

Investigating Microbial Diversity and UV Radiation Impact at the High-Altitude Lake Aguas Calientes, Chile

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ABSTRACT

The High-Lakes Project is funded by the NAI and explores the highest perennial volcanic lakes on Earth in the Bolivian and Chilean Andes, including several lakes ~6,000 m elevation. These lakes represent an opportunity to study the evolution of microbial organisms in relatively shallow waters not providing substantial protection against UV radiation. Aguas Calientes (5,870 m) was investigated (November 2006) and samples of water and sediment collected at 1, 3, 5, and 10 cm depth. An Eldonet UV dosimeter positioned on the shore records UV radiation and temperature, and is logging data year round. A UV SolarLight sensor allowed acquisition of point measurements in all channels at the time of the sampling. UVA, UVB, and PAR peaks between 11:00 am and 1:00 pm reached 7.7 mW/cm², 48.5 μW/cm², and 511 W/m², respectively. The chemical composition of the water sample was analyzed. DNA was extracted and DGGE analyses with bacterial and archaeal 16S fragments were performed to describe microbial diversity. Antibiotic resistances were established previously in similar environments in Argentine Andean wetlands. In order to determine these resistances in our samples, they were inoculated onto LB and R2A media and onto R2A medium containing either chloramphenicol, ampicillin or tetracycline. Bacterial was higher than archeal cell number determined by RT-PCR in all the samples, reaching maximum total values of 5x10⁵ cell mL⁻¹. DGGE results from these samples and Licancabur summit lake (5,916 m) samples were also compared. Eight antibiotic-resistant Gram negative strains have been isolated with distinct resistance patterns.

Keywords: High altitude lakes, microbial diversity, UV resistance, antibiotic resistance

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1. INTRODUCTION

We present data and results from an ongoing project of astrobiological high-altitude expeditions investigating the highest and least explored perennial volcanic lakes on Earth in the Bolivian and Chilean Andes¹, including several nearing and beyond 6,000 m in elevation. Due to their extreme conditions, and pristine characteristics, these lakes are natural laboratories for studying microbial diversity and their connection with environmental factors like UV radiation, oligotrophy and metal content. They provide the field data missing beyond 4,400 m to complete our understanding of terrestrial lakes and biota. Research on the effects of UV has been performed in lower altitude lakes^{e.g., 2, 3}). In addition, transparent water and high UV irradiance may maximize the penetration and effect of UV radiation^{4, 5}. These lakes represent an opportunity to observe the evolution of microorganisms in shallow waters that do not offer substantial UV protection. The main goal of that work is to identify the microbial community composition in Aguas Calientes (Fig. 1) and to test the UV and antibiotic resistances of some isolates to evaluate co-resistance mechanisms.



Figure 1. Laguna Aguas Calientes, 23.22°S, 67.41°W, 5870m, Northern Chile.



Figure 2. Licancabur Summit Lake photographed during the 2002 expedition from the crater rim, 22°50'S, 67°53'W, 5916m.

2. MATERIALS AND METHODS

2.1 Sampling and measurements

Aguas Calientes (23.22°S, 67.41°W, 5870 m) was investigated in November 2006 and a surface brine sample (AC-N°1) and sediment collected at 1, 3, 5, and 10 cm depth (AC-1 cm, AC-3 cm, AC-5 cm and AC-10 cm, respectively). Water and sediment samples from the Licancabur summit lake (22°50'S, 67°53'W, 5916 m) were collected during the 2002 and 2003 campaign performed in October⁶. All the samples were collected in sterile flasks and stored in between 5-10°C until further processing in the laboratory. The surface water samples from the Licancabur summit lake were collected following the same method. The chemical composition and physicochemical parameters of the water samples was analyzed in the laboratory using Inductively-Coupled Mass Spectrometry (ICPMS) and Ion Chromatography (IC). An Eldonet UV dosimeter positioned on the shore recorded UV radiation and temperature, and logged data year round. A UV SolarLight sensor allowed the acquisition of point measurements in all channels at the time of the sampling.

2.2 Total DNA extraction and quantification

DNA was extracted for 16S rRNA analysis. Between 200 and 700 ml of brine were filtered through 0.2 µm polycarbonate membranes (Nuclepore) and stored at -70°C. Filters were incubated with lysozyme, proteinase K and sodium dodecyl sulfate (SDS) in lysis buffer as described previously⁷. DNA was extracted with a High Pure PCR Template Preparation Kit. From the sediments, nucleic acid was extracted from 20 and 25 g of sediments re-suspended in the 0,9% NaCl solution and Tween 20%.

2.3 PCR amplifications and 16s rRNA analysis

The extracted genomic DNA was used as the target in the PCR to amplify the bacterial and archaeal 16S rRNA genes with thermocycler (Eppendorf Mastercycler gradient). Bacterial 550 bp and archaeal 624 fragments suitable for subsequent denaturing gradient gel electrophoresis (DGGE) analysis were obtained with the primer combination 358f with GC clamp plus 907r and 344f with GC clamp plus 915r respectively⁸⁻¹⁰. For nested PCR PARC21F-PARC1492R and fD2-rD2 were first used for archaeal and bacterial fragments respectively. These amplification products were then used as template in PCR as we describe above. A 6% polyacrylamide gel with a gradient of DNA-denaturant agent was casted by mixing two stock solutions of acrylamide containing 40% and 80% denaturant agent, using a two chambers gradient maker (100% denaturant agent is defined as 7 M urea and 40% deionized formamide) from CBS DGGE-2000 system (CBS Scientific Company). The gradient was overlaid with nondenaturant acrylamide in order to obtain well polymerized slots. Around 800 ng of PCR product was loaded for each sample (a maximum of 18 samples per gel) and the gels were run at 100 V and 60°C for 16 h for bacterial fragments and 250 V for 5 h, in 1x TAE buffer (40 mM Tris base [pH 7.4], 20 mM sodium acetate, 1 mM EDTA). The gels were stained with the nucleic acid stain SybrGold for 30 min, rinsed with 1x TAE buffer, removed from the glassplate to a UV transparent gel scoop (Sigma) and visualized with UV light.

Bands were excised from the gels and re-amplified as reported¹¹. PCR products were purified with the QIAquick PCR-Purification Kit (QIAGEN) and quantified in an agarose gel. About 10 to 20 ng were directly used for the sequencing reaction, using primer 358f without the GC-clamp. Sequences were sent to BLAST search (www.ncbi.nlm.nih.gov) to determine the closest relative in the database.

2.4 Real Time PCR

Real-time PCR was performed to quantify the 16S rRNA gene copies with cDNA target. Quantimix Easy SYG Kit (Biotools) was used with a machine Rotor-GeneTM 6200 (Corbett Research Pty Ltd). The reaction mixture contains 10 µl of SYBRB® Green PCR Master Mix (Biotools), 1 µl of cDNA, 1 µl of the universal primers for Bacteria and Archaea, and H₂O added to a total of 20 µl.

2.5 Strain isolation and identification

Bacteria were isolated by plating in solid Luria Bertani (LB) and R2A media and incubation at 28°C. Colonies were grouped into morphologically different types. Isolated strains were cultured in LB broth with shaking at 25°C. Genomic DNA extraction, 16S rDNA gene partial amplification and sequencing were carried out.

2.6 Antimicrobial Susceptibility

In order to determine the ATB resistances, the isolated strains were inoculated onto LB and R2A media containing either Amp (ampiciline) 10 g, Em (Erythromicin) 15 g, Clm (Chloram-phenicol) 30 g and Tc (Tetraciline) 30 g. After 24 hour of incubation, at 28°C, the bacterial colonies were counted. Tests were made by duplicate.

2.7 UV-B irradiation

In order to quantify the effect of UV on the strains, cells in mid log phase of growth were exposed to a UV-C lamp (253.7 nm, 30W) in a plate. After 10, 20, 40, 80 and 120 min of exposure, 100 µL of appropriate serial dilutions (10^{-3} to 10^{-6}) were spread onto LB-agar plates (9 cm in diameter) in duplicate and then incubated in the dark. The number of colony forming units (CFUs) was determined after 24 h of incubation at 28°C in the dark to prevent photoreactivation. Controls of unexposed samples were run simultaneously in darkness and calculations of the percentages of cell survival after each treatment were made relative to these controls. Results were expressed as percentage of survival, which indicate the percent of CFU counted in the irradiated samples versus the control without irradiation. TE_8 indicate the radiation exposure required to inactivate 92% of a bacterial population. The TE_8 values were calculated from the regression line of the exponential decrease of CFU with time. The r^2 of the regression is provided as an indication of the goodness of fit².

3. RESULTS AND DISCUSSION

3.1 Description of the studied environments

Both sampled lakes are located in volcano-tectonic and hydrothermal settings. The radiation characteristics of the surveyed Aguas Calientes lake and the temperature measured during sample collection are described in Table 1. The UV flux measured in the Licancabur' summit lake in 2003 campaign reached 89 W/m².

Table 1. Radiation and temperature measures acquired near the lake Aguas Calientes (5870 m), November 28, 2006

Time	UVA (mW/cm2)	UVB (µW/cm2)	PAR (W/m2)	Temp. (°C)
11:00	7	44.7	482	-1
12:00	7.526	49.5	509	2.3
12:30	7.721	48.5	512	5.6
12:45	7.715	48.5	511	6.2
13:00	7.709	48.8	512	3.8

The measured level of UVC is very small (not shown) because of the instrument specification.

ICP-MS results (Table 2) show that the Licancabur summit lake is enriched in some major rock forming elements: Na (67.5ppm), Mg (39.6ppm), Ca (230ppm), Al (1.75ppm), Fe (0.902ppm) compared with geothermal springs located at the base of Licancabur. In particular, Al is >10 times more abundant in the summit lake (a warm spring in LB has [Al]=0.00592 ppm)¹². If we assume that the fluids from the springs and summit lake have a similar composition, then these differences may result from increased water rock interaction as the fluid flowing up to the summit is allowed more time to react with local lithologies. IC results show a similar trend, but more notably with respect to local meteoric

waters (snowmelt analysis)¹². Aguas Calientes water samples shows from 1.4 to 2.3 folds higher contents of most of these rock forming elements, and up to 20 folds higher Al content than Licancabur summit lake samples. As and SO₄²⁻ contents are also higher. The pH of the Aguas Calientes water samples (4.9) may be the cause of higher rock dissolution.

Those results show the difference between those lakes located in volcano-tectonic and hydrothermal settings and other high altitude lakes where the microbial diversity in water and sediments have been recently studied in China^{13, 14}. Hydrothermal settings lakes show an upper than 10 rate sulphate/chloride compared with evaporitic high altitude lakes with lower than 1 rate sulphate/chloride.

3.2 Cell numbers by Real Time-PCR

In both studied systems, cells numbers are higher for bacteria than for archaea in water samples. The results do not show an UV limited cell number distribution in Aguas Calientes like in Licancabur lake where the organic count increases with sediment depth¹². In both sediment samples from Aguas Calientes the only microorganisms detected by Real Time PCR were archaeal cells in low content (Table 3). pH and higher ionic contents in Aguas Calientes may be the cause of lower cell numbers. Nevertheless, Nested-PCR used to perform DGGE analysis shows the presence of a limited bacterial population, also in sediment samples AC-1 and AC-5.

Table 2. Chemical analysis results from the brine sample AC-N°1 of Aguas Calientes lake (5,930 m) and Licancabur summit lake (5914 m).

Parameter	Units	AC-N°1	LC
As	mg/L	1,0	0,03
Ca	mg/L	249	230,0
Mg	mg/L	135	39,60
N03-	mg/L	0,97	0,08
K	mg/L	14,8	26,20
Na	mg/L	154	67,50
SO ₄ ²⁻	g/L	1,93	0,59
Cl-	mg/L	131	57,79
Fe	mg/L	1,24	0,90
Al	mg/L	36	1,75
Co	mg/L	0,19	ND
Cu	mg/L	0,36	ND
Mn	mg/L	3,94	0,04
Ni	mg/L	0,36	ND
P	mg/L	0,64	ND
Sr	mg/L	0,29	ND
Zn	mg/L	0,4	ND

Table 3. Physicochemical characteristics and cell number by Real Time PCR

System	Date	Sample Code	Sample type	pH	Temperature °C	TDS mg L ⁻¹	DAPI Cell count Cells mL ⁻¹	16S rRNA gene copy mL ⁻¹	
								Bacteria	Archaea
Licancabur Summit Lake	30-10-2002	LC-1	water	8.5	4.9	1050	1.94E+06	4,80E+04	1,30E+02
	and 04-11-2003	LC-2	water				1.61E+06	1,10E+04	6,50E+02
Laguna Aguas Calientes	28-11-2006	LC-2_05	water	4.7	-1 to 6.2	1240		1,20E+04	BDL
		AC-Nº1	water					4,90E+05	BDL
		AC-1	sediment					BDL	3,20E+02
		AC-3	sediment					1,90E+04	1,20E+02
		AC-5	sediment					BDL	1,90E+03
		AC-10	sediment				6,20E+04	9,60E+02	

TDS= Total dissolved solids

3.3 Microbial diversity fingerprint

In the sites studied (Aguas Calientes and Licancabur) DGGE fingerprint, from brine and sediment samples, present higher bacterial than archaeal diversity (data not shown). Those results were also found in other high altitude lakes^{13, 14}. Between 12-17% of Licancabur isolated sequences are unclassified at the phylum level, 60-72% at the genus level. *Alpha*, *beta* and *gammaproteobacteria* phyla were recovered from the Aguas Calientes lake, and *Bacteroidetes*, *Betaproteobacteria* and *Actinobacteria* from the Licancabur summit lake. In addition several sequences related to unidentified ones have also been retrieved. Only one phylogenetic group recovered is common between sites, *Betaproteobacteria* (Table 4 and 5). Many sequences (100% in Aguas Calientes samples and 60% in Licancabur summit lake) presented similarity lower than 95% with their closest cultured relative. Five of these sequences from the Licancabur samples are related to a previously defined *Bacteroidetes* cluster from Northern Chile salt flats, Salar de Atacama (2350 m), Salar de Ascotán (3990 m) and salar de Huasco (3800 m)¹⁵⁻¹⁷, and one more is related to an uncultured *Comamonadaceae* (*Betaproteobacteria*) also similar to a sequence isolated from Salar de Huasco¹⁷. One sequence from Aguas Calientes is related to the isolated from a fresh water lake Chungará (4520 m)¹⁷, Chile. The predominant Phylum in Laguna Aguas Calientes seems to be Proteobacteria, but most of the sequences (70%) are related to unidentified Bacteria and can not be assigned to known phylogenetic group. Microbial community composition in the Licancabur Lake reveals the dominance of *Cytophaga-Flavobacteria-Bacteroidetes* and *Proteobacteria* as recently reported in studies performed in Lake Chaka and Lake Quinghai, two athalassohaline lakes located on the Tibetan Plateau (NW China) at an altitude of 3200 m^{13, 14} and in altiplanic wetlands, Salar de Huasco and Salar de Ascotán located at an altitude of 3800 and 3900 m [7, 8], respectively with a wide range of salinities^{15, 17}. Similar results were also found by Farias et al in Argentinean High altitude Andean wetlands (unpublished data). These similarities probably reflects common environmental conditions, as water bodies located in the Tibetan Plateau and the Altiplano are athalassohaline and located at high altitude, with similar abiotic conditions including UV-B radiation and negative water balances.

As has been reported in similar environments¹⁷, the number of phylotypes observed in sediment samples from Aguas Calientes lake (Fig. 3, Table 4) is higher than in water samples.

Those results confirm the observation that, despite the diverse geological settings, physical and chemical properties, only a limited number of bacterial groups predominate¹⁴ in high altitude lakes studied. Aguas Calientes and Licancabur summit lake (> 5800 m), show an even more restricted microbial diversity considering only DGGE results.

Up to now, it was not possible to obtain good archaeal sequences from DGGE bands of Aguas Calientes samples.

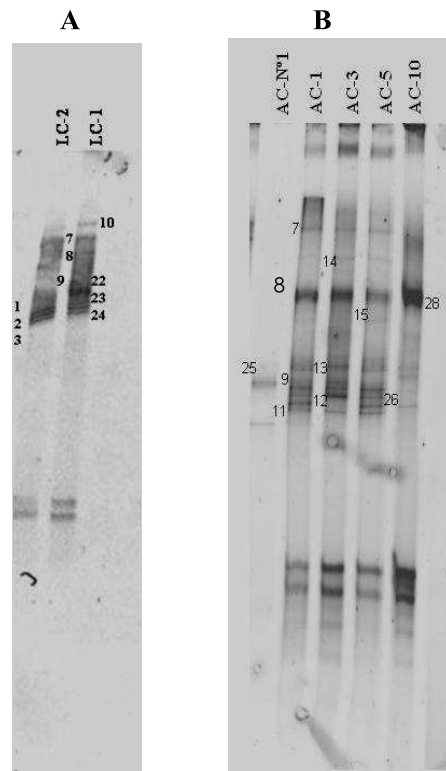


Figure 3. Denaturing Gradient Gel Electrophoresis DGGE (negative image) of the 16S rRNA gene fragments amplified with universal primers for Bacteria. A, Licancabur Summit Lake brine samples (LC-1 y LC-2); B, Aguas Calientes brine AC-Nº1, and sediment samples AC-1 cm , AC-3 cm, AC-5 cm, AC-10 cm.

Table 4. BLAST analysis of the sequences obtained from the DGGE analysis of 16S rRNA bacterial fragments of brine and sediment Aguas Calientes samples (Fig. 2)

<i>DGGE Band</i>	<i>Closest related microorganism</i>	<i>Accession number</i>	<i>Similarity %</i>	<i>Phylogenetic Group</i>	<i>Habitat of closest relative</i>
7	Uncultured bacterium clone oD10	EF034191	82	<i>Bacteria</i>	Bacterial community in an Arctic permafrost soil from Spitsbergen
8, 28	Uncultured beta proteobacterium clone Elb 263	AJ421938	87	<i>Betaproteobacteria</i>	Diversity and seasonal variability of beta-proteobacteria in biofilms of polluted rivers
9	<i>Skermanella</i> sp. 5416T-32	DQ672568	94	<i>Alphaproteobacteria</i>	Isolated from air
11	Uncultured gamma proteobacterium	AM116729	94	<i>Gammaproteobacteria</i>	Uranium mining waste sample, in the indigenous bacterial community
12, 13, 14	Uncultured bacterium clone Chun-s-91	EF632758	89, 85, 97	<i>Bacteria</i>	Microbial community in aquatic environments of the Andean Altiplano (northern Chile)
15	Uncultured bacterium clone KD8-79	AY218693	94	<i>Bacteria</i>	Bacteria in penguin droppings sediments from Ardley Island, Antarctica.
25	Uncultured bacterium clone YWB08	AB294277	89	<i>Bacteria</i>	Microbial community structure in deep coal seam groundwater
26	Uncultured bacterium clone MQ_B14	EF429636	88	<i>Bacteria</i>	Thermophilic cyanobacterial mats, geothermal springs in Luzon, The Philippines

3.4 Isolates characterization and antimicrobial susceptibility

Seventeen isolated were found by plating in solid Luria Bertani medium (LB) and R2A media. The colonies were grouped into different morphological types. Most of the isolates were gram Negative and ATB resistant Bacteria. The greatest susceptibility was found to tetracycline.

Phylogenetic identification of the isolated strains was determined by partially sequencing the 16S rRNA genes. Identified sequences are distributed in two bacterial phyla: *Firmicutes* (2 isolates) represented by *Staphylococcus epidermidis* strain S09; and *Gammaproteobacteria* (14 isolates) represented by *Pseudomonas* sp. Nj-55 and *Gammaproteobacterium* BT-P-1 (Table 3). *Gammaproteobacteria* and *Firmicutes* are usually isolated from these environments like Laguna Verde, Bolivia (4,340 m and Laguna Vilama (4,400 m altitude) and Laguna Azul (4,560 m) in Argentine¹⁸.

Antibiotic resistance patterns of isolates from Laguna Verde show that both strains were sensitive to kanamycin, rifampicin and nitrofurantoin. However, while one of the strains (LV3) was also sensitive to ampicillin and penicillin, the other one (LV3N) did not show sensitivity regarding those two antibiotics.

Table 5. BLAST analysis of the sequences obtained from the DGGE analysis of 16S rRNA bacterial fragments of brine and sediment Licancabur samples from 2002 and 2003 campaigns (Fig. 2).

<i>Band</i>	<i>Closest related microorganism</i>	<i>Accession number</i>	<i>Similarity %</i>	<i>Phylogenetic Group</i>	<i>Habitat of closest relative/cultured physiology</i>
2003: 5, 6	Uncultured beta proteobacterium clone ccsIm224	AY133088	83, 94	<i>Betaproteobacteria.</i>	TCE-contaminated site
	<i>Delftia acidovorans</i> isolate	AY367028	96	<i>Betaproteobacteria.</i>	
2003:30	As3-4				
	Uncultured beta proteobacterium clone AGCM38	AF477803	93	<i>Betaproteobacteria.</i>	Aerobic Granules
2003:33	Uncultured bacterium clone CCSD_RK1080_B15	AY820725	83	<i>Bacteria</i>	Rocks and Fluids From the Chinese Continental Scientific Drilling in China
2002: 1, 23	Uncultured lake bacterium P38.3	AY752085	94, 96	<i>Bacteria</i>	Bacterioplankton in two oligo-mesotrophic lakes
2002:3, 24	Actinobacterium MWH-Dar4	ASP565416	91, 91	<i>Actinobacteria</i>	Facultatively anaerobic and chemoorganotrophic. Optimal temperature is 37°C
2002:4, 5, 6, 18, 20, 21	Uncultured Flavobacterium sp.	AJ487534	96, 98, 90, 97, 97, 98	<i>Bacteroidetes</i>	Bacteroidetes cluster from Chilean salt flats close to <i>Psychrophlexus</i> genus
2002:7, 8	Bacterium c115	AB167243	90, 98	<i>Bacteria</i>	Chemostat enrichment from an aquifer soil as inoculum and phenol was supplied as sole carbon source
2002:9	Uncultured Comamonadaceae bacterium clone C-14	AF523051	91	<i>Betaproteobacteria</i>	Non-carbonated natural mineral water
2002:10	Cyclobacterium sp.	AY259502	88	<i>Bacteroidetes</i>	Bedrock aquifer contaminated with chlorinated solvent
2002:15	CFB group bacterium A0653	AF236016	86	<i>Bacteroidetes</i>	
2002:17	Uncultured prokaryote isolate	AY501502	85	<i>Prokaryotic environmental</i>	Salt marsh sediments along the East coast of the United States

Table 6. Phylogenetic characterization and ATB resistance pattern of the strains isolated from Aguas Calientes samples.

	Isolate	Closest related	Accession number	% 16S rDNA similarity	TE ₈ (min)	ATB Resistance			
						Em	Amp	Tc	Cm
Gamma Proteobacteria	BRU-N°1 (4)	<i>Pseudomonas</i> sp. Nj-55	AM409368	99	64.3	+	+	-	+
	BR-N°1 (3)	Gamma proteobacterium BT-P-1	AY539822	99	3	+	+	-	+
Firmicutes	AP-3cm (1)	<i>Staphylococcus epidermidis</i> strain S09	AY741152	99	ND	+	-	+	+

(+= Positive growth in presence of the ATB).

3.5 UV-C resistance

The percentage of survival versus the UV-C radiation doses is shown in Figure 4. Both isolates analyzed show further inhibition with larger doses of UV-C radiation. Significant differences in survival after UV-C irradiation were observed. Higher survival was observed in *Pseudomonas* sp. compared with the other gamma *Proteobacteria*, both isolated from brine samples. Similar UV-C resistance results were observed with *Proteobacteria* isolates from Lake Chaka¹⁴. Tested strains show no recovery of cells number after 120 min exposure under dark conditions. Similar low capacity for recovery have been found in the *Pseudomonas* sp. strain MF8 isolated from Laguna Pozuelos, Argentina (3600 m)¹⁹.

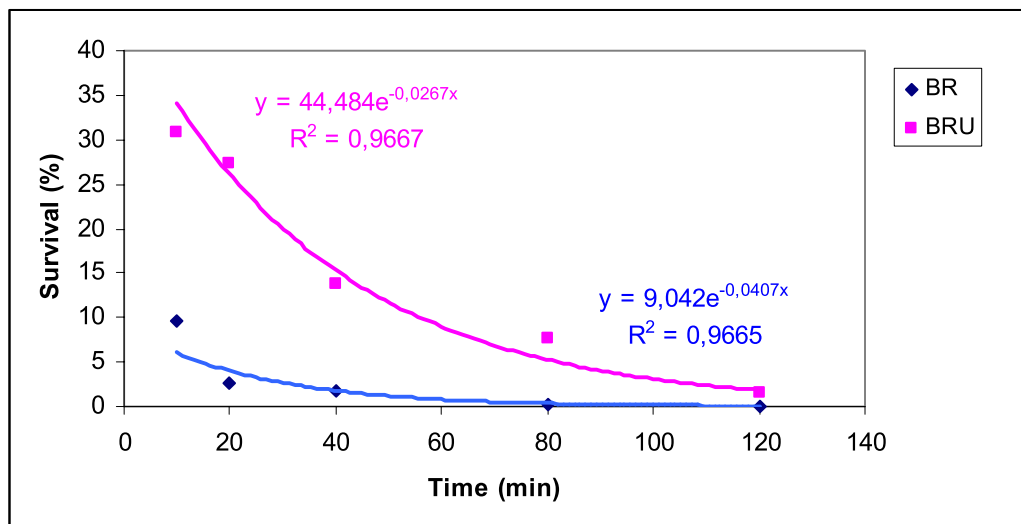


Figure 4. UV-C resistance of two isolates from Aguas Calientes (5,930 m) vs. exposure time.

These results would confirm that in extreme environments like high altitude wetlands there could be a correlation of multiresistances to UV radiation¹⁸. The presence of widespread resistance to ATB was unexpected in this pristine and

oligotrophic environment, where it is not predictable to find selection pressure for ATB resistances. Recently have been demonstrated in some isolates that the presence of ATB resistances would not be induced by mutagenesis¹⁸, one of the hypothesis proposed to explain the multiresistances relation to UV radiation.

4. CONCLUSIONS

Microbial diversity in high altitude andean lakes analyzed by a culture independent technique shows the presence of a limited number of bacterial groups and a high percentage of sequences that can not be assigned to any known phylogenetic group.

The isolates obtained from Aguas Calientes and Licancabur summit lake [8] belong to the *Gammaproteobacteria* and *Firmicutes* group. They denoted high ATB resistance.

Significant differences in survival after UV-C irradiation were observed. Higher survival was observed in *Pseudomonas* sp. compared with the other gammaproteobacteria, both isolated from brine samples.

More experiments should be performed to evaluate tendencies and to analyze DNA repair systems used.

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