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The Santa Pola saltern as a model for studying the microbiota of hypersaline environments

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Abstract

Multi-pond salterns constitute an excellent model for the study of the microbial diversity and ecology of hypersaline environments, showing a wide range of salt concentrations, from seawater to salt saturation. Accumulated studies on the Santa Pola (Alicante, Spain) multi-pond solar saltern during the last 35 years include culture-dependent and culture-independent molecular methods and metagenomics more recently. These approaches have permitted to determine in depth the microbial diversity of the ponds with intermediate salinities (from 10 ‰ salts) up to salt saturation, with haloarchaea and bacteria as the two main dominant groups. In this review, we describe the main results obtained using the different methodologies, the most relevant contributions for understanding the ecology of these extreme environments and the future perspectives for such studies.

Keywords

Hypersaline habitats
Salterns
Santa Pola
Haloarchaea
Halophilic bacteria
Microbial ecology
Metagenomics

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Introduction

Hypersaline habitats are widely distributed extreme environments in which the main life-limiting factor is their high salt concentration. However, other physico-chemical features may also reduce the growth of living organisms, such as temperature, pH, solar radiation, oxygen, nutrient availability, heavy metals and other toxic compounds (pesticides, chemicals), etc. (Rodriguez-Valera 1988). Overall, these factors may influence the biota of the hypersaline environments, that is limited to highly specialized eukaryotes, prokaryotes and phages (Ventosa 2006; de la Haba et al. 2011). The microbiota is dominated by well-adapted halophilic microorganisms that in many cases are polyextremophiles, with the ability to grow optimally not only at high salt concentrations, but also at high or low pH values plus high or low temperatures and to other of the above-mentioned features (Bowers et al. 2009; Bowers and Wiegel 2011; Mesbah and Wiegel 2012).

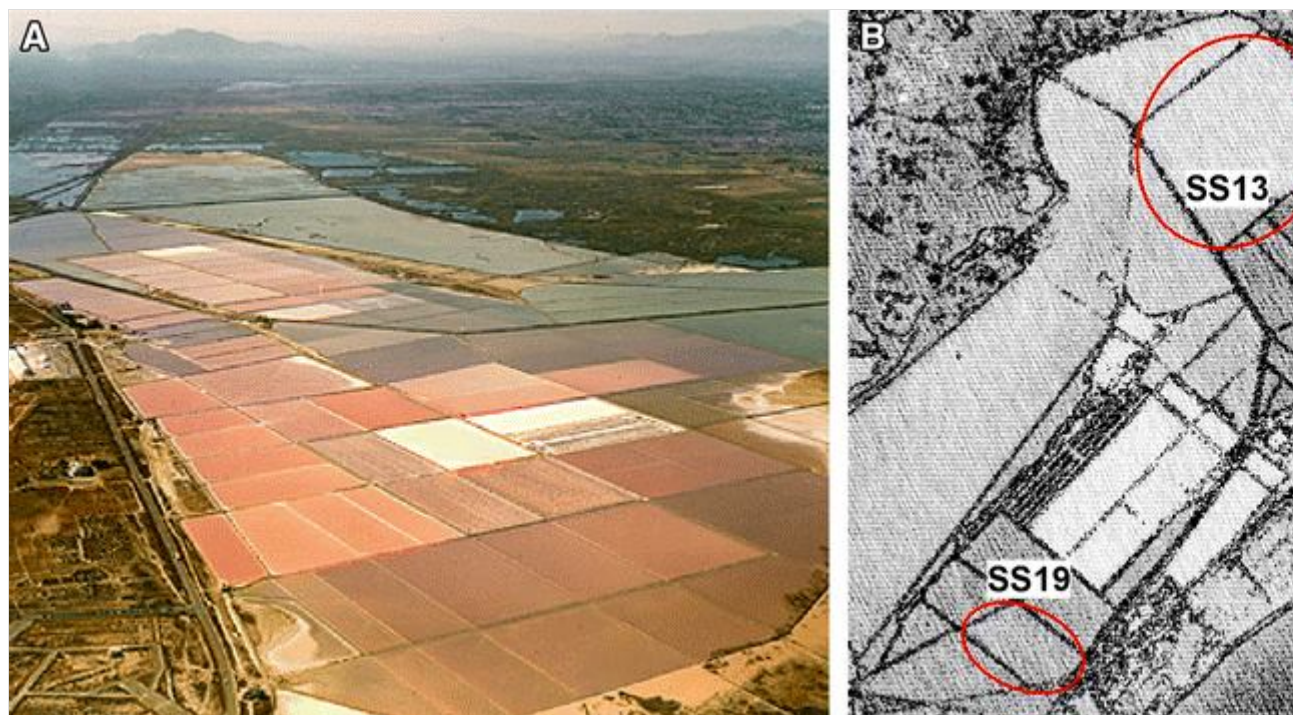
Hypersaline environments are represented by aquatic and terrestrial systems, as well as salted products, such as salted foods, hides, marine or rock salt, etc. (Ventosa 2006; Oren 2011). Most microbiological studies have been carried out on aquatic habitats, i.e., saline lakes (Dead Sea, Great Salt Lake, African, Chinese and Antarctic lakes, etc.) and salterns. Marine salterns constitute excellent models for the study of the microbial diversity and ecology of microorganisms at different salt concentrations. When they have a multi-pond system of salt production, they offer a wide range of salinities, from that of seawater to salt saturation. They are constituted by a series of shallow ponds

in which the water is periodically transferred from the lower salinity ponds (concentrators) to the ponds in which salts precipitate (crystallizers). The saturation of the different salts by evaporation of the water provokes the sequential precipitation of these salts: carbonate, gypsum and halite. Thus, three main domains are recognized: the carbonate domain ($70\text{--}140\text{ g l}^{-1}$), the gypsum domain ($220\text{--}290\text{ g l}^{-1}$) and the halite domain ($>290\text{ g l}^{-1}$) (Rodríguez-Valera 1988). One of these multi-pond salterns is located in Santa Pola, being probably the best studied hypersaline system in our planet with respect to its microbiology. In this review, we will focus on the most significant aspects that have been addressed in this saltern. However, we should indicate that several other salterns have also been investigated, but they are not the objective of this paper.

The Bras del Port saltern was built in the early twentieth Century over an ancient freshwater lagoon (Dulau 1983). It is a typical multi-pond saltern with a discontinuous salinity gradient. The saltern is located in Santa Pola, about 20 km South from Alicante, on the Spanish Mediterranean Sea coast. It is subjected to an arid Mediterranean climate characterized by low annual rainfall (200 mm year^{-1}) and moderate temperatures, with little oscillation between summer and winter (average temperatures of 26 and 12 °C for the warmest and coldest months, respectively) (Rodríguez-Valera et al. 1985). In each individual pond salt concentration is kept constant by a regulated artificial flow, so that in each pond the evaporation balances the input of less concentrated salt water (Fig. 1). The ponds are rectangular with an average surface area of approximately $20,000\text{ m}^2$ and a depth of 30 cm (Rodríguez-Valera et al. 1981; Ventosa et al. 1982).

Fig. 1

Aerial view of the Santa Pola saltern (a); schematic view of the saltern showing the ponds sampled to obtain the metagenomic datasets: SS13 (pond with 13 % salinity), SS19 (pond with 19 % salinity), SS33 (pond with 33 % salinity), SS37 (pond with 37 % salinity) (b)



The proportions of salts in Santa Pola saltern ponds are similar to seawater, except for those ponds where salts precipitate. However, environmental conditions oscillate significantly in ponds with different salt concentrations. The ponds with higher salinities have lower pH values and higher maximal temperatures, having a difference of one pH unit and 10 °C between ponds with saturated NaCl and those with less than 15 % salt concentration (Rodríguez-Valera et al. 1985). In ponds with lower salinities there is a high photosynthetic activity that causes a reduction in the CO₂ partial pressure, allowing an increase of pH in these ponds (Landry and Jaccard 1984). The total nitrogen and phosphorus concentrations increase at higher salinities, while the oxygen content is reduced since the saturation concentration decreases with salinity (Rodríguez-Valera et al. 1985).

Culture-dependent studies in Santa Pola saltern

Early studies on Santa Pola saltern were based on the isolation and characterization of microorganisms in pure cultures. Rodríguez-Valera et al. (Rodríguez-Valera et al. 1981, 1985) determined the changes in composition of microbial populations and distribution of taxonomic groups in ponds ranging from 10 % salts to salt saturation. Most organisms isolated from ponds with salinities over 15 % salts were halophilic. The unicellular algae *Dunaliella* and other eukaryotic organisms were observed. The populations of *Dunaliella* increased from 15 % salts, reaching large numbers between 20 and 30 % salts. Protozoa, other green algae and diatoms were observed in ponds

with up to 15 % total salts. Also mosquito larvae and some aquatic insects appeared. Large populations of *Artemia salina* (brine shrimp) appeared during certain times of the year, mainly in spring. Between 15 and 30 % salts, moderately halophilic bacteria (growing optimally in media with 3–15 % NaCl) and some fast-growing haloarchaea predominated as heterotrophic microorganisms. Among the first, the *Pseudomonas-Alteromonas-Alcaligenes* group (probably the current members of *Halomonas*, *Chromohalobacter* and related genera within the family *Halomonadaceae*) and *Vibrio* (currently *Salinivibrio*) were the most abundant taxonomic groups; Gram-positive cocci appeared mainly over 25 % salts. Phototrophic bacteria, both oxygenic and anoxygenic, were also found in this salinity range, with a predominance of *Halochromatium* and *Rhodospirillum*. In ponds with salinities over 30 % salts the microbial diversity was greatly reduced. The organisms found at the lower salt concentrations disappeared and instead large populations of haloarchaea developed (Rodríguez-Valera et al. ~~Rodríguez-Valera et al.~~ 1985).

During these early studies in the 1980s the number of validly described prokaryotic species names was low, making difficult the identification of new isolates and microbial ecology studies on hypersaline habitats. Besides, most studies on saline environments were carried out using similar complex growth media and sampling few hypersaline habitats. For these reasons, other approaches such as the use of numerical taxonomy and/or polar lipid comparative studies were carried out for the taxonomic characterization of the isolates from Santa Pola saltern (Ventosa et al. 1982, 1983; Torreblanca et al. 1986; Quesada et al. 1987; Montero et al. 1988; Moldoveanu et al. 1990). These and other chemotaxonomic and molecular techniques (Monteoliva-Sanchez et al. 1989; Ventosa 1993) permitted the taxonomic characterization of a large number of genera and species of archaea and bacteria from the Santa Pola saltern. Of particular interest are the studies describing the haloarchaeal genera *Haloarcula* and *Haloferax*, based on numerical taxonomy and the polar lipid composition (Torreblanca et al. 1986), and the square haloarchaeon *Haloquadratum* (Bolhuis et al. 2004; Burns et al. 2007) isolated finally in 2004 after many years of its discovery by microscopic observation in 1979 (Walsby 1980). Besides, the bacterial genera *Chromohalobacter* (Ventosa et al. 1989), *Salinicoccus* (Ventosa et al. 1990), and *Salinivibrio* (Mellado et al. 1996), classifying the previously described species *Vibrio costicola* (Garcia et al. 1987a, b) within a new genus, and more recently the extremely halophilic member of the *Bacteroidetes*, *Salinibacter* (Antón et al. 2002) were also described. Besides, several new species, some of them of great importance

since they have been used for understanding the molecular mechanisms of halophilism and several other molecular features, were originally described on the basis of strains isolated from the Santa Pola saltern (Table 1). We should also stress the importance of other studies based on strains from Santa Pola saltern that permitted the classification or delineation of some features of several haloarchaea and halophilic bacteria on the basis of nucleic acid studies (Gutierrez et al. 1989a; 1989b; 1990), their heavy metals and antimicrobial susceptibility and the use of the antimicrobial resistance as a genetic marker (Nieto et al. 1987; 1989a; 1989b; 1993; Garcia et al. 1987a, 1987b) or the production of the antimicrobial proteins designated as halocins (Rodriguez-Valera et al. 1982; Meseguer et al. 1986).

Table 1

Archaeal and bacterial species described and isolated from Santa Pola saltern

Species	NaCl range (%, w/v)	Optimal NaCl (%, w/v)	References
Domain <i>Archaea</i>			
<i>Haloarcula hispanica</i>	15-salt saturation	25	Juez et al. (1986)
<i>Haloferax mediterranei</i> (basonym: <i>Halobacterium mediterranei</i>)	7.5–27	17	Rodriguez-Valera et al. (1983); Torreblanca et al. (1986)
<i>Haloferax gibbonsii</i>	10-salt saturation	20–25	Juez et al. (1986)
<i>Haloferax lucentense</i>	10–30	25	Gutierrez et al. (2002)
<i>Haloquadratum walsbyi</i>	14-salt saturation	18	Burns et al. (2007)
Domain <i>Bacteria</i>			
<i>Halobacillus halophilus</i> (basonym: <i>Sporosarcina halophila</i>)	2–20	10	Claus et al. (1983); Ventosa et al. (1983); Spring et al. (1996)
<i>Salimicrobium album</i> (basonym: <i>Marinococcus albus</i>)	5–20	5–15	Hao et al. (1984); Yoon et al. (2007)
<i>Marinococcus halophilus</i>	0.5–20	5–15	Hao et al. (1984)
<i>Salinivibrio costicola</i> subsp. <i>costicola</i> (basonym: <i>Vibrio costicola</i>)	0.5–20	10	(Garcia et al. 1987a, 1987b); Mellado et al. (1996)

Species	NaCl range (% w/v)	Optimal NaCl (% w/v)	References
<i>Chromohalobacter marismortui</i>	1–30	10	Ventosa et al. (1989)
<i>Salinicoccus roseus</i>	0.9–25	10	Ventosa et al. (1990)
<i>Salinicoccus hispanicus</i> (basonym: <i>Marinococcus hispanicus</i>)	0.5–25	10	(Márquez et al. 1990); Ventosa et al. (1992)
<i>Halomonas salina</i> (basonym: <i>Deleya salina</i>)	2.5–20	5	Valderrama et al. (1991); Dobson and Franzmann (1996)
<i>Marinococcus halophilus</i>	0.5–30	5–15	Márquez et al. (1992)
<i>Salinibacter ruber</i>	15–NaCl saturation	20–30	Antón et al. (2002)
<i>Halomonas ilicicola</i>	2–17.5	10	Arenas et al. (2009)

Culture-independent studies in Santa Pola saltern

Despite advances in knowledge of halophilic microorganisms, most of the initial studies were performed using culture-dependent approaches and was clear that other techniques for the study of the microbial ecology in these environments were required. Early studies performed in Bras del Port salterns using molecular techniques such as fluorescence in situ hybridization (FISH) or PCR-fingerprinting approaches were focused on the study of the biodiversity in the crystallizer ponds (Benlloch et al. 1995, 2001; Antón et al. 1999, 2000). As expected, this hypersaline environment was shown to have a very low diversity but, surprisingly, the extremely hypersaline waters of the crystallizers showed less diversity by the direct 16S rDNA amplification methodology than by culture isolation (Benlloch et al. 1995). Also, most prokaryotes in the crystallizer ponds belonged to the domain *Archaea* and confirmed that Walsby's square bacteria belonged to this domain as previous phenotypic data indicated (Stoeckenius 1981; Kessel and Cohen 1982). In contrast, members of the genus *Haloarcula*, which had frequently been isolated from these ponds, represent less than 0.1 % of the total prokaryotic community (Antón et al. 1999). However, the contribution to the total community of members of the domain *Bacteria* was higher than expected from previous studies (Oren 1990).

On the other hand, the prokaryotic mortality due to viruses and bacterivores through the salinity gradient was estimated in different saltern ponds. Prokaryotic and viral abundance increased with the salinity, reaching 10^8 prokaryotic cells ml^{-1} and 10^9 virus-like particles (VLO) ml^{-1} at salinities higher than 25 %. It was known that the square haloarchaeon represented more than 25 % of the prokaryotic assemblage above 25 % salinity, so a lemon-shaped virus was found infecting this square archaeon and its abundance increased in the saltiest pond in correlation with this haloarchaeon (Guixa-Boixareu et al. 1996).

The abundance of prokaryotes, cell volume, prokaryotic heterotrophic production, chlorophyll *a*, and the abundance of heterotrophic flagellates, ciliates and phytoplankton were determined in several ponds of the Bras del Port saltern. Increases in salinity resulted in a progressive reduction in the abundance and number of different groups of eukaryotic microorganisms, but in an increase in biomass of prokaryotes. Maximal activity of phyto and bacterioplankton and chlorophyll *a* concentration were found at 10 % salinity. Another interesting fact that is derived from this study is that growth rates of heterotrophic prokaryotes decreased with increasing salinity and bacterivory was absent above 25 % salinity, whereas viral lysis appeared to be of minor importance throughout the gradient (Pedrós-Alió et al. 2000).

A molecular study in Santa Pola saltern analyzed the prokaryotic community along the salinity gradient by using an electrophoretic analysis of 5S rRNAs (Casamayor et al. 2000). This study revealed that the prokaryotic populations abundant in the ponds below 25 % salinity were neither flavobacteria nor haloarchaeal strains belonging to the genera *Halobacterium*, *Haloarcula* or *Halococcus*, instead members of *Proteobacteria* and *Firmicutes* were found. Finally, in the ponds above 30 % salinity none of the cultured halophilic archaea were detected (Casamayor et al. 2000).

Benlloch and coworkers (Benlloch et al. 2002) studied the prokaryotic diversity throughout the salinity gradient from Santa Pola saltern by 16S rDNA sequencing from both denaturing gradient gel electrophoresis (DGGE) and clone libraries and also culturing methods. This study showed that the abundance of bacterial and archaeal genera decreased along the gradient. At a 8 % salt pond, most sequences for *Bacteria* were related to organisms of marine origin. Thus, representatives of the *Alpha*-, *Beta*-, *Gamma*- and *Epsilonproteobacteria*, the *Cytophaga-Flavobacterium-Bacteroides* group

(CFB), high G + C *Firmicutes* and *Cyanobacteria* were found. In the 22 % salt pond *Alpha-* and *Gammaproteobacteria*, *Cyanobacteria* and CFB were the only groups found, and most of them were related to known halophilic genera. In the pond with 32 % total salt, only members of CFB were found, and most of the retrieved sequences clustered with *Salinibacter ruber*. With respect to *Archaea* in the lowest salinity ponds, the 16S rRNA sequences were related to environmental clones of Marine Archaea Group II (*Thermoplasmatales* relatives) and to unclassified branches of *Euryarchaeota*. Most of the clones in the three salinity ponds (with 8, 22 and 32 % salts) were related to different cultured strains of the family *Halobacteriaceae* (belonging to *Halorubrum* or *Haloarcula*) and finally most sequences from the crystallizer pond clustered with square haloarchaea (*Haloquadratum walsbyi*).

On the other hand, the microbial communities inhabiting Santa Pola saltern were analyzed and compared in parallel by four laboratories using SSU rRNA polymerase chain reaction (PCR)-based fingerprinting specifically denaturing gradient gel electrophoresis (DGGE), ribosomal internal spacer analysis (RISA) and terminal-restriction fragments length polymorphism (T-RFLP). Members of *Bacteria*, *Archaea* and *Eukarya* were retrieved from all salt concentrations. Two main, salinity-based groups of prokaryotes (from samples with 4–15 % and 22–37 % salts) were obtained. For eukaryotic microorganisms the two main groups detected were in samples with 4–5 % and 8–37 % salinity. *Archaea* showed the lowest number of operational taxonomic units (OTUs) in the lower salinity ponds. Although the particular taxonomic composition could vary among protocols, the general structure of the microbial assemblages was maintained (Casamayor et al. 2002).

Another study in these salterns was carried out by Estrada and coworkers (Estrada et al. 2004). They studied the diversity of prokaryotic and eukaryotic phytoplankton along the gradient of salinity using different community descriptors: chlorophyll *a*, HPLC pigment composition, flow cytometrically determined picoplankton concentration, taxonomic composition of phytoplankton (based on optical microscopy) and genetic fingerprint patterns of 16S (cyanobacteria- and chloroplast-specific primers) and 18S rRNA genes were determined for samples from ponds with salinities ranging from 4 to 37 %. A decrease in diversity with increasing salinity in both prokaryotic and eukaryotic microbes was confirmed. The number of elements of the different descriptors used were significantly correlated among themselves and negatively correlated with salinity.

Fluorescence in situ hybridization experiments showed that *Salinibacter* is an important component of the microbial community in saltern crystallizer ponds in the Bras del Port saltern and the analysis of the pigments extracted from ponds of this saltern showed that 5–7 % of the total prokaryotic pigment absorbance could be attributed to a carotenoid present in this bacterium. The red color of saltern crystallizer ponds may thus not only be due to red halophilic *Archaea* and to β -carotene-rich *Dunaliella* cells as previously assumed, but may contain a bacterial contribution as well (Oren and Rodríguez-Valera 2001).

Papke and coworkers (2003) used environmental PCR and cloning techniques to directly retrieve rhodopsin genes from three different salinity ponds in the Bras del Port saltern. Haloarchaeal rhodopsins are a diverse group of transmembrane proteins that use light energy to drive several cellular processes. They observed the presence of genes related to the rhodopsin in a wide range of salt concentrations, and they decreased with decreasing salinity, indicating that some haloarchaea are able to grow in a wide range of salt concentrations. On the other hand, other study suggested that the *Halorubrum* population is near linkage equilibrium and that random mating and recombination occur both within and (possibly at a somewhat reduced rate) between ponds of different salinities (Papke et al. 2004).

Using single cell sorting, saturated NaCl brine environments (32–35 %) of the South Bay Salt Works in Chula Vista in California (USA) and the Santa Pola saltern were compared. Both samples were quite different and included previously undetected organisms based on 16S rRNA sequences. *Archaea* dominated Santa Pola's community and its bacterial fraction consisted of the previously known *Salinibacter* lineages. The recently reported group of halophilic *Archaea*, *Nanohaloarchaea* was detected at both sites (Zhaxybayeva et al. 2013).

Metagenomic studies in Santa Pola saltern

Metagenomics of dominant prokaryotic species

Several metagenomic studies carried out in Santa Pola saltern have been focused on the crystallizer ponds, in which the microbial diversity is sharply reduced and is limited almost entirely to the square haloarchaeon *Haloquadratum walsbyi* as well as the extremely halophilic bacterium *Salinibacter ruber*. The first metagenomic study was carried out by Legault et

al. (2006). A total of ca. 2000 fosmid clones from environmental DNA were obtained from water column of the crystallizer pond, designated as CR30, and a total of 1029 sequences with more than 94 % nucleotide identity to the HSQ001 were considered as *Haloquadratum walsbyi*, a strain previously isolated from Santa Pola saltern. They recovered a large pangenome of *H. walsbyi* strain HBSQ001 and most of these metagenomic sequences exhibited synteny with this strain. The pangenome of this metapopulation revealed a remarkable gene pool of *H. walsbyi* in this habitat, at least twice the size of its genome (~3 Mb). Their accessory gene pool was located at relatively conserved regions of the genome that were less represented in the metagenome. Moreover, the accessory gene pool was enriched in signal transduction and gene regulation that suggested a key role in microadaptation to slightly different niches. Cuadros-Orellana et al. (2007) analyzed the genomic islands (GIs, regions in a genome considered hypervariable) in the genome of *H. walsbyi* HBSQ001, comparing it with complete sequences of fosmids found at or near the GIs. GI 2 and GI 4 had a high G + C content and were rich in mobile elements. GI 3 was a remnant of a lysogenic phage inserted in *H. walsbyi* HBSQ001 genome, but not present in most environmental lineages. These GIs contained genes involved in the transport of the nutrients across the membrane and detection of small molecules, probably reflecting the specialization of different genomes in the use of different compounds to coexist and avoid direct competition for resources. Besides, GI 1 was atypical compared to other GIs, because it had a G + C content similar to that of the genome and there were no mobile elements. GI 1 contained genes required to synthesize the rigid components of the cell envelope, reflecting probably a strategy of phage evasion. In a similar fashion the genome of *Salinibacter ruber* DSM 13855 was compared to metagenomic fragments obtained from crystallizer ponds of Chula Vista salterns, near San Diego, California (USA) and to the previous metagenomic fosmid ends obtained by Legault et al. (2006) (Pašić et al. 2009). Three GIs were found in *S. ruber*, GI 1 coded for cell surface polysaccharides, GI 2 contained genes involved in the biosynthesis of cell wall polysaccharide components, and GI 3 was rich in DNA-related enzymes.

Prokaryotic diversity along a salinity gradient

In 2011, Ghai et al. (2011) described the microbial community of Santa Pola saltern based on the analysis of the metagenomes obtained by pyrosequencing of the total prokaryotic DNA from the water of the two saltern ponds, one from an intermediate-salinity pond (with 19 % salinity, designated as SS19)

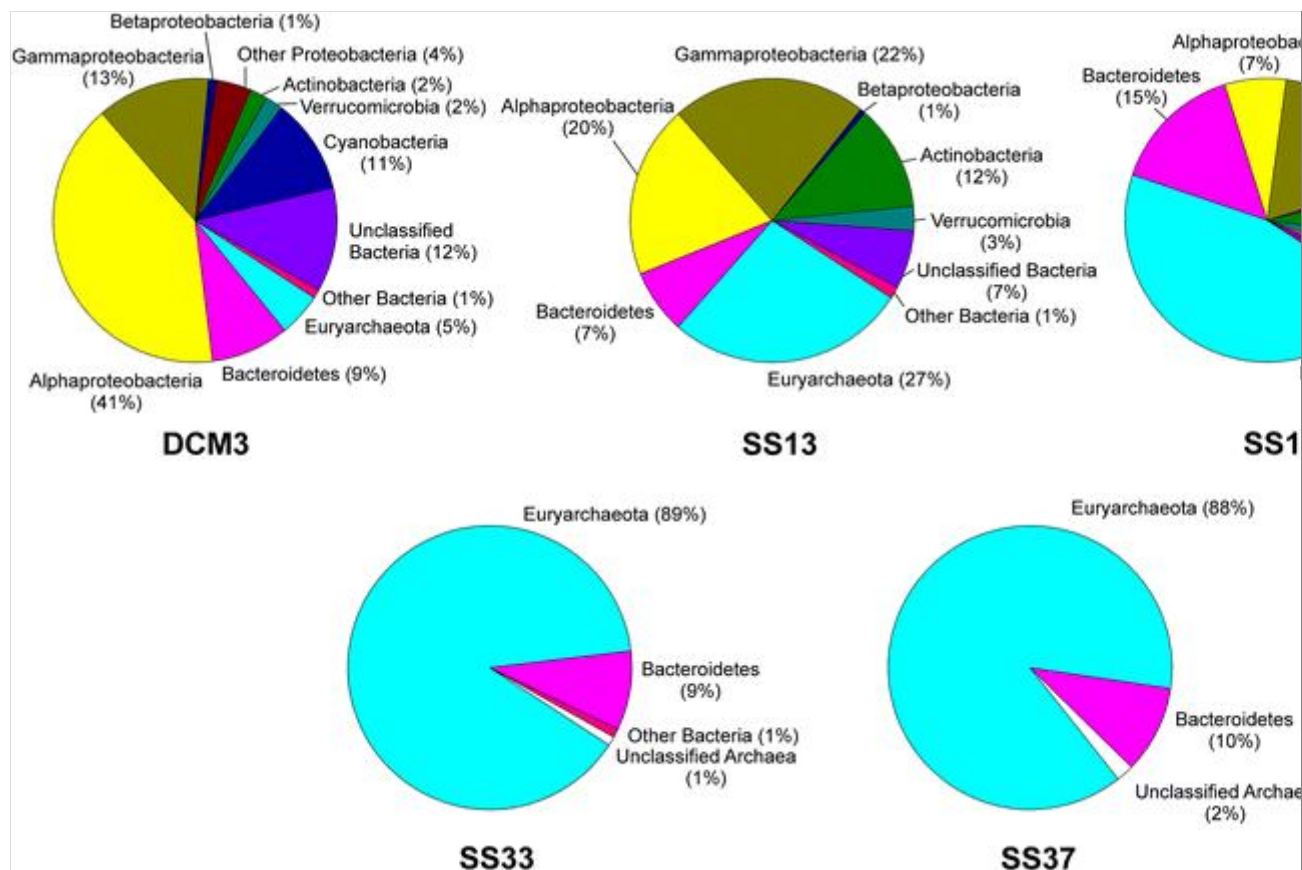
and another from a crystallizer pond (CR30 that in this specific sample had 37 % salinity and was identified as SS37) (Fig. 1). The metagenomes SS19 and SS37 had a dataset size of 475 and 309 Mb, respectively. This study reasserted the vast abundance of *H. walsbyi* in this saltern and revealed the presence of new abundant groups of microorganisms. In SS19 was found a group of low G + C *Actinobacteria*, related to the freshwater Luna1-A clade26 and a gammaproteobacterium related to the genera *Alkalilimnicola* and *Nitrococcus*. A high G + C and a low G + C euryarchaea were observed in SS19 (Ghai et al. 2011). Besides, in this study individual microbial cells were isolated from the SS37 saline water using fluorescence-activated cell-sorting. A partial single amplified genome (SAG) was sequenced turning out to be a novel archaeon, named *Candidatus Haloredivivus* sp. G17, which was abundant in the SS19 sample but not in SS37. *Candidatus Haloredivivus* has a low G + C content (~42 %), lower than *H. walsbyi* (47.9 %) and the recently described low G + C nanohaloarchaeon *Candidatus Nanosalina* (43.5 %). It showed a photoheterotrophic and polysaccharide-degrading lifestyle and is phylogenetically related to the *Nanohaloarchaea*. Based on the data acquired by Ghai et al. (2011), we have been able to isolate the abundant gammaproteobacterium, related to the genera *Alkalilimnicola* and *Nitrococcus*, and named it as *Spiribacter salinus* as we will describe in detail later (León et al. 2014).

Recently, a new metagenomic dataset obtained from an intermediate-salinity pond with 13 % salinity (designated as SS13) has been reported (Fernandez et al. 2013). This metagenome was sequenced using pyrosequencing, the same technology used by Ghai et al. (2011), and had 441 Mb of dataset size. We assessed the prokaryotic community structure of this lower salinity habitat (Fernández et al. 2014a). The 16S rRNA gene sequences analyzed from this metagenomic dataset were related to representatives of seven higher taxa, with *Euryarchaeota* (27 %), *Gammaproteobacteria* (22 %) and *Alphaproteobacteria* (20 %) as the most abundant representatives (Fig. 2). However, a high proportion of 16S rRNA gene sequences could not be classified at the genus level. Metagenomic reads from SS13 were assembled in contigs that revealed the presence of new groups of *Euryarchaeota*, related to reference genomes as *H. walsbyi* and other members of the *Halobacteriaceae*, and *Gammaproteobacteria*, related to *Spiribacter salinus*. Besides, proton pumps involved in a photoheterotrophic lifestyle like bacteriorhodopsin, xanthorhodopsin and proteorhodopsin gene sequences were found in SS13. The comparison of the metagenomic datasets of Santa Pola saltern showed

that bacteriorhodopsin, sensory rhodopsin and halorhodopsin (the last rhodopsin is a chloride pump) increased and proteorhodopsin decreased along the salinity gradient. In the SS13 pond, the nitrogen cycle was simplified to the assimilatory nitrate and nitrite reduction and the phosphate transport was higher than in ponds with lower (until 10 % of total salts) and higher (over 25 % of total salts) salinities but phosphonate uptake increased along the salinity gradient. Moreover, it was pointed out that haloarchaea known to accumulate ions inside their cells could be able to combine “salt-in” and “salt-out” strategies, due to sequences found related to the accumulation of compatible solutes such as glutamate or glycerol (Fernández et al. 2014a). Figure 2 summarizes the higher taxa determined in the metagenomic datasets of Santa Pola saltern in comparison with those reported for a metagenomic dataset obtained from the Mediterranean Sea. Besides, Table 2 shows the major genera that were retrieved from the metagenomic datasets obtained from ponds of Santa Pola saltern.

Fig. 2

Representation of high taxonomic levels affiliated to metagenomic 16S rRNA reads from the five datasets with different salinities. DCM3, Deep Chlorophyll Maximum (3 % salinity) from the Mediterranean sea; SS13, Santa Pola Saltern (pond with 13 % salinity); SS19, Santa Pola Saltern (pond with 19 % salinity); SS33, Santa Pola Saltern (pond with 33 % salinity); SS37, Santa Pola Saltern (pond with 37 % salinity). 16S rRNA genes were identified by comparing the datasets against the RDP database. Assigned sequences have an identity over 80 % and a minimum length of 100 bp

**Table 2**

Distribution of metagenomic 16S rRNA reads affiliated at genus level of the five metagen

Sample	DCM3		SS13		SS19
	<i>Pelagibacter</i> ^a	32.6 %	<i>Spiribacter</i>	4.8 %	<u><i>Halorubrum</i></u>
	<i>Prochlorococcus</i>	1.7 %	<i>Oceanicola</i>	3.5 %	<u><i>Haloquadratum</i></u>
	<i>Synechococcus</i>	1,5 %	<i>Roseovarius</i>	3.1 %	<u><i>Salinibacter</i></u>
	<i>Coralimargarita</i>	0.7 %	<i>Aquiluna</i> ^a	2.3 %	<u><i>Natronomonas</i></u>
	<i>Flavobacterium</i>	0.6 %	<i>Pseudoruegeria</i>	1.9 %	<i>Spiribacter</i>
			<i>Gracilimonas</i>	1.8 %	<i>Haloredivivus</i> ^a
			<i>Puniceicoccus</i>	1.5 %	<i>Puniceicoccus</i>
			<i>Alkalilimnicola</i>	1.2 %	<i>Arhodomonas</i>

Sequences were assigned to a specific genus if they shared over 95 % 16S rRNA sequ of 100 bp. Only those genera with more than 1 % of assigned sequences are shown

DCM3 Deep chlorophyll maximum (3 % salinity) from the Mediterranean sea, *SS13* S Saltern (pond with 19 % salinity), *SS33* Santa Pola Saltern (pond with 33 % salinity),

^a*Candidatus*

The genera with species that are known hyperhalophiles are underlined

Sample	DCM3	SS13	SS19
		Rhodoluna ^a 1.1 %	Aquiluna ^a
		<i>Agrococcus</i> 1.0 %	<i>Oceanicola</i>
		<i>Arhodomonas</i> 1.0 %	
		<u><i>Haloquadratum</i></u> 1.0 %	
Total number of sequences	2596	1552	
Number of genera	171	95	

Sequences were assigned to a specific genus if they shared over 95 % 16S rRNA sequ of 100 bp. Only those genera with more than 1 % of assigned sequences are shown

DCM3 Deep chlorophyll maximum (3 % salinity) from the Mediterranean sea, *SS13* S Saltern (pond with 19 % salinity), *SS33* Santa Pola Saltern (pond with 33 % salinity),

^a*Candidatus*

The genera with species that are known hyperhalophiles are underlined

More recently, two concentrator ponds from Santa Pola saltern with 19 and 33 % salinity (SS19 and SS33, respectively) were compared with a 21 % salinity pond from Isla Cristina saltern (designated as IC21), a saltern located in Southwest Spain, on the Atlantic ocean coast to explain the differences observed among these datasets (Fernández et al. 2014b). In IC21 the phylogenomic diversity was sharply reduced compared to SS19 dataset. At higher taxonomic levels *Euryarchaeota* was the predominant phylum in SS33 and IC21, but at genus level *Halorubrum* in IC21 and *Haloquadratum* in SS33 were the most abundant genera. The next predominant phylum was *Bacteroidetes* but the genera which recruited a higher proportion of 16S rRNA gene sequences differed in SS33 and IC21, in which *Salinibacter* and *Psychroflexus*, respectively, were the most abundant genera. The number of sequences related to bacteriorhodopsins and halorhodopsins observed were consistent with the abundance of *Haloquadratum* in SS19 and SS33 and of *Halorubrum* in IC21 dataset. About nitrogen cycle, similar results to SS13 were found in IC21, SS19 and SS33 datasets. Besides, an incomplete cycle of sulfate was observed in IC21, SS19 and SS33 datasets although in IC21 and SS19 a complete dissimilatory sulfate reduction was detected. In IC21 compared to SS19 and SS33 were detected more sequences related to

phosphate cycle and less for genes involved in the utilization of phosphonate. SS19 and SS33 datasets had higher numbers of sequences related to the synthesis of compatible solutes compared to IC21, such as betaine, glutamate and trehalose. Furthermore, it is suggested that the differences among these three datasets might be caused by local ecological conditions which were reflected in a different microbial community, such as the dominance of sequences related to *Halorubrum* in IC21 and to *Haloquadratum* in SS19 and SS33, which led to features like a lower number of sequences related to the synthesis of compatible solutes and in the utilization of phosphonate in the Isla Cristina dataset. The causes of the variation among samples are still unknown, but previous surveys carried out in hypersaline environments indicated that the microbial structure was highly influenced by the differences in the ionic composition of the brine (Pagaling et al. 2009; Grant et al. 2011; Boujelben et al. 2012; Podell et al. 2013).

Diversity of phages

For a long time, only the halophages His1 and SH1 that infect *Haloarcula hispanica* were known (Tang et al. 2002). Nowadays, several metagenomic studies of halophage populations have been performed in the crystallizer pond CR30. Santos et al. (2007) reconstructed the nearly complete genome of EHP-1, an environmental halophage, not yet isolated, from fosmid libraries from the purified 37 kb DNA obtained from the sample. The genome sequence had a size of 35 kb and a G + C content lower than those of other previously characterized halophages, around 51 %. The G + C content and codon usage in EHP-1 was similar to that of *H. walsbyi* (G + C content of 47.9 %), the most abundant microorganism in the crystallizer ponds and therefore perhaps could be the host for EHP-1. Subsequently, the metagenomic viral DNA from CR30 was used to construct two metaviromic dsDNA libraries, one in fosmids and one in plasmids, and were sequenced using PCC1FOS™ vector sequencing primers (Epicentre) and pBluescript SK primers BlueF and BlueR. Assembled metagenomic sequences showed a high number of single nucleotide polymorphisms (SNP) revealing a certain degree of diversity in the halophage populations (Santos et al. 2010). However, the halophage community of this metavirome exhibited some conserved characteristics like terminases and WD40/YVTN (The YVTN-type repeat domain is also found in archaeal surface layer proteins that protect cells from extreme environments) with metaviromes from high salt concentration ponds (27–30 % salinity) in San Diego saltern (CA, USA), located 10,000 km away. Moreover, the dinucleotide frequency analysis and the G + C content of the CR30

metavirome allowed the clustering of sequences in different groups and the speculation of their putative hosts. In 2011, Santos et al. analyzed the viral communities in CR30 through a metatranscriptomic approach. Contigs with a high viral level expression were included in five different groups, two groups with sequences of high G + C content haloarchaea and *S. ruber* (HVS-1 and HVS-2), a third group with sequences of *H. walsbyi* (HVS-4) and the groups HVS-3 and HVS-5. Interestingly, the viral groups that could infect high G + C content haloarchaea and *Salinibacter* representatives, which are minor components in this environment, had the highest expression level. Furthermore, samples from CR30 were submitted to stress conditions (UV-radiation and osmotic shock) and in the metatranscriptomes obtained under these stress conditions was observed that archaea were more sensitive than bacteria to electromagnetic radiation or dilution, since archaeal viruses increased the expression under these stress conditions. In addition, Garcia-Heredia et al. (2012) constructed fosmid libraries from CR30 and the fosmid DNA enabled to reconstruct the sequence of 42 almost complete viral genomes. Cluster of phage genomes supported by tetranucleotide frequency analysis, codon usage and the presence of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) protospacers, allowed the assignment to their possible hosts as *H. walsbyi*, *S. ruber* and a nanohaloarchaeon, covering most of the prokaryotic community of this habitat.

Through the metagenomic approach it has been observed that certain genomic regions are underrepresented in all of the genomes of one species when are compared to metagenomes from the environments in which this species is present, these regions are defined as metagenomic islands (MGIs), and these MGIs in saturated brine environments are mainly involved in adaptation to phage sensitivity and organic carbon degradation (Cuadros-Orellana et al. 2007; Wilhelm et al. 2007; Frias-Lopez et al. 2008). Therefore, Rodriguez-Valera et al. (2009) proposed the constant-diversity dynamics (CD) model to explain how phages are involved in maintaining prokaryotic diversity in these ecosystems. The periodic-selection model predicts that the best adapted organisms to the environment are selected and would expand and replace other organisms, but the dominant lineage would be replaced periodically by other type due to advantageous mutations or environmental changes (Koeppel et al. 2008). Instead, the CD model propose that no dominant lineage population will be observed because phage predation is responsible for maintaining the diversity among closely related lineages and this will result in each lineage acquiring different but complementary metabolic and ecological capabilities to

exploit the niche. Therefore, it is expected that the community exploits resources more efficiently and thus the ecosystem functioning becomes more efficient (Rodríguez-Valera et al. 2009).

AQ1

From metagenomics to pure culture: *Spiribacter salinus*

Sequence assembly from the metagenomic datasets of the Santa Pola ponds with different salinities provided many contigs related to a group of *Gammaproteobacteria* and giving consistent hits to the genomes of both *Alkalilimnicola ehrlichii* MLHE-1 and *Nitrococcus mobilis* Nb-231. Genomic fragments originated from metagenomic assembly and principal component analysis (PCA) on the normalized tetranucleotide frequencies of these contigs suggested that this might represent an abundant and as yet unknown bacterium or group of bacteria (Ghai et al. 2011; Fernández et al. 2014a; 2014b).

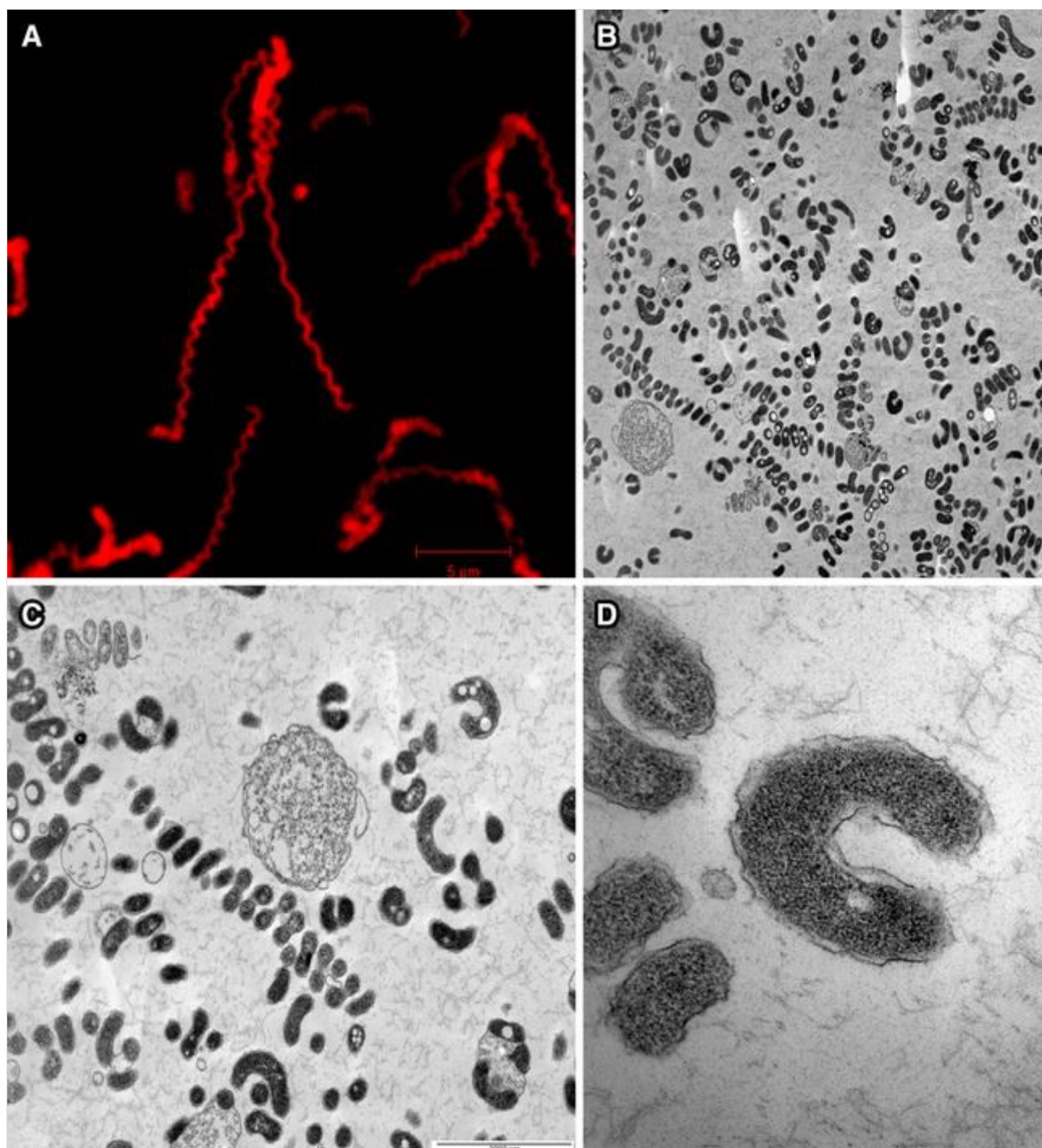
In an effort to culture these novel microbes, different oligotrophic media were designed and an extensive sampling and culturing was undertaken from water of ponds from Santa Pola saltern and from another saltern located in Isla Cristina, Southwest Spain. One strain, designated as strain M19-40, phylogenetically related to the genera *Alkalilimnicola* and *Arhodomonas*, showing a 16S rRNA gene sequence similarity of 94.9 % to *Alkalilimnicola*, was isolated from Isla Cristina saltern. It showed a 16S rRNA percentage of similarity equivalent to that reported previously on the basis of metagenomic studies for the contigs that represented the abundant gammaproteobacterium in the intermediate-salinity ponds with 13–19 % total salts (León et al. 2014). Actually, the largest contigs obtained from the 19 % metagenome from Santa Pola saltern were clearly associated with strain M19-40, confirming the abundance of this strain in this environment. However, many of these contigs had lower similarities while still being syntenic to strain M19-40, indicating that there might be other species or related taxa present in significant amounts in this habitat. This strain has been recently described as a new genus and species, for which the name *Spiribacter salinus* has been proposed (León et al. 2014).

The complete genomes of *Spiribacter salinus* M19-40 and of another related strain, “*Spiribacter*” sp. UAH-SP71, isolated from Santa Pola saltern were sequenced using Illumina HiSeq 2X 100-bp paired-end (PE) reads and Pacific Biosciences 3- to 5-Kb reads and assembled into single contigs (López-Pérez

et al. 2013). The total genome sizes were 1.74 and 1.93 Mb, respectively (the smallest genomes described within the *Ectothiorhodospiraceae*), with G + C contents of 62.7 and 63.9 %, and a single rRNA operon. The comparison of the genome of strain M19-40 with the closest available complete genome (*Alkalilimnicola ehrlichii* MLHE-1) showed that *Spiribacter salinus* is simplified in its metabolic versatility, as it misses the chemolithotrophic and carbon fixation pathways. *Spiribacter salinus* showed characteristics observed in oligotrophic microbes with streamlined genomes that reach high population densities in aquatic environments. The recruitment of the genome from the available metagenomes of hypersaline waters confirms that *Spiribacter salinus* is a very abundant microbe in intermediate salinities decreasing sharply its abundance at both high and low salinities, being able to grow in media containing 10–25 % (w/v) NaCl and optimally in a medium containing 15 % (w/v) NaCl. When strain M19-40 was grown on liquid medium at 37 °C cells adopted short and thin curved rods forms on young cultures but they produced long spiral cells at the stationary phase, with large polyalkanoate inclusion bodies (Fig. 3) (León et al. 2014).

Fig. 3

Confocal (a) and transmission electron microscopy (b–d) micrographs showing the morphology of cells of a pure culture of *Spiribacter salinus* M19-40



The cellular fatty acid profile of *Spiribacter salinus* M19-40 was characterized by the fatty acids $C_{18:1} \omega 6c/C_{18:1} \omega 7c$ (60.6 %), $C_{16:0}$ (13.4 %), $C_{10:0} 3\text{-OH}$ (6.4 %) and $C_{12:0}$ (5.7 %) as the major fatty acids. This fatty acid profile is quite different from that reported for *Alkalilimnicola*, for which the major fatty acids are $C_{18:1} \omega 7c/C_{18:1} \omega 9c$, but $C_{18:1} \omega 6c$ (major fatty acid determined in strain M19-40) is absent; besides, the other major fatty acids present in strain M19-40 are not found in species of *Alkalilimnicola*. Similarly, the major fatty acids present in strain M19-40 are also absent in species of the genera *Arhodomonas* and *Alkalispirillum*, for which the major fatty acids are $C_{18:1} \text{cys11}$ and $C_{18:1}$, respectively, both absent in strain

M19-40 (León et al. 2014). The polar lipids found in strain M19-40 are phosphatidylglycerol, phosphatidylethanolamine, a phosphoglycolipid, a phosphoaminoglycolipid and three phospholipids. The presence of phosphatidylglycerol, and phosphatidylethanolamine was also reported for *Alkalilimnicola halodurans* but not the other lipids, showing a different polar lipid profile with respect to this related bacterium (León et al. 2014).

Spiribacter salinus M19-40 appeared to have the “salt-out” strategy to balance the high environmental salinity. ~~Six different ABC-type~~ Several glycine betaine transport systems were found in its genome sequence suggesting that this compound has an important role in its osmoregulation. The complete *ectABC* gene cluster involved on the biosynthesis of ectoine was also found. In response to osmotic stress bacteria can also accumulate K^+ as an osmoregulatory solute and pH regulator. The uptake of K^+ is catalyzed by multiple uptake systems. *Spiribacter salinus* only showed the gene cluster *trkAH* that codes for the Trk transport system. The genome of strain M19-40 was found to contain rhodopsin-coding genes suggesting an additional energy source when light is available (López-Pérez et al. 2013).

Concluding remarks

Multi-pond salterns that create a gradient of salt concentrations are excellent models for studying the microbial diversity of hypersaline habitats. One of such salterns that has been extensively studied for more than 35 years is located in Santa Pola, near Alicante (Spain), being probably the best known hypersaline environment in our planet. Many studies, based on the techniques available at each period, have permitted to determine in depth the microbial diversity of the water of the saltern ponds, initially based on culture-dependent methods, later on culture-independent molecular methods and more recently on metagenomics. Metagenomic studies have determined that the most concentrated NaCl saturated ponds with ca. 37 % total salts (crystallizers) are dominated by *Euryarchaeota* (mainly the square haloarchaeon *Haloquadratum walsbyi*) and the nanohaloarchaea; in addition, a lower percentage by the bacterium *Salinibacter ruber*. In the ponds with intermediate salinity (13-19 % total salts) the prokaryotic diversity is high, represented by seven higher taxa. In contrast to the crystallizers, the number of genera and species is higher in these intermediate ponds. An abundant taxon in these intermediate-salinity ponds is the gammaproteobacterial group represented by the recently isolated and characterized species *Spiribacter salinus*, but perhaps this is not the only species and several other related

species or genera within the family *Ectothiorhodospiraceae* may be present. Besides, *Euryarchaeota* are also abundant in intermediate-salinity ponds, but the predominant species in the crystallizer and concentrator ponds are different, with *Haloquadratum* and *Halorubrum* as the most abundant genera, respectively. Besides, new groups of halophilic archaea not yet isolated or described have been observed on intermediate-salinity ponds.

The Santa Pola saltern has permitted scientists the discovery of new and interesting microbes, such as the red-pigmented extremely halophilic bacterium *Salinibacter ruber* and the square haloarchaeon *Haloquadratum walsbyi*, isolated about 25 years after its original observation in brine samples by A.E. Walsby (~~1980~~)(1980). The recent discovery and isolation in pure culture of the new gammaproteobacterium *Spiribacter salinus* opens new possibilities for studies on moderately halophilic bacteria. The study of this bacterium may permit a better knowledge of the microbial ecology and the adaptive mechanisms of microorganisms in their natural habitats. Previous studies on moderately halophilic bacteria have been carried out using model organisms such as representatives of the genera *Halomonas*, *Chromohalobacter*, *Halobacillus* or *Salinivibrio*, which are fast-growing bacteria easily isolated in complex laboratory media but that do not constitute a large proportion of the microbiota of salterns. Future studies should be based on organisms that like *S. salinus* represent abundant populations of the hypersaline habitats, although the less abundant microbes may also play an important role on the ecosystem and are worth detailed study in order to understand the microbial ecology of these habitats. If used in innovative way, metagenomics can result in the identification of previously unknown microorganisms and their eventual cultivation.

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