identified only as Bacteria (77 OTUs in Cahuelmó, 67 in Porcelana hot spring, and 137 in Porcelana Geyser). These OTUs were included in a "Novel" bin in Figure 10 (dark red). Sequences in Porcelana hot spring that could not be identified in the GreenGenes database ranged between 7.1% and 30.6%. In Porcelana geyser, the proportion of unidentified sequences ranged between 4.6% and 35.2%. In Cahuelmó hot spring, the unidentified sequences ranged between 1% and 17.7%.

The OTUs affiliated to phyla with abundances less than 5% of total in all samples were grouped together as a bin named "Other" in Figure 10. These included Acidobacteria, Actinobacteria, Chlamydiae, Chlorobi, Firmicutes, Fusobacteria, Nitrospira, OP10, Spirochaetes, Thermodesulfobacteria, and Verrucomicrobia. The OTUs under the "Other" classification were higher in Porcelana Geyser than in Porcelana or Cahuelmó hot springs (Figure 10A, B and C). In Geyser, the large number of OTUs with less than 5% of representativeness in the total community explains the high OTU richness showed in Table S1.

In Cahuelmó, a patchy pattern can be observed among samples at the right side of the grid (Figure 10A), in which dominance alternates between families such as Thermaceae (Deinococcus-Thermus) and Halomonadaceae (Proteobacteria). On the left side of the grid, on the other hand, a rather constant community composition was observed. In this side of the grid Proteobacteria, Deinoccocus-Thermus, Bacteroidetes, "Other" and Cyanobacteria co-dominated with smaller proportions of the assemblage made up by "novel" and Cloroflexi sequences. Abundance of novel sequences was considerably lower compared to Porcelana and Geyser communities, which might suggest that the sea influence, and anthropogenic contamination, in Cahuelmó could have replaced these "novel" groups with a more ubiquitous community members. In contrast to the other hot springs, Cyanobacteria were not the only dominant group,

and Chloroflexi were detected in small proportions and only in some samples (Fig. 10A), suggesting that external sources of carbon input might be more relevant than primary production in this hot spring.

In Geyser, the two most abundant OTU groups were Cyanobacteria and Bacteroidetes (Sphingobacteriales). Three main Cyanobacterial OTUs were apparent (*Mastigocladus sp.*, *Leptolyngbya sp*., and unknown cyanobacterium). It is remarkable that Chloroflexi was almost irrelevant in this mat (Figure 10B). The genus *Meiothermus* was present in low abundance in several samples of this hot spring. The community composition was fairly constant across all samples with only a couple of exceptions.

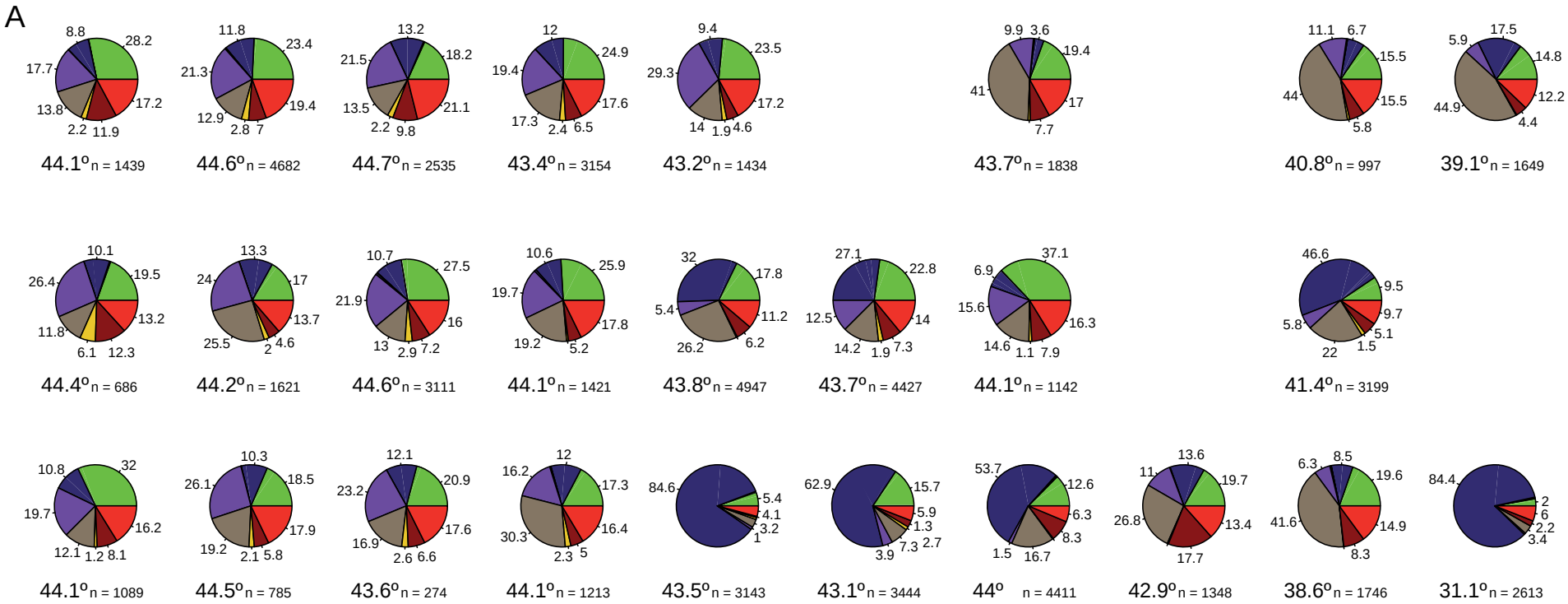
In Porcelana, phyla Cyanobacteria and Chloroflexi were the most abundant taxa while Bacteroidetes, Planctomycetes, and Deinococcus-Thermus were found in lower abundances. Although the temperature difference in the entire grid was only of 7.8 ºC, there were two clearly different patterns in community composition: Chloroflexi were more abundant at the highest temperatures, while Cyanobacteria radically increased their abundance until completely dominating at 55 to 45º C. Figure 10C shows how Chloroflexi dominated the upper left region of the mat, coinciding with the highest temperatures, while Cyanobacteria dominated the right side of the mat, which are the lowest temperature samples. Abundance of Deinococcus-Thermus showed a small increase with increasing temperature, with a higher relative abundance of 8.8% at 59.3º C, but were present in all samples in Porcelana (Figure 10C).

OTUs identified as Cyanobacteria and Chloroflexi were detected in the three hot springs (Figure 9), but they were rather dominant in Porcelana hot spring and Geyser, and not in Cahuelmó. Microbial mat in Geyser, however, showed higher abundance of Cyanobacteria in all samples collected, and Chloroflexi was rarely present (Figure 10B). On the other hand, Cahuelmó showed a very different community structure, as already revealed in the MDS analysis in Figure 8. Cyanobacteria were detected in less proportion than in Porcelana hot spring and Geyser. *Synechococcus* related OTUs were detected in most samples (Figure 9). Chloroflexi

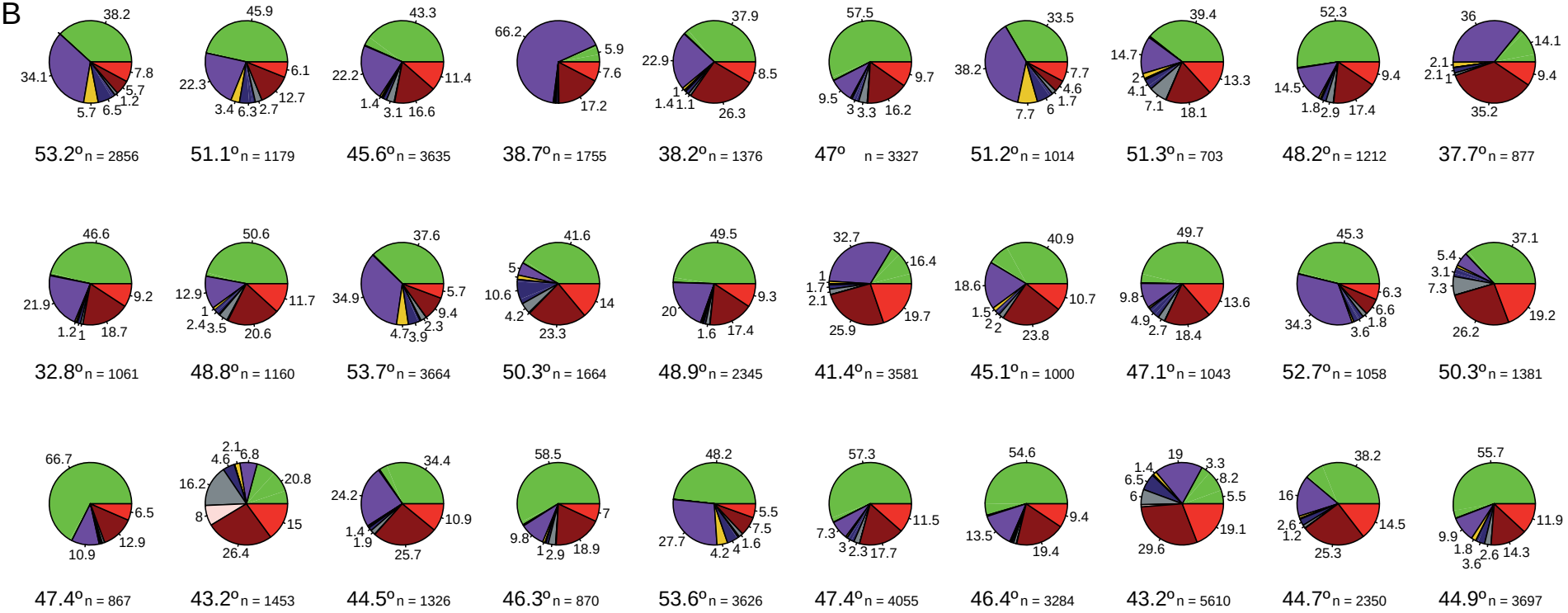
was barely detected, suggesting again that the microbial mat at this hot spring was subject of different environmental conditions.

The primary producers phyla of Cyanobacteria and Chloroflexi are both ubiquitous mat-forming phototrophs in neutral hot springs (Wang et al., 2013). In this study, the Cyanobacteria included oxygenic phototrophs such as genera *Synechococcus*, *Calothrix*, *Leptolyngbya*, *Nostoc* and *Mastigocladus*, whereas the latter included mixotrophs that performs anoxigenic photosynthesis (Liu et al., 2011) such as genera *Chloroflexus*, *Caldilinea*, and *Thermomicrobium.*

The variation in the identities of species among sites time was well described by Anderson et al. (2011), who distinguished two types of diversity shifts: variation and turnover. The difference between these two types of change is mostly due to the rare species. The first is non-directional, meaning that in a given spatial or temporal extent, the compared samples are discrete and are not connected to each other. Turnover, on the other hand, requires the investigator to define a specific gradient or directionality. Figure 8 shows the variation of community structure, by comparing diversity differences among three different hot springs separated by several kilometers from each other, with clear environmental distinctions. Figure 10 shows the patchiness were turnover at a microscale can be studied in order to describe the shift patterns in the fine-scale of microbial mats according to a narrow range temperature gradient.


A
8.8 28.2 17.7 13.8 17.2 2.2 11.9
44.1º n = 1439
11.8 23.4 21.3 12.9 19.4 2.8 7
44.6º n = 4682
13.2 18.2 21.5 13.5 21.1 2.2 9.8
44.7º n = 2535
12 24.9 19.4 17.6 17.3 2.4 6.5
43.4º n = 3154
9.4 23.5 29.3 17.2 14 1.9 4.6
43.2º n = 1434
9.9 3.6 19.4 41 17 7.7
43.7º n = 1838
11.1 6.7 15.5 44 15.5 5.8
40.8º n = 997
5.9 17.5 14.8 44.9 12.2 4.4
39.1º n = 1649
10.1 19.5 26.4 13.2 11.8 6.1 12.3
44.4º n = 686
13.3 24 17 13.7 25.5 2 4.6
44.2º n = 1621
10.7 27.5 21.9 16 13 2.9 7.2
44.6º n = 3111
10.6 25.9 19.7 17.8 19.2 5.2
44.1º n = 1421
32 17.8 5.4 11.2 26.2 6.2
43.8º n = 4947
27.1 22.8 12.5 14 14.2 1.9 7.3
43.7º n = 4427
6.9 37.1 15.6 16.3 14.6 1.1 7.9
44.1º n = 1142
46.6 9.5 5.8 9.7 5.1 22 1.5
41.4º n = 3199
10.8 32 19.7 16.2 12.1 1.2 8.1
44.1º n = 1089
10.3 26.1 18.5 17.9 19.2 2.1 5.8
44.5º n = 785
12.1 20.9 23.2 17.6 16.9 2.6 6.6
43.6º n = 274
12 17.3 16.2 16.4 30.3 2.3 5
44.1º n = 1213
84.6 5.4 4.1 1 3.2
43.5º n = 3143
62.9 15.7 5.9 1.3 3.9 7.3 2.7
43.1º n = 3444
53.7 12.6 6.3 1.5 16.7 8.3
44º n = 4411
13.6 19.7 26.8 13.4 17.7
42.9º n = 1348
6.3 8.5 19.6 41.6 14.9 8.3
38.6º n = 1746
84.4 2 6 2.2 3.4
31.1º n = 2613
Cyanobacteria
Bacteroidetes
Proteobacteria
Deinococcus–Thermus
Chloroflexi
Novel
Other

B


38.2 7.8 5.7 1.2 6.5 5.7 34.1
45.9 6.1 12.7 2.7 6.3 3.4 22.3
43.3 11.4 16.6 3.1 1.4 22.2
66.2 5.9 7.6 17.2
37.9 8.5 26.3 1.1 1.4 22.9
57.5 9.7 16.2 3.3 3 9.5
33.5 7.7 4.6 1.7 6 38.2
39.4 13.3 18.1 7.1 4.1 2 14.7
52.3 9.4 17.4 2.9 1.8 14.5
36 14.1 9.4 35.2 1 2.1 2.1
53.2º n = 2856
51.1º n = 1179
45.6º n = 3635
38.7º n = 1755
38.2º n = 1376
47º n = 3327
51.2º n = 1014
51.3º n = 703
48.2º n = 1212
37.7º n = 877
46.6 9.2 18.7 1 1.2 21.9
50.6 11.7 20.6 3.5 2.4 1 12.9
37.6 5.7 9.4 2.3 3.9 4.7 34.9
41.6 14 23.3 4.2 10.6 5
49.5 9.3 17.4 1.6 20
32.7 16.4 19.7 25.9 2.1 1.7 1
40.9 10.7 23.8 2 1.5 18.6
49.7 13.6 18.4 2.7 4.9 9.8
45.3 6.3 6.6 1.8 3.6 34.3
37.1 19.2 26.2 7.3 3.1 5.4
32.8º n = 1061
48.8º n = 1160
53.7º n = 3664
50.3º n = 1664
48.9º n = 2345
41.4º n = 3581
45.1º n = 1000
47.1º n = 1043
52.7º n = 1058
50.3º n = 1381
66.7 6.5 12.9 10.9
2.1 6.8 20.8 15 26.4 8 16.2 4.6
34.4 10.9 25.7 1.9 1.4 24.2
58.5 7 18.9 2.9 1 9.8
48.2 5.5 7.5 1.6 4 4.2 27.7
57.3 11.5 17.7 2.3 3 7.3
54.6 9.4 19.4 13.5
19 3.3 8.2 5.5 19.1 29.6 6 6.5 1.4
38.2 14.5 25.3 1.2 2.6 16
55.7 11.9 14.3 2.6 1.8 3.6 9.9
47.4º n = 867
43.2º n = 1453
44.5º n = 1326
46.3º n = 870
53.6º n = 3626
47.4º n = 4055
46.4º n = 3284
43.2º n = 5610
44.7º n = 2350
44.9º n = 3697
Cyanobacteria
Bacteroidetes
Chloroflexi
Proteobacteria
Planctomycetes
Actinobacteria
Novel
Other

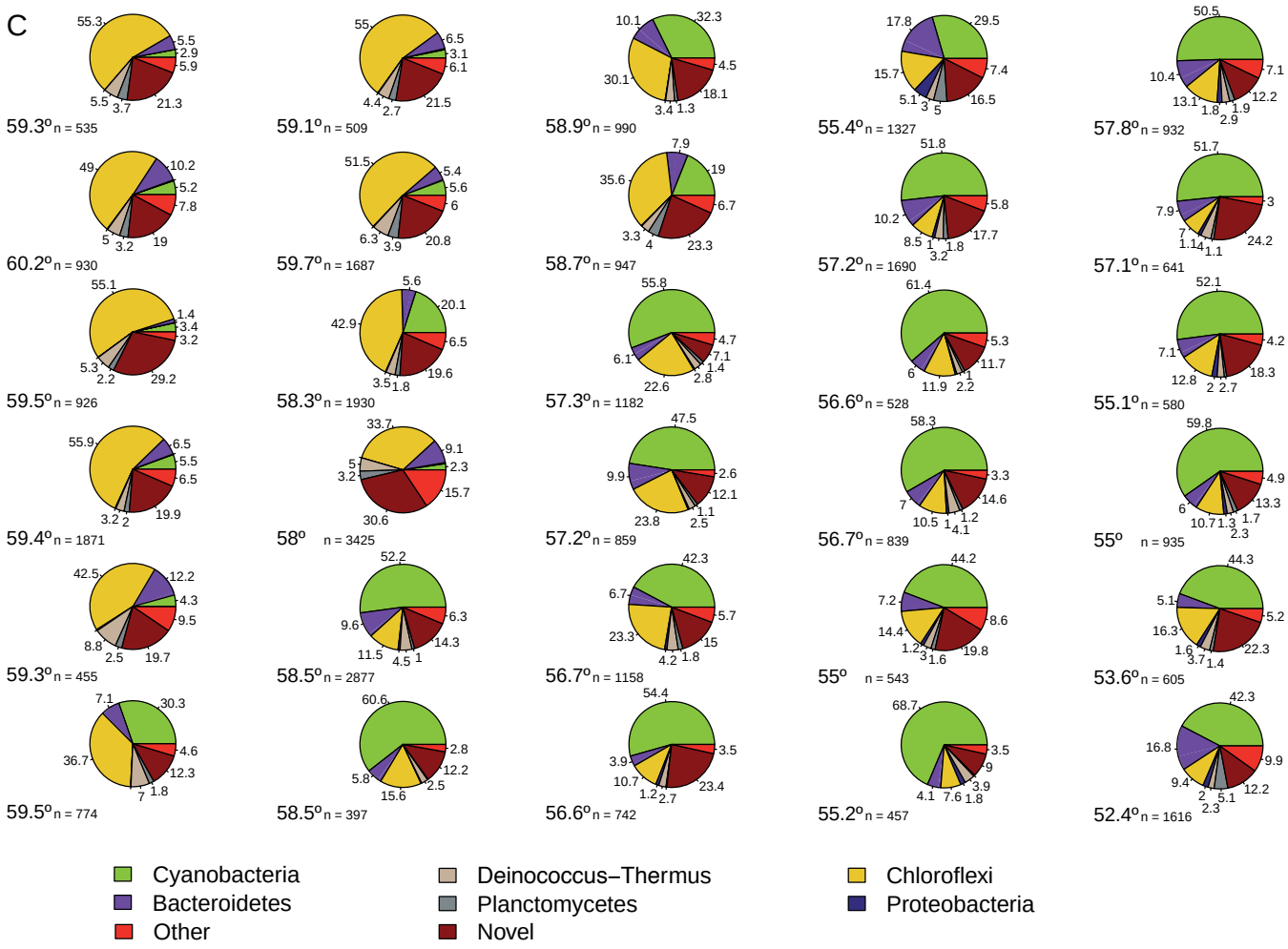


Figure 10: Taxonomic composition of bacterial communities in each sample. (A) Cahuelmó hot spring. (B) Porcelana geyser. (C) Porcelana hot spring.

Our results confirm a turnover pattern between both primary producers, as can be observed in Porcelana hot spring in Figure 11A (>55º C). In this figure, a negative relationship was determined in the ratio of Chloroflexi and Cyanobacteria regarding temperature. Cahuelmó and Geyser showed the lower ratio of Chloroflexi/Cyanobacteria, and Figure 11B and C showed that both populations lacked a relationship with temperature. This suggests a temperature-related turnover of both primary producers in the mat occurring at about 55-56 ˚C, but not at lower temperatures, where Cyanobacteria dominated. Cyanobacteria members showed a relative abundance decrease above 55° C threshold, especially in Stigonematales and Oscillatoriales members. These two Cyanobacterial groups showed different abundance decay when temperature increased, which may have ecological importance in the ecosystem. While Stigonematales members maintained their abundances until a drastic drop close to 60° C, Oscillatoriales diminished slowly as temperature increased. These results also suggests that the optimal temperature for Cyanobacteria might be around 45° C (Fig 11B). On the contrary, OTUs corresponding to Chloroflexi showed an overall increase with temperature, and were responsible for the turnover pattern observed in Figure 11A over 55º C. The genera *Bellilinea* and *Caldilinea* substituted each other along the temperature gradient, while *Chloroflexus* relative abundance increased drastically above 50 ˚C, where overcome Cyanobacteria (Figure 11C).

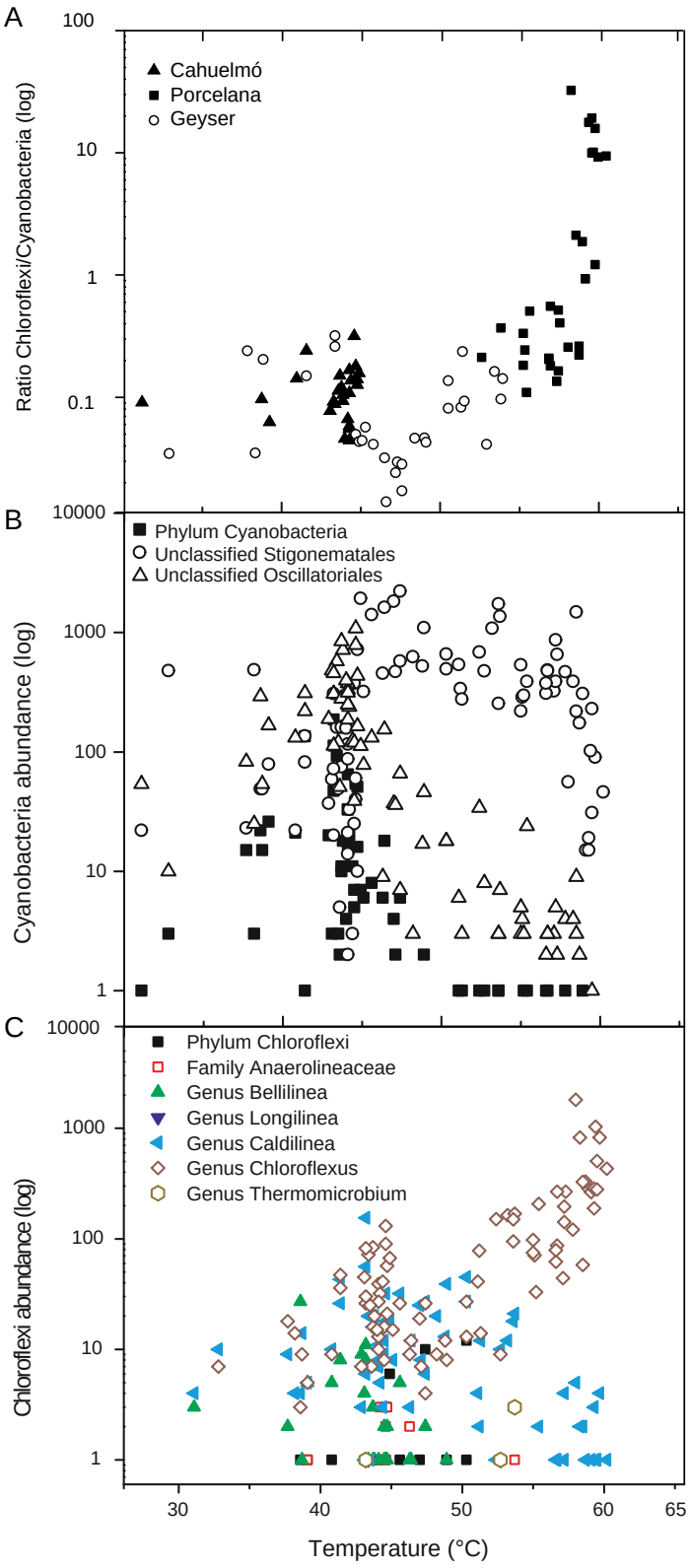


Figure 11: Scatter plots of primary producer-related OTU abundances recovered from the three hot spring grids versus temperature. (A) Ratio of Chloroflexi to Cyanobacteria with respect to temperature. (B) Abundance of different Cyanobacterial groups versus temperature. (C) Abundance of Chloroflexi according to temperature.

Other ecological relationships between thermophilic phototrophic primary producers has been previously reported in hot springs elsewhere. Skirnisdottir et al. (2000) described the presence of Stigonematales at approximately 65º-70º C in a neutral, slightly sulfide-rich microbial mat dominated by *Chloroflexus spp.* Roeselers et al. (2007), Sompong et al. (2005), Allewalt et al. (2006), and Miller et al. (2006 and 2009) also described the presence and interaction of *Synechococcus spp.*, and heterocystous cyanobacteria *Fischerella* and *Scytonema* species at around 60º C, but also the presence of *Chloroflexus spp*. This co-existence of phototrophs has been the subject of differing theories. Klatt et al. (2011) proposed that Cyanobacteria and Chloroflexi absorbed light at different wavelengths: Cyanobacteria perform oxygenic photosynthesis using the visible light spectrum, but chlorophototrophic groups such as *Roseiflexus spp*. have the potential to harvest near infrared light (850 to 900 nm light), explaining the presence of both in the same mat. The relative abundances of individual OTUs belonging to Chloroflexi and Cyanobacteria were rather offset with respect to each other, resulting in a negative relationship with temperature (Figure 11B and C). Similarly in Yellowstone National Park, Miller et al. (2009) stated that if different lineages of Cyanobacteria and Chloroflexi had co-adapted along a thermal gradient, it would be expected to observe tight niche overlap between members of both groups. Instead, they observed a strong negative correlation between the relative abundances of cyanobacteria and Chloroflexi as temperature increased close to 60º C, rejecting the co-adaptation hypothesis. On the other hand, a study of Wang et al. (2013) in hot springs of the Tibetan Plateau discussed that within the temperature range of 75–55º C, a positive correlation was observed between Cyanobacteria and Chloroflexi-like bacteria, suggesting that the later could be heterotrophic and may be dependent on organic carbon synthesized by Cyanobacteria for their growth. However, below 55º C the abundance of Cyanobacteria was negatively correlated with Chloroflexi, suggesting that Chloroflexi may become photoautotrophic and compete against Cyanobacteria for available nutrients and/or physical space.

In our study, results from Porcelana hot spring give sufficient data to confirm a turnover of both phototrophic primary producers, and a thermal niche clearly bordered at <55º C for Cyanobacteria, particularly *Mastigocladus,* and >55º C for Chloroflexi, principally *Chloroflexus,* outcompeting for space and light. Patchiness in Geyser did not show the same pattern, revealing the importance of sampling design and the microheterogeneity of bacterial community structure within a fine scale analysis. Cahuelmó showed a different community structure, as Cyanobacteria or Chloroflexi were not the only major components of the community. In Cahuelmó, Gammaproteobacteria, particularly *Halomonas* showed a high abundance in a patchy configuration (shown in pink in Figure 10A). The metabolic nature of Gammaproteobacteria suggests that microbial mat in Cahuelmó is mostly heterotrophic, and that organic carbon input might not be dependent solely on primary production. A possible explanation to this could be the fjord water exposure, which could contribute organic matter to the mats.

Regarding the heterotrophic groups, Proteobacteria was the most diverse considering richness and abundance, as previously mentioned. Most OTUs in the three hot springs belonging to class Alphaproteobacteria were found only at temperatures below 45º C (Fig 12A), and unclassified OTUs represented the largest number of reads. In general, BLAST identity percentage of unclassified OTUs was less than 60%, and particularly widely diverse within the class Alphaproteobacteria. The lowest relative abundance of Alphaproteobacteria was detected in Porcelana hot spring, represented by genus *Elioraea,* a slightly thermophilic, facultatively chemolithoorganotrophic genus found in hot springs in the Azores, which relative abundance increased with temperature up to 50º C, its optimum growth temperature (Alburquerque et al., 2008). The abundance of tentatively affiliated OTUs decreased with increasing temperature, with the exception of *Elioraea,* Also, the genera *Roseomonas* (thermotolerant) and *Rubritepida* (thermophilic), both heterotrophic aerobic bacteria commonly found in hot springs worldwide (Dong et al., 2014; Alarico et al., 2002), maintained their abundances to the highest temperatures, as their optimal growth ranged between 40º and 50° C.

Within class Betaproteobacteria, representatives of the order Burkholderiales were the most ubiquitous and abundant of all in the three hot springs. Bohórquez et al. (2012) and Jiménez et al. (2012) reported the dominance of this group in thermal water bacterial communities from acidic hot springs in Colombia. Some OTUs affiliated to the slightly thermophilic, chemolithoorganotrophic Betaproteobacteria belonging to family Comamonadaceae and in particular to the genus *Tepidimonas* (Moreira et al., 2000), were only found at 45˚C. Burkholderiales are commonly associated to nitrogen fixation in the rhizosphere (Masson-Boivin et al., 2009) and could probably have a role in the nitrogen cycle in these ecosystems. The remaining groups were much less represented (Figure 12B).

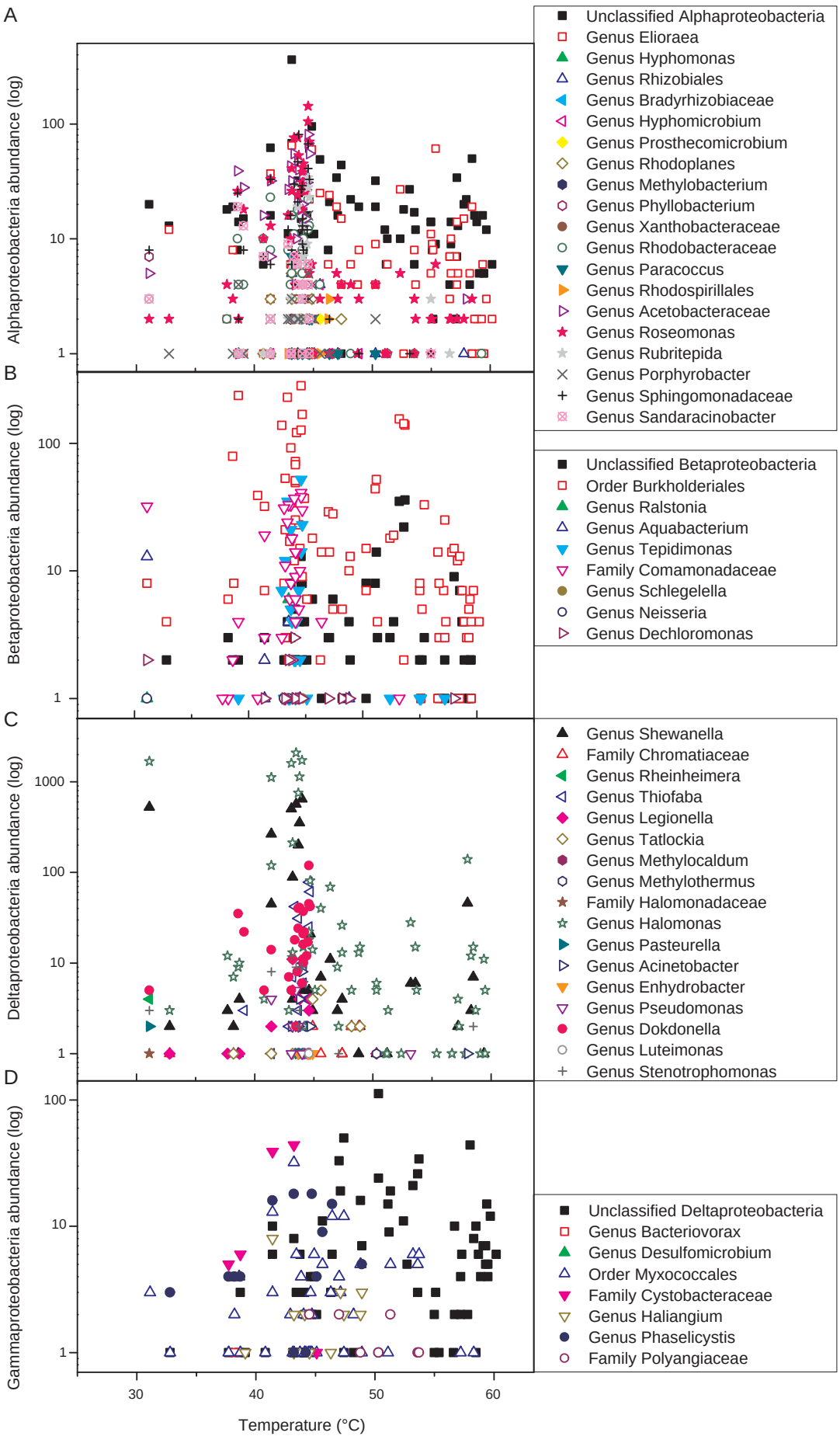
Class Gammaproteobacteria was represented mainly by genera *Dokdonella*, a mesophilic soil bacterium (Cunha et al., 2006); and *Thiofaba*, an obligately chemolithotrophic, thermophilic bacterium. Both OTUs showed an abundance peak around 45 ˚C in this study (Fig 12C), and have been detected in hot springs elsewhere (Mori et al., 2008).

Most OTUs belonging to class Deltaproteobacteria could not be assigned to lower taxonomic level within that class. The Order Myxococcales showed a wide temperature range below 50º C, and an abundance peak at a temperature close to 45º C, coinciding with the optimum growth temperature range of 45º - 50º C described by Iizuka et al. (2006) in Japan. The family Cystobacteraceae and its genus *Phaselicystis*, a heterotrophic mesophilic soil bacterium (García et al., 2009), were only found below 45 ˚C. Family Polyangiaceae and the genus *Haliangium*-like, a chemoautotrophic halophilic bacterium isolated from a coastal saline environment (Fudou et al., 2002) were present in a range of slightly higher temperatures (40-50º C and 45-54º C, respectively) in the three hot springs (Fig 12D). This is particularly interesting because there are no records of this genus at high temperatures.

In phylum Bacteroidetes, order Sphingobacteriales was the most abundant from 38º to almost 70º C. Also, a positive relationship could be observed between an unknown member of the Bacteroidetes group and temperature (Fig. 12E). Genus *Thermonema* was detected in high

abundance at >55º C, as mentioned earlier. Also, *Flexibacter*, a typical mesophilic heterotrophic genus that can be isolated from a variety of habitats including anoxic, acidic and sulphide-rich environments (Bernardet and Grimont, 1989) as well as families Cytophagaceae, and Chitinophagaceae were abundant in the three hot springs between 37 and 50 ˚C (Fig. 12E). OTUs related to potentially thermophilic, halophilic, heterotrophic bacterium isolated from submarine hot springs in Iceland (Alfredsson et al., 1988) that were closely related to the genus *Rhodothermus*, was abundant above 50º C (Figure 12E) in the three hot springs.

In the phylum Deincoccus-Thermus, genus *Meitothermus* was the most abundant at all temperatures sampled, and largely abundant in Cahuelmó. This heterotrophic thermophile can be found in natural or artificial hot environments, at a temperature range of 35º to 68º C (Albuquerque et al., 2010). Genus *Thermus*, much less abundant, was also detected in a narrower temperature range than *Meiothermus*, of approximately 38º to 55º C. Both genera are commonly found in hot springs (Brock and Freeze, 1969; Kristjansson and Alfredsson, 1983).


A
Alphaproteobacteria abundance (log)
100
10
1
Unclassified Alphaproteobacteria
Genus Elioraea
Genus Hyphomonas
Genus Rhizobiales
Genus Bradyrhizobiaceae
Genus Hyphomicrobium
Genus Prosthecomicrobium
Genus Rhodoplanes
Genus Methylobacterium
Genus Phyllobacterium
Genus Xanthobacteraceae
Genus Rhodobacteraceae
Genus Paracoccus
Genus Rhodospirillales
Genus Acetobacteraceae
Genus Roseomonas
Genus Rubritepida
Genus Porphyrobacter
Genus Sphingomonadaceae
Genus Sandaracinobacter
B
Betaproteobacteria abundance (log)
100
10
1
Unclassified Betaproteobacteria
Order Burkholderiales
Genus Ralstonia
Genus Aquabacterium
Genus Tepidimonas
Family Comamonadaceae
Genus Schlegelella
Genus Neisseria
Genus Dechloromonas
C
Deltaproteobacteria abundance (log)
1000
100
10
1
Genus Shewanella
Family Chromatiaceae
Genus Rheinheimera
Genus Thiofaba
Genus Legionella
Genus Tatlockia
Genus Methylocaldum
Genus Methylothermus
Family Halomonadaceae
Genus Halomonas
Genus Pasteurella
Genus Acinetobacter
Genus Enhydrobacter
Genus Pseudomonas
Genus Dokdonella
Genus Luteimonas
Genus Stenotrophomonas
D
Gammaproteobacteria abundance (log)
100
10
1
Unclassified Deltaproteobacteria
Genus Bacteriovorax
Genus Desulfomicrobium
Order Myxococcales
Family Cystobacteraceae
Genus Haliangium
Genus Phaselicystis
Family Polyangiaceae
30
40
50
60
Temperature (°C)

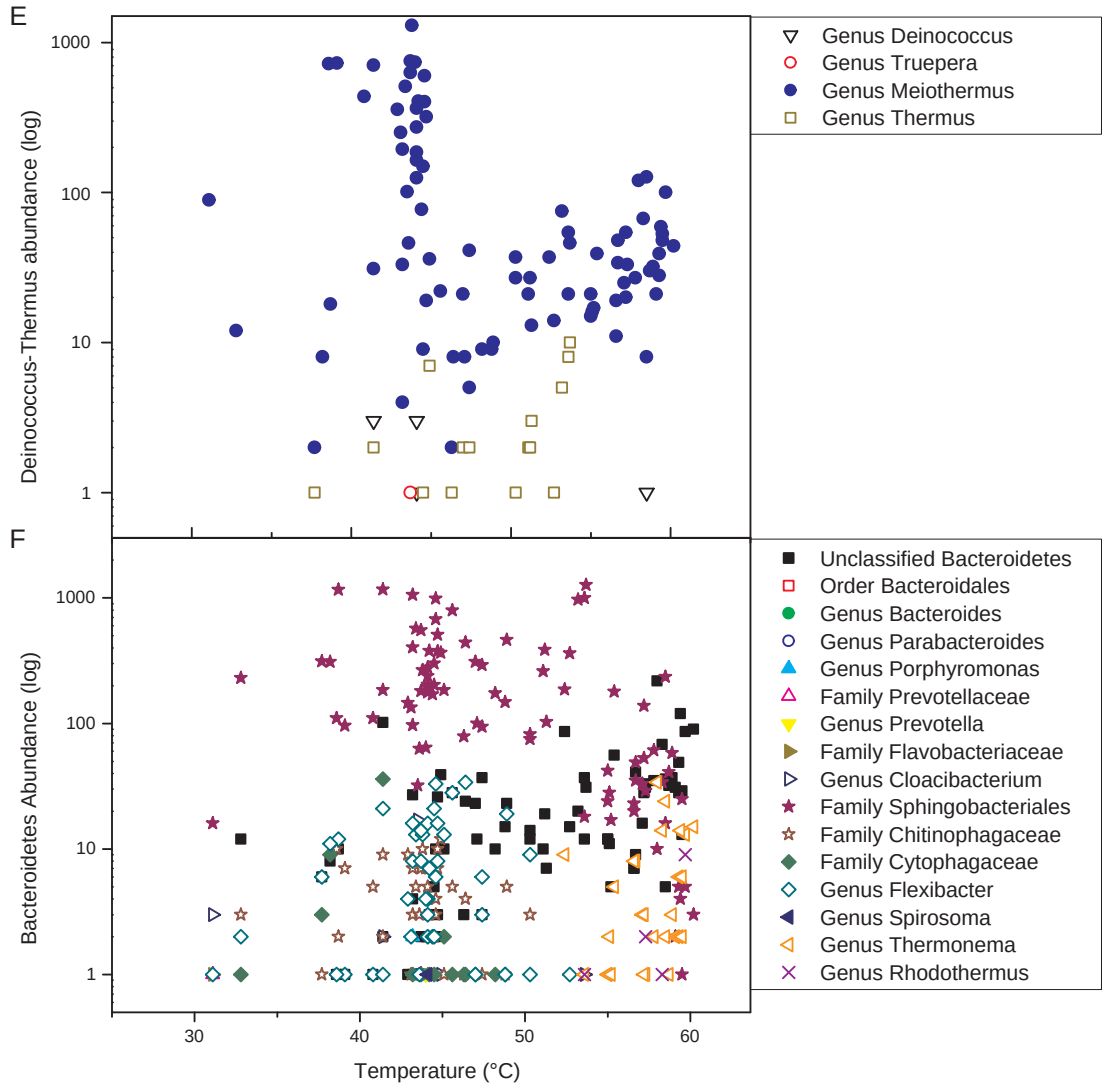


Figure 12: Scatter plots of relative abundance of most abundant groups in all hot springs versus temperature. (A) Alphaproteobacteria. (B) Betaproteobacteria. (C) Gammaproteobacteria. (D) Deltaproteobacteria. (E) Bacteroidetes. (F) Deinococcus-Thermus.

Alltogehter, our results suggest that the three hot springs studied shared less than 15% of the total OTUs detected, belonging to phyla Acidobacteria, Actinobacteria, Bacteroidetes, Chlamydiae, Chloroflexi, Chlorobi, Cyanobacteria, Deinococcus-Thermus, Firmicutes, Fusobacteria, Nitrospira, OP10, Planctomycetes, Proteobacteria, Spirochaetes, Thermodesulfobacteria, and Verrucomicrobia. Porcelana hot spring and Geyser were more similar to each other, probably due to their proximity and environment resemblance, while Cahuelmó, was strongly affected by human activities and seawater tides changing composition

and structure compared to the other two pristine thermophilic microbial mats. Bacterial diversity did not show any relationship to temperature. Richness, however, was negatively related to temperature, as both Richness and Chao1 diminished when temperature increased in all hot springs. Some OTUs belonging to heterotrophic phyla such as Bacteroidetes, Proteobacteria and Deinococcus-Thermus showed a temperature-related turnover. Most heterotrophic OTUs showed a clear peak of abundance at 45º C in the three hot springs. In Porcelana hot spring a turnover was observed between the later two phyla close to 55-56º C, dividing Cyanobacteria (lower than 55º C) from Chloroflexi (over 55º C).

Extreme ecosystems that are characterized by high dominance of particular organisms do not require extensive sampling to determine the main elements of their community structure than do less extreme environments. However, when exhausting mapping of the community structure is intended in an extreme ecosystem, the sampling size needed would be much larger than is generally used (Skirnisdottir et al., 2000). Microbial ecologists are needed to consider such sampling issues in order to effectively obtain representative snapshots of these interesting natural environments.

Acknowledgments

The authors are thankful for the sponsorship given by Fundación Huinay (Huinay Scientific Field Station www.huinay.cl) and their staff, who made possible the visits to the hot springs. This is the publication number 64 of Fundación Huinay. This work was supported by the cooperation project MIT-PUC, and projects FONDECYT nº 1110696 and FONDAP 15110009 funded by CONICYT, Chile.

REFERENCES

Adams M. W. W. and Kelly R. M. 1995. Enzymes from microorganisms in extreme environments. Chem. Eng. News Arch 73(51):32-42

Alarico S., Rainey F. A., Empadinhas N., Schumann P., Nobre M. F., da Costa M. S. 2002. *Rubritepida flocculans gen. nov., sp. nov.*, a new slightly thermophilic member of the alpha-1 subclass of the Proteobacteria. Syst Appl Microbiol. 25(2):198-206.

Albuquerque L., Rainey F. A., Nobre M. F. and da Costa M. S. 2008. *Elioraea tepidiphila gen. nov., sp. nov.*, a slightly thermophilic member of the Alphaproteobacteria. Int. J. Syst. Evol. Microbiol. 58:773–8.

Albuquerque L., F. a Rainey, M. F. Nobre, and M. S. da Costa. 2010. *Meiothermus granaticius sp. nov.*, a new slightly thermophilic red-pigmented species from the Azores. Syst. Appl. Microbiol. 33:243–246.

Alfredsson G. A., Kristjánsson J. K., Hjorleifsdottir S. and Stetter K. O. 1988. *Rhodothermus marinus, gen. nov., sp. nov.*, a thermophilic, halophilic bacterium from submarine hot springs in Iceland. J. Gen. Microbiol. 134:299–306.

Allewalt J. P., Bateson M. M., Revsbech N. P., Slack K. and Ward D. M. 2006. Effect of temperature and light on growth of and photosynthesis by *Synechococcus* isolates typical of those predominating in the octopus spring microbial mat community of Yellowstone National Park. Appl. Environ. Microbiol. 72:544–550.

Anderson M. J., Crist T. O., Chase J. M., et al. 2011. Navigating the multiple meanings of β diversity: a roadmap for the practicing ecologist. Ecology letters, 14:19–28.

Arahal D. R. and Ventosa A. 2006. The Family Halomonadaceae. In M. Dworkin et al. (eds). *Prokaryotes*. 6:811-835.

Baker, G.C., Smith, J.J. and Cowan, D.A. 2003. Review and re-analysis of domain-specific 16S primers. J Microbiol Meth. 55:541-555.

Becker J. M., Parkin T., Nakatsu C. H., Wilbur J. D. and Konopka A. 2006. Bacterial activity, community structure, and centimeter-scale spatial heterogeneity in contaminated soil. Microb. Ecol. 51:220–31.

Bernardet J. and Grimont P. A. D. 1989. Deoxyribonucleic acid relatedness and phenotypic characterization of *Flexibacter columnaris sp. nov., nom. rev., Flexibacter psychrophilus sp. nov., nom. rev.*, and *Flexibacter maritimus* Wakabayashi, Hikida, and Masumura. 1986. Int. J. Syst. Bacteriol. 39:346–354.

Bhaya D., Grossman A. R., Steunou A.-S., Khuri N., Cohan F. M., Hamamura N., Melendrez M. C., Bateson M. M., Ward D. M. and Heidelberg J. F. 2007. Population level functional diversity in a microbial community revealed by comparative genomic and metagenomic analyses. ISME J. 1:703–13.

Bohorquez, L. C., Delgado-Serrano, L., López, G., Osorio-Forero, C., Klepac-Ceraj, V., Kolter, R., Junca H, Baena, S, Zambrano, M. M. 2012. In-depth characterization via complementing culture-independent approaches of the microbial community in an acidic hot spring of the Colombian Andes. Microb Ecol, 63(1):103–115.

Brock T. D. 1969. Vertical zonation in hot spring algal mats. Phycologia 8:201–205.

Brock T. D. and Freeze H. 1969. *Thermus aquaticus gen. n. and sp. n.*, a non-sporulating extreme thermophile. J Bacteriol. 98(I), 289–297.

Caporaso J. G., Kuczynski J., Stombaugh J., Bittinger K., Bushman F. D., Costello E. K., Fierer N. et al. 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods. 7:335-336.

Casamayor E. O., Pedrós-Alió C., Muyzer G.and Amann R. 2002. Microheterogeneity in 16S Ribosomal DNA-defined bacterial populations from a stratified planktonic environment is related to temporal changes and to ecological adaptations. Appl Environ Microbiol, 68:1706–1714.

Chao A. 2005. Species richness estimation. In N. Balakrishnan, C. B. Read, and B. Vidakovic (eds). Encyclopedia of Statistical Sciences. Wiley, NY. pp 7909–7916

Ciccarelli, F. D., Doerks, T., Von Mering, C., Creevey, C. J., Snel, B., & Bork, P. (2006). Toward automatic reconstruction of a highly resolved tree of life. Science. 311(5765):1283–1287.

Collini E., Osores M. S., Folch A., Viramonte J. G., Villarosa G. and Salmuni G. 2013. Volcanic ash forecast during the June 2011 Cordón Caulle eruption. Nat Hazards. 66:389–412.

Cuecas A., Portillo M. C., Kanoksilapatham W. and Gonzalez J. M. 2014. Bacterial Distribution Along a 50 °C Temperature Gradient Reveals a Parceled Out Hot Spring Environment. Microb Ecol. DOI: 10.1007/s00248-014-0437-y

Cunha S., Tiago I., Pires A. L., da Costa M. S. and Veríssimo A. 2006 *Dokdonella fugitiva sp. nov.*, a Gammaproteobacterium isolated from potting soil. Syst. Appl. Microbiol. 29:191–6.

Demergasso C., Chong G., Galleguillos P., Escudero L., Martínez-Alonso M. and Esteve I. 2003. Microbial mats from the Llamará salt flat, Northern Chile. Rev. Chil. Hist. Nat. 76:485–499.

De León K. B., Gerlach R., Peyton B. M. and Fields M. W. 2013. Archaeal and bacterial communities in three alkaline hot springs in Heart Lake Geyser Basin, Yellowstone National Park. Front. Microbiol. 4:330.

De Los Ríos A., Ascaso C., Wierzchos J., Fernández-valiente E. and Quesada A. 2004. Microstructural Characterization of Cyanobacterial Mats from the McMurdo Ice Shelf, Antarctica. Appl. Environ. Microbiol. 70:569–580.

DeSantis T. Z., Hugenholtz P., Larsen N., Rojas M., Brodie E. L., Keller K. et al. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol 72: 5069–5072.

Dong, L., Ming, H., Yin, Y.-R., Duan, Y.-Y., Zhou, E.-M., Nie, G.-X. Feng, H.-C., Liu, L. and Li, W.-J. 2014. *Roseomonas alkaliterrae sp. nov*., isolated from an alkali geothermal soil sample in Tengchong, Yunnan, South-West China. Antonie van Leeuwenhoek, 105(5), 899–905

Dunckel A. E., Cardenas M. B., Sawyer A. H. and Bennett P. C. 2009. High-resolution in-situ thermal imaging of microbial mats at El Tatio Geyser, Chile shows coupling between community color and temperature. Geophys. Res. Lett. 36:1–5.

Edgar R. C., Haas B. J., Clemente J. C., Quince C., Knight R. 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 27(16):2194-2200

Everroad R. C., Otaki H., Matsuura K. and Haruta S. 2012. Diversification of Bacterial Community Composition along a Temperature Gradient at a Thermal Spring. Microbes and Environments, 27(4):374–381.

Franklin R. B. and Mills A. L. 2003. Multi-scale variation in spatial heterogeneity for microbial community structure in an eastern Virginia agricultural field. FEMS Microbiol. Ecol. 44:335–46.

Franklin R. B., Blum L. K., McComb A. C. and Mills A. L. 2002. A geostatistical analysis of small-scale spatial variability in bacterial abundance and community structure in salt marsh creek bank sediments. FEMS Microbiol. Ecol. 42:71–80.

Franks J. and Stolz J. F. 2009. Flat laminated microbial mat communities. Earth-Science Rev. 96:163–172.

Fudou R., Jojima Y., Iizuka T. and Yamanaka S. 2002. *Haliangium ochraceum gen. nov., sp. nov*. and *Haliangium tepidum sp. nov*.: Novel moderately halophilic myxobacteria isolated from coastal saline environments. J. Gen. Appl. Microbiol. 48:109–115.

Garcia R. O., Reichenbach H., Ring M. W. and Müller R. 2009. Phaselicystis flava gen. nov., sp. nov., an arachidonic acid-containing soil myxobacterium, and the description of Phaselicystidaceae fam. nov. Int. J. Syst. Evol. Microbiol. 59:1524–30.

Garrity G. M., Holt J. G., Hatchikian E. C., Olivier B., García J.-L. 2001. Phylum BIII Thermodesulfobacteria *phy. nov.* In D. R. Boone et al. (eds). *Bergey's Manual of Systematic Bacteriology.* Srpinger Science + Business media, NY. pp 389-393.

Ghosh D., Bal B., Kashyap V. K. and Pal S. 2003. Molecular Phylogenetic Exploration of Bacterial Diversity in a Bakreshwar (India) Hot Spring and Culture of *Shewanella*-related thermophiles. Appl Environ Microbiol, 69(7):4332–4336.

Gumerov V. M., Mardanov A. V., Beletsky A. V., Bonch-Osmolovskaya E. A. and Ravin N. V. 2011. Molecular analysis of microbial diversity in the Zavarzin Spring, Uzon Caldera, Kamchatka. Microbiology 80:244–251.

Hanada S. and Pierson B. K. 2006. The Family Chloroflexaceae. In M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, E. Stackebrandt (eds). *Prokaryotes*. Springer, Singapore. 7:815-842.

Huang Q., Dong C. Z., Dong R. M., Jiang H., Wang S., Wang G., Fang B., Ding X., Niu L., Li X., Zhang C., Dong H. 2011. Archaeal and bacterial diversity in hot springs on the Tibetan Plateau, China. Extremophiles 15:549–63.

Hughes J. B., Hellmann J. J., Ricketts T. H. and Bohannan B. J. M. 2001. Counting the Uncountable : Statistical Approaches to Estimating Microbial Diversity. Appl Environ Microbiol, 67:4399–4406.

Hugenholtz P., Pitulle C., Hershberger K. L., Pace N. R. 1998. Novel division level bacterial diversity in a Yellowstone hot spring. J. Bacteriol. 180:366–76.

Iizuka T., Tokura M., Jojima Y., Hiraishi A., Yamanaka S. and Fudou R. 2006. Enrichment and phylogenetic analysis of moderately thermophilic Myxobacteria from hot springs in Japan. Microbes Environ. 21:189–199.

Jiménez D. J., Andreote F. D., Chaves D., Montaña J. S., Osorio-Forero C., Junca H., Zambrano M. and Baena S. 2012. Structural and functional insights from the metagenome of an acidic hot spring microbial planktonic community in the Colombian Andes. PloS One, 7(12):e52069.

Jørgensen B. B. and Nelson D. C. 1988. Bacterial zonation, photosynthesis, and spectra light Distribution in Hot Spring Microbial Mats of Iceland. Microb Ecol. 16:133–147.

Kaye J. Z., Márquez M. C., Ventosa A. and Baross J. A. 2004. *Halomonas neptunia sp. nov., Halomonas sulfidaeris sp. nov., Halomonas axialensis sp. nov.* and *Halomonas hydrothermalis sp. nov.:* halophilic bacteria isolated from deep-sea hydrothermal-vent environments. Int. J. Syst. Evol. Microbiol. 54:499–511.

Klatt C. G., Liu Z., Ludwig M., Kühl M., Jensen S. I., Bryant D. A. and Ward D. M. 2013. Temporal metatranscriptomic patterning in phototrophic Chloroflexi inhabiting a microbial mat in a geothermal spring. ISMEJ. 7:1775–1789.

Kristjansson J. K. and Alfredsson G. A. 1983. Distribution of *Thermus spp.* in Icelandic hot springs and a thermal gradient. Appl. Environ. Microbiol. 45:1785–1789.

Lara L. E. 2009. The 2008 eruption of the Chaitén Volcano, Chile: a preliminary report. Andean Geology 36(1):125-129.

Lau M. C. Y., Aitchison J. C. and Pointing S. B. 2009. Bacterial community composition in thermophilic microbial mats from five hot springs in central Tibet. Extremophiles, 13(1):139–49

Letunic I. and Bork P. 2007. Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. Bioinformatics. 23(1):127–128.

Li Z. and Brand J. 2007. *Leptolyngbya nodulosa sp. nov*. (Oscillatoriaceae), a Subtropical marine cyanobacterium that produces a unique multicellular structure. Phycologia 46:396–401.

Liu Z., Klatt C. G., Wood J. M., Rusch D. B., Ludwig M., et al. 2011. Metatranscriptomic analyses of chlorophototrophs of a hot-spring microbial mat. ISME J 5:1279–1290.

Mackenzie R., Pedrós-Alió C. and Díez B. 2013. Bacterial composition of microbial mats in hot springs in Northern Patagonia: variations with seasons and temperature. Extremophiles. 17:123–136.

Madigan M. T., Martinko J. M. and Parker J. 1997. Brock Biology of Microorganisms. 8th edition, Prentice Hall NJ USA.

Madigan M. T. and Marrs B. L. 1997. Extremophiles. Scientific American. 4:82-87

Magurran A. E. 1988. Ecological Diversity and Its Measurement. 179 pp. Princeton University Press, NJ USA.

Magurran A. E. 2004. Measuring Biological Diversity. Chapter 5. Blackwell Publishing, Cornwall, UK. pp. 131-160.

Magurran A. E. and Henderson P. A. 2003. Explaining the excess of rare species in natural species abundance distributions. Letters to Nature 422:714-716.

Masson-Boivin C., Giraud E., Perret X. and Batut J. 2009. Establishing nitrogen-fixing symbiosis with legumes: how many rhizobium recipes? Trends Microbiol. 17:458–466.

Mardanov A. V., Gumerov V. M., Beletsky A. V., Perevalova A. A., Karpov G. A., Bonch-Osmolovskaya E. A. and Ravin N. V. 2011. Uncultured archaea dominate in the thermal groundwater of Uzon Caldera, Kamchatka. Extremophiles 15:365–72.

Meyer-Dombard D. R., W. Swingley, J. Raymond, J. Havig, E. L. Shock, and R. E. Summons. 2011. Hydrothermal ecotones and streamer biofilm communities in the Lower Geyser Basin, Yellowstone National Park. Environ. Microbiol. 13:2216–31.

Miller S. R., Strong A. L., Jones K. L., Ungerer M. C. 2009a. Bar-coded pyrosequencing reveals shared bacterial community properties along the temperature gradients of two alkaline hot springs in Yellowstone National Park. Appl Environ Microbiol 75: 4565–4572.

Miller S. R., Williams C., Strong A. L. and Carvey D. 2009b. Ecological specialization in a spatially structured population of the thermophilic cyanobacterium *Mastigocladus laminosus*. Appl. Environ. Microbiol. 75:729–34.

Moreira C., F. a Rainey, M. F. Nobre, M. T. da Silva, and M. S. da Costa (2000) Tepidimonas ignava gen. nov., sp. nov., a new chemolithoheterotrophic and slightly thermophilic member of the beta-Proteobacteria. Int. J. Syst. Evol. Microbiol. 50 Pt 2:735–42.

Mori K., and K. Suzuki (2008) Thiofaba tepidiphila gen. nov., sp. nov., a novel obligately chemolithoautotrophic, sulfur-oxidizing bacterium of the Gammaproteobacteria isolated from a hot spring. Int. J. Syst. Evol. Microbiol. 58:1885–91.

Myers N., R. A Mittermeier, C. G. Mittermeier, G. A da Fonseca, and J. Kent. (2000) Biodiversity hotspots for conservation priorities. Nature 403:853–8.

Nakagawa T. and Fukui M. 2002. Phylogenetic characterization of microbial mats and streamers from a Japanese alkaline hot spring with a thermal gradient. J. Gen. Appl. Microbiol. 48:211–22.

Nakagawa T. and Fukui M. 2003. Molecular Characterization of Community Structures and Sulfur Metabolism within Microbial Streamers in Japanese Hot Springs. Appl. Environ. Microbiol. 69:7044–7057.

Oksanen J., Blanchet F. G., Kindt R., Legendre P., Minchin P. R., O'Hara R. B., Simpson G. L., Solymos P., Stevens M. H. H., Wagner H. (2012) Vegan: Community Ecology Package. R package version 2.0-5. http://CRAN.R-project.org/package=vegan

Oren A. (2004) Prokaryote diversity and taxonomy: current status and future challenges. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 359:623–638.

Oren A. (2006) The Genera *Rhodothermus, Thermonema, Hymenobacter* and *Salinibacter*. In *Prokaryotes*. Eds. M Dworkin, S Falkow, E Rosenberg, K-H Schleifer, E Stackebrandt. 7:712-738. Springer, Singapore.

Pedrós-Alió, C. (2007). Dipping into the Rare Biosphere. Science, 315, 192.

Pentecost A. (2003) Cyanobacteria associated with hot spring travertines. Can. J. Earth Sci. 1457:1447–1457.

Portillo M. C., Sririn V., Kanoksilapatham W. and Gonzalez J. M. 2009. Differential microbial communities in hot spring mats from Western Thailand. Extremophiles 13:321–331.

Preheim S. P., Perrotta A. R., Martin-Platero A. M., Gupta A. and Alm E. J. 2013. Distribution-Based Clustering: Using Ecology to Refine the Operational Taxonomic Unit. Appl Environ Microbiol 29(71):6593-6603.

Price M. N., Dehal P. S. and Arkin A. P. 2010. FastTree 2-Approximately Maximum-Likelihood Trees for Large Alignments. Plos One 5(3):e9490

Pueyo S. 2006. Diversity: between neutrality and structure. OIKOS, 112, 392–405.

R Core Team. 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

Ramsing NB, Ferris MJ, Ward DM. 2000. Highly ordered vertical structure of *Synechococcus* populations within the one-millimeter-thick photic zone of a hot spring cyanobacterial mat. Appl. Environ. Microbiol. 66:1038–49.

Roeselers G., Norris T. B., Castenholz R. W., Rysgaard S., Glud R. N., Kühl M. and Muyzer G. 2007. Diversity of phototrophic bacteria in microbial mats from Arctic hot springs (Greenland). Environ. Microbiol. 9:26–38.

Schloss P. D., Westcott S. L., Ryabin T., et al. 2009. Introducing Mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75(23): 7537–41.

Sharp C. E., Brady A. L., Sharp G. H., Grasby S. E., Stott M. B., and Dunfield P. F. 2014. Humboldt's spa: microbial diversity is controlled by temperature in geothermal environments. ISME J. 8:1166–1174.

Skirnisdottir S., Hreggvidsson G. O., Hjörleifsdottir S., Marteinsson V. T., Petursdottir S., Holst O. and Kristjansson J. K. 2000. Influence of Sulfide and Temperature on Species Composition and Community Structure of Hot Spring Microbial Mats. Appl. Environ. Microbiol. 66:2835–2841.

Sompong U., Hawkins P. R., Besley C. and Peerapornpisal Y. 2005. The distribution of cyanobacteria across physical and chemical gradients in hot springs in northern Thailand. FEMS Microbiology Ecology 52:365–76.

Stamatakis A, Hoover,P. Rougemont J. (2008) A Rapid Bootstrap Algorithm for the RAxML Web-Servers, Systematic Biology, 75(5): 758-771.

Stegen J. C., Lin X., Konopka A. E. and Fredrickson J. K. 2012. Stochastic and deterministic assembly processes in subsurface microbial communities. The ISME Journal, 6(9), 1653–64.

Stern C. R. 2004. Active Andean volcanism: its geologic and tectonic setting. Rev. Geol. Chile 31(2): 161:206.

Steunou A.-S., Bhaya D., Bateson M. M., Melendrez M. C., Ward D. M., Brecht E., Peters J. W., Kühl M. and Grossman A. R. 2006. In situ analysis of nitrogen fixation and metabolic switching in unicellular thermophilic cyanobacteria inhabiting hot spring microbial mats. PNAS 103:2398–403.

Steunou A.-S., Jensen S. I., Brecht E., Becraft E. D., Bateson M. M., Kilian O., Bhaya D., Ward D. M., Peters J. W., Grossman A. R. and Kühl M. 2008. Regulation of nif gene expression and the energetics of N2 fixation over the diel cycle in a hot spring microbial mat. ISME J. 2:364–78.

Tekere M., Lötter A., Olivier J., Jonker N. and Venter S. 2011. Metagenomic analysis of bacterial diversity of Siloam hot water spring, Limpopo, South Africa. African J. Biotechnol. 10:18005–18012.

Tenreiro S., M. F. Nobre, and M. S. D. A. Costa. 1995. *Thermus silvanus* sp. nov. and *Thermus chliarophilus* sp. nov., Two New Species Related to *Themus ruber* but with Lower Growth Temperatures. Int. J. Syst. Bacteriol. 45:633–639.

Tobler D. J., and L. G. Benning. 2011. Bacterial diversity in five Icelandic geothermal waters: temperature and sinter growth rate effects. Extremophiles 15:473–85.

Urbieta M. S., González-Toril E., Giaveno M. A., Aguilera-Bazán A., Donati E. R. 2014. Archaeal and bacterial diversity in five different hydrothermal ponds in the Copahue region in Argentina. Syst. Appl. Microbiol. 37:429–41.

Van der Meer M. T. J., Schouten S., Jaap S., Damsté S., De Leeuw J. W., Ward D. M., and Damste J. S. S. 2003. Compound-specific isotopic fractionation patterns suggest different carbon metabolisms among *Chloroflexus*-like bacteria in hot-spring microbial mats. Appl. Environ. Microbiol. 69:6000–6006.

Van Gemerden, H. 1993. Microbial mats: A joint venture. Mar. Geol. 113:3–25.

Vick T. J., Dodsworth J. A., Costa K. C., Shock E. L. and Hedlund B. P. 2010. Microbiology and geochemistry of Little Hot Creek, a hot spring environment in the Long Valley Caldera. Geobiology. 8:140–54.

Wang Q. G., Garrity M., Tiedje J. M., Cole J. R. 2007. Naive Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. Appl Environ Microbiol. 73(16):5261-7.

Wang S., Hou W., Dong H., Jiang H., Huang L. and Wu G. 2013. Control of Temperature on Microbial Community Structure in Hot Springs of the Tibetan Plateau, 8(5).

Ward D. M., Ferris M. J., Nold S. C. and Bateson M. M. 1998. A Natural View of Microbial Biodiversity within Hot Spring Cyanobacterial Mat Communities. Microbiol. Mol. Biol. Rev. 62:1353–1370.

Watve M. G. and Gangal R. M. 1996. Problems in measuring bacterial diversity and a possible solution. Appl Environ Microbiol. 62, 4299–301.

Weltzer M. L. and Miller S. R. 2013. Division-specific differences in bacterial community assembly of two alkaline hot spring ecosystems from Yellowstone National Park. Microb Ecol, 65, 537–40.

Supplementary Material

Table S1: Temperature of microbial mat samples, total 16S reads per sample, and number of OTUs at 0.97 identity.

Hot spring	Sample	Temperature (ºC)	Total reads	OTUs (0.97)
Porcelana	F6	57.8	922	54
	F7	55.4	1316	45
	F8	58.9	943	48
	F9	59.1	474	45
	F10	59.3	512	42
	F1	57.1	626	34
	F2	57.2	1675	56
	F3	58.7	918	63
	F4	59.7	1596	63
	F5	60.2	881	60
	E6	55.1	552	40
	E7	56.6	505	29
	E8	57.3	1170	55
	E9	58.3	1910	68
	E10	59.5	909	37
	D1	55	893	59
	D2	56.7	825	47
	D3	57.2	817	35
	D4	58	3398	76
	D5	59.4	1838	60
	D6	53.6	573	52
	D7	55	501	53
	D8	56.7	1131	54
	D9	58.5	2838	80
	D10	59.3	441	48
	E1	52.4	1605	66
	E2	55.2	432	26
	E3	56.6	690	38
	E4	58.5	361	28
	E5	59.5	758	46
Cahuelmó	A1	44.1	1340	81
	A2	44.6	4630	136
	A3	44.7	2369	115
	A4	43.4	2948	107
	A5	43.2	1380	91
	A7	43.7	1834	107
	A9	40.8	991	75

	A10	39.1	1622	99
	B1	44.4	653	74
	B2	44.2	1583	97
	B3	44.6	3090	107
	B4	44.1	1413	88
	B5	43.8	4944	104
	B6	43.7	4419	135
	B7	44.1	1132	92
	B9	41.4	3197	109
	C1	44.1	1035	82
	C2	44.5	779	80
	C3	43.6	271	53
	C4	44.1	1201	86
	C5	43.5	3143	63
	C6	43.1	3440	79
	C7	44	4408	86
	C8	42.9	1338	93
	C9	38.6	1734	100
	C10	31.1	2613	78
Geyser	G1	53.2	2841	53
	G2	51.1	1169	61
	G3	45.6	3575	143
	G4	38.7	1751	80
	G5	38.2	1354	85
	G6	47	3248	109
	G7	51.2	1010	41
	G8	51.3	700	69
	G9	48.2	1195	75
	G10	37.7	868	74
	H1	32.8	1049	65
	H2	48.8	1147	89
	H3	53.7	3624	78
	H4	50.3	1623	111
	H5	48.9	2317	96
	H6	41.4	3565	162
	H7	45.1	985	83
	H8	47.1	1020	92
	H9	52.7	1051	56
	H10	50.3	1378	98
	I1	47.4	862	51
	I2	43.2	1436	122
	I3	44.5	1232	93
	I4	46.3	802	80

I5	53.6	3596	67
I6	47.4	3997	120
I7	46.4	3266	120
I8	43.2	5591	194
I9	44.7	2326	111
I10	44.9	3676	134

Chapter IV

Biogeography of thermophilic communities in hot spring microbial mats in a latitudinal gradient in the Americas.

Roy Mackenzie, German Marchandon, Lorena Uribe, Maria Estrella Alcaman, Carlos Pedrós-Alió, Beatriz Díez

Abstract

The biogeography of microorganisms has been a matter of debate for several decades. With the development of high throughput sequencing techniques this question can now be addressed. We analyzed the bacterial composition of microbial mats in hot springs (pH 6-8) spanning a latitudinal gradient from northern Costa Rica to Deception Island in Antarctica. Hot springs can be considered as "hot islands" in a "cold ocean," and we could assess the relative importance of local environmental factors versus bacterial dispersal limitation on the taxonomic composition of the mats. Communities at the highest temperatures (60-70 degrees) were very similar regardless of distance, indicating that at extreme temperatures, dispersal limitation was not a factor. These communities were dominated by a single OTU of Chloroflexi despite the thousands of km of separation. At lower temperatures (<50 degrees), however, distance was progressively more important. Different cyanobacterial *Mastigocladus*-like OTUs dominated in the northern and southern hemispheres at intermediate temperatures (40-60ºC). Finally, at the coldest temperatures, the dominant cyanobacteria belonged to different genera such as *Leptolyngbya*, *Phormidium* or *Limnothrix*. Other dominant bacterial OTUs were *Chloroflexus*-like and, to a lesser extent, Alphaproteobacteria, Sphingobacteria, and Thermales. Overall, distance and temperature were similarly important in determining the composition of the mats. The distance decay relationship was significant at different spatial scales: global, local and regional, but not at the continental scale. Biogeography of bacteria is, therefore, the consequence of both dispersal limitation and environmental effects.

INTRODUCTION

Whether microorganisms have a biogeography or not has been a matter of active debate for several decades (Finlay 2002, O'Malley 2008). The traditional view was nicely summarized by L.G.M. Baas Becking (building on ideas of M.W. Beijerinck) in 1934, as "everything is everywhere, but the environment selects" (de Wit and Bouvier 2006). This statement assumes that microorganisms do not experience dispersal limitation. They are found all over the Planet and, in principle, they can be isolated in pure culture from any sample. The local environmental conditions, however, determine which ones actually grow and are abundant at each particular habitat. Finlay (2002) extended this position by remarking that microorganisms are very small and have extremely large populations. These two properties would explain their dispersal without barriers. And he and colleagues used the case of ciliate morphospecies as an example.

Other scientists considered that morphospecies did not provide sufficient resolution to discriminate "true" biological species and, therefore, morphospecies were lumping together very different populations and failing to recognize differential distribution. Thus, Whitaker et al. (2003) used the ITS region of *Sulfolobus* isolates to demonstrate an effect of distance on the distribution of different phylotypes in acidic hot springs. Likewise, Papke et al. (2003) found similar results with *Synechoccocus* ITS sequences retrieved directly from neutral hot springs, by cloning and qPCR. And Miller et al. (2007) did a similar study with *Fischerella-Mastigocladus* strains. The three studies analyzed particular bacteria from hot springs. Thus, these authors claimed, when proper taxonomic resolution was used, dispersal limitation was found to be important for bacteria. At least for these bacterial specialists of hot springs.

Martiny et al. (2006) reviewed the situation and proposed a framework defining four possible alternative situations. 1) Microorganisms are distributed at random in space. This would be the null hypothesis. 2) Environmental variation (contemporary conditions) is the only factor determining species distributions. This would be equivalent to the Baas-Becking statement. 3) Dispersal limitation (and thus historical events) is the main factor, and 4) both

environmental factors and dispersal limitation are important. These authors reviewed the 10 studies available at the time and found that in the two studies analyzing space scales larger than 10000 km, distance was the only significant factor. For the two studies analyzing scales of less than 1 km, the environment was the only significant factor. Finally, the six studies with intermediate spatial scales showed mixed results.

Hot springs are particularly adequate for these approaches (Papke et al. 2003, Whitaker et al. 2003, Miller et al. 2007, Takacs-Vesbach et al. 2008; Sharp et al 2014). They can be considered as "hot islands" surrounded by "cold oceans". The thermophilic bacteria and archaea inhabiting such hot islands cannot grow in the cold environments surrounding them. Therefore, whether they are present everywhere (in all hot springs) or not is a good test of the relevance of both environmental factors and dispersal limitation.

In the previous studies, the targeted organism was either isolated or its rDNA amplified from several spots with different temperatures in several hot springs, many times with different pH and chemistry. Moreover, the samples where the target organism did not appear were eliminated from the study. This treatment of the data does not consider the abundance of the organisms at the different spots. In effect, it only considers presence-absence data. But the difference between presence and absence is a subtle one for microorganisms. The so-called rare biosphere includes literally thousands of taxa in extremely low abundance (Sogin et al. 2006). The deeper the sampling and sequencing, the more taxa are retrieved (Pedrós-Alió 2012). What is the sequencing effort needed to determine that a given organisms is not present? On top of this, presence-absence data eliminate the effect of the environment on the abundance of the target organism. At one temperature, the organism may be the dominant one and at a few degrees lower temperature it may be part of the rare biosphere. Yet, the previous approach would treat all the occurrences as equivalent. We think that considering the abundance, the effects of both distance and environment should be more apparent. In the particular case of hot springs, presence-absence data basically eliminate the likely effects of temperature on community composition and, thus, of the most important environmental factor in hot springs.

Not surprisingly, no effects of the environment were found in three of the studies (Martiny et al., 2006, Tacaks-Vesbacch et al., 2008). Sharp et al. (2014) sampled hot environments covering a temperature range of over 90º C surveying the microbial diversity from several hot environment from New Zealand and Canada, and concluded that species richness and diversity indices were strongly correlated to temperature.

Similar temperature effects were found by Miller et al. (2009) in hot springs from Yellowstone National Park, were they probed the niche differentiation of dominant *Mastigocladus* populations due to temperature, and proposed that such sympatric differentiation could represent a source of innovation and evolution in thermophilic microbial populations.

Another point is that different bacteria coexisting in the same habitat may be subject to different degrees of either dispersal limitation or environmental selection, depending on their life history traits. Thus, we analyzed the whole community. This allowed evaluation of the effects of distance and environment on the whole community composition, on the one hand, and on individual members of it on the other. Finally, thanks to the distribution of our samples, we could also analyze whether the mat composition followed any latitudinal patterns.

Material and Methods

Study area and sampling

Table 1 shows the samples taken together with date, location, and a few environmental parameters. We chose two sites in Northern Costa Rica's Cordillera de Guanacaste: Hornitas de Miravalles (MV) and Río Negro (RN); two more sites at the Cordillera Central, Bajo las Peñas (BP) and Santa Teresita, and two more in the southern Cordillera de Talamanca: Rocas Calientes (RC) and Río Cotón. Río Cotón did not have any substantial mats at the time of sampling and the mats from Santa Teresita produced very few sequences and were discarded. In Northern Chile, we sampled different spots at El Tatio Geyser Field (GA). In Southern Chile we

collected samples from three different hot springs in Northern Patagonia: Cahuelmó (C), Porcelana hot spring (P) and Porcelana Geyser (G). Finally, we sampled a mat in Kroner Lake, Deception Island, Antarctica (K). The distances between samples within systems and among systems can be seen in Figure 1.

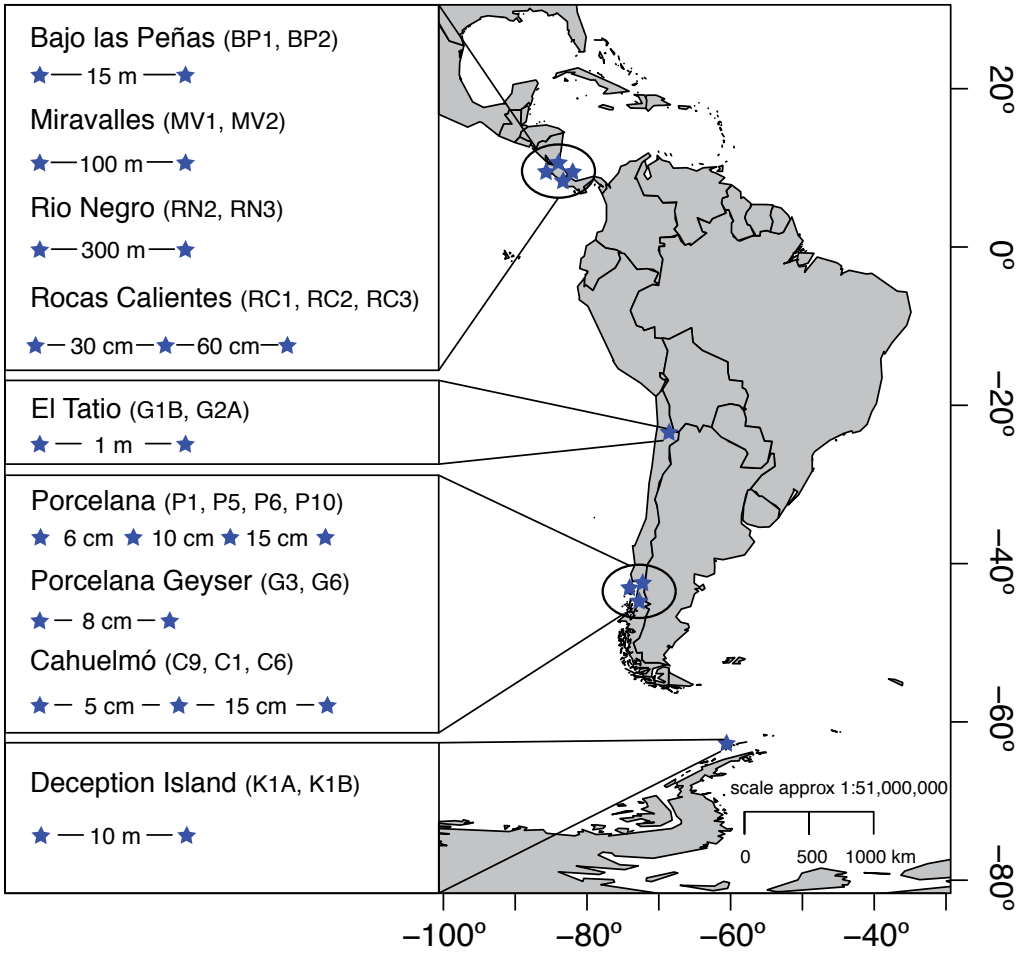


Figure 1: Map of the nine sites sampled in Central and South America, and in Antarctica. The left side shows the distance between the different samples collected in each site and the codes used in the paper.

Table 1. Location, selected environmental parameters, number of tags per sample after cleaning and quality filtering, and OTU richness before and after normalization by rarefaction. Singletons and doubletons represented less than 1% in all samples. (*) Number and percentage of tags from raw data after denoising and quality filtering.

Sample	Spring	Geographical area	Date	Latitude	Longitude	Temperature (ºC)	pH	Conductivity µS/cm	Tags (% of total)*	OTUs	OTUs Normalized
		Central America		**North**	**West**						
RN2	Río Negro	North Costa Rica	25 Jan 2012	10º 44' 26.4"	85º 21' 03.13"	55	6	1120	27630 (79.4)	710	638
RN3	Río Negro	North Costa Rica	25 Jan 2012	10º 44' 26.4"c	85º 21' 03.13"	59	6	1100	34361 (71.6)	249	220
MV1	Miravalles	North Costa Rica	24 Jan 2012	10º 42' 33.5"	85º 11' 43.9"	49	6	811	27076 (75.8)	269	248
MV2	Miravalles	North Costa Rica	24 Jan 2012	10º 42' 33.5"	85º 11' 43.9"	42	7	713	47177 (79.2)	434	327
BP1	Bajo las Peñas	Central Costa Rica	20 Jan 2012	9° 59' 37.2"	83° 48' 055"	50.2	7	>1990	25146 (79.5)	284	264
BP2	Bajo las Peñas	Central Costa Rica	20 Jan 2012	9° 59' 37.2"	83° 48' 055"	37.2	8		22615 (66.2)	357	339
RC1	Rocas Calientes	South Costa Rica	28 July 2012	9°18´10.11”	83°17´52.07”	63	6	2265	23975 (67.9)	147	144
RC2	Rocas Calientes	South Costa Rica	28 July 2012	9°18´10.11”	83°17´52.07”	60			22936 (77.3)	262	253
RC3	Rocas Calientes	South Costa Rica	28 July 2012	9°18´10.11”	83°17´52.07”	60	6.2	2310	48136 (79.9)	429	309
		South America		**South**	**West**						
G1B	El Tatio Geyser	North Chile	5 Dec 2010	22° 19.772'	68° 00.584'	70	7.1	2550	42223 (69.5)	69	57
G2A	El Tatio Geyser	North Chile	6 Dec 2010	22° 19.524'	68° 00.481'	54	6.8		21018 (74.2)	434	433
C9	Cahuelmó	South Chile	12 Mar 2011	42º 15’ 11.8’’	72º 22’ 4.4’’	40.8	7	119	23557 (76.5)	379	368

C1	Cahuelmó	South Chile	12 Mar 2011	42º 15’ 11.8’’	72º 22’ 4.4’'	44.1	7	119	30039 (76.1)	444	393
C6	Cahuelmó	South Chile	12 Mar 2011	42º 15’ 11.8’’	72º 22’ 4.4’'	43.1	7	119	21455 (82.9)	114	113
P10	Porcelana	South Chile	15 Mar 2011	42º 27’ 29.1’’	72º 27’ 39.3’’	59.5			20757 (76.8)	93	93
P1	Porcelana	South Chile	15 Mar 2011	42º 27’ 29.1’’	72º 27’ 39.3’’	52.4	6 - 7	1413	65905 (79.5)	211	131
P5	Porcelana	South Chile	15 Mar 2011	42º 27’ 29.1’’	72º 27’ 39.3’’	59.5			41414 (74.9)	126	93
P6	Porcelana	South Chile	15 Mar 2011	42º 27’ 29.1’’	72º 27’ 39.3’’	55.1			33436 (84.1)	307	247
G3	Porcelana Geyser	South Chile	19 Mar 2011	42º 24' 51"	72º 29' 2.2"	53.7	7 - 8	1717	31815 (81.5)	175	155
G6	Porcelana Geyser	South Chile	19 Mar 2011	42º 24' 51"	72º 29' 2.2"	41.4			40005 (74.9)	431	342
		Antarctica		**South**	**West**			**Salinity (ppt)**			
K1A	Antarctica	Deception Island	02 Feb 2013	62° 58′ 52″	60° 34′ 79″	33	6.1	11.8-17	43578 (80)	590	438
K2A	Antarctica	Deception Island	02 Feb 2013	62° 58′ 52″	60° 34′ 79″	32	6.6	29,5	201055 (74.9)	1316	634

Water from above the mats was collected and preserved for different chemical analyses. Temperature was measured at each point before the extraction of samples using a digital thermometer (Oakton, model 35607-85) or an environmental mercury thermometer. pH was determined with either a pH meter of with indicator paper to the closest integer. None of the samples was strongly acidic (pH<6) or alkaline (pH>8). Conductivity was determined with a conductivity meter (Oakton, model 35607-85), and dissolved oxygen was measured using an oxymeter (YSI, model 550I). Samples were collected with a core borer sampler and stored in sterile cryovials. Samples from Northern Chile (El Tatio), Northern Patagonia and Antarctica were kept in liquid nitrogen until DNA extraction in the laboratory. Samples from Costa Rica were preserved at -80ºC until laboratory processing. Extraction of nucleic acids from samples was done as described by Mackenzie et al. (2013). Briefly, samples were thawed and approximately 200 µl of microbial mat was bead-beaten for rapid and thorough homogenization. Nucleic acids were extracted using a modified phenol: chloroform: IAA protocol, and purified using Amicon Ultra-15 (Millipore, MA, USA). DNA integrity and concentration was determined in a Nanodrop (Thermo Scientific, DE, USA).

454-pyrosequencing and noise removal

DNA samples were sent to Research and Testing Laboratory (Lubbock, Texas, USA) for amplification of the 16S rRNA gene. Tag-pyrosequencing was done with Roche 454 Titanium platform following manufacturer protocols (454 Life Science). Primers 28F (5'-GAGTTTGATCNTGGCTCAG) and 519R (5'- GTNTTACNGCGGCKGCTG) were used for amplification of the hypervariable regions V1, V2 and V3, and approximately 450 bp long tags were obtained. Dowd et al. (2008) described the subsequent PCR and sequencing. A total of 1 174 804 tags were retrieved in raw data.

The raw tag-sequences were processed using QIIME (Caporaso et al. 2010). Briefly, to reduce sequencing errors and their effects, the multiplexed reads were first trimmed, quality-filtered and assigned to the corresponding sample. The filtering criteria included a perfect match to the sequence barcode and primer, at least 400 bp in length, a quality score window of 50 bp and a minimum average quality score of 28. Additionally, denoising was used to reduce the amount of erroneous OTUs. The sequences were then clustered into Operational Taxonomic Units (OTUs) based on the relatedness of the sequences (97% similarity) using the UCLUST package. Afterward, a representative sequence from each OTU was selected. To identify potential chimera sequences, the dataset was subjected to the ChimeraSlayer implemented in Mothur (Schloss et al., 2011). Then, taxonomy assignment was made with QIIME by searching the representative sequences of each OTU against the SILVA 16S/18S rDNA non-redundant reference dataset (SSURef 108 NR) (Quast et al. 2013) using the Basic Local Alignment Search Tool (BLAST). As sequences retrieved were not long enough to ensure a low quantity of matches by chance in reference sequences from database, a stringent e-value significance threshold of 0.03 was chosen. Chimeras, chloroplasts, Eukarya and Archaea related sequences were removed from the output fasta file, that was used for building a table with the OTU abundance of each sample and the taxonomic assignments for each OTU. The final number of tags was reduced after this processing to 895 309.

Richness and diversity of 454 pyrosequecing data

Richness (S) was computed as the total number of OTUs (97% similarity) in each sample. Estimates of total richness (S), Chao1 richness, Shannon diversity (H'), Evenness (J'), Simpson, Inverted Simpson and the accumulation curves were calculated using the "vegan" package (Oksanen et al. 2013, accessed on October 2013) of the free software R v 3.1.1 (R Core Team 2013). The map and coordinate locations were made using the "maps" package (accessed

in August 2014). Rank-abundance plots were done using the "BiodiversityR" package (Kindt and Coe, 2005, accessed in October 2013).

Multidimensional scaling (MDS) was done with Primer-E software v6.1.2. Data was transformed to square root before calculation of Bray-Curtis similarities.

RESULTS AND DISCUSSION

Sampling and methodological considerations

Our sampling was designed to encompass a range of distances among sampling sites of almost nine orders of magnitude, from 5 cm between different parts of the same mat in Cahuelmó (Northern Patagonia) to 8450 km between Antarctica and Central America. In addition, we collected our samples approximately along the same latitude, so that hypotheses about changes in diversity with latitude could also be tested (Figure 1). Within these two considerations we tried to get a nested design. This could not be perfect because of the peculiarities of the thermal springs at each site. Thus, the largest distance between samples at Rocas Calientes (Costa Rica) could only be 60 cm, due to the reduce size of the mat, while similar mats could be sampled 300 m apart along the Río Negro River (Costa Rica). Likewise, we could sample two different sites in Northern Costa Rica (Río Negro and Miravalles) and three in Southern Chile (Porcelana Geyser, Porcelana spring and Cahuelmó), but only mats in one site in Antarctica (Deception Island). Despite these imponderables, we obtained a reasonably balanced distribution of pairwise distances across the whole range, from local to continental.

Samples were collected in different years and, to a certain extent, in different seasons. This might have introduced unaccounted variability. However, we think this factor did not override the effects of environmental variability and distance that we were looking for. In general, the taxonomic composition of hot spring microbial mats is not known to change substantially with seasons (but see Briggs et al. 2014). Second, all samples from the Southern

Hemisphere were collected in the same season (summer). Third, even though samples from Costa Rica were collected in January and July of 2012, seasonal variations are relatively mild along the Costa Rican cordilleras (the Costa Rican National Meteorology Institute http://www.imn.ac.cr/ reported a mean air temperature of 26º C in both months). Fourth, we demonstrated that in the Southern Chile hot springs, where seasonal changes in the ecosystem are very well marked, the differences among springs were larger than those between seasons. And, moreover, a substantial part of the seasonal variability could be accounted for by differences in temperature of the water (Mackenzie et al. 2013). Therefore, we assume that differences due to time of sampling would either be included in the effects of temperature, or would not obscure the effects of environment and distance.

Another potential source of variation was the chemical composition of the water and the geological substrate. The three main studies carried out in thermal springs did not find an effect of the chemistry of the water or the geological setting on the phylogeny of the studied microbes (Papke et al. 2003, Whitaker et al. 2003, Takacs-Vesbach et al. 2008). Bacteria from springs with different chemistry were closer together than those of other springs with similar chemistry. This included factors such as pH, sulfate, sulfide or arsenic. Despite differences in temperature, this did not affect the phylotypes of *Synechoccocus*, *Sulfolobus* or *Sulfurihydrogenibium* living in the same area. Since we considered the whole community composition, temperature was the main factor contributing to the composition of mats. In summary, we trust that comparisons can be carried out confidently among all samples and that temperature can be taken as the most influential environmental variable.

We chose pyrosequencing of 16S rDNA fragments as the method to analyze diversity. Even though it provided less sequences, and at a higher cost, than alternative high throughput sequencing methods, the amplicons were substantially longer and the quality criteria used for accepting or rejecting tags are now well established (Quince et al. 2011). We processed all the samples in one single 454-pyrosequencing plate and carried out the same processing protocol with all of them. The number of clean tags per sample ranged between 20757 and 48136 (Table

1). The exceptions were one Antarctic sample where over 200000 tags were retrieved and the Santa Teresita sample where only 1564 tags were obtained. The latter sample was eliminated from further analysis. In order to normalize the number of tags per sample we carried out rarefaction to 20757 reads (see suplementary Figure 1). These numbers were enough to determine most patterns in diversity and compare samples robustly.

Diversity changes with temperature and latitude

Using the 97% similarity criterion, we found between 69 (at El Tatio, 70˚C, the hottest mat) and 710 (in a 55 ˚C sample from Río Negro) OTUs per sample. Less than 1% of OTUs were singletons or doubletons in each sample (Table 1). The only exception was the Antarctic sample K2A where over 200000 reads had been obtained. In this one we found 1316 OTUs (Table 1). After rarefaction, the number of OTUs ranged between 57 and 638 per sample. The number of OTUs was negatively correlated with temperature when the whole data set was considered (r^2 = 0.340, p = 0.004; Figure 2A). This relationship was also significant in samples from South America (p = 0.043), but not in samples from Central America (p = 0.51) when analyzed separately (data not shown). When samples above and below 50 ˚C were analyzed separately, the lower temperature samples also showed a significant negative relationship with temperature (Figure 2C). The variability explained was higher (r^2 = 0.464) but the significance was lower (p = 0.018). For the upper temperature samples, the relationship was not significant.

On the other hand, there was no significant relationship of richness with latitude (p = 0.903; Figure 2B) for the global data set. We also analyzed the results with latitude separating samples above and below 50 ˚C. In this case, the lower temperature samples did show a significant relationship with latitude (r^2 = 0.576, p = 0.029, Figure 2D) while the higher temperature samples did not (p = 0.107, data not shown). Neither evenness alone (supplementary figure 2), nor two indices that integrate richness and evenness (Inverted

Simpson and Shannon, data not shown) presented a significant relationship with either temperature or latitude.

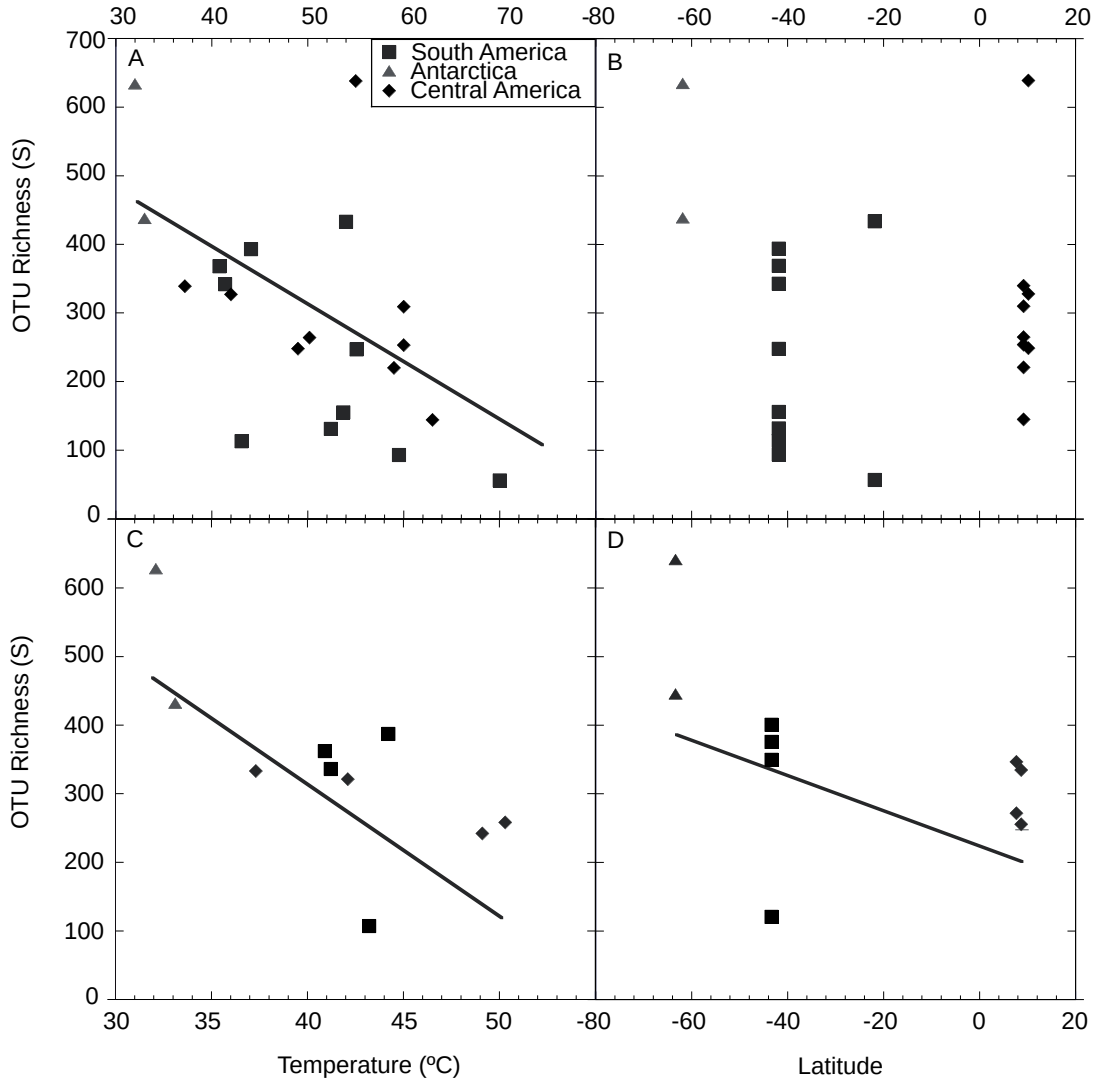


Figure 2: A) OTU richness versus temperature for all samples (slope = -9.336, p = 0.004, R2 = 0.308). B) OTU richness versus latitude (p = 0.903). C) OTU richness versus temperature for samples < 50˚C (slope = -16.483, p = 0.018, R2 = 0.464. D) OTU richness versus latitude for samples < 50˚C (slope = -2.702, p = 0.029, R2 = 0.505). Negative latitudes correspond to the southern hemisphere.

When the identity of the OTUs was compared, extremely few OTUs (26 out of 4295 or 0.6% of the total) were shared by mats in the three continents (Figure 3). Antarctic samples shared only between 2 and 3% of their OTUs with the other mats. The American springs, in turn, shared 422 OTUs, which accounted for 22 to 29% of the OTUs in Central and South America respectively. This translated into Central America having 75% of OTUs unique to the continent. These numbers were 70% for South America and 93% for Antarctica. We were expecting Antarctica to share a larger percentage of OTUs with South rather than with Central America if distance were an important factor. However, surrounding environment dynamics are very different in Antarctica, and the mesophilic mats in this hot spring are flooded with 1-2º C seawater during high tides, so bacteria that thrive under such temperature shifts might also be psychrotolerant to survive.

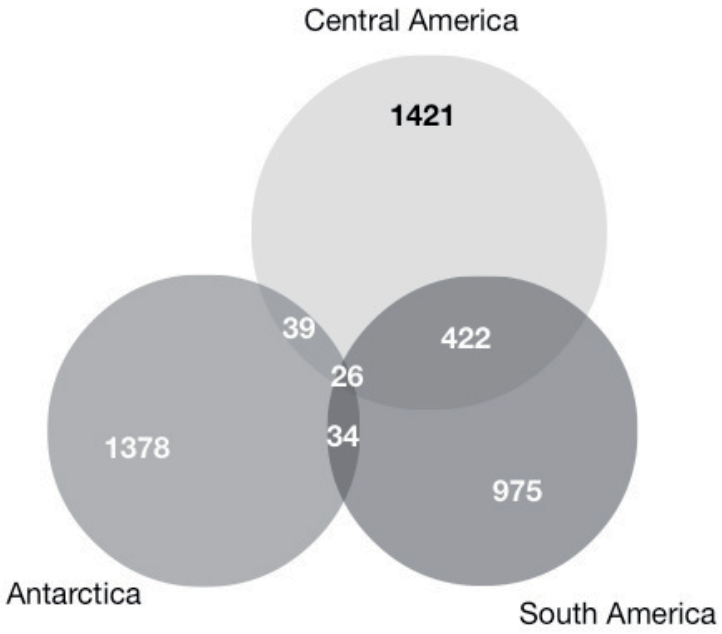


Figure 3: Venn diagram of samples grouped according to their continent of origin: Central America, South America or Antarctica.

The main bacterial Classes represented in each spring are shown in Figure 4 as heat maps (detailed numbers in supplementary Table ST1). The two most abundant groups were the phototrophic genera *Chloroflexus* and *Mastigocladus*. Both genera have been reported to be dominant in many other springs in America (Bohórquez et al., 2012; Becraft et al., 2011; Miller et al., 2009; Finsinger et al., 2008; Miller et al., 2007; Fernández-Turiel et al., 2005; among others) and hot springs in other continents (Wang et al., 2013; Everroad et al., 2012; Sompong et al., 2005; Kristjansson and Alfredsson, 1983). The next groups in abundance were the heterotrophic Proteobacteria, Thermus-Deinoccocus and Bacteroidetes, which have been also found in hot springs elsewhere (Bohórquez et al., 2012; Everroad et al., 2012; Sompong et al., 2005). Cyanobacteria, Proteobacteria, Thermus-Deinococcus and Bacteroidetes were reported in hot springs Cahuelmó, Porcelana, and Porcelana geyser in our previous study (Mackenzie et al. 2013). Some groups were significant in a few springs but absent or rare in others, such as Armatimonadia, Planctomycetaceae, Chlorobea, Acidobacteria or Ignavibacteria. Some characteristic members of thermal springs such as the Aquificae were found in very low abundance.

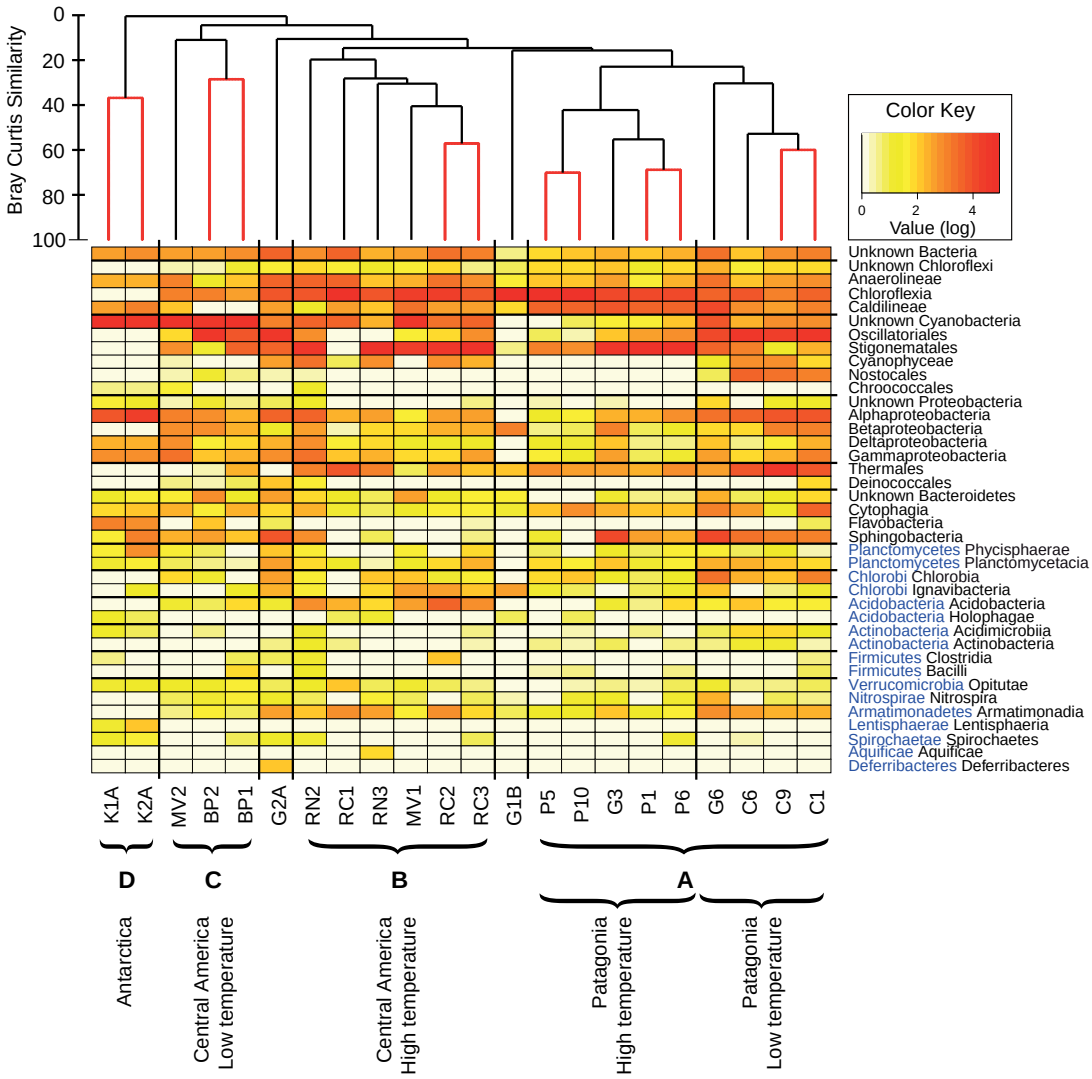


Figure 4. Class level composition of the different samples grouped with a dendrogram based on the Bray-Curtis similarity. Heatmaps indicate abundance (in log scale). The dendrogram organized samples in a few clusters whose locale and temperature is indicated in the lower part of the figure. Branches in red correspond to samples without significant differences among them.

Similarity among bacterial communities

Bray-Curtis distances were used to calculate similarity among communities and a MDS was carried out (Figure 5). The horizontal axis was obviously related to temperature, since the hottest mats were all to the right and the coldest (Antarctica) to the left. The vertical axis

separated samples from the Southern (below) and Northern (above) Hemispheres. There was a clear tendency for the mats with highest temperatures (above 50 degrees) to be closer together than the cooler ones. In about half of the systems, samples from the same system showed similarities above 50%. This was the case with Kroner Lake (Antarctica), Cahuelmó (Patagonia, Chile), Rocas Calientes, and Bajo las Peñas (Costa Rica). Other systems, however, had very different mat communities, such as Miravalles, Río Negro (Costa Rica), Porcelana Geyser (Patagonia, Chile) and El Tatio (Northern Chile). The four Porcelana spring samples were all above 40% similarity, but samples with closer temperature P5 and P10 (both 59.5º C) and P1 and P10 (2.7º C difference) formed two clusters of more than 60% similarity (dendrogram in Figure 4).

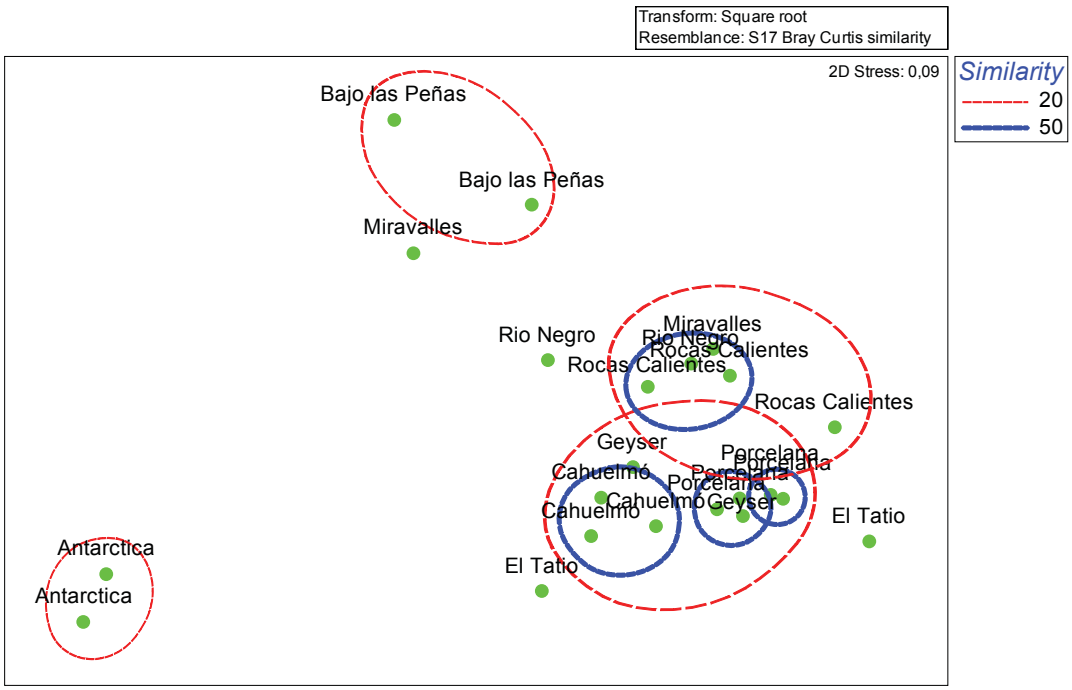


Figure 5. MDS analysis based on Bray-Curtis similarities. The hottest samples are to the right and the coolest ones to the left. The vertical axis separates southern hemisphere samples (below) from those of the northern hemisphere (above).

The analysis was repeated with the most abundant members of the community alone (Figure 6). As could be expected, the most important taxa were all primary producers:

Chloroflexii at the higher temperatures and Cyanobacteria at the lower ones. It was remarkable that one single *Chloroflexus* OTU (OTU_2354) was the dominant microorganism in both Central and South American mats at the highest temperatures (>50º C). This was part of the reason for the high temperature mats to be similar to each other, regardless of the origin. These results might appear to contradict those of Papke et al. (2003), Whitaker et al. (2003), and Tacaks-Vesbach et al. (2008). As already mentioned, these three studies found different phylotypes of cultures of *Sulfolobus*, and amplified sequences of *Synechoccocus* and *Sulfurihydrogenibium*, consistent with the existence of dispersal barriers. Our sequences, however, were only about 450 bp long. The former authors had to resort to much higher resolution taxonomy to find those patterns. In effect, they used the highly variable ITS region to determine the different phylotypes. It is possible that we would also have found different phylotypes at this higher taxonomic resolution. What this implies is that the time scales necessary for local genetic drift to result in different taxa at the gross 97% level are too long to prevent dispersal, even at the large scales we analyzed. This was not the case in our study at lower temperatures. A similar pattern was reported by Sharp et al. (2014) for hot spring microbial communities, who found that the relationship between bacterial diversity and richness with temperature was weakest at temperatures over 55 degrees.

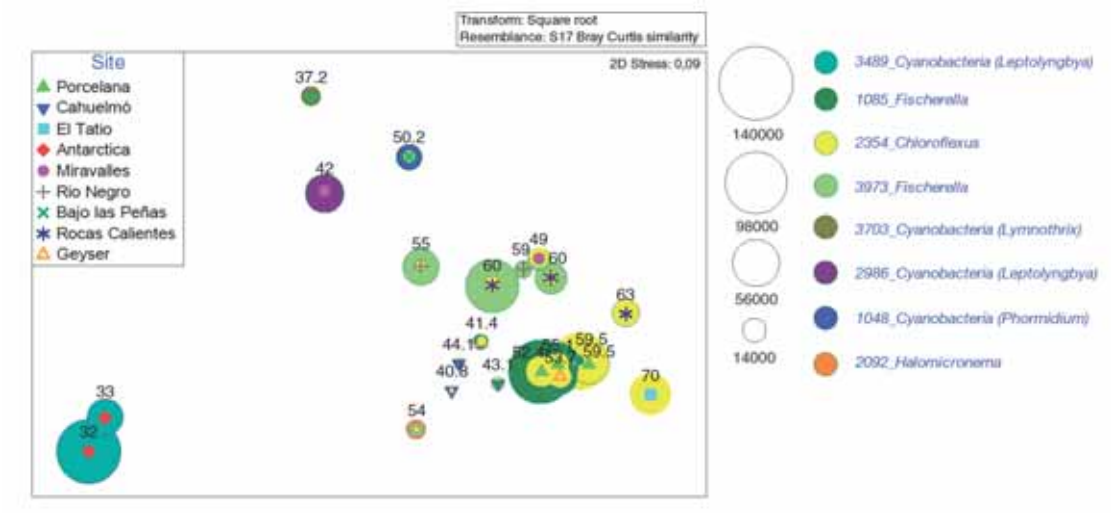


Figure 6. MDS analysis based on Bray-Curtis similarities showing the most abundant OTUs. These were all phototrophic bacteria, identified as either Chloroflexi or Cyanobacteria. The size of the dots indicates the relative abundance of each group in each sample.

At lower temperatures, there was a shift from Chloroflexi to Cyanobacteria. The transition took place at slightly different temperatures in the different systems. Thus, in Porcelana Spring the two samples at 59.9º C were dominated by Chloroflexi while the two samples at 52-55 degrees were dominated by Cyanobacteria, while still retaining a significant amount of Chloroflexi. At Rocas Calientes, in turn, the 63 ˚C sample was dominated by Chloroflexi, but the remaining two samples were dominated by Cyanobacteria despite having a temperature close to 60 degrees. The shift took place at a few degrees lower temperature at Porcelana than at Rocas Calientes. At Cahuelmó, finally, samples with temperatures between 40 and 44 ˚C were still dominated by Chloroflexi.

While the higher temperature samples were dominated by one single Chloroflexi OTU, the intermediate samples had a different dominant cyanobacterium depending on whether they came from Central or South America. In both cases it was a *Mastigocladus*- like phylotype: OTU_3973 in Central America and OTU_1085 in South America. We could confirm the dominance of this branching morphotype of the subsection V of Cyanobacteria in these samples by microscopy. Members of subsection V of Cyanobacteria form a monophyletic group with the

most complex morphology, including heterocysts, akinetes and branching filaments (Kastovsky and Johansen, 2008; Tomitani et al. 2006). Thermophilic *Fischerella* and *Mastigocladus* strains commonly form monotypic masses of filaments in neutral pH and alkaline hot springs throughout the world (Miller et al. 2007).

At the lowest temperatures (<50º C) the dominant Cyanobacteria belonged to different genera: *Leptolyngbya*, *Limnothrix* or *Phormidium*. In the case of Bajo las Peñas (Costa Rica), two different genera (*Limnothrix* and *Phormidium*) were found in each of the samples collected, despite the relatively short distance (a few meters) separating them. On the other hand, two different OTUs of *Leptolyngbya* were found in Antarctica (OTU_3489) and Miravalles (OTU_2986). Obviously, at the lower temperatures, distance does not seem to have a strong influence on mat dominance. The three genera belong to Subsection III, Oscillatoriales, and in particular to the *Lyngbya-Plenctonema-Phormidium* (LPP) group, a poorly resolved group of non-branching filamentous cyanobaceria without specialized cells such as heterocysts or akinetes (Kastovsky and Johansen, 2008). The phylogeny of this group is confusing and the group is not monophyletic. However, the sequences found in the present study are sufficiently different from each other to determine that different organisms colonized the different low temperature mats.

The similarity among samples is shown as a dendrogram in Figure 4, where the contribution of both the autotrophic and heterotrophic groups of bacteria to the differences can be appreciated. Samples from the same system and temperature were usually not significantly different (indicated by red branches in the dendrogram). The Southern Chile samples clustered together (cluster A) but separated into two sub clusters formed by samples with higher (52–60º C) and lower (41–44º C) temperatures respectively. The former included all samples from Porcelana spring and the hottest sample from Porcelana Geyser, while the latter included all samples from Cahuelmó plus the cooler samples from Geyser. It is important to emphasize that Cahuelmó hot spring is exposed to wind and brackish water from the fjord, therefore microbial mats are subjected to environmental conditions that affect bacterial community structure

differently, as previously reported in Mackenzie et al. (2013). The difference in Cahuelmó was mainly due to OTUs 2125 and 210, associated to *Leptolyngbya spp.* and *Meiothermus spp*., respectively. Samples from Central America formed two clusters. One (cluster B) was formed by samples from Rocas Calientes and Río Negro with temperatures between 49 and 63 degrees, plus the hotter sample from Miravalles. The other cluster (cluster C) included samples from Bajo las Peñas and the cooler sample from Miravalles. Thus, both Central and South American samples formed a high and a low temperature cluster.

Antarctic samples formed their own very distinct cluster (cluster D) and the Northern Chile samples from El Tatio were very different in temperature and community composition both between themselves and with the other samples. This dendrogram, therefore, is consistent with a significant influence of both geography and temperature on the mat community composition. The influence of these two factors was tested with Mantel tests (Table 2). Both variables had a significant influence and, moreover, when partial tests excluding the influence of the other factor were done, both temperature ($r = 0.392$, $p < 0.001$) and geographical distance ($r = 0.412$, $p < 0.001$) were still highly significant. Although Mantel coefficients cannot be used to estimate the percentage of the variability of the dependent variable explained by the independent variables (Legendre and Fortin 2010), the partial coefficient for geographical distance was slightly higher than that for temperature.

Table 2. Influence of geographic distance and temperature on bacterial community composition (by means of Bray-Curtis dissimilarity) with Mantel tests.

Factor	**r**	**p-value**
Temperature	0.4078	0.001
Geographic Distance	0.4263	0.001
Temperature [Geographic Distance]	0.3919	0.001
+Geographic Distance [Temperature]	0.4115	0.001

r is the Mantel statistic, and *p-value* is the significance of the tests. Factors in brackets means that their effects were removed from the analysis.

The patterns seen in the distribution of high abundance OTUs (Figure 6) were also apparent in Figure 4. But further differences could be found by examining other groups of bacteria. Within the A cluster, for example, Caldilineae were more important at higher temperatures while Thermales and Alphaproteobacteria were more abundant at lower ones. Another example was the higher abundance of Anaerolineae and Gammaproteobacteria in the Central than in the South American mats. These groups have been found in hot springs elsewhere. Thermophilic Alpha and Gammaproteobacteria were reported in a slightly acidic hot spring in the Colombian Andes (29º C, over 3000 meters above sea level, Jiménez et al., 2012), through metagenomic analysis, with a relative abundance as high as 40 and 30% of total sequences, respectively. In that study, Alphaproteobacteria were related to autotrophic and heterotrophic, sulfur oxidizing mesophilic bacteria able to grow at pH between 2.5–6, and Gammaproteobacteria were associated to microorganisms that can grow on reduced inorganic sulfur and iron compounds as the energy sources. Class Thermales in this study was mainly represented by *Meiothermus*-like OTUs, which have also been found in many hot springs

elsewhere. Portillo et al. (2009) and Kanokratana et al. (2004) reported up to 11% of total sequences belonging to this group in sediments at 50–57º C from hot springs in Thailand, and Skirnisdottir et al. (2000) also found high relative abundance of *Meiothermus* in the heterotrophic fraction of microbial mats at 65–70º C in Icelandic hot springs.

Distance-decay relationships

Several authors have tested the decay in similarity of microbial communities with increasing geographic distance (Martiny et al., 2011; Bell, 2010; Ramette and Tiedje, 2007; among others). Naturally, environmental conditions are expected to become increasingly different with distance. If the microorganisms within a community are adapted to local conditions, communities are likely to become increasingly different as microorganisms organize according to their niche requirements (Bell 2010). Recently, Sharp et al. (2014) made a comprehensive study of 165 hot springs in New Zealand and Canada to quantify the influence of temperature and pH on microbial mats of geochemically diverse hot springs.

Having found that geographical distance had a significant influence on the similarity in community composition, we analyzed this effect at different space scales by constructing distance-decay curves (Figure 7). The hot spring mat communities displayed a highly significant negative relationship (slope = -0.265, $p < 0.0001$). The percentage of the variability explained by distance was 15%. When we calculated the regressions separately for each space scale, the distance-decay relationship was significant at the local (slope = -0.210, $p = 0.0055$) and regional scales (slope = -0.486, $p = 0.0105$), but not at the continental scale ($p = 0.674$). These results can be compared to those of Martiny et al. (2011) who analyzed ammonia-oxidizing populations in marshes distributed over a range of space scales similar to ours. The length of the rRNA fragments analyzed was also similar (around 460 bp). Differences between the studies include: i) they analyzed only one guild while we analyzed the whole community, ii) they used conventional cloning and Sanger sequencing thus obtaining circa 5000 sequences in

total, while we obtained almost 900000 by pyrosequencing. Despite these differences, the general patterns we found were the same: all relationships were significant except for the continental scale, which was not. Also in accordance with Martiny et al. (2011), the slopes of the global (-0.265) and local (-0.210) scales were similar to each other and not as steep as the regional scale (-0.486). However, the slopes in the present study were much steeper.

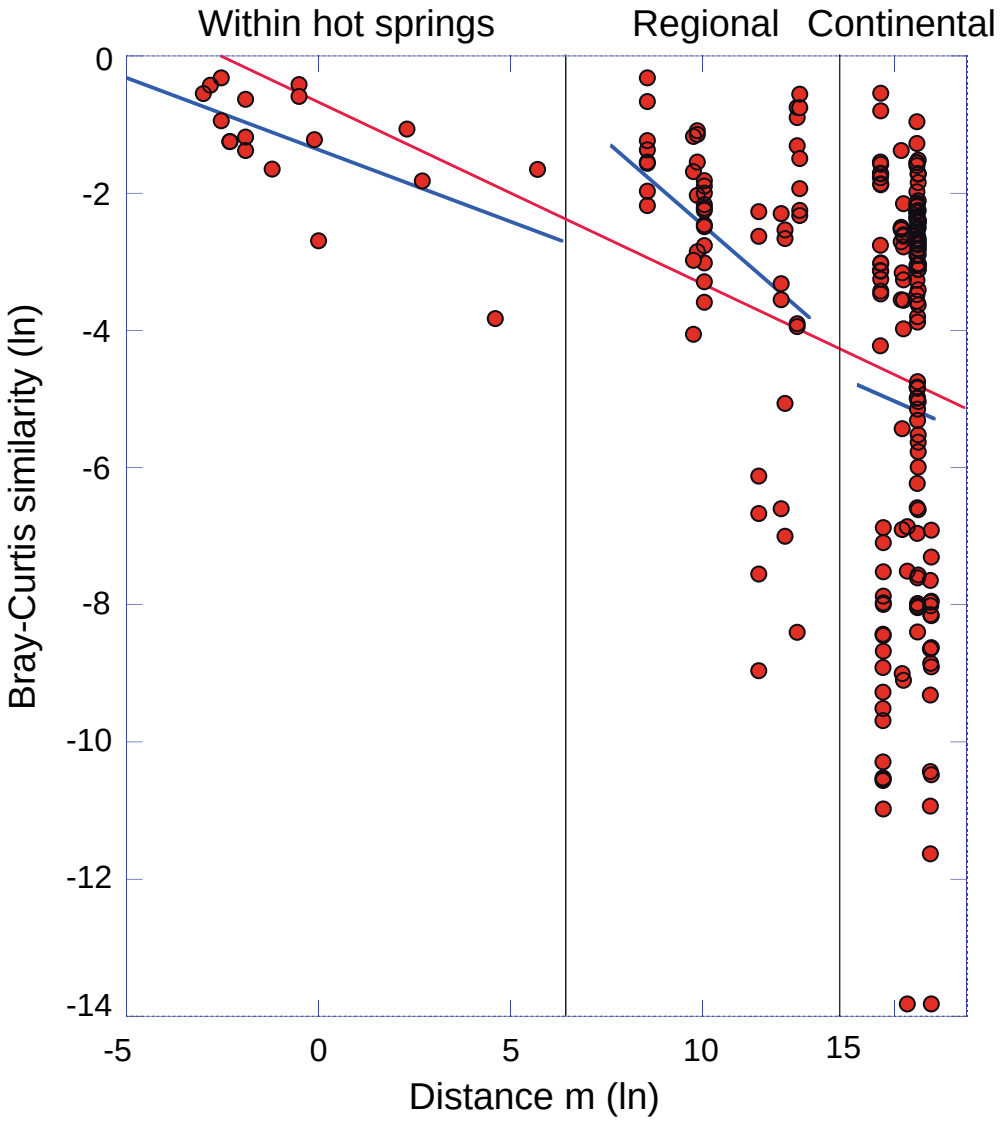


Figure 7. Distance-decay curves for the hot spring microbial mat communities. The blue line shows the linear regression considering all the data (slope = -0.265, $p < 0.0001$, $R^2 = 0.153$). The red lines show the regressions considering only the data within each spatial scale: local (slope = -0.210, $p = 0.006$, $R^2 = 0.354$), regional (slope = -0.486, $p = 0.011$, $R^2 = 0.099$) or continental (slope = -0.247, $p = 0.674$, $R^2 = -0.005$). Except the line for the continental scale, the other three have slopes significantly less than zero.

Distance-temperature relationships

We did a similar analysis for the relationship between Bray-Curtis similarities and temperature differences for each pair of samples. We calculated the regressions both with linear and log-log data. Both were significant and showed negative slopes (slope = -0.014, p = 0.001 and slope = -0.193, p = 0.0004 respectively, supplementary figure 3A and B). In the log-log plots, however, the two Antarctic samples had such large differences with all the other samples that the data set was not adequate for regression analysis (there was heteroscedasticity, for example). When we repeated the regression excluding these two samples the slope was significant: the similarity between samples decreased as the temperature difference increased (slope = -0.348, p = 0.005, supplementary figure 3C). We calculated regressions separately for samples below and above 50˚C. In this case, the relationship for the cooler samples was significant, but that for the hotter samples was not significant (supplementary Figure 3D and E, respectively). For the cooler samples, temperature differences explained 28% of the variability in Bray-Curtis similarity.

CONCLUSIONS

The patterns detected were different at different temperatures. At the higher temperatures (>50˚C) we found a significant decrease in richness both with increasing temperature and latitude, while there was no significant change at lower temperatures. Community composition was very similar for high temperature mats and these were dominated by a single anoxygenic phototrophic Chloroflexi OTU. This indicates that there were no dispersal barriers for this organism. On the other hand, community composition in Central and South American mats was increasingly different as temperatures were lower. At intermediate temperatures, different OTUs from the same photoautotrophic cyanobacterial genus (*Mastigocldus*-like) were dominant in the two continents and at the lowest temperature, the

dominant OTUs belonged to different genera (*Leptolyngbya*, *Meiothermus*, *Ruegeria*, Sphingobacteriales, among others). This relationship with temperature was not only reflected in the dominant organisms, but the community similarity decreased as temperature difference between samples increased. We speculate that at high temperatures, this environmental factor overrides any other environmental differences and the same organisms can colonize hot springs in distant areas. As temperatures decrease, other environmental factors may become important in determining the organisms that can grow and become abundant.

On top of the effect of temperature, we also detected a significant effect of distance. The partial Mantel tests revealed both temperature and distance to be significant factors. The distance decay relationship was significant at the global, local and regional scales (but not at the continental scale). Altogether, our analysis showed how distance and environment interact, producing patterns in community composition that vary at different spatial scales and temperatures.

Acknowledgments

Sampling was funded by grants from INACH T15-10 (Antarctica), FONDECYT nº 1110696, Fundación San Ignacio del Huinay including three cooperation projects with CSIC, 2012CL, 2013CL0017, and 2014CL18 (Southern Chile), FONDECYT nº 1110696 (El Tatio), and cooperation project CSIC-CRUSA (Costa Rica). We thank Lorena Escudero, Mónica Vásquez, and Martin Polz for help in the field.

Work in Chile was funded by FONDECYT grant nº 1110696 and in Barcelona by grant MarineGems CTM2010-20361 from the Spanish Ministry of Economy and Competitiveness.

REFERENCES

Becraft E. D., F. M. Cohan, M. Kühl, S. I. Jensen, and D. M. Ward. 2011. Fine-scale distribution patterns of Synechococcus ecological diversity in microbial mats of Mushroom Spring, Yellowstone National Park. Appl. Environ. Microbiol. 77:7689–97.

Bell T. 2010. Experimental tests of the bacterial distance-decay relationship. ISME J. 4:1357–65.

Bohorquez, L. C., Delgado-Serrano, L., López, G., Osorio-Forero, C., Klepac-Ceraj, V., Kolter, R., … Zambrano, M. M. 2012. In-depth characterization via complementing culture-independent approaches of the microbial community in an acidic hot spring of the Colombian Andes. Microbial Ecology, 63(1), 103–15.

Briggs BR, Brodie EL, Tom LM, Dong H, Jiang H, Huang Q, Wang S, Hou W, Wu G, Huang L, Hedlund BP, Zhang C, Dijkstra P, Hungate B A. 2014. Seasonal patterns in microbial communities inhabiting the hot springs of Tengchong, Yunnan Province, China. Environ. Microbiol. 16:1579–91.

Brock T. D. 1967. Life at high temperatures. Science 158:1012–1019.

Caporaso JG, Lauber CL, Walters WA, Berg-lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R. 2010. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. PNAS 108:4516–4522.

Cho J., and J. M. Tiedje. 2000. Biogeography and Degree of Endemicity of Fluorescent *Pseudomonas* Strains in Soil. Appl. Environ. Microbiol. 66:5448–5456.

Clarke A., and K. J. Gaston. 2006. Climate, energy and diversity. Proc. Biol. Sci. 273:2257–66.

De Wit R., and T. Bouvier. 2006. “Everything is everywhere, but, the environment selects”; what did Baas Becking and Beijerinck really say? Environ. Microbiol. 8:755–8.

Dowd S. E., Callaway T. R., Wolcott R. D., Sun Y., McKeehan T., Hagevoort R. G., and Edrington T. S. 2008. Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). BMC Microbiol. 8:125.

Everroad R. C., H. Otaki, K. Matsuura, and S. Haruta. 2012. Diversification of Bacterial Community Composition along a Temperature Gradient at a Thermal Spring. Microbes Environ. 27:374–381.

Fernandez-Turiel J. L., M. Garcia-Valles, D. Gimeno-Torrente, J. Saavedra-Alonso, and S. Martinez-Manent. 2005. The hot spring and geyser sinters of El Tatio, Northern Chile. Sediment. Geol. 180:125–147.

Ferris M. J., and D. M. Ward. 1997. Seasonal distributions of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 63:1375–81.

Fierer N. 2008. Microbial biogeography: patterns in microbial diversity across space and time. In: *Accessing Uncultivated Microorganisms: from the Environment to Organisms and Genomes and Back*. K. Zengler (editor). ASM Press, Washington DC pgs. 95-115. 1

Fierer N., and R. B. Jackson. 2006. The diversity and biogeography of soil bacterial communities. Proc. Natl. Acad. Sci. U. S. A. 103:626–31.

Finlay B. J. 2002. Global dispersal of free-living microbial eukaryote species. Science 296:1061–3.

Finsinger K., I. Scholz, A. Serrano, S. Morales, L. Uribe-Lorio, M. Mora, A. Sittenfeld, J. Weckesser, and W. R. Hess. 2008. Characterization of true-branching cyanobacteria from geothermal sites and hot springs of Costa Rica. Environ. Microbiol. 10:460–73.

Fuhrman J. a, J. a Steele, I. Hewson, M. S. Schwalbach, M. V Brown, J. L. Green, and J. H. Brown. 2008. A latitudinal diversity gradient in planktonic marine bacteria. Proc. Natl. Acad. Sci. U. S. A. 105:7774–8.

Fuhrman J. A. 2009. Microbial community structure and its functional implications. Nature 459:193–9.

Hillebrand H. 2004. On the generality of the latitudinal diversity gradient. Am. Nat. 163:192–211.

Jiménez DJ, Andreote FD, Chaves D, Montaña JS, Osorio-Forero C, Junca H, Zambrano MM, Baena S. 2012. Structural and functional insights from the metagenome of an acidic hot spring microbial planktonic community in the Colombian Andes. PLoS One 7:e52069.

Kanokratana P., S. Chanapan, K. Pootanakit, and L. Eurwilaichitr. 2004. Diversity and abundance of Bacteria and Archaea in the Bor Khlueng Hot Spring in Thailand. J. Basic Microbiol. 44:430–44.

Kassen R., and P. B. Rainey. 2004. The ecology and genetics of microbial diversity. Annu. Rev. Microbiol. 58:207–31.

Kastovsky J. and Johansen J. 2008. *Mastigocladus laminosus* (Stigonematales, Cyanobacteria): phylogenetic relationship of strains from thermal springs to soil-inhabiting genera of the order and taxonomic implications for the genus. Phycologia 47(3):307–320.

Kindt R. and Coe R. 2005. Tree diversity analysis: a manual and software for common statistical methods for ecological and biodiversity studies. World Agroforestry Centre.

Kristjansson J. K., and G. A. Alfredsson. 1983. Distribution of Thermus spp. in Icelandic hot springs and a thermal gradient. Appl. Environ. Microbiol. 45:1785–1789.

Legendre, P. and M-J Fortin. 2010. Comparison of the Mantel test and alternative approaches for detecting complex multivariate relationships in the spatial analysis of genetic data. Molec Ecol Resources 10: 831-844.

Liu Z., C. G. Klatt, J. M. Wood, D. B. Rusch, M. Ludwig, N. Wittekindt, L. P. Tomsho, S. C. Schuster, D. M. Ward, and D. a Bryant. 2011. Metatranscriptomic analyses of chlorophototrophs of a hot-spring microbial mat. ISMEJ, 5:1279–90.

Lomolino M. V, B. R. Riddle, and J. H. Brown. 2006. The Science of Biogeography, p. 3–12. In *Biogeography*. Sinauer Associates, Inc, Sunderland, Massachussets.

Mackenzie R., C. Pedrós-Alió, and B. Díez. 2013. Bacterial composition of microbial mats in hot springs in Northern Patagonia: variations with seasons and temperature. Extremophiles 17:123–36.

Magurran, A. 2004. Measuring Biological Diversity. Blackwell Publishing, Oxford, UK.

Martiny J. B. H., B. J. M. Bohannan, J. H. Brown, R. K. Colwell, J. A. Fuhrman, J. L. Green, M. C. Horner-Devine, M. Kane, J. A. Krumins, C. R. Kuske, P. J. Morin, S. Naeem, L. Ovreås, A. Reysenbach, V. H. Smith, and J. T. Staley. 2006. Microbial biogeography: putting microorganisms on the map. Nat. Rev. Microbiol. 4:102–12.

Martiny J. B. H., J. A. Eisen, K. Penn, S. D. Allison, and M. C. Horner-Devine. 2011. Drivers of bacterial β -diversity depend on spatial scale. PNAS 108:7850–7854.

Miller S. R., R. W. Castenholz, and D. Pedersen. 2007. Phylogeography of the thermophilic cyanobacterium *Mastigocladus laminosus*. Appl. Environ. Microbiol. 73:4751–9.

Miller S. R., A. L. Strong, K. L. Jones, and M. C. Ungerer. 2009. Bar-coded pyrosequencing reveals shared bacterial community properties along the temperature gradients of two alkaline hot springs in Yellowstone National Park. Appl. Environ. Microbiol. 75:4565–72.

Nübel U., M. M. Bateson, V. Vandieken, A. Wieland, M. Kühl, and D. M. Ward. 2002. Microscopic Examination of Distribution and Phenotypic Properties of Phylogenetically Diverse Chloroflexaceae -Related Bacteria in Hot Spring Microbial Mats Microscopic Examination of Distribution and Phenotypic Properties of Phylogenetically Diverse Chlo. Appl. Environ. Microbiol. 68:4593–4603.

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., and O'Hara, R. B. 2013. Vegan: Community Ecology Package. R package version 2.1-27/r2451.

O'Malley M. 2008. "Everything is everywhere: but the environment selects": ubiquitous distribution and ecological determinism in microbial biogeography. Stud. Hist. Philos. Biol. Biomed. Sci. 39:314–25.

Papke R. T., N. B. Ramsing, M. M. Bateson, and D. M. Ward. 2003. Geographical isolation in hot spring cyanobacteria. Environ. Microbiol. 5:650–9.

Papke R. T., and D. M. Ward. 2004. The importance of physical isolation to microbial diversification. FEMS Microbiol. Ecol. 48:293–303.

Pedrós-Alió C. 2012. The rare bacterial biosphere. Annu. Rev. Marine Sci. 4: 449-466.

Portillo M. C., V. Sririn, W. Kanoksilapatham, and J. M. Gonzalez. 2009. Differential microbial communities in hot spring mats from Western Thailand. Extremophiles 13:321–31.

Quast C., E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, and F. O. Glöckner. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41:D590–6.

Quince, C., A. Lanzen, R.J. Davenport, P.J. Turnbaugh. 2011. Removing Noise From Pyrosequenced Amplicons. BMC Bioinformatics 12:38

Ramette A., and J. M. Tiedje. 2007. Biogeography. An emerging cornerstone for understanding prokaryotic diversity, ecology and evolution. Microb. Ecol. 53:197–207.

Roeselers, G., Norris, T. B., Castenholz, R. W., Rysgaard, S., Glud, R. N., Kühl, M., & Muyzer, G. 2007. Diversity of phototrophic bacteria in microbial mats from Arctic hot springs (Greenland). Environ Microbiol, 9(1), 26–38.

Sharp C. E., A. L. Brady, G. H. Sharp, S. E. Grasby, M. B. Stott, and P. F. Dunfield. 2014. Humboldt's spa: microbial diversity is controlled by temperature in geothermal environments. ISME J. 8:1166–74.

Schloss PD, Gevers D, Westcott SL. 2011. Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. PLoS One 6:e27310.

Skirnisdottir, S., Hreggvidsson, G. O., Hjorleifsdottir, S., Marteinsson, V., Petursdottir, S., Holst, O., & Kristjansson, J. K. 2000. Influence of Sulfide and Temperature on Species Composition and Community Structure of Hot Spring Microbial Mats. Appl Environ Microbiol, 66(7), 2835–2841.

Sogin ML, Morrison HG, Huber JA, Welch DM, Huse SM, Neal PR, Arrieta JM, Herndl GJ. 2006. Microbial diversity in the deep sea and the underexplored "rare biosphere" Proc. Natl. Acad. Sci. 103:12115–12120.

Sompong U, Hawkins PR, Besley C, Peerapornpisal Y. 2005. The distribution of cyanobacteria across physical and chemical gradients in hot springs in northern Thailand. FEMS Microbiol. Ecol. 52:365–76.

Takacs-Vesbach C., K. Mitchell, O. Jackson-Weaver, and A.-L. Reysenbach. 2008. Volcanic calderas delineate biogeographic provinces among Yellowstone thermophiles. Environ. Microbiol. 10:1681–9.

Tomitani, A., A.H. Knoll, C.M. Cavanaugh & T. Ohno. 2006. The evolutionary diversification of cyanobacteria: Molecular–phylogenetic and paleontological perspectives. PNAS 103: 5442–5447.

Wang S., W. Hou, H. Dong, H. Jiang, L. Huang, G. Wu, C. Zhang, Z. Song, Y. Zhang, H. Ren, J. Zhang, and L. Zhang. 2013. Control of Temperature on Microbial Community Structure in Hot Springs of the Tibetan Plateau. PLoS One 8:e62901.

Ward D. M., M. J. Ferris, S. C. Nold, and M. M. Bateson. 1998. A Natural View of Microbial Biodiversity within Hot Spring Cyanobacterial Mat Communities. Microbiol. Mol. Biol. Rev. 62:1353–1370.

Whitaker R. J., D. W. Grogan, and J. W. Taylor. 2003. Geographic barriers isolate endemic populations of hyperthermophilic archaea. Science 301:976–8.

Supplementary materials

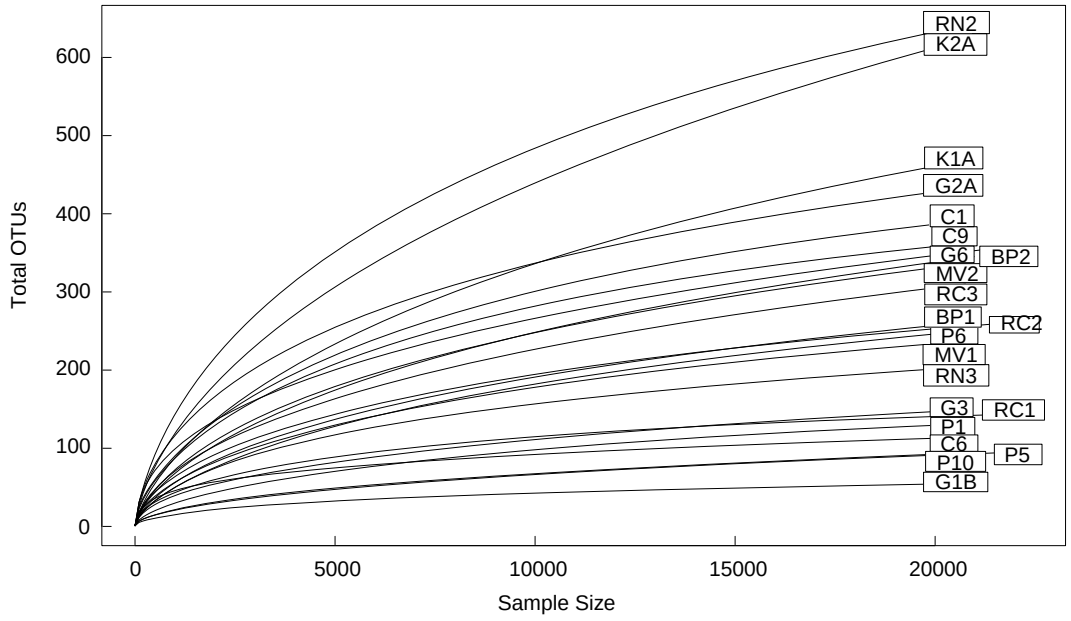
Supplementary Table 1 (ST1). Relative abundance of class level taxa in the different samples (in number of tags).

	South America											Antarctica					Central America				
Taxa	C9	C1	C6	P1	P5	P6	P10	G3	G6	G1B	G2A	K1A	K2A	BP1	BP2	MV1	MV2	RN2	RN3	RC1	RC2	RC3
Unknown Bacteria	319	585	67	64	43	112	64	132	863	3	1324	162	349	298	144	169	145	340	140	1114	900	540
Unknown Chloroflexi	41	31	28	13	32	41	33	69	136	6	22	0	0	9	1	23	1	37	16	23	49	3
Anaerolineae	195	255	57	27	64	100	66	149	753	28	1475	98	102	78	11	103	402	1108	80	1047	734	346
Chloroflexia	485	1154	2054	3710	18623	3694	18468	3798	1390	19895	2310	0	0	187	520	5453	549	2382	2005	13806	4758	2664
Caldilineae	182	609	335	1539	1124	1591	1294	2327	2988	42	153	194	382	0	0	869	53	11	79	142	157	156
Unknown Cyanobacteria	368	332	98	27	0	66	4	27	1786	0	392	16548	11694	15136	8889	8588	17666	1029	123	1532	713	1613
Oscillatoriales	6356	10417	11026	159	4	273	1	80	4286	0	7625	0	0	2721	9599	27	37	322	0	0	32	347
Stigonematales	18	103	603	14743	452	14063	269	9648	1475	3	1521	0	0	1520	7	4703	291	11304	16989	0	10362	13854
Cyanophyceae	233	38	270	0	0	0	0	0	13	0	225	0	0	0	0	0	1	660	231	4	317	95
Nostocales	943	543	1373	0	0	0	0	0	4	0	1	0	0	3	8	0	1	1	0	0	0	0
Chroococcales	0	0	0	0	0	0	0	0	0	0	0	2	3	0	0	0	23	16	0	0	0	0
Unknown Proteobacteria	12	13	0	0	1	0	0	1	50	0	5	29	11	2	9	0	1	15	0	0	0	2

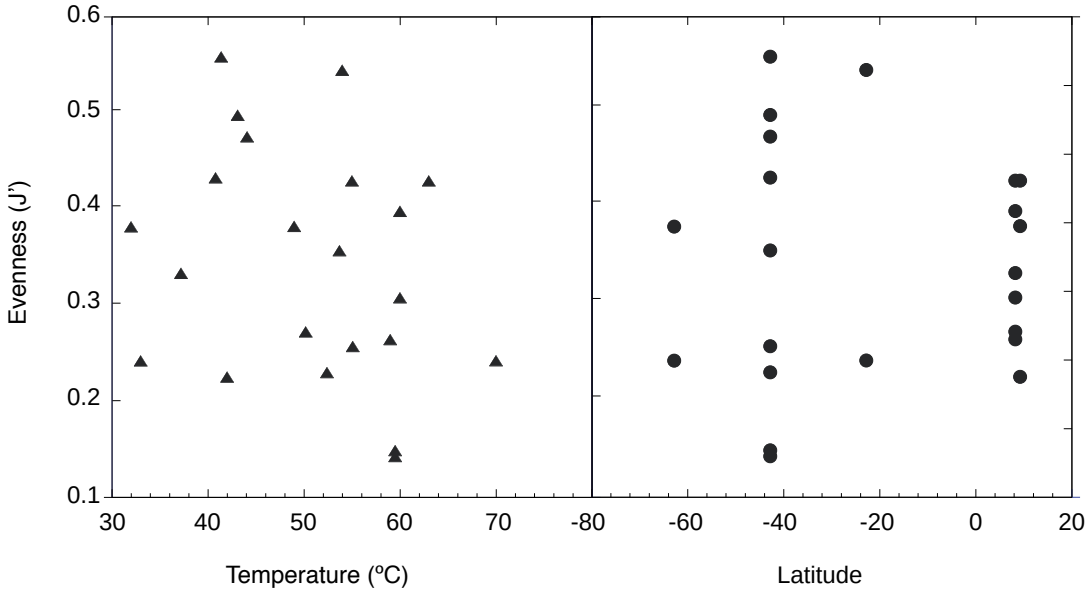
Alphaproteobacteria	1875	1688	1306	86	16	257	27	128	900	0	1618	2656	6316	90	373	22	383	1339	144	94	227	157
Betaproteobacteria	616	490	46	5	2	18	4	425	33	460	14	0	0	125	252	44	346	209	34	1	86	85
Deltaproteobacteria	21	132	2	3	12	3	8	67	62	0	94	129	97	31	20	11	325	284	51	23	13	11
Gammaproteobacteria	95	423	43	6	24	7	16	152	212	0	273	350	319	104	81	46	681	743	110	78	37	163
Thermales	8193	1788	1890	136	241	241	215	193	176	83	0	0	0	122	1	6	0	402	474	2441	227	84
Deinococcales	0	37	0	0	0	0	0	0	0	0	54	0	0	5	1	0	2	26	0	0	0	0
Unknown Bacteroidetes	14	32	4	2	0	3	0	9	135	29	226	12	23	13	358	208	26	51	6	14	16	25
Cytophagia	11	1165	173	63	75	52	256	103	423	7	50	48	82	108	27	16	121	89	4	8	21	25
Flavobacteria	0	4	0	0	0	0	0	0	0	0	4	446	271	0	83	0	0	0	0	0	0	1
Sphingobacteria	506	603	875	150	7	105	0	3495	3830	0	2614	30	412	56	219	0	120	251	5	0	0	3
Phycisphaerae	13	1	4	6	4	25	0	13	23	0	61	19	364	0	5	21	27	23	0	0	0	42
Planctomycetacia	55	33	108	9	36	20	20	69	131	0	203	8	20	1	3	13	4	38	1	15	38	92
Chlorobia	64	379	108	6	49	13	54	15	942	0	195	0	0	0	8	78	36	23	78	0	7	26
Ignavibacteria	3	9	0	5	8	10	4	0	60	186	98	0	11	14	0	153	0	21	50	0	156	71
Acidobacteria	23	28	67	3	0	34	0	7	22	0	7	0	0	41	4	171	9	287	34	123	1645	255
Holophagae	0	0	0	0	0	0	4	0	0	4	0	18	6	0	0	0	0	0	9	5	0	0
Acidimicrobiia	33	17	48	2	0	0	1	0	6	0	0	11	4	0	3	0	0	7	0	0	0	3
Actinobacteria	9	1	8	0	2	3	1	4	3	0	3	0	0	0	0	0	0	13	0	2	0	1
Clostridia	0	3	0	0	0	0	0	0	0	0	4	3	0	5	0	0	0	8	0	0	77	0
Bacilli	0	4	0	0	1	3	2	0	0	0	0	0	0	37	0	0	0	18	0	0	0	0

Opitutae	3	4	2	1	0	4	1	2	9	0	6	9	12	27	12	8	13	16	5	75	4	2
Nitrospira	5	2	0	0	0	9	10	13	87	1	17	0	0	29	9	6	5	4	20	0	0	5
Armatimonadia	128	90	209	12	17	25	12	77	282	4	212	0	0	4	23	28	2	74	153	278	248	37
Lentisphaeria	0	0	0	0	0	0	0	0	0	0	0	7	61	0	0	0	0	0	0	0	0	0
Spirochaetes	0	0	1	0	0	7	0	0	0	0	16	14	26	6	0	0	0	8	0	0	0	4
Aquificae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	35	0	0	0
Deferribacteres	0	0	0	0	0	0	0	0	0	0	54	0	0	0	0	0	0	0	0	0	0	0

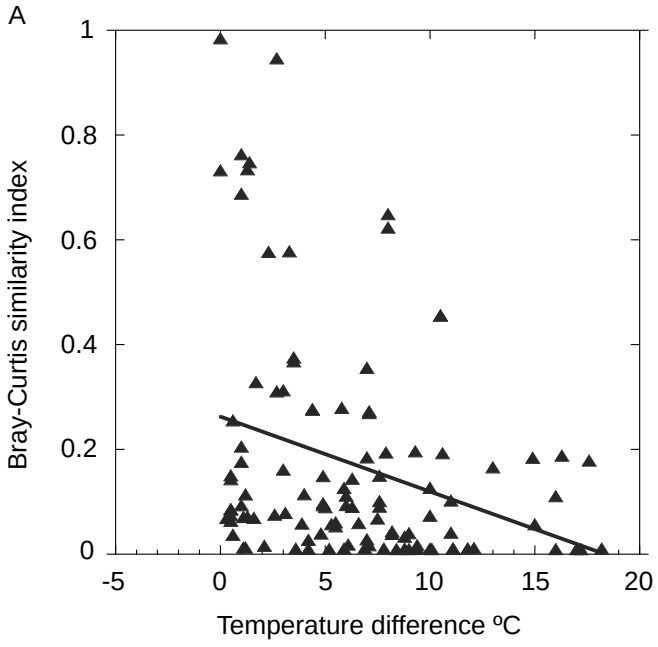
Supplementary Figure 1. Rarefaction curves for the 22 samples analyzed.

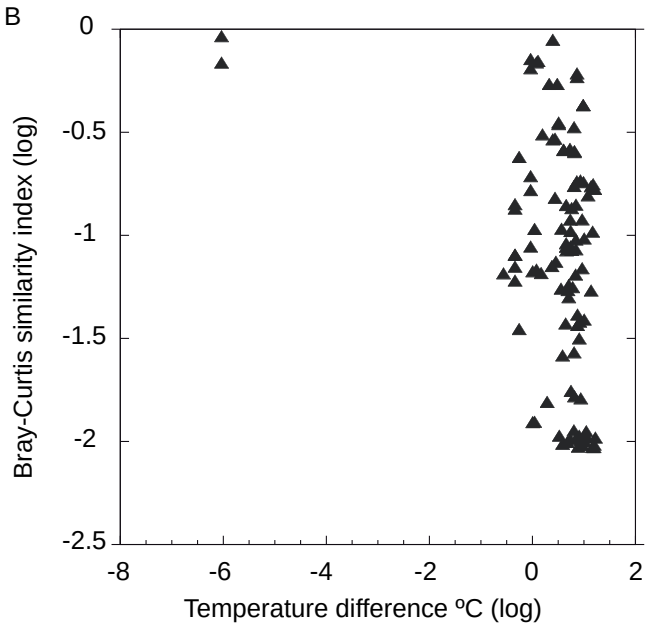


Supplementary Figure 2. Plots of evenness versus temperature and latitude. None of the relationships was significant

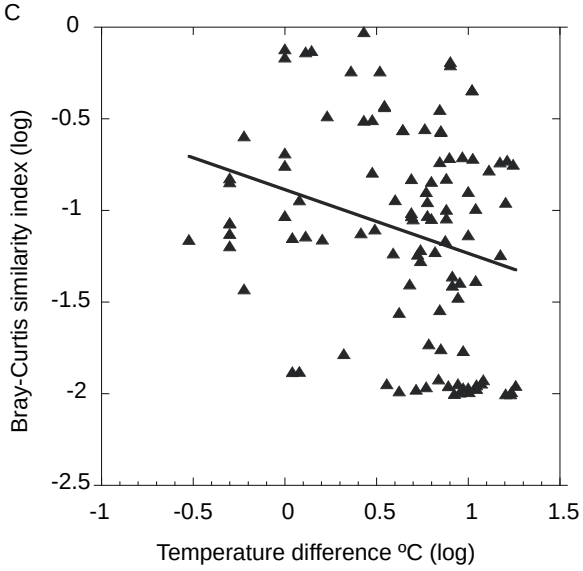


Supplementary Figure 3. Decay in community similarity with increasing difference on temperature of the samples: A) Linear scatter plot of Diversity and Temperature difference of all samples (slope = -0.014, p = 0.001); B) log-log scatter plot of Diversity and Temperature difference of all samples (slope = -0.193, p = 0.0004); C) log-log scatter plot of Diversity and Temperature difference of all samples with exception of those from Antarctica (slope = -0.347, p = 0.005, R2 = 0.062). The two Antarctic points were excluded from the analysis). D) Scatter plot of

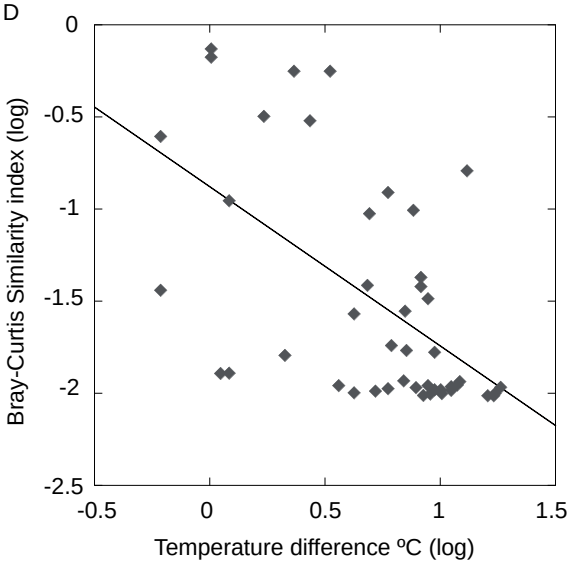




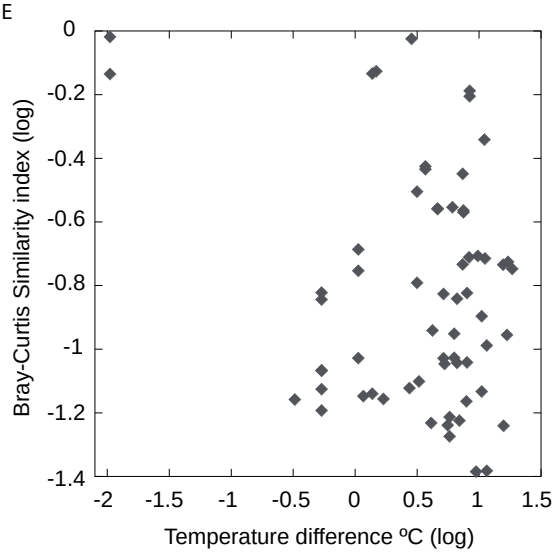
C


0
-0.5
-1
-1.5
-2
-2.5
Bray-Curtis similarity index (log)
-1
-0.5
0
0.5
1
1.5
Temperature difference ºC (log)

D


0
-0.5
-1
-1.5
-2
-2.5
Bray-Curtis Similarity index (log)
-0.5
0
0.5
1
1.5
Temperature difference ºC (log)

E


0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
-1.4
Bray-Curtis Similarity index (log)
-2
-1.5
-1
-0.5
0
0.5
1
1.5
Temperature difference ºC (log)

GENERAL DISCUSSION

General Discussion

Diversity of bacteria in hot springs has been widely studied all over the world (Sharp et al., 2014; Hou et al., 2013; Roeselers et al., 2007; Ward et al., 1998) by means of 16S rRNA, a molecule that provides a view of what microbial taxa are present in a given sample because it is an excellent phylogenetic marker (Pace, 1997). Techniques used to assess the entire taxonomic complexity of thermophilic microbial communities have evolved rapidly in the last decades, from pure cultures (Brock and Freeze, 1969), to fingerprinting patterns (Ferris et al., 1993), to the current high throughput sequencing (Sharp et al., 2014). In the course of the present thesis, these methodologies have been used to characterize microbial mats from pristine hot springs in Patagonia, and to compare them to hot springs from Central America and Antarctica at different diversity depths.

Description and value of hot spring microbial mats in Patagonia

As described in the introduction, due to the logistic difficulties to access and sample some of the hot springs studied in this thesis, an exploratory expedition became essential in the first place to tune and calibrate the sampling procedures. Microbial mats along thermal gradients of hot springs from Chilean North Patagonia (Porcelana and Cahuelmó hot springs, and Porcelana geyser) were sampled, and a physico-chemical outline was obtained by determining the environmental parameters (temperature and pH) together with a microbiological picture of the thermophilic prokaryotic communities thriving in them. The hydrogen ion concentration was neutral or slightly acidic in the three hot springs and along transects, therefore pH was not considered as a factor influencing community diversity changes. Hot springs in this study ranged between 30–70 ºC, hence they were classified as thermal and hyperthermal (Pentecost et al., 2003). It has been widely reported that temperature is one of the most important environmental factors controlling the activities and evolution of organisms (Brock, 1967; Charlier and Droogmans, 2005), therefore we focused on the relationship of bacterial community diversity shifts according to thermal gradients in hot springs as a model

study. Additionally, prokaryotes in hot springs are not subject of predators with the exception of phages (Breitbart et al., 2004), which simplifies the study of temperature and geographic isolation as pressure factors shaping bacterial community structure.

Three collection and transport methods were tested and compared to preserve genetic material in microbial mat samples during the trip back to laboratory: Liquid nitrogen in a dry-shipper, RNAlater (Qiagen) as recommended by the manufacturer, and paraformaldehyde at low concentration (<4%). We considered cost and effort to carry to the sites, and legitimacy of results. Both RNAlater and liquid nitrogen gave similar results, but liquid nitrogen showed more consistency among samples in fingerprinting analysis when considering replicates (data not shown). Despite the logistic effort and cost that a dry-shipper full with liquid nitrogen meant, the Huinay Scientific Station support eased this option and further samplings were made using this method.

Scanning electron microscopy images showed the complexity of community structure and phenotypic diversity at the different temperatures, and we found a large dominance of at least two filamentous microorganisms in most samples that were characterized later on. Cocci observed specially in microbial mat from Geyser showed a close resemblance with nanobacteria reported by Folk (1993), in travertines in hot springs from Viterbo, Italy. Travertines are carbonate deposits, formed by enhanced bioprecipitation of minerals in a bacterial matrix. Large travertine deposits are formed in this manner, and may represent a niche for the nanobacteria in Porcelana Geyser as they were in the Viterbo hot spring, explaining the resemblance of SEM images. Images obtained by epifluorescence microscopy using DAPI staining were used for total cell counts, which were two– and fourfold higher for bacilli and cocci, respectively, than those obtained by Inagaki et al. (2001) in Steep Cone hot spring, Yellowstone National Park. The percent of live cells, according to the method used, was constant among samples and ranged between 15 and 17%, almost twice as that reported by Janssen et al. (2002) (9% active cells) with the same epifluorescence method in pasture soils from USA. This result revealed the high activity and potential metabolic fitness of microorganisms in the studied microbial mats, compared with known high productivity soils such as those used as pastures. Biovolume

measurements revealed that two filamentous morphotypes were differently distributed according to temperature. Thick filaments (2 μm diameter) were detected mainly in samples with low temperature ranging from 46-38.5° C and were observed in the three hot springs. On the other hand, thin filaments (0.5 μm diameter) were detected in samples with higher temperature (>47° C), and had little presence in Porcelana Geyser.

Enrichment cultures obtained during the first expedition were stored in dependencies of the Universidad de Concepción, and were lost in the 2010 Earthquake in the same city. Hence, no isolate was available for further studies. By means of molecular fingerprinting using 16S ribosomal RNA (DGGE), when looking at each hot spring MDS we observed a cluster of low temperature and a cluster of high temperature samples. An MDS analysis of the three hot springs together showed that each hot spring clustered separately regardless of the temperature of the samples.

A second expedition in winter and a third in summer to the same hot springs (both in 2009) allowed us to compare diversity changes between seasons. We attempted to follow the changes in bacterial diversity by obtaining microbial mats from as close to the previous points sampled as possible. We faced some difficulties due to the geothermal variations in hot springs from one season to the next. In Cahuelmó, the most anthropogenically influenced spring, the few people who managed to get to the springs usually changed the course of water channels to fill pools with hot water, and in doing so, some of the mats previously sampled were inexistent or dead-dry. In Porcelana hot spring, there were several hot water sources that turned on and off during the year, causing a temperature difference in previously sampled microbial mats. In Geyser, the travertine deposits grew in height as much as its own weight allowed it, therefore columns collapsed and the entire microbial mats were differently exposed to hot water from one season to the next. Despite all of these naturally occurring inconveniences, we managed to follow microbial mats according to the temperature of the interstitial water in situ, and their macroscopic traits such as color and size.

Using DGGE, seasonal shifts in bacterial diversity of microbial mats sampled in 2009 were analyzed in the three hot springs with a temperature range of 39 ºC to 68 ºC. Three major bacterial groups were detected by Sanger sequencing of DGGE bands in all springs: Phyla Cyanobacteria and Bacteroidetes, and Order Thermales. Proteobacteria, Acidobacteria and Green Non-Sulfur Bacteria were also detected in small amounts and only in some samples. A specific cyanobacterial fingerprint by DGGE showed that thermophilic filamentous heterocyst-containing *Mastigocladus* were the dominant Cyanobacteria in Porcelana Hot Spring and Geyser, and *Calothrix* in Cahuelmó, followed by the filamentous non-heterocyst *Leptolyngbya* and *Oscillatoria*. *Synechococcus*-like sequences were detected only in two samples in Cahuelmó, which constitutes a significant difference with the extensive reports of *Synechococcus* dominated mats in Yellowstone National Park (USA), for example in Octupus Spring (Allewalt et al., 2006) and Mushroom Spring (Becraft et al., 2011)

Mastigocladus and Oscillatoriales cyanobacteria (including other members of the LPP group) have been found in hot springs elsewhere (Miller et al., 2007; Papke et al., 2003, respectively), and this study showed the presence and dominance of *Mastigocladus spp.* at higher temperatures (>55° C) than reported in other natural hot springs (Miller et al., 2009; Sompong et al., 2005). We considered that these filamentous phototrophic organisms were related to those observed previously by SEM, and because of their dominance and wide distribution in the studied microbial mats along temperature gradients and seasons, their primary producer ecological role in these phototrophic microbial mats is likely the main carbon input to the mat ecosystems.

Overall, again fingerprinting analysis of the three springs with universal bacterial primers showed high similarities within each hot spring despite differences in temperature, but Porcelana Hot Spring and Porcelana Geyser were rather similar to each other, likely due to a common geological substrate given their geographical proximity. This was even more evident with specific cyanobacterial primers. Community structure showed little difference between seasons, as described for non-tropical thermal environments (Briggs et al., 2014; Lacap et al.,

2009), which suggests that temperature of thermal water had greater influence on shaping bacterial community structure than the meteorological conditions of Patagonia at this latitude (42° South).

We hope that the description and publication in the scientific literature of the diversity of these mats, will add scientific interest to this remote area of Patagonia. This added value should encourage both conservation and sustainable touristic use that will bring well-needed resources to the local human population,

Spatial heterogeneity, latitude relationships and biogeography of bacterial diversity at different scales

In summer 2011, a fourth expedition was made to these hot springs, and again we attempted to obtain microbial mat samples of similar traits as previously sampled. We had seen the temperature influence within and among hot springs, and considering the patchiness and high heterogeneity of microbial mats, we designed a fine-scale sampling that would allow us to better understand the spatial distribution of bacterial diversity in these environments, and its relationship to temperature. By high throughput sequencing (Illumina Tags) of 30 samples obtained in a 150 square centimeter grid configuration in each hot spring microbial mat, we improved the depth of microbial taxa characterization, and observed three different spatial patterns. In Porcelana hot spring, a spatial turnover of primary producers along a small temperature gradient (8° C difference) was observed between Cyanobacteria and Chloroflexi. Different temperature preferences were observed for both phototrophic groups in the analyzed mat, as it was clear that a ~58° C temperature threshold divided the mat in two colored hemispheres (green for Cyanobacteria and orange for Chloroflexi). On the other hand, Porcelana geyser showed a similar diversity among samples, despite the steep temperature difference from one sample to the next (20º C). Microbial community structure in geyser exhibited slight variations along the microbial mat, which was dominated mostly by Cyanobacteria and Bacteroidetes. Cahuelmó hot spring showed patches dominated by

Gammaproteobacteria, Thermales, or Cyanobacteria, in a thermal gradient of over 12º C. Although relationship between diversity and temperature at the fine-scale was not very clear, these results suggested that for a comprehensive understanding of bacterial community structure in a thermophilic microbial mat, steep environmental gradients had to be considered as they might influence the community structure, increasing the microheterogeneity in samples. Therefore, when analyzing bacterial diversity, several samples must be extracted to detect diversity shifts at the fine-scale in order to extrapolate results to the rest of the microbial mat.

In 2012, several representative samples from the three grids were chosen for pyrosequencing (454) together with samples from hot springs in Costa Rica, El Tatio in Atacama Desert, and Deception Island in Antarctica. Temperature in samples ranged widely, Antarctic samples were the coolest (32º C) and those from El Tatio the hottest (70º C). We observed that samples above 50º C showed no relationship between temperature and diversity or richness. On the contrary, samples below 50º C showed a significant negative relationship between the same variables: richness and diversity decreased with increasing temperature. High temperature samples were dominated by phototrophic OTUs regardless of their geographic distance, suggesting that temperature as selective pressure overrides other environmental factors, and geographic barriers are not preventing thermophilic microorganisms from colonizing distant hot springs. As temperatures decreased, other environmental factors may become important in determining the organisms that can grow and become abundant.

The distance decay relationship was significant at the global, local, and regional scales. Results suggest that hot spring bacteria at high temperature are less dispersal limited, and that distance scale and temperature range have a different influence on shaping the community structure.

Altogether, a larg part of the studies of thermophilic bacteria inhabiting hot springs have been carried out in Yellowstone National Park, USA (extensively reviewed by authors such as Brock and Ward, see references). Other studies have been done in Nakabusa hot springs in Japan (Nakagawa and Fukui, 2002; Kubo et al., 2011; Everroad et al., 2012), New Zealand (largely

sampled by Sharp et al., 2014; Papke et al., 2003), Thailand (Hayashi et al., 1994; Sompong et al., 2005; Cuecas et al., 2014), China and the Philippines (Hongmei et al., 2004; Lacap et al., 2007); El Coquito spring in Colombian Andes (Bohorquez et al., 2012; Jiménez et al., 2012), Russia and Iceland (Perevalova et al., 2008; Reigstad et al., 2009), among other parts of the world. The high volcanic activity of the Andes in the American Southern Cone makes it a suitable place for studies of thermophiles, although only a handful of microbiological studies can be cited in the region (Urbieta et al., 2014; Willis et al., 2013), since most investigations has been conducted on the geology of the hot springs (i.e. Fernández-Turiel et al., 2005 Glennon and Pfaff, 2003 in El Tatio; Sepúlveda et al., 2004 in Patagonia; and Rey et al., 1995 in Deception Island in Antarctica). Characterization of these unique microbial mats allowed us to include the Antarctic, and Central and South America, in the literature of thermophilic bacterial distribution in natural hot springs. At a regional scale, this work increases the knowledge of our biodiversity, which represents a natural source of genetic resources. By using such a large latitudinal range of springs, finally, we could determine some ecological patterns expressed by bacterial communities.

GENERAL CONCLUSIONS

- Characterization of the microbial mats of the three hot springs from Northern Patagonia revealed that bacterial communities were dominated by a few populations, usually belonging to phyla Cyanobacteria (*Mastigocladus*-like) and Chloroflexi (*Chloroflexus*-like), and to a lesser extent by Proteobacteria and Bacteroidetes.

- Analysis of bacterial community structure of the three hot springs in Patagonia showed a higher similarity between hot springs with common environmental conditions.

- No substantial seasonal diversity changes were observed, suggesting little influence of weather on bacterial community structure.

- *Synechococcus*-like sequences were detected only in Cahuelmó hot spring, in contrast with that reported for other hot springs in North America, suggesting a marine influence to this spring.

- A turnover was observed between dominating Cyanobacteria and Chloroflexi in close relationship with temperature at a fine scale. Chloroflexi dominated over 58º C and Cyanobacteria dominated under 58º C.

- Microheterogeneity analysis showed that some mats exhibited patches of bacterial communities largely dominated by a few populations in close proximity to bacterial communities dominated by completely different bacterial groups.

- Latitude had no influence on diversity or richness of thermophilic bacterial communities at a continental scale.

- OTUs belonging to Cyanobacteria and Chloroflexi showed a wide geographic distribution at high temperature (>50º C), suggesting less geographic barriers among hot springs at a continental scale.

- Bacterial populations at a temperature range lower than 50º C showed higher dispersal limitations, as diversity and richness were negatively related to both distance and temperature.

- Overall, distance decay relationship was significant at the local and regional scales, but not at continental scale, suggesting that geographic distance is important only to some extent.

REFERENCES

Allewalt J. P., M. M. Bateson, N. P. Revsbech, K. Slack, and D. M. Ward. 2006. Effect of Temperature and Light on Growth of and Photosynthesis by *Synechococcus* Isolates Typical of Those Predominating in the Octopus Spring Microbial Mat Community of Yellowstone National Park. Appl. Environ. Microbiol. 72:544–550.

Becraft E. D., F. M. Cohan, M. Kühl, S. I. Jensen, and D. M. Ward. 2011. Fine-scale distribution patterns of Synechococcus ecological diversity in microbial mats of Mushroom Spring, Yellowstone National Park. Appl. Environ. Microbiol. 77:7689–97.

Breitbart M, Wegley L, Leeds S, Schoenfeld T, Rohwer F. 2004. Phage Community Dynamics in Hot Springs. Appl. Environ. Microbiol. 70:1633–1640.

Brock T. D., and H. Freeze. 1969. *Thermus aquaticus gen. n.* and *sp. n.*, a Non-sporulating Extreme Thermophile. J. Bacteriol. 98:289–297.

Brock TD. 1967. Life at high temperatures. Science. 158:1012–1019.

Charlier D., and Droogmans L. 2005. Microbial life at high temperature, the challenges, the strategies. Cell. Mol. Life Sci. 62:2974–84.

Fernandez-Turiel J. L., M. Garcia-Valles, D. Gimeno-Torrente, J. Saavedra-Alonso, and S. Martinez-Manent. 2005. The hot spring and geyser sinters of El Tatio, Northern Chile. Sediment. Geol. 180:125–147.

Folk R. L. 1993. SEM imaging of bacteria and nanobacteria in carbonate sediments and rocks. J. Sediment. Petrol. 63:990–999.

Glennon J. A., and R. M. Pfaff. 2003. The Extraordinary Thermal Activity of El Tatio Geyser Field , Antofagasta Region , Chile. GOSA Trans. 8:31–77.

Hou W., S. Wang, H. Dong, H. Jiang, B. R. Briggs, J. P. Peacock, Q. Huang, L. Huang, G. Wu, X. Zhi, W. Li, J. A. Dodsworth, B. P. Hedlund, C. Zhang, H. E. Hartnett, P. Dijkstra, and B. A. Hungate. 2013. A comprehensive census of microbial diversity in hot springs of Tengchong, Yunnan Province China using 16S rRNA gene pyrosequencing. PLoS One 8:e53350.

Miller SR, Castenholz RW, Pedersen D. 2007. Phylogeography of the thermophilic cyanobacterium Mastigocladus laminosus. Appl. Environ. Microbiol. 73:4751–9.

Pace NR. 1997. A Molecular View of Microbial Diversity and the Biosphere. Science 276:734–740.

Papke RT, Ramsing NB, Bateson MM, Ward DM. 2003. Geographical isolation in hot spring cyanobacteria. Environ. Microbiol. 5:650–9.

Pentecost A., B. Jones, and R. W. Renaut. 2003. What is a hot spring? Can. J. Earth Sci. 40:1443–1446.

Perevalova A. a, T. V Kolganova, N.-K. Birkeland, C. Schleper, E. a Bonch-Osmolovskaya, and A. V Lebedinsky. 2008. Distribution of Crenarchaeota representatives in terrestrial hot springs of Russia and Iceland. Appl. Environ. Microbiol. 74:7620–8.

Rey J., L. Somoza, and J. Martínez-Frías. 1995. Tectonic, volcanic, and hydrothermal event sequence on Deception Island (Antarctica). Geo-Marine Lett. 15:1–8.

Roeselers G., T. B. Norris, R. W. Castenholz, S. Rysgaard, R. N. Glud, M. Kühl, and G. Muyzer. 2007. Diversity of phototrophic bacteria in microbial mats from Arctic hot springs (Greenland). Environ. Microbiol. 9:26–38.

Sepúlveda F., K. Dorsch, A. Lahsen, S. Bender, and C. Palacios. 2004. Chemical and isotopic composition of geothermal discharges from the Puyehue-Cordon Caulle area (40.5US), Southern Chile. Geothermics 33:655–673.

Sharp C. E., A. L. Brady, G. H. Sharp, S. E. Grasby, M. B. Stott, and P. F. Dunfield. 2014. Humboldt's spa: microbial diversity is controlled by temperature in geothermal environments. ISME J. 8:1166–74.

Ward D. M., M. J. Ferris, S. C. Nold, and M. M. Bateson. 1998. A natural view of microbial biodiversity within hot spring cyanobacterial mat communities. Microbiol. Mol. Biol. Rev. 62:1353–70.

Willis, G., Hedrich, S., Ñancucheo, I., Johnson, D.B., Donati, E.R. 2013. Microbial diversity in acidic anaerobic sediments at the geothermal Caviahue-Copahue System, Argentina. Adv. Mater. Res. 825, 7–10.