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Bacterial and Geochemical Composition of Thrombolites from Lake Sarmiento, Torres del Paine National Park of Chilean Patagonia

Jorge R. Osman[a], Pabla Viedma[a], Jorge Mendoza[b], and Davor Cotoras[a]

[a]Laboratorio de Microbiología y Biotecnología, Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Independencia, Chile; [b]Laboratorio de Química de Suelos, Departamento de Química Inorgánica y Analítica, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Independencia, Chile

ABSTRACT

Microbialites are organo-sedimentary structures formed as minerals precipitate due to the metabolic activity of microorganisms. They can be differentiated by their internal mesostructure into stromatolites and thrombolites. Lake Sarmiento, located in the Patagonia region of southern Chile, is a sub-saline alkaline lake in which living submerged and sub-fossil thrombolites are present. A submerged thrombolite was collected and one of the fragments was deposited in an experimental aquarium for 1.5 years, in order to examine possible changes to its biological and chemical composition. The bacterial biodiversity was examined using Illumina sequencing of PCR-amplified 16S V4 rRNA genes from total extracted DNA. The chemical structure was studied using XRD and bench chemical methods. The results show that in the living submerged and aquarium thrombolite samples, the *Proteobacteria, Verrucomicrobia*, *Planctomycetes* and *Bacteroidetes* phyla dominated the bacterial communities, which were similar at the upper taxonomic level. However, differences between the samples were detected at deeper classification levels (class, genus). Interestingly, no changes in the carbonate composition of the thrombolites were observed after culturing during 1.5 years. This study is the first to provide new insights into the bacterial community composition of thrombolites from this site. The thrombolites from Lake Sarmiento are active and contain a unique bacterial community composition. Further studies, including greater sampling and greater variety of experimental conditions *in vitro* (aquarium) will be helpful to create a global understanding of the microbial composition and formation of the thrombolites from Lake Sarmiento.

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Bacterial community; hypersaline lakes; thrombolites; 16S rRNA

Introduction

Microbialites are organo-sedimentary structures formed through the metabolic activity of microorganisms that cause the precipitation of minerals such as carbonates, silicates and sulfates (Gleeson et al. 2016; Louyakis et al. 2017). Microbialites can be differentiated by their internal mesostructure into stromatolites (well-lamninated) and thrombolites (unlaminated) (Gleeson et al. 2016; Mobberley et al. 2012). The key microstructures of the thrombolites are lumps or clots (Shapiro 2000). However, the stromatolites contain sheets of sediments and minerals formed by layers of bacteria (Solari et al. 2010). Some of these sheets have been dated to approximately 3.5 billion years ago. Found in seas and saline lakes, they are formed by bacterial communities, which trap sediments and induce precipitation of minerals. They are considered one of the first key evidences of life (Allwood et al. 2006; Solari et al. 2010). In this way, they provide insight into the nature and diversity of ecosystems and the existence of microbial metabolisms contributing to the evolution of biogeochemical cycles (Gleeson et al. 2016).

Microbialites produce a range of carbonate precipitates that result from the interaction of the biological activities of microorganisms and environmental conditions. The degree of lithification depends on the balance between dissolution and precipitation. These factors are controlled by the equilibrium between photosynthesis, sulfate reduction, respiration and sulfide oxidation (Visscher and Stolz 2005). The relationship between the composition of the prokaryotic community and the processes of mineral precipitation in the thrombolites has been the subject of a long-standing debate (Myshrall et al. 2010). Dupraz and Visscher (2005) suggest that the critical factors that support carbonate precipitation are due principally to microbial metabolic activities such as the production of exopolymer substances (EPS) and the saturation state of the solution. On the other hand, the sequestration of dissolved CO_2 by photosynthetic organisms increases the pH in the local microenvironment, promoting carbonate precipitation (Dupraz et al. 2009; Warden et al.

CONTACT Davor Cotoras dcotoras@uchile.cl Laboratorio de Microbiología y Biotecnología, Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Independencia, Santiago 1025000, Chile
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2016). Recently, metagenomic and metatranscriptomic studies have been conducted to identify the microorganisms and metabolic processes associated with microbialites. These studies suggest that microbialites may share key pathways related to carbonate precipitation, regardless of environmental conditions (Casaburi et al. 2016).

Currently, there are few places in the world where microbialites thrive. They are restricted to oligotrophic ecosystems such as (1) "alkaline lakes" [e.g., Lake Van, Turkey (Lopez-García et al. 2005), Crater Lake Satonda, Indonesia (Benzerara et al. 2010), Pyramid Lake, USA (Arp et al. 1999)], (2) "hypersaline lakes" and wetlands (e.g., Lago Vermelha, Brazil (Spadafora et al. 2010), Cuatro Ciénagas, México (Breitbart et al. 2009), Lake Clifton, Australia (Gleeson et al. 2016; Warden et al. 2016), La Brava wetlands, Chile (Farías et al. 2014), Highborne Cay, Bahamas (Mobberley et al. 2012; Myshrall et al. 2010), Lake Amarga, Chile (Solari et al. 2010; Soto 2016) and (3) "freshwater" [e.g., Ruidera Pools Natural Park, Spain (Santos et al. 2010)]. However, only two sites containing active thrombolites (Lake Clifton, Australia and Highborne Cay, Bahamas) have been microbiologically studied. These works show that bacterial communities of the thrombolites were mainly composed of *Proteobacteria*, *Bacteroidetes* and *Firmicutes* phyla in Lake Clifton, Australia (Gleeson et al. 2016) and of *Cyanobacteria* and *Proteobacteria* phyla in Highborne Cay, Bahamas (Mobberley et al. 2012; Myshrall et al. 2010).

These two lakes have served as study models. For example, it has been reported that Lake Clifton has been subjected to a steady increase in salinity since the first measurements published in the early 1970s (Williams and Buckney 1976). This has potentially altered the dominant microbial communities in the system and has restricted the formation of thrombolites (Gleeson et al. 2016).

Lake Sarmiento in Chilean Patagonia is the place where living thrombolites have been found in the southernmost region of the world (Solari et al. 2010), so it is of great interest to know their microbial and geochemical composition. Lake Sarmiento is part of the Torres del Paine National Park (51°03′00″S; 72°45′01″W). This National Park comprises approximately 242,000 hectares and has been declared a biosphere reserve by UNESCO because of the high diversity of landscapes, glaciers, valleys, snowfields, lakes and lagoons (Soto et al. 1994).

Lake Sarmiento is an elliptical closed basin with a surface area of 86 km^2 and a shoreline of 78.3 km, lying at an elevation of 75 m (Solari et al. 2012) with a maximum depth of 340 m (Airo 2010). It is an oligotrophic and alkaline lake with a mean salinity of 1.9 mg l^{-1}, a pH of 8.3 to 8.7, and a mean surface water temperature varying between 6.2 °C (winter) and 12.2 °C (summer) (Campos et al. 1994; Soto and Zúñiga 1991). The climate of the region is characterized as cold steppe with average annual air temperature of 7 °C and precipitation 700 mm.

Studies of Airo (2010) and Solari et al. (2010) revealed the presence of subfossil and submerged (living) thrombolites in Lake Sarmiento. Due to the permanent decrease in water level, dead subfossil thrombolites are massively exposed along the coast of Lake Sarmiento with heights of up to 8 m. As well, living thrombolites are actively growing under the surface of lake water (Airo 2010; Solari et al. 2010). However, the microbial communities and the chemical composition have been poorly studied.

Lake Sarmiento, as well as other oligotrophic ecosystems in the world, needs to be subjected to a deep scientific investigation, due to its distinctive characteristics, which could allow finding new evolutionary or adaptive mechanisms and development of new biotechnological applications. For this reason, it is important to study and preserve these sites, in order to increase the knowledge of the ecology of these ecosystems (Paul and Mormile 2017).

It is of great importance to determine the prokaryotic community structure and chemical composition of the thrombolites due to the fact that environmental alterations, such as climate change and anthropogenic interventions, could produce changes in their chemical and microbiological composition and be determining factors in the precipitation of different mineral forms (Warden et al. 2016).

Studies simulating artificial microcosms of microbialites under laboratory conditions are limited. Among them are microbialites from Exuma Sound, Bahamas (Havemann and Foster 2008), microbialites from the alkaline Lake Alchichica, Mexico (Couradeau et al. 2011) and the stromatolites from coastal Lagoa Vermelha, Brazil (Vasconcelos et al. 2014). These models help to understand the biochemical processes involved in the formation of microbialites and simulate possible anthropogenic and environmental influences in this type of ecosystem. There are no studies of maintenance of thrombolites in aquariums. Hence, advances in new sequencing technologies may help to estimate the microbial diversity and community composition of thrombolites from Lake Sarmiento.

Based on the above background, the following questions arise: are the microbial communities of thrombolites and/or microbialites from other regions of the world similar to those of Lake Sarmiento? Is it possible to maintain the mineralogical and bacterial composition of a thrombolite under experimental laboratory conditions? Do Lake Sarmiento thrombolites harbor bacterial groups that have been reported as calcium carbonate precipitation inducers?

In this study, we hypothesized that the bacterial community composition of Lake Sarmiento thrombolites is similar to that of microbialites from other saline aquatic systems. We also propose that it is possible to preserve the bacterial composition by maintaining the conditions in laboratory aquariums, simulating the water chemistry of Lake Sarmiento. Therefore, the bacterial diversity of living and maintained thrombolites in the aquarium was studied by sequencing the hypervariable V4 region of the 16S rRNA gene. The chemical composition of both thrombolite samples was analyzed by XRD mineralogical assays. The objectives of this study were: (i) to compare the bacterial populations of Lake Sarmiento thrombolites with those of microbialites from other sites, (ii) to compare the bacterial diversity of underwater (living) thrombolites with the bacterial diversity of a thrombolite maintained in an aquarium

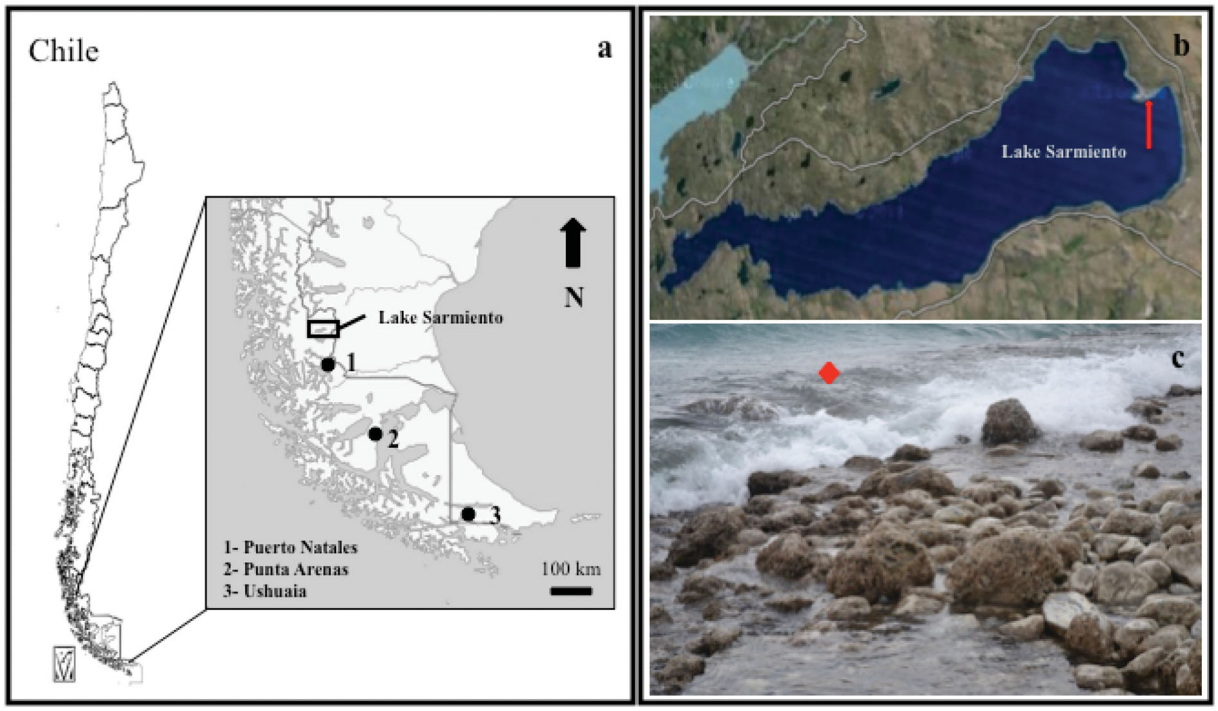

Figure 1. Location of the sampled thrombolite at Lake Sarmiento (a) Map showing location of Lake Sarmiento, approximately 80 km north of Puerto Natales in Southern Chile. (b) Google map image showing the sampling area, located at the northwest area of Lake Sarmiento. (c) Picture of the shoreline of Lake Sarmiento, where the red rhomboid indicates the approximate sampling site of living thrombolites.

(iii) study the chemical and mineralogical composition of thrombolite samples and (iv) identify bacterial taxa potentially involved in carbonate precipitation and the formation of thrombolites.

Materials and methods

Samples collection

Samples of subaerial fossil thrombolites (1 m height above the ground) and living submerged thrombolites (15 cm depth under water) were obtained on the shore of the small peninsula in the northeast of Lake Sarmiento (51°03′00″S; 72°45′01″W) in March 2016 at the Torres del Paine National Park in Chilean Patagonia (Figure 1). Samples were picked up with gloves and sterile scalpel and tweezers to minimize possible contaminations, placed in sterile bags and kept at 4 °C until processing. The samples are named as LTHR (underwater living thrombolite), STHR (subfossil thrombolite) and AQTHR (aquarium thrombolite) for the fragment of the living thrombolite (Figure 2), deposited in an aquarium of $23 \times 17 \times 23$ cm and maintained during 18 months before chemical and genetic analyses. Only the LTHR and AQTHR samples were used for genetic analyses.

Aquarium conditions

This aquarium was illuminated with a 2 W – 120 lumens Led lamp, producing light at all wavelengths of the solar spectrum. The photoperiod was 12 h in the daytime. A synthetic solution was used, constituted in g l^{-1} by NaCl, 0.1; K_2SO_4, 0.02; $MgSO_4$, 0.12; $NaHCO_3$, 1.3; K_2HPO_4, 0.02; Na_2HPO_4, 0.2; $CaCl_2$, 0.06 and $(NH_4)_2SO_4$, 0.01, pH 8.5, simulating the water composition of Lake Sarmiento (Campos et al. 1994). For water recirculation a submersible pump of 2.5 W with a maximum flow of 150 L h^{-1} was used. Each week, the loss of water due to evaporation was compensated with distilled water. Once a month, the temperature (21.2 ± 1.9 °C) and pH (9.0 ± 0.1) were measured. To keep the salinity and composition of the solution almost constant, 20% of the total volume was replaced, adding fresh synthetic solution, monthly.

Calcium and magnesium determination

A thrombolite sample was dissolved in hydrochloric acid (0.2 mol l^{-1}) for 24 h, followed by filtration. Calcium and magnesium measurements were performed in the obtained solution by Atomic Absorption Spectrophotometry with flame using a Perkin Elmer, Model 3110, Atomic Absorption Spectrophotometer, USA (Perkin Elmer, Norwalk, CT, USA). The solution was diluted to determine Ca at 442.7 nm in the linear range from 0.1 to 5 mg l^{-1} and Mg at 285.2 nm in the linear range from 0.01 to 0.5 mg l^{-1}.

Carbonate determination

Between 0.2 and 0.3 g (±0.0001 g) of dry thrombolite (60 °C for 48 h) was weighed, in duplicate. The sample was treated with 25 ml of dilute hydrochloric acid (0.2 mol l^{-1}) and kept overnight. Subsequently, an aliquot of 5 ml of the solution was titrated with sodium hydroxide (0.2 mol l^{-1} NaOH) to neutralize the residual acid (which was not neutralized by the carbonate).

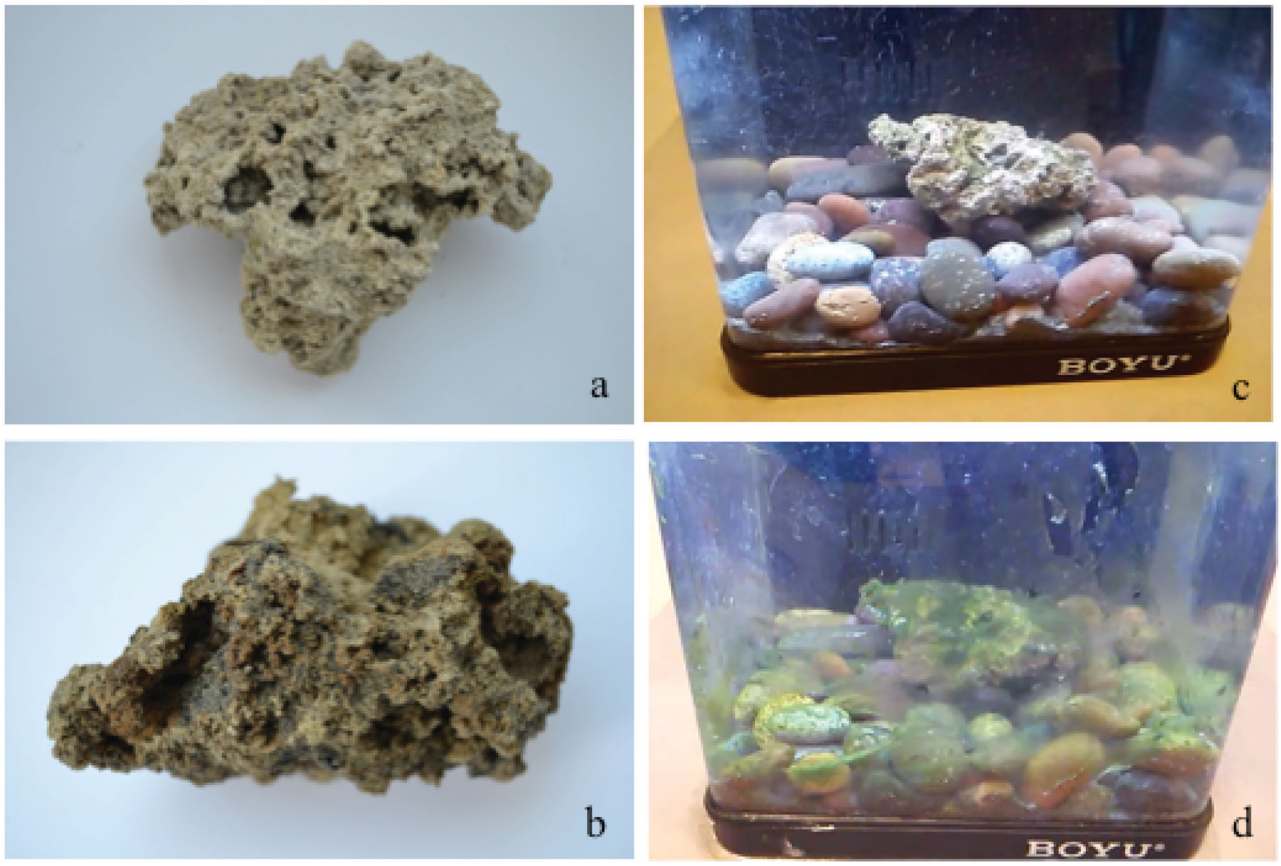

Figure 2. Images of the (a) subfossil thrombolite, (b) underwater living thrombolite (field) and the underwater living thrombolite at laboratory aquarium at (c) day 0 and (d) after 1.5 years.

Mineralogical analysis using XRD

The samples were pulverized to obtain fine particles and then evaluated using a XRD Bruker D8 Advance Diffractometer with LynxEye Detector (CuKα1, 40KV, 30 mA, 0.5 sec, step size 0.02 degree, 2–80 degree in 2 Theta, locked coupled scan without rotation). The diffraction spectrum obtained was then compared with standard spectra (Liu et al. 2007). Mineral phase identification was carried out using the ICDD PDF-2 Database. Quantitative phase analysis was performed using TOPAS 4.2 software (Bruker 2009).

Geochemical modeling

Based on the composition data of Lake Sarmiento water determined by Campos et al. (1994) and Ríos and Soto (2009), a calculation of chemical speciation was carried out to establish the main species present in the aquatic environment. For this purpose, the SpecE8 program within the Geochemist's Workbench program v. 11.0.8 (Rockware, Golden, Colorado, USA) was used. The saturation index (SI) in Lake Sarmiento was calculated using the MINTEQ database (thermo_minteq.dat). The SI allows the prediction of the thermodynamically stable mineral phases that may exist under the experimental conditions.

DNA extraction and tag sequencing process

About 1 cm of each underwater living (LTHR) or aquarium cultured (AQTHR) thrombolite was sectioned from the surface using a sterile scalpel. Total genomic DNA was extracted from 0.25 g of each sample using the Power Soil DNA Isolation Kit (MO BIO Laboratoties, Inc.). PCR and sequencing were completed according to the protocol used by the Center for Genomics and Bioinformatics, Faculty of Sciences of the Universidad Mayor (Chile) and described below. PCR was performed on 1–5 ng of total extracted DNA, targeting the V4 hypervariable region of the 16S rRNA bacterial genes using the 515 F and 806 R primer pairs (Caporaso et al. 2011). Three replicates of each performed PCR were amplified in 35 µL final volume with Taq buffer 1× final concentration, 2 nmol l^{-1} of $MgCl_2$, 0.3 nmol l^{-1} of dNTPs, 0.3 µmol l^{-1} of each primer and 2.5 units of GoTaq Flexi DNA polymerase (Fermelo). Amplification was performed under the following conditions: 94 °C for 3 min followed by 28 cycles of 94 °C for 30 s (denaturation), 57 °C for 1 min (annealing), 72 °C for 1.5 min (polymerization) and a final extension of 72 °C for 10 min. The three generated amplicons were pooled and quantified using a standard qPCR assay using a Library Quant Kit Illumina (Kapa) according to manufacturer instructions and sequenced using a 300 cycles Illumina Miseq kit (Caporaso et al. 2011).

Data analysis

Using Mothur software, raw sequences were trimmed and selected for length (>200 bp) and for the absence of ambiguous bases, homopolymers longer than eight base pairs and more than one primer mismatch (Schloss et al. 2009). The 5' and 3' adaptors were removed from the sequences using Cutadapt (Martin 2011). Chimeras were screened and removed using UCHIME (Edgar et al. 2011). The remaining sequences were classified using the Silva NGS website with Silva database release 123 (Quast et al. 2012; Yilmaz et al. 2014). We also assigned the sequences to Operational Taxonomic Units (OTUs) at 97% similarity and calculated the Shannon and Chao1 indexes using Qiime (Caporaso et al. 2011). Sequences were normalized to the same number of reads using the *sub.sample* option in Mothur (Schloss et al. 2009).

Microbialite samples comparison with other world regions

The bacterial communities of the LTHR and AQTHR samples were compared at the phylum level with those from microbialites (stromatolites and/or thrombolites) thriving in different regions of the world. The bacterial relative abundance of microbialite communities from four different regions was examined using Unweighted Pair Group Method with Arithmetic mean (UPGMA) based on Bray–Curtis distance at 95% similarity. The samples correspond to thrombolytic mats of four types (black (A1), beige (A2), pink (A3) and button (A4)) from Highborne Cay in the Bahamas (Mobberley et al. 2012) as well as two aquarium cultured stromatolites (B1 and B2) and one field-sampled stromatolite (B3) from Alchichica Crater Lake, Puebla State of Mexico (Couradeau et al. 2011), one microbialite (C) sample from la Brava in Northern Chile (Farías et al. 2014) and two sections from a thrombolite from the outer (D1) and inner (D2) surfaces from Lake Clifton in Western Australia (Gleeson et al. 2016).

Samples database deposition

The raw DNA sequences from the two sequenced samples were deposited in the SRA database under the accession number SRP140680.

Results

Elemental and mineralogical composition

XRD analysis showed that the thrombolite samples were mostly composed of crystalline phases, with some variation in proportions among the samples (Table 1). As expected, carbonates were the major minerals in all samples. The living thrombolite, obtained under the lake water (LTHR), showed predominantly magnesium calcite (87.8%). In the sample of thrombolite maintained in an aquarium for 1.5 years (AQTHR), magnesian calcite (75.8%) was predominant. This mineral was similar to the magnesium calcite existing in the LTHR sample. However, in the sample of subfossil thrombolite (STHR) two minerals predominated: Mg-rich calcite (58.5%) and calcite (24.5%). Other minerals such as albite, anorthite, nimite and quartz were also detected, but in lower abundances (Table 1). The presence of clay minerals in these thrombolites may be associated with detritus entrapment. An unidentified amorphous phase was noted in the samples, being prominent (ca. 10%) in the subfossil thrombolite sample (STHR).

Table 1. Relative abundances (%) of the major minerals composing the subfossil (STHR), underwater living (LTHR) and cultured (AQTHR) thrombolites revealed by XRD analyses.

Mineral	Formula	STHR	LTHR	AQTHR
Albite, disordered	$Na(Si_3Al)O_8$	4.0 (1)	0	10.4 (5)
Albite, Ca-rich, ordered	$(Na,Ca)Al(Si,Al)_3O_8$	0	3.2 (2)	0
Anorthite, Na-rich	$Na_{0.33}Ca_{0.67}Al_{1.67}Si_{2.33}O_8$	0	0	2.1 (3)
Calcite, Mg-rich	$Mg_{0.1}Ca_{0.9}CO_3$	58.5 (1)	0	0
Calcite, syn	$CaCO_3$	24.5 (1)	0	0
Magnesium calcite, syn	$Mg_{0.06}Ca_{0.94}CO_3$	0	87.8 (4)	0
Calcite, magnesian	$Mg_{0.1}Ca_{0.9}CO_3$	0	0	75.8 (5)
Nimite	$(Ni,Mg,Al)_6(Si,Al)_4O_{10}(OH)_8$	0	0	0.83 (9)
Quartz, syn	SiO_2	2.1 (2)	9.0 (3)	10.6 (2)
Amorphous		10.9	0.01	0.28

*In brackets (% error).

Elemental chemical analysis confirmed the presence of carbonate, Ca and Mg showing no important differences among LTHR, AQTHR and STHR samples. Almost 50% of the composition of the thrombolites belonged to carbonate and 30% to Ca. The Mg contents were 1.8% for the STHR and LTHR samples and 1.6% for the AQTHR sample (Table 2). The formulas of the carbonates, calculated from the chemical analyses, confirm that the composition of the minerals of the LTHR and AQTHR samples were similar to each other. For the STHR sample, the ratio estimated by XRD between carbonates Mg-rich calcite (70.5%) and calcite (29.5%) was used to calculate the Mg-rich calcite formula. It was found that this mineral had a molar fraction of Mg of 0.13 (Table 2).

Simulation of Lake Sarmiento aqueous chemistry

Saturation index values were calculated for carbonate minerals with the equilibrium-based SpecE8 program (Geochemist's Workbench), based on the composition data of Lake Sarmiento water determined by Campos et al. (1994) and Ríos and Soto (2009). The speciation yielded information for several carbonate-related minerals (Table S.1). According to the composition, temperature, pH, and ionic strength of the water in contact with the microbialites, favorable conditions existed for the formation of carbonate minerals. The calculated SI values indicate that the water conditions remained supersaturated with respect to dolomite, calcite, aragonite, magnesite, huntite, and vaterite (SI > 0.0). Considering this, it is possible that the aquatic environment plays a fundamental role in the composition of the microbialites isolated from Lake Sarmiento. The results of the simulation were consistent with the experimentally determined mineralogical composition, which showed a predominance of calcite, magnesian calcite, magnesium calcite and high Mg-calcite in the samples analyzed.

Table 2. Chemical analyses of Lake Sarmiento thrombolites.

Lake Sarmiento Thrombolite	Carbonate (%)[a]	Ca (%)[b]	Mg (%)[b]	Calculated formula
Subfossil thrombolite (STHR)	50.5	30.6	1.8	$Mg_{0.13}Ca_{0.85}CO_3$ (70.5 %)[c] $CaCO_3$ (29.5%)[c]
Underwater living thrombolite (LTHR)	49.6	31.3	1.8	$Mg_{0.09}Ca_{0.91}CO_3$
Cultured thrombolite (AQTHR)	49.2	29.1	1.6	$Mg_{0.08}Ca_{0.92}CO_3$

[a]Acid base titration method.
[b]AAS (Atomic Absorption Spectrometry).
[c]Ratio estimated by XRD.

Table 3. Number trimmed sequences, richness and diversity indices for each sample.

High quality sequences						Normalized sequences[a]			
Sample	Number of sequences	OTUs	Chao1	% Coverage	Shannon index	OTUs	Chao1	% Coverage	Shannon index
AQTHR	46,579	464	567	83%	4.32	–	–	–	–
LTHR	56,801	630	800	79%	4.66	603	750	80%	4.66

OTU: Operational Taxonomic Unit.
[a]The sequences numbers of the LTHR sample was normalized to 46,579 using *sub.sample* in Mothur (Schloss et al. 2009).

Sequencing of bacterial communities

A total of 120,861 raw sequences (average length 151 bp) were obtained after Illumina Miseq sequencing of PCR amplified 16S rDNA genes from total extracted DNA from our two samples. After the trimming process, 92% of the sequences remained. A total of 2,895 sequences (1.1%) and 4,566 sequences (1.0%) were detected as chimeras for the AQTHR and the LTHR samples, respectively and subsequently removed. The LTHR sample contained 56,801 sequences after cleaning, while the AQTHR sample had 46,270 sequences. Remaining sequences of each sample were considered as high-quality reads and used for further analyses.

Bacterial richness, diversity and community composition

The numbers of Operational Taxonomic Units (OTUs) were defined by a 97% similarity cutoff. We obtained 467 OTUs for the AQTHR sample and 630 OTUs for the LTHR sample (Table 3). The Chao1 estimator shows 567 and 800 absolute species in samples AQTHR and LTHR, respectively, revealing >82% identification of the total OTU population for the AQTHR sample and >78% for the LTHR sample. The diversity in the samples, calculated by the Shannon index, revealed a higher diversity in the LTHR sample than in the AQTHR sample. Normalization of the sequences from LTHR sample to 46,579 did lower the OTUs and Chao1 richness estimators. However, the Shannon diversity index remained relatively stable (Table 3).

The normalized sequences of the AQTHR and LTHR samples were classified with SILVA database release 123 in the SILVA NGS website. Sequences from the two samples could be classified into 18 phyla dominated by the *Proteobacteria*, *Planctomycetes*, *Verrucomicrobia*, *Bacteroidetes*, *Gemmatimonadetes* and *Acidobacteria* (Figure 3). Unclassified sequences at this level represent <1% of the total sequences. The most dominant phyla in the AQTHR sample were the *Proteobacteria* (26%) and the *Planctomycetes* (23%), whereas in the LTHR sample this phylum represents 13% of the total sequences. In sample LTHR, the most abundant bacteria belong to the

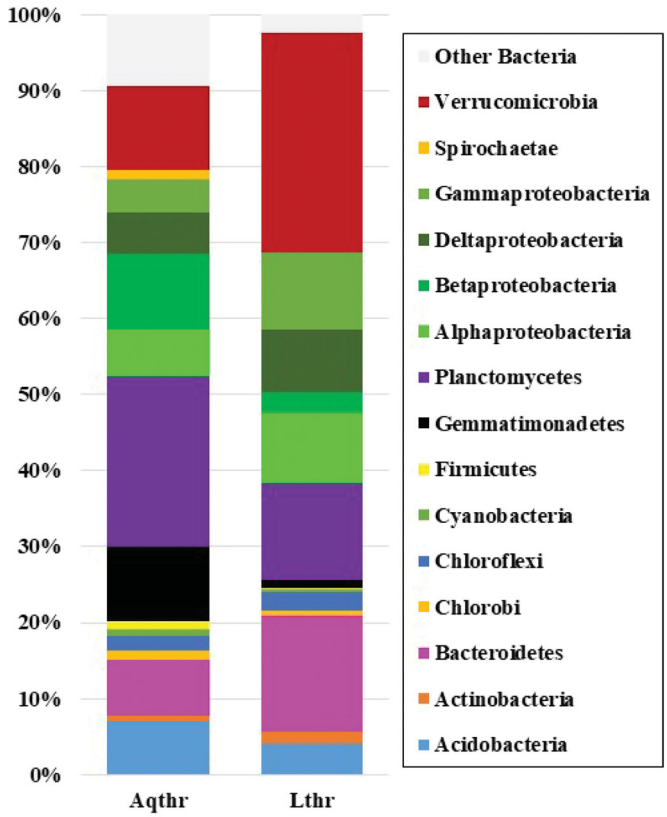

Figure 3. Bacterial phyla composition of the aquarium cultured (AQTHR) and underwater living (LTHR) thrombolite samples from Lake Sarmiento.

Proteobacteria phylum (30%), followed by the *Verrucomicrobia* phylum (29%). This last was found to represent 11% of the total sequences of the AQTHR sample. Members of the *Gemmatimonadetes* phylum were found to be more dominant in the AQTHR sample (9.8%) than in the LTHR sample (1%), whereas members of the *Bacteroidetes* phylum are more common in LTHR sample (15%) than in AQTHR sample (7.2%). The relative abundance of the *Chloroflexi* phylum was 2.06% in the AQTHR sample and 2.4% in the LTHR sample (Figure 3).

Within the *Proteobacteria* phylum (Figure 4), the *Gammaproteobacteria* class was more abundant in the

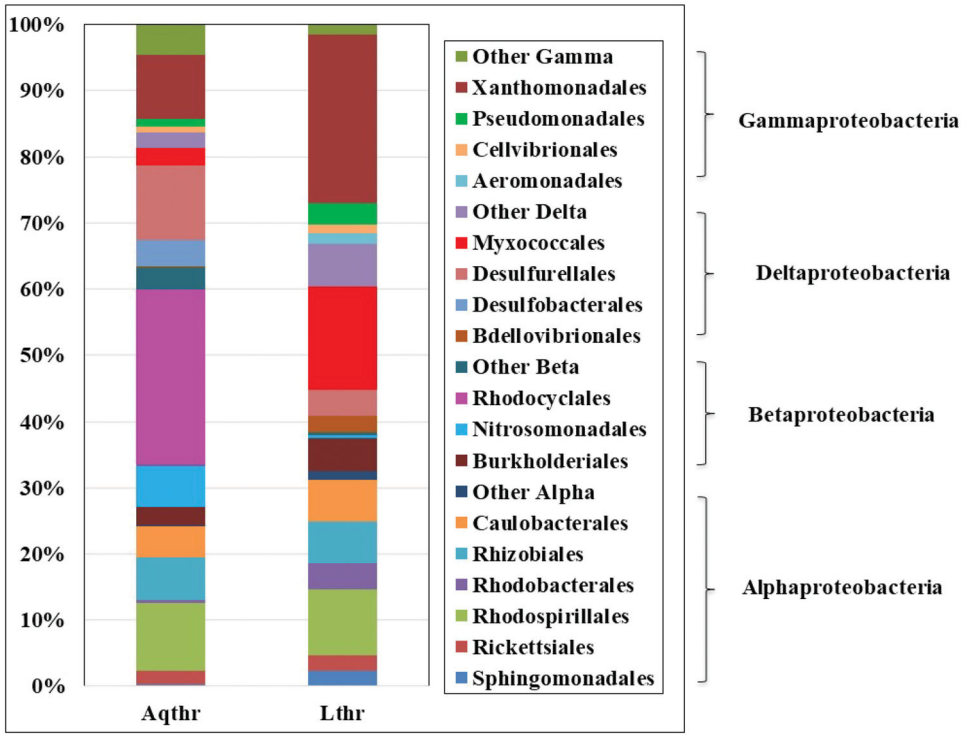

Figure 4. Bacterial classes composition of the aquarium cultured (AQTHR) and underwater living (LTHR) thrombolite samples from Lake Sarmiento.

LTHR sample than in the AQTHR. Within the *Gammaproteobacteria* class, the *Xanthomonadales*, *Pseudomonadales* and *Cellvibrionales* were found in both samples. These groups were present in higher proportions in the thrombolite sample LTHR. Within the *Deltaproteobacteria* class, the thrombolite AQTHR shows higher proportions of *Desulfurellales* and *Desulfobacterales* orders. However, the abundance of the *Myxococcales* order was three-fold higher in the LTHR sample. Within the *Betaproteobacteria* class, the orders *Rhodocyclales* and *Nitrosomonales* had higher relative abundances in the AQTHR sample than in the LTHR sample. In the *Alphaproteobacteria* class, the *Rhodobacteriales* and *Sphingomonadales* orders were found to be more abundant in the thrombolite sample LTHR. However, the *Rhodospirillales* and *Rickettsiales* were found at similar levels of relative abundance in both samples.

At the genus level, between 14% and 17% of the sequences belong to currently unknown bacterial genera (Table S. 2). The most abundant genera (≥1%), are the *Chthoniobacter* (10.80%), *Luteolibacter* (4.02%), *Haloferula* (1.96%), and *Haliangium* (1.85%) in the LTHR sample and an uncultured *Gemmatimonadaceae* genus (15%), *Denitratisoma* (6.14%), SM1A02 (*Phycisphaeraceae* family) (4.59%), *Opitutus* (3.09%) and H16 (*Desulfurellaceae* family) (2.83%) in the AQTHR sample (Table S.2). A total of 349 bacterial genera in common were found between the two samples. However, the AQTHR and LTHR samples contain individual unique genera, represented by 504 and 410 genera, respectively.

Bacterial communities from Lake Sarmiento were compared with those from other environments, shown in Figure 5. The different regions were found to contain bacterial communities that were distinct at the phylum level. However, the LTHR and AQTHR samples from Lake Sarmiento clustered together in a group detached from the clusters of samples used for comparison. The AQTHR and LTHR thrombolite samples from Lake Sarmiento clustered closest to the C microbialite sample from La Brava of Northern Chile, which was dominated by *Proteobacteria*, *Chloroflexi*, *Firmicutes*, *Verrucomicrobia* and *Spirochetes* phyla. The thrombolytic mat samples (A2–A4) from the Bahamas clustered together, having higher abundance of *Alphaproteobacteria* (>40%) than the samples in this study and undetectably low abundances of *Verrucomicrobia* and *Planctomycetes* although these phyla were detected in the LTHR and AQTHR samples of Lake Sarmiento. The thrombolytic A1 sample from the Bahamas clustered together with the B1 and B3 samples from Alchichica Lake in Mexico, principally due the differences in abundance of *Cyanobacteria*. It was found that *Firmicutes* were present in significant proportions (>25%) in the D2 sample from Lake Clifton in Western Australia and the B2 sample from Alchichica Lake in Mexico, which contributed to these samples clustering together. Principally, the *Alphaprotecobacteria* and *Bacteroidetes* dominated the D1 sample from Lake Clifton in Western Australia, setting it apart of the other samples.

Discussion

The bacterial community composition from the sub-saline Lake Sarmiento thrombolite was examined by sequencing the 16S rRNA gene. A submerged thrombolite fragment was analyzed and compared to a fraction of the original, which was cultured in a laboratory aquarium for 1.5 years. In this

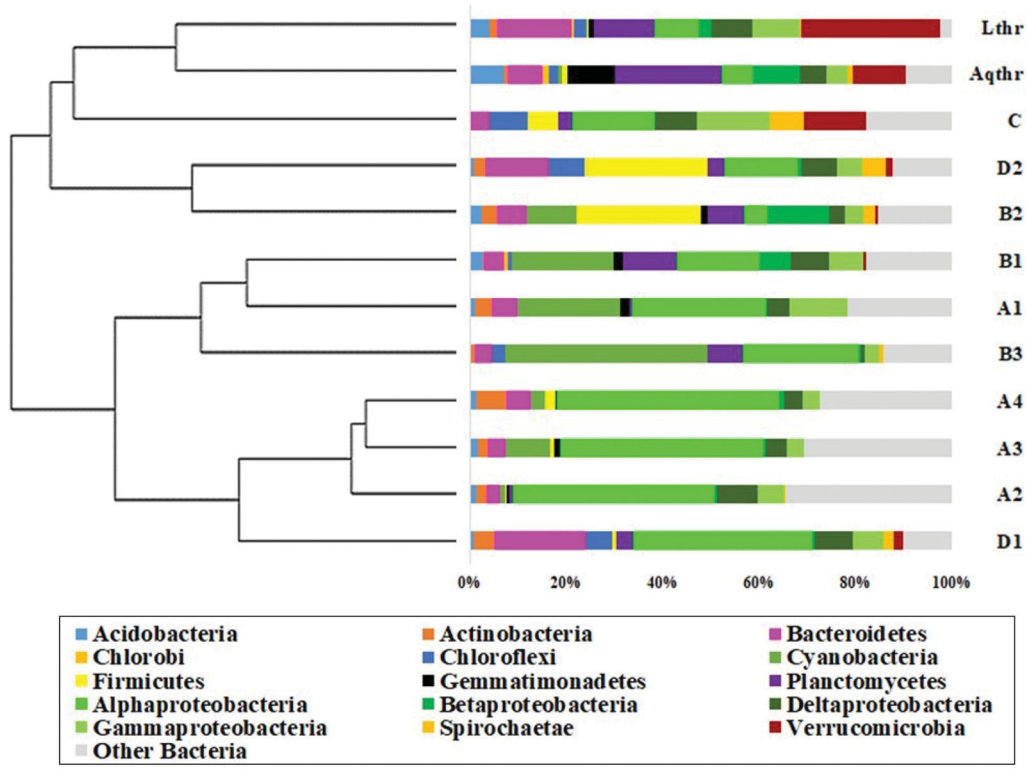

Figure 5. Comparison of Lake Sarmiento samples with other worldwide sites at the phylum level. An UPGMA clustering using the Bray-Curtis distance at 95% was constructed. The histograms represent the bacterial composition at phylum level based on the relative abundance. The samples correspond to four thrombolytic mats types: black (A1), beige (A2), pink (A3) and button (A4) from Highborne Cay in Bahamas (Mobberley et al. 2012). Two aquarium cultured (B1 and B2) and one field B3) stromatolites from the Alchichica Crater Lake, Puebla State of Mexico (Couradeau et al. 2011), one microbialite (C) sample from la Brava in Northern Chile (Farías et al. 2014) and two thrombolites from outer (D1) and inner (D2) surface from Lake Clifton in Western Australia (Gleeson et al. 2016).

work, the chemical composition of the thrombolite fragment was characterized and the bacterial communities were compared with those existing in microbialites from other regions of the world.

Lake Sarmiento is a natural water basin located in the National Park Torres del Paine (Chilean Patagonia) and one of the unique places in the world where thrombolites are developed. The advances of new scientific technologies are helpful to study those structures formed by microbial communities and allow researchers of diverse disciplines to understand ecological traits as climate change and anthropogenic pollution influence the natural world. Preserving the original conditions and the natural resources of this ecosystem is extremely important for the maintenance of the ecologic equilibrium of this area.

Thrombolites from Lake Sarmiento have been subjected to geological and paleontological studies (Airo 2010; Solari et al. 2010, 2012) during the last ten years. However, there are no papers that report a detailed examination of the bacterial community composition in these structures. Previous studies focused on the identification of biotic and abiotic factors controlling the microscopic and macroscopic formation of the microbialites at Lake Sarmiento and the relationships between the morphological development and the properties of associated microbial communities (Airo 2010) or on the formation of microbialite carbonates as paleoclimatic indicators (Solari et al. 2010).

Almost the full extent of the coastline of Lake Sarmiento is underlain by massive carbonate buildings that occur approximately 8 m above the current level of the lake, due to the decrease in lake level. The microbial communities of living thrombolites are now growing below the surface of the water. Solari et al. (2010) reported that living thrombolites are composed predominantly by magnesium calcite (87.8%). The chemical analyses carried out in our work are also consistent with this identification. The thrombolite fragment maintained for 1.5 years in an aquarium in a synthetic solution of similar composition to the water of Lake Sarmiento did not show major changes in chemical composition and mineralogy. In contrast, the presence of two carbonate minerals in the subfossil thrombolite (Mg-rich calcite and calcite) was not expected. However, chemical analyses showed that the subfossil sample had a composition similar to living thrombolite. These results suggest that once the living thrombolites are exposed to the air due to the decrease in the level of the lake, they could undergo a process of diagenesis and recrystallization, which may alter their mineralogy (Pace et al. 2016; Paul et al. 2016). However, it is also possible that these results might indicate that the composition of dissolved minerals in the lake has changed over time.

The presence of the main anions and cations in the water has been correlated with the type of minerals that can be formed in the different types of modern lacustrine microbialites (Chagas et al. 2016; Valdespino-Castillo et al. 2018; Zeyen et al. 2017). This is consistent with the prediction of the formation of different calcium and magnesium carbonates (dolomite, calcite, aragonite, magnesite, huntite, and

vaterite), obtained by modeling with the Geochemist's Workbench software, using the chemical composition described for Lake Sarmiento. For a long time the Mg/Ca ratio has been considered as a main determinant of carbonate mineralogy in aqueous environments. For Mg/Ca >2 the production of aragonite, high Mg-carbonates and dolomite has been frequently found (Chagas et al. 2016; Zeyen et al. 2017). These observations agree, in general, with our results, since the Mg/Ca ratio was approximately 3.5 (Campos et al. 1994; Ríos and Soto, 2009) and the thrombolites were composed mainly of magnesium calcite. However, the Lake Sarmiento thrombolites are different from modern microbialites in other lakes (for example Lake Clifton), where Mg/Ca ratios are similar, but aragonite predominates (Zeyen et al. 2017).

Other environmental factors may also be important in the formation of microbialites. It is interesting to consider that Lake Sarmiento and Lake Amarga are part of the same hydrologic system. Both lakes have adequate conditions for microbialite development (close basins, higher evaporation than precipitation and contribution of carbonate to the lake water through leaching of the underlying rocks) (Solari et al. 2010). However, their microbialites are quite different. Thrombolites, formed by magnesium calcite, are present in Lake Sarmiento, whereas stromatolites, constituted primarily by aragonite, occur in Lake Amarga. Solari et al. (2010) suggested that salinity could be an important factor in determining the type of microbialite. Higher salinity favors stromatolites, and lower salinity favors thrombolites. In addition, other environmental conditions may have a determining role. For example, Warden et al. (2019) recently highlighted the importance of groundwater flow to the formation of modern thrombolitic microbialites.

The 16S rRNA gene sequencing of the thrombolite samples (LTHR and AQTHR) show >90% of the total coverage of the bacterial communities at the phylum level (Figure 3) and captures the majority of the bacterial richness present in the samples (Figure S.1). To prevent an overestimation of the bacterial richness and diversity, we used a pairwise similarity threshold of 97% to reduce sequencing errors, and we normalized the number of sequences for both samples to reduce overestimation of the bacterial communities (Schloss et al. 2009). The calculations of the number of OTUs defined by a 97% cutoff reveals a higher richness in the LTHR sample (630 OTUs) than in the AQTHR sample (464 OTUs). These values were found to be in the same broad range observed in marine thrombolite mats from the Bahamas (Mobberley et al. 2012). The diversity, represented by the Shannon index, indicates that the LTHR sample is slightly more diverse than the aquarium AQTHR sample (4.66 vs. 4.32) (Table 3). This small difference may be due to more stable conditions in the aquarium, excluding some native bacterial taxa associated with the field thrombolite. Contrary to this, the study performed by Couradeau et al. (2011) in Lake Alchichica (Mexico) found much more diverse microbial communities in the microbialites maintained for two years in an aquarium than the lake microbialiltes.

It was also observed that the composition of the bacterial community of all these microbialites differed little at the phylum level (Figure 5). Interestingly, the sample of the microbialite called "C" from the Atacama Desert, in northern Chile, was most similar to the samples from this study, suggesting a possible relationship with geographic location (Hanson et al. 2012). However, the environmental conditions of the two sites are not similar, since desert samples are exposed to high solar radiation, extreme temperature fluctuations, and high concentrations of arsenic, among others (Farías et al. 2014).

It was found that the thrombolite mat samples from Highborne Cay, Bahamas (Mobberley et al. 2012) and the D1 sample from Lake Clifton, Australia (Gleeson et al. 2016) were the samples most dissimilar to those from Lake Sarmiento (Figure 5). This is not surprising, due to the low oxygen levels of the mat samples from the Bahamas. The D2 sample from the inner surface of a thrombolite from Lake Clifton, Australia (Gleeson et al. 2016) was found to be closest to the stromatolite B2 (aquarium sample) from Lake Alchichica, which contains almost the same proportions (~25%) of *Firmicutes* (Figure 5). *Firmicutes* are considered important drivers in microbialite formation, because they facilitate calcium carbonate precipitation through metabolic activity and by producing EPS (Dupraz and Visscher 2005).

Interestingly, we also observed that samples from the same geographic locations (A and D samples) did not cluster together, suggesting that other factors, such as microbiolite pigmentation or structure (inner or outer surface) might be influencing the bacterial community composition. Therefore, if everything is everywhere and the environment selects (Baas Becking 1934; de Wit and Bouvier 2006), nearby lakes with different abiotic environmental conditions would have microbial communities that are not any more similar than lakes that are far apart. However, in this case, microbial communities from lakes which are geographically close together but exposed to different abiotic environmental conditions are clustered together. Therefore, this indicates that physical proximity is an important factor in determining the composition of the community and suggests that not everything is everywhere (Osman et al. 2018).

This also seems to occur with the microbial communities of microbialites of the Lake Amarga of Torres del Paine. Despite the geographic proximity of this lake to Lake Sarmiento, Soto (2016) reported a clearly different microbial composition in stromatolites of Lake Amarga. This difference may be due to the unlike water composition, especially their salinity and pH (Solari et al. 2010). However, the comparison should be made carefully because of methodological differences. While this work employed Illumina high-throughput DNA sequencing, Soto (2016) used denaturing gradient gel electrophoresis (DGGE)-cloning.

The bacterial composition of Lake Sarmiento samples was dominated by *Proteobacteria*, *Verrucomicrobia*, *Planctomycetes* and *Bacteroidetes*. In contrast, the *Cyanobacteria* phylum was present at low abundances. The LTHR sample contained 0.5% of *Cyanobacteria* and the AQTHR sample 2%. The *Spirulina* genus was identified in the AQTHR sample (1%). This genus was also observed as part of the microbial composition of microbialites of a

lagoon of Tikehau Atoll in French Polynesia (Abed et al. 2003). Other studies, comprising the examination of microbialites, also reported significantly low abundances of this phylum in their composition (Farías et al. 2014; Gleeson et al. 2016). The variability in the abundance of *Cyanobacteria* in different microbialites may be mainly due to physicochemical factors, especially the concentration of the major ions in water and mineral content (Valdespino-Castillo et al. 2018).

The *Proteobacteria* phylum was found to predominate in both LTHR and AQTHR samples; however, differences were observed at the examined class and genus levels. This phylum has been also reported to dominate in microbialites from Highborne Cay, Bahamas (Mobberley et al. 2012; Myshrall et al. 2010) and Alchichica Lake in Mexico (Centeno et al. 2012).

The *Alphaproteobacteria* class differed little between the LTHR (9.1%) and the AQTHR samples (6.8%). Six dominant orders were found in the samples (*Rhodospirillales*, *Rhodobacterales*, *Rhizobiales*, *Caulobacterales*, *Rickettsiales* and *Sphingomonadales*), with the *Sphingomonadales* and *Rhodobacterales* orders being the lowest in the AQTHR sample (Figure 4). Members of this group are also part of the microbial communities of thrombolite mats from Highborne Cay, Bahamas (Mobberley et al. 2013). Members of *Alphaproteobacteria* class are ubiquitous in marine microbial communities and could contribute to carbon fixation at depths where *Cyanobacteria* are not present due to the reduced light levels and the presence of sulfide (Louyakis et al. 2017).

Sphingomonadales were found in lower abundances as part of the diversity of other thrombolites (Gleeson et al. 2016; Mobberley et al. 2013). This group is part of the marine aerobic anoxygenic phototrophic bacteria and captures light using bacteriochlorophyll a and several carotenoids that serve as auxiliary pigments, which can be found on the surface of the structures of the thrombolites (Zheng et al. 2011).

Some bacteria of the *Rhodobacterales* and *Rhizobiales* orders were also reported in other studies as being sulfide oxidants and nitrogen fixers, playing a role in the nutrient cycle and carbonate precipitation, and consequently, they can influence thrombolite formation and accretion (Gleeson et al. 2016; Havemann and Foster, 2008; Louyakis et al. 2017; Myshrall et al. 2010; Mobberley et al. 2013). However, they are cosmopolitan and might be also found in non-lithifying habitats (Myshrall et al. 2010).

Members of the *Betaproteobacteria* class were also found in the samples. This class was observed as part of the composition of the bacterial community of giant microbialites from Lake Van, Turkey (López-García et al. 2005) and thrombolites from Lake Clifton, Western Australia (Warden et al. 2016).

Within the *Gammaproteobacteria* class, the *Xanthomonadales* order dominated both samples (Figure 4). They have also been described as part of the bacterial community of thrombolite mats from Highborne Cay, Bahamas (Mobberley et al. 2013) and Lake Clifton, Western Australia (Gleeson et al. 2016). Members of this order have been associated with nitrogen metabolism and carbonate dissolution (Chon et al. 2010; Gleeson et al. 2016).

The *Deltaproteobacteria* class varied from 5.4% to 8.4% in the AQTHR and the LTHR samples, respectively. Within this group, the *Myxococcales* and *Bdellovibrionales* orders were found to be of greater abundance in the LTHR sample; however, members of the *Desulfobacterales* and *Desulfurellales* orders were more abundant in the AQTHR sample (Figure 4; Table S.2). Mobberley et al. (2013) reported that members belonging to the *Deltaproteobacteria* class (*Desulfobacterales* and *Desulfovibrionales* orders) carried genes associated with the dissimilatory sulfate reduction pathways. Altogether, members from this group might play an important function in the thrombolite formations, especially by action of the Sulfate Reducing Bacteria (SRB), being implicated as having an important role in biologically induced carbonate precipitation (Baumgartner et al. 2006; Dupraz et al. 2009; Gallagher et al. 2012). In addition, part of the H_2S consumed by the anoxygenic photosynthesis may come from the activity of the SRB (Couradeau et al. 2011), represented by this bacterial group.

Field and cultured thrombolites from Lake Sarmiento also contain diverse and abundant heterotrophic bacteria belonging to the *Verrucomicrobia*, *Planctomycetes*, *Gemmatimonadetes*, *Chloroflexi* and, *Bacteroidetes* phyla (Figure 3). Members of these groups may induce carbonate dissolution due to the respiration of organic matter and production of protons (Dupraz and Visscher 2005) or promote carbonate precipitation by liberating cations sequestered by EPS and other macromolecules (Couradeau et al. 2011). For example, the *Bacteroidetes* phylum was found to dominate in hypersaline systems including microbial mats (Casaburi et al. 2016), microbialites (Mobberley et al. 2013) and soils (Lauber et al. 2009). They are known for their ability to uptake and degrade a wide range of high molecular weight biopolymers (Church 2008), and they seem to proliferate under anoxic and high salinity conditions (Farías et al. 2014). Members of the *Chloroflexi* phylum, were represented by <3% of the total abundance in the samples of this study. They may contribute to the photosynthesizing capacity of the community (Gleeson et al. 2016).

At the genus level, the bacterial communities of the LTHR and AQTHR thrombolytic samples were found to vary in their proportions (Table S.2). The most abundant genera in the LTHR sample were *Chthoniobacter* (*Verrucomicrobia* phylum), *Luteolibacter* (*Verrucomicrobia* phylum), *Haloferula* (*Verrucomicrobia* phylum) and *Haliangium* (*Proteobacteria* phylum). Genera belonging to the *Verrucomicrobia* phylum have a great ecological importance in soils, where their abundances can exceed 20% of the total bacterial community (Bergmann et al. 2011). However, little is known about their ecological and metabolic characteristics due to the low cultivation rate (Hugenholtz et al. 1998). Members of this phyla are present in marine (Cardman et al. 2014) and hypersaline mat (Spring et al. 2015) ecosystems. *Chthoniobacter* and *Opitutus* were the second most abundant genera in the AQTHR sample. They are present in soil and freshwater environments (Spring et al. 2016). The *Chthoniobacter* genus was also found as part of the most abundant and diverse phylotype belong to

the *Verrucomicrobia* phylum in stromatolites from the pool Pozas Azules at Cuatro Ciénagas, México (Bonilla-Rosso et al. 2012). The *Haliangium* genus was also detected. They are halophilic bacteria and have been isolated from saline soil ecosystems (Fudou et al. 2002). This suggests that these microbes can accomplish an important role in the ecology of the thrombolites from Lake Sarmiento, due to the current salt concentration in the water and a possible increase in salinity as a result of the continuous decrease in the water level of this lake (Campos et al. 1994; Soto et al. 1994).

In the AQTHR sample, the *Denitratisoma* (*Proteobacteria* phylum), *Opitutus* (*Verrucomicrobia* phylum), uncultured *Gemmatimonadaceae*, (*Gemmatimonadetes* phylum) and uncultured *Phycisphaeraceae* (*Planctomycetes* phylum) were found to be abundant (Table S.2). The *Denitratisoma* genus represented 10% of the total abundance of the sequences. It has been reported to grow under strictly anaerobic conditions and to participate in the reduction of nitrate to a mixture of dinitrogen monoxide and dinitrogen, with the intermediate accumulation of nitrite (Fahrbach et al. 2006).

Members of the *Cytophagaceae* family were found to comprise between 6% and 7% of the total relative abundance in the AQTHR and LTHR samples, respectively (Table S.2). This group is one of the largest families of the *Bacteroidetes* phylum and its members were associated with nitrogen-fixing activity in the digestion of macromolecules such as polysaccharides or proteins. An uncultured genus belonging to the *Gemmatimonadaceae* family was detected in about 15% of the total relative abundance in the AQTHR sample, suggesting that the conditions of this microcosm were ideal for the growth of members of this genus. Uncultured *Gemmatimonadaceae* were also detected in thrombolites from Highborne Cay, Bahamas (Mobberley et al. 2012) and were reported to be little studied due to slow growth under laboratory conditions.

Comparing between bacterial communities of microbialites is a challenge, due to the inherent heterogeneity of these systems: irregular structure, varieties of niches with different physical and chemical parameters at microscale. A better understanding of the effect of these parameters can be achieved using larger samples and more detailed information on the physical and chemical characteristics of each lake. Also, metagenomic analyses will help to evaluate the patterns of microbial activities in microbialites.

The experimental aquarium designed using adjusted laboratory conditions can potentially help in future studies to predict chemical and physical environmental changes in order to evaluate microbial community variations and understand their responses to metabolic pathways associated with the formation of thrombolites.

Conclusions

The results obtained in this study lead the following conclusions:

1. The sequencing of the V4 region of the 16S rRNA gene revealed the composition of the bacterial community of Lake Sarmiento thrombolite. Based on an UPGMA tree construction, it was found that its composition is similar to the bacterial profiles of other microbialites present in different regions of the world, such as Highborne Cay in Bahamas, Alchichica Crater Lake, Puebla State of Mexico, La Brava in Northern Chile and Lake Clifton in Western Australia. The greatest similarity was found with the microbialites of Lake La Brava and Lake Clifton.
2. A thrombolite maintained 1.5 years in an aquarium simulating the water chemistry of Lake Sarmiento had a bacterial community similar to that found in a living thrombolite extracted from this lake at the phylum level. However, differences between the communities were observed at the genus level. It was found that the *Proteobacteria*, *Verrucomicrobia*, *Planctomycetes* and *Bacteroidetes* phyla dominated the bacterial communities of both samples. No significant changes in the carbonate composition and mineralogy were observed after maintenance of the thrombolite in an aquarium.
3. Bacterial orders possibly involved in carbonate precipitation and the formation of thrombolites, such as *Rhodospirillales*, *Rhodobacterales,* *Rhizobiales*, and *Chloroflexi*, which include photosynthetic bacterial groups were identified. In addition, sulfate reducing bacteria, such as *Desulfobacterales* and *Desulfurellales* orders were found. The ability of SRB to increase the alkalinity of the microenvironment may promote the precipitation of calcium carbonate. Those SRB were found in higher proportions in the aquarium-cultured sample, in were the laboratory conditions were much more stable.

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Disclosure statement

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