Extremophiles (2008) 12:505–518
DOI 10.1007/s00792-008-0154-x

ORIGINAL PAPER

Prokaryotic diversity of a Tunisian multipond solar saltern

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Received: 6 November 2007 / Accepted: 25 February 2008 / Published online: 29 March 2008

Abstract 16S rRNA gene clone libraries were separately constructed from three ponds with different salt concentrations, M2 (15%), TS38 (25%) and S5 (32%), located within a multipond solar saltern of Sfax. The 16S rRNA genes from 216 bacterial clones and 156 archaeal clones were sequenced and phylogenetically analyzed. 44 operational taxonomic units (OTUs) were generated for *Bacteria* and 67 for *Archaea*. Phylogenetic groups within the bacterial domain were restricted to *Bacteroidetes* and *Proteobacteria*, with the exception that one cyanobacterial OTU was found in the TS38 pond. 85.7, 26.6 and 25.0% of the bacterial OTUs from M2, TS38 and S5 ponds, respectively, are novel. All archaeal 16S rRNA gene sequences were exclusively affiliated with *Euryarchaeota*. 75.0, 60.0 and 66.7% of the OTUs from, respectively, M2, TS38 and S5 ponds are novel. The result showed that the Tunisian multipond solar saltern harbored novel prokaryotic diversity that has never been reported before for solar salterns. In addition, diversity measurement indicated a decrease of bacterial diversity and an increase of archaeal diversity with rising salinity gradient, which was in agreement with the previous observation for thalassohaline systems. Comparative analysis showed that prokaryotic diversity of Tunisian saltern was higher than that of other salterns previously studied.

Keywords Solar saltern · Molecular diversity · *Bacteria* · *Archaea* · 16S rRNA genes

Communicated by L. Huang.

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Introduction

Hypersaline ecosystems are among the most extreme environments on earth. Solar saltern ponds are an example of an environmental gradient, which has been extensively studied. These consist of a series of interconnected shallow ponds in which the concentration of salts increases as seawater evaporates, providing a wide set of ecological niches for halophilic microorganisms. Because of the limitation of traditional cultivation methods, which are not sufficient for culturing most *Bacteria* and *Archaea* (Hugenhotz et al. 1998), the use of culture-independent methods for studying prokaryotic diversity based on direct PCR amplification of the gene encoding small-subunit 16S rRNA from environmental DNA sample has proved to be a powerful means of microbial identification and evaluation

of diversity (Amann et al. 1995; Benlloch et al. 2001; Burns et al. 2004; Ovreas et al. 2003). Previous studies on the microbiology of hypersaline environments showed that halophilic members of the domain *Archaea* were dominant, whereas those of the domain *Bacteria* represented minor components (Litchfield et al. 2002; Ochsenreiter et al. 2002; Ovreas et al. 2003). Nevertheless, other studies have demonstrated that members of the *Bacteria* domain play an important role in hypersaline environments (Antón et al. 2000).

The multi-pond solar saltern of Sfax (Tunisia) was located in the south of Sfax City (Central-Eastern coast of Tunisia, about 34°39′N and 10°42′E), covers nearly 1,500 hectares area divided into several ponds along 12 km seacoast. The climate of the region is characterized by low rainfall (230 mm/year), temperate temperature (average temperature of 15 and 33°C for the hottest and coldest months, respectively) and a high evaporation rate (1,800 mm/year). This saltern consists of a series of shallow evaporation ponds (20–70 cm deep), connected by pipes and canals, and designed for the production of NaCl by evaporation from seawater, known as preparation, concentrator and crystallizer ponds, depending on their salt concentration and composition. There are no previous reports on the microbial communities of this hypersaline environment. In this study we are describing and comparing bacterial and archaeal communities of three saltern ponds with large differences in salt concentration, using culture independent molecular approaches. We demonstrate that salt concentration is an important parameter governing distribution and ecology of microbial communities. A comparative analysis to other previous studies was made.

Materials and methods

Sample collection and physico-chemical analysis

Three samples were collected aseptically from three different salinity ponds, respectively: concentrator pond M2 (15%), crystallizer ponds TS38 (25%) and S5 (32%) (Fig. 1) in May 2005. The samples from each pond were the average of ten sub-samples spanning the whole pond. The representative samples were centrifuged at 12,000*g* for 10 min, washed with sterile phosphate-buffered saline (PBS) and stored at −20°C. The total salt concentrations were determined at 120°C by drying 10 ml of water brines, pH and redox potential (Eh) were measured with a pH/redox meter equipped with a Metrohm platinum combination electrode (Met Rohm). Electrical conductivity (EC) and turbidity were measured with a conductivimeter (Amel 123) and a turbidity meter (HACH, 2,100 N), respectively. Dissolved O_2 concentration was measured by an oxymeter (YSI 57). Chlorine content was determined by titration (Skoog et al. 1996). The Ca^{2+} and the Mg^{2+} were determined by the volumetric method after complexation (Harris 1997). The SO_4^{2-} was determined with the gravimetric method (Belcher et al. 1954). The K^+ and the Na^+ were determined with flame photometry (Sherwood 410) (Golterman and Clymo 1971).

Genomic DNA extraction, PCR amplification of 16S rRNA genes, cloning and sequencing

Cell biomass samples from M2, TS38 and S5 ponds were collected by centrifugation of 30 ml of water sample. Pellets were diluted in 200 µl of TE buffer (10 mM Tris HCl—1 mM EDTA, pH 8) and then incubated at 90°C for 10 min. After cooling in ice, enzymes were added and genomic DNA extraction was achieved as described by Chouari et al. (2003). The 16S rRNA genes were amplified using bacterial-specific forward 008F (Hicks et al. 1992) or archaeal-specific primer 21F (DeLong 1992), combined with the universal reverse primer 1390R (Zheng et al. 1996). The PCR thermal profile was as follows: initial denaturation at 94°C for 5 min, primer annealing at 59°C for 1 min, and extension at 72°C for 1.5 min. The final elongation step was extended to 15 min. Under these conditions, a single PCR product of 1.4 kb was obtained. The 16S rRNA amplicons were cloned using a TA cloning kit (pGEM-T Easy vector; Promega) in accordance with the manufacturer's instructions. The resulting 16S rRNA gene-containing clones were grown in Nunc microliter plates containing 150 µl of Luria-Bertani medium supplemented with 5% glycerol and ampicilline (100 µg ml^{-1}) (Chouari et al. 2003). Plasmid extraction and 16S rRNA gene sequencing were performed as described by Artiguenave et al. (2000).

Phylogenetic analyses

The 16S rRNA gene sequences were compared to those of the GenBank and EMBL databases by advanced BLAST searches from the National Center for Biotechnology Information. Chimeric sequences were searched using the procedure described by Juretschkoo et al. (2002). Sequences were analyzed by using the ARB software package (http://www.arb-home.de). All sequences showing more than 1,200 nucleotides were imported and automatically aligned into the ARB database; the resulting alignments were manually checked and corrected when necessary. Phylogenetic placement was done in comparison with reference sequences representing the main lines of descent in the domain *Bacteria* and *Archaea* using the ARB program. Tree topology was evaluated using

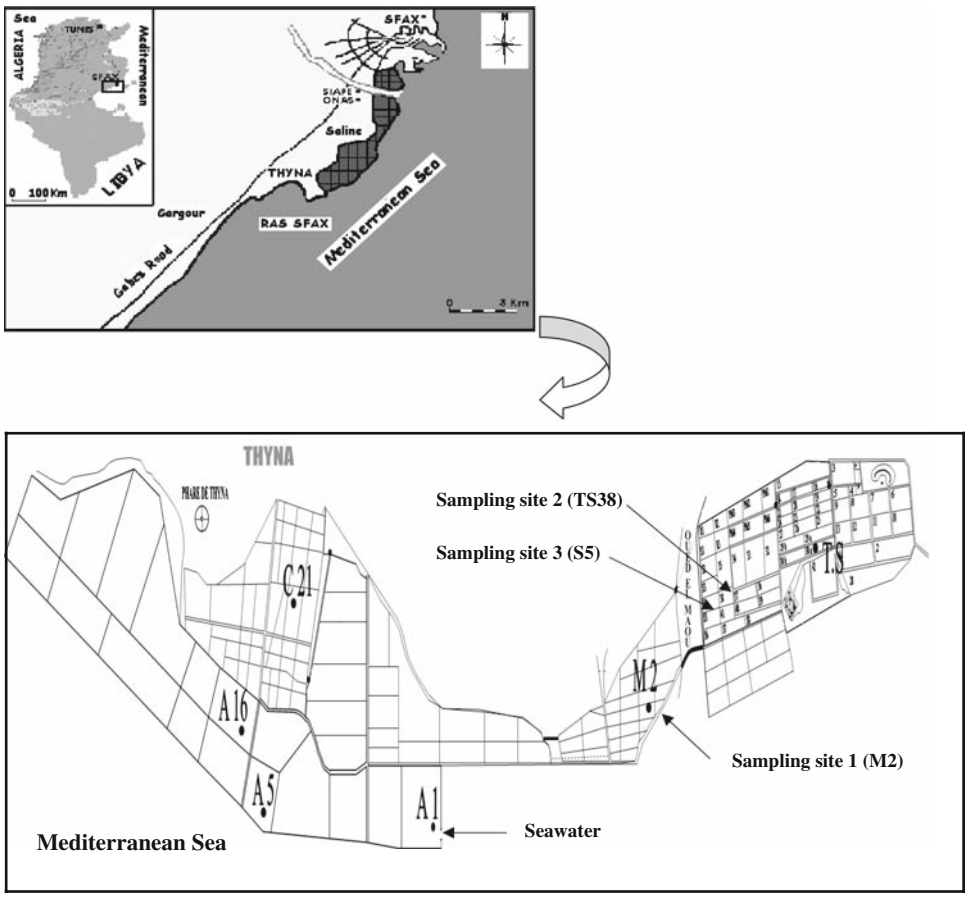

Fig. 1 Location map of the solar saltern of Sfax (Tunisia) and sampling points

Neighbor-Joining (NJ) method with Jukes and Cantor corrections and maximum parsimony.

Statistical analysis

In order to evaluate and compare prokaryotic diversity within the three ponds, 16S rRNA clone sequences showing ≥97% sequence similarity were grouped into the same OTU. Bacterial and archaeal diversity of the three ponds were assessed by calculating diversity index, comparing OTUs composition and analyzing phylogenetic distribution of 16S rRNA clones in each library. DOTUR software (http://www.plantpath.wisc.edu/fac/joh/dotur.html) (Schloss and Handelsman 2005) was used to calculate various diversity indices and richness estimations: the Shannon index (H′), evenness (J′) (Hughes et al. 2001; Magurran 1996), the reciprocal of Simpson's index (Magurran 1996) and Chao species-richness (Chao 1987). Coverage of clone libraries was calculated as described by Good (1954). All sequence data were accumulated in collector's curves, which were established to compare the relative diversity and coverage of each library by plotting the number of OTUs versus the number of clones. The OTUs compositions of libraries were compared using the Sorensen index (Magurran 1996).

Nucleotide sequence accession numbers

Sequences reported in this study have been submitted to EMBL, GenBank databases under accession numbers CU467268 to CU467484 for the bacterial clone sequences, and CU467113 to CU467267 for the archaeal clones sequences.

Results

Physico–chemical analysis of brines samples

The physico–chemical analysis of water samples from the three ponds of the solar saltern of Sfax (Tunisia) are reported in Table 1. The average temperature at the sampling sites was 15°C. The pH was near neutral. The Eh values reflected the reductive effect of the water brines. Sodium and chloride were the most abundant ions.

Sequence analysis and phylogenetic distribution of bacterial 16S rRNA sequences

A total of 216 16S rRNA gene sequences were obtained from three bacterial clone libraries. These sequences were

Table 1 Physico–chemical characteristics of the three ponds studied

Pond	M2	TS38	S5
Physical parameters			
Salinity (%)	15.00	25.00	31.00
pH	8.16	7.80	7.40
EC (mS cm^{-1})	170.00	190.00	140.00
Turbidity (NTU)	13.00	80.00	130.00
Dissolved O_2	2.60	1.04	0.94
Eh (mV)	−60.00	−30.00	−25.00
Major cation and anion (g/liter)			
Na^+	63.30	80.65	92.65
Ca^{2+}	0.27	0.04	0.03
Mg^{2+}	0.99	5.54	11.60
Cl^-	97.30	138.00	145.00
K^+	1.96	8.56	15.65
SO_4^{2-}	12.80	20.90	36.00

grouped into 44 OTUs distributed as follows: 21, 15 and 8 OTUs in M2, TS38 and S5 ponds, respectively (Tables 2, 3, 4). Operational taxonomic unit distribution within the bacterial libraries was restricted to *Bacteroidetes* (63.63% of OTUs) and *Proteobacteria* (34% of OTUs) (Table 5; Fig. 2).

M2 pond

The 48 bacterial clones sequences recovered from the M2 pond were grouped into 21 OTUs. These OTUs were affiliated to the *Bacteroidetes* and *Proteobacteria* divisions (Table 5). The *Bacteroidetes* division represented the predominant phylogenetic group within the clone library, encompassing 71.43% of the total of the obtained OTUs (15 OTUs). *Flavobacteriales* and *Sphingobacteriales*-affiliated 16S rRNA sequences predominated. The *Proteobacteria* (28.57%), represented by six OTUs, constituted the second phylogenetic group. They are distributed among two subclasses, the *gamma* subclass representing an important group (four OTUs) and the *alpha subclass* represented by only two OTUs (Table 5). No 16S rRNA gene sequences from M2 pond were affiliated with cultured microorganisms with ≥97% sequence similarity. Three OTUs are affiliated with uncultivated microorganisms in public databases with ≥97% similarity, and 18 OTUs (85.7% of OTUs) showed <97% sequence similarity with closely related bacterial 16S rRNA gene sequences and constituted novel OTUs. Two clones from the M2 pond are affiliated with cultivated species *Psychroflexus torquis* and *Roseovarius tolerans* (Table 2). The latter is an aerobic phototrophic bacterium abundant in marine environments, where it is involved in the degradation of organic sulfur compounds (Gonzalez et al. 1999).

TS38 pond

The 16S rRNA gene sequences recovered from TS38 bacterial clone library made up 15 OTUs affiliated with two major phylogenetic groups, *Bacteroidetes* (seven OTUs) and *Proteobacteria* (seven OTUs). *Cyanobacteria* was represented with only one OTU, affiliated with *Euhalothece* sp. (AJ000709) with 99.39% sequence similarity (Table 3). The three phylogenetic groups were composed of 16S rRNA sequences affiliated with cultured microorganisms with ≥97% sequence similarity as well as uncultured organisms. Four novel OTUs were affiliated with *Bacteroidetes* and *Proteobacteria*, respectively (Table 5). In contrast to the M2 pond, *Bacteroidetes* was dominated by *Sphingobacteriales*, represented by the genus *Salinibacter*. *Salinibacter ruber* was isolated from the crystallizer pond of a Spanish solar saltern (Antón et al. 2000, 2002; Oren et al. 2002).

S5 pond

The 86 16S rRNA gene sequences recovered from S5 pond clone library were distributed within two phylogenetic groups; *Bacteroidetes* was represented by 81 sequences (six OTUs), formed by far the most important fraction of the clone sequences and OTUs (75% of the total OTUs). Like TS38 pond, *Bacteroidetes* was exclusively represented by *Sphingobacteriales* (Table 4). The *Proteobacteria* represented only five sequences (two OTUs). Two and four OTUs of *Proteobacteria* and *Bacteroidetes,* respectively, were affiliated with cultivated microorganisms with ≥97% sequence similarity (Table 5).

The vast majority of 16S rRNA gene sequences recovered from TS38 and S5 clone libraries were affiliated with *Sphingobacteriales* (*Salinibacter*). The other 16S rRNA gene sequences obtained from these two ponds were closely related to 16S rRNA sequences affiliated with the *Rhodobacterales* and *Rhizobiales* orders and uncultured species of the order *Chromatiales*, *Ectothiorhodospiraceae*, a group of haloalkaliphilic purple sulfur bacteria (Stackebrandt et al. 1984). In contrast to the *Chromatiaceaea* which accumulates intracellular elemental sulfur, all strains of the family *Ectothiorhodospiraceae* produce external globules of elemental sulfur during photosynthetic sulfide oxidation (Imhoff 1984). *Ectothiorhodospiraceae* were obtained from marine, hypersaline and haloalkaline environments. *Ectothiorhodospiraceae* requires saline and alkaline growth conditions (Imhoff and Suling 1996).

Sequence analysis and phylogenetic distribution of archaeal 16S rRNA sequences

A total of 156 16S rRNA gene sequences were obtained from three archaeal clone libraries, resulting in 67 OTUs. OTU

Table 2 Affiliations of the bacterial and archaeal 16S rRNA gene sequences obtained from M2 pond sample

Sequence representative of OTUs	No. of clones in library	Microorganism or clone with highest 16S rRNA sequence similarity		
		Taxon	Accession no.	% similarity
Alpha-*Proteobacteria*				
185ZG07	1	*Roseovarius tolerans*	Y11551	96.9
185ZA11	2	Uncultured *Rhodobacteriaceae* bacterium	AF513928	97.95–98.91
Total 2	3			
Gamma-*Proteobacteria*				
185ZE12	2	Uncultured *gamma Proteobacterium*	AF452606	96–96.53
185ZB01	15	Uncultured *gamma Proteobacterium*	AF452606	96.3–96.82
185ZC08	2	Uncultured *Ectothiorhodospiraceae*	AF513947	97–97.65
185ZF11	2	Uncultured *Ectothiorhodospiraceae*	AF513947	96.54–96.9
Total 4	21			
Bacteroidetes				
185ZC06	1	*Psychroflexus torquis*	AY167318	96.87
185ZD12	1	Benzene mineralizing consortium	AF029041	92
185ZA04	4	Benzene mineralizing consortium	AF029041	91.29–92.76
185ZA12	1	Benzene mineralizing consortium	AF029041	90.44
185ZH07	1	Uncultured *Flavobacterium* sp.	AJ487527	96.08
185ZE08	1	Uncultured *Flavobacterium* sp.	AJ487527	96.27
185ZC09	1	Uncultured *Flavobacterium* sp.	AJ487527	96.66
185ZG06	1	Uncultured *Flavobacterium* sp.	AJ487527	96.46
185ZG05	1	Uncultured *Flavobacterium* sp.	AJ487527	96.88
185ZG08	1	Uncultured *Flavobacterium* sp.	AJ487527	89.38
185ZG10	1	Uncultured *Flavobacterium* sp.	AJ487527	89.79
185ZE02	2	Uncultured *Flavobacterium* sp.	AJ487527	90.53
185ZA07	1	Uncultured *Flavobacterium* sp.	AJ487527	90.77–91.04
185ZE05	4	Uncultured bacterium EKH0–12	AF142895	97.31–97.53
185ZA08	3	*Flavobacteriaceae* bacterium Gaa02	AY319330	92.13–92.98
Total 15	24			
Archaea				
186ZA11	24	Halophilic archeon PalaeaII	AJ276863	91–92
186ZC04	1	Halophilic archeon PalaeaII	AJ276863	91
186ZC11	1	Halophilic archeon PalaeaII	AJ276863	91
186ZD05	1	Halophilic archeon PalaeaII	AJ276863	90
186ZC10	1	Halophilic archeon PalaeaII	AJ276863	93
186ZA12	1	Haloarcheon CSW4 (*Haloquadratum Walsbyi*)	AY498649	94
186ZA06	1	Haloarcheon CSW4 (*Haloquadratum Walsbyi*)	AY498649	98
186ZC03	6	Haloarcheon CSW4 (*Haloquadratum Walsbyi*)	AY498649	99
Total 8	36			

distribution was 8, 20 and 39 OTUs in M2, TS38 and S5 ponds, respectively. Phylogenetic analysis indicated that archaeal sequences were exclusively affiliated with the *Euryarchaeota* (Fig. 3). A total of 20 OTUs were affiliated with cultivated species with ≥97% sequence similarity, they represented 25, 40 and 25.64% of M2, TS38 and S5 clone libraries (Table 5). They were distributed among different genera of the *Halobacteriaceae* such as *Haloquadratum, Halorubrum* and *Halobacter* (Tables 2, 3, 4; Fig. 3). Species representatives of the latter genera have been repeatedly isolated from many crystallizers worldwide (Antón et al. 2000; Benlloch et al. 2001; Ochsenreiter et al. 2002; Burns et al. 2004, 2007). Of the total OTUs within M2, TS38 and S5 75, 60 and 66.66%, respectively, are novel (Table 5).

Table 3 Affiliations of the bacterial and archaeal 16S rRNA gene sequences obtained from TS38 pond sample

Sequence representative of OTUs	No. of clones in library	Microorganism or clone with highest 16S rRNA sequence similarity		
		Taxon	Accession no.	% similarity
Alpha-*Proteobacteria*				
SFA1F121	1	*Bacterium* DG941	AY258087	94.46
SFA1E041	1	*Nitratireductor aquibiodomus*	AF534573	99.39
Total 2	2			
Gamma-*Proteobacteria*				
SFA1E061	3	Uncultured *Ectothiorhodospiraceae*	AF513947	97–97.78
SFA1B041	1	Uncultured *Ectothiorhodospiraceae*	AF513947	96.97
SFA1C021	2	*Halomonas venusta*	AJ306894	98.67
SFA1D101	3	*Pseudomonas stutzeri*	AJ312172	99.70
SFA1F061	1	*Shewanella* sp.Hac319	DQ307732	99.78
Total 5	10			
Bacteroidetes				
SFA1H111	1	*Salinibacter ruber*	AF323502	93.65
SFA1E091	2	*Salinibacter ruber*	AF323502	97–98.08
SFA1D071	35	*Salinibacter ruber*	AF323502	94.83–95.5
SFA1B081	2	*Salinibacter ruber*	AF323502	98.01
SFA1E011	9	Halophilic eubacterium EHB-2 (*Salinibacter ruber)*	AJ242998	97.12–99.93
SFA1D031	19	Halophilic eubacterium EHB-5 (*Salinibacter ruber)*	AJ242997	99.56–99.93
SFA1A021	1	Uncultured *Bacteroidetes* bacterium	AY862786	98.15
Total 7	69			
Cyanobacteria				
SFA1B051	1	*Euhalothece* sp.'MPI 95AH10'	AJ000709	99.39
Archaea				
186ZG09	1	*Haloquadratum walsbyi*	AY676200	93
186ZE12	1	*Haloquadratum walsbyi*	AY676200	96
186ZH08	1	*Haloquadratum walsbyi*	AY676200	92
186ZH04	12	*Haloquadratum walsbyi*	AY676200	98–99
186ZG01	2	*Haloarchaeon* CSW1(*Haloquadratum Walsbyi*)	AY498641	94
186ZE04	5	*Haloarchaeon* CSW1(*Haloquadratum Walsbyi*)	AY498641	97–98
186ZE02	5	*Haloarchaeon* CSW1(*Haloquadratum Walsbyi*)	AY498641	95–96
186ZG12	1	*Haloarchaeon* CSW1(*Haloquadratum Walsbyi*)	AY498641	98
186ZE07	1	Uncultured *Haloquadratum* sp.	AY987833	93
186ZF06	1	Uncultured *Haloquadratum* sp.	AY987833	97
186ZF12	1	Uncultured *Haloquadratum* sp.	AY987833	95
186ZH05	1	Uncultured *Haloquadratum* sp.	AY987833	98
186ZH06	1	*Halorubrum* sp. A29	DQ309088	94
186ZG08	1	*Halorubrum* sp. A29	DQ309088	98
186ZF01	1	*Halorubrum* sp. A29	DQ309088	98
186ZE01	1	*Halorubrum* sp. A29	DQ309088	96
186ZH03	1	*Halorubrum* sp. A29	DQ309088	95
186ZH09	1	*Halorubrum tibetense*	AF435111	97
186ZF04	1	*Halobacter utahensis*	AF071880	90
186ZH10	1	*Halobacter utahensis*	AF071880	93
Total 20	40			

Table 4 Affiliations of the bacterial and archaeal 16S rRNA gene sequences from S5 pond sample

Sequence representative of OTUs	No. of clones in library	Microorganism or clone with highest 16S rRNA sequence similarity		
		Taxon	Accession no.	% similarity
Alpha-*Proteobacteria*				
SFB1B081	1	*Nitratireductor aquibiodomus*	AF534573	99.46
Gamma-*Proteobacteria*				
SFB1A031	4	*Pseudomonas stutzeri*	AJ312172	99.63–99.85
Total 1	4			
Bacteroidetes				
SFB1B031	1	*Halophilic eubacterium* EHB-2 (*Salinibacter ruber)*	AJ242998	99.56
SFB1H011	1	*Halophilic eubacterium* EHB-5 (*Salinibacter ruber)*	AJ242997	98.91
SFB1A101	61	*Halophilic eubacterium* EHB-5 (*Salinibacter ruber)*	AJ242997	99.41–99.63
SFB1C051	1	*Salinibacter ruber*	AF323502	96.28
SFB1F121	2	*Salinibacter ruber*	AF323502	93.55–93.91
SFB1C081	15	*Salinibacter ruber*	AF323502	98.38–99.85
Total 6	81			
Archaea				
SFE1C061	1	*Haloquadratum walsbyi*	AY676200	92.66
SFE1F051	1	*Haloquadratum walsbyi*	AY676200	97.93
SFE1G061	1	*Haloquadratum walsbyi*	AY676200	95.38
SFE1A101	9	*Haloquadratum walsbyi*	AY676200	99.36–99.77
SFE1B081	1	*Haloquadratum walsbyi*	AY676200	93.78
SFE1B121	1	*Haloarchaeon* CSW1 (*Haloquadratum Walsbyi*)	AY498641	94.65
SFE1B051	1	*Haloarchaeon* CSW1(*Haloquadratum Walsbyi*)	AY498641	96.38
SFE1E061	2	*Haloarchaeon* CSW1 (*Haloquadratum Walsbyi*)	AY498641	94.32–96.57
SFE1C051	3	*Haloarchaeon* CSW1 (*Haloquadratum Walsbyi*)	AY498641	98.73–99.33
SFE1A011	5	*Haloarchaeon* CSW1 (*Haloquadratum Walsbyi*)	AY498641	96.57–96.72
SFE1D091	1	Uncultured *Haloquadratum* sp.	AY987831	95.40
SFE1D061	12	Uncultured *Haloquadratum* sp.	AY987831	99.18–99.85
SFE1E111	2	Uncultured *Haloquadratum* sp.	AY987831	97–98.43
SFE1F111	1	Uncultured *Haloquadratum* sp.	AY987833	96.96
SFE1D071	2	Uncultured *Haloquadratum* sp.	AY987833	97.07–98.39
SFE1H051	1	Uncultured *Haloquadratum* sp.	AY987833	94.01
SFE1A061	1	*Halorubrum* sp. A29	DQ309088	98.06
SFE1D121	1	*Halorubrum* sp. A29	DQ309088	95.46
SFE1B101	1	*Halorubrum* sp. A29	DQ309088	95.39
SFE1A041	1	*Halorubrum* sp. A29	DQ309088	98.77
SFE1C031	1	*Halorubrum* sp. A29	DQ309088	91.12
SFE1F021	1	*Halorubrum* sp. A29	DQ309088	99
SFE1G051	11	*Halorubrum* sp. A29	DQ309088	98.14–98.89
SFE1C081	1	*Halorubrum* sp. A29	DQ309088	97.46
SFE1A091	1	*Halorubrum* sp. A29	DQ309088	94.13
SFE1H031	1	*Halorubrum* sp. A29	DQ309088	94.18
SFE1C071	1	*Halorubrum* sp. A29	DQ309088	93.67
SFE1C041	3	*Halorubrum tibetense*	AF435111	97.62–98.14
SFE1F061	1	*Halorubrum tibetense*	AF435111	94.49
SFE1E091	1	*Halorubrum tibetense*	AF435111	92.98
SFE1G071	1	*Halorubrum tibetense*	AF435111	96.88
SFE1B091	1	*Halorubrum tibetense*	AF435111	91.67
SFE1F081	1	*Halorubrum tibetense*	AF435111	97

Table 4 continued

Sequence representative of OTUs	No. of clones in library	Microorganism or clone with highest 16S rRNA sequence similarity		
		Taxon	Accession no.	% similarity
SFE1C091	1	*Halorubrum tibetense*	AF435111	91.85
SFE1A051	1	*Halobacter utahensis*	AF071880	96.72
SFE1E071	1	*Halobacter utahensis*	AF071880	92.76
SFE1F091	1	*Halobacter utahensis*	AF071880	92.36
SFE1G121	1	*Halobacter utahensis*	AF071880	91.12
SFE1B111	2	*Halobacter utahensis*	AF071880	95.06
Total 39	80			

Table 5 Distribution of bacterial and archaeal OTUs within the three ponds

	Bacteria					*Archaea*
	Proteobacteria		*Cyanobacteria*	*Bacteroidetes*	Total	
	Alpha	*Gamma*				
M2 pond						
Number of sequences	3	21	0	24	48	36
Total OTUs	2	4	0	15	21	8
OTUs (%)	9.52	19.05	0	71.43	100	ND
OTUs $\geq$ 97% cultivated[a]	0	0	0	0	0	2
OTUs (%)	0	0	0	0	0	25.00
OTUs $\geq$ 97% not yet cultivated[b]	1	1	0	1	3	0
OTUs (%)	4.76	4.76	0	4.76	14.28	0
Novels OTUs[c] (< 97%)	1	3	0	14	18	6
OTUs (%)	4.76	14.28	0	66.66	85.7	75
Coverage (%)	79					83.33
Diversity (H′)[d]	2.78					1.16
Dominance (1/D)[e]	11					2.17
Evenness (J')	2.01					1.28
Species richness (Chao)	38					23.00
TS38 pond						
Number of sequences	2	10	1	69	82	40
Total OTUs	2	5	1	7	15	20
OTUs (%)	13.34	33.34	6.66	46.66	100	ND
OTUs $\geq$ 97% cultivated[a]	1	3	1	4	9	8
OTUs (%)	6.66	20.00	6.66	26.66	60	40
OTUs $\geq$ 97% not yet cultivated[b]	0	1	0	1	2	0
OTUs (%)	0	6.66	0	6.66	13.32	0
Novels OTUs[c] (< 97%)	1	1	0	2	4	12
OTUs (%)	6.66	6.66	0	13.33	26.66	60
Coverage (%)	91.47					60
Diversity (H')[d]	1.87					2.5
Dominance (1/D)[e]	4.20					9
Evenness (J′)	1.55					1.92
Species richness (Chao)	23.00					80
S5 pond						
Number of sequences	1	4	0	81	86	80

Table 5 continued

	Bacteria					*Archaea*
	Proteobacteria		*Cyanobacteria*	*Bacteroidetes*	Total	
	Alpha	*Gamma*				
Total OTUs	1	1	0	6	8	39
OTUs (%)	12.5	12.5	0	75	100	ND
OTUs ≥ 97% cultivated[a]	1	1	0	4	6	10
OTUs (%)	12.5	12.5	0	50	75	25.64
OTUs ≥ 97% not yet cultivated[b]	0	0	0	0	0	3
OTUs (%)	0	0	0	0	0	7.70
Novels OTUs[c] (< 97%)	0	0	0	2	2	27
OTUs (%)	0	0	0	25	25	66.66
Coverage (%)	95.4					63.00
Diversity (H′)[d]	0.98					3.04
Dominance (1/D)[e]	1.88					11.62
Evenness (J′)	0.75					1.91
Species richness (Chao)	11					106.6

[a, b] >97% similarity sequence to the most related sequence having a cultivable or with no cultivable representative

[c] <97% similarity sequence to any known sequence

[d] Shannon index

[e] Reciprocal of Simpson index

Diversity measure

Diversity coverage, Shannon index, dominance (Simpson index), evenness and species richness estimators (Chao-1) are shown in Table 5. Diversity coverage calculated using the Good formula was ranged from 79 to 95.4% for bacterial clone libraries and 60–83.33% for archaeal clone libraries. The same trend was observed by rarefaction curves obtained by plotting the number of OTUs observed against the number of clones sequenced (Figs. 4, 5). The decrease of number of OTUs detection rate shown in the curves representing M2 and TS38 ponds for archaeal clone libraries, and the S5 pond for bacterial clone libraries indicated that the major fraction of the diversity in these libraries was detected. These curves indicated that bacterial populations were the least diverse in the S5 pond and the most diverse in the M2 and TS38 ponds, whereas archaeal populations were the most diverse in the S5 pond. These observations are supported by bacterial diversity parameters, such as Shannon index, species richness estimators and evenness (Table 5). Species richness suggests a clear trend for increasing in archaeal populations from M2 to S5 ponds and a decreasing trend for bacterial populations. Similarities of OTUs composition in the 6 clone libraries as calculated using the Sorensen index ranged from 0 to 0.3478. There was no OTUs in common between bacterial or archaeal libraries of M2 and S5 pond samples, and between M2 and TS38 archaeal libraries and only a few OTUs were common between bacterial libraries of M2 and TS38 (similarity index of 0.1). However, a relatively high similarity index was found between the archaeal and bacterial libraries of TS38 and S5 (similarity index of 0.3 and 0.3478, respectively). Thus, the M2 pond is different from the two other ponds TS38 and S5 in terms of diversity indices, composition and OTUs distribution. LIBSHUFF analysis (Singleton et al. 2001) was used to compare the six clone libraries and to determine if they are significantly different. For all clone libraries P value was low (0.001), indicating a significant difference in community composition between the three pond samples with 95% confidence. This difference could be correlated with the selective pressure of salt concentration, which increases from M2 to S5 ponds.

Discussion

In the present study, we described microbial diversity among both *Bacteria* and *Archaea* domains within three ponds of gradually increasing salinity ranging from 15% (M2) to 31% (S5). The microbial communities in the three studied ponds were different in terms of diversity indices and phylogenetic distribution of the 16S rRNA sequences. *Proteobacteria* and *Bacteroidetes* of the *Bacteria* domain represented predominant phylogenetic groups with a large number of clones affiliated with *Bacteroidetes* phylum. The

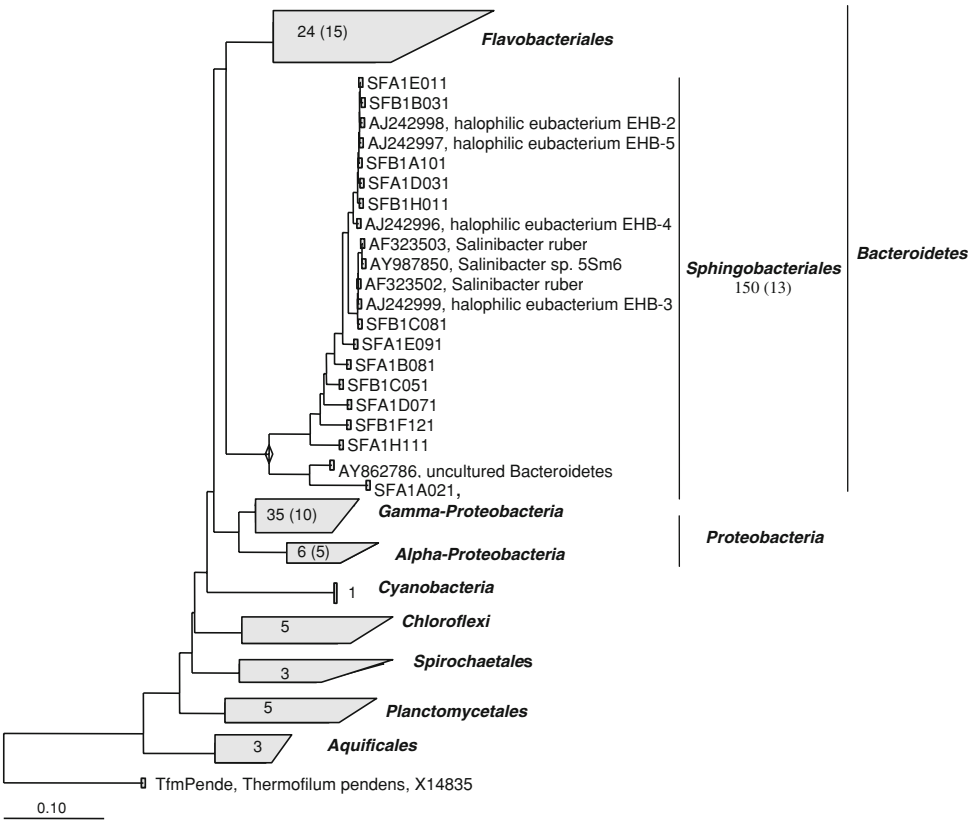

Fig. 2 Evolutionary distance dendrogram constructed using the NJ method and showing the affiliation of the environmental bacterial 16S rRNA gene sequences recovered from the three ponds (M2, TS38 and S5). The total number of OTUs is indicated in brackets. Trees were calculated using the ARB software package, as described in experimental procedures. The scale bar corresponds to a 10% estimated difference in nucleotide sequence positions. *Thermofilum pendens (X14835)* was used as an outgroup

differences between the three ponds indicated that microbial diversity may be strongly influenced by physical and chemical parameters in the three sites, particularly differences in salt concentration and in ions specification. Indeed, the *Flavobacteriales* were present only in M2 pond while the *Sphingobacteriales* were specifically found in both TS38 and S5. The rate comparison of the different minerals showed that in M2 pond, the Na^+/K^+ (32.29), Na^+/SO_4^-(4.94) and Na^+/Ca^{++} (234.44) were widely different from those found in the both ponds TS38 and S5, respectively, 9.42–5.92, 3.85–2.57 and 2016–3088. Moreover, oxygen concentration and reductive conditions were higher in M2 pond. As a consequence, *Flabobacteriacaea* seemed to be dependent on potassium, calcium and sulfates concentrations and not adapted to high salinities compared to the *Sphingobacteriales.*

Demergasso et al. (2004) observed that the relative abundance of *Bacteroidetes* increased with salinity and concluded that hypersaline waters apparently constituted an important environment for *Bacteroidetes* particularly the *Flavobacteriales* and *Sphingobacteriales*. *Bacteroidetes* are frequently found in microbial mats and sediments, and may play an important role in organic polymer degradation (proteins and polysaccharides: cellulose, pectin and chitin) as well as cell remains (Cottrell and Kirchman 2000).

Benlloch et al. (2002) have studied the community's diversity of the solar saltern in Santa Pola (Spain). They noted that in the 22% salt pond, *alpha* and *gamma Proteobacteria*, *Cyanobacteria* and *Bacteroidetes* were the only representative groups found. In the 32% salt pond, only *Bacteroidetes*-affiliated sequences were found, and almost all the retrieved sequences are affiliated with *Salinibacter ruber*. Recently, the bacterial diversity of Korean solar saltern (33% salt) and Maras salterns in the Peruvian Andes (25–31% salt) were analyzed (OTUs were defined at 97% similarity). Result showed that the majority of clones obtained from the Korean solar saltern (53%) belonged to *Bacteroidetes*, *alpha*, *gamma* and *delta Proteobacteria* groups. All the bacterial clones recovered from Maras salterns were closely related to each other and to the *gamma Proteobacteria* "*Pseudomonas halophila*". In these two salterns no *Salinibacter* clone was detected (Maturrano et al. 2006; Park et al. 2006).

With regard to the *Archaea* population, it could be noticed that all the 16S rRNA gene sequences obtained were affiliated with the *Halobacteriales* order of the *Euryarchaeota*. We did not detect any 16S rRNA gene sequence affiliated with *Crenarchaeota* or *Korarchaeota*. As previously noted in other coastal salterns (Anton et al. 2000; Benlloch et al. 2001; Rossello-Mora et al. 2003; Bolhuis et al. 2004; Walsby 2005; Fendrihan et al. 2006;

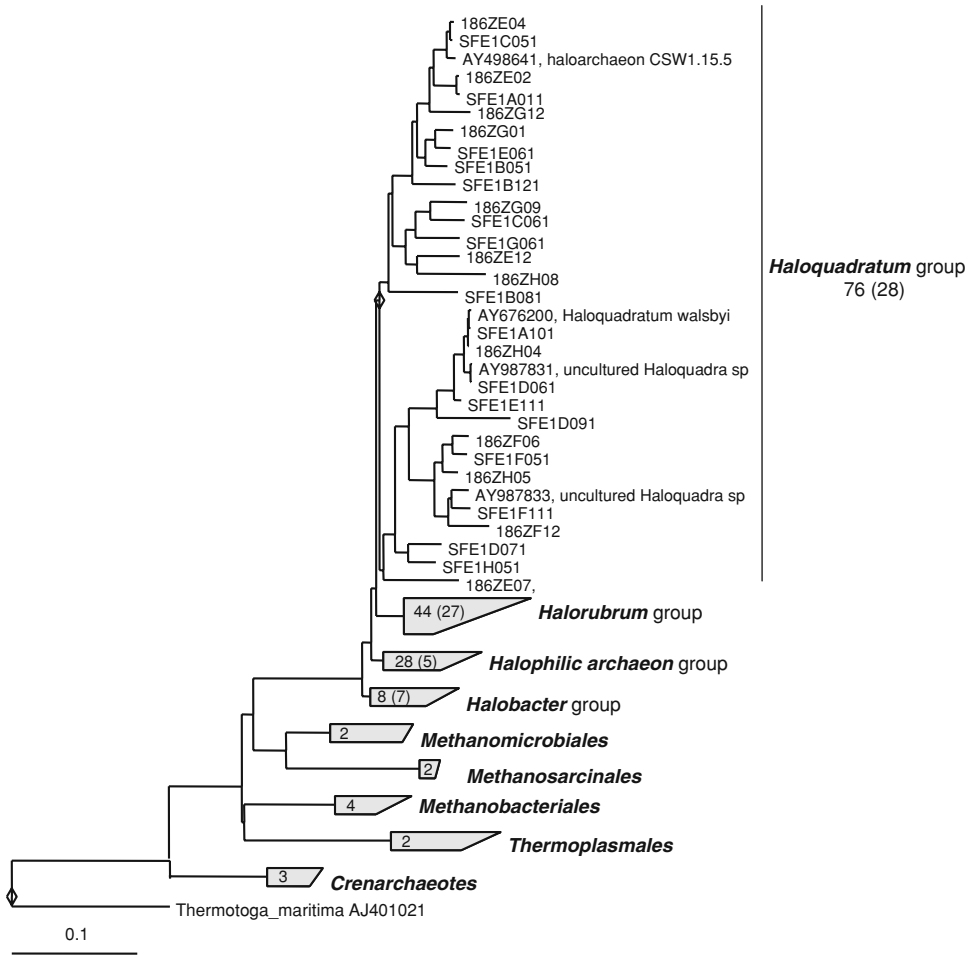

Fig. 3 Evolutionary distance dendrogram constructed using the NJ method and showing the affiliation of the environmental archaeal 16S rRNA gene sequences recovered from the three ponds (M2, TS38 and S5). The total number of OTUs is indicated in brackets. Trees were calculated using the ARB software package, as described in experimental procedures. The scale bar corresponds to a 10% estimated difference in nucleotide sequence positions. *Thermotoga maritima* (AJ401021) was used as an out group

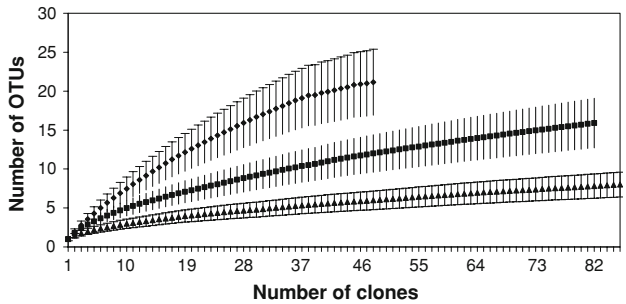

Fig. 4 Rarefaction curve generated for16S rRNA genes in bacterial clones' libraries from. M2 pond (*filled diamond*), TS38 pond (*filled square*) and S5 pond (*filled triangle*). The *error bars* give the 95% confidence intervals

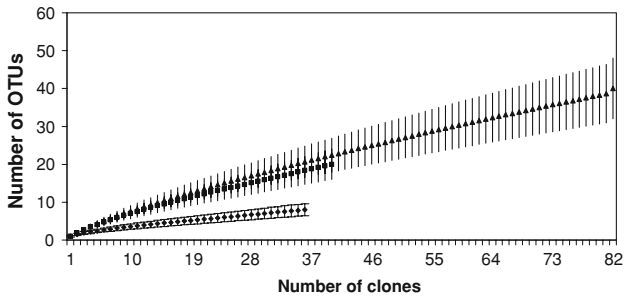

Fig. 5 Rarefaction curve generated for16S rRNA genes in archaeal clones' libraries from pond M2 (*filled diamond*), pond TS38 (*filled square*) and pond S5 (*filled triangle*). The *error bars* give the 95% confidence intervals

Maturrano et al. 2006), the prokaryotic community of Tunisian crystallizers ponds (TS38 and S5) was dominated by *Haloquadratum walsbyi* which was frequently found in large solar salterns number, notably in the ponds located at the end of the concentrating process, leading to NaCl precipitation. *Haloquadratum walsbyi* was isolated from a variety of geographical locations such as Western Europe, Australia, Western Asia and South America (Burns et al. 2004; Oren 2002). Other studies have assessed the diversity of halophilic *Archaea* in Korean (33% salt), Secovlje (25–30% salt) and Maras salterns (25–31% salt) (OTUs were defined at 97% similarity). The results showed that most sequences detected from Korean and Secovlje salterns were grouped within the *Halorubrum* branch, where *Haloquadratum walsbyi* was rare, although it was repeatedly-reported to dominate crystallizers' communities

Table 6 *Archaeal* diversity indices for Tunisian salterns compared with Santa Pola salterns, Secovlje saltern and Ston salterns

	Tunisian salterns		Santa Pola salterns		Secovlje salterns (25–30%)	Ston salterns (25–30%)
	TS38 pond (25–30%)	S5 pond (31–34%)	22% pond	32% pond		
Diversity (H')	2.50	3.04	1.00	1.67	2.24	0.49
Dominance (SI′)	9.00	11.62	ND	ND	0.14	0.75
Species richness (Chao)	80.00	106.60	ND	ND	27.00	3.00
	This study	This study	Benlloch et al. (2002)		Pasic et al (2005)	Pasic et al (2007)

ND not determined

(Pašić et al. 2005, 2007; Park et al. 2006). For Maras saltern, the archaeal assemblage was dominated by the square *Archaea Haloquadratum walsbyi* and *Halobacterium* sp (31% of clones) (Maturrano et al. 2006).

Considering the Shannon-Weaver indices, our results were comparable to those of Benlloch et al. (2002) who analyzed prokaryotic diversity of a solar saltern pond with salinity gradient varying between 22 and 32% and defined OTUs as the number of DGGE band or T-RFLP gels. In their study, Shannon-Weaver indices estimated for archaeal diversity were between 1.00 and 1.67 in the Spanish Santa Pola solar salterns. Moreover, for bacterial diversity, these indices were equal to 1.89 and 0.56 for the two solar salterns ponds. Compared to these results, the level of diversity observed in the present study is slightly higher than that found in Spanish Santa Pola solar saltern study.

Archaeal diversity indices estimated in this study and compared to other salterns (Table 6) indicated that the diversity was higher in Tunisian 16S rRNA library, than Santa Pola, Secovlje and Ston salterns. Tunisian and Spanish salterns were similar because of Mediterranean location and/or arid climate, allowing sufficient solar energy for year-round solar salt production. In area with no dry season like Secovlje and Ston, solar salterns functioning was limited to the dry summer season and in shallow ponds size not exceeding 10 cm in depth compared to 30–50 cm in the other salterns studied (Pašić et al. 2005). Furthermore, the Croatian Ston saltern showed lower diversity than the Secovlje salterns. This fact could be related to the relatively more oligotrophic water source (Pacic et al. 2007).

Our work consolidates the results found in salterns from different geographic regions that differ in their bacterial and archaeal diversity (Benlloch et al. 2002; Sorensen et al. 2005). It was noticeable that the climate may play an important role on the microbial communities found even in these extreme environments (Pasic et al. 2007).

Our results showed that the sequences affiliated with *Salinibacter ruber* and *Haloquadratum walsbyi* were dominant in the TS38 and S5 ponds; this may be explained by the fitness of specialized prokaryotic populations in this harsh habitat. Halophilic *archaea* with their red carotenoid pigments increase light absorption of the brines and promote evaporation by increasing temperature (Norton and Grant 1988; Oren and Truper 1990). *Haloarchaea* in solar salterns are known to promote crystallization of halite. Their cells could serve as templates for the nucleation of halite crystals and their subsequent development (Lopez and Ochoa 1998). Indeed, at scale laboratory, it was demonstrated that the salt production decreased in the absence of *Haloarchaea* (Javor 2002).

Salinibacter ruber and *Haloquadratum walsbyi* were the microorganisms found frequently at high concentration in climax saltern crystallizer communities. They were sequenced to unravel how these microorganisms were adapted to this hostile ecological niche, and how they were able to survive within this permanent stress (Mongodin et al. 2005, Legault et al. 2006). *Salinibacter ruber* was remarkably similar to that of the hyperhalophilic *Archaea*. Several genes and gene clusters may also be derived by lateral transfer, or may have been laterally transferred to *Haloarchaea* (Mongodin et al. 2005). *Haloquadratum walsbyi* contains large "accessory or adaptive" gene pools (Legault et al. 2006).

In conclusion, this culture-independent study reports on the microbial diversity, using 16S rRNA gene sequences analyses within three Tunisian saltern ponds where salinity gradients increased. The diversity of the *Archaea* and *Bacteria* found in the three studied ponds of a solar saltern showed a decrease of bacterial diversity and an increase of archaeal diversity with rising salinity gradient. *Bacteria* domain clones were represented exclusively by *Proteobacteria* and *Bacteroidetes*. *Archaea* domain clones were closely related to the *Halobacteriales* order (phylum *Euryarchaeota* class *Halobacteria*). These microbial communities were different from other worldwide salterns. This difference was attributed to the weather conditions as well as the nutrient-enriched water source. The development of novel culturing technical strategies allowing new group isolations of uncultured microorganisms using molecular approaches is an essential step to improve understanding microbial diversity in hypersaline environments.

Acknowledgments We are very grateful to Mr. S. Cure for reading the manuscript, Professor A. Rebai (Centre of Biotechnology in Sfax, Tunisia) for constructive discussion, and the technical assistance of the Genoscope sequencing and bioinformatics teams. We thank also the staff of the solar saltern of Sfax for allowing access to the saltern and sampling.

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