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Effect of a cyanobacterial community on calcium carbonate precipitation in Puente del Inca (Mendoza, Argentina)

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The involvement of cyanobacteria in the precipitation process forming calcium carbonate was studied in samples collected at a geothermal spring located in an area close to Puente del Inca (Mendoza, Argentina). In the summer season profuse cyanobacterial growth is observed at Puente del Inca in areas exposed to sunlight and over which thermal water flows. Differences in cellular structure allowed the recognition of strains of *Oscillatoria, Spirulina, Plectonema,* and *Nostoc*, *Oscillatoria* and *Spirulina* being the dominant species. The mass cultivation of *Oscillatoria* sp. was obtained using a new culture medium (BW_3) PI which was formulated according to the chemical composition of the thermal water. On a dry-weight basis the biomass concentration was 0.88 g L^{-1} at pH 7.5 and 0.44 g L^{-1} with a free pH evolution after 11 days of incubation. The increase of pH associated with *Oscillatoria* sp. growth triggered calcium carbonate precipitation at values higher than 8.1. The events observed under laboratory conditions are likely to occur in situ as a consequence of cyanobacterial growth in the saturated thermal water of Puente del Inca.

Key words: Cyanobacteria, *Spirulina*, *Oscillatoria*, calcium carbonate, biomineralization, geothermal spring, Puente del Inca, Argentina

Introduction

Puente del Inca is a geological formation normally flowed over by thermal waters saturated in CO_2 and enriched in calcium salts. The main components of Puente del Inca is travertine. Travertine is an accumulation of calcium carbonate in springs (karstic, hydrothermal), small rivers, and swamps, formed mainly by incrustation (cement precipitation and/or biochemical precipitation). These deposits can form on higher and lower plants, but most commonly on algae (blue green, green), mosses, hepatics and on insect larva caparaces (JULIA 1983).

Living organisms contribute to the precipitation of minerals, a phenomenon generally referred to as biomineralization (BROWN et al. 1994, INAGAKI et al. 1997, JULIA 1983, SKLERYK et al. 1997, WESSELS and BÜDEL 1995). The involvement of cyanobacteria in the

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formation of carbonate minerals has been extensively studied in marine systems (SCHULTZE-LAM and BEVERIDGE 1994). It has also been shown that cyanobacteria can mediate the formation of calcite in fresh water environments and, indeed, are essential to the process (BATHURST 1975). In waters containing abundant calcium ions in which dissolved CO_2 is present predominantly as bicarbonate, cyanobacteria provide nucleation sites for mineral formation to commence (SCHULTZE-LAM and BEVERIDGE 1994).

At Puente del Inca when the conditions are propitious, mainly in the summer months, important cyanobacterial growth is observed in areas subjected to thermal water level oscillations. It therefore appeared purposeful to analyze the possible effect of cyanobacterial growth on the formation of precipitating salts, particularly calcium salts, leading to the formation of travertine deposits, which are the main components of Puente del Inca. Furthermore, a medium based on the chemical composition of the thermal waters was devised in order to isolate and cultivate the cyanobacteria present at the location site.

Material and Methods

Study site

Puente del Inca is situated in the Andes mountains of Mendoza, Argentina at 32° 50' S, 69° 55' W and an altitude of 2720 m above sea level. Puente del Inca possesses historic value and is also a popular tourist attraction that was exploited four decades ago for its thermal baths having healing properties and a hotel was even established at the site. At the moment, only the ruins of the facilities remain after an avalanche occurred. Underneath the formation flows the Cuevas River (Fig. 1.1). On the right margin, five thermal mineral springs are present from which ground water saturated in CO_2 emerges. In this place, travertine is deposited as the result of a readjustment process, in which the high content of dissolved calcium of the spring water is deposited as $CaCO_3$ to form travertine.

Sample collection and water analysis

Periodic visits every three months during a period of two years were done at Puente del Inca. In every visit, samples containing microalgal material were collected at the surface of Puente del Inca, under the bridge and also in areas over which thermal waters flow. The samples were examined in situ under light microscopy for the presence of cyanobacteria and their morphology was noted, followed by transportation to the laboratory in polyethylene containers kept at 5 °C in a water ice bath.

Horizontal temperature profiles in the direction of the flow starting from the thermal source were estimated using an electrode Orion Research digital ionanalizer/501, with a pH electrode and temperature probe. Several chemical analyses of the thermal waters were performed at the Centro Regional de Aguas Subterráneas, Mendoza, Argentina coincidentally with microalgal sampling (Tab. 1). The thermal waters showed a high content of sodium, sulphates, bicarbonates and chlorates. Apart from sodium, the main cations were calcium, magnesium and potassium, which accounted for 99.7% of the total amount of cations present. The bicarbonate content accounted for 15.7% of the total amount of anions with traces of nitrate, fluorate and phosphate.

Fig. 1. The Puente del Inca bridge, view from the left margin of Cueva's river (1), zone underneath the Puente del Inca colonised by cyanobacteria, visible as dark green bands (arrow) (2), *Oscillatoria* sp. in BW_3PI medium. Scale bar = 20 µm (3), salt precipitation in non-agitated cultures of *Oscillatoria* sp. (a) with pH control at 7.5, (b) without pH control after 5 days of incubation and (c) without pH control after 25 days of incubation (4), patches of a broken layer three months old of *Oscillatoria* sp. biomass associated with calcium carbonate developed in medium BW_3PI (5).

Tab. 1. Chemical composition of Puente del Inca thermal water and BW_3PI medium

Thermal water		BW_3PI	
Mineral	Amount (mg L^{-1})*	Constituent	Amount (g L^{-1})
Ca	990 ± 99.00	$NaHCO_3$	1.5
Mg	175 ± 27.12	K_2HPO_4	0.75
Na	4200 ± 210.00	$MgSO_4$	0.8666
K	180 ± 18.90	Na_2SO_4	0.6
HCO_3	1590 ± 270.30	$CaCl_2$	0.4
SO_4	1750 ± 157.50	NaCl	11.1
Cl	6750 ± 303.75	$NaNO_3$	0.5
Si	12 ± 0.84	$FeCl_3$	0.024
Fe	0.2 ± 0.022	EDTA	0.085
Mn	0.5 ± 0.047	$ZnSO_4$	0.004
F	12 ± 0.66	$Na_2B_4O_7$	0.008
NO_3	12 ± 1.20	Na_2MoO_4	0.002
B	12 ± 0.402	$MnSO_4$	0.004
As	1 ± 0.025	$NaVO_3$	0.0001
PO_4	0.001 ± 0.0006	$CoCl_2$	0.001
		$NiSO_4$	0.0002
		$CuSO_4$	0.002

* Means ± standard deviation of three analysis

Culture methods

For isolation procedures the following media were used: BG11, Z_8, Zarrouk (ZARROUK 1966), and BW_3 (SILVA et al. 1989). Based on the chemical composition of major ions of the thermal waters and taking BW_3 as a basal medium, salt additions were made in order to devise an artificial spring water medium called BW_3 PI (Tab. 1) which was later used to isolate and cultivate the cyanobacteria. This medium was prepared in the following five separate solutions: (a) K_2HPO_4 and $NaHCO_3$ adjusted to pH 7.7 with NaOH, (b) $CaCl_2$ and $MgSO_4$, (c) NaCl and KNO_3, (d) $FeCl_3$ + 2 mols EDTA per mol Fe, (e) other salts + 1mol EDTA per mol metal, and sterilized at 121 °C for 20 min and then mixed after cooling in the following order: a + b + c + d + e.

Three samples by duplicate were collected every visit at the locations previously described and 5 mL aliquots were inoculated in 250 mL Erlenmeyer flasks containing 25 mL of the isolation media and then incubated without agitation. When growth was observed 5 mL of the culture broth was transferred to 50 ml of medium BW_3PI and the flasks were incubated under photoautrophic conditions. After 15 days the culture broths were diluted and spread on plates of BW_3PI medium solidified with 1.5% agar. Single colonies were picked up and suspended in fresh BW_3PI medium for microscopic observation. Of the strains isolated only one was able to develop in mass cultures using the medium BW_3PI.

For mass cultures 50 mL of an actively growing culture, raised from a single colony, were inoculated and grown batch-wise in one litre Roux bottles containing 500 mL of the culture medium. Several Roux bottles by duplicate were included in the experiment to estimate cellular growth, to follow the pH evolution and to analyse the effect of pH on the growth of the cyanobacterial strain. The cultures were allowed to stand still without aeration and were continuously illuminated by a set of three fluorescent lamps with an average intensity of 13.3 W m^{-2} at the surface level. The light intensity was measured by a Kipp and Sonnen total radiation solarimeter. The cultures were incubated at 30 °C in a temperature controlled room under an atmosphere of the following composition 97% N_2: 3% CO_2. The effect of pH on cellular growth was analyzed in the range 7.5 to 10 with manual control of pH every 72 h using HCl 2 N for a period of 36 days. The pH was measured with an Orion pHmeter. The biomass was estimated by chlorophyll a determination and expressed on dry weight basis using the following coefficient: biomass dry weight (g L^{-1})/chlorophyll a (g L^{-1}) = 6.71, experimentally determined. For chlorophyll a estimation, the cells were centrifuged, washed twice with distilled water and extracted with 80% acetone and incubated for 30 minutes at 37 °C and then for 16 hours at 4 °C (SILVA et al. 1989).

The experimental design to analyze the relation between cyanobacterial growth and salt precipitation consisted of three bottles of 500 ml containing 250 to 300 mL of BW_3PI medium, each one inoculated with 50 mg dry weight biomass of one of the cyanobacteria isolated under laboratory conditions. A fourth bottle of BW_3PI medium was left uninoculated as an abiotic mineralization control. In one bottle, pH was controlled manually at 7.5 every 24 hours, and in the remaining two, the pH was left uncontrolled, one bottle being analyzed after five days of incubation, and the other after 25 days of incubation. $CaCO_3$ precipitation was chemically analyzed according to the procedure of PENTECOST (1998).

Results and Discussion

Ecology of cyanobacterial mat formation

When the stream of thermal water coming from the spring reached areas exposed to sunlight, a commonly green jelly-like material covered the areas flowed over by the waters. In those areas, with an average light intensity of 800 W m^{-2}, algal growth was slight. However, growth was denser in areas receiving indirect sun light such as those under the bridge with an intensity of 30 W m^{-2} (Fig. 1.2).

Upon desiccation, colour changes in the algal mats from regular green and blue-green to yellow green were observed. This chlorosis may result from a preferential decomposition of chlorophyll a and persistence of other pigments (beta-carotens, phycobiliproteins) induced by low temperatures.

The temperature of the thermal waters varied from 36 °C at the spring to 14 °C at an average distance of 80 meters in all directions from the spring. In winter time, the temperature dropped to 5 °C over the same distance. After surge, the water ran in a multi- branch pattern overflowing Puente del Inca. In some shallow branches, the algal mats were submerged and laid on the bottom. In deeper branches the algal growth developed on the margins. Algal growth was also observed in sloping areas flowed over by thermal waters and in areas with high gas to liquid transfer rates created by solids protruding above the water level in the stream. At such places algae can readily attach, grow and positively influence carbonate

precipitation (GOLUBIC 1969, CHAFETZ and FOLK 1984). Underneath the algal mat, a layer of mineral deposit was observed. The living blue-green alga on the locations mentioned before might have induced the precipitation and entrapment of mineral material, which forms a mixed lamina up to 1 mm thick.

Characterization of cyanobacterial community

The algal material collected at Puente del Inca location was analyzed by direct microscopy and showed the presence of cyanobacteria belonging to several genera. Cyanobacteria were identified according to RIPPKA et al. (1979) based on structural properties determinable by light microscopy in order to characterize the genera involved.

Differences in cellular structure allowed the recognition of the following genera: *Oscillatoria, Plectonema, Spirulina* and *Nostoc*, *Oscillatoria* and *Spirulina* being the dominant species. The organic component in Puente del Inca also included the presence of diatoms, cysts of flagellates, green algae and bacteria. However, cyanobacteria were the predominant microorganisms.

Concerning the cyanobacterial community in situ, a variation was observed over a given period of time and also with the season, similarly to other locations in the world (REEDERS et al. 1998, RICHMOND 1988). This became evident after several visits to the site. In the case of *Spirulina* sp., changes in the environmental temperature played an important role in its viability. The strong fluctuation in temperature between the spring and the flowing water, which normally occurred in the winter months, markedly affected its growth. *Spirulina* sp. was the predominant species in illuminated areas very close to the thermal spring at 36 °C. Meanwhile, at locations further away from the thermal source and in winter months *Spirulina* sp. declined and was overtaken by *Oscillatoria* sp., which was probably better adapted to growth at lower temperatures.

Oscillatoria sp. is a filamentous cyanobacterium with cells of about 4–5 μm in width (Fig. 1.3), which morphologically vary from small isolated filaments, to filaments densely arranged in a mucilagenous film. In samples taken in situ, at microscopy level, the cellular trichome was surrounded by a thin mucilagenous sheath to which particles of calcium carbonate adhered. It may be argued that calcium carbonate was accumulated by precipitation and entrapment. First, as a result of photosyntesis, calcium carbonate precipitated on the sheaths and then calcium carbonate particles became trapped among the trichomes.

Cyanobacterial growth under laboratory conditions and its effect on pH and salt precipitation

The chemistry of the water plays an important role in determining the organisms that can live and grow within a spring (CHAFETZ and FOLK 1984), and for that reason a new medium based on the composition of the major ions present in the water spring was devised. Using the medium BW_3PI (Tab. 1) all the cyanobacteria identified by microscopy in situ were isolated in the laboratory; however, stable mass cultures were only obtained with the strain of *Oscillatoria* under an atmosphere of 97% N_2: 3% CO_2. In the case of *Spirulina* a complete variation of the helix form was observed growing with straight trichomes. The straight variants showed no tendency to revert to the helical form after subcultures in the laboratory. Similar behaviour has been reported for *Spirulina platensis* isolated in an alka-

line coastal salt-flat (LEWIN 1980) and in strains held in a culture collection (RICHMOND 1988).

The photosynthetic growth of cyanobacteria in environments rich in calcium carbonate proceeds according to the following reaction (PENTECOST 1998):

$$CO_2 + H_2O + CaCO_3 \leftrightarrows Ca^{++} + 2\ HCO_3^-$$

When the equilibrium moves to the left as a result of evaporation or consumption of CO_2 by photosynthesis, $CaCO_3$ precipitates. It can be assumed that a similar reaction is developed by *Oscillatoria* sp. growing in the CO_2 saturated thermal waters of Puente del Inca. As a consequence of *Oscillatoria* sp. growth, the pH values increase, favouring further the precipitation of calcium carbonate.

Some features of the above events, mainly those related with organic processes, were confirmed in the laboratory using mass cultures of *Oscillatoria* sp. developed in BW_3PI medium. In the experiments with free evolution of pH, the initial pH 7.5 of the cultures increased to 9.8 within 30 days, and finally reached a maximum of 10 in 30–36 days. It was observed that salt precipitation was significantly profuse only when the pH values exceeded 8.1 (Fig. 2). However, at this point, the growth of *Oscillatoria* sp decreased by 50% (Fig. 3). The decline in growth was sharp first, the biomass concentrations were 0.88 g L^{-1} at pH 7.5 and 0.44 g L^{-1} at pH 8.05 and then slowed down, a further 50% diminution in biomass occurring when the pH values rose from 8 to 9.7 after 31 days of incubation.

Experiments with manual control of pH at 7.5 in non-agitated cultures, and with free evolution of it were conducted to analyze the degree of salt precipitation induced by the growth of *Oscillatoria* sp. (Fig. 1.4). In the container with pH control no precipitation was observed (Fig. 1.4 a). However, visible precipitation was evident in the cultures without pH control after 5 days of incubation (Fig. 1.4 b), which increased clearly after 25 days of incubation (Fig. 1.4 c). The abiotic mineralization control showed no precipitation.

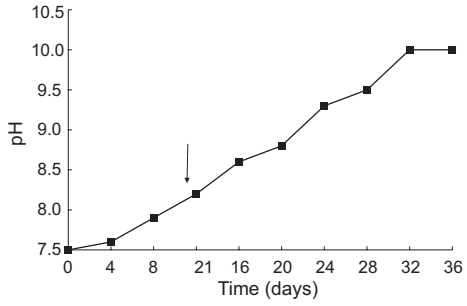

Fig. 2. pH variation associated with *Oscillatoria* sp. growth and salt precipitation. The arrow indicates the precipitation starting point in cultures incubated for 36 days. Each point is the mean of two experiments in duplicate.

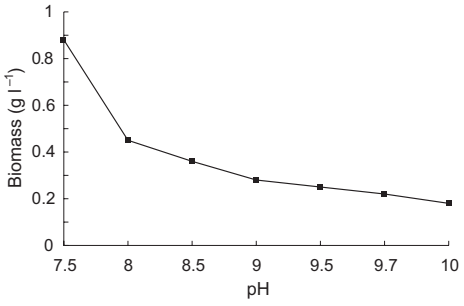

Fig. 3. Effect of pH on growth of *Oscillatoria* sp. Each point is the mean of two experiments in duplicate.

The precipitation of calcium carbonate was positively confirmed by chemical analysis and light microscopy. For light microscopy, biomass samples of *Oscillatoria* sp. developed in BW_3PI, were taken from the interface gas-liquid and placed on a microscope slide with the setting of a cover slip and the areas surrounding the trichomes were observed for mineral material. The mineral surrounding the cells was readily identified. Calcium carbonate was solubilized by the addition of HCl, leaving an organic residue completely free of mineral. The material composed of *Oscillatoria* sp. biomass associated with calcium carbonate formed a layer in the upper part of the vessel 0.5 mm thick which was stable for long periods of time (Fig. 1.5).

Conclusions

Our study shows that the growth of cyanobacteria can assist the precipitation of calcium carbonate forming mineralised layers stable for long periods of time. The events described above under laboratory conditions are likely to occur at Puente del Inca as a consequence of cyanobacterial growth in the CO_2 saturated thermal waters. One might consequently speculate that the conservation of Puente del Inca is in part due to the growth of cyanobacteria inducing the precipitation of calcium carbonate to form travertine. Although these results give an initial insight into the precipitation of calcium carbonate induced by *Oscillatoria* sp., in order to appreciate the extent of biomineralization in situ, additional growth experiments should be conducted with pH control at values currently observed when the thermal water flows from the spring source to variable distances of the bridge together with timely microscopic analyses of the travertine sediments of Puente del Inca to characterize fully the role of cyanobacteria as calcium carbonate precipitating agents at this location.

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