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Bacterial diversity and activity along a salinity gradient in soda lakes of the Kulunda Steppe (Altai, Russia)

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Abstract Here we describe the diversity and activity of sulfate reducing bacteria along a salinity gradient in four different soda lakes from the Kulunda Steppe (South East Siberia, Russia). For this purpose, a combination of culture-dependent and independent techniques was applied. The general bacterial and SRB diversity were analyzed by denaturing gradient gel electrophoresis (DGGE) targeting the 16S rDNA gene. DNA was used to detect the microbial populations that were present in the soda lake sediments, whereas ribosomal RNA was used as a template to obtain information on those that were active. Individual DGGE bands were sequenced and a phylogenetic analysis was performed. In addition, the overall activity of SRB was obtained by measuring the sulfate reduction rates (SRR) and their abundance was estimated by serial dilution. Our results showed the presence of minor, but highly active microbial populations, mostly represented by members of the *Proteobacteria*. Remarkably high SRR were measured at hypersaline conditions (200 g L^{-1}). A relatively high viable count indicated that sulfate reducing bacteria could be highly active in hypersaline soda lakes. Furthermore, the increase of sodium carbonate/bicarbonate seemed to affect the composition of the microbial community in soda lakes, but not the rate of sulfate reduction.

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Introduction

Soda lakes are a specific type of salt lake with high to extremely high carbonate alkalinity, a pH from 9 to 11, and a moderate to extremely high salinity. They are spread all over the world, but located, as most inland salt lakes, in arid and semi-arid areas where the evaporative climate favors accumulation of salts in local depressions. The major ions in soda lake brines are sodium, carbonate/ bicarbonate, chloride and sulfate, whereas calcium is virtually absent and magnesium only present at very low concentrations. In contrast to other alkaline environments, such as low-salt alkaline ("cement") springs, soda lakes maintain a stable alkaline pH due to the high buffering capacity of the soluble carbonates. These double extreme conditions (i.e. high pH and high salinity) make soda lakes a unique ecosystem.

In the last decade, special attention has been given to the investigation of the microbial communities in soda lakes using both traditional isolation methods (Duckworth et al. 1996; Sorokin et al. 2004; Sorokin and Kuenen 2005b) and molecular biology techniques (Humayoun et al. 2003; Rees et al. 2004; Scholten et al. 2005; Foti et al. 2007). Few reviews (Zavarzin et al. 1999; Jones et al. 1998) summarize these results, showing that soda lakes contain representatives of the major trophic and phylogenetic groups of prokaryotes, and that they can be considered as autonomous systems, in which cycling of nutrients is close to complete. The most well-studied soda lakes are those located in the East African Rift Valley (Duckworth et al. 1996; Rees et al. 2004), in the Libyan desert (Wadi Natrun)(Imhoff et al. 1979) and in North America, i.e. Mono Lake (California) (Humayoun et al. 2003; Scholten et al. 2005a) and Soap Lake (Washington) (Sorokin et al. 2007a). There are also data available on soda lakes in India (Wani et al. 2006) and

Central Asia (Sorokin et al. 2004; Gorlenko et al. 2004; Ma et al. 2004). This last region accommodates a great number of steppe soda lakes, especially in the Inner Mongolia (North east China) and south Siberia (Transbaikal region, Tuva Republic and Kulunda Steppe).

Except for the study conducted by Issancheko et al. (1951) between 1933 and 1935, almost nothing is known about the microbial communities in soda lakes of the Kulunda Steppe. In contrast to tropical African lakes and deep stratified North American lakes, the Central Asian lakes are very shallow and usually very small, in which the total concentration of salts and chemistry may differ considerably among nearby lakes as well as during the year. Furthermore, lakes are subjected to unstable water and temperatures regimens between −40°C and +40°C, causing frequent fluctuations of the water level and salinity.

It has been hypothesized that the diversity of metabolic types of prokaryotes decreases with salinity for thermodynamic reasons (Oren, 1999). Several studies have been performed on systems with saline gradients (Sörensen et al. 2004; Nübel et al. 2000; Casamayor et al. 2002), especially estuaries (Crump et al. 2004; Schultz and Ducklow 2001), to investigate the influence of salinity on the composition of the microbial community. However, estuaries have very low salinities compared to salt lakes or salterns (Benlloch et al. 2002; Sörensen et al. 2004). Commonly, thalassohaline ecosystems are object of investigation, in which sodium, magnesium, chloride and sulfate are the major components of the brines. In soda lakes sodium carbonate/bicarbonate is the major salt in solution, which has different physical and chemical properties, such as being a two times weaker electrolyte than sodium chloride. For this, a less energetically expensive osmotic adaptation is needed than demanded for life in NaCl brines (Sorokin and Kuenen 2005b). Nevertheless, organisms inhabiting soda lakes have to deal with high alkaline conditions, which represent an extra stress factor. Therefore, it was of interest to study the microbial community composition in soda lakes, and how this was affected by an increase in sodium carbonate/bicarbonate concentration.

Since the sulfur cycle is one of the most active cycles in soda lakes, special attention was given to the sulfate-reducing bacterial community (SRB). It has been hypothesized (Oren 1999; Ollivier et al. 1994) that specific groups of SRB, i.e. complete oxidizers, stop growing at salt concentrations above 130 g L^{-1}. So, it was of interest to investigate the presence of these different phylogenetic groups of sulfate-reducers along a salinity gradient.

In this study we investigated the bacterial diversity and the major actively growing groups, based on ribosomal RNA determination, in four soda lakes with increasing salinity. For the first time the dominant active populations were detected in these extreme environments by targeting

the 16S rRNA gene from the lake sediments. A combination of culture-dependent and -independent techniques was applied to investigate the SRB community. Identification of sulfate-reducers was done by DGGE analysis of the 16S rRNA genes, while the activity was determined by measuring the sulfate reduction rates (SRR).

Our results showed the presence of less abundant, but highly active microbial populations, mostly represented by members of the *Proteobacteria*. The detection of active SRB from sediment samples by targeting the 16S rRNA gene was not completely successful. However the remarkably high sulfate reduction rates (SRR) measured at hypersaline conditions (200 g L^{-1}) and the relatively high viable counts indicated that this group of organisms was active in these hypersaline soda lakes. Furthermore, an increase of salinity seemed to affect the composition of the microbial community in soda lakes, but did not affect the rate of active processes, such as sulfate reduction.

Materials and methods

Site description

The sampled lakes are located in the southern part of the Kulunda Steppe, in south-eastern Siberia (Altai, Russia), along the north-eastern border with Kazakhstan, about 320 km south–west of Barnaul (Altai capital), near the village Mikhaylovskiy (Fig. 1). The only information on the geology and chemistry of this area is available from the results of the Kulunda Expedition of the Russian Academy of Sciences taken place between 1933 and 1935 (Issachenko 1951). Lakes present in this region are generally very small and shallow, except for the large chloride–sulfate lakes with neutral pH in the north, such as Kulunda Lake, Lake Burlinskoe, and Lake Big Yarovoe. In the south of the Kulunda Steppe, the lakes are much smaller and some of them belong to the soda lake type. The concentration and nature of the salts present as well as their sediment structure may differ even between closely situated lakes. Except for Cock Lake, all investigated lakes (see Table 1) are located close to each other, i.e. within a diameter of 25 km. They belong to two systems: the Tanatar and the Bitter Lakes System, and both are part of a larger system, which is locally known as the "Salt Lake Steppe". Although Cock Lake does not belong to the above-mentioned systems of lakes being situated ca 50 km north–west, it is very similar for its characteristics to the lakes in the Bitter Lake System. Characteristic feature of all the investigated lakes is the mineral deposition of ancient trona (i.e., sodium carbonate/bicarbonate), which confer the high salinity and alkalinity. Cock Lake was the first source of commercial soda in the Soviet Union since

Fig. 1 Location of the investigated soda lakes in the Kulunda Steppe (Altai, Russia). **a** Large scale map including Cock Lake. The lakes within the *square* are shown in detail in Fig. 1b. **b** Detailed map showing lakes Tanatar-1 (*T1*), Tanatar-3 (*T3*), and Bitter-1
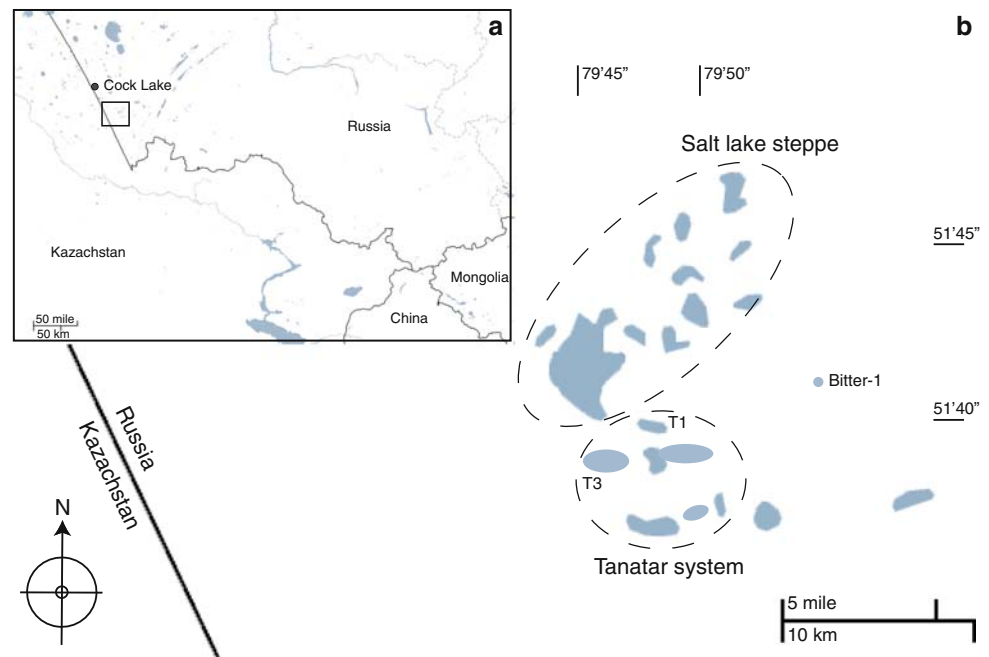
Table 1 Chemical parameters of the investigated soda lakes and viable count numbers of SRB

Sediment sample (depth in cm)	Coordinates		Name	Na^+ g L^{-1}	pH	Alkalinity (M)		CH_4 ml L^{-1}	SO_4^{2-} mM	SRR µmol dm^{-3} day^{-1}	H_2 (viable cells cm^{-3}	Acetate (viable cells cm^{-3})
	Longitude	Latitude				CO_3^{2-}	Total					
C (nbw)	79.15°E	52.10°N	Cock Lake	60	10.30	0.85	0.90	0.44	0.84	0.2	10^5	10^6
C-1 (0–2)								0.73	0.84	116		
C-2 (3–10)								0.13	7.9	7.4		
C-3 (11–20)								0.07	2.6	4.8		
T3 (nbw)	79.75°E	51.65°N	Tanatar-3	112	10.13	0.90	1.125	0.74	74	0.47	10^6	10^6
T3-1 (0–10)								0.44	74	68		
T3-2 (10-20)								0.79	83	236		
T1 (nbw)	79.81°E	51.66°N	Tanatar-1	200	10.0	1.30	1.57	0.010	221	12	10^3	10^6
T1-1 (0–5)								0.016	221	153		
T1-2 (6-20)								0.004	242	104		
B1 (nbw)	79.90°E	51.67°N	Bitter Lake-1	200	10.20	2.25	2.825	3.33	6.6	92	10^3	10^4
B1-1 (0-4)								0.59	6.6	1426		
B1-2 (5-11)								0.70	5.6	43		

CH_4 and SO_4^{2-} concentrations and SRR were measured for the near bottom water (nbw) and for different sediment layers (first column). In the last two columns the enumeration of SRB has been estimated from positive tubes of serial dilutions on H_2 or rich acetate medium, respectively. The top 5 cm of the sediment samples were used as inoculum. 0.6 M Na^+ and 4 M Na^+ were used for Cock Lake (*C*) and lake Tanatar-3 (*T3*) and for lake Tanatar-1 (*T1*) and lake Bitter-1 (*B1*), respectively

1923, while Tanatar system is still actively mined for sodium carbonate. Cock Lake and Tanatar-3 presented a sandy-clay type of sediment, whereas lakes Tanatar-1 and Bitter-1 presented a black-clay type of sediment, with a strong sulfide smell. Sediment samples were taken from the mentioned lakes with increasing salinity in July 2007. The physical and chemical parameters of the lakes are presented in Table 1.

Chemical analysis and sampling

Field measurements included pH, conductivity, and carbonate alkalinity in brine water. pH and conductivity were measured using a field meter supplemented with pH and conductivity probes (WTW, model pH/cond340, Weilheim, Germany). The pH probe was calibrated in saline buffers containing 0.5–2 M total Na^+ (Sorokin 2005). The

conductivity probe was calibrated in NaCl and carbonate buffer with pH 10 from 10 to 80 g L^{-1}. At higher values, the samples were diluted with distilled water. Alkalinity was measured by titration with 1 M HCl using phenolphthalein (carbonate alkalinity) and methyl orange (bicarbonate alkalinity) as indicators. The salt concentration inferred from the conductivity measurements was verified by gravimetry in the laboratory. Samples were taken for nucleic acid extraction, sulfate reduction rate measurements and viable counts. Sediment cores (top 20 cm) and overlaying water were sampled manually along the littoral, using a corer. Sediment cores were divided into different layers, based on color, and stored in 50 ml Falcon tubes. Samples for nucleic acid extraction were mixed with RNA*later* (Ambion, UK) in a 1:1 volume ratio. All tubes were kept at 4°C during transportation to the laboratory, and stored at −20°C until further analysis.

Sulfate reduction rates

Sulfate-reduction rate (SRR) experiments were conducted in situ and measured in 5-ml syringes capped with butyl rubber stoppers using the ^{35}S-SO_4^{2-} methodology (Lein et al. 2002). After 1–2 days of incubation at ambient temperature, the sediments were fixed with 10 M KOH and further processed in the laboratory. Additional analysis of total SO_4^{2-} and CH_4 content in the sediment pore water was performed in the laboratory after centrifugation of 2 cm³ sample using ion-exchange chromatography (Biotronic) for sulfate and by gas chromatography for methane.

Additional experiments to study the influence of pH/salt and nutrient additions on indigenous SRB populations were performed in the laboratory with sediment slurries (top 5 cm layer) from Cock Lake and Tanatar-1. Nutrient addition experiments were performed with native sediment slurries after addition of nitrogen (100 μM NH_4Cl), phosphorus (10 μM K_2HPO_4) and electron donors such as hydrogen, lactate and acetate (1 mM) as substrates. The influence of pH and salt was examined after separation of the sediment solids from the pore brines by centrifugation. The influence of pH was investigated by suspending the sediments in buffers containing 5 mM sodium sulfate, varying the pH between 7 and 11 and keeping the original salinity. The influence of salt was investigated at pH 10 with increasing concentration of sodium carbonates. The SRR rates were measured in the same way as for the in situ measurements.

Quantification of sulfate-reducing bacteria by serial dilutions

Quantification of haloalkaliphilic SRB was performed by serial decimal dilutions in Hungate tubes. The top 5 cm sediment layers were used as inoculum. Low-salt (0.6 M total Na^+) and high-salt (4 M total Na^+) carbonate medium at pH 10 (Sorokin and Kuenen 2005a) was used with either H_2 or acetate (20 mM plus 0.5 g L^{-1} yeast extract) as an energy source. The inoculation was done directly in the field. Growth was monitored within 4–8 weeks incubation at room temperature by estimating the sulfide production (Trüper and Schlegel 1964). Tubes with the highest positive dilutions were then subjected to DGGE analysis.

Nucleic acids extraction

Prior to the nucleic acid extraction, RNA*later* was removed by 1 min centrifugation at 5,000 rpm. The sediments were then washed three times with 1 M NaCl and 10 mM Tris (pH 7.5) to lower the pH and the salt concentration. Genomic DNA was extracted from circa 2 g of sediment using the UltraClean Soil DNA Extraction Kit (MoBio Laboratories, USA), following the manufacturer's instructions with minor modification. Two hundred microlitres of 0.1 M $AlNH_4(SO_4)_2$ was added to the sediment to remove potential PCR inhibitors, such as humic acids (Braid et al. 2003). For RNA extraction, 7 g of sediments were divided in 2-ml screw cap tubes and washed as described above. After the washing step, bead beating and the addition of 120 μl of preheated MBER® (Max Bacterial Enhancement Reagent, Invitrogen, The Netherlands) were added to each tube and incubated for 4 min at 95°C. Cells were lysed by adding 0.7 ml TRIzol® (Invitrogen, The Netherlands), a mono-phasic solution of phenol and guanidine isothiocyanate and homogenized for 45 s, followed by 5 min incubation at room temperature to allow the complete dissociation of nucleo-proteins complexes. The TRIzol® reagent maintains the integrity of the RNA during sample homogenization, while disrupting cells and dissolving cell components. After homogenization cold chloroform was added (0.1 ml/0.5 ml TRIzol®). Subsequently, the samples were vigorously shaken manually for 15 s and incubated at room temperature for 3 min followed by 15 min centrifugation at 10,000 rpm and 4°C. The aqueous upper phase was then transferred to a new tube, 0.3 ml cold isopropanol was added and mixed to precipitate the RNA. After 10 min incubation at room temperature, the pellet was collected by centrifugation at 10,000 rpm for 10 min and washed with 75% (v/v) ethanol. Samples were then vortexed and centrifuged for 5 min at 8,000 rpm. The supernatant was discarded, while the pellet was air dried and dissolved in 40 μl RNA-free water. Subsequently, DNA traces were removed using the Turbo DNA free kit (Ambion, UK) and the resulting

DNA-free RNA was purified with the Qiagen RNAeasy MiniEluteKit (Qiagen, the Netherlands), following manufacturer's instructions. All reagents, plastic and glassware were RNAase free.

RT-PCR, PCR and DGGE

RNA was converted to cDNA by reverse transcriptase using the iScript cDNA Synthesis Kit$^{®}$ (BioRad, The Netherlands), following the manufacture's instructions. The purity of the extracted RNA was checked by 16S rRNA gene amplification using RNA as template; no PCR product was obtained indicating the absence of contaminating DNA. From the genomic DNA and cDNA. a fragment of the 16S rRNA gene suitable for DGGE, was amplified. A nested PCR approach was used for the amplification of the 16S rRNA gene. First the whole gene was amplified using the primer GM3F/GM4R (Brinkhoff et al. 1998). The product was then excised from the agarose gel, purified using the Qiagen Gel Extraction Kit (Qiagen, the Netherlands) and used as a template for the amplification of the a gene fragment suitable for DGGE using primer combination 341F + GC/907R (Schäfer and Muyzer 2001). To detect sulfate-reducing bacteria, six primers pairs specific for the 16S rRNA gene of different SRB groups (Table 2; Daly et al. 2000) were used as described by Dar et al. (2005). PCR products of each amplification step were excised from the gel and purified before the following PCR step to avoid non-specific amplification. The obtained PCR products were then subjected to DGGE analysis. DGGE was performed as described by Muyzer et al. (1993), using a denaturing gradient of 35 to 60% denaturants in 8% polyacrylamide gel. Individual bands were excised, reamplified, and run again on a denaturing gradient gel to check their purity. PCR products for sequencing were purified using the Qiaquick PCR purification kit (QIAGEN).

Comparative sequence analysis

The sequences were first compared to sequences stored in GenBank using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST). Subsequently, the sequences were imported into the ARB software program (Ludwig et al. 2004), automatically aligned, and added to a phylogenetic tree using the Quick-add tool. Sub-trees were then built using the Neighbor Joining algorithm with automatic selected correction settings.

Nucleotide sequence accession number

The sequences determined in this study were deposited in GenBank under the following accession numbers: EF622422–EF622488.

Results

Physical–chemical characterization of the sediments

The investigated lakes were less than 1 km in diameter and had a maximum depth of 1 m. The salinities varied from 60 to 200 g L^{-1} and the pH from 10 to 10.3 (see Table 1). The dominant salt in solution was sodium carbonate/bicarbonate and the total alkalinity was in the range of 0.9–2.82 M. Sulfate concentrations in sediment pore waters correlated with total salt and in general were very high except for Bitter Lake in which, despite the extremely high salinity, the sulfate concentration was nearly depleted (Table 1). Free sulfide (up to 0.5 mM) was also detectable in the pore waters of the top 5 cm sediment layer in Tanatar-1 and Bitter Lake. In Bitter Lake the highest methane content was measured in the sediments and even in the brines, suggesting that both sulfate reduction and methanogenesis are active.

Nucleic acids extraction and DGGE analysis of 16S rRNA genes

Nucleic acids extraction and PCR amplification of the 16S rRNA gene was successful for all analyzed samples. PCR amplification using specific primers for SRB groups was successful with most of the samples when targeting group 1 (*Desulfotomaculum*, DFM), group 5 (*Desulfosarcina*, DCC) and group 6 (*Desulfovibrio–Desulfomicrobium*, DVV). One, four and no positive amplifications were obtained targeting group 2 (*Desulfobulbus*, DBB), group 4 (*Desulfobacter* DSB), and group 3 (*Desulfobacterium*, DBM), respectively. In some cases amplification products were identified as non-specific, resulting in the detection of different phylogenetic groups than the targeted ones (see Fig. 4).

Amplicons obtained from genomic DNA and from cDNA (obtained after reverse transcription of the RNA) were then analyzed by DGGE. An average of 4 bands was observed for both DNA- and RNA-derived profiles (Fig. 2) using primers for the 16S rRNA genes of Bacteria. The DNA- and RNA-derived profiles were found to be highly different when analyzing the same samples (Fig. 2).

DGGE analysis of PCR products obtained with the primer pairs specific for the 16S rRNA genes of different

Table 2 Closest relatives of the detected sequences

Bands	Closest relative	Similarity %	Acc nr	Source
Alfa-proteobacteria				
C-3_DVV *	UncAl431	99.2	AY293404	Travertine Terraces at
	Brevundimonas sp.	99.15	AY145543	Yellowstone Hot Springs; fresh water
Beta-proteobacteria				
T3-2*; B-1*	*Massilia* sp.	99.6	DQ177478	Alkaline permafrost
C-2 *	*Tepidimonas aquatica*	99.6	AY324139	Hot water tank
Gamma-proteobacteria				
C-2_DCC**	UncHTC042	100	AB010848	Deep Sea
	Acinetobacter junii	100	AB101444	Activated Sludge
B-1 *	*Acinetobacter venetianus*	99.2	AJ295007	
T3-1*	*Moraxella osloensis*	99.1	AJ505859	Lake water
E_C_H$_2$**	*Halomonas sp.EF43*niHail3	97	AF275709	Hailaer soda lake
		95	AF280796	Hungarian soda lake
T3-2*; B-1*	*Stenotrophomonas* sp	∼99 to ∼88.5	AJ002814	Collemobola gut
	UniBac65		X92132	African soda lakes
Delta-Proteobacteria				
C-1_DCC**; T1-2*; T3-1** ;T1-1**; B-1_DCC**	ML28_DCC1	94–98.5	AY245462	Mono Lake
	D. propionicus	93–94.75	DQ067422	Great Salt Lake
T1-1_DCC*;T1-2_DCC; B-1_DCC*; T3-1_DSB**; T1-1_DSB**				
T3-1_DBB**	*Desulfobulbus propionicus*	99.8	AY548789	Sulfate-reducing fluidized-bed bioreactor
C-1_DVV**	*Desulfonatronum thiodismutans*	98	AF373920	Mono Lake
T3-1_DVV **	*Desulfonatronum lacustre*	98.6	AF418171	Soda lake
Actinobacteria				
C-2*	UncMicr4	99.81	AY569297	Microbial mat
	Microbacterium sp.35	99.81	AF306542	Cellulolithic bacteria from Refuse Landfill
C-2; T3-1*	Re0sea	99.7	AJ309523	Brine sea-water
	Propionibacterium acnes	98	AB108481	Blood
C-1_DFM**; T3-1_DFM**	UncAc655	98	DQ432396	African soda lakes
Bacillus				
T3-2_DFM*	*Bacillus thuringensis*	91.8	DQ078742	Chinese cabbage
C-3_DFM*; T3-2_DFM*	*Lactobacillus curvatus*	99.3–100	AY281292	
C-1_DCC*	UncLow16	90.5	AF507895	Mono Lake
Clostridia				
E_B_Ac **; T1-1**	UncLow98	87.7/ 85.5	DQ432226	African soda lakes
	Haloanaerobium sp.	85 / 82	AJ309520	Brine-sea water
E_T1_Ac**; E_T1_H$_2$**; T1-1_DVV**; T1-1_DVV**; T3-2_DFM**	IRB1	99.1–100	DQ631799	Soap Lake
	A. metalliredigenes	97.5	AY137848	Leachate ponds
C-2_DFM** ; C-3_DFM**	UncLow 12	92.5–97.6	AF507890	Mono Lake
C-3_DFM**	ML21_DFM1	99.2	AY245477	Mono Lake
C-2_DVV**;T3-2_DVV**; B-1**	UncLow69	93–96.7	DQ206412	Mono Lake
C-3*; B-1_DVV**; C-3_DVV**	UncLow11	95.2	AF507879	Mono Lake
T1-1_DFM**; T3-2_DVV**	UncB3880	94.5–94.2	AJ295640	Uranium mill tailing
	A. californiensis	83-84	AY064217	Mono Lake
B-1_DVV*	*N.halophilum*	92.4	AJ271451	Lake Magadi

Table 2 continued

Bands	Closest relative	Similarity %	Acc nr	Source
B-1; B-1_DVV*	*E.saburreum*	98.4	AF287777	Oral strain
C-3_DVV*	*Selenemonas sputigena*	94	AF287793	Oral strain
Cyanobacteria				
C-1*	*Oscillatoria neglecta*	98.4	AB003168	Hot spring, New Zealand
T3-1**	*Leptolyngbya* sp.	97.3	AB039012	Long Island, USA
Deinococcus–Thermus				
C-1*	IC_M_5a	96.8	DQ028374	Alkaline ikaite columns
	Deinococcus murrayi	83.6	Y13043	Hot spring
Bacteroidetes–Chlorobi				
T3-1*; B-2*	*Hymenobacter aerophilus*	90	AJ276901	Air
C-2**; T1-1**	Unc25804	95.7	DQ015831	Lake water, Antarctica
C-3*; B-1**	UncB33772	95–99.8	AF449772	Mono Lake
	UncChl57	84	AF445702	Travertine terrace

* Sequence retrieved from RNA-derived profiles

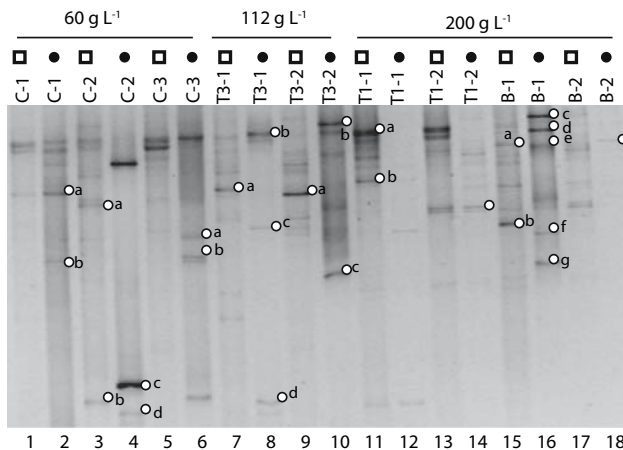** Sequence retrieved from DNA-derived profiles

Fig. 2 DGGE analysis of the bacterial 16S rRNA gene from sediment samples of the investigated soda lakes from the Kulunda Steppe. *Lanes 1–6* Cock Lake; *lanes 7–10* lake Tanatar-3; *lanes 11–14* lake Tanatar-1; *lanes 15–18* lake Bitter-1. Sediment depths have been analyzed for each lake (see Table 1). DNA (*open square*)- and RNA (*filled circle*)-derived profiles of the same samples are situated close to each other. Bands indicated by a *white dot* have been excised from the gel and sequenced

SRB groups (results not shown) gave complex patterns for both group 1 and 6, whereas for group 2 and 4 just one dominant band was detected, corresponding to *Desulfobulbus propionicus* and *Desulfosalina propionicus* respectively. From the highest positive tube of the dilution series for the SRB enrichment, 1 and 5 bands were obtained, using hydrogen or acetate as electron donor, respectively. Almost all sequences grouped with members of the low G + C gram-positive bacteria and not to SRB of the *Delta-proteobacteria*.

The DGGE analysis of the different soda lakes did not show a decrease in the number of bands at different sediment depths or along the salinity gradient, except for the RNA-derived profiles obtained for Lake Tanatar-1 and Bitter-1 (Fig. 2). Rather, the composition of bacterial taxa decreases at higher salinities (Fig. 3). In Cock Lake, the lowest saline lake (60 g L^{-1}), ten phylogenetic groups were detected, in Tanatar-3 (112 g L^{-1}), eight groups and in Tanatar-1 (200 g L^{-1}) and Bitter Lake (200g L^{-1}), 3 and 5, respectively (see Fig. 3).

Microbial diversity along a salinity gradient

From the different DGGE gels obtained targeting the 16S rRNA gene, a total of 72 partial sequences have been analyzed phylogenetically. An overview of the obtained results is shown in Table 2 and Fig. 4.

The less saline Cock Lake showed the highest bacterial taxa richness (Fig. 3). The top 10 cm sediments were characterized by the presence of sequences affiliated to the halo (alkaliphilic) Delta-proteobacterial SRB, such as *D. propionicus* and *Desulfonatronum thiodismutans*, to the Gamma-proteobacterial *Acinetobacter junii* and to the actinobacterium *Propionibacterium acnes*. The sequences retrieved from the RNA-derived DGGE profiles, thus presumed to correspond to the active population, belonged to the Cyanobacteria (i.e. *Oscillatoria neglecta*), *Deinococcus*, Actinobacteria and *Beta-proteobacteria* (i.e. *Tepidimonas aquatica*). The deepest sediment layer (10–20 cm) was mostly characterized by sequences related to uncultured low G + C organisms. From RNA-based DGGE's, sequences related to the alphaproteobacterium

Fig. 3 Microbial community composition along the salinity gradient. *CL* Cock Lake, *T3L* lake Tanatar-3, *T1L* lake Tanatar-1, *BL* lake Bitter-1. *Alfa Alphaproteobacteria, beta Beta-proteobacteria, gamma Gamma-proteobacteria, delta Delta-proteobacteria, Bacter-Chlor Bacteroidetes/Chlorobii, Cyano Cyanobacteria*
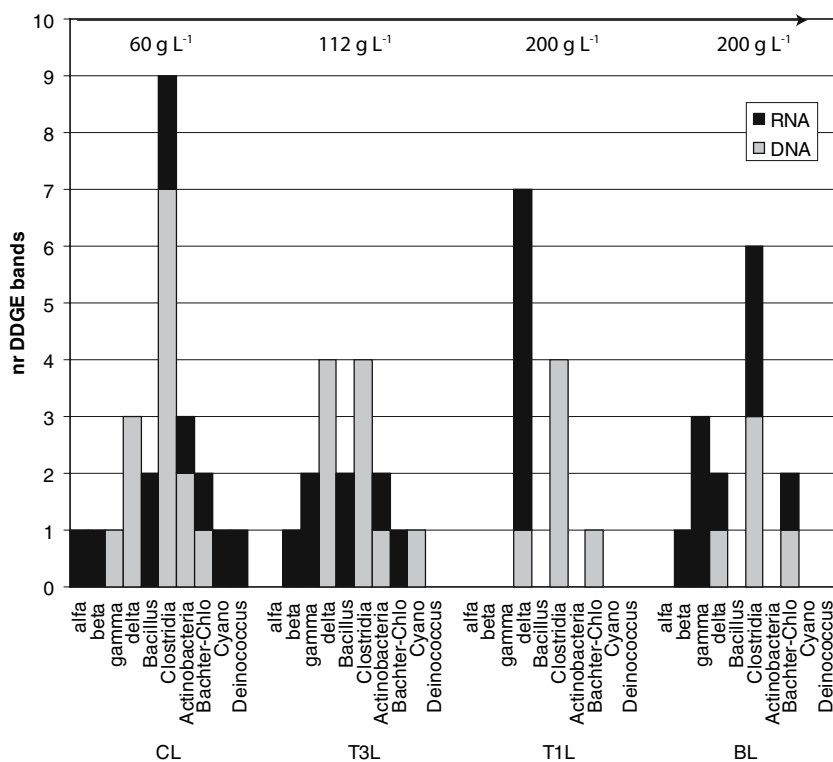
Brevundimonas and to uncultured green sulfur bacteria (Fig. 2; lane 6; band a) were observed.

In lake Tanatar-3 (112 g L^{-1}) members of the *Alpha-proteobacteria* and *Deinococcus–Thermus* were detected. Sequences retrieved from DNA-based profiles were affiliated to the *Delta-proteobacteria* (*D. propionicus* and *D. lacustre*), Actinobacteria, Clostridia and Cyanobacteria (*Leptolyngbya* sp., i.e., Fig. 2, lane 7, band a)). The sequences presumed to correspond to the active population were related to the actinobacterium *P. acnes*, the gamma-proteobacterium *M. osloensis* and *Stenotrophomonas* sp., the betaproteobacterium *Massilia* sp. (Fig. 2) and to the *Bacillus* group.

The lakes with highest salinity, i.e. Lake Tanatar-1 and Bitter-1 (200 g L^{-1}), showed the poorest bacterial diversity (Fig. 3). Both were mostly represented by low G + C organisms and Delta-proteobacterial SRB related to *D. propionicus*. The latter were predominant in the RNA-based DGGE's obtained by targeting the SRB groups specific 16S rRNA gene (data not shown). The top 4 cm sediment of Lake Bitter-1 was found to be particularly rich in sequences belonging to the *Proteobacteria* (see Fig. 2, lane 17 and Table 2) and all of them were retrieved from the RNA-derived profiles.

Remarkably, almost all identified sequences were related to organisms isolated from halo (alkaliphilic) environments (Fig. 4).

Sulfate-reducing activity

High sulfate reducing-activity was observed in the near bottom water and in the sediment layers of all four investigated soda lakes (Table 1). The rates were comparable with previous measurements (Foti et al. 2007) and, in general, corresponded to characteristic values of eutrophic marine sediments (Lein et al. 2002). Salinity seems to have no effect on the sulfate reduction process in the studied soda lakes. In fact, the highest SRR (1.426 10^3 μmol dm^{-3} day^{-1}) were observed in Bitter Lake, at highest salinity conditions (200 g L^{-1}).

Results of the sediment slurry experiments to investigate the influence of pH and salt concentration suggested that the SRB populations in low-salt Cock Lake were limited by electron donors, and not by inorganic nutrients, i.e. nitrogen and phosphor, while in high-salt Tanatar-1 lake both inorganic nutrients, and electron donors were moderately stimulating (Fig. 5a). Furthermore, both populations were

Fig. 4 Phylogenetic trees based on the 16S rRNA gene. The *open squares* and the *closed circles* represent sequences retrieved from DNA- and RNA-derived DGGE profiles, respectively. The *stars* indicate sequences or organisms originated from (hyper) saline/alkaline habitats. The initial of the lake is followed by the sediment layer, and eventually by the SRB-specific group set of primers used (see Table 2). **a** *Proteobacteria*, **b** Clostridia, **c** Actinobacteria, **d** Bacilli, **e** Bacteroidetes–Chlorobi

A

C-2_DCC (EF622450) □
GamPro30 HTC042 (AB010848) *
Acinetobacter junii (AB101444)
Acinetobacter venetianus (AJ295007)
B-1_Bac_e (EF622444) ●
T3-1_Bac_b (EF622431) ●
Moraxella osloensis (AJ505859)
UniBac25 (X92135) *
unidentified Hailaer soda lake bacterium (AF275709) *
E_C_H2 (EF622485) □
Halomonas sp. EF43 (AF280796) *
Thioalkalimicrobium sibiricum (AF126549)
Ectothiorhodospira sp. (AM180482)
Thioalkalivibrio jannaschii (AF329083)
T3-2_Bac_c (EF622436) ●
B-1_Bac_g (EF622446) ●
B-1_Bac_f (EF622445) ●
Br_Z21 (AF506995) *
Br_Z23 (AF506993) *
Stenotrophomonas sp. (AJ002814)
UniBac64 (X92132)
T3-2_Bac_b (EF622435) ●
Massilia sp. Tibet–IIU22 (DQ177478)
B-1_Bac_c (EF622442) ●
uncultured bacterium (AY627604)
C-2_bac_d (EF622427) ●
Tepidimonas aquatica (AY324139)
C-3_DVV_b (EF622475) ●
uncultured bacterium (AY293404)
Brevundimonas sp. AP–5 (AY145543)
Br_38 (AF507006) *
Br_z43 (AF507008) *
15 — uncultured bacteria (EF622448-51-52-54-55-56-58) □ ●
Desulfosalina propionicus (DQ067422) *
Desulfosarcina variabilis (M26632)
Desulfobulbus propionicus (AY548789)
T3-1_DBB (EF622457) □
Desulfonatronum thiodismutans (AF373920)
C-1_DVV (EF622472) □
Desulfonatronum lacustre (AF418171) *
T3-1_DVV (EF622477) □
Desulfonatronum cooperativum (AF418171) *
Desulfonatronovibrio hydrogenovorans (X99234) *

0.10

B

6 — Tindallia and relatives *
E_T3_H2 (EF622486) □
E_T3_Ac (EF622488) □
uncultured bacterium IRB 1 (DQ631799) *
T1-1_DVV_a (EF622480) □
T1-1_DVV_b (EF622481) □
T3-2_DFM_a (EF622467) □
Halanaerobium sp. KT–8–13 (AJ309520)
Red Sea bacterium KT–2K23 (AJ309524)
uncultured bacterium (DQ432226) *
E_B_Ac (EF622487) □
T1-1_Bac_a (EF622437) □
B-1_DFM (EF622471) □
C-3_DFM_e (EF622464) □
C-3_DFM_c (EF622462) □
C-3_DFM_a (EF622460) □
uncultured bacterium (AF507890) *
C-3_DFM_b (EF622461) □
ML21_DFM1 (AY245477) *
ML21_DSV11 (AY245475) *
C-3_DFM_d (EF622643) □
T1-1_DFM (EF622470) □
T3-2_DVV_b (EF622479) □
uncultured bacterium KF–Gitt2–16 (AJ295640)
Anaerobranca californiensis (AY064217) *
uncultured bacterium (AJ431343)
Selenomonas sputigena (AF287793)
C-3_DVV_c (EF622476) ●
T3-2_Bac_a (EF622434) □
uncultured bacterium (DQ206412) *
uncultured bacterium (AJ431344) *
uncultured bacterium (AF507879) *
B-1_Bac_a (EF622440) □
uncultured bacterium (DQ432330) *
uncultured bacterium (AF507883) *
C-3_Bac_b (EF622429) ●
C-2_DVV (EF622473) □
T3-2_DVV_a (EF622478) □
C-3_DVV_a (EF622474) □
B-1_DVV_a (EF622482) □
uncultured bacterium (AF507888) *
uncultured bacterium (DQ432150) *
Natronoanaerobium aggerbacterium (AJ271452) *
Natronoanaerobium halophilum (AJ271451) *
B-1_DVV_c (EF622484) ●
B-1_Bac_d (EF622443) ●
Eubacterium cf. saburreum C27KA (AF287777)
B-1_DVV_b (EF622483) ●
Johnsonella ignava (X87152)

0.10

C

T3-1_Bac_d (EF622433) ●
Red Sea bacterium KT–2K12 (AJ309523) *
Red Sea bacterium KT–2K38 (AJ309527) *
C-2_Bac_b (EF622425) □
Propionibacterium acnes (AB108481)
Aeromicrobium alkaliterrae (AY822044) *
Rhodococcus sp. (X80616)
Dietzia natronolimnaea (X92157) *
C-2_Bac_c (EF622426) ●
uncultured Microbacterium sp. (AY569297) *
Microbacterium sp. I33 (AF306542)
Cellulomonas sp. (Y09656)
Streptomyces sodiiphilus (AY236339)
Br–15 (AF507010) *
uncultured bacterium (AF507852) *
T3-1_DFM (EF622466) □
uncultured bacterium (DQ432396)
ANLIso2 *
C-1_DFM (EF622459) □
bacterium Chibacore 1500 (AB038407)

0.10

D

Halobacillus salinus (AY505517) *
Bacillus halodurans (AB031210) *
Bacillus alcalophilus (AF078812) *
Bacillus clausii (AY152839) *
Bacillus thuringiensis (DQ078742)
T3-2_DFM_b (EF622468) ●
Bacillus cereus (AE017264)
low G+C Gram–positive bacterium HTE856 (AB010843) *
C-3_DFM_f (EF622465) ●
Lactobacillus curvatus (AY281292)
T3-2_DFM_c (EF622469) ●
Lactobacillus sakei (AB183697)
uncultured bacterium (AY862794)
uncultured low G+C Gram–positive bacterium (AF507895) *
C-1_DCC_b (EF622449) ●

0.10

E

Alkaliflexus imshenetskii (AJ784993)
Hymenobacter aerophilus (AJ276901)
Hymenobacter sp. Tibet–IIU11 (DQ177475) *
T3-1_Bac_c (EF622432) ●
uncultured bacterium (DQ351728) *
uncultured bacterium (AY922213) *
B-2_Bac (EF622447) ●
uncultured bacterium (DQ015831)
uncultured bacterium (AY553111) *
T1-1_Bac_b (EF622438) □
uncultured bacterium (AF449772) *
C-2_Bac_a (EF622424) □
Salinibacter ruber (AY531230) *
Rhodothermus marinus (AF217499)
Chlorobium chlorochromatii (AJ578461)
Chlorobium phaeobacteroides (AJ290832)
uncultured bacterium (DQ463733)
C-3_Bac_a (EF622428) ●
B-1_Bac_b (EF622441) □
uncultured bacterium (AF445702) *

0.10

active between pH 8 and 10 (Fig. 5b), but differed in salt optimum-tolerance (Fig. 5c), although not as much as might be expected from the actual difference in salt content in the two lakes.

The presence of active SRB populations in sediments of the investigated lakes was confirmed by relatively high viable counts obtained both at high and low-salinities (Table 1). However, the heterotrophic cultures with acetate and yeast extract proved to be unstable during further transfers. In contrast, autotrophic enrichments with H_2 were active and transferable even at 4 M of total Na^+ with prominent development of relatively large vibrio's resembling the extremely natronophilic sulfite-disproportionating isolate ASO3-1, that previously was isolated from Kulunda soda lakes (Foti et al. 2007).

Discussions

Microbial diversity along a salinity gradient

Here we describe, for the first time, the diversity and activity of microbial communities in general and SRB communities in particular along a salinity gradient in different soda lakes. For this purpose we used DGGE analysis of 16S rRNA gene fragments obtained from genomic DNA (as a measure for diversity) and from rRNA (as a measure for activity). In addition, sulfate reduction rates were measured. Salinity did affect the general microbial composition, but not the sulfate reducing activity. As reported in other studies (Wu et al. 2006; Bennloch et al. 2002), the number of DGGE bands did not show a negative correlation with increasing salinity, rather a decrease in the richness of bacterial genera was observed. This suggests an increase of the microdiversity, within the remaining phylogenetic group, at higher salinities.

At the highest salinities, the dominant actively growing populations were belonging to the *Delta* and *Gammaproteobacteria*. The latter were also detected previously in hypersaline lakes (Wu et al. 2006; Bennloch et al. 2002), together with organisms belonging to the *Alphaproteobacteria*. In the literature, little information can be found about the microbial diversity along salt gradients in athalassophilic habitats; most of the studies have been conducted either on estuaries, with overall salinities 10–100 times lower than in hypersaline lakes, or on sea solar salterns. Furthermore, in most of these studies water and brines were the target and not the sediments, making comparison difficult or impossible.

The highest bacterial diversity was observed in Cock Lake (60 g L^{-1}) and Tanatar-3 (120 g L^{-1}) with actively growing populations affiliated to members of the *Proteobacteria* and bacilli. At the lowest salinity, a sequence
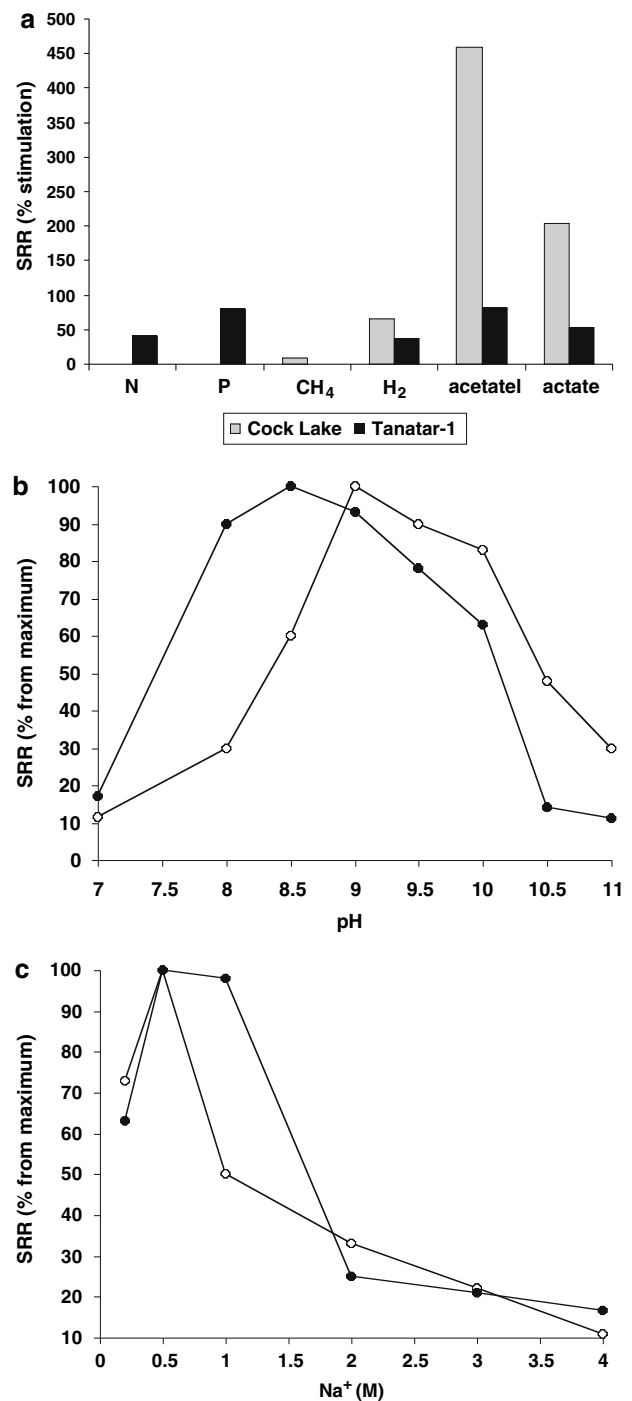

Fig. 5 Influence of nutrients (**a**), pH (**b**), and salt (**c**) on SRR in the top sediment layer of Cock Lake (*gray columns* or *open circles*) and lake Tanatar-1 (*black columns* or *closed circles*), incubated for 48 h at 30°C. pH profiles were measured at 0.6 M total Na^+ for the Cock Lake sediments, and at 2 M Na^+ for the lake Tanatar-1 sediments. Salt profiles were measured at pH 10 with sodium carbonate/bicarbonate

corresponding to the cyanobacterium *Oscillatoria neglecta* was detected, which is probably responsible of the bulk of primary production. Interesting was the detection of one

sequence closely related the N_2-fixing cyanobacterium *Leptolyngbia* sp. PCC7104. So far, no cyanobacteria capable in N_2-fixation have been detected or isolated from soda lakes, and so it is still not clear which organisms are responsible for this process. Hydrolytic (bacilli, *Bacteroidetes*), lipolytic (*Stenotrophomons* sp.), and fermentative (Propionibacteria, Clostridia, lactobacilli) bacteria were also detected as actively growing. Interesting was the identification in Cock Lake of two sequences related to the *Beta-proteobacteria* (*Tepidimonas aquatica*) and to an uncultured *Chlorobium*-like organism, both of which might be involved in the sulfur and carbon cycle. *Tepidimonas aquatica* is a slightly thermophilic, facultatively chemo-lithoheterotrophic bacterium able to oxidize thiosulfate and tetrathionate, and able to grow up to pH 8.5 (Freitas et al. 2003). Since the phylum green sulfur bacteria (GSB) are known to be a homogeneous physiological group, it can be assumed that members of this group inhabiting soda lakes might be involved in the sulfur cycle. This shows the necessity of looking more closely at the anaerobic photo-trophic green sulfur bacteria in soda lakes. This *Chlorobium*-like sequence was detected in the deepest sediment layer, in which low light intensity and high sulfide are favorable for GSB. Unexpectedly the chemolithoautrophic sulfur-oxidizing bacteria of the genera *Thioalkalivibrio* and *Thioalkalimicrobium* were not detected in this study despite the fact of high viable numbers (up to 10^6 cell cm^{-3}) and the isolation of 12 extremely natronophilic *Thioalkalivibrio* strains in pure culture (D. Sorokin, unpublished results). This clearly shows the limitation of the DGGE analysis method, which detects major components, but is less suitable than cloning when microbial diversity is analyzed.

At the highest salinities a decrease of the bacterial genera richness was observed, with the actively growing groups in these lakes represented by the *Delta* and *Gammaproteobacteria*, together with a minority of Clostridia (*Natronoanaerobium halophilum*). Saprophytic (*Acinetobacter* sp.), lipolytic (*Stenotrophomonas* sp.) and saccharolytic (*Eubacterium* sp., Bacteroidetes) bacteria were the dominant active groups in the Bitter Lake sediments.

Only few of the identified sequences were related to culturable organisms. Unexpectedly, none of those have been described as tolerant halo-alkaliphilic or halo-alkali-tolerant (e.g. *Stenotrophomonas* sp., *Massilia* sp., *Brevundimonas* sp.). Still, the majority (90%) of the identified sequences were related to representatives, i.e. uncultured organisms, from alkaline and saline environments (Fig. 4), indicating that many haloalkaliphilic phylotypes remain unknown. One such example is a new cluster of Actinobacteria, from which the first culturable representative recently has been described. Strain ANL-

iso2 was enriched and isolated from Kulunda Steppe soda lake sediments using isobutyronitrile as the only substrate. It is specialized in the degradation of C3-C6 aliphatic nitriles (Sorokin et al. 2007b).

Sulfate reduction rates (SRR) and sulfate reducing community

Remarkably high SRR values have been observed in all four investigated soda lakes, especially in the top organic-rich sediment layer, with the exception of the sandy sediments of Tanatar-3, in which we measured the highest SRR in the deepest layer. The highest SRR were found in Bitter Lake-1, an organic-rich hypersaline lake. In contrast to other studies (Sörensen et al. 2004; Brandt et al. 2001), in which a remarkable decrease of the sulfate-reducing activity at salt concentrations greater than 12% was documented, we did not observe inhibition of SRR by increasing salinity. Brandt et al. investigated three different stations located in the moderate and hypersaline arms of the Great Salt Lake, Utah. A difference of factor 10 was observed between the stations at concentrations 120 and 274 g L^{-1} of NaCl. Sörensen (2004) studied the salinity responses of the benthic microbial communities in the Solar salterns (Israel) and they also observed a strong inhibition of the sulfate reduction activity at salinity of 215 g L^{-1}. In case of the soda lakes investigated in this study, the highest SRR were found at the highest salinity. To the best of our knowledge, these high SRR have never been measured before in hypersaline soda lakes. Only Sorokin et al. (2004) reported a higher measurement in a Mongolian soda lake, but at lower salinity (60 g L^{-1}). Life at high salt concentrations is energetically costly and organisms with a dissimilatory metabolism, which yield low energy, e.g. autotrophic nitrification, methanogenesis and complete-oxidizer SRB, may not be able to deal with high salinities for thermodynamic reasons (Oren 1999). However, soda lakes are a specific type of saline lake in which $NaHCO_3/Na_2CO_3$ is the major salt in solution, a two times weaker electrolyte than sodium chloride, demanding approximately two times less energy to adapt to osmotic stress (Sorokin and Kuenen 2005b). Furthermore, at concentrations higher than 2 M total Na^+, sodium carbonates are present only in their undissociated form, causing less stress than sodium chloride, which is fully dissociate up to saturation (5 M Na^+). This might be one of the reasons of the observed unexpectedly high SRR in the investigated hypersaline soda lakes.

In a recent work conducted on two different soda lakes, i.e. Mono Lake and Searles Lake, Kulp et al. (2007) found different results. They observed a reduction of the SRR in Mono Lake with increasing salinity and no SRR at all was

measured in Searles Lake, in which the sulfate reduction activity seemed to be inhibited by the high concentrations of borate. However, they did impose the salinity gradients in the laboratory, without analyzing similar lakes with increasing salinities, as we did.

Even if we measured the highest SRR at the highest salt conditions, SRR incubation experiment conducted at different salt concentration (see Fig. 5c) showed the optimum at 1 M total Na$^+$, which was two times lower than the actual salt concentration. Furthermore, the optimum pH measured in the similar experiment was also lower (pH 8.5–9) than the natural pH, suggesting that the indigenous SRB population is living under stress in these extreme environments. Spiking experiments demonstrated that the SRB populations are limited by availability of electron donors at both salinities, but especially in low-salt lakes, whereas nitrogen and phosphate were required only in high-salt sediments. These results might be explained by a higher microbial activity and nutrient turnover in the low-salt Cock Lake. Most likely acetate is utilized only by a small, but highly active population, which might correspond to the detected *D. propionicus*–like organisms. In fact, at the highest salt concentrations, at which the highest SRR were measured, this group of organisms has been persistently detected by molecular analysis, both by DNA- and RNA-based approaches. *D. propionicus*, a complete oxidizing halophilic SRB (Kjeldsen et al. 2007), can grow at salinity up to 190 g L^{-1}, using propionate as a carbon source and electron donor. Since propionibacteria have also been detected in the investigated lakes, there might be a trophic link between these groups of primary and secondary anaerobes in soda lake sediments. In our previous study (Foti et al. 2007), the predominance of the *Desulfosalina*-like sequences was also observed when targeting the *dsr*B gene, indicating the relevance of these bacteria in soda lakes of the Kulunda Steppe. So far, complete oxidizing SRB have never been isolated from soda lakes with extreme salinity, therefore it is important to identify these organisms and to infer their functional role in saline lakes.

One of the goals of this research was to determine the diversity and activity of SRB communities along a salinity gradient. In general, this question was only partly answered due to non-specific PCR amplification, resulting mainly in the detection of non-SRB low G + C Gram-positive bacteria. This was also observed in another study conducted on Mono Lake (Scholten et al. 2005), suggesting that the specific amplification of the 16S rRNA gene from six SRB-groups is not recommended in environments rich in low-G + C organisms. It is possible that preferential amplification of the DNA template from low G + C content occurred due to the easier dissociation into single DNA strand than higher G + C templates, resulting in a bias in the favor of the former group (Suzuki and Giovannoni

1996). On the other hand, it could also be that low G + C bacteria, like acetogenic clostridia, out-number SRB at hypersaline alkaline conditions, since it is known that the acetogenic haloalkaliphiles benefit from sodium-based energetics (Häse et al. 2001). This was also observed by DGGE analysis and microscopic analysis of the highest positive tubes of the serial dilutions in SRB media, which revealed the predominance of Clostridia even at the highest dilutions. The discrepancy between observed high SRR rates/SRB viable count and the detection of SRB by molecular techniques might in fact imply that sulfate-reducers are represented by very active, but numerically minor populations in soda lake sediments.

Concluding, in this work we investigated the bacterial diversity and activity in sediments along a salinity gradient in different soda lakes of the Kulunda Steppe in south-eastern Siberia. For the first time the actively growing bacterial groups, based on ribosomal RNA determination, have been detected by culture-independent techniques with special attention to SRB representing one of the most active bacterial functional groups in this extreme habitat. A new cluster of complete oxidizing SRB seems to be active in hypersaline soda lakes. In agreement with previous studies a decrease of the general bacterial diversity has been observed with increasing salinity, in contrast to the sulfate reduction activity, which seems to be well adapted even to saturating concentrations of sodium carbonates.

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