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Bacterial Communities' Structure in a High mountain lake during the ice-free season: cultural and PCR-TGGE investigations

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ABSTRACT:"La Caldera" is a high mountain lake located in Sierra Nevada, Spain. For its position and characteristics it is considered as an important "sensor" for global changes. Water samples were collected during the ice-free season in order to observe changes in the bacterial community structure. Experiments were carried out complementing culture and culture-independent methods. Strong variations of the bacterial communities were recorded along the sampling period in particular regarding number and phylogenetic relationships of the microorganisms detected. At the season beginning, bacterial counts were rather high but strains belonged only to β- and γ-Proteobacteria. By contrast, in the middle of the season, possibly due to external inputs, total number of bacteria decreased but other phylogenetic groups, such as α–proteobacteria, Actinobacteria and Firmicute appeared. On the whole, Proteobacteria were the dominant microbiota. Bacterial diversity and presence of most abundant species were analysed by PCR-TGGE. Sequencing of TGGE bands confirmed that prevalent Bacteria populations were evolutively close to Proteobacteria with predominance of α- and β-Proteobacteria. Analysis of diversity and functional organization suggested that "La Caldera" bacterial microbiota, typical of oligotrophic and/or extreme environments with low habitability, was characterized by a community organized in an adequate distribution of dominant and resilient species. Although rather specialized, the community presented a sufficient functionality and flexibility to react to changing and stressing conditions. However, along the ice-free season, the community evolved showing more specialized populations with less adaptation abilities. This is the first work complementing cultural and cultural-independent methods for the "La Caldera" lake.

Key words: High Mountain Lake, Bacterial community, Cultural methods, PCR-TGGE, global changes

INTRODUCTION

High mountain lakes are normally located in remote areas exposed to low temperature, high ultraviolet irradiation, long ice cover periods, and fluctuating hydrology. These abiotic factors may influence development and structure of microbial communities (Straškrabová *et al*., 1999). In addition, these water bodies are hardly affected by human activity and usually submitted to severe nutrient limitation. However, they receive external contribution, sometimes related to the global climate change, of atmospheric pollutants, dust, other inorganic and organic aerosols and microorganisms, which could contribute to their nutrient input and can cause variations in the microbial community structure (Mladenov *et al*., 2011; Morales-Baquero *et al*., 1999; Reche *et al*., 2009). Food web in high mountain lakes is generally rather short and its main development occurs within few months during the ice-free period only (Cruz-Pizarro *et al*., 1994; Morales-Baquero *et al*., 1999). In this context, bacteria sometimes constituted a minor component of the plankton community, in terms of abundance and production, in contrast to the situation often reported in others oligotrophic conditions (Medina-Sánchez and Villar-Argaiz, 2004). By contrast, other studies indicated that harsh conditions, in particular low temperature, could have little effects on bacterial growth in alpine lakes (Karlsson *et al*., 2001). Thus, it is difficult to generalize about the key factors determining bacterial growth and development of microbial communities' structure in high mountain lakes clear-water.

However, for better comprehension of the influence of these factors, the first mandatory step is

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a deep characterization of the microbiota in order to assess microbial numerical significance, to identify most relevant species and to understand phylogenetic relationships. Since the large majority of environmental microorganisms cannot be isolated in pure cultures (Lewis, 2007; Zhao *et al*., 2008), traditional culture-based methods, only provide limited information on the microbial communities (Hugenholtz *et al*., 1998; Lewis, 2007; Webster *et al*., 2001). Thus, to obtain a complete overview of presence, diversity and role of microorganisms in the studied ecosystem, culture-independent approaches are needed to complement traditional culture-based microbiological methods. "La Caldera" is an oligotrophic high mountain lake, remotely located in the Sierra Nevada Mountains, in South of Spain. The lake is rather close to the Sahara Desert and suffers a significant input of Saharan dust (Morales-Baquero *et al*., 2006; Pulido-Villena *et al*., 2006). It lacks of littoral vegetation and vertebrate life and hydrological loading together with evaporation seem to be the only responsible for its seasonal and annual water level fluctuations (Villar-Argaiz *et al*., 2001). In addition, lake water is highly transparent and receives considerable UV irradiance (Carrillo *et al*., 2002). For its location and characteristics, as reported for some other remote lakes, "La Caldera" could be considered as a "sensor" of global change (Mladenov *et al*., 2011). However, little attention has been paid to the analysis of the bacterial community structure in this ecosystem and diversity and distribution of its microbial communities remain poorly defined (Medina-Sánchez and Villar-Argaiz, 2004; Reche *et al*., 2009). Moreover, to the best of our knowledge, no study has been carried out, regarding the structure of its bacterial communities, complementing the information obtained by cultural-independent and cultural methods.

In this study, the bacterial community structure and composition of "La Caldera" lake was investigated, by both cultural methods and PCR-TGGE fingerprint analysis, during the ice-free period. Phylogenetic analysis by 16S rDNA sequencing of representative strains is also reported.

MATERIALS & METHODS

"La Caldera" is a rather shallow, medium size (20,000 m²) and remote oligotrophic high mountain lake situated above the tree line (Medina-Sánchez and Villar-Argaiz, 2004) located in the Sierra Nevada Mountain on siliceous bedrock in a glacial cirque at 3050 m asl. The lake is usually covered by ice from end-October to beginning-July. The lake was sampled at the beginning (July 7, 2010, C1) and in the centre (August 26, 2010, C2) of the ice-free season. Average water temperature (°C), dissolved oxygen (mg L^{-1}) and pH were 6.1, 8.8, 7.1 and 16, 6.2, 6.9 for sample C1 and C2, respectively. Samples were taken in sterile bottles at 50 cm depth as reported by Sommaruga and Casamayor (2009). Samples, collected in three different sites across the lake and pooled together to obtain an integrated sample (Hervas and Casamayor, 2009), were stored at 4 ºC and protected from light until their transfer to the laboratory for filtration through a Millipore V2 filtration system equipped with sterile membrane (0.22 µm, Millipore, USA).

In order to maximize the isolation of diverse groups of heterotrophic bacteria (Männisto and Häggblom, 2006), cultures were obtained placing the membranes in the following culture media: Tryptic Soy Agar (TSA, Panreac); 10% diluted TSB + 1.5% agar (w/v) (TSD) and Winogradsky salt medium amended with 0.5% (w/v) glucose and 1.5% agar (w/v) (MMW). Plates were incubated aerobically in the dark at 5 and 15 ºC for up to 2 weeks and checked daily. Pure cultures of bacteria grown on the different media were obtained by streak plate method. In order to discharge evident replicate of the same isolate, preliminary tests were carried out considering some morphological characteristics (shape, colour, morphology, aspect and dimensions) and Gram staining. Tests allow obtaining 53 different isolates that were stored at -80 ºC as glycerol 20% (v/v) stocks.

A fresh cultured (24 h) colony of each isolate was lysed as previously described by Sanchez-Peinado *et al*., 2008. The cleared lysate (4 µl) was used as templete for amplification to which were added 46 µl of PCR reaction mixture. PCR reaction was kept as previously described (Molina-Muñoz *et al*., 2007) using the universal primers fD1 and rD1 (Weisburg *et al*., 1991) from Sigma-Aldrich (USA) and AmpliTaq Gold polymerase (Applied Biosystems). PCR products were run on 1% (w/v) agarose gels and bands of expected size (approximately 1.5 Kb) were purified using the Quiaquick II kit (Quiagen, Germany). Nucleotide sequences were performed by the DNA Sequencing Service of Instituto de Parasitologia y Bioquimica Lopez-Neyra (CSIC, Granada). Water (1 L) was filtered through nitrocellulose membranes (0.22 µm, Millipore, USA). Total DNA was extracted from membrane filters as follows. Each membrane was suspended in ca. 2 ml of sterile water and triturated grossly with a sterile pipette tip; tubes were then vigorously stirred by vortex (IKA, Germany) in order to re-suspend DNA. The suspension was transferred to a clean microcentrifuge tube and used for DNA extraction by the commercial kit MasterPureTM Complete DNA and RNA Purification Kit (Epicentre® Biotechnologies, USA) accordingly to the manufacturer instructions.

For the total Bacteria community, a two-steps PCR (nested PCR) approach was selected for specific

amplification of the V3 hypervariable region of the 16S rRNA gene of Bacteria, as previously described by other authors for TGGE or DGGE fingerprinting (Calderón *et al*., 2011; Gómez-Silván *et al*., 2010; Molina Muñoz *et al*., 2009). Extracted DNA (2-5 ng) was used as a template for a first PCR carried out using the universal primers fD1 and rD1 (Weisburg *et al*., 1991) to amplify the 16S-rRNA gene. Second step: subsequently, nested PCR was performed using as template 1 µl of the first PCR product and the universal primers GC-P1 and P2, amplifying the V3 hypervariable region of the 16S rRNA gene (Muyzer *et al*., 1993). Conditions for each PCR reactions were as previously described Molina-Muñoz *et al*., (2009).

For *Pseudomonas*-related communities, extracted DNA (2-5 ng) was used as a template for the first PCR, using the taxon-specific primers F311Ps and R1459Ps described by Milling *et al*., (2004) for the amplification of *Pseudomonas* partial 16S rRNA genes. PCR reaction was carried out as previously described by Milling *et al*., (2004), except for the annealing temperature which was optimized at 53°C. Subsequently, 1 µl of a 1/30 dilution of the first PCR product was used as a template for the nested PCR, using universal primers targeting the V3 region (Muyzer, 1999) as previously described Molina-Muñoz *et al*., (2009). TGGE runs were done on a TGGE Maxi system (Whatman-Biometra). Denaturating gels (6% PAGE with 20% deionised formamide, 2% glycerol and 8 M urea) were made and run with 2x TAE buffer. The temperature gradient applied for efficient separation of bands was optimized at 43-63 °C (Vílchez *et al*., 2007). The gels were run at 125 V for 18 h. For *Pseudomonas* fingerprinting the temperature gradient was optimized at 38-50 ºC. Gel bands were visualized by silver staining using the Gel Code Silver Staining kit (Pierce), following the manufacturer´s indications with slight modifications as reported before Vilchez *et al*., (2007). Band patterns generated by TGGE were normalized, compared and clustered using the Gel Compar II image analysis software, version 5.102 (Applied Maths). For cluster analysis, TGGE profiles were compared using a band assignment independent method based on the Pearson product moment correlation coefficient. This analysis uses the whole densitometric curve, taking into consideration band intensity. Dendograms relating band pattern similarities were automatically calculated with UPGMA algorithms (Unweighted pair group method with arithmetic mean). Significance of UPGMA clustering was estimated by calculating the cophenetic correlation coefficients (Sokal and Rolf, 1982).

Based on the TGGE fingerprints, several theoretical indexes were calculated to analyze population richness and evenness in the samples. Range weighted richness indexes (*Rr*), which provide an estimation of the level of microbial diversity in environmental samples, were calculated based on the total number of bands in each TGGE pattern (N) and the temperature gradient (°C) between the first and the last band of each pattern (Tg), as described by Marzorati *et al*., (2008). The resulting values were divided by 100 (Gómez-Silván *et al*., 2010) to keep an order of magnitude analogous to that of the *Rr* index as originally described for DGGE by Marzorati *et al*., (2008). To render a graphical representation of the evenness of the bacterial communities in the samples, Pareto-Lorenz distribution curves were draw based on the TGGE fingerprints, as previously described Marzorati *et al*., (2008). The bands in each TGGE lane were ranked from high to low based on intensity levels. The cumulative normalized band intensities for each TGGE lane were plotted against their respective cumulative normalized number of bands. The curves were numerically interpreted by the functional organization index (Fo), given by the horizontal y-axis projection on the intercept with the vertical 20% x-axis line (Marzorati *et al*., 2008). The calculation of the Fo indexes allows for the evaluation of the functional redundancy of the microbial communities analyzed by fingerprints methods (Marzorati *et al*., 2008).

Portions of individual bands on silver stained TGGE gels were picked up with sterile pipette tips, placed in 10 µl of filtered (0.22 µm) and autoclaved water, and directly used for reamplification with the appropriate primers. PCR products were purified by gel running and extraction with the Quiaex-II kit (Quiagen). DNA recovered was directly used for automated sequencing in an ABI PRISM 3100 Avant genetic analyzer. DNA sequences were analyzed using the biocomputing tools provided on-line by the European Bioinformatics Institute (http:D D www.ebi.ac.uk). The BLASTn program (Altschul *et al*., 1997) was used for preliminary sequence similarity analysis. The ClustalX version 2.0.3 software (Jeanmougin *et al*., 1998) was used for the aligning of sequences. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura *et al*., 2007). A p-distance based evolutionary tree was inferred using the Neighbor-Joining algorithm (Saitou and Nei, 1987). The bootstrap test was conducted to infer the reliability of branch order (Felsenstein, 1985), with a round of 1,000 reassemblings. Bootstrap values below 50% are not shown in the tree. ANOVA and pair-wise multiple comparison procedure (Tukey test) were carried out using the software SigmaStat (Jandel Scientific, CA, USA).

RESULTS & DISCUSSION

Counts and colony isolation were obtained from agar plates using various culture media and incubated at 5 and 15 ºC as suggested for cultivable heterotrophic bacteria from cold environment by Mannisto and Haggblom (2006). As for the bacterial counts, noticeable differences were recorded between the two samples for TSA and TSD. Surprisingly, for all the media, no statistical differences were observed within the two incubation temperatures. In addition, as expected for a minimal medium, the lowest counts were obtained on MMW (Table 1). It is worth nothing that, in term of scale, our results were markedly below than those reported by others both for the same mountain lake and for other similar oligotrophic water bodies. Actually, on average, our counts were in-between 10^2 and 10^3 cells/ml while in general high mountain lakes, including "La Caldera", showed bacterial abundance of about 10^5-10^6 (Crump *et al*., 2003; Mladenov *et al*., 2008; Pernthaler *et al*., 1998; Pulido-Villena *et al*., 2003; Reche *et al*., 2009; Sommaruga and Casamayor, 2009). However, in our case we counted cultivable bacteria while in the mentioned works total bacterial living cells were calculated. It is generally known, from the very early works carried out in the sixties and seventies, that big difference could be noted between total microscopic, direct viable and cultural counts particularly in aquatic environments. Phylogenetic affiliation of the 53 isolates, 38 from C1 and 15 from C2, was assessed by comparative analysis of 16S rDNA. According to their evolutionary distances, the isolates grouped in five phylogenetic lineages: α-, β- and γ-Proteobacteria, Firmicutes and Actinobacteria (High GC Gram positive bacteria) (Fig. 1). However, big quantitative and qualitative differences were recorded between the two samples showing a community

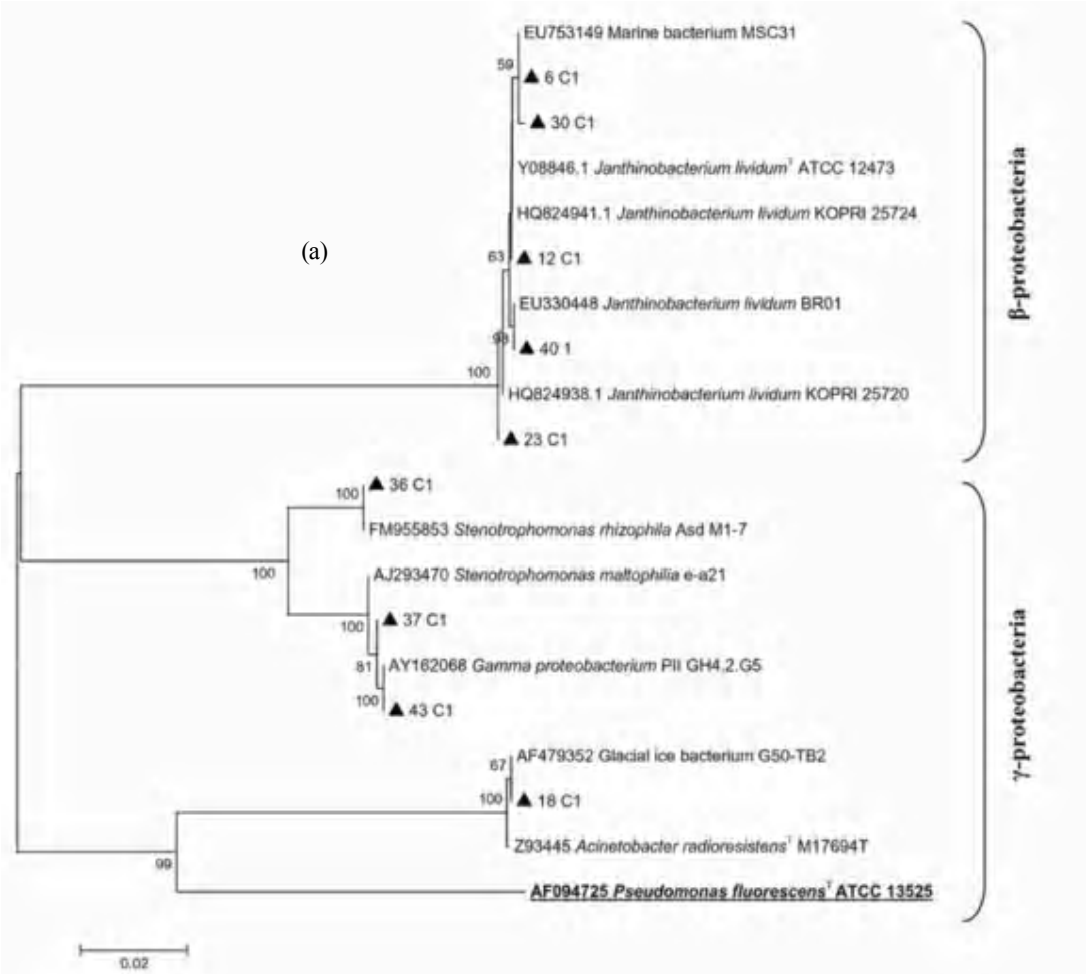

Fig .1. Neighbour-Joining phylogenetic trees based on the sequence of the gene encoding 16S rRNA (16S rDNA) The phylogenetic trees showing the positions of 38 strains (▲) analyzed from first sampling (July 7th) and the most similar sequences retrieved from the EMBL database. a) Sequences phylogenetically close to β- and γ- Proteobacteria. b) Sequences phylogenetically close to the γ-Proteobacteria of the Family Pseudomonadaceae. T = type strain. The scales bar indicates a 2% (a) and 0.2% (b) divergence. Bootstrap values over 50% are shown in nodes

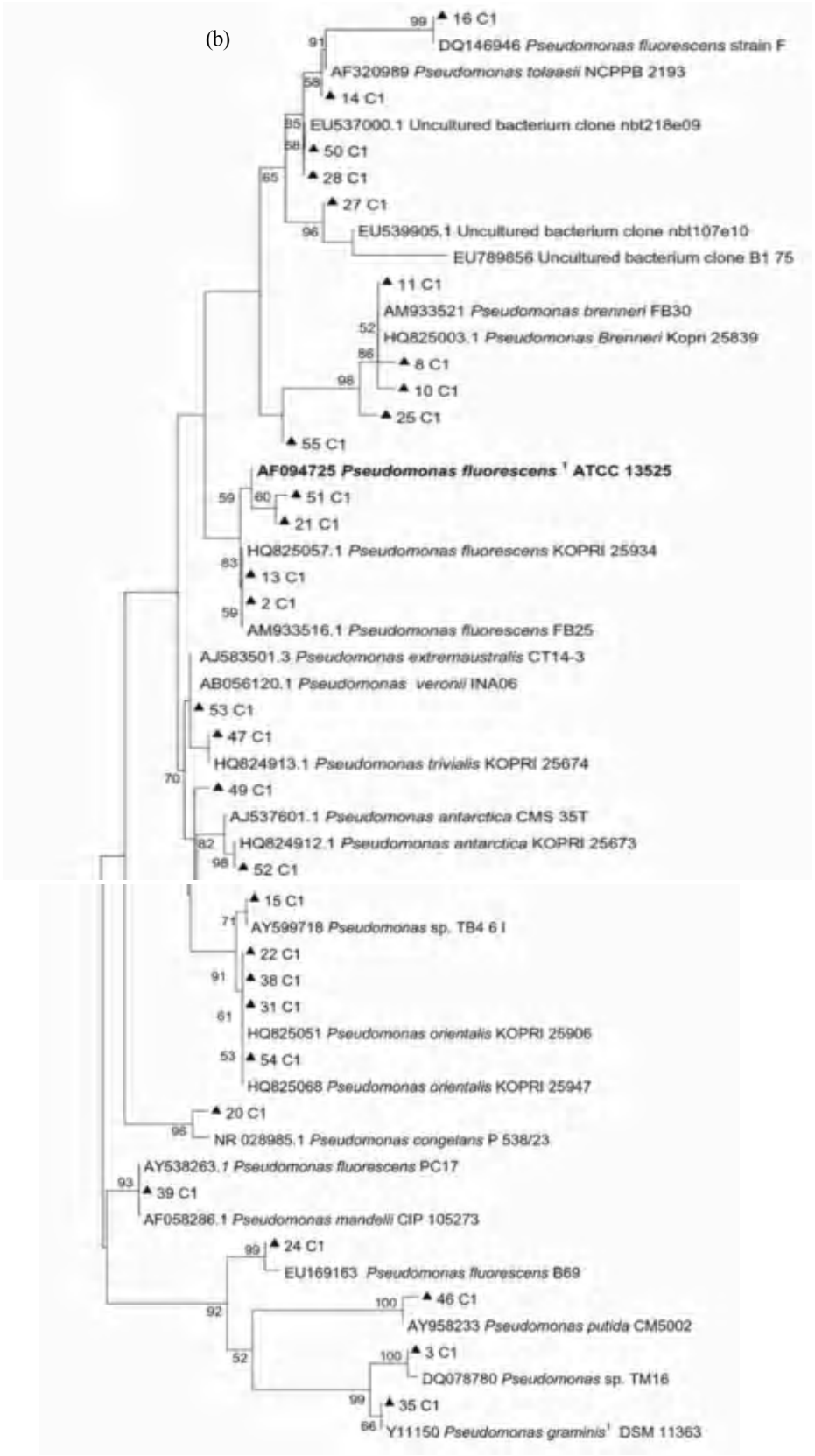
(b)
16 C1
DQ146946 Pseudomonas fluorescens strain F
AF320989 Pseudomonas tolaasii NCPPB 2193
14 C1
EU537000.1 Uncultured bacterium clone nbt218e09
50 C1
28 C1
27 C1
EU539905.1 Uncultured bacterium clone nbt107e10
EU789856 Uncultured bacterium clone B1 75
11 C1
AM933521 Pseudomonas brenneri FB30
HQ825003.1 Pseudomonas Brenneri Kopri 25839
8 C1
10 C1
25 C1
55 C1
AF094725 Pseudomonas fluorescens [T] ATCC 13525
51 C1
21 C1
HQ825057.1 Pseudomonas fluorescens KOPRI 25934
13 C1
2 C1
AM933516.1 Pseudomonas fluorescens FB25
AJ583501.3 Pseudomonas extremaustralis CT14-3
AB056120.1 Pseudomonas veronii INA06
53 C1
47 C1
HQ824913.1 Pseudomonas trivialis KOPRI 25674
49 C1
AJ537601.1 Pseudomonas antarctica CMS 35T
HQ824912.1 Pseudomonas antarctica KOPRI 25673
52 C1
15 C1
AY599718 Pseudomonas sp. TB4 6 I
22 C1
38 C1
31 C1
HQ825051 Pseudomonas orientalis KOPRI 25906
54 C1
HQ825068 Pseudomonas orientalis KOPRI 25947
20 C1
NR 028985.1 Pseudomonas congelans P 538/23
AY538263.1 Pseudomonas fluorescens PC17
39 C1
AF058286.1 Pseudomonas mandelii CIP 105273
24 C1
EU169163 Pseudomonas fluorescens B69
46 C1
AY958233 Pseudomonas putida CM5002
3 C1
DQ078780 Pseudomonas sp. TM16
35 C1
Y11150 Pseudomonas graminis [T] DSM 11363

Table 1. Viable counts obtained from C1 and C2 samples, on different culture media (TSA, TSD and MMW) at 15 and 5°C

	Microbial Growth (CFU/mL x 10^2)					
	TSA		TSD		MMW	
	15ºC	5ºC	15ºC	5ºC	15ºC	5ºC
C1	$74.5\pm19.1^{a,1}$	$55.0\pm11.3^{a,1,2}$	$46.5\pm14.8^{a,1,2}$	$38.5\pm12.7^{a,2}$	$7.5\pm2.1^{a,3}$	$3.0\pm1.4^{a,3}$
C2	$14.0\pm2.8^{b,1}$	$8.0\pm1.4^{b,1,2}$	$11.5\pm4.9^{b,1,2}$	$7.0\pm1.4^{b,2,3}$	$4.0\pm1.4^{a,3}$	$2.5\pm0.7^{a,3}$

Data are means of 3 replicates ±SD. Column means followed by the same superscript letter and line means followed by the same superscript number, were not statistical different ($P<0.05$) as determined by the Tukey test.

evolution during the ice-free season. In C1, only β- and γ-Proteobacteria were present and with higher number of isolates. By contrast, C2 showed all the five lineages but with very low number of isolates. Appearance of isolates affiliated to α –Proteobacteria, Firmicutes and Acinetobacteria, that were not revealed in C1, could be related to possible inputs of new species from the melting ice and seasonal perturbations such as dust and precipitation (Mladenov *et al*., 2008; Reche *et al*., 2009). Even if big yearly and seasonal numerical variations of the bacterial population in "La Caldera" was already stated for the ice-free period (Pulido-Villena *et al*., 2003; Reche *et al*., 2009) till now no information was available concerning the microbial groups involved in the seasonal community evolution.

On the whole, the lake was dominated by Gram-negative bacteria with few Gram-positive strains detected in the second samples, only. It is known that the majority of aquatic bacteria and most from cold environments are gram-negative (Weiss *et al*., 1996), thus, again, the occurrence of gram positive strains in the second sample could be attributed to external inputs. Moreover, it has been stated that, in cold environments, presence of many *Pseudomonas* strains, as occurred in C1, could strongly inhibit Gram-positive bacteria (Männisto and Häggblom, 2006). Very low presence of *Pseudomonas* in C2 could have permitted growth of Gram positive bacteria. Considering both samples, the majority of the strains (91%) were included as Proteobacteria being α-, β- and γ-proteobacteria the 1.9, 19.9 and 69.2%, respectively (Fig. 1 and 2).

The only α-Proteobacteria isolated was closely related to *Brevundimonas* sp. (Fig. 2). Most of the β-Proteobacteria was evolutively close to the Class Burkholderiales (family Oxalobacteraceae) with various strains of *Janthinobacterium lividum* and *Massilia timonae*. Moreover, strains 16b and 20b were related to the family Comamonadaceae.

The majority of γ-Proteobacteria strains (30) were related to members of the genus *Pseudomonas* and only few strains were related to *Stenotrophomonas*, *Psychrobacter* and *Acinetobacter*. As for *Pseudomonas,* most strains were affiliated to *P. fluorescens* with few others strains belonging to the *P. putida* and *P. jessenii* lineages. It is worth nothing that 95.5% of the *Pseudomonas* strains were isolates from C1 (Fig. 1b).

The strains belonging to Firmicutes (class Bacilli) were affiliated to *Planococcus* and *Bacillus* while those belonging to *Actinobacteria* were closely related to *Arthrobacter* and *Clavibacter* (Fig. 2). Since no work is available dealing with cultivable bacteria from "La Caldera", comparison of our results with those of others is quite difficult. However, some considerations are possible taking into account similar environments. First of all, high mountain lakes in temperate regions sometime have similar characteristic (i.e. temperature, U.V. exposition, oligotrophy) of lakes located in Polar or sub-Polar Regions but at lower altitude. Moreover, various phylogenetic groups and even species, among those detected in our work (i.e. *Pseudomonas*, *Janthinobacterium* and *Stenotrophomonas*), seem to be globally distributed from Antarctica to the Artic Polar Circle including various high mountains locations (Bai *et al*., 2006; Lee *et al*., 2011; Pesciaroli *et al*., 2012; Spring *et al*., 2000; Van Trappen *et al*., 2002).

However, the comparisons of culture-independent and culture-dependent methods to obtain phylogenetic information clearly demonstrated that the culturable fraction of a bacterial population alone is neither quantitatively nor qualitatively representative of the total microbial community in the respective environment (Felske *et al*., 1999; Spring *et al*., 2000; Wagner *et al*., 1993). Therefore, the community structure will be discussed through the results obtained by PCR-TGGE. The molecular approach revealed significant structure differences in the two samples; this was recorded for both total Bacteria and *Pseudomonas* communities (Fig. 3 and 4). Actually, Pearson coefficient-based analysis allowed for the identification of two separated clusters corresponding to C1 and C2. Actually, cluster similarity was 54.35 and 53.28 % for total bacteria and *Pseudomonas*, respectively (Fig. 3a and 4a). Similar results were

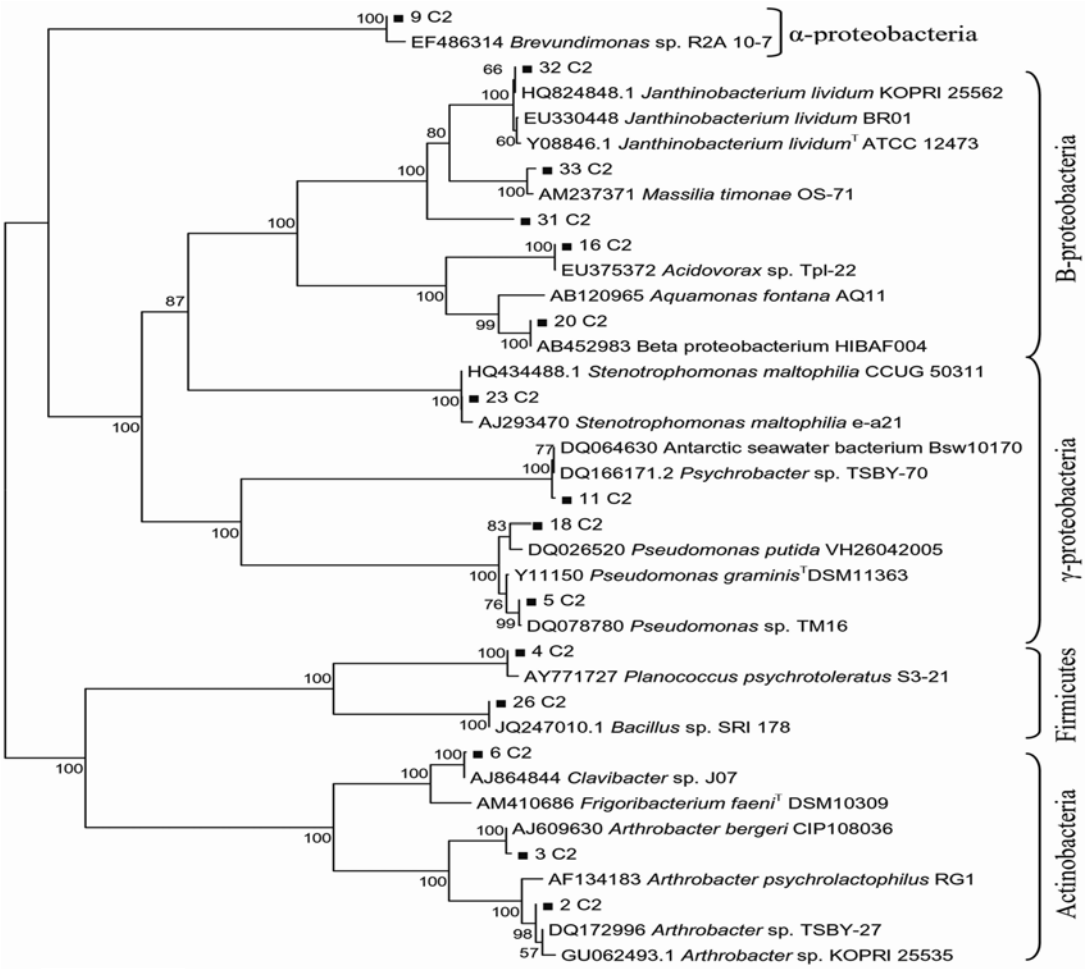

Fig. 2. Neighbour-Joining phylogenetic trees based on the sequence of the gene encoding 16S rRNA (16S rDNA)
The phylogenetic trees showing the positions of 15 strains (■) analyzed from second sampling (August 26th) and the most similar sequences retrieved from the EMBL. T = type strain. The scales bar indicates a 2% divergence. Bootstrap values over 50% are shown in nodes

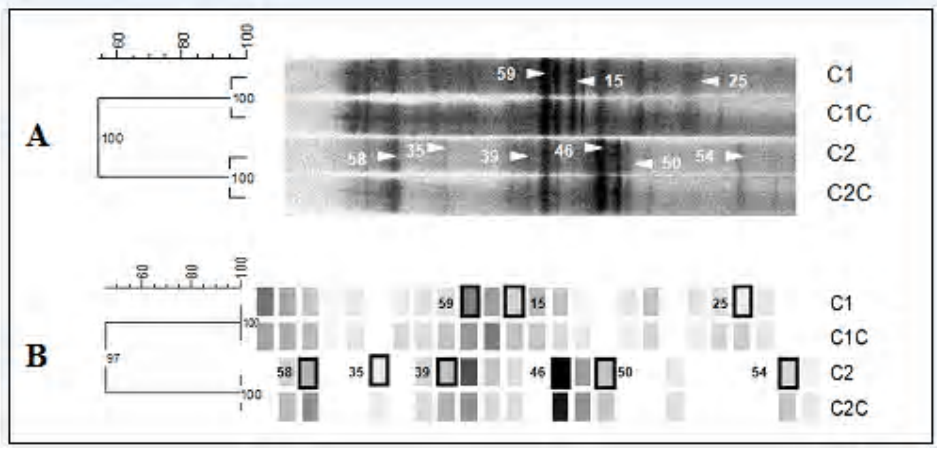

Fig. 3. Community structure of the bacteria in the two samples (C1 and C2) analyzed by TGGE profiling
(A) Pearson coefficient-based analysis of the band patterns generated from two samples analyzed. (B) Dice coefficient-based analysis of band patterns generated from two sampling analyzed with presence/absence matrix. **C1C** and **C2C** are duplicate TGGE run of samples C1 and C2 for method reproducibility

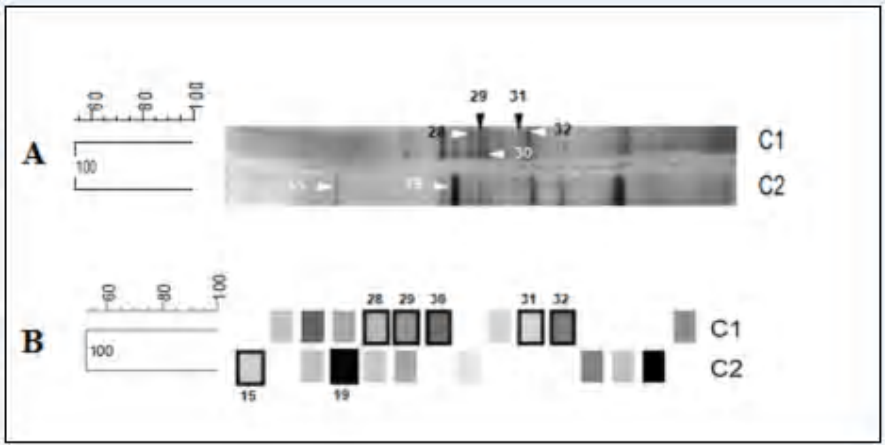

Fig. 4. Community structure of *Pseudomonas* in the two samples (C1 and C2) analyzed by TGGE profiling
(A) Pearson coefficient-based analysis of the band patterns generated from two samples analyzed. (B) Dice coefficient-based analysis of band patterns generated from two sampling analyzed with presence/absence matrix.

obtained by the analysis with Dice coefficient (Fig. 3b and 4b).

Thus, the evolution of both total Bacteria and *Pseudomonas* communities within the ice-free season appeared very similar. Possibly, the harsh environmental changes occurring in the lake, equally affected all the microbial communities. As for total Bacteria community, 25 different band classes (20 and 14 from C1 and C2, respectively; with 9 bands in common) were identified by the image analysis in the TGGE fingerprints (Fig. 3). Since only nine bands were common to both samples, more than 50% of the bacterial diversity revealed in C1 was not detected in C2. Possibly, these bacteria disappeared or become minority. The Range weighted richness indexes (*Rr*) can be related with the environmental microbial diversity. According to Marzorati *et al*., (2008), thresholds of $Rr < 10$ and $Rr > 30$ define communities with low- and high-diversity, respectively. In our case, since average *Rr* was ca 3.5±0 in both samples, microbial diversity was very low as generally recognised for oligotrophic and/or extreme environments (Marzorati *et al*., 2008). It is worth noting that *Rr* depends both on the number of bands and on band distribution along the profile. Very habitable environments can host many different organisms and genetic variability. Consequently, a wide gradient would be needed to describe total diversity (Marzorati *et al*., 2008). In our case, we obtained both low number of bands and incomplete profile coverage within the gradient showing low environmental habitability.

Regarding the functional organization (*Fo*), the indices values of the samples indicated a community with some dominant species, showing high number of individuals, and others presenting lower abundance. The *Fo* value of C1 (47%) reflected the community ability to be organized in a rather adequate distribution of dominant and resilient microorganisms. Although rather specialized (45%>*Fo*<80%), the community had sufficient functionality and flexibility to react to changing and stressing conditions (Marzorati *et al*., 2008). *Fo* value of C2 (43%), even not very different from that of C1, indicated a more specialized community with less adaptation abilities.

As for *Pseudomonas*, a total of 15 different band classes (10 and 9 from C1 and C2, respectively, with four bands in common) were identified by the image analysis (Fig. 4). Again, in the second sample, apparently, lot diversity disappeared or become minority. *Pseudomonas* community diversity was even lower than that recorded for total Bacteria being average *Rr* values ca 1.3±0.1. The calculation of *Fo* for only a part of complex community has scarce meaning. Therefore, it was not calculated for *Pseudomonas* community. Our results, showing scarce environmental habitability of the "La Caldera" at least for low specialised microorganisms, also indicated that this lake apparently had a low carrying capacity, being unable to support a large number of individuals and lot of different species. However, significant variations occurred along the ice-free period, and the above described phenomena were much more evident in the second sample. Even if, as said, comparison between cultural and culture-independent methods is not always helpful, it is worth nothing that, in our case the two studies gave converging information.

Predominant TGGE bands were successfully reamplified and sequenced from TGGE gels,

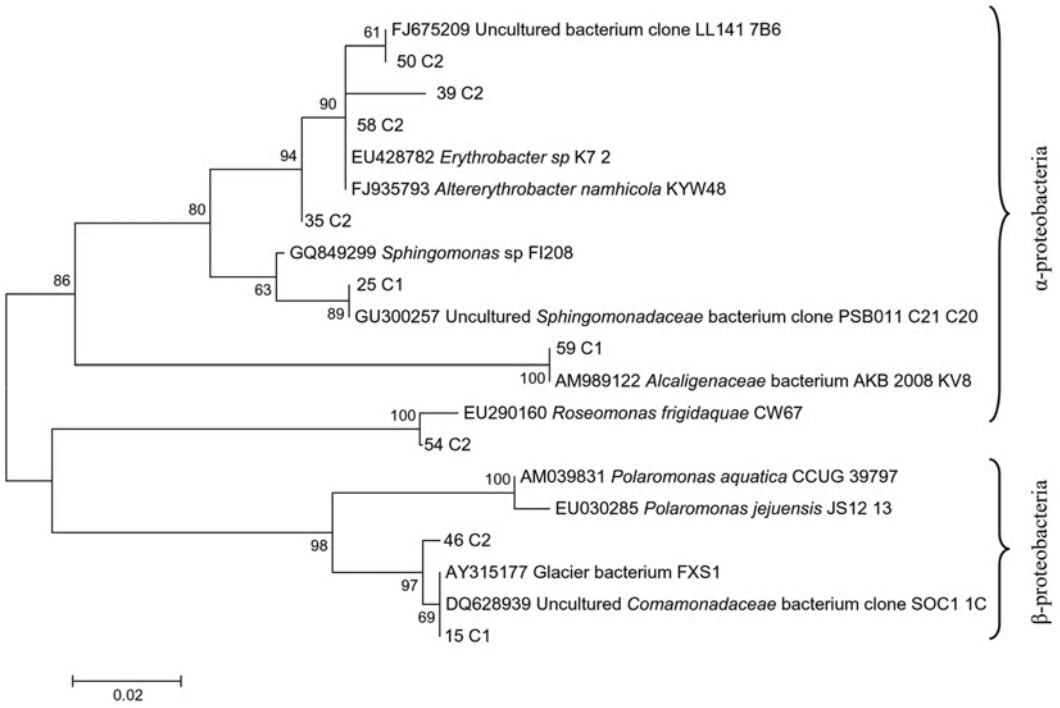

Fig. 5. Neighbor-joining phylogenetic tree
showing the positions of 9 bacterial sequences from re-amplified TGGE bands and the most similar sequences retrieved from the EMBL database, based on *ca*. 200 nt length of sequences. The scale bar indicates a 2% divergence. Bootstrap values over 50% are shown in nodes

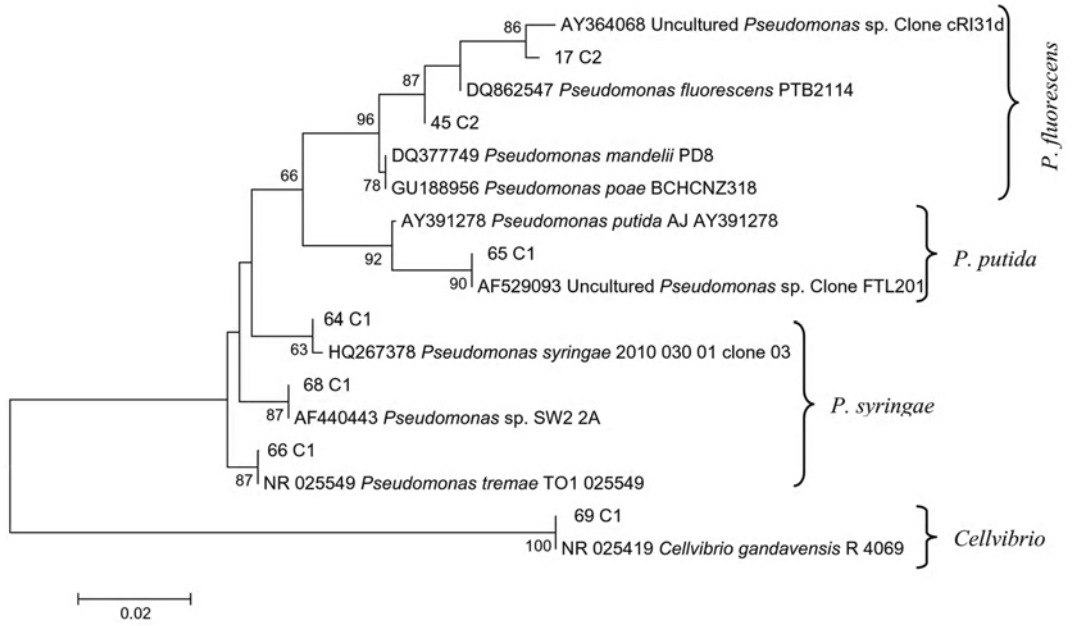

Fig. 6. Neighbor-joining phylogenetic tree
showing the positions of 7 *Pseudomonas* sequences from re-amplified TGGE bands and the most similar sequences retrieved from the EMBL database, based on *ca*. 200 nt length of sequences. The scale bar indicates a 2% divergence. Bootstrap values over 50% are shown in nodes

corresponding to dominant Bacteria populations in "La Caldera" lake. Five bands out of nine were present in both samples (Fig. 3). All sequences were related to Protobacteria with a main group associated to α-Proteobacteria and a minority cluster related to β-Proteobacteria (Fig. 5). Thus, apparently, Protobacteria were the predominant bacterial group in the lake. This result somehow confirmed those obtained by the cultural methods even if no γ-Proteobacteria were detected with the universal primers. By contrast, using the specific primers for *Pseudomonas*, various TGGE bands were reamplified, sequenced and affiliated to various species (i.e. *P. fluorescens, P. putida, P. syringae*) of this genus (Fig. 6). The meaning of these apparently contrasted results is that, dominant microbial groups are absent or poorly represented in cultivation experiments (Benlloch *et al*., 2001; Burns *et al*., 2004; Hugenholtz *et al*., 1998). Thus, γ-Proteobacteria (and among them *Pseudomonas* species) could be not predominant in the "La Caldera" lake even if under cultural conditions they could emerge or prevail. It is worth noting that the comparison of cultural and cultural-independent methods, reducing the limits of both techniques, gave more detailed and complemented information of the bacterial structure of this peculiar site as already demonstrated for other environments (Burns *et al*., 2004; Sánchez-Peinado *et al*., 20104).

CONCLUSION

In this work, identification of the principal microbial groups in the "La Caldera" lake was obtained complementing cultivation-independent and cultural methods. Structure and evolution of the bacterial community was studied within the ice-free season. The use of simple cultivation techniques, in combination with molecular methods, permitted to isolate and identify representatives of significant members of the microbial community of the studied environment for further investigations. This work demonstrated that cultivation still remains a useful tool for assessing ecology, diversity and dynamics of microbial communities.

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