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Retinal-binding proteins mirror prokaryotic dynamics in multi-pond solar salterns

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Summary

Microbial opsin (i.e. retinal-binding protein) dynamics has been studied along a salinity gradient in Santa Pola solar salterns (Alicante, Spain) by using culture-independent approaches and statistical analyses. Five ponds of salinities ranging from 18% to above 40% were sampled nine times along a year. Forty three opsin-like sequences were retrieved by denaturing gradient gel electrophoresis and clustered into 18 different phylogroups, indicating that their diversity was higher than expected according to previous data. Moreover, the statistical correlation between environmental factors controlling microbial community structure and dynamics of environmental rhodopsin proteins indicated almost identical temporal fluctuations between the opsin-related sequences and their corresponding putative 'producers' in nature. Although most sequences were related to others previously detected in hypersaline environments, some pond-specific opsins putatively belonged to previously uncharacterized hosts. Furthermore, we propose that subtle changes in the bacteriorhodopsin 'retinal proton binding pocket', which is key in the photocycle function, could be the molecular basis behind a fine 'photocycle-tuning' mechanism to avoid inter/intra-species light-competition in hypersaline environments.

Introduction

Light-driven microbial rhodopsins are a widespread family of proteins whose function is to transform visible light into a physiological response. Rhodopsins are formed by a seven-helix transmembrane protein bound to a retinal chromophore, and are widely distributed in microorganisms inhabiting photic waters. Microbial retinal-binding proteins* (RBPs) in hypersaline systems mainly include outward-directed proton pumps, such as bacteriorhodopsin (BR) and related proteins, the inward-directed chloride pump halorhodopsin (HR), and sensory rhodopsins involved in light sensing for phototaxis (Oren, 2002). In these RBPs, light excites the retinal chromophore and subsequently a change of the native protein configuration occurs in a cyclic succesion of intermediate states leading the photocycle process. As a result, certain ions are released through the cell membrane to accomplish several functions, including ATP synthesis, flagellar motion or osmoregulation. Other prokaryotic rhodopsins, currently under characterization, have been recently detected, such as the xenorhodopin, a sensory RBP found in *Anabaena* sp. (Ugalde *et al*., 2011), and the NaR, a new type of microbial rhodopsin found in marine Flavobacteria, which acts as a light-driven sodium pump (Inoue *et al*., 2013).

*Note: The names BR, XR and ActR refer to the protein bound to retinal. The proper names of unbound proteins are bacterio-opsin, xantho-opsin and actino-opsin. Here, with the exception of BR purified from salterns, we work with the retinal-binding proteins coding genes and their translated sequences, which subsequently lack retinal. However, to avoid a tedious system of symbols, the following abbreviations will be

used throughout the text: BR for bacteriorhodopsin/bacterio-opsin, XR for xanthorhodopsin/xantho-opsin, ActR for actinorhodopsin/actino-opsin and RBP for the retinal-binding proteins as a whole.

Microbial rhodopsins were first discovered in extremely halophilic *Archaea*, such as *Halobacterium salinarum*, and later in other members of the family Halobacteriaceae, such as *Haloquadratum walsbyi*, the 'square' archaeon that dominates many hypersaline environments (Bolhuis *et al*., 2006). One of the best characterized archaeal rhodopsins is BR, whose functional features, together with the photoelectric, photochemical and photochromic properties, make it a suitable model for applied technologies, such as optic sensors and artificial retina (Birge *et al*., 1999; Hampp *et al.,* 2000; Wagner *et al*., 2013).

In the past decades, new types of light-driven proton pumping proteins, analogous to BR, were also found in the bacterial domain: proteorhodopsin (PR), xanthorhodopsin (XR) and actinorhodopsin (ActR). PRs were discovered in the surface waters of the oceans by means of metagenomics (Béja, 2001; Rusch *et al*., 2007; Fuhrman *et al*., 2008). XR is produced by *Salinibacter ruber* (Balashov *et al*., 2005), an extremely halophilic bacterium broadly distributed among different hypersaline environments (Antón *et al*., 2000, 2002, 2008). XR, besides the retinal chromophore, possesses the carotenoid salinixanthin acting as light-harvesting antenna and providing energy from a wider spectral range (Balashov *et al*., 2005). Although in past years several XR-coding genes have been found in *Gammaproteobacteria*, *Flavobacteria* or *Betaproteobacteria* members (López-Pérez *et al*., 2013; Riedel *et al*., 2013; Oh *et al*., 2011), there is no evidence of a light-harvesting carotenoids in any of them yet. More recently, ActRs, associated with uncultured *Actinobacteria* have been recovered from freshwaters, estuarine and lagoon sites (Sharma *et al*., 2007, 2008) and also from hypersaline waters (Ghai *et al*., 2011).

Many rhodopsins from halophilic microorganisms (Otomo *et al*., 1992; Kamekura *et al*., 1998; Yatsunami *et al*., 2000; Brown *et al*., 2001; Balashov *et al*., 2005; Luecke *et al*., 2008) have been exhaustively characterized. However, only a few studies have been focused on natural ecosystems. In particular, Oren (1983) and Javor (1983) analyzed the BR production by the haloarchaeal populations inhabiting two hypersaline environments: The Dead Sea and Guerrero Negro saltern ponds (Baja CA Sur, Mexico), respectively. Later, Papke *et al*., (2003) and Pašić *et al*., (2005) studied the diversity of BR genes in Mediterranean and Adriatic solar salterns, respectively. Recently, Dillon *et al*., (2013) studied the prokaryotic community structure and the BR genes recovered from three ponds of increasing salinity in the Exportadora de Sal (ESSA) salterns (Baja CA, MX), finding new bacterio-opsins, some of them "pond-specific".

Here, we have analyzed RBP diversity in five hypersaline interconnected ponds of increasing salinity from Santa Pola salterns (Spain), spanning a salt gradient from 18% to more than 40%. These ponds were two medium concentrators (CMs: CM1 and CM2), a brine concentrator (CCAB), and two crystallizers (CRs: CR30 and CR41). Previously, we had studied the particular physicochemical and microbial diversity characteristics of these same five ponds (Gomariz *et al*., 2014) and found that the microbial community structure was strongly correlated with ionic composition and meteorological factors (Gomariz *et al*., 2014). The microbial community was dominated by the well-known halophilic prokaryotes *Hqr. walsbyi* and *S. ruber*, together with members of the recently reported groups of uncultured *Nanohaloarchaeota*, low GC *Actinobacteria* and a new group of ubiquitous, previously unrecognized *Bacteroidetes* generalists. In addition, our data indicated that

103 although both crystallizers were fed with the same water, different "pond-specific"
104 phylotypes were detected together with significant variations in microbial community
105 structure and temporal dynamics. Overall, we observed that variation in archaeal and
106 bacterial diversity was much higher than expected for solar salterns, considered to date as
107 steady-state systems.
108 The main goal of the work presented here was to assess RBP temporal dynamics along a
109 year and correlate it with microbial dynamics and environmental parameters. Overall, we
110 found that RBP diversity followed the same trends as their putative microbial producers. In
111 addition, some of the BR-like sequences obtained displayed amino acid changes in
112 homologous positions of the predicted 'retinal-binding pocket' which could be involved in
113 photocycle-tuning mechanisms.
114
115 **Results and discussion**
116 ***Diversity of archaeal bacteriorhodopsins and BR quantification in solar saltern ponds***
117 Around 30 sequences related to genes coding for bacteriorhodopsins were obtained from
118 the DGGE analysis (Figure S1; see experimental procedures for more details). These
119 sequences were clustered into 16 groups according to their phylogenetic relatedness based
120 on nucleotide similarities (Table S1). Each one of the resulting groups corresponded to a
121 single and robust branch (hereafter named as 'phylogroup') in the phylogenetic tree shown
122 in Figure 1. As shown in Table 1 and Figure 1, a large proportion (80%) of BR-like
123 sequences, recovered in nearly all the samples, were clearly related to *Hqr. walsbyi*
124 bacterio-opsin SbopI (phylogroups 9 to 15) and *Halorubrum* sp. archeo-opsin (groups 1

and 6) with amino acid similarities above 60% in the fragment analyzed. Sequences from phylogroups 2 to 5 were, surprisingly, related to BR-genes from microorganisms which had not been previously detected in these samples by 16S rRNA gene amplification (Gomariz *et al*., 2014), such as *Natrinema*, *Halosimplex*, *Halonotius* and *Halorhabdus* representatives. These rhodopsins were only retrieved from specific ponds in a few samples (see Table 1).

Around 7% of the RBP sequences obtained in this work (Table 1) had been previously detected in Santa Pola salterns (Papke *et al*., 2003). In the case of BR sequences, despite the geographical distance, ≈20% and 60% of the sequences were similar to those from Lake Tyrrell in Australia (Podell *et al*., 2013) and ESSA saltern ponds in Baja California, Mexico (Dillon *et al*., 2013).

In addition to the diversity study, an analysis of the BR protein production was accomplished by SDS-PAGE (Figure S2), where a highly stained protein band of nearly 26 kDa (molecular weight determined for purified BRs) was detected in all the samples analyzed. MALDI-TOF analysis showed a peptide sequence (9 amino acids) identical to a fragment of the 'chain A' present in the BR (SbopI) from *Hqr. walsbyi*. Given that SbopI is a predominant protein in the *Hqr. walsbyi* membrane (Fusetti and Poolman, unpublished), and this haloarchaeon represents up to 60% of the microbial communities in evaporation ponds from solar salterns (Antón *et al*., 1999), it is very likely that the observed 26 kDa band corresponds indeed to SbopI.

The concentration of bacteriorhodopsin in the analyzed samples, determined by densitometry from SDS-PAGE images, ranged from 20 to 340 μg/ml (Table S2). The

highest amounts of BR corresponded to the summer samples (June 1, June 2 and July) from the crystallizer ponds CR30 and CR41. As discussed below, there was a strong correlation between BR production, PAR, salinity and archaeal numbers, whose values were very high in these summer samples. Previous works in the Dead Sea and Guerrero Negro saltworks (Javor *et al*., 1983; Oren *et al*., 1983) attributed the high BR concentration to the oligotrophic nature of these environments. The crystallizer pond waters studied here contained considerable amounts of both organic and inorganic nutrients, as well as dense prokaryotic communities (Gomariz *et al*., 2014, and unpublished results). However, according to Burns and colleagues (2007), magnesium ions, that can reach up to 2 M in these environments (Gomariz *et al*., 2014), could form complexes with dissolved nutrients, decreasing their availability to microbes. In addition, oxygen solubility decreases in these extremely hypersaline ponds. The combination of these factors could act in promoting the production of BR to obtain an additional energy supply.

Finally, it is also known that members from the recently described phylum Nanohaloarchaeota have been shown to present a photoheterotrophic lifestyle (Ghai *et al*., 2011) since they present genes which would code for BR proteins. We did not detect nanohaloarchaeal rhodopsin sequences since the PCR primers used here were designed before the discovery of this phylum in 2012.

Diversity of bacterial rhodopsins

After DGGE analysis, 13 sequenced bands were related to bacterial rhodopsins (Figures S3-4): XR (9 bands) and ActR (4 bands) (Table 1). XRs were detected in all the samples analyzed (Figure S3) and were all classified into one group according to their relatedness.

169 XR sequences showed amino acid similarities between 91% and 100% with *S. ruber* XR,
170 thus suggesting that they most likely correspond to *Salinibacter* producers. However, it is
171 possible that some of them could be related to different bacteria. In fact, it has been
172 recently discovered by means of single-cell genomics, that the ubiquitous hyperhalophilic
173 *Bacteroidetes* phylotype BC3, present in Santa Pola salterns, harbors a XR-coding gene
174 (Gomariz *et al*., 2014; SAG AB577O10). Other halophilic bacteria, such as the recently
175 described *Spiribacter* sp. (a moderately halophilic Gammaproteobacterium), also possesses
176 XR (López-Pérez *et al*., 2013). However, *Spiribacter* representatives were not detected in
177 the ponds analyzed here and its XR presents a similarity below 50% when compared with
178 the *S. ruber* XR.
179 The ActR sequences obtained here were related (77-81% similarities) to the
180 actinorhodopsin of *Candidatus* `Aquiluna rubra´, isolated from arctic fjord seawaters
181 (Kang *et al*., 2012). ActR sequences were detected from spring to early summer only in
182 low-medium salinity ponds CM1 and CM2 (212.5 to 350.13 g/L total salts) (Figure S4).
183 However, the detection of *Actinobacteria*-related phylotypes was not always coupled with
184 the presence of ActR sequences. One possible explanation for this paradox could rely on
185 the fact that ActR sequences are also known to be produced by *Gammaproteobacteria*, as
186 it occurs in other aquatic systems (Martinez-Garcia *et al*., 2012), maybe due to lateral gene
187 transfer events, as dicussed below. Members of *Gammaproteobacteria* were detected in
188 CM ponds (Gomariz *et al*., 2014).
189 ***Comparison between RBP and prokaryotic and environmental dynamics***

One of the main findings in this study was that both RBP and prokaryotic diversity dynamics were nearly parallel in the five ponds analyzed (Figure 2). RBP and prokaryotic diversities, determined by Shannon indices (H, Table S2), showed temporal fluctuations in each pond. Low and medium salinity ponds (CMs and CCAB) showed higher BR and archaeal diversity fluctuations than the crystallizers, which were more stable (Figure 2A). In addition, the highest Shannon index H values for BR and *Archaea* were found in the brine concentrator CCAB in June2 samples (H~2.2 for BR and H~2.4 for *Archaea*, respectively). Equivalent H indices for BRs were also determined in another medium-high salinity pond (Pond 9) from ESSA salterns (Dillon *et al*., 2013) while lower indices (H~1.4) had been previously observed in a 'single-sample' study carried out in Santa Pola and Portoroz salterns (Papke *et al*., 2003, Pašić *et al*., 2005).

Data from both the prokaryotic community of Santa Pola salterns (Gomariz *et al*., 2014) and rhodopsin diversity were arranged according to their environmental optima as a result of the CCA analysis (Figure 3 and Table S3). The term "optima" does not refer to growth parameters but to the biplot site where a particular phylotype presents the highest possibility to be found. In the CCA biplots: (i) those sequences located in the center of the diagram correspond to sequences retrieved from nearly all the samples; (ii) the distance between two sequences represents the probability of recovering them from the same sample given that they share their environmental optima; and (iii) the relative position of RBP and 16S rRNA sequences allows the suggestion of the relationship between rhodopsins and their putative producers. For instance, in the CCA space, a given *Haloquadratum* phylotype would be the most likely "producer" of its closest BR sequence.

Accordingly, different *Haloquadratum* and *Halorubrum*-related phylotypes appeared near their respective BRs, along the salinity gradient (Figure 3). These results were in agreement with previous diversity studies where *Sbop* gene clone libraries retrieved the expected sequences according to the analysis of the 16S rRNA genes (Papke *et al*., 2003, Pašić *et al*., 2005). However, there were also some intriguing findings since some of our RBP sequences were related to microorganisms that had not been detected in the same samples by the 16S rRNA approach, as discussed above and as was also reported by Dillon *et al*., (2013). Some of these "orphan" RBPs were pond-specific and month-specific (Table 1). Together with database limitations, this finding could be due to lateral transfer of RBP coding genes. Indeed, it is well known that microbial rhodopsins present a patchy distribution in prokaryotes (Frigaard *et al*., 2006), and in particular, in haloarchaeal lineages, due to lateral gene transfer processes (Sharma *et al*., 2007). This mechanism allows the organisms receiving RBP-coding genes to exploit more efficiently the energy sources available in the environment.

Contrary to BR, XR diversity was quite stable over the year in all the samples although the diversity of *Salinibacter*-related phylotypes was increasing along the salinity gradient (Figure 2B). Indeed, most of the obtained XR sequences were located in the center of the CCA diagram (Figure 3), showing their widespread distribution in the analyzed ponds. This observation could be indicating that, as discussed above, other bacterial members, apart from *Salinibacter* representatives, could contain XR-coding genes. Sequences related to ActR were placed, as expected, next to actinobacterial phylotypes in the low salinity optima conditions from the CCA biplot.

234 Regarding the biological-environmental data relationships, redundancy detrented analysis
235 (RDA) showed that the ionic composition (especially ammonium concentration), together
236 with PAR and the light hours per day, were the most significant environmental parameters
237 affecting the BR production in the ponds analyzed (Figure 4 and Table S4). Besides
238 salinity, meteorological factors were highly correlated with RBP diversity, prokaryotic
239 community abundance and prokaryotic diversity as well. In particular, BR and archaeal
240 diversities, as well as archaeal numbers, were strongly correlated with light, temperature
241 and solar radiation (Gomariz *et al*., 2014). However, bacterial rhodopsin diversities were
242 negatively correlated with bacterial diversity and showed a highly positive correlation with
243 calcium concentration and bacterial counts.

244 ***"Photocycle-tuning" of BR-like proteins***

245 BR alignments revealed that some of the amino acids in the obtained sequences changed
246 with respect to the closest rhodopsin relatives available in databases. Over the years, it has
247 been demonstrated that changes affecting certain amino acids could lead to changes in the
248 rhodopsin photocycle, such as displacement of the maximun absorption peak,
249 modification of the average life of the intermediates in the photocycle or a proton-pumping
250 direction inversion (Brown *et al*., 2001; Luecke *et al*., 2001; Sanz *et al*., 2001; Chaumont
251 *et al*., 2008 , Sudo *et al*., 2011).

252 In our study, although some amino acids involved in the 'retinal-binding pocket' (Trp137,
253 Trp182, Trp189, Thr89 and Asp115) were quite conserved, others (such as Glu194-
254 Gly200, in helix loop F-G, and Glu204-Val210, in helix G) presented modifications which
255 could play an important role in the photocycle. In particular, Glu194, Gly200 and Glu204

256 are known to be important residues in transduction signaling, color tuning or in the
257 regulation of certain functions such as energy production and motility (Shimono *et al*.,
258 2003; Sanz *et al*., 2011; Sudo *et al*., 2006; Sudo *et al*., 2011). Other amino acids, such as
259 Arg82 and Trp86 (in helix C), also play an important role in proton channel release;
260 however, we did not analyze this region of the protein since the amplified BR fragment
261 ranged from amino acid 90 to position 222. Taking into account the amino acid
262 similarities, our BR sequences were clustered in different groups (0-XIII), corresponding
263 to different putative types with respect to their response to light. Therefore, we will refer to
264 these 'functional groups' as 'photocycle-tuning' varieties based on their changed amino
265 acids (Table 2).
266 The relationship between BR phylogroups and functional varieties showed some
267 interesting features. For instance, sequences related to SbopI, within phylogroup 11,
268 presented different functional varieties (functional groups 0 and VII in Figure 1). In the
269 environment, this fact could probably portray a scenario where different *Haloquadratum*
270 coexisting lineages would be differentially specialized in the use of light in order to avoid
271 direct intraspecific competition, as discussed in Legault *et al*. (2006) and Cuadros-Orellana
272 *et al*. (2007). Additionaly, specific BR sequences related to different species (e.g.
273 *Halorubrum* spp. and *Haloquadratum* spp.) and thus clustered in different phylogroups
274 (phylogroups 6 and 7, respectively), belonged to the same functional group XIII. Indeed,
275 this variety (group XIII) was the most widely distributed among the saltern ponds analyzed
276 (Figure 5); while the remaining varieties presented a particular distribution along the
277 different environmental gradients (Figures 2 and 5). In addition, the CCA analysis (Figure

3) revealed that meteorological factors were correlated with the occurrence of the different varieties. For instance, two *Halorubrum* BR-related sequences (25br5 and 23br3), included in the same phylogroup (6) but in different functional varieties (XI and VI), were detected in different and opposite temperature optima conditions (Figure 3). Therefore, even though no significant temporal variations of phylogroup diversity was observed (Figure S5), we detected temporal fluctuations of the functional varieties (Figure 4). This points to a fine tuning of light exploitation which is most likely driven by environmental conditions.

Conclusion

The present study shows coherent spatial and temporal parallelisms between retinal-binding proteins and prokaryotic communities in solar salterns. A high number of different opsin-coding gene sequences has been successfully collected, mainly because this work has been carried out over a wide period of time. In fact, as Dillon and colleagues (2013) suggested "a global undersampling could be the main reason of a currently limited 'bop' environmental data set". Our results point to the existence of a spatial and temporal arrangement of different 'functional varieties' of BRs that could be part of a strategy to avoid light-competition in saturated brines. Future studies could thus focus on elucidating the implications of this "photocycle-tuning" in light-adaptation strategies of both bacterial and archaeal rhodopsin producers in hypersaline environments.

Experimental procedures

Collection and characterization of samples

Samples from five interconnected ponds of increasing salinity (CM1, CM2, CCAB , CR30 and CR41) from Santa Pola saltworks were taken during 2006, on the following dates: 23

January (JN), 7 March (MR), 26 April (AP), 6 June (J1), 27 June (J2), 25 July (JL), 5 September (SP), 2 October (OC) and 28 November (NV). Changes in prokaryotic communities in these samples and their relationship with 16 different environmental parameters have been described previously (Gomariz *et al*., 2014).

Bacteriorhodopsin quantification

Environmental water samples (500 ml) were centrifuged at 16,000 rpm (rotor JLA-16, centrifuge Beckman Coulter Avanti J-25) 15 min, at 4ºC. Then, pellet cells were washed and concentrated in basal salt solution, 500 µl SW25% (Rodríguez-Valera *et al*., 1983). Subsequently, cells were centrifuged at 13,000 rpm (Biofuge, HERAUS) and lysed in 500 µl mQ water. Then, cells lysates were sonicated (Bandelin electronic, Sonoplus GM2200) in three cycles of 5 s in ice to avoid heating of the membranes. Finally, membrane suspensions were lyophilized (Telstar, Cryodos 5) and kept at -20ºC until their electrophoretic analysis.

Total membrane proteins (50 µg) from environmental samples were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) following the protocol described in Laemmli *et al*. (1970). For each sample, two different amounts of protein (25 µg and 50 µg, dry weight) were analyzed. For one of the samples, a protein band with a molecular weight similar to that of bacteriorhodopsin (26 kDa) was cut with a sterile blade and analyzed by Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-tof), which unambiguously indicated the presence of a BR-like peptide. Thus, based on these results, we assumed that all the bands with the expected molecular weight

corresponded to BR. BR concentration was estimated by densitometry of these bands in SDS-PAGE images by means of the LabWorks 4.0 software (UVP).

PCR-DGGE analysis of rhodopsin genes

DNA purified (100 ng) extracted from environmental samples as described in Gomariz *et al*., 2014, was used to amplify RBP coding genes using specific primers designed in this study (Table S5). Each PCR reaction contained $MgCl_2$ 1.5 mM, Tris-HCl 10 mM pH 9.0, KCl 50 mM, 200 μM of each dNTP (deoxynucleotide triphosphate), 0.2 M of each primer, 1 U Taq DNA polymerase I and DNA template (100 ng) in a final volume of 50 μl. Each PCR reaction was performed in a PTC-100 (Peltier-Effect Cycling) using PCR programs specific for each amplified gene (see Table S6 for more details). After amplification, the presence of heteroduplex and chimeric molecules was minimized by a 'reconditioning PCR' step (Thompson *et al*., 2002). Finally, the 'reconditioned' PCR products were purified with the Gel Band Purification Kit (GE Healthcare) and quantified by using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

DGGE was performed by using the D-Code System (Bio-Rad). PCR products were loaded onto 6% (w/v) polyacrylamide gels with 45% to 65% urea-formamide denaturing gradient in 1X TAE buffer and subjected to 16-18 h of electrophoresis at 60 °C and 70 V. After electrophoresis, the gels were stained with SYBR Green (1:10,000) (Fluka) for 15 min, washed in TAE 1X for 30 minutes and visualized using a computer image analyzer Typhoon 9410 (Amersham Biosciences). Nearly all different bands were cut, and re-amplified with the same primer pairs. Then a DGGE was run to check that each band corresponded to a single PCR product. Bands were then, checked for presence of single

band by DGGE and then sequenced at the Technical Services Unit of the University of Alicante using a GeneAmp PCR System 2400 and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The obtained rhodopsin sequences were compared with the GenBank protein database using the BLASTx software at the National Center of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) (Altschul *et al*., 1997). These sequences have been deposited in the GenBank database with Accession Numbers KM226176- KM226204, KM226164- KM226171, KM226172- KM226175 for BR, XR and ActR sequences, respectively. Finally, RBP sequences were classified in different groups according to their phylogenetic signals and a maximum likelihood (ML) tree was constructed (100 bootstrap replicates) using Geneious 6.0 software Biomatters, Ltd (www.geneious.com) in order to evaluate the evolutionary relationships among them.

Additionally, bacteriorhodopsin nucleotide sequences were translated using Expasy translate tool (www.expasy.org/translate) and aligned with database bop sequences by means of Clustal Omega software program (http://www.ebi.ac.uk/Tools/msa/clustalo/) in order to analyze changes in retinal-binding pocket amino acids.

Multivariate statistical analysis

In order to correlate the biological data (bacterial and archaeal abundances, amount of BR, prokaryotic and rhodopsin diversities) with environmental parameters (temperature, pH, salinity and sodium, potassium, calcium, magnesium, ammonium, sulfate, chloride, carbonate and phosphate concentration) from Gomariz *et al*., (2014), redundancy detrented analysis (RDA) and canonical correspondence (CCA) multivariate analyses were carried out using the CANOCO 4.5 software package program (ter Braak and Smilauer, 2002).

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515 **Figure legends**

516 Figure 1: Phylogenetic tree of bop sequences retrieved from five hypersaline saltern ponds.
517 Maximum likelihood (ML) tree was constructed with 100 bootstrap using Geneious 6.0
518 software. Each tree branch corresponds with 16 phylogroups while "photocycle-tuning"
519 are colored according to their proton pumping pocket amino acids changed (Table 2). Non-
520 colored sequences are shorter than the rest and PBP amino acids are not available to
521 evaluate possible changes.

522 Figure 2: Temporal variation of retinal-binding protein (dashed lines) with prokaryotic
523 community diversity in each pond from January to November in 2006. In panel A:
524 *Archaea* (black) and BR-like protein (white) diversity (circles); HQR-related phylotypes
525 (black) and SbopI-like protein (white) diversity (squares). In panel B: *Bacteroidetes*
526 (black) and *Proteobacteria* (gray) diversity (squares); *S. ruber* (black), XR-like,
527 xanthorhodopsin (white) diversity (circles). Finally, Actinobacteria (black) and ActR
528 (gray) diversity (asterisks). A Shannon index 0 value correspond to only one registered
529 OTU sequence, while empty samples are obviously non-sequence detection.

530 Figure 3: Canonical correspondence analysis (CCA) ordination biplot of phylotypes
531 (symbols) and environmental parameters (arrows) from Gomariz *et al*., 2014. Retinal-
532 Binding Proteins (BR, XR and ActR; described in Table 1) are represented as colored
533 cross-hatched symbols with related phylotypes (archaeal in section A and bacterial in
534 section B).

535 Figure 4: Redundancy detrented analysis (RDA) canonical ordination biplot of
536 environmental and biological parameters. DAPI, *Archaea* and *Bacteria* abundances are
537 represented by the logarithm of the number of cells per milliliter and the prokaryotic
538 community (all of them taken from Gomariz *et al*., 2014) and retinal-binding proteins (BR
539 and XR) diversity by Shannon diversity indices (H). RDA yielded two synthetic canonical
540 axes (RDA1 and RDA2) that explained 69.2% of data variance.
541 Figure 5. Spatio-temporal distribution of "Photocycle-tuning" varieties (detailed in Table
542 2) in each of the analyzed ponds.

Table 1: Retinal-binding coding protein sequences retrieved in this study. Closest cultured and uncultured relative similarities are indicated as well the pond and month where/when the sequences were detected in (Pond and month detected). Ponds: Medium concentrators, CMs (CM1 and CM2), brine concentrator, CCAB and crystallizers, CRs (CR30 and CR41).

			BACTERIORHODOPSIN-LIKE PROTEIN				
Phylogroup	*Photocycle-tuning protein*	*Sequences*	Closest cultured relative (Genbank Access Number) Closest uncultured relative (Genbank Access Number)	*Authors*	*Similarity (%)*	*Detected in*	
						Pond	*Month*
1	**III**	18BR1	bacteriorhodopsin, partial [uncultured bacterium] (ADJ38522.1)	Dillon et al., 2013	89%	CM1 CM2	J1-J2 AB, J2-OC
			bacteriorhodopsin [*Halorubrum kocurii*] (WP_008848850.1)	Podell et al., 2013	69%		
2	**VIII**	19BR5	bacteriorhodopsin, partial [uncultured Halobacteriales archaeon] (AAZ76796.1)	Dillon et al., 2013	82%	CCAB	All months (except SP and NV)
			bacteriorhodopsin, partial [*Halorhabdus* sp. A001] (AGV98942.1)	Yang,J et al., unpublished	54%		
3	**VI**	19BR8	bacteriorhodopsin, partial [uncultured bacterium] (ADJ38539.1)	Dillon et al., 2013	55%	CCAB	J2-OC
			bacteriorhodopsin, partial [*Halosimplex carlsbadense*] (ABT17454.1)	Sharma et al., 2007	57%		
4	**II**	18BR3	bacteriorhodopsin, partial [uncultured haloarchaeon] (AHF27668.1)	Dillon et al., 2013	90%	CMs	J1-J2 and NV
			bacteriorhodopsin, partial [*Natrinema* sp. enrichment culture clone ABDH2] (ACO36736.1)	Tohty et al., 2009	67%		
5	**XII**	25BR4	bacteriorhodopsin [uncultured archaeon A07HN63] (WP_023502072.1)	Podell et al., 2013	91%	CR41	EN
			bacteriorhodopsin [*Halonotius sp*. J07HN4] (WP_021041057.1)	Burs et al., 2009	98%		
6	**XIII**	18BR6	bacteriorhodopsin, partial [uncultured haloarchaeon] (AGZ13717.1)	Dillon et al., 2013	73-96%	CM1	AB
	0	23BR3,4				CR30	JL-SP and NV
	XI	25BR5	bacteriorhodopsin [*Halorubrum californiense*] (WP_008440455.1)	Presente et al., 2008	61-84%	CR41	EN
7	**XIII**	23BR2 18BR5, 23BR9, 25BR2	bacteriorhodopsin, partial [uncultured haloarchaeon] (AGZ13712.1)	Dillon et al., 2013	94-100%	CRs	Every months
			bacteriorhodopsin precursor (Squarebop I) [*Haloquadratum walsbyi* DSM 16790](YP_656801.1)	Pfeiffer et al., 2008	58-62%	CM2 and CRs	Every months
8		19BR9	bacteriorhodopsin, partial [halophilic archaeon F7] (ABY53427.1)	Dillon et al., 2013	55%	CCAB	J2-NV
			bacteriorhodopsin, partial [*Natrinema altunense*] (AAS87571.1)	Xu et al., unpublished	55%		
9		25BR3	bacteriorhodopsin, partial [uncultured Halobacteriales archaeon] (AAR12473.1)	Papke et al., 2003	64%	CR41	Every months
			bacteriorhodopsin precursor (Squarebop I) [*Haloquadratum walsbyi* DSM 16790](YP_656801.1)	Pfeiffer et al., 2008	60%		

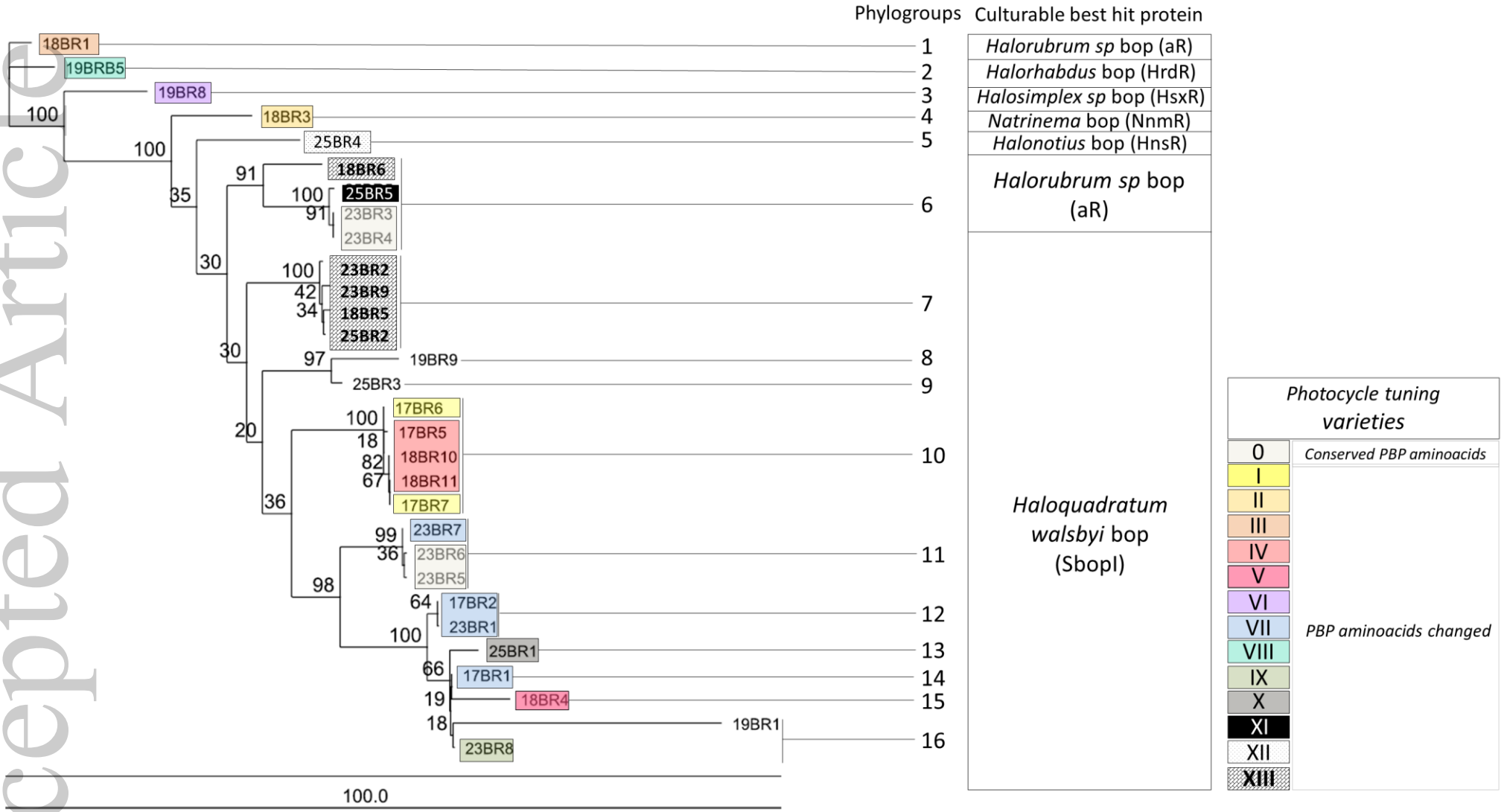
Table 1 (cont.): Retinal binding coding protein sequences retrieved in this study. Closest cultured and uncultured relative similarities are indicated as well the pond and month where/when presence of the sequences were detected in (Pond and month detected). Ponds: Medium concentrators, CMs (CM1, CM2), brine concentrator, CCAB and crystallizers, CRs (CR30 and CR41).

			BACTERIORHODOPSIN-LIKE PROTEIN				
Phylogroup	*Photocycle tuning protein*	*Sequences*	Closest cultured relative (Genbank Access Number) Closest uncultured relative (Genbank Access Number)	*Authors*	*Similarity (%)*	*Detected in*	
						Pond	*Month*
10	**I**	17BR6,7	bacteriorhodopsin, partial [uncultured haloarchaeon](AHF27622.1)	Podell et al., 2013	91-100%	CMs	All
	IV	17BR5, 18BR10,11	bacteriorhodopsin precursor (Squarebop I) [*Haloquadratum walsbyi* DSM 16790](YP_656801.1)	Pfeiffer et al., 2008	81-83%		
11	**0**	23BR5, 6	bacteriorhodopsin, partial [uncultured haloarchaeon] (AGZ13722.1)	Dillon et al., 2013	97-99%	CRs	All
	VII	23BR 7	bacteriorhodopsin precursor (Squarebop I) [*Haloquadratum walsbyi* DSM 16790](YP_656801.1)	Pfeiffer et al., 2008	68-71%		
12	**VII**	17BR2 23BR1	bacteriorhodopsin, partial [uncultured haloarchaeon] (AHF27592.1)	Dillon et al., 2013	99%	CMs and CR30	All
			bacteriorhodopsin precursor (Squarebop I) [*Haloquadratum walsbyi* DSM 16790](YP_656801.1)	Pfeiffer et al., 2008	92%		
13	**X**	25BR1	bacteriorhodopsin, partial [uncultured haloarchaeon] (AHF27701.1)	Dillon et al., 2013	78%	CR41	All
			bacteriorhodopsin precursor (Squarebop I) [*Haloquadratum walsbyi* DSM 16790] (YP_656801.1)	Pfeiffer et al., 2008	79%		
14	**VII**	17BR1	bacteriorhodopsin, partial [uncultured haloarchaeon] (AGZ13698.1)	Papke et al., 2003	99%	CMs	MR-OC
			bacteriorhodopsin precursor (Squarebop I) [*Haloquadratum walsbyi* DSM 16790](YP_656801.1)	Pfeiffer et al., 2008	97%		
15	**V**	18BR4	bacteriorhodopsin, partial [uncultured haloarchaeon] AHF27701.1	Dillon et al., 2013	76%	CMs	All
			bacteriorhodopsin precursor (Squarebop I) [*Haloquadratum walsbyi* DSM 16790](YP_656801.1)	Pfeiffer et al., 2008	75%		
16		19BR1	bacteriorhodopsin, partial [uncultured Halobacteriales archaeon]AAR12458.1	Papke et al., 2003	85%	CCAB	All
			bacteriorhodopsin precursor (Squarebop I) [*Haloquadratum walsbyi* DSM 16790](YP_656801.1)	Pfeiffer et al., 2008	85%		
	IX	23BR8	bacteriorhodopsin, partial [uncultured Halobacteriales archaeon]AAR12458.1	Dillon et al., 2013	99%	CR30	All
			bacteriorhodopsin precursor (Squarebop I) [*Haloquadratum walsbyi* DSM 16790](YP_656801.1)	Pfeiffer et al., 2008	99%		

Table 1 (cont.): Retinal binding coding protein sequences retrieved in this study. Closest cultured and uncultured relative similarities are indicated as well the pond and month where/when the seque
nces were detected in (Pond and month detected). Ponds: Medium concentrators, CMs (CM1, CM2), brine concentrator, CCAB and crystallizers, CRs (CR30 and CR41).

XANTHORHODOPSIN-LIKE PROTEIN (XR)						
Phylogroup	*Sequences*	Closest cultured relative (Genbank Access Number) Closest uncultured relative (Genbank Access Number)	*Authors*	*Similarity (%)*	*Presence*	
					Pond	*Month*
17	17XR2, 6, 8, 9, 10, 11 18XR2, 3, 6	Xanthorhodopsin [*Salinibacter ruber* DSM 13855] (YP_445623.1)	Mongodin et al., 2005	91-100%	All ponds	All samples
ACTINORHODOPSIN-LIKE PROTEIN (ActR)						
18	ActR1, 2, 3, 4	Putative actinorhodopsin, partial [*Candidatus* Aquiluna rubra] (ACN42845.1)	Sharma et al., 2009	77-84%	CM1 CM2	J1-JL J1-J2
		Actinorhodopsin, partial [uncultured bacterium] (CCQ25966.1)	Salka et al., 2014	77-81%		

Photocycle tuning protein	**96**	**115**	**137**	**182**	**185**	**186**	**189**	**194**	**200**	**204**	**210**	**212**	**215**	**Sequences**
0	D	D	W	W	Y	P	W	E	G	Y	I	D	A	23BR3, 4, 5 and 6
I	**H**	D	W	W	Y	P	W	E	G	Y	I	D	A	17BR6 and 7
II	D	D	W	W	Y	P	W	E	**D**	Y	I	D	A	18BR3
III	D	D	W	W	Y	P	W	E	**S**	Y	**M**	D	A	18BR1
IV	D	D	W	W	Y	P	W	E	**S**	Y	**V**	D	A	17BR5, 18BR10 and 11
V	D	D	W	**R**	Y	P	W	E	G	Y	I	D	A	18BR4
VI	D	D	W	W	Y	P	W	E	**S**	Y	**Y**	D	A	19BR8
VII	D	D	W	W	Y	P	W	E	G	Y	**V**	D	A	17BR2, 23BR1 and 7
VIII	D	D	W	W	Y	P	W	E	G	**K**	Y	D	A	19BR5
IX	D	D	W	W	Y	P	W	E	G	Y	**V**	D	A	23BR8
X	D	D	W	W	Y	P	W	**N**	G	**K**	**G**	**S**	A	25BR1
XI	D	D	W	W	Y	P	W	E	G	Y	**G**	**R**	**R**	25BR5
XII	**G**	D	W	W	Y	P	W	E	G	Y	I	D	A	25BR4
XIII	D	D	W	W	Y	P	W	E	**N**	Y	I	**G**	A	18BR5, 6; 23BR2, 9 and 25BR2



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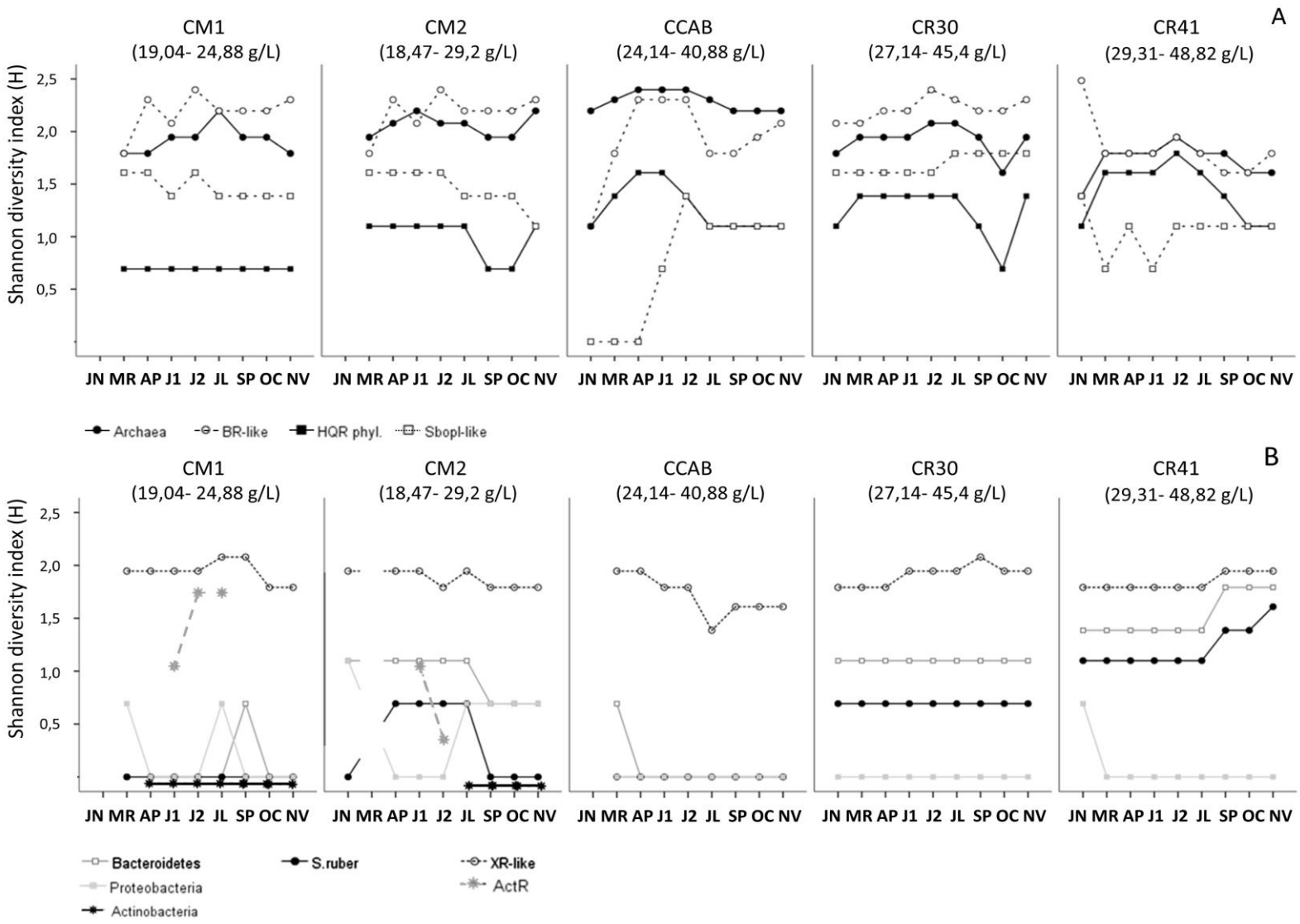

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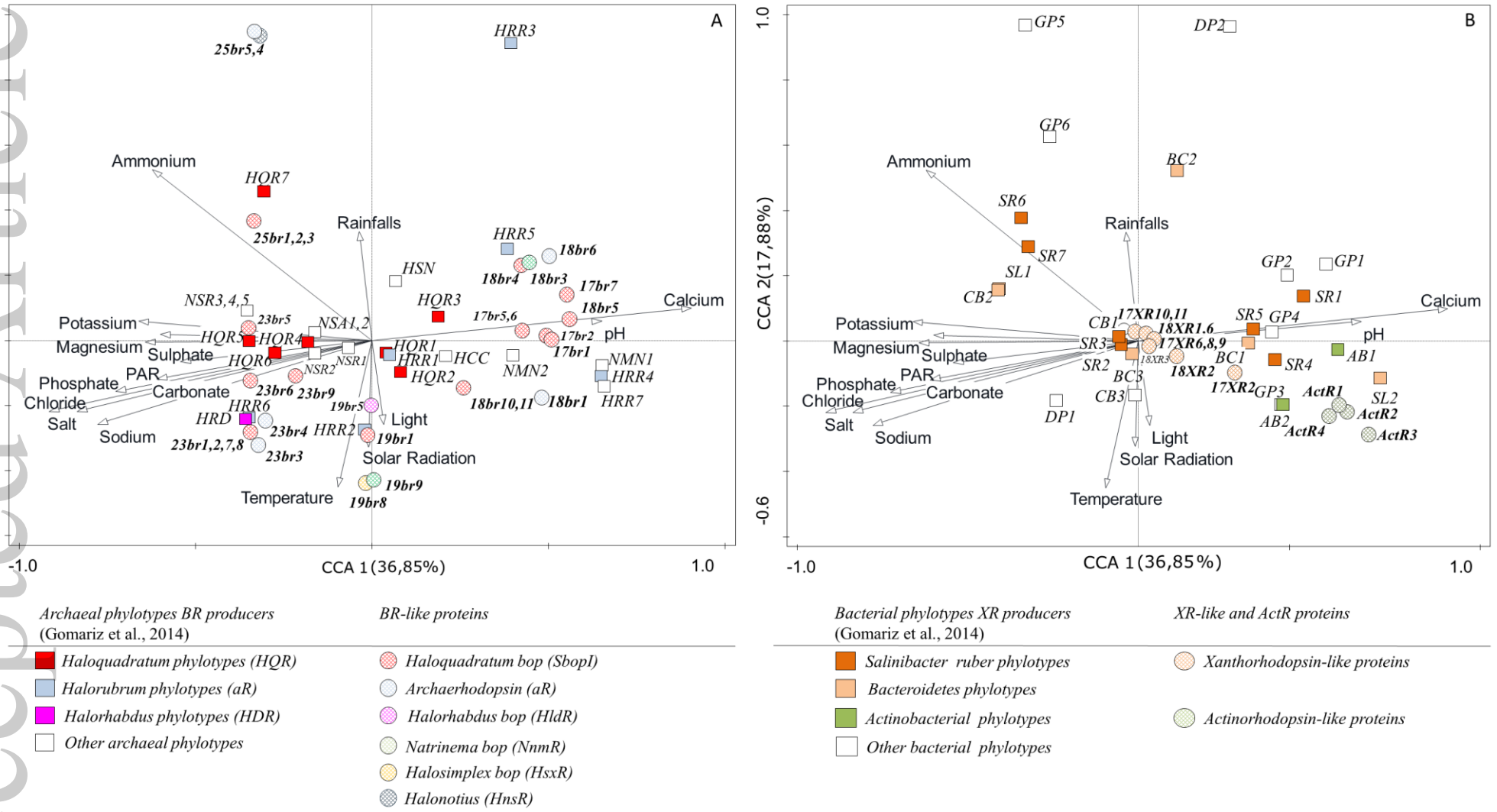

Archaeal phylotypes BR producers (Gomariz et al., 2014)

- *Haloquadratum phylotypes (HQR)*
- *Halorubrum phylotypes (aR)*
- *Halorhabdus phylotypes (HDR)*
- *Other archaeal phylotypes*

BR-like proteins

- *Haloquadratum bop (SbopI)*
- *Archaerhodopsin (aR)*
- *Halorhabdus bop (HldR)*
- *Natrinema bop (NnmR)*
- *Halosimplex bop (HsxR)*
- *Halonotius (HnsR)*

Bacterial phylotypes XR producers (Gomariz et al., 2014)

- *Salinibacter ruber phylotypes*
- *Bacteroidetes phylotypes*
- *Actinobacterial phylotypes*
- *Other bacterial phylotypes*

XR-like and ActR proteins

- *Xanthorhodopsin-like proteins*
- *Actinorhodopsin-like proteins*

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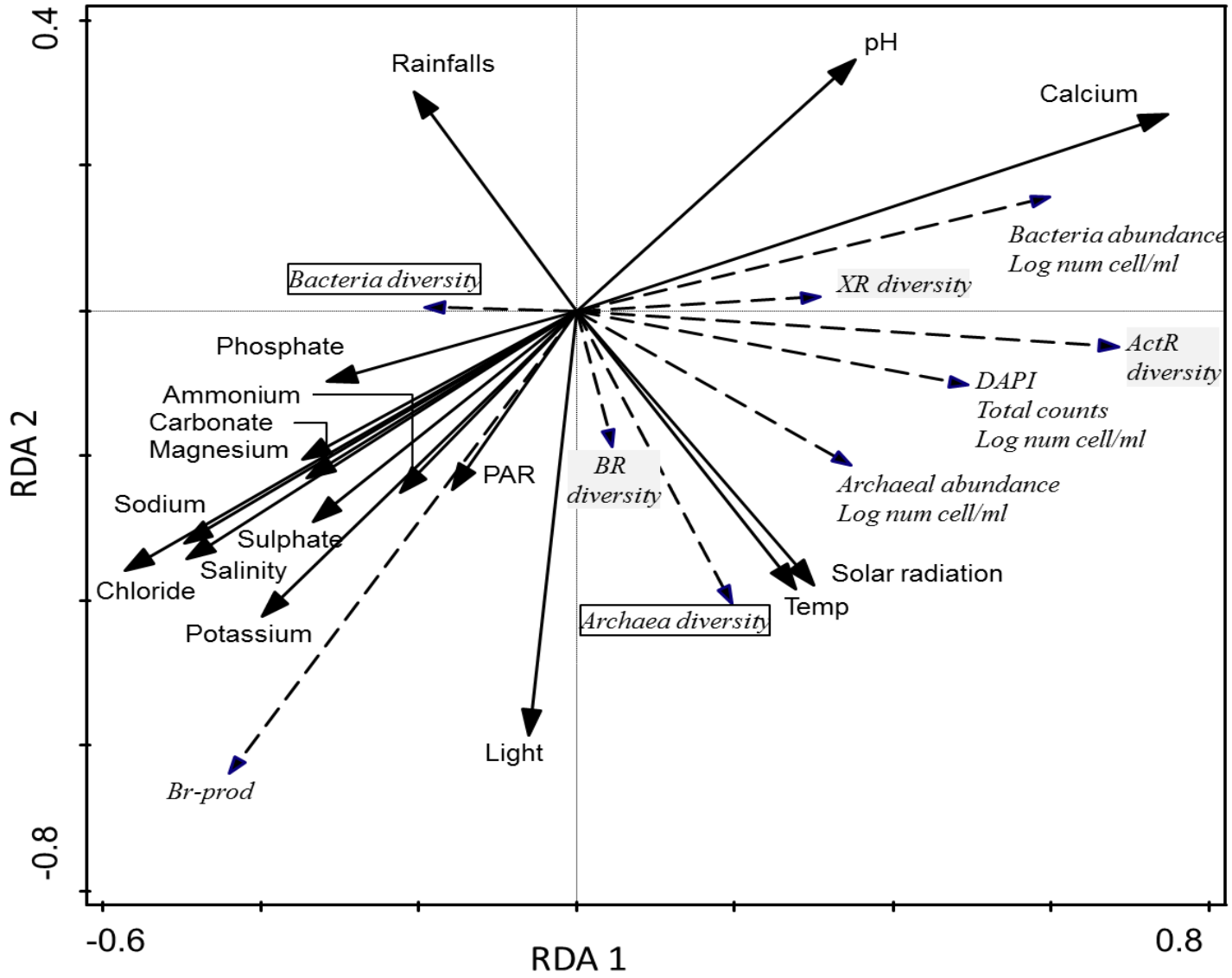

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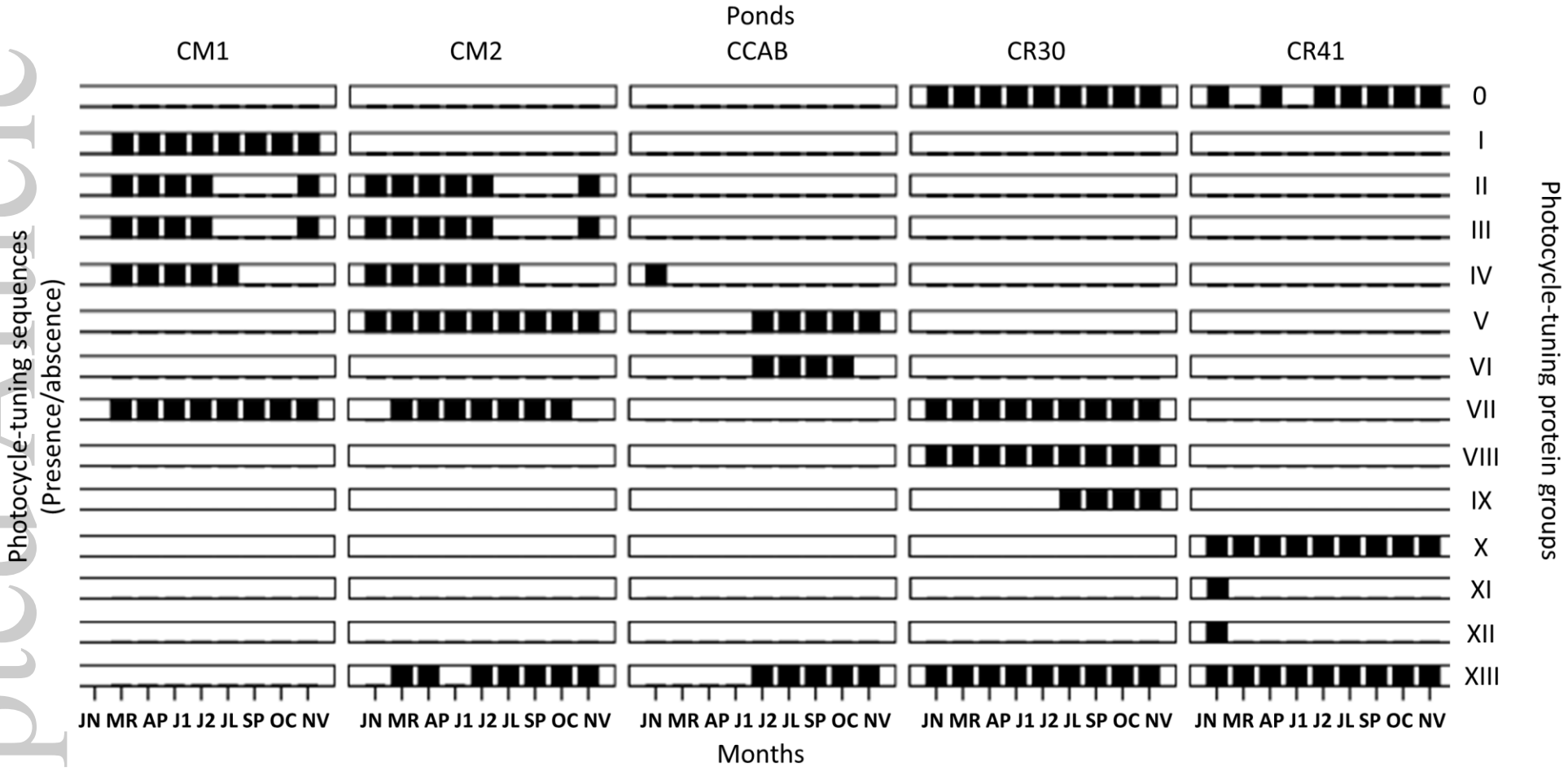

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