Dissimilatory Sulphate Reduction in Hypersaline Coastal Pans: An integrated microbiological and geochemical study

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Abstract

Here, we report on the spatial and temporal variation in sulphate-reducing bacterial community structure and activity in three hypersaline coastal pans. Community structure was determined using denaturing gradient gel electrophoresis (DGGE). Cluster analysis of DGGE patterns indicated that similar microbial populations were generally found in individual pans but varied from one pan to the other. Sulphate reducing bacteria (SRB) were quantified by competitive polymerase chain reaction based on the amplification of the dsrAB genes. Cell numbers and in situ sulphate reduction activities varied between seasons and pans but in general showed low variation in depth. Sulphate reduction activity was not correlated with microbial population size indicating that community composition is relevant for specific microbial processes. Principal component analysis coupled with correlation analyses suggested that salinity, sulphate concentration, C/N ratio and pH were the most important factors in explaining variations in SRB community composition. Most sequences derived from DGGE amplicons belonged to members of the Desulfobacteraceae and Desulfohalobiaceae families.

Introduction

Sulphate reducers use sulphate as an electron acceptor in anaerobic environments over the entire range of salt concentrations, from freshwater to hypersaline (Rabus *et al.,* 2000). Although sulphate reduction has been extensively studied, there are; however, only a handful of studies where sulphate reduction in extreme environments, including hypersaline systems, has been investigated (Brandt *et al*.; 2001; Foti *et al.,* 2007; Kjeldsen *et al.,* 2007). Moreover, the studies focus either on the microbial community structure or microbial activity. It is seldom where data are integrated to investigate if there is any correlation that exists between microbial community and

their activity. From diversity studies we know that sulphate reducing bacteria occur in five distinct lineages: the archaeal genus *Archaeoglobus* and the bacterial divisions *Firmicutes*, *Nitrospira*, *Proteobacteria* and *Thermodesulfobacteria* (Castro *et al.,* 2000). Furthermore, the majority of SRB described are members of the δ-*Proteobacteria* and it is also in this class that most halophilic SRB are found. How the SRB are distributed or behave *in situ* in hypersaline conditions; however, is little known. Halophilic SRB isolates are known from the families *Desulfohalobiaceae*, *Desulfonatronumaceae*, *Desulfobacteraceae* and *Desulfovibrionaceae*. In addition, a halophilic species belonging to the *Firmicutes* has also been described (Tardy-Jacquenod *et al.*, 1998). This distribution is reflected in diversity studies such as that carried out at Great Salt Lake (Kjeldsen *et al.*, 2007), where members of the *Desulfohalobiaceae* were dominant, but members of the *Desulfobacteraceae* were also detected in large numbers. In contrast, studies of deep hypersaline anoxic basins have shown that members of the *Desulfobulbaceae* (Daffonchio *et al.*, 2006) or the *Firmicute*s family *Peptococcaceae* (Van der Wielen *et al.*, 2007) may be dominant under certain conditions.

Although sulphate reduction is energetically less favourable than nitrate, manganese or iron reduction, the relative abundance of sulphate, particularly in seawater, makes sulphate reduction one of the dominant anaerobic respiratory processes [Froelich *et al.*, 1979; Goldhaber, 2003]. At hypersaline sites, evaporation of water creates favourable conditions for sulphate reducing bacteria, such as a concentrated supply of sulphate and decreased oxygen solubility. High rates of sulphate reduction (>6 $\mu mol.cm^{-3}.day^{-1}$) have been measured in hypersaline sediment (Brandt *et al.*, 2001) while rates of over 10 $\mu mol.cm^{-3}.day^{-1}$ have been measured in hypersaline microbial mats (Canfield and Marais, 1993; Caumette *et al.*, 1991; Jørgensen 1982). As such, their activity has a profound impact on the geochemical evolution of specific environments. Geochemical signatures from such environment are often used for the interpretation of paleo-environmental conditions such as evolution of oxygen on Earth (Canfield *et al.*, 2000) or for the understanding of global biogeochemical processes (Jørgensen 1982). Given the global significance, a clear understanding of geo-microbe interaction is necessary and is best provided by integrated studies in modern systems.

Here we present results from an integrated microbiological and geochemical investigation of sulphate reduction under hypersaline conditions. The study was conducted at Darling salt pans (Western Cape, South Africa) building upon previous studies on geochemical evolution of the saltpans and the nature of *in situ* sulphate reduction (Porter *et al.*, 2007; Sørensen *et al.*, 2004). The size and composition of the SRB community at the Darling pans were ascertained in order to gain a holistic view of sulphate reduction at these pans and to investigate any correlation between microbial community structure and their spatial geochemical signature.

Site Description

The Darling hypersaline pans are located 80km north of Cape Town, South Africa, 5-10km north to north-west of the town of Darling (Figure 1). This area lies on the semi-arid coastal lowlands of the Western Cape province. Average rainfall is 400-500mm per annum, occurring primarily during austral winter in the form of frontal rain. Summers are hot, dry and windy, resulting in arid conditions. The pans were estimated to have formed in the early Holocene, a time of climate change and lunette dune formation (Meadows and Baxter, 1999; 2001). Salt accumulation is preceded by the evaporation and transpiration of coastal rainwater, which precipitates and concentrates salts in catchment soils. Heavy rainfall results in these salts being flushed from the soil and carried to the pan. In addition, some pans are fed by older groundwater (Smith and Compton, 2004).

Rooipan (S 33.25740° E 18.32541°) and Zwartwater B (S 33.28670° E 18.27613°) were classified as hypersaline [43], while Slangkop (S 33.32020° E 18.28596°) was classified as highly saline. The hypersaline pans are each covered by a thick salt crust during the dry season (late spring to early autumn). The highly saline pans lack salt crusts, although thin and patchy regions of salt precipitation may occur on the surface at Slangkop.

Slangkop is 4ha in size and at 105-110 metres above mean sea level (mamsl), has the highest elevation of the pans studied. The pan sits in a dip at the foot of the Darling Hills, which is a source of water seepage into the pan. Sulphidic muds are present to depths of 6-8cm, mottled in places with brown mud, which becomes more dominant

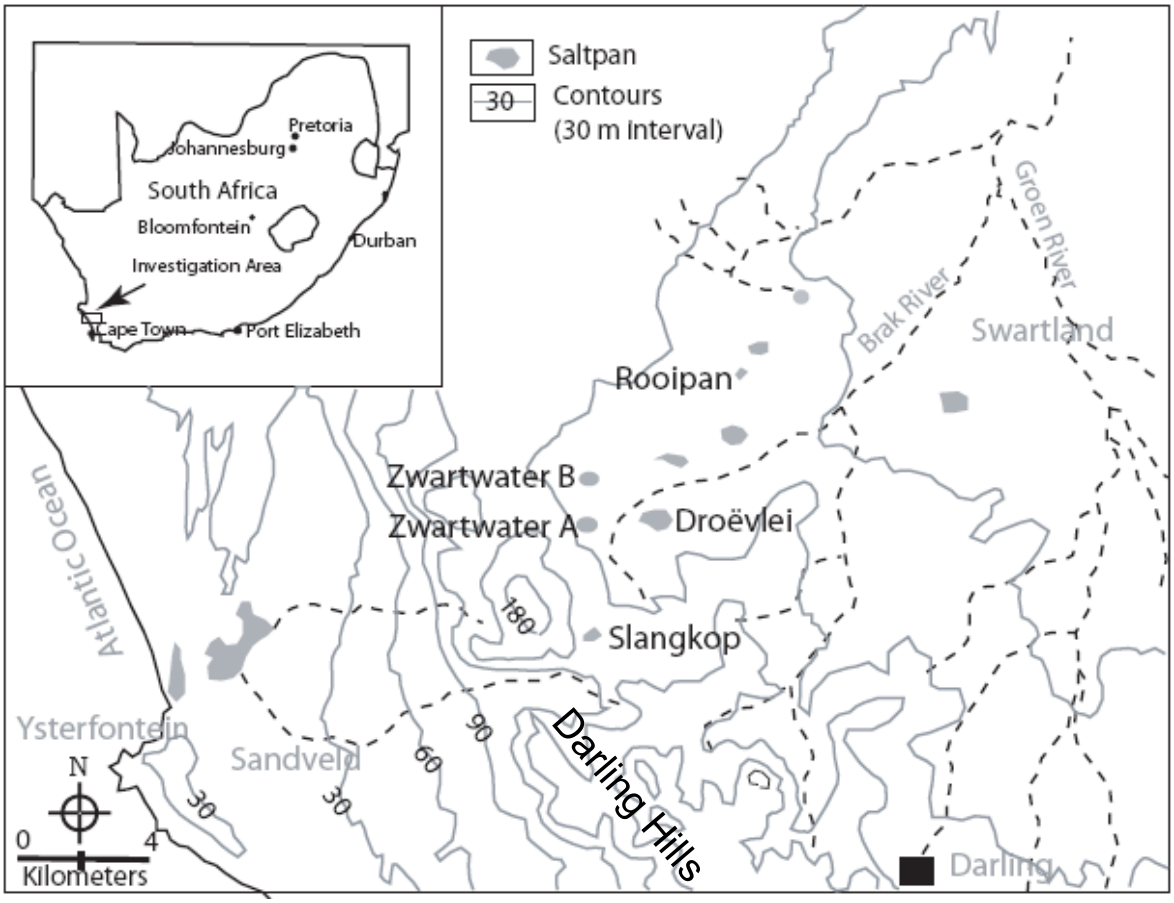

Figure 1

Figure 1. Location map of the Darling Pans, showing regional drainage pattern and topography.

at greater depths. Black sulphidic muds at the hypersaline pans extend to depths of 8-10cm, grading to green muddy sand below this. Thick salt crusts are present in the dry season, while pan surface waters often have a pink tinge. Rooipan and Zwartwater B are 3 and 2ha in size, respectively. Rooipan is at 60-65 mamsl, while the elevation of Zwartwater B is 70-75 mamsl. A spring to the north of Zwartwater B also provides an inflow of relatively fresh water at the surface.

Methods

Sampling and Storage

Seasonal sampling took place between over two years. A piston corer was used to obtain 12cm sediment cores from the Zwartwater B, Rooipan and Slangkop pans. Cores were sectioned at 1cm intervals and frozen at -80°C. For the purposes of DGGE analysis, three sections were selected from each core on the basis of peaks and troughs in the sulphate reduction rate profile determined *a priori* in separate cores collected at the same time. *In situ* sulphate reduction rates (SRR) were determined in whole core incubations using a radiolabel technique as described in Porter *et al.,* (2007).

DNA Extraction

DNA was extracted from 0.1g wet weight of sediment using the bead-beating method of Miller *et al.,* (1999). Bead-beating was carried out using the FastPrep FP120 bead beating system (Bio-101 Inc., La Jolla CA, USA). DNA concentrations were determined on a Nanodrop ND-1000 spectrophotometer. DNA aliquots were stored at -20°C.

Competitive Polymerase Chain Reaction

SRB were quantified by competitive polymerase chain reaction (cPCR) using a method based on that of Leloup *et al.,* (2004). In order to construct the competitor plasmid, DNA extracted from sample ZB1 (summer) was used as the template in the PCR-amplification of *dsr*AB genes with the dsr-1F/4R primerset (dsr-1F: ACS CAC TGG AAG CAC G; dsr-4R: GTG TAG CAG TTA CCG CA; (Wagner *et al.*, 1998)). The amplification was performed on a DNA thermal cycler (Techne TC-312). An initial denaturation step of 3 min at 94°C was followed by 30 cycles of denaturation at 94°C (30s), annealing at 54°C (30s) and extension at 72° (2 min). The amplification was

completed by a final extension step (72°C) of 3 min. The same amplification cycle was used to perform cPCR reactions.

PCR products were run on 2% agarose gels and the required band (~1900bp) cut out of the gel under UV light (365nm). DNA was extracted from the band using the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). Purified PCR products were cloned using the InsT/Aclone™ PCR Product Cloning Kit (Fermentas, Ontario, Canada). Recombinant plasmids showing inserts of the correct size were digested with *Bgl*II and *Bst*EII to confirm the presence of the restriction sites used by Leloup *et al.,* (2004). One such plasmid was named pSG1 and used to construct the competitor plasmid pSGA as described by Leloup *et al.,* (2004).

Aliquots of each DNA sample were diluted to 4ng/µl. Amplification, calibration and determination of band intensity were carried out as per Leloup *et al.,* (2004), taking into account the masses of the respective plasmids (pSG1: 5.31×10^{-18}g; pSGA: 4.75×10^{-18}g). Determination of cell counts assume a *dsrAB* copy number of one per organism (Leloup *et al.*, 2004). Specific SRR were calculated by dividing SRR (Porter *et al.*, 2007) by SRB community size.

Polymerase Chain Reaction for Denaturing Gradient Gel Electrophoresis

PCR of 16S rDNA for DGGE was carried out in duplicate using the δ-Proteobacteria-specific primer 385F (CCT GAC GCA GCG ACG CCG with 5'GC clamp: CGC CCG CCG CGC GGC GGG CGG GGC GGG GGC ACG GGC; (Amann *et al.*, 1995)) and the universal bacterial primer 907R (CCG TCA ATT CCT TTR AGT TT; (Lane, 1991)) on a DNA thermal cycler (Techne TC-312). A 1min initial denaturing period at 93°C was followed by a 15 cycle loop wherein the annealing temperature was decreased from 65°C to 58°C. One cycle consisted of a 30s denaturing step at 94°C, a 1min annealing step and a 45s extension step at 72°C. A second loop consisted of 20 cycles of a 30s denaturing step at 92°C, a 1min annealing step at 58°C and a 45s extension step at 72°C. The reaction was completed by a 5min final extension step at 72°C. Oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc.

Denaturing Gradient Gel Electrophoresis (DGGE) and Cloning

PCR products were analysed by DGGE on a 40 to 70% denaturing gradient. Prominent unique bands were excised from the gel and washed twice in DNase-free water before being placed in TE buffer for 48h in order to elute the DNA. Purity of the bands was confirmed by re-amplification of the eluted DNA and re-analysis by DGGE. Eluted DNA was re-amplified, with PCR products being extracted, purified and cloned as described above. Clones containing the required insert (as determined by amplification with the 385F/907R primer set) were sequenced by Inqaba Biotec (Cape Town, South Africa). Alternatively, recombinant plasmids were purified with Plasmix Minipreps (Talent, Trieste) and sequenced at the DNA Sequencing Unit of the Department of Molecular and Cell Biology, University of Cape Town. The nucleotide sequences were deposited in GenBank (accession numbers EU394681 – EU394700).

Phylogenetic Analysis

Phylogenetic affiliations of the 16S rDNA sequences were determined using the BLAST program of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/blast; (Altschul *et al.*, 1997)). Sequences affiliated with taxa containing SRB were aligned with reference sequences using ClustalX v1.83 (Thompson *et al.*, 1997). Phylogenetic trees were constructed by the neighbour-joining method with Jukes-Cantor correction in the Phylowin program (Galtier *et al.*, 1996) and visualised with TreeView v1.6.6. Bootstrap resampling was carried out with 500 replicates.

Statistical Analysis of DGGE patterns

DGGE bands were identified visually in Microsoft Windows Picture and Fax Viewer. Brightness and contrast were adjusted as needed in order to clearly identify bands. Bands were scored for relative density (0= absent; 5= most dense) and the scores collated into a data matrix. Statistical analysis of the data matrix was performed in Statistica v7.0 (Statsoft Inc.). Cluster analysis was performed by UPGMA and Ward's method, both with squared Euclidean distance. The data matrix was also used to carry out principal components analysis. Principal components contributing to 10% or more of the total variance were correlated against pH, salinity, sulphate concentration,

alkalinity, H_2S, organic carbon levels (C_{org}), $\delta^{13}C_{org}$, C_{org}:N, sulphate reduction rate (SRR), specific SRR and SRB community size.

Results

Competitive PCR yielded gene copy numbers per ng DNA of 1.29-5.02, 1.42-5.26 and 1.16-4.38 $x10^3$ at Zwartwater B, Rooipan and Slangkop respectively. This translates to SRB community sizes of 25-87, 27-97 and 20-73 $x10^6$ cells.cm^{-3} for the three respective pans (Figure 2). Changes in community size approximately paralleled changes in the measured SRR (Figure 3), albeit with lower variation in measurements with depth. Specific SRR were calculated from SRR and community size, the ranges being 5.6-57.6, 9.5-53.6 and 5.4-39.1 fmol.$cell^{-1}$.day^{-1} at Zwartwater B, Rooipan and Slangkop, respectively (Figure 3). Due to the larger variation in SRR than in cell numbers, depth profiles of specific SRR tended to reflect the SRR depth profiles from which they were determined. The highest depth integrated specific SRR for each pan were found in winter and spring, while the lowest values were found in summer and autumn.

Results of DGGE analysis are shown in Figure 4. DGGE bands from which δ-*Proteobacteria* sequences were obtained are indicated. Their phylogenetic affiliations are identified in Table 1. A neighbour-joining phylogenetic tree was constructed in which these sequences were compared to δ-proteobacterial reference sequences (Figure 5). Of the 20 δ-proteobacterial sequences, 9 were inferred to derive from members of the *Desulfobacteraceae*, 7 from the *Desulfohalobiaceae*, 3 from the *Desulfobulbaceae* and 1 from the *Geobacteraceae*. Eight sequences clustered with a "*Desulfosalina"* sequence to the exclusion of other *Desulfobacteraceae* sequences, while 6 sequences clustered with "*Desulfovermiculus halophilus"* and *Desulfohalobium utahense* sequences to the exclusion of other *Desulfohalobiaceae* sequences. For most sequences, the closest BLAST matches were from uncultured organisms.

Cluster analysis of DGGE banding patterns (Figure 4) revealed that microbial communities present in samples from a given pan tended to be more similar to each other than to communities in other pans. Also, near-surface communities of the two

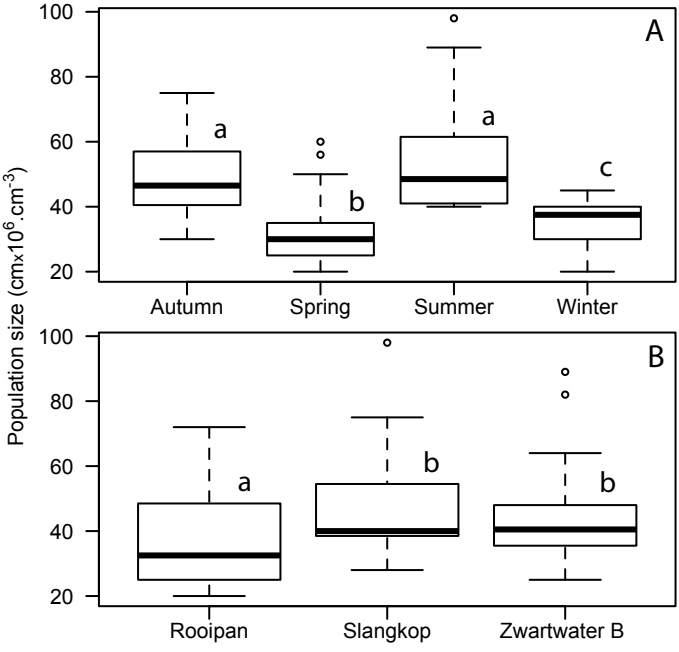

Figure 2. Seasonal abundance and variation of sulphate reducing bacteria with depth.

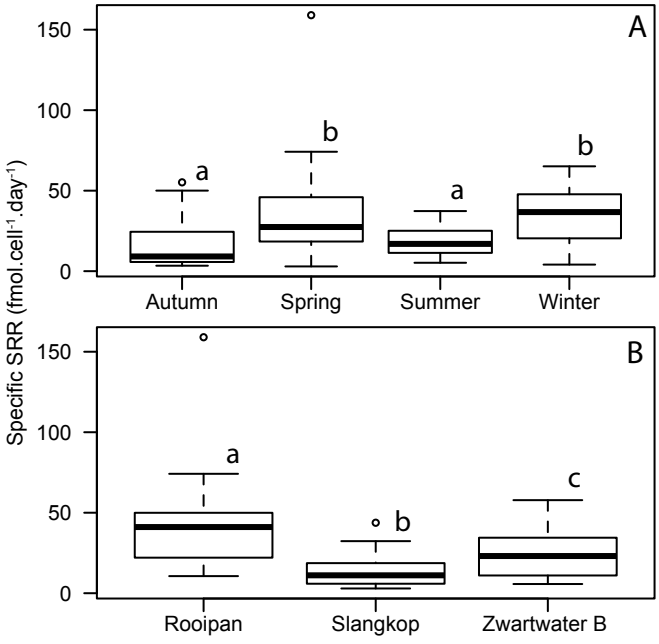

Figure 3. Seasonal depth profiles of measured Sulphate reduction rates (♦) and calculated specific sulphate reduction rates (●)

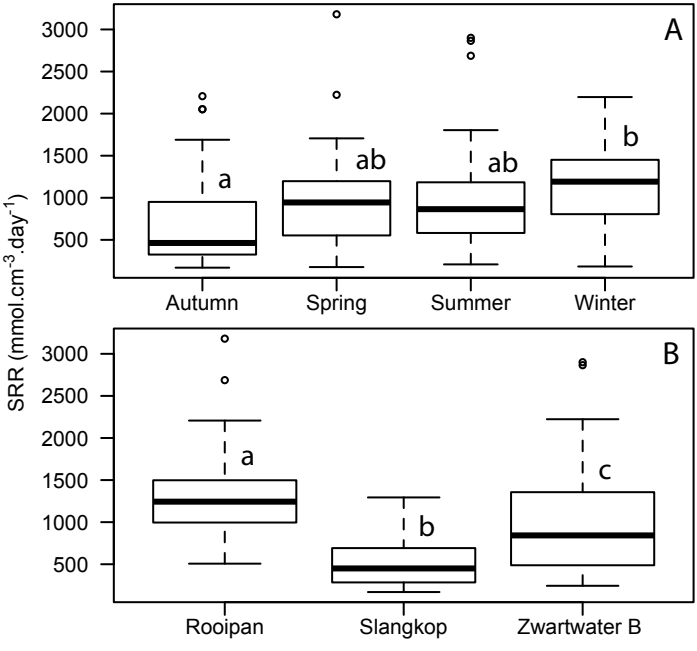

Figure 4. Cluster analysis of DGGE banding profiles by UPGMA with squared Euclidean distance. Scale bar indicate dissimilarities among samples. Bands cut out for cloning purposes are indicated. Lane labels indicate pan and depth of sample, where RA = Rooipan , SK = Slangkop and ZB = Zwartwater B. Seasons are indicated as follows: A = Autumn, B = Winter, C = Spring and D = Summer. Gels are shown in the negative for ease of viewing.

Table 1. Phylogenetic affiliation of sequenced DGGE bands

Closest Cultured Strain[a]	DGGE band sequence	Identity (%)
Desulfovermiculus halophilus (DQ139408)	M12	96
	S4[b]	90
	S2	90
	W11	90
	S5	88
	S9	88
Geobacter sulfurreducens (U13928)	S1[c]	82
Desulfosalina propionicus (DQ067422)	M6	97
	S7	97
	M8	96
	W12	94
	M11	94
	M7	94
	W5	93
	W10	92
Desulfosarcina variabilis (M34407)	M5	95
Desulfurivibrio alkaliphilus (EF422413)	S10[d]	89
	S13	89
Pelobacter acetylenicus (X70955)	W17	96
Desulfobulbus mediterraneus (AF354663)	W2	87

[a] Closest cultured strain pertains to each group of sequences.

[b] S2, S4 and W11 are 98% and S5 97% identical to clone SB-45 (AJ495690)

[c] 91% identical to clone LL68A (EF105906)

[d] S10 and S13 are respectively 97% and 96% identical to clone ST-12K3 (AJ347756)

Table 2 Results of correlation analysis of principal components against major variables (*see Table 3*)

Season/Component [a]	Variables in Best Correlation with Principal Component [b]
Winter (82.2%; 39)	
PC1 (25.3%)	
PC2 (19.0%)	H_2S (0.45)
PC3 (16.4%)	
PC4 (11.0%)	
PC5 (10.5%)	
Spring (74.0%; 27)	
PC1 (28.9%)	
PC2 (24.1%)	Salinity (0.48) SO_4^{2-} (0.93) $\delta^{13}C$ (0.74) C_{org}:N (0.68)
PC3 (21.0%)	
Summer (75.9%; 30)	
PC1 (34.6%)	pH (0.46) SO_4^{2-} (0.49) SRR (0.46)
PC2 (16.8%)	Salinity (0.77) SO_4^{2-} (0.50) C_{org} (0.63) C_{org}:N (0.66)
PC3 (13.9%)	SRR (0.74) Community Size (0.74)
PC4 (10.6%)	
Autumn (70.2%; 28)	
PC1 (42.4%)	pH (0.82) SO_4^{2-} (0.50) H_2S (0.45) C_{org}:N (0.51) Community Size (0.71)
PC2 (15.8%)	pH (0.52)
PC3 (12.0%)	Salinity (0.80)

PC1 to PC5 are principal components (only those contributing to 10% or more of the total variance are shown).

[a] Values in brackets after principal components indicate the variability explained by each component. Values in brackets after seasons indicates the variability explained by all components used and the number of DGGE bands scored)

[b] Variables listed are those deemed significant ($p<0.05$) by correlation analysis. Values in brackets are r^2 values

Table 3. Geochemical data used for correlating with principal components analysis of DGGE banding patterns[a]

Location	Season	Depth (cm)	Salinity (‰)	pH	Alkalinity (mM)	SO_4^{2-} (mM)	$\sum H_2S$ (mM)	C_{org} (%)	$\delta^{13}C$	C_{org}:N
Zwartwater B	Autumn	2	405	7.1	26.9	287.3	3.42	2.4	-22.3	14.1
		6	378	7.1	31.3	271.7	0.13	5.1	-23.4	14.7
		11	342	7.2	18.9	236.3	0.25	2.9	-22.3	23.6
	Winter	1	312	7.5	25.4	187.4	1.40	2.4	-22.5	14.6
		4	332	7.3	24.4	287.3	0.65	1.3	-22.6	23.7
		11	357	7.2	23.0	281.1	0.51	1.5	-22.1	26.5
	Spring	3	392	7.0	24.1	287.3	2.21	7.2	-22.1	12.4
		8	390	7.4	25.4	218.6	0.71	2.2	-21.9	16.9
		12	362	7.4	20.0	231.1	0.52	1.4	-22.2	22.7
	Summer	1	342	6.9	20.8	291.5	2.38	3.1	-21.9	15.2
		5	383	7.2	23.1	299.8	3.29	3.0	-21.9	22.6
		11	183	7.3	21.0	274.8	0.38	1.9	-22.7	24.6
Rooipan	Autumn	1	417	7.0	31.1	637.1	1.88	1.2	-21.4	48.0
		5	380	6.9	25.5	540.3	0.80	3.1	-21.2	49.9
		12	318	7.1	13.8	286.3	2.41	0.4	-22.6	32.0
	Winter	2	276	7.2	18.7	223.8	0.22	0.3	-22.2	26.4
		5	333	7.3	18.5	274.8	0.21	1.4	-22.8	19.6
		12	371	7.1	19.7	356.0	0.02	1.1	-22.8	22.3
	Spring	1	397	6.8	22.6	212.4	0.23	1.1	-20.9	22.3
		9	366	7.3	26.9	374.8	0.26	1.6	-21.1	25.8
		12	396	7.1	27.3	443.5	0.58	1.1	-20.7	45.0
	Summer	1	343	7.0	18.4	530.9	0.85	1.0	-21.8	33.3
		6	363	7.4	18.2	408.1	0.52	2.6	-21.0	24.4
		11	358	7.3	16.6	362.3	0.21	0.9	-21.9	40.2
Slangkop	Autumn	3	360	8.3	18.1	207.2	0.11	2.8	-22.4	13.0
		7	321	8.2	11.9	272.8	0.08	2.4	-23.1	12.6
		11	264	8.3	12.8	138.5	0.28	1.4	-23.4	11.8
	Winter	1	104	8.5	17.8	114.5	0.30	2.0	-22.4	10.3
		5	177	7.9	16.1	184.3	0.08	2.6	-22.4	10.2
		12	204	7.4	12.4	115.6	0.25	2.0	-23.1	12.8
	Spring	1	269	8.6	33.3	160.3	2.06	2.8	-22.2	10.5
		5	171	7.8	23.1	152.0	0.33	3.4	-22.5	10.9
		12	192	8.4	24.2	142.6	0.56	1.9	-22.5	10.8
	Summer	1	297	7.9	14.6	166.6	1.73	3.5	-22.7	10.1
		4	246	7.4	21.7	208.2	0.36	4.8	-22.3	10.7
		10	312	8.3	17.3	156.2	1.09	3.4	-22.7	12.5

[a] Data reproduced from [43]

hypersaline pans (Rooipan and Zwartwater B) tended to cluster together. Principal components analysis (Table 2) followed by correlation analysis with geochemical parameters (Table 3) revealed few significant correlations with the first principal component. Sulphate concentration and organic carbon reactivity correlated to the same principal components in spring, summer and autumn, with salinity also correlating to these principal components in spring and summer.

Discussion

The general microbiology of hypersaline environments has been extensively studied. For instance, many studies on the biogeochemistry and community composition of hypersaline microbial mats (Baumgartner *et al.,* 2006; Decker *et al.*, 2005; Fourçans *et al.*, 2004; Sørensen, *et al.*, 2004) and stratified communities within salt crusts (Oren *et al.*, 1995; Sørensen, *et al.*, 2004) have been reported. In addition, a number of novel species of halophilic SRB have been isolated (Caumette *et al.*, 1991; Krekeler *et al.*, 1997; Ollivier *et al.*, 1994). However, only few studies (Foti *et al.*, 1997; Kjeldsen *et al.*, 2007) have investigated the nature and activity of the sulphate-reducing microbial community.

The size of the SRB community at the Darling pans (1.9-9.7 $x10^7$ $cells.cm^{-3}$) is similar to those measured at Great Salt Lake by Brandt *et al.,* (2001) (2.2 $x10^7$-6.7 $x10^8$ $cells.cm^{-3}$) and from soda lakes (up to 10^8 $cells.cm^{-3}$) on the Kulunda Steppe in southeastern Siberia (Foti *et al.,* 2007). Furthermore, the measured community size at the pans is not significantly larger than what has been measured (2 x 10^5 - 2 $x10^6$ $cells.cm^{-3}$) in normal marine environments (Teske, *et al.*, 1996). The largest depth integrated (0-12 cm) community size at each pan was measured in summer and autumn, with a minima occurring in winters at all pans. The increase in community size in summer was mostly confined to increase in population in near surface samples when salt crust is at its thickest. In general the community size decreased slightly down-core with some profiles showing sub-surface maxima and stabilizing narrowly between 30 – 40 x 10^6 cell cm^{-3} at bottom depths. The observed decrease was consistent with decrease in sulphate and labile carbon concentration. Foti *et al.,* (2007) also observed a decrease in community size with depth, although section depths were larger than used here.

Each of the other two studies on hypersaline environments used different methods by which to enumerate SRB. While our study and that of Foti *et al.,* (2007) used molecular PCR-based methods (competitive PCR and real-time PCR, respectively), Brandt *et al.,* (2001) made use of a tracer-most probable number technique. The similarity in observed community size in each case may indicate that the accuracy of quantification using either of these methods may be similar. This is further evident in

the similar range of SRR and specific SRR determined here (169-3180 nmol.cm^{-3}.day^{-1} and 4.5-58.8 fmol.cell^{-1}.day^{-1} respectively) and by Brandt *et al.,* (2001) (363-6131 nmol.cm^{-3}.day^{-1}; 5-70 fmol.cell.day^{-1}).

The specific SRR values are close to what has been measured (3 – 11 fmol.cell^{-1}.day^{-1}) for sulphate reducers from normal marine environments under mesophilic conditions (Knoblauch *et al.*, 1999). For example, Kondo *et al* (2004) and Leloup *et al* (2004) both enumerated SRB in estuarine sediment using competitive PCR of *dsrAB* genes, and obtained specific SRR of 0.02-3.7 and 0.19-21.8 fmol.cell^{-1}.day^{-1} respectively. Calculated specific SRR at the salt pans varied with season with higher rates observed in winter. Interestingly this corresponds to rainy season that results in lower salinity. Furthermore, the highest depth integrated specific rates correspond to lowest measured depth integrated population size, and sulphate in each pan. We conclude that the microbial consortia at the pans are well adapted to high salt and are working under optimal conditions.

Specific rates increase under stress and SRB have been shown to up-regulate components of the sulphate reduction pathway as part of a salt stress response (Mouné *et al.*, 2003). Although it has been shown that sulphate reduction occurs *in situ* at extremely high salinities (Foti *et al.*, 2007; Porter *et al.*, 2007), strains isolated from hypersaline environments invariably show lower salt tolerance in pure culture and optimum growth occurs at salinities much lower than experienced *in situ* (Ollivier *et al.*, 1991). Thus *in situ* communities of SRB in hypersaline environments may be living under constant salt stress (Brandt *et al.*, 2001) resulting in increased specific SRR. However, given the similarity in the community size and specific SRR measured here compared to mesophilic normal marine environments, the salt stress may not be playing any significant role. Although the data available for specific SRR is extensive, different methods of varying accuracy have been used to determine SRR and SRB community size, complicating comparison of specific SRR data. Thus, in order to test this hypothesis, it may be necessary to study a number of hypersaline and normal marine environments using a standardised set of protocols.

The phylogenetic affiliations determined in this study show low sequence identities to cultured strains. For example, S10 and S13 cluster with members of the

Desulfobulbaceae but share <90% identity. Similarly low sequence identities have been reported elsewhere (Mouné *et al.*, 2003; Mussmann *et al.*, 2005; Nakagawa *et al.*, 2004]. If this is a real phenomenon, not a by-product of metagenomic sampling, then a large amount of diversity exists among SRB that has not yet been uncovered through culture methods. Evidently, several sequences obtained here with <90% similarity to cultured strains show 96-98% identity to clone sequences derived from other hypersaline sites (Eder *et al.*, 2002; Minz *et al.*, 1999). These conclusions are based on the use of only 20 partial rRNA sequences and that due caution is necessary. However, a similar phylogenetic distribution of sequences was obtained by Foti *et al.,* (Foti *et al.*, 2007) in a study of Siberian soda lakes. As in the present study, sequences clustered with "*Desulfosalina propionicus*" (*Desulfobacteraceae*) and within the *Desulfohalobiaceae*, while one sequence was closest to *Desulfobulbus*. Although the *Desulfobulbaceae* are not commonly halophilic, Daffonchio *et al.,* (2006) have demonstrated the presence of members of this family in deep hypersaline anoxic basins. The *Desulfobulbaceae* are mostly incomplete oxidisers of organic carbon, as are the *Desulfohalobiaceae* which are prominent in hypersaline environments.

In the present study, 6 sequences clustered with "*Desulfovermiculus halophilus"* (Beliakova *et al.*, 2006) and *Desulfohalobium utahense* (Goldhaber, 2003) to the exclusion of other *Desulfohalobiaceae* sequences. These 6 sequences vary widely in their identity (88-96%) to the reference sequences, although it is also noted that 4 of these sequences share 97-98% identity with clone SB-45, amplified from hypersaline pond sediment (Minz *et al.*, 1999). Interestingly, each of the corresponding DGGE bands (Figure 4) for the members of this cluster are most intense in the lanes associated with surface or near-surface sediment at Rooipan, while M12 is most strongly associated with surface sediment at Zwartwater B, the other hypersaline pan. Likewise, several members of the "*Desulfosalina*" cluster (see below; Figure 5) are most prominent at the middle depths (5-9cm) of the Rooipan and Zwartwater B samples. In terms of the geochemical data available (Table 2), the environmental ranges of *Desulfohalobiaceae* and *Desulfobacteraceae* overlap extensively, with only sulphate concentration serving as a predictor of their distributions. However, these two families would need to be enumerated separately before formulating a hypothesis regarding spatial progression of SRB at the pans.

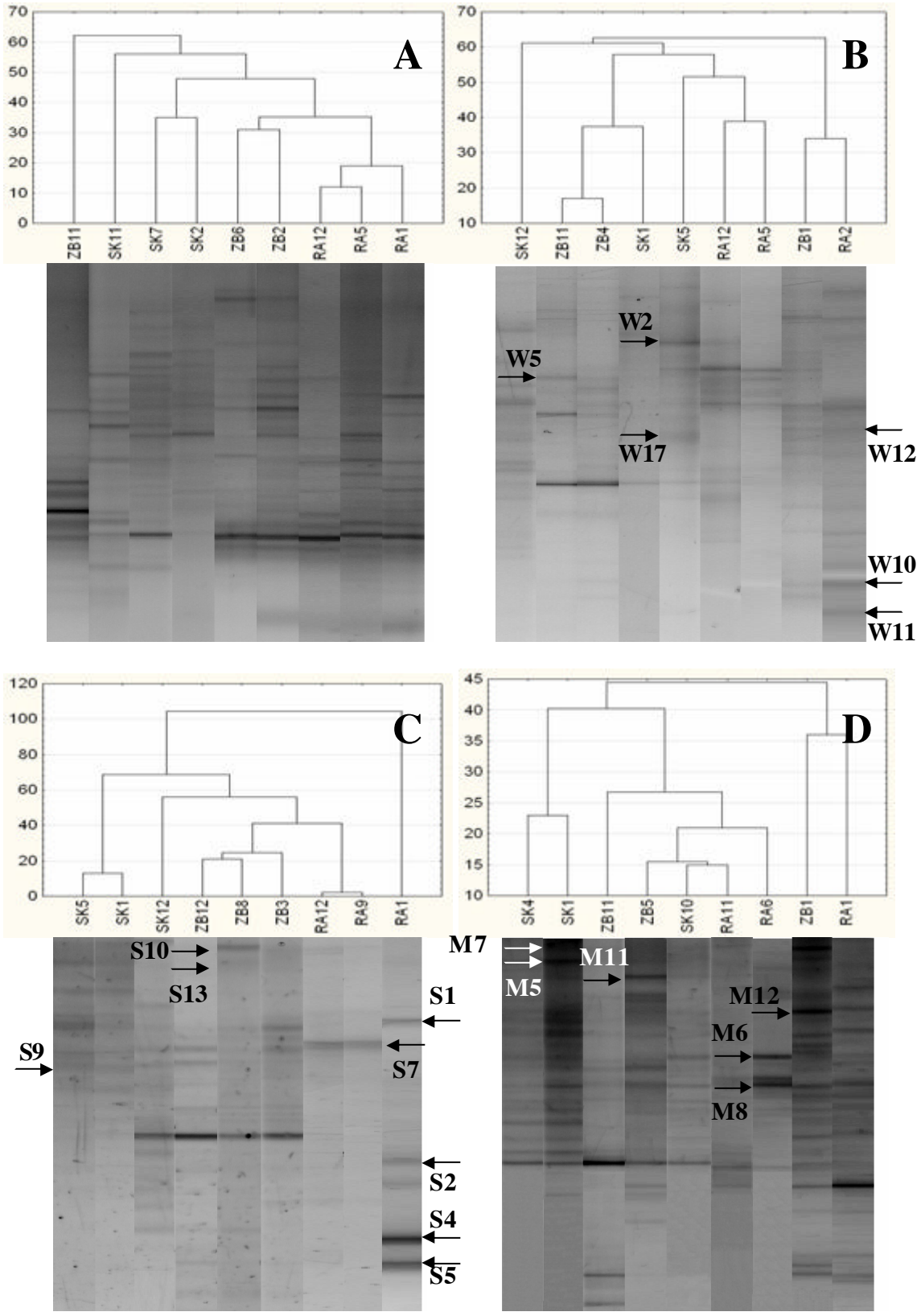

Figure 5. 16S rDNA based neighbour-joining tree of δ-Proteobacteria, with Escherichia coli as an outgroup. Bootstrap support (500 replicates) is shown at tree nodes. Bootstrap values <50% are not shown.

Several of the obtained sequences clustered with "*Desulfosalina propionicus*" strain PropA in the family *Desulfobacteraceae*, isolated from Great Salt Lake (Kjeldsen *et al.*, 2007) at a salinity of 270. Members of the *Desulfobacteraceae* oxidise organic compounds completely to CO_2 (Brenner *et al.*, 2005). It has been suggested (Oren *et al.*, 1995) that bioenergetic considerations would preclude the complete oxidation of organic compounds at high salinity. However, acetate has been shown to be a preferred substrate for sulphate reduction at the Darling pans and to significantly increase SRR at some sites (Porter, 2007). In addition, n-butyrate, which can only be used by complete oxidisers (Rabus *et al.*, 2000) was also a preferred substrate in some cases. Foti *et al.,* (2007) and Kjeldsen *et al.,* (2007) noted the presence of large numbers of *Desulfobacteraceae* in hypersaline sediments. Members of the *Desulfobacteraceae* had been previously identified at hypersaline sites (Minz *et al.*, 1999; Sørensen *et al.*, 2004) but at lower salinities and as components of a microbial mat. At the Darling pans, sulphate is mostly in vast excess and organic carbon is limited and largely unreactive (Porter *et al.*, 2007). Thus, we suggest that it is beneficial to SRB to obtain the maximum energy available from the limited organic matter and that this is achieved by the complete oxidation of organic compounds at the expense of increased sulphate consumption.

With relatively little sequence data available to correlate with the geochemical data (Table 2), the important factors affecting SRB community structure were determined by applying principal components analysis, followed by correlation analysis, to DGGE banding patterns. We note that with the exception of the winter period, sulphate concentration and organic carbon reactivity (C_{org}:N; $\delta^{13}C$) correlate to the same principal components (Table 2). In addition, in spring and summer these correlations also occurred together with a strong correlation of salinity to the particular principal component. A previous study (Porter *et al.*, 2007) has clearly demonstrated that sulphate reduction rates are affected by organic substrate addition and changes in salinity and sulphate concentration. Based on the above correlations, it appears that these parameters are also major factors affecting the SRB community structure. One possible conclusion is that distinct SRB subcommunities which have different ranges with respect to halotolerance, sulphate uptake regulation and organic substrate utilisation are present in the Darling pans. SRB community size also correlates strongly with community structure in summer and autumn. This may be

related to the previous point concerning subcommunities of SRB. Although there are no clear correlations between salinity, sulphate concentration and organic matter reactivity on the one hand and community size on the other, large community sizes may be indicative of sites where the best compromise is reached between the various environmental constraints acting on the SRB community.

Conclusion

The Darling pans are populated by a large well adapted SRB consortia carrying out high rates of sulphate reduction. Despite high stress enironment, members of the family *Desulfobacteraceae*, which carry out complete oxidation of organic carbon were identified. Community size and specific SRR rates measured were comparable to those found in other normal marine enviornments further suggesting that high salt concentrations may not be playing a significant role in distribution or activity of SRBs. In fact we show that the microbial consortia show signs of stress during winter when salinities are lower. Salinity, sulphate concentration and organic carbon reactivity are important parameters at these pans, affecting both the extent of sulphate reduction and the structure of the community carrying out this process.

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References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl Acids Res 25: 3389-3402

Amann RL, Ludwig W, Schleifer KH (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59: 143-169.

Baumgartner LK, Reid RP, Dupraz C, Decho AW, Buckley DH, Spear JR, Przekop KM, Visscher PT (2006) Sulfate reducing bacteria in microbial mats: Changing paradigms, new discoveries. Sediment Geol 185: 131-145

Beliakova E, Rozanova E, Borzenkov I, Tourova T, Pusheva M, Lysenko A, Kolganova T (2006). The new facultatively chemolithoautotrophic, moderately halophilic, sulfate-reducing bacterium *Desulfovermiculus halophilus* gen. nov., sp. nov., isolated from an oil field. Mikrobiologiya 75: 161-171.

Brandt KK, Patel BKC, Ingvorsen K (1999) *Desulfocella halophila* gen nov, sp nov, a halophilic fatty-oxidizing sulfate-reducing bacterium isoloated from sediments of the Great salt Lake. Int J Syst Bacteriol 49:193-200

Brandt K, Vester F, Jensen A, Ingvorsen K. (2001). Sulfate-reduction dynamics and enumeration of sulfate-reducing bacteria in hypersaline sediments of the Great Salt Lake (Utah, USA). Microb Ecol 41: 1-11.

Brenner DJ, Krieg NR, Staley JT (2005) The *Alpha-*, *Beta-*, *Delta-*, and *Epsilonproteobacteria*. In: Garrity GM, Boone DR, Castenholz RW (eds) Bergey's manual of systematic bacteriology, vol 2C, 2nd edn. Springer, Berlin Heidelberg New York

Canfield DE, Des Marais D (1993). Biogeochemical cycles of carbon, sulfur and free oxygen in a microbial mat. Geochim Cosmochim Acta 57: 3971-3984.

Canfield DE, Habicht KS, Thamdrup B (2000) The Archean sulfur cycle and the early history of atmospheric oxygen. Science 288: 658-661

Castro HF, Williams NH, Ogram A (2000). Phylogeny of sulfate-reducing bacteria. FEMS Microbiol Ecol 31: 1-9.

Caumette P, Cohen Y, Matheron R (1991). Isolation and characterization of *Desulfovibrio halophilus* sp. nov., a halophilic sulfate-reducing bacterium isolated from Solar Lake (Sinai). Syst Appl Microbiol 14: 33-38.

Caumette P, Matheron R, Raymond N, Relexans J-C (1994) Microbial mats in the hypersaline ponds of Mediterranean salterns (Salins-de-Giraud, France) FEMS Microbiol Ecol 13: 273-286

Daffonchio D, Borin S, Brusa T, Brusetti L, van der Wielen PWJJ, Bolhus H, Yakimov MM, D'Auria G, Giuliano L, Marty D, Tamburini C, McGenity TJ, Hallsworth JE, Sass AM, Timmis KN, Tselepides A, de Lange GJ, Hübner A, Thomson J, Varnavas SP, Gasparoni F, Gerber HW, Malinverno E, Corselli C, Biodeep Scientific Party (2006) Stratified prokaryote network in the oxic-anoxic transition of a deep-sea halocline. Nature 440: 203-207

Decker KLM, Potter CS, Bebout BM, Des Marais DJ, Carpenter S, Discipulo M, Hoehler TM, Miller SR, Thamdrup B, Turk KA, Visscher PT (2005) Mathematical simulation of the diel O, S and C biogeochemistry of a hypersaline microbial mat. FEMS Microbiol Ecol 52: 377-395

Eder, W., Schmidt, M., Koch, M., Garbe-Schonberg, D. and Huber, R. (2002) Prokaryotic diversity and corresponding geochemical data of the brine-seawater interface of the Shaban Deep, Red Sea. *Environmental Microbiology* **4**: 758-763

Foti M, Sorokin DY, Lomans B, Mussman M, Zacharova EE, Pimenov NV, Kuenen JG, Muyzer G (2007) Diversity, activity and abundance of sulfate-reducing bacteria in saline and hypersaline Soda Lakes. Appl Environ Microbiol 73: 2093-2100

Fourçans A, de Oteyza TG, Wieland A, Solé A, Diestra E, van Bleijswijk J, Grimalt JO, Kühl M, Esteve I, Muyzer G, Caumette P, Duran R (2004). Characterisation of functional bacterial groups in a hypersaline microbial mat

community (Salins-de-Giraud, Camargue, France). FEMS Microbiol Ecol 51: 55-70

Froelich PN, Klinkhammer G, Bender M, Luedtke N, Heath G, Cullen D, Dauphin P, Hammond D, Hartman B, Maynard V (1979). Early oxidation of organic matter in pelagic sediments of the eastern equtorial Atlantic: suboxic diagenesis. Geochim Cosmochim Acta 43: 1075-1090.

Galtier N, Gouy M, Gautier C (1996) SeaView and Phylo_Win, two graphic tools for sequence alignment and molecular phylogeny. Comput Applic Biosci 12: 543-548

Goldhaber, M. B. (2003). Sulfur-rich Sediments. In Sediments, Diagenesis and Sedimentary Rocks (ed. F. T. Mackenzie) Oxford, Elsevier-Pergamon. 7: 257-288.

Jakobsen TF, Kjeldsen KU, Ingvorsen K (2006) *Desulfohalobium utahense* sp. nov., a moderately halophilic, sulfate-reducing bacterium isolated from Great Salt Lake. Int J Syst Evol Microbiol 56: 2063-2069

Jonkers HM, Koh I-O, Behrend P, Muyzer G, de Beer, D (2005) Aerobic organic carbon minerilization by sulfate-reducing bacteria in the oxygen-saturated photic zone of a hypersaline microbial mat. Microb Ecol 49: 231-300

Jørgensen BB (1982). Mineralization of organic matter in the sea bed – the role of sulphate reduction. Nature 296: 643-645

Jørgensen BB (1994) Sulfate reduction and thiosulfate transformations in a cyanobacterial mat during a diel oxygen cycle. FEMS Microbiol Ecol 13: 303-312

Kjeldsen KU, Loy A, Jakobsen TF, Thomsen TR, Wagner M, Ingvorsen K (2007) Diversity of sulfate-reducing bacteria from an extreme hypersaline sediment, Great Salt Lake (Utah). FEMS Microbiol Ecol 60: 287-289

Knoblauch C, Jørgensen BB, Harder J, (1999) community size and metabolic rates of psychrophilic sulfate- reducing bacteria in arctic marine sediments; App Environ Microbiol, 65(9): 4230–4233.

Kondo, R., Nedwell, D., Purdy, K. and De Queiroz Silva, S. (2004). Detection and Enumeration of Sulphate-Reducing Bacteria in Estuarine Sediments by Competitive PCR. *Geomicrobiology Journal* **21**: 145-157.

Krekeler D, Sigalevich P, Teske A, Cypionka H, Cohen Y (1997). A sulfate-reducing bacterium from the oxic layer of a microbial mat from Solar Lake (Sinai), *Desulfovibrio oxyclinae* sp. nov. Arch of Microbiol 167: 369-375.

Lane DJ (1991). 16/23S rRNA sequencing. In Nucleic acid techniques in bacterial systematics. (ed E. Stackebrandt and M. Goodfellow) pp. 113-175. Wiley, Chichester, England

Leloup J, Quillet L, Oger C, Boust D, Petit F (2004). Molecular quantification of sulphate-reducing microorganisms (carrying *dsrAB* genes) by competitive PCR in estuarine sediments. FEMS Microbiol Ecol 47: 207-214.

Meadows M, Baxter A (1999). Late Quaternary Palaeoenvironments of the southwestern Cape, South Africa: a regional synthesis. Quat Int 57/58: 193-206.

Meadows M, Baxter A (2001). Holocene vegetation history and palaeoenvironments at Klaarfontein Spring, Western Cape, South Africa. Holocene 11: 699-706.

Miller D, Bryant J, Madsen E, Ghiorse W (1999). Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. Appl Environ Microbiol 65: 4715-4724.

Minz D, Flax JL, Green S, Muyzer G, Cohen Y, Wagner M, Rittman B, Stahl DA (1999). Diversity of sulfate-reducing bacteria in oxic and anoxic regions of a microbial mat characterised by comparative analysis of dissimilatory sulfite reductase genes. Appl Environ Microbiol 65: 4666-4671.

Mouné S, Caumette P, Matheron R, Willison JC (2003). Molecular sequence analysis of prokaryotic diversity in the anoxic sediments underlying cyanobacterial mats of two hypersaline ponds in Mediterranean salterns. FEMS Microbiol Ecol 44: 117-130.

Mukhopadhyay A, He Z, Alm EJ, Arkin AP, Baidoo EE, Borglin SC, Chen W, Hazen TC, He Q, Holman H-Y, Huang K, Huang R, Joyner DC, Katz N, Keller M, Oeller P, Redding A, Sun J, Wall J, Wei J, Yang Z, Yen H-C, Zhou J, Keasling JD (2006). Salt stress in *Desulfovibrio vulgaris* Hildenborough: an integrated genomics approach. J Bacteriol 188: 4068-4078.

Mussmann M, Ishii K, Rabus R, Amann R (2005). Diversity and vertical distribution of cultured and uncultured Deltaproteobacteria in an intertidal mud flat of the Wadden Sea. Environ Microbiol 7: 405-418.

Nakagawa T, Nakagawa S, Inagaki F, Takai K, Horikoshi K (2004) Phylogenetic diversity of sulfate-reducing prokaryotes in active deep-sea hydrothermal vent chimney structures. FEMS Microbiol Lett 232: 145-152

Nieman W (1981). Climate of the coastal lowlands of the Western Cape. In Proceedings of a Symposium on Coastal Lowlands of the Western Cape. (ed E. Moll). University of the Western Cape, Cape Town

Ollivier B, Hatchikian C, Prensier G, Guezennec J, Garcia J (1991). *Desulfohalobium retbaense* gen. nov., sp. nov., a halophilic sulfate-reducing bacterium from sediments of a hypersaline Lake in Senegal. Int J Syst Evol Microbiol 41: 74-81.

Ollivier B, Caumette P, Garcia J, Mah R (1994) Anaerobic bacteria from hypersaline environments. Microbiol Rev 58: 27-38

Oren A, Kühl M, Karsten U (1995) An endoevaporitic microbial mat within a gypsumcrust: zonation of phototrophs, photopigments and light penetration. Mar Ecol Prog Ser 128: 151-159

Oren A (1999). Bioenergetic aspects of halophilism. Microbiol Mol Biol Rev 63: 334-348.

Porter D, Roychoudhury AN, Cowan D (2007). Dissimilatory sulfate reduction in hypersaline coastal pans: Activity across a salinity gradient. Geochim Cosmochim Acta 71: 5102-5116

Porter, D. (2007) An integrated geochemical and microbiological investigation of sulphate reduction in hypersaline pans. Unpublished PhD Thesis, University of Cape Town, South Africa. pp. 293

Rabus R, Hansen T, Widdel F (2000). The dissimilatory sulfate- and sulfur-reducing prokaryoyes. In The Prokayotes: An evolving electronic resource for the microbiological community. (ed. M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer and E. Stackebrandt) Springer-Verlag, New York

Smith M, Compton JS (2004). Origin and evolution of major salts in the Darling pans, Western Cape, South Africa. Appl Geochem 19: 645-664.

Sørensen KB, Canfield DE, Oren A (2004). Salinity responses of benthic microbial communities in a Solar Saltern (Eilat, Israel). Appl Environ Microbiol 70: 1608-1616.

Sørensen KB, Canfield DE, Teske AP, Oren A (2005). Community composition of a hypersaline endoevaporitic microbial mat. Appl Environ Microbiol 71: 7352-7365.

Tardy-Jacquenod C, Magot M, Patel BKC, Matheron R, Caumette P (1998) Desulfotomaculum halophilum sp. nov., a halophilic sulfate-reducing bacterium isolated from oil production facilities. Int J Syst Bacterial 48: 333-338

Teske AC, Muyzer WG, and Ramsing NB, (1996) Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. Appl. Environ. Microbiol. 62:1405–1415.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl Acids Res 24: 4876-4882

Van der Wielen PWJJ, Heijs SK (2007) Sulfate-reducing prokaryotic communities in two deep hypersaline anoxic basins in the Eastern Mediterranean deep sea. Environ Microbiol 9: 1335-1340

Wagner M, Roger AJ, Flax JL, Brusseau GA, Stahl DA (1998). Phylogeny of dissimilatory sulfite reductases supports an early origin of sulphate respiration. J Bacteriol 180: 2975-2982.