

## BACTERIAL COMMUNITY SHIFTS OF A HIGH MOUNTAIN LAKE IN RESPONSE TO VARIABLE SIMULATED CONDITIONS: AVAILABILITY OF NUTRIENTS, LIGHT AND OXYGEN

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### ABSTRACT

We studied bacterial population composition shifts by exposing natural water samples to variable simulated environmental conditions. The samples were taken from Lake Jori XIII (2640 m a.s.l), an oligo-to mesotrophic cold freshwater lake, located in the eastern Swiss Alps. The Jori lakes are characterized as remote, unpolluted high mountain lakes with a long period of ice cover and typically low nutrient concentrations. Culture independent techniques (PCR-based analyses) were used for detection and molecular characterization of a large number of bacteria most of which are still uncultivable. Bacterial community shifts over three ecological conditions (nutrients, light and oxygen availability) were detected by using Temporal Temperature gradient Gel Electrophoresis (TTGE) of a PCR-amplified part of the 16S rRNA gene. The bacterial populations responded differently to the variable conditions, as revealed by TTGE pattern shifts during the experiment.

**Key words:** Temporal Temperature gradient Gel Electrophoresis (TTGE), ARB, small subunit ribosomal RNA gene (SSU rRNA gene), alpine freshwater lake Jori XIII, PCR

### INTRODUCTION

Lake Jori XIII is located in Canton Graubiinden, Switzerland. This lake has a maximum depth of 10.9 m and its surface area is approximately 1 5400 m<sup>2</sup>. Lake XIII presently is one among 22 medium and small lakes in the Jori catchment. Some of them are several thousand years old, and others were formed when the glacier retreated during the last 150 years. The habitats are still evolving. They are situated in the eastern part of Swiss Alps, at 46°46'N latitude and 9°58'E longitude, at altitudes between 2489 m to 2750 m above sea level. The iron-rich area is almost free of vegetation. Several lakes (for example Lakes I, II and III) are still influenced by streamlets from the melting glacier, whereas many others, which are mostly situated on higher places, are no longer under glacier influence, including Lake Jori XIII (Figure 1). Remote, unpolluted high mountain lakes are ecosystems in which their hydrochemical conditions are characterized by extremely variable fluctuations and often sudden changes. These intrannual and interannual environmental conditions

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Photo by Kurt Hanselmann

Figure 1. View of Lakes Jori I, II, III, XII and XIII during summer, ice-free period.

can be related to the atmospheric or climate variability. The alpine lakes can be considered as relatively simple aquatic ecosystems with short food chains, restricted environmental variables and low species diversity.

Organisms living in these habitats are challenged by harsh temperatures (below freezing to +15°C maximally), by seasonal and diurnal radiation differences between darkness and high levels of UV, by nutrient deprivation and bursts during snow melt phases, and by anoxia and darkness in the hypolimnion below a long lasting ice cover. During the short summer period, the epilimnion experiences high diurnal temperature fluctuations, strong light penetration and high UV radiation (Bothwell *et al.* 1994). Maximal water temperature during this period can reach 15°C but it could suddenly drop to 0°C in case of snow fall.

Bacteria are known to thrive under extremely broad-range conditions including such that would kill other organisms. They are further able to perform various metabolic processes, and their capabilities are more diverse compared to higher organisms. Therefore, we focused on the adaptation of the bacterial community in this extreme habitat. We were interested in how the bacterial population composition shifts under fluctuating environmental conditions. Normally, in the environments which get exposed to diurnal changes and annual variation in physicochemical and trophic conditions, the communities are forced to adapt quickly.

The bacterioplankton community structure changes were studied *in vitro* by simulating three environmental determinants i.e. light, oxygen, and nutrients. Temporal Temperature gradient Gel Electrophoresis (TTGE) has been used to follow the bacterial community composition changes. TTGE is a method which does not require a cloning step for profiling microbial populations, therefore, it is a fast monitoring method. The small subunit (SSU) ribosomal RNA gene is widely used as a comparative phylogenetic marker (Ward *et al.* 1990; Muyzer *et al.* 1993). The ribosomal RNA gene has several features that make it an ideal phylogenetic marker. First, as the backbone inside the ribosome it is ubiquitous in the living world.

Further it has the same function in all organisms, it is functionally homologous. Also it is more sensitive to mutations than most of the other genes since its gene product is used directly without any further translation into a protein where some mutations could keep silent. The gene sequence itself has regions of broad homology but also of heterogeneity compared with different organisms (it is moderately well-conserved) which makes it easy to align it in almost all levels of phylogeny. Concerning the nucleic acid length of the rRNA gene, the sequence of the SSU rDNA has the advantage that it is long enough for a reasonable alignment but quite shorter than the sequence of the large subunit rDNA (LSU rRNA gene) which makes it cheaper and faster to work with. The community shifts were monitored by comparing the changes of DNA fingerprints of PCR-generated 16S rDNA fragments in a TTGE gel. The electrophoretic separation of the community DNA molecules in TTGE is based on the temperature gradient. The separated bands can then be excised, PCR-amplified, and sequenced to identify the members of the community. This technique has been applied already for assessment and monitoring of bacterioplankton community dynamics in diverse aquatic environments (Bosshard *et al.* 2000; Pearce 2000; Casamayor *et al.* 2002).

The three environmental determinants chosen - nutrient supply, light, and redox condition - strongly affect biological processes. Combinations of these factors were used for *in vitro* simulations. The results illustrate the population flexibility and further support the validity of the selective adaptation hypothesis for ecosystems under harsh and highly variable environmental conditions (Elena and Sanjuan 2003).

## MATERIALS AND METHODS

### Sample collection

Sampling was carried out in late autumn in October 2002 in Lake Jb'ri XIII. Samples were taken from the lake water column, at 5 different depths of 1, 3, 5, 7, and 9 meters, respectively. Water samples were collected with a 500 ml Niskin sampler. The sampler was pre-rinsed with the lake water from the desired depths. The actual water samples were filled in sterilized 500 ml glass bottles and were kept at low temperature in a cooling box for transportation.

Sub samples for the experiments under the different conditions were taken in the laboratory (Table 1). Volumes of 100 ml for each experiment were placed aseptically in sterile 200 ml glass bottles. All samples were incubated at 4°C under a combination of the following conditions: with or without additional nutrients, in the light and in the dark, with oxygen (stirring) and anoxically (Figure 2). The final concentration after adding nutrients was 10 fold diluted Luria Bertani (LB) medium consisting of: tryptone 0.5 g l<sup>-1</sup>, yeast extract 1.0 g l<sup>-1</sup>, and NaCl 0.5 g l<sup>-1</sup>. The pH was adjusted to 7.2. A sample set was incubated in the dark (the bottles were wrapped in aluminum foil). Samples that needed light were incubated under two 15 Watt tube lamps (Osram) in a 30 x 30 x 70 cm<sup>3</sup> refrigerator, with continuous illumination. Aerobic incubation was done on stirrer plates. Each bottle of this

Table 1. Experimental overview of the community changes *in vitro*.

Sample <sup>a)</sup> from depth (m)	Nutrients added <sup>b)</sup>				No nutrients			
	dark		light		dark		light	
	oxic	anoxic	oxic	anoxic	oxic	anoxic	oxic	anoxic
1	*		*		*		*	
3		*		*		*		*
5	*		*		*		*	
7		*		*		*		*
9	*		*		*		*	

\*: experiments carried out

a) Samples were collected on 1<sup>st</sup> October, 2002

b) 10-fold diluted LB medium



Figure 2. Incubation of samples treated under a series of different simulated conditions at 4°C.

treatment was stirred continuously using a magnetic bar and the bottles were covered by sterile cotton plugs. For anoxic treatment, the bottles were purged with O<sub>2</sub>-free N<sub>2</sub> gas, completely filled and tightly sealed. Resazurin was used as redox indicator in the anaerobic cultures.

#### **Nucleic acid extraction, PCR, and TTGE analysis for community profiling**

Preparation for DNA extraction of initial samples (without treatment) was processed directly in the field laboratory. Water samples of 75 ml were filtered

aseptically through 0.22  $\mu\text{m}$  pore size filters (Millipore GVWP, 25 mm in diameter) using a sterile syringe and a "Swinnex®" disc filter holder (Millipore, SXOO 025 00). The filters were placed in sterile 1.5 ml tubes and kept at  $-20^{\circ}\text{C}$  until further processing, i.e. DNA extraction, PCR amplification and TTGE analyses.

After 2 weeks of incubation, the cultures were harvested for total genomic DNA extraction, PCR amplification, and further TTGE analysis. Cells were collected by filtration using 0.22  $\mu\text{m}$  pore size filters (Millipore, GVWP, 25 mm in diameter). The sample quantity varied, depending on the turbidity of the culture. Normally, 40 to 50 ml from the treatments with nutrients and 75 ml from cultures without added nutrients were filtered.

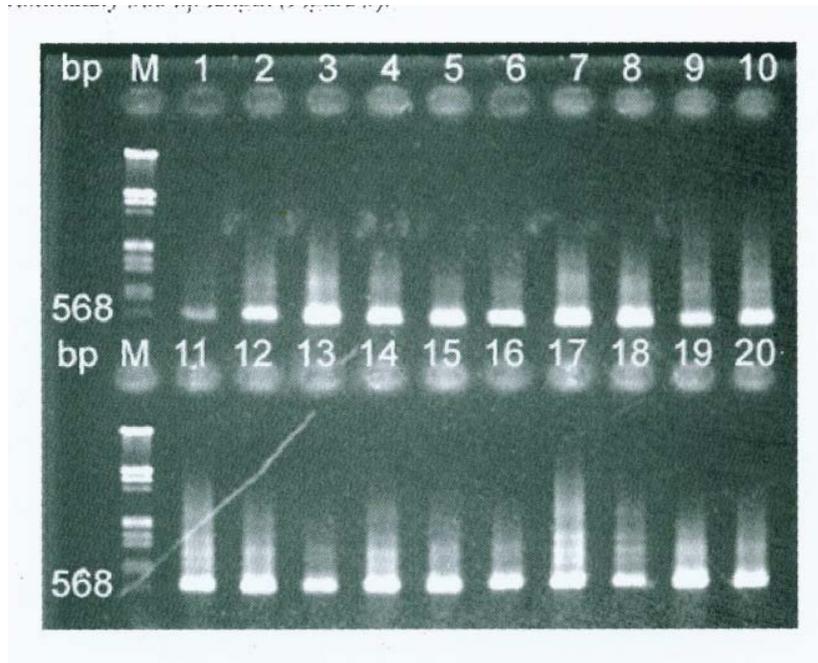
Samples were treated applying protocol B of the Qiagen DNeasy® Tissue Kit for Gram positive bacteria with an additional bead beating lysis step. The microorganisms on the filters were rinsed with lysis buffer and further processed according to the Qiagen protocol. The cells were lysed with lysozyme and disrupted physically by bead beating. Bead beating was done by adding 100 mg of glass beads (0.1 mm in diameter) to each tube and shaking at 80% of maximum speed in a Retsch MM2000 bead beater (F. Kurt Retsch GmbH & Co. KG, 42781 Haan, Germany) for 1 min. The extracted DNA was diluted in  $100 \times$  sterilized  $\text{H}_2\text{O}$ . Tubes containing extracted genomic DNA were stored at  $-20^{\circ}\text{C}$  until further processing.

To amplify the 16S rRNA gene fragments from the total community DNA, we used the general bacterial primers 27f (= S-D-Bact-0008-b-S-20; 5'-AGA GTT TGA TCM TGG CTC AG) and 1524r (= slightly modified 1525r, S-D-Bact-1524-a-A-18; 5'-AAG GAG GTG ATC CAR CCG) (Lane 1991). PCR reactions were prepared in a volume of 25  $\mu\text{l}$ , containing (final concentration):  $\text{dH}_2\text{O}$ , *Taq* buffer (1x) (Sigma), 0.1  $\text{mg ml}^{-1}$  DNase-free bovine serum albumin (Amersham, Pharmacia Biotech Inc.), 0.2 mM dNTPs, 200 nM of each primer, 40  $\text{U ml}^{-1}$  *Taq* Polymerase (Sigma), and approximately 50-100 ng template DNA. PCR was performed with a Techne Thermocycler (Techne LTD, Duxford Cambridge, U.K.).

PCR was run under the following conditions: initial denaturation at  $94^{\circ}\text{C}$  for 130 sec. The next steps were 10 cycles of  $94^{\circ}\text{C}$  for 15 sec,  $63^{\circ}\text{C}$  for 30 sec and lowering the temperature by  $0.5^{\circ}\text{C}$  in every cycle (touch down), then  $72^{\circ}\text{C}$  for 80 sec with increasing the duration by 1 sec every cycle. These steps were followed by 20 cycles of  $94^{\circ}\text{C}$  for 15 sec,  $56^{\circ}\text{C}$  for 30 sec,  $72^{\circ}\text{C}$  for 90 sec with increasing the period by 1 sec every cycle followed by a final extension step at  $72^{\circ}\text{C}$  for 10 min. PCR products were analyzed by electrophoresis in a 1% agarose gel and 0.5x TAB running buffer [20 mM Trizma base, 10 mM Glacial acetic acid, and 1 mM  $\text{Na}_2\text{EDTA}$ ]. First PCR amplifications yielded approximately 1500 bp length of the 16S rRNA gene (data not shown).

The primary PCR products were used as templates for the nested PCR. This method was used to increase the sensitivity and signal strength of the PCR amplification. Universal primers 357f (slightly modified) (= S-D-Bact-341-b-S-17; 5'-CCT ACG GGA GGC AGC AG) and GC-907r (= GC-Univ-907-a-A-20; 5'-CGC CCG CCG CGC GCG GCG GGC GGC GCG GGC GCA CGG GGC G CCG TCA ATT CMT TTR AGT TT) (Lane 1991; Muyzer *et al.* 1998) were used to amplify

the 16S rRNA gene fragments. PCR was performed with initial denaturation at 94° for 5 min, followed by 75°C for 15 sec. The next steps were 20 cycles of 94°C f 20 sec, annealing at 65°C - lowered by 0.5°C every cycle- for 30 sec (touch down and elongation temperature 72°C for 1 min. These steps were followed by 15 cycl< of 94°C for 20 sec, 52°C for 30 sec, 72°C for 70 sec, and a final extension step ; 72°C for 10 min. Nested PCR amplification yielded 16S rRNA gene fragments c approximately 560 bp length (Figure 3).



Figures. Products after nested PCR with 357f and GC-907r of the 16S rRNA gene fragments. Only a part of the PCR products from several samples in this study is shown. M: Marker (A DNA digested with *EcoRI/HinfII*).

(1-5: samples from depth 1 m; 1: initial, 2: HDO, 3: HLO, 4: NDO, 5: NLO; 6-10: samples from depth 3 m, 6: initial, 7: HDA, 8: HLA, 9: NDA, 10: NLA, 11-15: samples from depth 5 m; 11: initial, 12: HDO, 13: HLO, 14: NDO, 15: NLO; 16-20: samples from depth 7 m, 16: initial, 17: HDA, 18: HLA, 19: NDA, 20: NLA).

Initial: initial population, before treatments were applied. H: 10-fold diluted LB medium added, N: no nutrients added, D: incubation in the dark, L: incubation in the light, A: anoxic condition, O: oxic condition.

TTGE was carried out in a DCode™ Mutation Detection system (Bio-Rad laboratories). 10 µl of the PCR samples and 10 µl of 2x loading buffer (70% [v/v] glycerol, 0.05% [w/v] bromophenol blue) were loaded onto 6% polyacrylamide gels (acrylamide: N,N'-methylene bis-acrylamide 37.5:1 [w/w]; 7 M urea, 1x TAB). The gels were run at temperatures starting at 54°C and raising to 64°C, temperature ramp rate was +1.1°C h<sup>-1</sup>, the voltage was 90 V (4.0 V/cm), and running time was about 9 h. The gels were stained in 1 ng ml<sup>-1</sup> ethidium bromide solution for 15 min, stained in water for 45 min, visualized, and photographed under UV illumination (X = 312 nm).

#### •quencing of the 16S rRNA genes from PCR-TTGE bands and phylogenetic tree construction

The representative bands of the dominant bacteria which appeared distinctly in TTGE gels were excised and DNA was recovered by putting the gel fragments together with 20 µl of H<sub>2</sub>O into a 1.5 ml tube, freezing twice, and taking the supernatant. PCR amplification was performed by using 341f and the non-GC-907r primers. PCR products were bidirectionally sequenced using ABI Prism® Big Dye™ 3.1 (Applied Biosystems). The PCR products were purified by centrifugation in spin filter devices (Microcon YM 100, Millipore, Bedford, Mass., USA), and were used for a 10 µl single PCR reaction: 5 to 20 ng DNA template, 3 µl Big Dye Applied Biosystems and 3 µl of 1.5 µM primer were used. After the sequencing run, the products were purified with Sephadex G-50 (Amersham, Pharmacia Biotech AB) and loaded onto the sequencing machine (ABI Prism 377 DNA sequencer).

The BLAST Search tool available from NCBI (<http://www.ncbi.nlm.nih.gov/blast>) (Altschul *et al.*, 1998) was used to list the closest neighbors of the sequences. The new 16S rRNA gene sequences were imported to the SSU rRNA database retrieved from the Technical University of Munich (release January 2004) using the RB phylogeny program (<http://www.arb-home.de/>) (Ludwig *et al.*, 2004) and aligned automatically employing the Fast Aligner V1.03 of the ARB software environment. Subsequently the alignment was corrected manually according to the secondary structure. The sequences were then added to the general tree of the ARB SSU rRNA database by calculating distances with a maximum-parsimony algorithm and using the appropriate SAI (Sequence Associated Information) filters.

## RESULTS AND DISCUSSION

During the short summer period and autumn, the lake was homothermic i.e. the lake water masses were completely mixed. Therefore, the physical and chemical parameters of the lake water column did not differ much among various depths (Table 2). The initial TTGE profile from depth of 1 m is the same as those from depths of 3 m, 5 m, 7 m, and 9 m. The community patterns of Lake Jari XIII differ

Generally, it can be seen that incubations under high nutrient conditions showed fewer bands than the initial community and also fewer when compared to those without nutrients addition. Low numbers of TTGE bands indicate a limited number of species, i.e. only those microorganisms from the community which possess the highest competitive ability, are growing well. Environments with low nutrients lead to a more diverse species composition, and community shifts between the initial and the final state are less pronounced. Cultures kept in the light did not yield distinctly different patterns from those ones kept in the dark regardless whether they were grown with or without nutrient addition, except for the aerobic condition with no additional nutrients.

Certain bacterial communities develop different life strategies as an adaptation to various environmental conditions. Their population would be present predominantly even under markedly different environmental pressures which results in similar TTGE banding patterns. However, it cannot be ensured that those co-migrating bands have the same sequence identity. Therefore, it is helpful to identify the communities appearing in the TTGE bands. In this experiment, the representative TTGE bands of dominant communities were recovered and sequenced. The bands are phylogenetically affiliated to *Alpha*-, and *Beta-Proteobacteria*, *Actinobacteria*, chloroplasts, and candidates of division OP 10 (Figure 5). The communities are phylogenetically diverse and respond quickly to various environmental fluctuations.

Some sequences represented by bands A, B, C, 6 and 8, showed very low similarity values (86 to 94 % sequence similarities) to known 16S rDNA sequences of cultured bacteria present in the databank (Table 3). Cultivation efforts for those microorganisms could become valuable concerning their physiological properties.

The results suggest that community competition and adaptation processes are common in ecosystems exposed to strong physicochemical fluctuations. Physiological abilities of the microorganisms present in their gene pool allow the community to respond rapidly to environmental changes and thus ensure the functioning of microbially-mediated processes in the habitat. Microorganisms possessing a high physiological flexibility, so-called niche generalists, will dominate the population in their communities under various conditions (Elena and Sanjuan 2003), while niche specialists will get selected under more specific conditions. Environmental changes express themselves as physical and chemical instabilities, which exert selection pressures for different microorganisms. These changes promote the dynamic microbial community transitions in fluctuating environments (Rainey and Travisano 1998).

added) rather than to NLO. Under low nutrient conditions, the majority of TTGE bands still appeared. Some became less intense after the two-week incubation period. A few bands are missing at the end of the experiment (e. g. bands A and B).

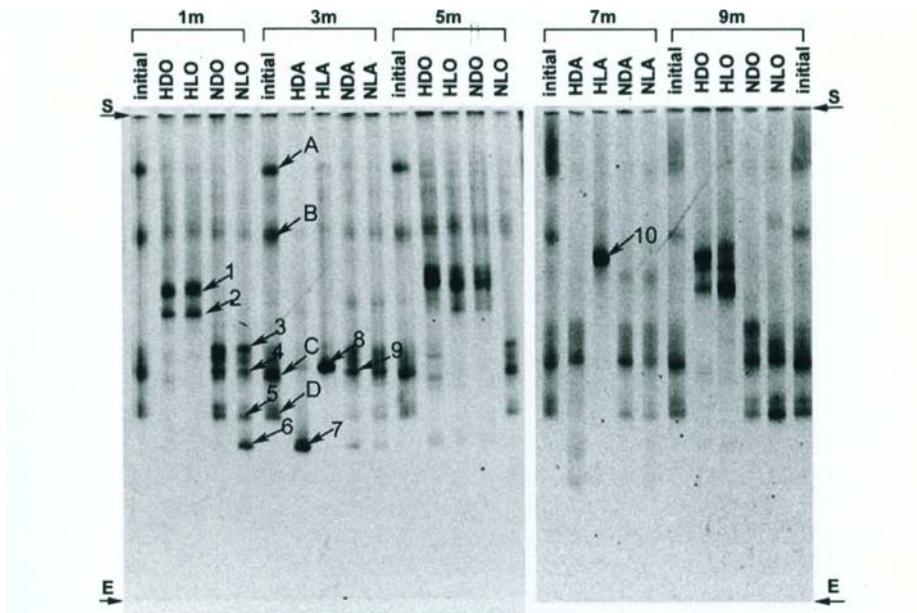


Figure 4. TTGE patterns of 16S rDNA fragments amplified by nested PCR from total bacterial community DNA of Lake Jöri XIII from distinct depths incubated at different environmental conditions (except "initial").

Initial: initial population, before treatments were applied. H: 10-fold diluted LB medium added, N: no nutrients added, D: incubation in the dark, L: incubation in the light, A: anoxic condition, O: oxic condition. S: start of gels, E: end of gels.

The bacterial diversity decreases when the community is exposed to high nutrient concentrations and anoxic conditions. This can be detected from experiments designated HDA and HLA at depths of 3 m and 7 m, respectively. In these treatments, light apparently is an essential determinant. In the light, bands 8 (3m depth) and 10 (7m depth) became prominent, in the dark, band 7 (3m depth). Based on NCBI-BLAST of 16S rRNA gene sequences, the closest known relative of band 7 (community exposed to HDA treatment) was an anaerobic, sulfate reducing bacterium, *Desulfotomaculum* sp. DEM-KMe99-2, AJ276565 (with 88% sequence similarity). In the HLA culture, band 8 dominated the community, which is phylogenetically related to the alphaproteobacterium Sphingomonadaceae bacterium N, DQ497241 (99% sequence similarity). The sequence of band 10 was closest related to the betaproteobacterium LI2-55 from Lake Loosdrecht water column, Netherlands, AJ964892 (97% sequence similarity).

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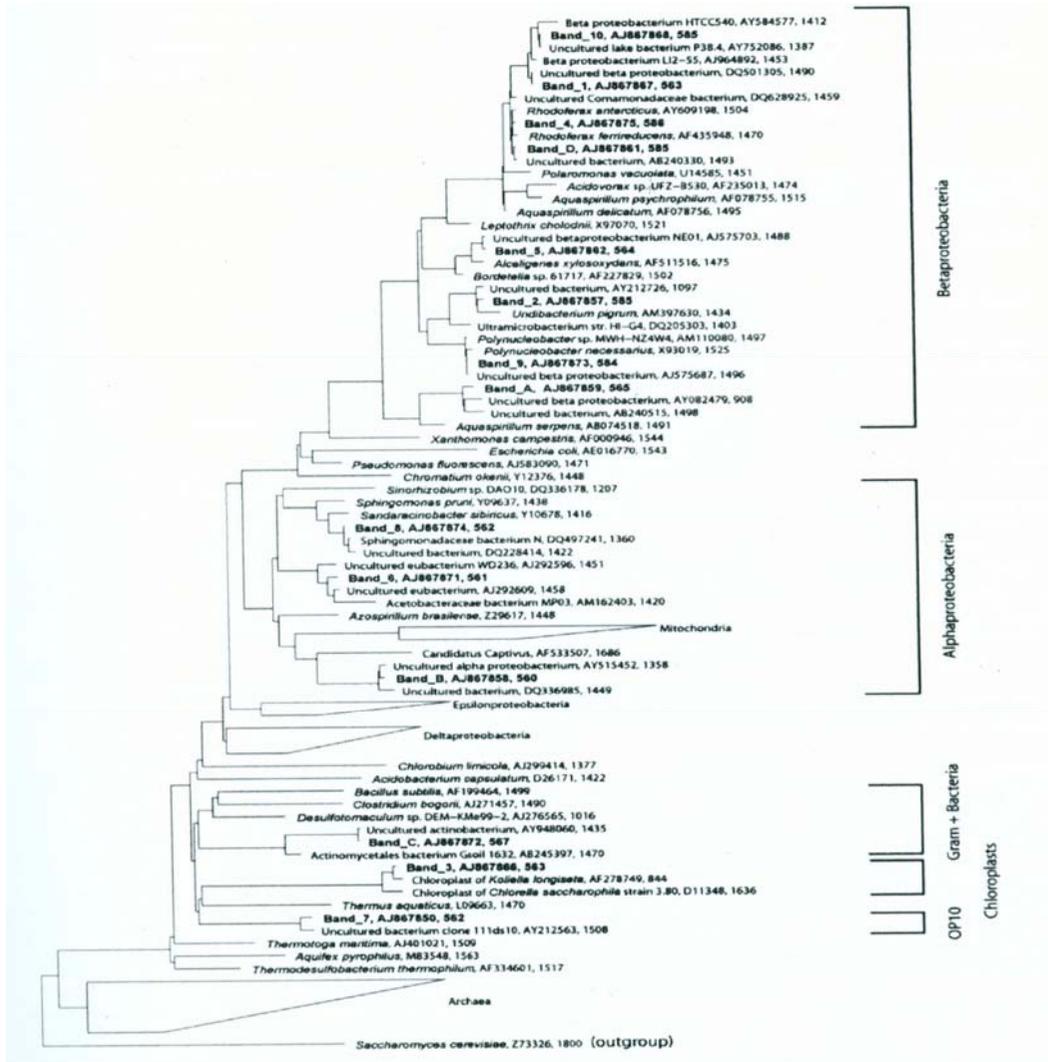


Figure 5. Phylogenetic affiliation of representative dominant TTGE bands of water samples from Lake Jori XII1 incubated at different environmental conditions. *Nei* sequences are written in bold (**Band xx, accession no., length lbp**). Closest known relatives are written in italic, followed by their accession nos. and sequence lengths (bp). A yeast 18S rRNA gene of *Saccharomyces cerevisiae* is used as outgroup. The scale bar indicates 10% sequence divergence

Table 3. Identification of representative dominant TTGE bands of Lake Jöri XIII to their closest known relatives obtained from BLAST search from NCBI.

Band designation	Length (bp)	Subgroup	Closest known species, accession no.	% Similarity
Band A	565	<i>Beta-Proteobacteria, unclassified Betaproteobacteria</i>	<i>Ultramicrobacterium</i> str. HI-G4, DQ205303	91
Band B	560	<i>Alpha-Proteobacteria, Rhizobiales</i>	<i>Sinorhizobium</i> sp. DAO10, DQ336178	86
Band C	567	<i>Actinobacteria</i>	<i>Actinomycetales</i> sp. strain Gsoil, AB245397	91
Band D	585	<i>Beta-Proteobacteria, Comamonadaceae</i>	<i>Rhodoferax ferrireducens</i> strain DSM 15236, CP000267	98
Band 1	563	<i>Beta-Proteobacteria, Comamonadaceae</i>	<i>Acidovorax</i> sp. strain R-25076, AM084035	97
Band 2	585	<i>Beta-Proteobacteria, Janthinobacteriaceae</i>	<i>Cenibacterium arsenoxidans</i> , AY728038	97
Band 3	564	Chloroplast organelles	<i>Chlorella saccharophila</i> (strain 3.80) chloroplast, D11348	97
Band 4	586	<i>Beta-Proteobacteria, Comamonadaceae</i>	<i>Rhodoferax antarcticus</i> strain Fryx1, AY609198	97
Band 5	564	<i>Beta-Proteobacteria, Alcaligenaceae</i>	<i>Bordetella</i> sp. strain 61717, AF227829	97
Band 6	561	<i>Alpha-Proteobacteria, Acetobacteraceae</i>	Acetobacteraceae bacterium MP03, AM162403	94
Band 7	565	<i>Clostridia, Peptococcaceae</i>	<i>Desulfotomaculum</i> sp. DEM-KMe99-2, AJ276565	88
Band 8	562	<i>Alpha-Proteobacteria, Sphingomonadaceae</i>	Sphingomonadaceae bacterium N, DQ497241	99
Band 9	584	<i>Beta-Proteobacteria, Polynucleobacter</i>	<i>Polynucleobacter</i> sp. MWH-NZ4W4, AM110080	99
Band 10	585	<i>Beta-Proteobacteria, Comamonadaceae</i>	Betaproteobacterium LI2-55, AJ964892	97

## CONCLUSIONS

It is possible to follow the bacterial community shifts by applying the PCR-based molecular technique of Temporal Temperature Gradient Gel Electrophoresis (TTGE). The physicochemical determinants applied in the experiments triggered the changes in the bacterial community composition originating from a high mountai

lake Jo'ri XIII. 16S rRNA gene sequencing and phylogenetic analysis revealed that the predominant populations are affiliated to the Alpha- and Beta- subgroups of the Proteobacteria, to Actinobacteria (high GC Gram + bacteria), chloroplasts and candidates of division OP 10.

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