

Accepted Manuscript

Title: Diversity of extremely halophilic cultivable prokaryotes in Mediterranean, Atlantic and Pacific solar salterns: Evidence that unexplored sites constitute sources of cultivable novelty

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PII: S0723-2020(15)00023-5
DOI: <http://dx.doi.org/doi:10.1016/j.syapm.2015.02.002>
Reference: SYAPM 25676

To appear in:

Received date: 24-11-2014
Revised date: 3-2-2015
Accepted date: 5-2-2015

Please cite this article as: T. Viver, A. Cifuentes, S. Díaz, G. Rodríguez-Valdecantos, B. González, J. Antón, R. Rosselló-Móra, Diversity of extremely halophilic cultivable prokaryotes in Mediterranean, Atlantic and Pacific solar salterns: evidence that unexplored sites constitute sources of cultivable novelty, *Systematic and Applied Microbiology* (2015), <http://dx.doi.org/10.1016/j.syapm.2015.02.002>

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1 **Diversity of extremely halophilic cultivable prokaryotes in Mediterranean, Atlantic and**
2 **Pacific solar salterns: evidence that unexplored sites constitute sources of cultivable**
3 **novelty**

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24 **Key Words:** halophilic, MALDI-TOF MS, large-scale cultivation, OTUs, OPUs, salterns.

25

25

26 **Abstract**

27 The culturable fraction of aerobic, heterotrophic and extremely halophilic microbiota retrieved
28 from sediment and brine samples of eight sampling sites in the Mediterranean, Canary Islands
29 and Chile was studied by means of a tandem approach combining large-scale cultivation,
30 MALDI-TOF MS targeting whole cell biomass, and phylogenetic reconstruction based on 16S
31 rRNA gene analysis. The approach allowed the identification of more than 4,200 strains and a
32 comparison between different sampling sites. The results indicated that the method constituted
33 an excellent tool for the discovery of taxonomic novelty. Four new genera and nine new species
34 could be identified within the archaeal family *Halobacteriaceae*, as well as one new bacterial
35 species, and a representative of *Salinibacter ruber* phylotype II, a group that had been
36 refractory to isolation for the last fifteen years. Altogether, the results indicated that in order to
37 provide better yields for the retrieval of novel taxa from the environment, performance of non-
38 redundant environment sampling is recommended together with the screening of large sets of
39 strains.

40

41 **Introduction**

42 Culture-dependent microbiology suffers from being empirical and time and effort intensive, but it
43 is essential to basic science and biotechnology [7]. In addition, obtaining pure cultures of the
44 vast majority of microorganisms in the environment is difficult due to slow growth, metabolic
45 needs or the incapacity to find appropriate media [17], as well as additional microbial
46 interactions that could be related to the modification of their connections with the environment,
47 other prokaryotes or viruses [23]. Therefore, there is a need to develop strategies to culture
48 organisms in the laboratory, and this is a prerequisite for biodiscovery [23]. The search for
49 novelty by means of culture techniques can be approached using different methodologies, such
50 as large-scale cultivation, innovative culturing strategies or enrichment by micromanipulation
51 [17]. One of the important advantages of large-scale cultivation is that the extent of any novelty
52 may be related to the extent of the screening itself.

53 The exhaustive studies on 16S rRNA gene sequences as a measure of the microbial
54 diversity thriving on the Earth have led to a compilation of a vast database, which currently
55 contains more than 3.5 million environmental sequences [52]. The current measurements of the
56 extent of diversity indicate that 0.5 to 2 million species may exist in the biosphere and that this is
57 an achievable amount for classification purposes [52]. On the other hand, it seems that there is
58 a redundancy in the environments studied, and that perhaps the search for novelty might be
59 more successful in unexplored systems [52]. This may also hold true for the cultivable fraction,
60 and perhaps unexplored environments should be studied in order to retrieve novel strains.
61 Additionally, large-scale cultivation may also be successful in retrieving members of the rare
62 biosphere [38].

63 The screening of large sets of organisms may require extensive (and to some extent
64 expensive) work by means of genetic studies, such as partial sequencing of 16S rRNA genes
65 [54], molecular fingerprints [16], phenotypic analyses, fatty acid [13] or polar lipid profiles [24,
66 44], and infrared mass spectroscopy [51]. Of special relevance, given its relatively low cost and
67 reliable screening of a large number of cultures, is MALDI-TOF mass spectrometry using whole
68 cell biomass [50]. This approach has been shown to be very effective in sorting almost 290,000
69 clinical isolates in a relatively short period of time, as well as in the identification of rare bacterial
70 species that may be implicated in pathogenesis [46]. Moreover, this technique was successfully
71 applied for the identification of clusters of isolates in a given environmental sample as single but
72 non-clonal species [34].

73 The different disciplines that can benefit from large culture screenings range from very
74 applied sciences, such as biotechnology, to taxonomy which is one of the most fundamental
75 disciplines. Actually, taxonomic practices changed drastically at the beginning of this century
76 when species descriptions based on a single isolate overtook those with two or more strains
77 [48]. In the International Journal of Systematic and Evolutionary Microbiology, between June
78 2013 and June 2014, 82% of the published species descriptions included one strain, 8.3% had
79 two strains, 5.3% had three strains and 3.6% had four or more strains. The tolerance for
80 classifying taxa with a single isolate has greatly increased the speed of describing cultured
81 diversity. However, the description of a given taxon based on just one representative has been
82 criticized as inaccurate scientific practice [10,14] because these descriptions may not reflect the
83 actual diversity of the taxon. However, others have justified this practice since the whole
84 biological diversity must be described with reasonable speed [12]. In order to overcome the
85 difficulties in isolating several organisms of the same taxon, the screening of large sets of
86 cultures may be of help.

87 Hypersaline environments, such as crystallizer ponds of solar salterns, are extreme
88 environments characterized by a reduction of microbial diversity with increasing salt
89 concentrations [32]. The dominant organisms inhabiting these environments belong to the
90 archaeal domain, whereas members of the bacterial domain are generally less abundant
91 [5,19,20,32]. Molecular microbial ecology studies have revealed the archaeal taxa
92 *Haloquadratum walsbyi* (the so-called “square archaeon”) and the recently described
93 *Nanohaloarchaea* [19] as highly abundant. On the other hand, *Halorubrum*, *Haloferax*,
94 *Halobacterium* and *Haloarcula* were the dominant genera recovered by cultivation techniques
95 [49]. The most abundant bacterial genera thriving in such environments, as revealed by both
96 culture-dependent and -independent methods, were *Salinibacter* and *Salicola* [5,33]. In general,
97 diversity studies have been performed mostly in brines [5,15,20,34], with very few in
98 corresponding sediments [29].

99 Most of the current studies on the diversity of halophilic microorganisms in hypersaline
100 systems have been performed by means of culture-independent molecular techniques, such as,
101 for example, on either 16S rRNA gene diversity [20] or by metagenomic approaches [15].

102 Despite the fact that molecular studies describe to a great extent the taxonomic and genetic
103 diversity of the key players in their environments, they have failed to culture living organisms
104 that can be potentially important sources of information for biotechnological, pharmaceutical and
105 even taxonomic purposes. Culturing techniques may satisfy the needs of many microbiologists,
106 as exemplified very well by the statement of Steve Giovannoni that “Nothing beats actually
107 having the organism in culture” [8].

108 In the current study, the isolation and identification of over 4,200 extremely halophilic
109 strains from eight different locations in the world are presented by means of a tandem approach
110 using Matrix-Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry (MALDI-
111 TOF/MS) and 16S rRNA gene sequencing. The study confirmed that the approach was very
112 suitable for understanding the diversity of the culturable fraction, as well as for isolating rare
113 representatives of known taxa. Moreover, the results pointed to the fact that extending the
114 studies to scarcely explored (e.g. hypersaline sediments in comparison to brines) or as yet
115 unexplored sites (e.g. South American salterns) enhanced the success of retrieving
116 representatives of novel taxa.

117

118 **Materials and methods**

119 *Samples and processing*

120 Sediment and brine samples for this study were obtained from eight different solar salterns:
121 S'Avall (AV) and Campos (CA), both from the island of Mallorca, and Formentera (FM), all three
122 located in the Balearic Islands; Janubio (LZ) and Fuerteventura (FV) both located in the Canary
123 Islands; La Trinitat (ST) in Tarragona, and Santa Pola (SP) in Alicante, both on the east coast of
124 the Spanish peninsula; and Lo Valdivia (LV) located on the coast of Curicó in Chile (Table 1). At
125 each location the samples were taken from two different crystallizers. Brines were collected in 1
126 L sterile flasks from three different sampling points in the ponds. Triplicates of the sediment
127 samples were taken with methacrylate cores, as previously reported [28]. Samples were
128 transported to the laboratory within 24-48 h after collection and processed immediately. Brines
129 were directly diluted and plated. The three sediment cores were initially sliced, the first 0.5 cm
130 and the overlying salt crust were removed, and the following 30 cm were homogenized and
131 further diluted for cultivation purposes.

132

133 *Growth media, plating and isolation*

134 In all cases, a surface-spread plating method was used to isolate aerobic heterotrophic extreme
135 halophiles. One milliliter of homogenized sediment or 1 mL of brines were used to prepare the
136 serial dilutions (to 10^{-5}) in seawater medium (SW) at a salt concentration of 25% [43]. All
137 samples and their respective dilutions were plated in duplicate on SW at two different salt
138 concentrations: 20% and 30%. In both cases, Yeast Extract (YE, Cultimed Panreac Química
139 S.A.) was added at a final concentration of 0.05% as a carbon and energy source. Plates were

140 incubated at room temperature (22 °C) for at least one month until growth was observed.
141 Approximately 100 colonies from each sample (i.e. each of the duplicate samples of brines or
142 sediments, and at the two respective growth conditions) were selected taking into account
143 different size, morphology and color in order to obtain the largest diversity possible. Selected
144 colonies were brought to pure culture by re-streaking them on solid media ensuring the recovery
145 of a single morphology for each. For storage purposes, individual isolates were grown in liquid
146 medium (SW 20% and 30% with 0.05% YE), and the resultant suspensions were mixed with
147 40% (v/v) glycerol and stored at -80 °C. Subculturing of the glycerolated strains reactivated
148 approximately 95% of the collection checked.

149

150 *MALDI-TOF analyses*

151 The initial screening of the isolated strains was carried out with MALDI-TOF MS using whole cell
152 biomass, as previously published [34]. All isolates were refreshed by replicating them onto agar
153 plates with their respective isolation media (i.e. 20% or 30% SW with 0.05% YE). Cells were
154 grown until the colony size was approximately 1 mm in diameter. A small amount of biomass (1-
155 2 mg) was picked from the agar plates with a 1- μ L sterile plastic loop, and deposited onto a
156 ground steel 384-target plate (Bruker Daltonik Leipzig, Germany). Samples were overlaid with 2
157 μ L of matrix solution (saturated solution of α -cyano-4-hydroxy-cinnamic acid in 50% acetonitrile
158 and 2.5% trifluoroacetic acid) and air dried at room temperature. Measurements were
159 performed with an Autoflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Leipzig,
160 Germany) equipped with a 200 Hz Smartbeam laser. Spectra were recorded in the linear,
161 positive mode at a laser frequency of 200 Hz within a mass range from 2000 to 20,000 Da. The
162 IS1 voltage was 20 kV, the IS2 voltage was maintained at 18.7 kV, the lens voltage was 6.50
163 kV, and the extraction delay time was 120 ns. For each spectrum, approximately 500 shots at
164 different positions of the target spot were collected and analyzed. The spectra were externally
165 calibrated using the Bruker Bacterial Test Standard (*Escherichia coli* extract including the
166 additional proteins RNase A and myoglobin). Calibration masses were as follows: RL29 3637.8
167 Da; RS32, 5096.8 Da; RS34, 5381.4 Da; RL33meth, 6255.4 Da; L29, 7274.5 Da; RS19,
168 10,300.1 Da; RNase A, 13,683.2 Da; myoglobin, 16,952.3 Da. Spectra analyses were carried
169 out with BioTyper software 3.0 (Bruker Daltonics) and were used to construct similarity
170 dendrograms. Each single similarity cluster in the dendrograms was regarded as an operational
171 taxonomic unit (OTU), and this was the minimal unit used for further identification by means of
172 16S rRNA gene sequence analysis.

173

174 *PCR amplification and sequencing of 16S rRNA genes*

175 16S rRNA gene PCR amplification of the selected isolates was performed by taking a small
176 amount of biomass with a sterile toothpick and directly suspending it in the PCR mix. The
177 reaction mix (50 μ L final volume) contained 5 μ L of 10x Ex Taq™ buffer (20 mM MgCl₂), 1 μ L of

178 each forward and reverse primers (10 μ M each), 4 μ L of dNTP Mix 10x (25 μ M each) and 0.25
179 μ L Taq polymerase TaKaRa Ex Taq™ (Takara Bio Inc, Japan; 5 units/ μ L). Amplification for the
180 *Bacteria* domain was conducted using the universal [24] primers GM3 (5'-
181 AGAGTTTGATCATGGCTCAG-3') and S (5'-GGTTACCTTGTTACGACTT-3'). For the archaeal
182 domain the primers used were 21F (5'-TTCCGGTTGATCCTGCCGGA-3' [11] and 1492R (5'-
183 TACGGYTACCTTGTTACG-3' [25]. The amplification reaction was performed in a
184 Mastercycler® gradient (Eppendorf, Germany) using the following steps: one denaturing cycle at
185 94 °C (5 min) and 35 cycles of: 94 °C (1 min), 55 °C (30 s), 72 °C (2 min); and a final extension
186 step at 72 °C (10 min). Electrophoresis was performed in a 1% agarose gel, and visualization
187 was carried out after staining with ethidium bromide. PCR products were purified with MSB®
188 Spin PCRapace (INVITEK GmbH, Berlin), following the manufacturer's indications, and then
189 sent for sequencing to Secugen S.L. (Spain). The sequences have been deposited in the public
190 repositories with the entries LN649797 to LN650054.

191

192 *Tree reconstructions*

193 Sequences were reviewed, corrected and assembled using Sequencher v4.9 software (Gene
194 Codes Corp., USA). Alignments and tree reconstructions were performed using the ARB
195 software package version 5.5 [30]. The new sequences were added to the reference datasets
196 SILVA REF111 and LTP115 [42, 53], respectively, and aligned using the SINA tool (SILVA
197 Incremental Aligner, [41]) implemented in the ARB software package. Final alignments were
198 manually improved following the reference alignment in ARB-editor. Complete sequences were
199 used to reconstruct *de novo* trees using the neighbor-joining algorithm, while the partial
200 sequences were added into a pre-existing tree using the ARB-Parsimony tool, both
201 implemented in the ARB software package. Sequences were grouped in operational
202 phylogenetic units (OPUs) as an alternative to using strict cut-off values of identity thresholds in
203 order to identify isolated clades derived from the phylogenetic tree topology that produce
204 biologically meaningful units [16,29]. An OPU was considered as the smallest clade containing
205 one or more amplified sequences affiliating together with reference sequences available in the
206 public repositories. When possible, the OPU should include a type strain sequence present in
207 the LTP database [53], and for identity values >98.7% with type strain sequences the amplicons
208 were considered to belong to the same species using this conservative threshold, as previously
209 recommended [47]. On the other hand, for the identity values <98.7% and >94.5% with the
210 closest relative type strain 16S rRNA gene sequence of the same OPU, the amplicons were
211 considered to be the same genus (according to Yarza et al. [52]) but from a different
212 unclassified species.

213

214 *Statistical analyses*

215 The presence or absence of isolates detected for each OTU was coded as a binary matrix and
216 imported into the statistical program. Data ordination was undertaken considering location and

217 type of sample (sediment or brines). Non-metric multi-dimensional scaling (nMDS) was
218 performed using PRIMER 5 software version 5.2.8 (PRIMER-E Ltd., UK) and the previous
219 matrix distance was elaborated using the Euclidean distance. Rarefaction curves were
220 calculated using PAST software version 1.82b [22]. Good's coverage values were also
221 calculated in order to estimate the diversity coverage of the strain collection [21].

222

223 **Results**

224 *Isolates and MALDI-TOF MS analyses*

225 A total of 32 different samples (sediments and brines of two crystallizer ponds in each of the
226 eight sampled salterns) were screened for the cultivable fraction of heterotrophic aerobic
227 extreme halophilic microbiota. In all cases, the salinities in the crystallizer ponds were higher
228 than 27%, ranging between 27% in ST2 and 37.6% in LV2 (Table 1). Cultivation yields from the
229 different samples and media were very variable, ranging between 3.2×10^4 colony forming units
230 per milliliter (CFU/mL) in FM brines (on 30% salinity medium) and 2.05×10^6 CFU in FV brines
231 (with the 20% salinity medium) (Supplementary Table S1). Unexpectedly, no growth was
232 obtained at 30% SW for the FV sample. It was intended to cover the widest diversity range
233 possible by selecting all colonies with distinguishable morphologies, sizes and colors from the
234 incubated agar plates at SW salt concentrations of 20% and 30%, from their respective brines
235 or sediments, with a minimum of 77 strains for each sample and condition. A total of 5,076
236 isolates were recovered, with a minimum of 378 isolates from FV and a maximum of 792 from
237 CA. More than 720 isolates were isolated from five samples (SP, AV, CA, LZ and LV).

238 All isolates were analyzed by whole-cell MALDI-TOF/MS within the 4 weeks following
239 their isolation to pure cultures. Spectrometric profiles were manually inspected and only those
240 with a stable baseline and good signals were considered for further analysis. After sieving the
241 profiles, the discarded fraction ranged between 3.5% and 22% (SP and AV, respectively) of the
242 initial dataset. Poor baselines could have been due to the salt present in the culture medium,
243 but for pragmatic reasons bad profiles were discarded. The number of valid spectra was
244 approximately 86% of the total measured (Supplementary Table S1). In order to generate a
245 global dendrogram (Supplementary Figure S1) and select representative strains, dendrograms
246 for each location were constructed (Supplementary Figures S2 to S9). Independent clusters of
247 profiles were recognized as different operational taxonomic units (OTUs) following similar
248 criteria in previous studies [34]. In general, two different major clusters (with the exception of LV
249 and AV) at each location could be determined that, upon phylogenetic inference, could be
250 distinguished as *Bacteria* or *Archaea* (Supplementary Figures S2 to S9), respectively. For
251 further analysis, members of both domains were treated independently. The global archaeal
252 dendrogram (Supplementary Figure S1A) was constructed with 1,017 representative profiles
253 with a total of 73 OTUs: 46 OTUs were formed from isolates originating in only one location; 18
254 OTUs were from two to three locations; and 9 OTUs from four or more locations. In this regard,
255 OTU 23 consisted of isolates from the eight solar salterns analyzed. Furthermore, 24 OTUs

256 were represented only in sediment isolates, and 4 OTUs only from brine isolates. On the other
257 hand, the global bacterial dendrogram (Supplementary Figure S1b) was constructed with 1,226
258 profiles and exhibited a much simpler composition where only 6 OTUs could be distinguished. It
259 was remarkable that the LV and AV samples did not render any bacterial isolate. OTU 74
260 harbored the majority of the profiles (1,161 strains isolated from all samples except LV and AV).
261 Five OTUs embraced isolates from both sediment and brine samples, and the other one was
262 composed of strains originating only from sediment samples.

263

264 *Affiliation of the OTUs corresponding to the archaeal fraction*

265 Since it was intended to construct a spectra database of extreme halophilic microorganisms, a
266 large set of representative strains from the samples studied initially (LV, CA, AV and SP) was
267 selected for 16S rRNA gene sequencing. For this purpose, an attempt was made to cover the
268 maximum diversity in each dendrogram. One strain within each OTU was selected for
269 sequencing of its almost complete 16S rRNA gene, and two or more additional strains only for
270 partial sequencing. For the latter studied samples (ST, FM, LZ, and FV), the sequencing effort
271 was reduced significantly as most of the OTUs detected could be readily identified (Table 1).
272 The representatives of each OTU were used to reconstruct a domain phylogeny and recognize
273 the different OPUs present in the samples.

274 From the archaeal phylogenetic reconstruction (Figure 1), 35 OPUs could be identified
275 that affiliated with 15 distinct putative genera and 25 species within the family *Halobacteriaceae*,
276 using the conservative thresholds of 94.5% [52] for the genus category, and 98.7% for species
277 [47]. Among them, four putative novel genera and 17 additional novel species were recognized
278 (11 with identity values below 98.1% with their closest relative sequence of an existing type
279 strain; Figure 1, Table 2). The OPUs affiliated with the genera *Halorubrum* (*Hrr.*; 2,251 isolates),
280 *Haloarcula* (*Har.*; 126 isolates), *Haloterrigena* (*Htg.*; 121 isolates), *Halolamina* (*Hlm.*; 94
281 isolates), *Haloplanus* (*Hpn.*; 94 isolates), *Haloferax* (*Hfx.*; 83 isolates), *Halonotius* (*Hns.*; 61
282 isolates), *Natronomonas* (*Nmn.*; 60 isolates), *Halovivax* (*Hvx.*; 53 isolates), *Halomicrobium*
283 (*Hmc.*; 51 isolates), *Halogeometricum* (*Hgm.*; 38 isolates), *Halobellus* (*Hbs.*; 17 isolates),
284 *Halorientalis* (*Hos.*; 13 isolates), *Natronoarchaeum* (*Nac.*; 12 isolates) and *Halobacterium* (*Hbt.*;
285 3 isolates) (Figure 1). Since colony selection was not random (as the highest diversity possible
286 was sought by identifying different colony shapes) no diversity indices could be deduced.
287 However, when analyzing the rarefaction curves (Supplementary Figure S10), they were
288 already saturated when the collection size was ~300 colonies. In all samples, the number of
289 colonies in the study largely exceeded this number and in most of them it was double.
290 Moreover, the *minimum sample size* (i.e. the smallest number of colonies to be selected from
291 each sample to obtain enough representativeness of the total cultivable) recommended for each
292 sample collection [31] (Supplementary Table S2) was exceeded between two to four fold.
293 Altogether, the results agreed with the calculated Good's indices that, in all cases, were greater
294 than 95.8% of the total expected culturable diversity. Therefore, we could be confident that a

295 considerable large fraction of the culturable diversity was covered under the conditions of this
296 study.

297 The branch comprising the *Halorubrum* genus was the most represented and accounted
298 for 2,251 strains representing 52% of the total, and 71% of the archaeal isolates. Moreover, with
299 this genus 14 out of the 35 OPU of this domain could be affiliated. Among the 14 OPU of this
300 lineage, one putative new genus (OPU 14) and eight putative new species of *Halorubrum*
301 (OPUs 2, 3, 6, 7, 10, 11, 12 and 13) could be identified. OPU 14, with 27 isolates, appeared as
302 an isolated branch, and the closest relative was *Hrr. tibetense* with a 92.3% 16S rRNA
303 sequence identity. The remaining OPU detected affiliated with classified *Halorubrum* species
304 with identity values above 98.7%. Most of the OPU were present in two or more locations, and
305 OPU 8 was the only one detected in one sample (LV). The clade comprising *Hrr. californiense*
306 (OPUs 1, 2 and 3), with 955 isolates, was the largest (23.1% of the total and 42.4% of the
307 genus) and was present in high numbers at all locations except LV. Contrarily, LV showed
308 higher representation of OPU 7, 8, and 9 that were closely related to *Hrr. coriense* (112
309 isolates; OPU 7) and *Hrr. litoreum* (211 isolates; OPU 8 and 9). Almost all OPU affiliating with
310 *Halorubrum* were isolated from both brines and sediments. Interestingly, OPU 14 was isolated
311 only from sediment samples in CA, SP and LZ.

312 The branch comprising the genus *Haloarcula* was the second most diverse and
313 accounted for 126 strains that represented 3.1% of the total, and 4.3% of the archaeal isolates.
314 The lineage harbored seven OPU, four of which (OPUs 24, 25, 26 and 28) were putative new
315 species, and one was different enough to be considered as a putative new genus (OPU 27 with
316 93.8% identity to the closest type strain *Har. salaria*). The presence of *Har. hispanica* (13
317 isolates in OPU 22), *Har. salaria* (66 isolates in OPU 23, 24, 25, 26 and 27) and *Har.*
318 *marismortui* (47 isolates in OPU 28) species could also be identified. However, this genus was
319 unevenly represented as only LV, FV and FM samples contained these isolates. LV exhibited
320 the highest OPU diversity, and OPU 22, 25, 26 and 28 were exclusively found in this location.
321 Similarly, OPU 23 and 27 were exclusive to FV (Table 3[CJR1]). All other archaeal branches
322 detected were represented by only one OPU, and the representatives of the genera *Haloferax*,
323 *Halolamina* and *Haloplanus* were isolated in four or more locations.

324 All samples rendered between 11 to 15 OPU, except for LV that showed the highest
325 richness with 22 OPU (Table 2). In general, brines showed smaller numbers of OPU than
326 sediments. The former presented a minimum of 9 OPU at CA and a maximum of 18 OPU at
327 LV, whereas sediments presented a minimum of 11 at CA and LZ, and a maximum of 21 OPU
328 at LV. Only ST exhibited the same number of OPU in both brines and sediments. In this
329 regard, 26 of the 35 archaeal OPU were isolated from both sediment and brine. *Hbt. noricense*
330 (OPU 31) was a unique group recovered only from brines, and was only present in LV.
331 Contrarily, the putative new genus OPU 14, as well as OPU 17 (*Hgm. rufum*), OPU 21
332 (*Natronoarchaeum* sp.), OPU 23 (*Haloarcula* sp.), OPU 25 (*Haloarcula* sp.) and OPU 26
333 (*Haloarcula* sp.), were only isolated from sediment samples.

334 In some cases, different OTUs (i.e. clusters based on MALDI-TOF MS profiles) affiliated with
335 the same OPU (i.e. unique phylogenetic clades affiliating the new isolates with reference
336 sequences; Supplementary Figure S1). For example, OPU 1 embraced OTUs 5, 22 and 28.
337 However, the reconstruction based on the 16S rRNA gene showed that each OTU represented
338 slightly distinct lineages within the OPU, indicating that they could represent different
339 populations of the same species. Contrarily, there were few cases (OTUs 29, 32 and 65) where
340 the isolates of the same cluster affiliated with two different OPUs (e.g. OTU 29 affiliated with
341 OPUs 10 and 11 that corresponded to *Hrr. arcis* with 96.2% and *Hrr. aidingense* with 97.9%
342 sequence identities, respectively). However, in all such cases, a detailed observation of the
343 MALDI-TOF MS clustering topology (Supplementary Figure S11) showed two slightly different
344 subpopulations that clustered below the threshold settings.

345

346 *Affiliation of the OTUs corresponding to the bacterial fraction*

347 The bacterial set of isolates was much less diverse (Figure 2). All isolates affiliated with five
348 genera, with *Salinibacter* (1,163 isolates) being the most commonly retrieved organism,
349 followed by the very low occurrence of *Salicola* (21 isolates), *Halovibrio* (5 isolates), *Rhodovibrio*
350 (31 isolates), and *Pontibacillus* (10 isolates). The percentages of bacterial isolates varied
351 between the different locations and ranged between 26.7% (FM) and 64.6% (FV) (Table 1).
352 Surprisingly, no bacterium could be isolated from more than 1,188 strains at the LV and AV
353 locations. *Salinibacter ruber* was the most retrieved species among the bacterial isolates with
354 nearly 95% of the total (corresponding to OPUs 36 and 37). Interestingly, one isolate of OPU 37
355 affiliated with the sequence of the hitherto uncultured phylotype II (EHB-2) of *S. ruber* species
356 [5]. Sequences from genus *Rhodovibrio* (OPU 40) were retrieved in FM and CA, *Salicola* (OPU
357 38) in ST and SP, *Halovibrio* (OPU 39) in ST and *Pontibacillus* (OPU 41) in SP. OPU 38,
358 affiliating with *S. marasensis* (DQ019934), possibly represented a novel species of the genus
359 *Salicola* with 97.7% 16S rRNA sequence identity with the closest relative.

360

361 *Detection of putative novel taxa*

362 A total of 22 unique groups were detected among the 41 OPUs identified in the *Archaea* and
363 *Bacteria* domains (Figures 1 and 2), and they had 16S rRNA gene identities below conservative
364 thresholds with their closest relatives for species and genus (98.7% and 94.9% identity levels,
365 respectively). These comprised 53% of the total, and could represent 18 new species (labeled
366 with a white circle, Figures 1 and 2), and four new genera (labeled with a black circle, Figure 1).
367 Only one putative new species occurred in the bacterial domain. The majority of putative new
368 taxa were simultaneously isolated from different locations, such as OPUs 2, 3, 10, 11, 12, 14,
369 15, 19 and 20 that were common to at least three different locations (Table 2). The single
370 southern hemisphere sample (LV) provided the highest number of new taxa, where 14 of the 22
371 potential new taxa were isolated, nine of which were shared by other samples (OPUs 2, 3, 7,

372 11, 12, 13, 15, 24 and 32; Figure 1). The remaining five species were exclusive to this sample
373 (OPUs 21, 25, 26, 28, and 34; Figure 1).

374 Analyses of the Euclidean distances between the different diversity measurements,
375 plotted as nMDS (Figure 3), showed that sediment diversity was coincident with that of the
376 overlaying brines. The diversity measurements of the LV, SP and ST samples exhibited larger
377 differences compared to those observed in the island samples (AV, CM, FM, LZ, and FV).
378 Among the samples studied, those from Chile (LV) exhibited the highest diversity and
379 heterogeneity.

380

381 Discussion

382 In this study, a comprehensive analysis is presented for the species retrieved from eight solar
383 salterns distributed among different locations in the Spanish Mediterranean, Canary Islands'
384 Atlantic and Chilean Pacific coasts by means of standard culture methods. A collection of 5,085
385 isolates was compiled and their MALDI-TOF/MS profiles were obtained. For pragmatic reasons,
386 approximately 16.5% inadequate profiles were discarded and a final set of 4,243 strains was
387 processed. This study may be regarded as one with the largest set of identified cultures
388 obtained from environmental samples. Although this culture set may seem small compared to
389 the one of 284,899 clinical isolates [46], it is comparable to the 3,626 isolates from bottled
390 natural mineral water identified by random amplified polymorphic DNA (RAPD) fingerprinting
391 and 16S rRNA gene analyses [16]. MALDI-TOF/MS profiling has been shown to be very
392 advantageous for analyzing the microbial diversity of the cultured fraction of environmental
393 samples [34]. This technique has also been applied to the study of isolates from sewage sludge
394 [45], PCB-contaminated sediments [25], intra-specific diversity of *S. ruber* [4], and identification
395 of 845 yeast strains isolated from grape musts [1].

396 The values obtained for the different indices used (i.e. rarefaction curves and Good's
397 coverage) gave us the confidence that most of the cultivable diversity was sampled using the
398 culture media and conditions established for this work. The tandem study combining MALDI-
399 TOF/MS and 16S rRNA gene sequencing rendered a total of 41 different OPUs, of which 22
400 could be regarded as putative new species according to their genealogic affiliation and identity
401 with the closest related type strain sequences (Figures 1 and 2). This observation was
402 reinforced by previous reports indicating that single clusters in the MALDI-TOF/MS dendrogram
403 (OTUs) can be regarded as individual species [34]. The diversity observed was in accordance
404 with haloarchaea shown to be the principal prokaryotic component of hypersaline habitats [2],
405 and the fact that bacteria (despite having been underestimated for decades) could constitute up
406 to 20% of their total diversity [5]. Our isolates were distributed among 35 distinct archaeal and 6
407 bacterial OPUs or species.

408 The most frequently retrieved bacterial species was *S. ruber*, which has been reported
409 to be the most relevant member of this domain thriving in brines [5] and is widely distributed in

410 many hypersaline systems worldwide [3]. One of the most remarkable results from this survey
411 was the unexpected successful isolation of representatives of phylotype II (OPU 37) (EHB-2;
412 [5]). This phylotype was reported to co-occur with *S. ruber* (EHB-1) in lower amounts, but has
413 been refractory to pure culture for more than a decade [5]. The large number of isolates
414 belonging to this taxon (over 1,100) permitted the recognition of two members of the second
415 phylotype (Figure 2), and was an example of the benefits of large-scale cultivation approaches.
416 It was remarkable that neither the Mallorcan AV nor the Chilean LV samples rendered a single
417 bacterial isolate. These results were very surprising because *Salinibacter* had been isolated in
418 previous studies from AV [35], and sequences of this bacterium and others had been retrieved
419 by a culture-independent pyrosequencing approach (unpublished data). This phenomenon
420 cannot be easily explained but could be related to either the culture media used (although this is
421 improbable given the previous isolation successes), or that the organisms in the samples were
422 in a “viable but not cultivable” state [36]. Other bacterial isolates were representatives of known
423 halophiles but to a much lesser extent, and some of them, such as *Salicola* and “*Pseudomonas*
424 *halophila*”, are of high relevance in hypersaline environments, with the latter actually being a
425 member of *Halovibrio denitrificans* [33].

426 The archaeal fraction was more diverse than the bacterial component, and all cultures
427 were members of the *Halobacteriaceae* [37]. Members of the genus *Halorubrum* were by far the
428 most frequently recovered in all samples. Actually, this genus accounts for the largest number
429 of species with validly published names within the *Halobacteriaceae* family [37], has been
430 exhaustively studied by means of multilocus sequence analysis (MLSA) and genome analyses,
431 and is a prominent example for understanding the genetic properties of the archaeal species
432 [18]. In fact, the members of this group have also been reported to be the most recovered
433 culture types in similar environments [6,35]. In all cases, most of the retrieved species of this
434 genus were related to *Hrr. californiense*, which was originally described from a crystallizer pond
435 at the Cargill Solar Salt Plant in California [40]. This species was especially relevant in numbers
436 in the Mediterranean and Atlantic sites, although it was present in all samples (Figure 1, Table
437 3[CJR2]). On the other hand, relatives of *Hrr. coriense* and *Hrr. litoreum* had a major relevance in
438 the Chilean samples. The second most recovered genus was *Haloarcula*, which is also known
439 for being a readily culturable haloarchaeon [6,35]. The remaining 13 cultured genera were less
440 abundant.

441 Almost all OPUs affiliated with known genera but, surprisingly, 22 of the 41 OPUs could
442 constitute new species considering the minimal conservative threshold of 98.7% (Table 2) 16S
443 rRNA gene identity [47]. However, even if this threshold was considered too conservative, 14 of
444 these OPUs shared 16S rRNA gene identities <98.1% with their closest relative type strains.
445 Moreover, among the putative new species, four of them exhibited identity values <94.5% with
446 the closest relative type strains, which is a threshold that can be considered to discriminate
447 between different genera [53]. The observation that approximately 50% of the detected OPUs
448 could be regarded as new unclassified taxa makes the approach of large-scale screening a
449 good source of taxonomic novelty.

450 It is remarkable that all taxa detected in brine samples were also retrieved from their
451 corresponding sediment fraction. Contrarily, not all taxa retrieved from sediments could be
452 isolated from their corresponding brines. In this case, sediments appeared to be a source of
453 higher diversity yields of aerobic heterotrophic extreme halophilic taxa compared to brines. The
454 sediments studied here were most probably anaerobic given their moody structure [CJR3](fine-
455 grained sediments exhibit a very low oxygen penetration which occurs only in the first mm, [9]),
456 their blackish color (because of the formation of FeS due to sulfate respiration), and that the first
457 0.5 cm (out of a 30 cm deep core) had been discarded. Actually, oxygen may already be a
458 limiting factor for aerobiosis in brines given its low solubility [2]. Hypersaline sediments are
459 much more diverse than the overlaying brines, containing larger amounts of bacterial
460 representatives and lower amounts of the archaeal domain [28]. However, among the archaeal
461 representatives, a significant proportion of the taxonomic diversity may correspond to
462 *Halobacteriales* that coexist with other methanogenic extreme halophilic archaea [28]. Not much
463 is known about the role of *Halobacteria* in anaerobic sediments, or whether they only occur as
464 inactive cells that have been sedimented from the overlaying brines. However, some
465 *Halobacteria* have been demonstrated to grow anaerobically by either fermentation or anaerobic
466 respiration using alternative electron acceptors, such as nitrate, dimethyl-sulfoxide or fumarate,
467 among others [2]. The fact that a larger diversity was retrieved in this study from the sediments
468 compared to the overlaying brines at each site might be related to either the higher abundances
469 of cells in the former or to the higher diversity in ecological niches given the distinct availability
470 of substrates and electron acceptors.

471 Finally, it was also remarkable that the largest source of diversity occurred in the
472 Chilean samples, from where most of the novel taxa could be retrieved, some of which were
473 exclusive to this site (i.e. OPUs 21, 25, 26, 28, and 34). As already hypothesized, studying
474 unexplored sites avoiding environmental sampling redundancy may constitute a source of
475 discovery for microbial novelty [52]. The Chilean salterns of Lo Valdivia were the most remote in
476 this study, and both the water origin and the artisanal operation for the salt production and
477 harvest may be responsible for the larger and novel diversity observed. In this regard, the
478 Chilean saltern operation differs significantly from the other salterns studied. Chilean salterns
479 are constructed with small ponds (approximately 50 m³) and water is manually transferred
480 between ponds of different salinities. The other salterns contain much larger brine bodies
481 (greater than 1,500 m³) and water is transferred through inlets with nearly continuous brine
482 feeding.

483 Altogether, the results of this study indicated that the strategy of screening large sets of
484 isolates constituted a proportional source of novelty. In addition, success in finding new taxa
485 may be enhanced by sampling as yet unexplored sites (such as LV here), or poorly studied
486 sources (such as hypersaline sediments here). The tandem approach combining MALDI-
487 TOF/MS and 16S rRNA gene sequencing allowed cultivable diversity to be studied at a
488 relatively low cost. Moreover, the large-scale screening of cultures provided an excellent
489 approach for gathering more than single strains representing new species from distinct samples

490 and sampling sites. This approach may help to avoid the important problems of understanding
491 intraspecific diversity promoted by the current practice of classifying taxa based on only a single
492 isolate [48].

493

494 **Acknowledgements**

495 The current study was funded with the scientific support given by the Spanish Ministry of
496 Economy through the projects CGL2012-39627-C03-01 and CGL2012-39627-C03-03, which
497 were also supported with European Regional Development Fund (FEDER) funds, and the
498 preparatory phase of the Microbial Resource Research Infrastructure (MIRRI) funded by the EU
499 (grant number 312251). TVP acknowledges the predoctoral fellowship of the Ministerio de
500 Economía y Competitividad of the Spanish Government for the FPI fellowship (Nr BES-2013-
501 064420) supporting his research activities. Finally, the authors acknowledge the help and
502 access to their infrastructures of all the salterns sampled in the study: Salines de Campos
503 (Oliver Baker); Salines de S'Avall (Family Zaforteza-Dezcallar); Salinas de Formentera S.L.
504 (David Calzada); Salinas del Bras del Port; Salinas de Janubio and Salinas del Carmen (David
505 Calzada); Salines de la Trinitat (Mateu Lleixà); Salinas de Lo Valdivia (Alejandro Chaparro, Sal
506 de Mar & Turismo Pacífico Central SpA).

507

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663

663 **Tables and Figures:**

664

665 Table 1. Solar salterns, location and salinity of the sampled ponds, percentage of the isolates
 666 corresponding to the archaeal and bacterial domains, number of partial and complete
 667 sequences of the 16S rRNA gene, number of OTUs and OPUs detected in sediment and brine
 668 samples and at each location.

669

670

Solar Saltern	Location and coordinates	Sampling date	% Salinity		Nr. OTUs	Archaea (%)	Bacteria (%)	Partial sequences	Complete sequences	Nr. OPUs			
			Cr. 1	Cr. 2						S	B	TOTAL	
Trinitat (ST)	Tarragona 40°34'22"N 0°39'13"E	June - 2010	29	27	18	65.3	34.7	23	0	14	14	14	
Santa Pola* (SP)	Alicante 38°11'5"N 2°37'46"W	June - 2010	32.8	34.4	23	54.5	45.5	27	11	14	10	15	
S'Avall* (AV)	Sant Jordi, Mallorca (IB) 39°19'26"N 2°59'22"E	October - 2010	28	31.5	13	100	0	35	8	13	10	13	
Campos* (CA)	Campos, Mallorca (IB) 39°20'46"N 2°59'57"E	October - 2010	33	31	14	66.4	33.6	12	11	10	9	11	
Formentera (FM)	Formentera (IB) 38°43'34"N 1°24'14"E	July - 2012	36	34	11	73.3	26.7	5	1	12	11	13	
Janubio (LZ)	Yaiza, Lanzarote (CI) 28°55'47"N 13°49'51"W	July - 2012	33.8	35	15	59.7	40.3	7	1	11	7	11	
Carmen (FV)	El Carmen, Fuerteventura (CI) 28°27'30"N 13°56'30"W	July - 2012	28	29.5	10	35.4	64.6	1	2	14	5	14	
Lo Valdivia* (LV)	Boyeruca, Chile 34°42'16"S 72°1'4"W	December - 2011	36.8	37.6	25	100	0	54	24	21	18	22	
						16.1 ^B	69.3 ^B	40.9 ^B	164 ^A	56 ^A	13.6 ^B	10.5 ^B	14.1 ^B

671 S: sediments; B: brines. ^ATotal, ^BMean, *Initial set of solar salterns analyzed, Cr: crystallizer pond, IB: Balearic Islands, CI: Canary

672 Islands

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674

674

675 Table 2. Distribution of isolates according to their origin and 16S rRNA gene sequence identity

676 with the closest relative type strains.

677

	Nº OPU	% Similarity	Number of isolates from solar salterns																
			Spanish Peninsula				Balearic Islands						Canary Islands				Chilean Coast		
			ST		SP		AV		CA		FM		LZ		FV		LV		
S	B	S	B	S	B	S	B	S	B	S	B	S	B	S	B				
<94.9%	14	92.3			10								13						
	15	93.2											16					10	5
	27	93.8													6	4			
	16	94.3	6	1					7	3					15	13			
95%-98.1%	26	95																10	
	28	95.1																23	24
	35	95.2			6	7													
	10	96.2	4	20	21				12				36		6				
	25	96.3																20	
	34	96.6																21	3
	20	97.2	4	8	15	1	10		10	5			24	12	5				
	21	97.2																12	
	38 ^B	97.7			9	12													
11	97.9			15				14	2	13	4			11			5	9	
24	98.1									10	4						1	10	
98.2%-98.7%	3	98.2	17	7	19	36	51	23	32	31	7	18	17	25	7			11	15
	6	98.2			25	16							15	19	2				
	19	98.3	5	8	4	1			25	5			18	11				14	3
	12	98.4	7	9	56		30	91	15	13		15			9	5		5	4
	7	98.5													1			38	73
	32	98.7					27											23	3
	2	98.7	15	9	20	12	54	47	23	20	8	15	20	15	14	6		5	5
	13	98.7					7					14	27		2	4		9	3
98.8%-100%	9	98.8	12	3			2	5				5	10					25	63
	23	98.9													5				
	29	98.9	6	1			6					12	26						
	1	98.9	21	11	37	17	32	21	45	57	10	20	31	15	12	14		10	8
	37 ^B	99	1		1														
	5	99	3	10	17						7	15							
	30	99.1	5	13	13	3						26							
	8	99.1																26	60
	33	99.2			5	18	35	3				14	22						
	22	99.3																1	12
	39 ^B	99.4	5																
	18	99.6	9	6					4	13	9			27				10	5
	17	99.6														13		32	
	40 ^B	99.6								22	4	5							
	41 ^B	99.6			3	7													
4	99.7	7	18	10	7	6	75	20	56	9	6	20	21	4					
31	99.7																		3
36 ^B	99.8	55	75	69	229			62	119	108	2	87	153	63	143				
TOTAL number of isolates			182	199	355	366	270	299	297	321	467	180	324	271	153	176	321	308	
Total number of new species per solar saltern			7	7	10	6	8	5	8	6	5	5	8	5	10	4	15	12	
			7BS		6BS + 4S		5BS + 3S		6BS + 2S		5BS		5BS + 3S		4BS + 6S		12BS + 3S		
Number of new species per solar saltern <98.2%			3	3	5	2	3	2	4	2	2	1	4	1	4	1	8	5	
			3BS		2BS + 3S		2BS + 1S		2BS + 2S		1BS + 1S		1BS + 3S		1BS + 3S		5BS + 3S		

678 ^BBacteria OPU[CJR10]

679

679

680 Figure legends:

681 FIGURE 1. Phylogenetic reconstruction based on 16S rRNA genes of the haloarchaeal isolates
682 and their closest representative type strains. The percentage sequence identity of each OPU
683 with the closest relative is indicated in brackets, and the type strain sequence used to calculate
684 the identities is framed in grey. In addition, sequences <94.9% were considered as putative new
685 genera (black star), and <98.7% as putative new species (white star). Novel taxa occurring in
686 the Chilean sample are indicated with a white circle when co-occurring in other sampling sites,
687 and a black circle when exclusive to this location. The numbering of the OTUs for each OPU is
688 given in the second column, and the third column indicates the location where the OPU was
689 present, and the number of isolates recovered in sediment (S) and brine (B) samples is in
690 brackets. [CJR11]

691

692 FIGURE 2. Phylogenetic reconstruction based on 16S rRNA genes of the bacterial isolates and
693 their closest representative type strains. The percentage sequence identity of each OPU with
694 the closest relative is indicated in brackets, and the type strain sequence used to calculate the
695 identities is framed in grey. In addition, sequences <94.9% were considered as putative new
696 genera (black star)[CJR12], and <98.7% as putative new species (white star). The numbering of
697 the OTUs for each OPU is given in the second column, and the third column indicates the
698 location where the OPU was present, and the number of isolates recovered in sediment (S) and
699 brine (B) samples is in brackets. [CJR13]

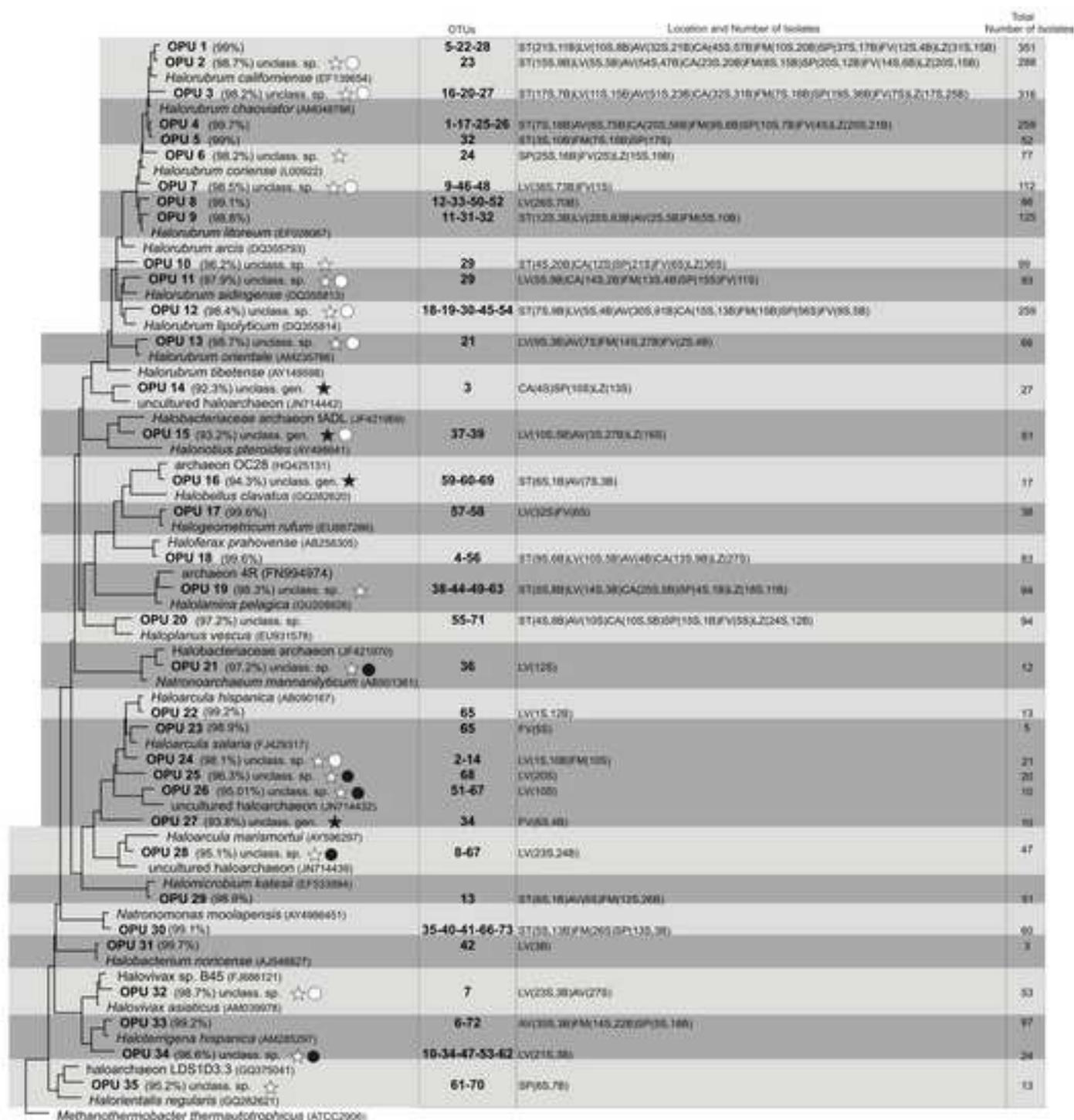
700

701 FIGURE 3. nMDS (non-metric multi-dimensional scaling) analysis based on Euclidean distances
702 considering the presence or absence of isolates for each OTU by location and type of sample
703 (sediment or brine). Squares indicate insular and triangles mainland samples. The abbreviations
704 of the symbols are: Trinitat (ST), Santa Pola (SP), Avall (AV), Campos (CA), Formentera (FM),
705 Janubio (LZ), Carment (FV) and Lo Valdivia (LV). The suffix -S indicates sediments and -B
706 indicates brines.

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