RESEARCH ARTICLE

Diversity and distribution of *Halomonas* in Rambla Salada, a hypersaline environment in the southeast of Spain

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Received 29 July 2013; revised 21 September 2013; accepted 11 October 2013.

DOI: 10.1111/1574-6941.12237

Editor: Iks Laanbroek

Keywords
diversity; salinity; halophiles; *Halomonas*; denitrification; hypersaline habitat.

Abstract

We have studied the diversity and distribution of *Halomonas* populations in the hypersaline habitat Rambla Salada (Murcia, southeastern Spain) by using different molecular techniques. Denaturing gradient gel electrophoresis (DGGE) using specific primers for the 16S rRNA gene of *Halomonas* followed by a multivariate analysis of the results indicated that richness and evenness of the *Halomonas* populations were mainly influenced by the season. We found no significant differences between the types of samples studied, from either watery sediments or soil samples. The highest value of diversity was reached in June 2006, the season with the highest salinity. Furthermore, canonical correspondence analysis (CCA) demonstrated that both salinity and pH significantly affected the structure of the *Halomonas* community. *Halomonas almeriensis* and two denitrifiers, *H. ilicicola* and *H. ventosae* were the predominant species. CARD-FISH showed that the percentage of *Halomonas* cells with respect to the total number of microorganisms ranged from 4.4% to 5.7%. To study the functional role of denitrifying species, we designed new primer sets targeting denitrification *nirS* and *nosZ* genes. Using these primers, we analyzed sediments from the upwelling zone collected in June 2006, where we found the highest percentage of denitrifiers (74%). *Halomonas ventosae* was the predominant denitrifier in this site.

Introduction

Rambla Salada is a saline habitat located in Murcia (southeastern Spain) that has been declared a protected area by the Murcian regional government (BORM 10 September 1998), a place of local community interest (LIC) for the European Union and a protected wildfowl zone (ZEPA). Rambla Salada is an athalassohaline environment because its salinity is mainly derived from Miocene gypsiferous marls in the Fortuna basin (Muller & Hsü, 1987). Rambla Salada includes soil, water and sediments with different salt concentrations. Our group has described three new halophilic bacterial species isolated from Rambla Salada: *Halomonas ramblicola* (Luque *et al.*, 2012a), *H. cerina* (González-Domenech *et al.*, 2008) and *Idiomarina ramblicola* (Martínez-Cánovas *et al.*, 2004a). We have also proved that the archaeal community inhabiting this area is very influenced by salinity (Oueriaghli *et al.*, 2013).

Halomonas is the type genus of the family *Halomonadaceae* belonging to the class *Gammaproteobacteria*. The genus *Halomonas* comprises, at the moment of this study, 83 species (Parte, 2013). They are Gram-negative, rod-shaped and nonsporulated chemoorganotrophs. Few species of this genus have fermentative metabolism, whereas most of them possess a respiratory metabolism, using oxygen or nitrate and nitrite as electron acceptors. Among these latter, there are some able to denitrify, a characteristic of taxonomic importance in the genus *Halomonas* (González-Domenech *et al.*, 2010). Members of this genus are widely distributed throughout different kinds of hypersaline environments. Moreover, the genus *Halomonas* represents a high percentage of strains either isolated or

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detected by molecular means in these habitats (Martínez-Cánovas *et al.*, 2004b; Cui *et al.*, 2008; Shapovalova *et al.*, 2008; Shi *et al.*, 2012). Nevertheless, in spite of the apparent predominance of this genus in Rambla Salada, the attempts to detect it by molecular means using general primers for bacteria have been unsuccessful (Oueriaghli *et al.*, 2012).

The development and application of culture-independent tools have made a detailed and more thorough investigation of microbial biodiversity avoiding the bias associated with culturing. Denaturing gradient gel electrophoresis (DGGE) of PCR amplified DNA fragments is one of the most commonly used molecular approaches to determine the presence and distribution of a particular microbial community in natural and engineered environments. The usefulness of DGGE lies on the assumption that different sequences will migrate to different positions in DGGE gels based on their melting behavior (Lerman *et al.*, 1984; Muyzer *et al.*, 1993; Jackson & Churchill, 1999). Moreover, DGGE gives an overview of the diversity of predominant community members because the band intensity is related to the numbers of microorganisms that are present at a specific site. *In situ* hybridization techniques, such as fluorescence *in situ* hybridization (FISH) and catalyzed reported deposition *in situ* hybridization (CARD-FISH) provide quantitative information about microbial populations (Wagner *et al.*, 2003; Amann & Bernhard, 2008). 16S rRNA gene sequence analysis is of paramount importance in taxonomic resolution in bacteria (Stackebrandt *et al.*, 2002), so its use is widespread as a target in the mentioned approaches. In addition, other genes, that is, genes participating in the denitrification pathway, are becoming common probes to assess metabolically determined populations.

We undertook a molecular analysis of the community of the genus *Halomonas* inhabiting different sampling areas of the Rambla Salada during various seasonal periods. For this purpose, we used specific primers for the 16S rRNA gene of this genus, as well as other oligonucleotides designed *ad hoc* to specifically study the denitrifying population of *Halomonas*. This molecular study demonstrated that the diversity of the genus *Halomonas* depended on the different salt concentrations and on the pH in Rambla Salada and that the species *H. almeriensis*, *H. ilicicola* and *H. ventosae* were predominant among the *Halomonas* sequenced population.

Materials and methods

Sample collection and physicochemical determinations

We collected a total of 18 samples from three different sites in Rambla Salada (Murcia, south-eastern Spain) on three separate occasions during two years (June 2006, and February and November 2007). The samples were as follows: soil and watery sediment from the Finca La Salina (riverbed zone), watery sediment from the water-transfer conduit between the Tagus and Segura rivers (transfer zone) and watery sediment from a saline groundwater spring (upwelling zone) (Table 1). The samples were aseptically taken and stored in sterile polycarbonate tubes at 4 °C until study in the laboratory (always within 24 h after collection). The physicochemical parameters pH, oxygen and salinity of each sampling location were determined by Luque *et al.* (2012b) and Oueriaghli *et al.* (2013).

DNA extraction and PCR amplification

DNA was extracted from 10 g of the soil and watery sediments using a PowerMax Soil DNA Isolation Kit (MO

Table 1. Physicochemical parameters in Rambla Salada in the three areas sampled during the three seasons in which samples were taken

				Physicochemical parameters		
Sites	Co-ordinates	Sample*	Season	O_2 (mg L^{-1})	Salinity (g L^{-1})	pH
Site 1: Riverbed zone	38°07′34.44″N; 1°07′11.13″W	S1A, S2A	June 2006	10.2	44.4	6.3
		S4A		4.5	83	6.8
		S1B, S2B	February 2007	17.2	18.6	8.2
		S4B		14.3	20.2	8.1
		S1C, S2C	November 2007	13	11.8	8.3
		S4C		7.9	22.2	8.0
Site 2: Transfer zone	38°07′30.23″N; 1°07′42.22″W	S3A	June 2006	1.5	62.1	8.7
		S3B	February2007	10.5	34	8.1
		S3C	November 2007	20.6	29	8.3
Site 3: Upwelling zone	38°07′29.09″N; 1°07′42.15″W	S7A, S8A	June 2006	1	140	7.1
		S7B, S8B	February 2007	0.6	157.6	6.7
		S7C, S8C	November 2007	4	151.2	7.2

Data from Luque *et al.* (2012b) and Oueriaghli *et al.* (2013).

*Type of sample: S1 and S2 soil sample; S3, S4, S7 and S8 watery sediments.

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BIO Laboratories) and following the manufacturer's protocol. The PCR primers for specific amplification of 16S rRNA gene of *Halomonas* were 16F27 (Lane, 1991) and Hlm474-R primers (Yoshie *et al.*, 2004), as forward and reverse oligonucleotides, respectively. Regarding the denitrification genes, the design of primers was based on the DNA sequences of the *nirS* and *nosZ* genes obtained from thirteen denitrifying species of *Halomonas* (González-Domenech *et al.*, 2010). The alignment was performed using the CLUSTAL X program (Thompson *et al.*, 1997).

The length of the specific amplified region for both 16S rRNA genes and denitrification genes ranged from 300 to 500 bp with the aim of combining provided information and resolution of DGGE technique (Sheffield *et al.*, 1989). A 40-bp long GC clamp (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3′) was attached to the 5′ end of the forward primer for *nos*Z gene and of the reverse primers for 16S rRNA gene and *nir*S genes to obtain PCR fragments suitable for DGGE analysis (Muyzer *et al.*, 1998). Primers sequences are described in Table 2.

PCRs were performed with 20–100 ng of the extracted DNA into 50 μL of total reaction volume, with 10 pmol of each primer (Sigma), 0.2 mM of dNTP mix (Bioline 29), 2 mM of $MgCl_2$ 25 mM (Applied Biosystems), 5× PCR Buffer Gold 10× (Applied Biosystems), and 2.5 units of AmpliTaq Gold polymerase (Applied Biosystems, Life Technologies, Carlsbad, CA). The reactions were carried out as follows: 5-min initial denaturation of DNA at 94 °C, followed by 30 cycles of 1-min denaturation at 94 °C, 1-min primer annealing at the temperature specific for each primer pair (Table 2), and 3-min extension at 72 °C. Amplification was completed by a final extension step at 72 °C for 30 min to avoid the generation of heteroduplex (Janse *et al.*, 2004). PCR products were first visualized in a 1.5% (w/v) agarose gel in TBE 1× buffer by ethidium bromide staining and then purified using Amicon Ultra-0.5 mL Centrifugal Filters (Eppendorf, Hamburg, Germany).

DGGE analysis

The PCR products obtained were subjected to DGGE analysis performed as described by Muyzer *et al.* (1998). Denaturing gradient gel electrophoresis was carried out with a DCODE™ system (Universal mutation detection system, Bio-Rad) using a denaturing gradient of 35% to 60% in the case of 16S rRNA gene, and a denaturing gradient of 40% to 70% for the denitrifying genes. The denaturing gradient was prepared with 7 M urea and 40% (v/v) formamide in a 6% polyacrylamide gel. Each sample (1000–1600 ng of DNA) was loaded on the gel and run for 15 min at 200 V and 16 h at 100 V in TAE (Tris-acetate-EDTA) buffer 1× (pH 7.4) at 60 °C. Following electrophoresis, the gels were incubated in a 1 : 10 000 dilution of a stock solution of SYBR Gold (Invitrogen-Molecular Probes) for 45 min and then rinsed in Milli-Q water before being photographed using a Bio-Rad GelDoc station. Individual bands were excised, re-amplified as mentioned previously and run again on a denaturing gradient gel to check their purity. PCR products were purified using the Illustra® GFX DNA and Gel Band Purification (GE Healthcare®) before being sequenced with an ABI PRISM dye-terminator, cycle-sequencing, ready-reaction kit (Perking-Elmer) and an ABI PRISM 377 sequencer (Perking-Elmer) according to the manufacturer's instructions.

Phylogenetic study

The sequences obtained were compared with sequences available in the GenBank database using BLAST search to determine their identity percentage with most related species (top BLAST hits with e-values $< 1 \times 10^{-5}$ were used for band assignation). Our sequences and the same region of those related were aligned using the CLUSTAL X program (Thompson *et al.*, 1997), and a phylogenetic analysis was carried out by the neighbor-joining (NJ) method with MEGA (Molecular Evolutionary Genetics Analysis) version 5 software (Tamura *et al.*, 2011) in the case of 16S rRNA

Table 2. Oligonucleotides used in this study for *Halomonas* spp. (16S rRNA gene) and the denitrifying strains of this genus (*nirS* and *nosZ* genes)

Gene	Oligonucleotide	Annealing temp	Primer sequence (5′-3′)	Length of amplicon	References
16S rRNA gene	16F27 Hlm474-R	60 °C	AGAGTTTGATCMTGGCTCAG +CTGTGRGTGATGTCCTTCCT	515 bp	Lane (1991) Yoshie *et al.* (2004)
nirS	Sdgge-F Sdgge-R	55 °C	GAGTCCAACCAGATCGT +GGAGGTCTCGTCCATGGT	472 bp	This study This study
nosZ	Zdgge-F Zdgge-R	52 °C	+CGACATCATGAAGACG GATRTAGCGGGTCAGCT	486 bp	This study This study

*M = A or C; R = A or G; Y = C or T.

+CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG. GC composition from Sheffield *et al.* (1989) and Muyzer *et al.* (1998).

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gene, and with the corresponding program from the Phylogeny Inference PHYLIP package version 3.68 (Felsenstein, 2008) for the *nir*S and *nos*Z genes. Genetic distances were calculated by the Kimura two-parameter model (Kimura, 1980). In addition, we used three species of the family *Halomonadaceae*, *Carnimonas nigrifaciens* CTCBS1^T, *Cobetia marina* DSM 4741^T and *Salinicola socius* SMB35^T as outgroups to reconstruct the phylogeny from 16S rRNA gene, whereas we chose the denitrifying species *Pseudomonas aeruginosa* PA01 for the same purpose, but with the phylogenies derived from *nir*S and *nos*Z denitrification genes. In this latter case, we also included the sequences of the corresponding gene from all the type strains of *Halomonas* species characterized previously as denitrifiers by our research group (González-Domenech *et al.*, 2010). Finally, we determined the reliability of each inferred tree by using bootstrap values based upon 1000 replications.

DGGE fingerprints analysis

The band patterns generated by DGGE were normalized and compared using the FPQUEST v.5.101 software (Bio-Rad). Clustering analysis was performed by determining the Pearson and Dice coefficients. Pearson coefficient uses the whole densitometry curve, taking into consideration the intensity of each band, whereas the Dice coefficient is based on presence/absence of the bands. Dendrograms linking band pattern similarities were automatically calculated with UPGMA algorithm (Unweighted Pair Group Method with Arithmetic Mean). Significance of UPGMA clustering was estimated by calculating the cophenetic correlation coefficients (Sokal & Rohlf, 1962).

Richness and evenness estimations

Data derived from the presence/absence of bands and from their intensity were exported from the program FPquest to determine the corresponding indexes. The Shannon–Weaver (*H'*, diversity) and Simpson's (*SI'*, dominance) indexes (Shannon & Weaver, 1963; Magurran, 1996) were calculated for each DGGE lane using the following equations:

$$H' = \sum_{i=1}^{S} p_i \ln p_i$$

$$SI' = \sum (p_i)^2$$

S represents the total number of bands in a given DGGE lane and $p_i = n_i/N$, Where n_i is the band intensity for individual bands, and N is the sum of the intensities of bands in a DGGE lane.

The range-weighted richness index (*Rr*) provides an estimation of the level of richness in an environmental sample, and it was calculated according to the following equation:

$$Rr = (N^2 \times D_g)$$

N represents the total number of bands in each DGGE pattern, and *Dg* is the denaturing gradient comprised between the first and the last band of each pattern (Marzorati *et al.*, 2008).

The Pareto-Lorenz (PL) distribution curves were used to render a graphic representation of the evenness of the *Halomonas* population. For this determination, we used the DGGE fingerprints as previously described by Marzorati *et al.* (2008). The bands in each DGGE lane were ranked from high to low based on their intensity levels. The cumulative normalized numbers of bands were represented in the *x*-axis, and their respective cumulative normalized band intensities were represented in the *y*-axis. The 45° diagonal represents the perfect evenness of a community. To numerically interpret the PL curves, we calculate the functional organization index (*Fo*), scoring the *y*-axis with the vertical 20% *x*-axis (Marzorati *et al.*, 2008).

In addition, STATGRAPHICS *plus* version 3.2 (STSC, Rockville, MD) was used for the calculation of analyses of variance (ANOVA), considering a level of 95% ($P < 0.05$) as statistical significant.

Multivariate statistical analysis

To establish the influence of environmental variables upon *Halomonas* diversity, a detrended correspondence analysis (DCA) and a canonical correspondence analysis (CCA) using CANOCO 4.5 (Biometris, Wageningen, the Netherlands) was applied. The environmental variables were salinity, pH, and oxygen. A Monte Carlo test was used to verify the ordination axes significance among the environmental variables and the species data and among the environmental variables and the sampling seasons (June, 2006, February and November, 2007). Significance of the CCA axes was tested with 499 unrestricted permutations to test the null hypothesis that the *Halomonas* profiles were unrelated to the environmental variables. The effects of environmental variables were selected according to their significance level ($P < 0.05$) prior to permutation (Salles *et al.*, 2004; Sapp *et al.*, 2007). Ordination biplots were used to represent the effect of environmental variables on the *Halomonas* community structure. The environmental factors were represented as

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arrows: the length of the arrows indicated the relative importance of that environmental factor, while the angle between each arrow and the nearest axis indicated the closeness of the relationship between each other.

CARD-FISH

CARD-FISH was performed according to Pernthaler *et al.* (2004). Samples were fixed with 2% (w/v) paraformaldehyde overnight at 4 °C. Cells were immobilized on filters, then embedded in 0.1% (w/v) agarose and finally permeabilized by treatment with 10 mg mL^{-1} lysozyme in 50 mM EDTA, 100 mM Tris/HCl for 1 h at 37 °C (Pernthaler *et al.*, 2002). Filter sections were cut and hybridized with 50 ng μL^{-1} horseradish peroxidase (HRP)-labeled oligonucleotide probe (Hlm474-R; Yoshie *et al.*, 2004) in buffer hybridization (2 : 20 for each section), 35% (w/v) formamide for 2.5 h at 35 °C. For amplification, we used tyramide (1 mg mL^{-1}) labeled with FITC (Pernthaler *et al.*, 2004). Counterstaining of CARD-FISH preparations was performed with 4′, 6′-diamidino-2-phenylindole (DAPI, final concentration 1 mg mL^{-1}). Strains phylogenetically related to *Halomonas* were used as controls. Cells were visualized with a Leica TCS-SP5 Confocal Laser Scaning microscope (CLSM). From each sample, CARD-FISH stained cells were counted with 20 randomly selected frames using IMAGEJ software (http://rsb.info.nih.gov/ij; Rhasband, 2010).

Nucleotide sequence accession numbers

The sequences reported in this study have been submitted to the GenBank database under accession numbers JX997648 to JX997691 and KC415600 to KC415608.

Results

Physicochemical determinations

Table 1 shows the physicochemical parameters at three different sites in Rambla Salada during the three seasons in which we took samples (data from Luque *et al.*, 2012b; Oueriaghli *et al.*, 2013). In general, these parameters (salinity, pH, and oxygen) showed both spatial and temporal variation. The samples taken at the Finca la Salina (riverbed zone) and at the water-transfer conduit between the Tagus and Segura rivers (transfer zone) were less saline than those obtained in the samples from the saline groundwater spring (upwelling zone). The salt concentration was higher in June 2006, a season characterized by low rainfall. The pH values varied seasonally in the riverbed zone (S1, S2, and S4), showing a decrease in June 2006 (pH between 6.3 and 6.8) compared with February and November 2007, both of which were slightly alkaline. As far as oxygen is concerned, there were no wide temporal variations, but the values were lower in the samples from the upwelling site (S7 and S8) and in the sample from the transfer site (S3A) collected in June 2006. The environmental parameters in the upwelling zone, this zone is a saline sulfur pond characterized by a high chloride content and high proportion of sulfates, were practically constant during all three seasons studied. This water source tends to remain constant throughout the year (Oueriaghli *et al.*, 2013).

Analysis of the diversity and distribution of the *Halomonas* community by DGGE fingerprinting

DGGE patterns of the 18 different samples were compared by using FPQUEST software. The dendrogram based on the Pearson coefficient (Fig. 1a), reveals two clusters at 34% similarity level: cluster 1 includes the majority of the samples taken in February 2007 and cluster 2 includes nearly all the samples taken in June 2006 and November 2007, as well as two samples from February 2007. Cluster analysis using the Dice coefficient (Fig. 1b) shows three clusters at a 40% of similarity corresponding with the three seasons studied. Dice analysis also confirms the separation of the samples from the upwelling zone (samples S7 and S8) in a different branch for each sampling season. Finally, fingerprint analysis using the FPQUEST software detected a total of 54 band classes in the 18 samples analyzed.

Distribution of the *Halomonas* populations based on 16S rRNA gene sequences

A total of 44 DGGE bands were successfully re-amplified and sequenced (around 500 bp each) from the 54 band classes detected. We have compared the sequences of bands to the same region of the 16S rRNA genes retrieved from the GenBank database. The results are shown in Table 3. DGGE bands of *Halomonas* were mainly associated with denitrifying species (*H. campisalis*, *H. kenyensis*, *H. nitroreducens*, *H. saccharevitans*, *H. ventosae*, *H. ilicicola* and *H. sabkhae*). In some cases, such as *H. ventosae*, the identity percentages were higher than 99%. In addition, we have built a phylogenetic tree (Fig. 2) that shows two main clusters, phylotypes I and II, related to different strains of *Halomonas*. Phyloptype I includes *H. ventosae*, *H. nitroreducens*, *H. saccharevitans*, *H. stenophila*, *H. salina*, *H. halmophila*, *H. almeriensis* and *H. sabkhae*. *Halomonas campisalis* and *H. kenyensis*, which are haloalkaliphilic denitrifying species, constitute a separate phylotype together with *H. ilicicola*.

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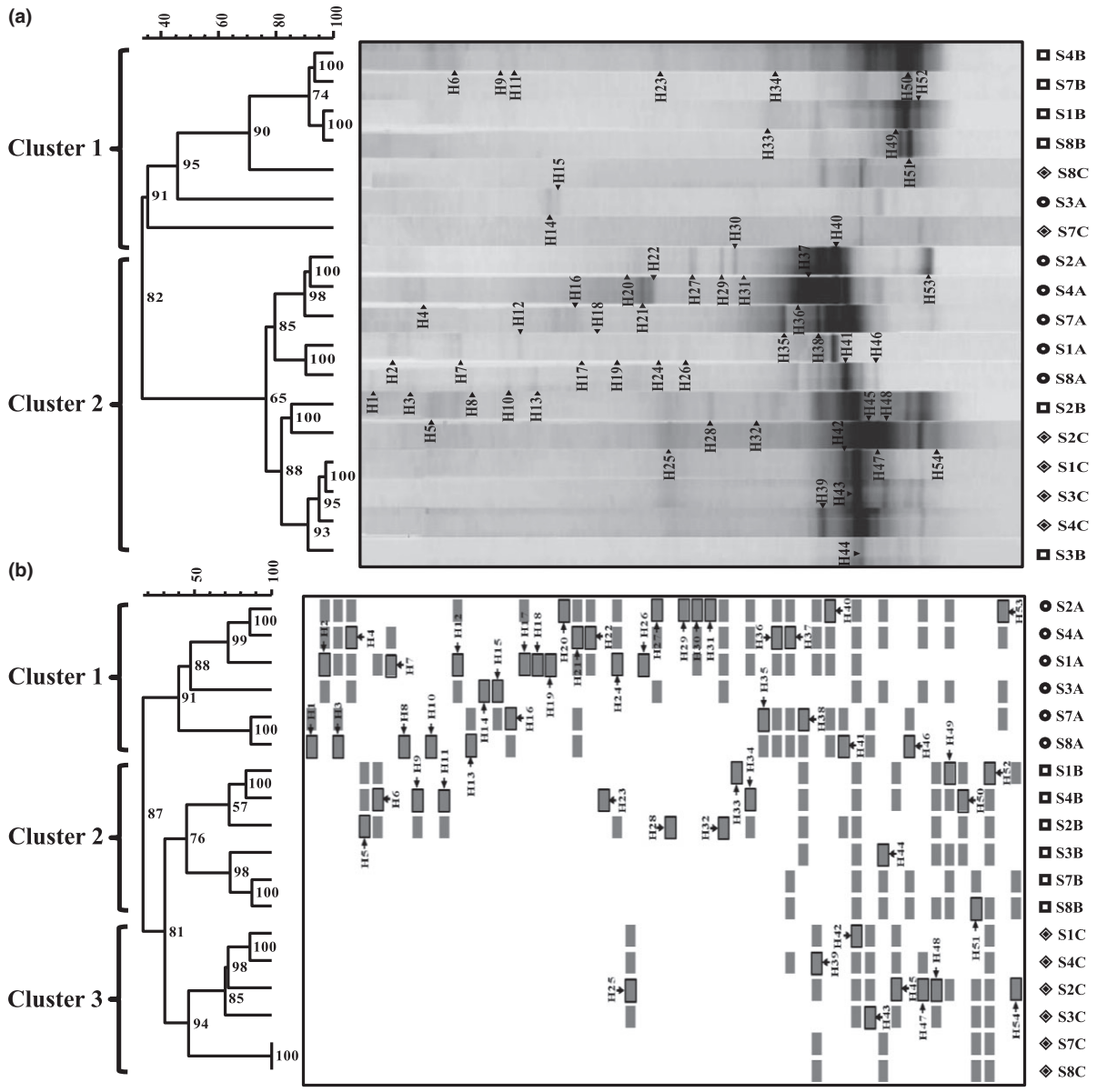

Fig. 1. The *Halomonas* population in Rambla Salada analyzed by DGGE. (a) Pearson-coefficient-based analysis; (b) Dice-coefficient-based analysis. ➔ Bands re-amplified and sequenced to conduct the phylogenetic study. The scale bar indicates the percentage of similarity. Numbers in nodes represent the cophenetic correlation coefficient values. ● Samples A (S1A to S8A) from June 2006; ◘ Samples B (S1B to S8B) from February 2007; ◈Samples C (S1C to S8C) from November 2007. Origin and type of each sample is indicated in Table 1.

The denitrifying community made up the predominant group within the *Halomonas* population in Rambla Salada in all sampling sites (62% of the total population). *Halomonas almeriensis*, *H. ilicicola,* and *H. ventosae* were the most abundant species with the respective percentages of 25%, 22%, and 20%. Bands of *H. almeriensis* predominated in all the sampling seasons with percentages of 22%, 16%, and 38% of relative abundance in June 2006 and February and November 2007, respectively. *Halomonas ventosae*, whose bands were grouped together in a common cluster within the phylotype I (Fig. 2), was found in high proportion in samples taken in June 2006 from the riverbed zone (52.6% of the total bands in this season), while the values were lower in February and November 2007 (0.6% and 5.7%, respectively). *Halomonas halmophila* (H16, H35 and H37) was mainly detected in June 2006, *H. saccharevitans* (H47) was only detected in June 2006 and November 2007, *H. nitroreducens* (H46) was present in samples taken in June 2006 and February 2007, and *H. salina* (H54) and *H. stenophila* (H45) were

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Table 3. 16S rRNA gene, *nir*S and *nos*Z genes sequences obtained from the bands in DGGE gel and percentages of identity with the closest phylogenetic strain

Band class	Accession No.	Closest relative (Accession No.)*	Identity (%)	Environmental source of the closest strain
16S rRNA gene				
H2	JX997648	*H. ventosae* M4-15 (JQ811031)	99	Hypersaline habitat, Rambla Salada, Murcia, Spain
H3	JX997649	*H. ventosae* M4-38 (JQ811054)	99	Hypersaline habitat, Rambla Salada, Murcia, Spain
H4	JX997650	*H. ventosae* M4-38 (JQ811054)	99	Hypersaline habitat, Rambla Salada, Murcia, Spain
H7	JX997651	*H. ventosae* M4-22 (JQ811039)	99	Hypersaline habitat, Rambla Salada, Murcia, Spain
H8	JX997652	*H. sabkhae* 5-3^T (NR_044217)	99	Ezzemoul sabkha, north-eastern Algeria
H10	JX997653	*H. sabkhae* 5-3^T (NR_044217)	99	Ezzemoul sabkha, north-eastern Algeria
H12	JX997654	*H. ventosae* M4-22 (JQ811039)	99	Hypersaline habitat, Rambla Salada, Murcia, Spain
H13	JX997655	*H. sabkhae* 5-3^T (NR_044217)	99	Ezzemoul sabkha, north-eastern Algeria
H16	JX997656	*H. halmophila* ATCC 19717^T (NR_042064)	100	Dead Sea, Jordan to the east and Israel to the west
H17	JX997657	*H. ventosae* M4-22 (JQ811039)	100	Hypersaline habitat, Rambla Salada, Murcia, Spain
H18	JX997658	*H. ventosae* M4-38 (JQ811054)	100	Hypersaline habitat, Rambla Salada, Murcia, Spain
H19	JX997659	*H. ventosae* M4-15 (JQ811031)	100	Hypersaline habitat, Rambla Salada, Murcia, Spain
H20	JX997660	*H. ventosae* M4-38 (JQ811054)	99	Hypersaline habitat, Rambla Salada, Murcia, Spain
H21	JX997661	*H. ventosae* M4-38 (JQ811054)	100	Hypersaline habitat, Rambla Salada, Murcia, Spain
H22	JX997662	*H. ventosae* M4-22 (JQ811039)	99	Hypersaline habitat, Rambla Salada, Murcia, Spain
H24	JX997663	*H. ventosae* M4-15 (JQ811031)	99	Hypersaline habitat, Rambla Salada, Murcia, Spain
H26	JX997664	*H. ventosae* M4-38 (JQ811054)	99	Hypersaline habitat, Rambla Salada, Murcia, Spain
H27	JX997665	*H. ventosae* M4-38 (JQ811054)	99	Hypersaline habitat, Rambla Salada, Murcia, Spain
H29	JX997666	*H. ventosae* M4-22 (JQ811039)	99	Hypersaline habitat, Rambla Salada, Murcia, Spain
H30	JX997667	*H. ventosae* M4-38 (JQ811054)	99	Hypersaline habitat, Rambla Salada, Murcia, Spain
H31	JX997668	*H. ventosae* M4-22 (JQ811039)	99	Hypersaline habitat, Rambla Salada, Murcia, Spain
H32	JX997669	*H. ventosae* M4-15 (JQ811031)	99	Hypersaline habitat, Rambla Salada, Murcia, Spain
H33	JX997670	*H. ilicicola* SP8^T (NR_044436)	98	Solar salterns of Santa Pola, Alicante, Spain
H34	JX997671	*H. ventosae* M4-22 (JQ811039)	99	Hypersaline habitat, Rambla Salada, Murcia, Spain
H35	JX997672	*H. halmophila* ATCC 19717^T (NR_042064)	99	Dead Sea, Jordan to the east and Israel to the west
H36	JX997673	*H. ventosae* M4-22 (JQ811039)	100	Hypersaline habitat, Rambla Salada, Murcia, Spain
H37	JX997674	*H. halmophila* ATCC 19717^T (NR_042064)	99	Dead Sea, Jordan to the east and Israel to the west
H38	JX997675	*H. almeriensis* M3-47 (JQ810968)	99	Hypersaline habitat, Rambla Salada, Murcia, Spain
H39	JX997676	*H. ventosae* M4-22 (JQ811039)	99	Hypersaline habitat, Rambla Salada, Murcia, Spain
H40	JX997677	*H. ventosae* M4-38 (JQ811054)	100	Hypersaline habitat, Rambla Salada, Murcia, Spain
H41	JX997678	*H. almeriensis* M3-47 (JQ810968)	100	Hypersaline habitat, Rambla Salada, Murcia, Spain
H42	JX997679	*H. almeriensis* M3-47 (JQ810968)	100	Hypersaline habitat, Rambla Salada, Murcia, Spain
H43	JX997680	*H. almeriensis* M3-47 (JQ810968)	100	Hypersaline habitat, Rambla Salada, Murcia, Spain
H44	JX997681	*H. campisalis* M2-61 (JQ810888)	98	Hypersaline habitat, Rambla Salada, Murcia, Spain
H45	JX997682	*H. stenophila* B-100 (HM357129)	99	Saline soil, Sabinar saline-wetland, Almeria, Spain
H46	JX997683	*H. nitroreducens* 12CR (EF613114)	99	Fuente de Piedra, Málaga, southern Spain
H47	JX997684	*H. saccharevitans* whb37 (FJ444983)	98	Weihai Solar Saltern, China
H48	JX997685	*H. ilicicola* SP8^T (NR_044436)	97	Solar salterns of Santa Pola, Alicante, Spain
H49	JX997686	*H. ilicicola* SP8^T (NR_044436)	97	Solar salterns of Santa Pola, Alicante, Spain
H50	JX997687	*H. ilicicola* SP8^T (NR_044436)	97	Solar salterns of Santa Pola, Alicante, Spain
H51	JX997688	*H. kenyensis* AIR-2^T (AY962237)	98	Soda lake, Kenya
H52	JX997689	*H. ilicicola* SP8^T (NR_044436)	97	Solar salterns of Santa Pola, Alicante, Spain
H53	JX997690	*H. kenyensis* AIR-2^T (AY962237)	98	Soda lake, Kenya
H54	JX997691	*H. salina* XJSL6-8 (GQ903437)	96	Sedimentary soil, salt lake, Xinjiang, China
*nir*S gene				
S1	KC415605	*H. ventosae* Al12^T (FJ686160)	94	Saline soils in Jaén, south-eastern Spain
S2	KC415606	*H. fontilapidosi* 5CR^T (FJ686147)	95	Soil sample from Fuente de Piedra, Málaga, Spain
S3	KC415607	*H. fontilapidosi* 5CR^T (FJ686147)	94	Soil sample from Fuente de Piedra, Málaga, Spain
S4	KC415608	*H. fontilapidosi* 5CR^T (FJ686147)	95	Soil sample from Fuente de Piedra, Málaga, Spain
nosZ gene				
Z1	KC415600	*H. cerina* R53 (GQ384060)	93	Hypersaline habitat, Rambla Salada, Murcia, Spain

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Table 3. Continued

Band class	Accession No.	Closest relative (Accession No.)*	Identity (%)	Environmental source of the closest strain
Z2	KC415601	*H. koreensis* JCM 12237^T (FJ686170)	93	Solar saltern of the Dangjin area, Korea
Z3	KC415602	*H. cerina* R53 (GQ384060)	93	Hypersaline habitat, Rambla Salada, Murcia, Spain
Z4	KC415603	*H. cerina* R53 (GQ384060)	93	Hypersaline habitat, Rambla Salada, Murcia, Spain
Z5	KC415604	*H. cerina* R53 (GQ384060)	95	Hypersaline habitat, Rambla Salada, Murcia, Spain

*In brackets, accession number of the closest relatives for each corresponding gene.

found in February and November 2007. *Halomonas sabkhae* (H8, H10 and H13) was only detected in samples from upwelling zone taken in June 2006. The species belonging to phylotype II, *H. campisalis* (H44), *H. ilicicola* (H33, H48, H49, H50, and H52) and *H. kenyensis* (H51 and H53) (Fig. 2) were mainly detected in samples from February to November 2007, (Fig. 3). Finally, the bands H1, H5, H6, H9, H11, H14, H15, H23, H25, and H28 were detected in the three seasons, but we were unable to sequence them successfully.

Analysis of diversity indexes

The diversity and richness of the *Halomonas* community in Rambla Salada depended on the sampling season. The average richness index (*Rr*) values ranged from 54.43 ± 26.09 to 3.29 ± 3.08 depending on the sampling time, with high and medium average values in the samples from June 2006 and February 2007, and low in November 2007 (Table 4).

The evaluation of the functional organization of the *Halomonas* community was performed by calculating the *Fo* index (Table 4) and drawing the Pareto-Lorenz distribution curves (Fig. 4). Twenty percent of the bands from samples taken in June 2006 presented *Fo* values of 80%, 45.12%, and 72.82% for the riverbed, river-transfer and upwelling zones, respectively, 65.98% being the average value of the cumulative normalized band intensities for each zone against their respective cumulative normalized number of bands (Fig. 4a). Twenty percent of the bands detected in February, 2007 represented the 61.05%, 39%, and 56.84% (52.29% on average) of the accumulative band intensities for each zone (Fig. 4b) and, finally, 59.04%, 52.38%, and 32.38% (47.93% on average) were the values of the accumulative band intensities for the 20% of the bands from samples of each mentioned site taken in November 2007 (Fig. 4c).

The Shannon–Weaver Index values (*H*') were 2.04 ± 0.16, 1.73 ± 0.15 and 1.47 ± 0.20 for the samples obtained in June 2006, February and November 2007, respectively. The Simpson Index (*SI*') represents species dominance and is inversely proportional to the Shannon–Weaver index; its values for each season were 0.17 ± 0.04, 0.22 ± 0.03, and 0.27 ± 0.03, respectively. ANOVA analysis ($P < 0.05$) revealed that there were significant differences in the diversity indexes from one season to another (Table 4).

Relationship between environmental parameters and the composition of the *Halomonas* communities

The detrended correspondence analysis (DCA) showed that the data exhibited a unimodal response to the environmental variables (Lepš & Šmilauer, 2003), and therefore, we performed a CCA analysis using CANOCO 4.5. The eigenvalues, the cumulative percentage variance of species data and the cumulative variance in the species–environment relationship along the three axes of the CCA analysis are depicted in Table S1. Based on the 5% level in a partial Monte Carlo permutation test, the value for salinity and pH were significant ($P < 0.05$), providing 78% and 43%, respectively, of the total CCA explanatory power. Therefore, the data concerning the environmental factors contributing to the model were ranked in the following order: salinity, pH, and finally oxygen. Species-environment correlation for the first two axes was more than 0.91, suggesting that *Halomonas* phylotypes were strongly correlated with these environmental factors. The first axis was negatively correlated with salinity ($P = 0.002$), and the second axis was correlated positively with pH ($P = 0.004$).

Figure 5 shows the influence of the environmental variables upon the diversity of the *Halomonas* community (Fig. 5a) in the three seasons studied and also in relation to the sampling site (Fig. 5b). As seen in Fig. 5a, there were three different groups: the group A included all the bands corresponding to *H. ventosae*. The projection of any given taxon along an axis shows where the taxon is more abundant according to the rank of the variable. Therefore, *H. ventosae* correlated negatively with pH and slightly positively regarding salinity. The group B, correlated positively with pH and oxygen, including *H. almeriensis* (H38, H41, H42, and H43), *H. ilicicola* (H33, H48, H49, H50, and H52), *H. saccharevitans* (H47), *H. salina* (H54), *H. stenophila* (H45) and some unidentified sequences (H5, H6, H9, H11, H23, H25, and H28). Finally, the group C, which correlated positively with salinity, included *H. campisalis* (H44), *H. halmophila* (H16, H35, and H37), *H. kenyensis* (H51, H53), *H. nitroreducens* (H46), *H. sabkhae* (H8, H10,

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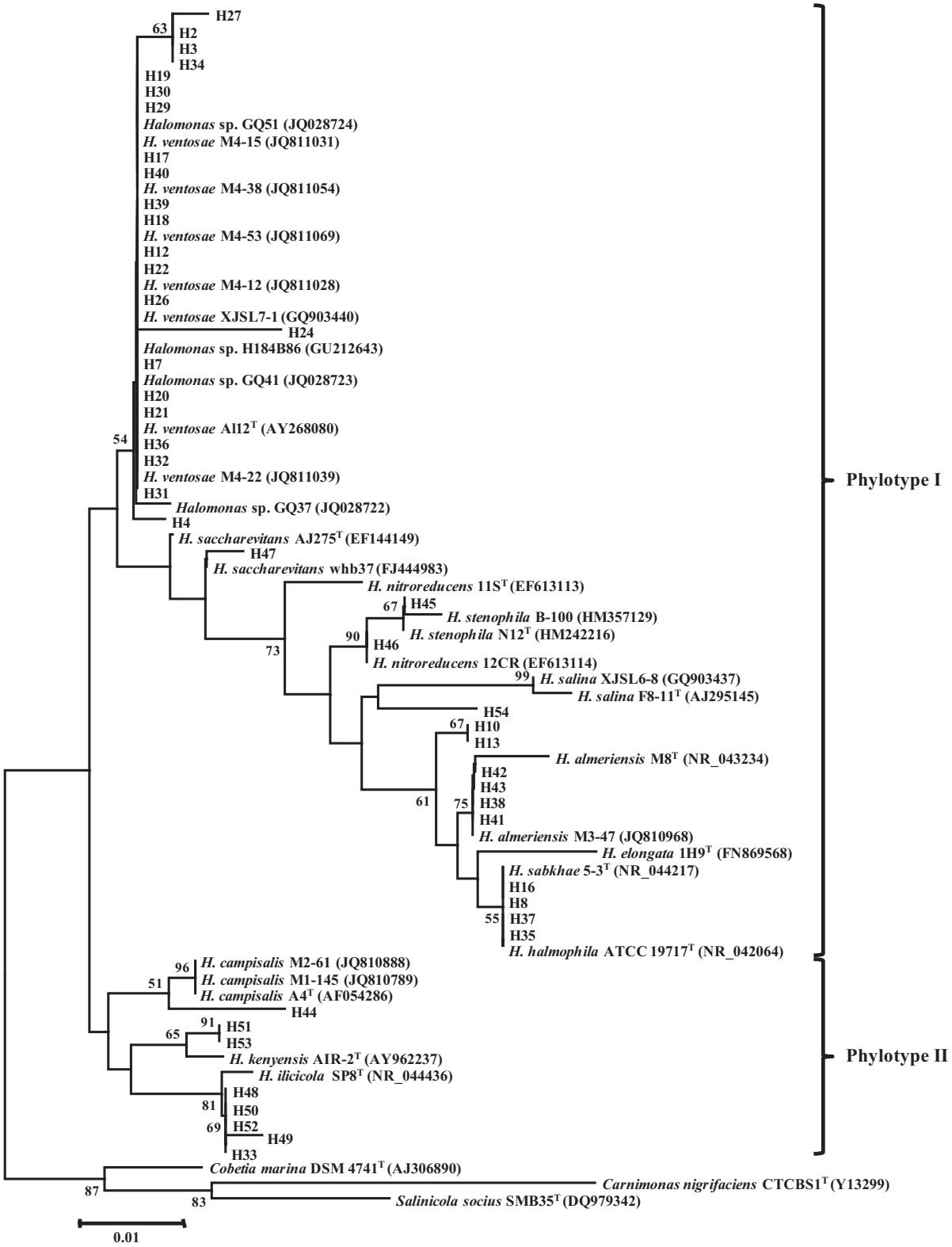

Fig. 2. Neighbor-joining phylogenetic tree based on the sequences of the V1-V3 hypervariable region of the 16S rRNA gene reamplified from the DGGE gels compared with other members of the genus *Halomonas* retrieved from the databases. Bootstrap values > 50% are shown as nodes. *Cobetia marina*, *Carnimonas nigrifaciens* and *Salinicola socius* were used as outgroups.

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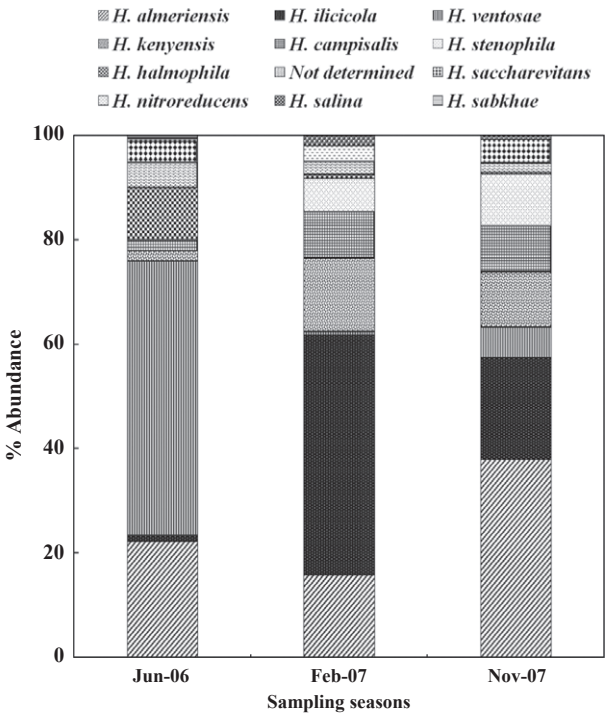

Fig. 3. Abundance of the *Halomonas* population determined by PCR-DGGE analysis.

and H13) and an unidentified sequence (H1). CCA confirmed the clear separation of upwelling zones (S7 and S8) from the other sites studied (Fig. 5b). Samples from S7 and S8 were the nearest to the salinity arrow, demonstrating their strong relationship with this factor. The other sampling sites were influenced positively by pH and oxygen, and negatively by salinity, with the exception of the samples from riverbed zone (S1A, S2A, and S4A), taken in June 2006, which were influenced positively by salinity and oxygen but negatively by pH.

Quantitative analysis of *Halomonas*

An average of 6.7×10^8 total microbial cells mL^{-1} were detected with DAPI staining in Rambla Salada (7.1×10^8, 6.1×10^8 and 6.7×10^8 cells mL^{-1} in June 2006, February 2007 and November 2007, respectively). CARD-FISH using specific primers for *Halomonas* showed the maximum counts from samples taken in June 2006 (4.1×10^7 cells mL^{-1}), medium values in February 2007 (3.2×10^7 cells mL^{-1}) and the lowest in November 2007 (2.9×10^7 cells mL^{-1}); the average value was 3.4×10^7 cells mL^{-1}. Cells stained with DAPI and FITC are shown in Supporting Information, Fig. S1.

Specific PCR-DGGE approach to analyze the denitrifying community of *Halomonas*

The denitrifying community was studied in the upwelling zone in June 2006. Figure 6 presents the corresponding DGGE patterns and the phylogenetic trees based on *nir*S and *nos*Z gene fragments. The bands obtained from DGGE gels with *nir*S primers were related to three denitrifiers of this genus: strain R53 of *H. cerina* and the type strains of *H. fontilapidosi* and *H. ventosae* (Table 3). Bands assigned to *H. ventosae* were the most intense. We also detected new denitrifying species (band Z1-Z5) with *nor*Z primers. None of the bands yielded sequences linked to alkaliphilic denitrifying species of *Halomonas*, which forms a distinct clade within the phylogenetic trees (Fig. 6a and b).

Discussion

In this work, we describe the diversity, distribution and the possible ecological role of *Halomonas* populations at different sites in Rambla Salada. For this purpose, we used DGGE analysis of the 16S rRNA genes specific for *Halomonas* and *nirS* and *nosZ* genes specific for denitrifying *Halomonas*. We have also studied the influence of spatial and temporal environmental variables (pH, oxygen, and salinity) by means of a multivariate analysis. In addition, we have sequenced the different bands to determine their phylogenetic affiliation. Finally, we have used the CARD-FISH technique to quantify the *Halomonas* population inhabiting Rambla Salada during the three seasons in which we took the samples (June 2006, and February and November 2007).

Table 4. Diversity indexes in the three seasons studied

	Season			
Indexes	Jun-06	Feb-07	Nov-07	LSD
Range-weighted richness (*Rr*)	54.43 ± 26.09*	12.57 ± 12.17	3.29 ± 3.08	0.0002
Functional organization (*Fo*)	65.98%*	52.29%	47.93%	0.009
Shannon (*H'*)	2.04 ± 0.16*	1.73 ± 0.15	1.47 ± 0.20	0.0068
Simpson (*SI'*)	0.17 ± 0.04*	0.22 ± 0.03	0.27 ± 0.03	0.0456

LSD: least significant difference at $P < 0.05$.
*Significant differences among the three seasons.

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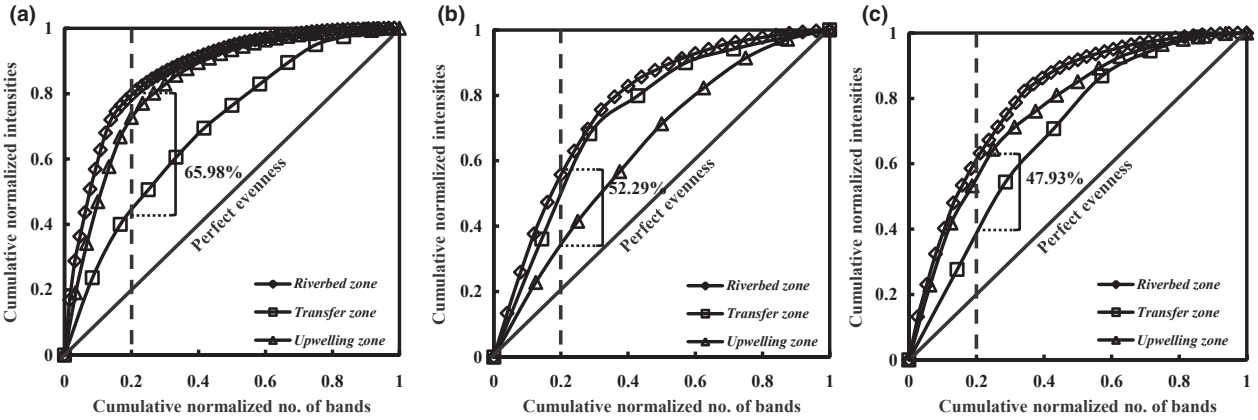

Fig. 4. Pareto-Lorenz distribution curves based on the DGGE fingerprints of the *Halomonas* community in the different sampling zones in (a) June 2006, (b) February 2007 and (c) November 2007. The vertical projection at the 0.2 χ- axis are plotted to determine Pareto values (*Fo*).

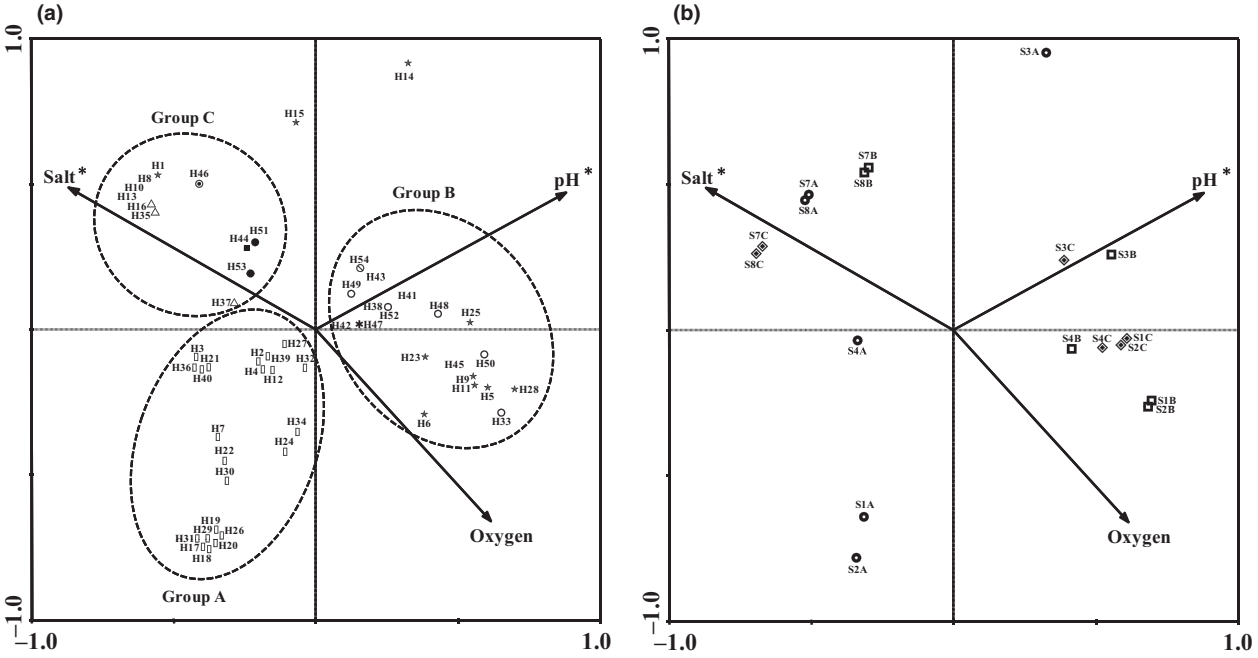

Fig. 5. Canonical correspondence analysis (CCA) ordination diagram (biplot) of environmental variables [salinity (salt), pH, and oxygen] vs. (a) band class (letters) related to *Halomonas* species and (b) the sampling site and season [A (June 2006◉), B (February 2007◘), and C (November 2007◈); origin of sampling sites S1-S8 shown in Table 1]. Environmental variables are indicated by arrows, where those marked with asterisks are significant ($P < 0.05$). ⌑: *H. almeriensis*; ■: *H. campisalis*; ❖: *H. halmophila*; ○: *H. ilicicola*; ●: *H. kenyensis*; ⊙: *H. nitroreducens*; Δ: *H. sabkhae*; ✱: *H. saccharevitans*; ⊘: *H. salina*; ☼: *H. stenophila*; ▯: *H. ventosae*; ☆: Not determined.

Analysis of the diversity and distribution of the *Halomonas* community by DGGE fingerprinting

Halomonas is one of the most frequent genera isolated by traditional methods from saline and hypersaline environments (Martínez-Cánovas *et al.*, 2004b; Xu *et al.*, 2007; Cui *et al.*, 2008), even when these habitats simultaneously possess other extreme conditions, such as very alkaline or acidic pH (Shapovalova *et al.*, 2008; Shi *et al.*, 2012). Rambla Salada does not represent an exception for this trend and, in fact, in this habitat, Luque *et al.* (R. Luque, V. Béjar, E. Quesada, F. Martínez-Checa and I. Llamas, unpublished data) have isolated a high number of *Halomonas* species. Moreover, the CARD-FISH technique using a specific probe for *Halomonas* showed that the percentage of cells pertaining to such genus was 5.1% compared with the total microbial population. Nevertheless, in

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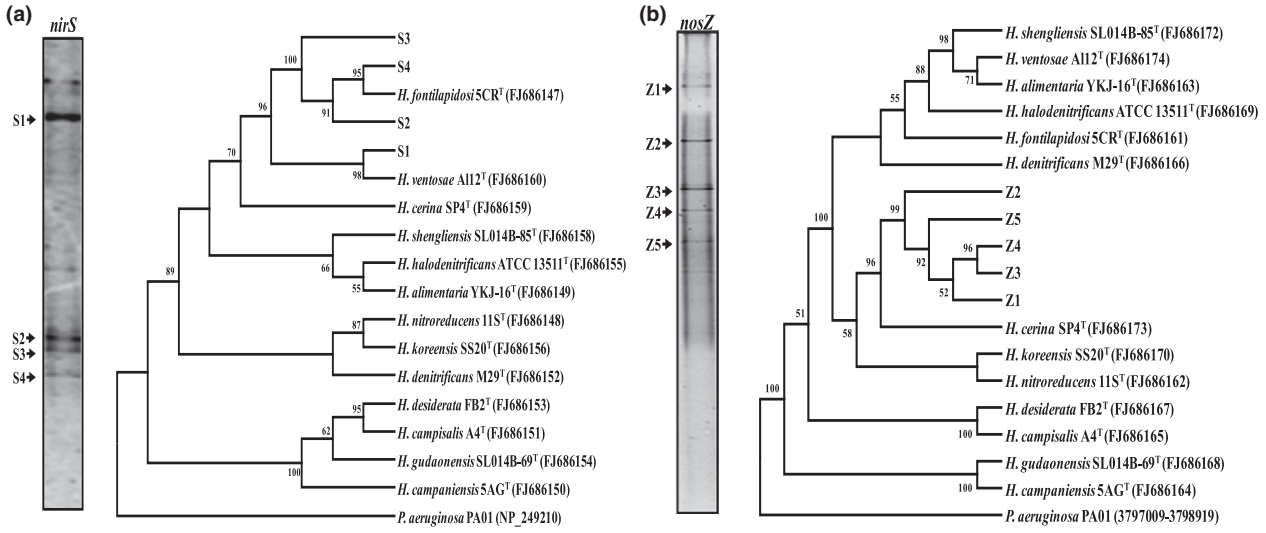

Fig. 6. DGGE fingerprints and phylogenetic affiliation of the excised gel bands corresponding to (a) *nir*S gene; (b) *nos*Z gene, after using the specific PCR primers for the denitrifying species of *Halomonas*.

spite of the apparent predominance of this genus, the attempts to detect it by molecular means in Rambla Salada using general primers for bacteria have been unsuccessful (Oueriaghli *et al.*, 2012). It is known that a species contributing < 1% of the total population would not be readily detected by direct PCR from samples and further DGGE analysis (Muyzer, 1999; Dar *et al.*, 2005), but this is not the case of *Halomonas* which, according to our results, represents 5.1% of the total microbial population. This fact may well be due to the bias produced by the PCR amplification (Neufeled & Mohn, 2006).

We have followed a novel strategy to overcome the difficulties associated with the use of general primers of *Bacteria*. Firstly, we conducted a search in the literature and found the 16S rRNA gene oligonucleotide probe Hlm474-R, which targeted most of the members of the genus *Halomonas* in previous FISH experiments (Yoshie *et al.*, 2004). In addition, we combined this oligonucleotide with the primer 16F27 (Lane, 1991) to perform specific PCRs. Although 16F27 is widely used in the amplification of ribosomal genes in a great number of genera, its combination with the specific oligonucleotide Hlm474-R as a pair of PCR primers only reported sequences affiliated to the genus *Halomonas* and in any case amplified related taxa, such as *Cobetia* or *Carnimonas*, which were used in our work as negative controls.

The clustering of PCR-DGGE patterns showed different results depending on the coefficient used in the fingerprint analysis. The dendrogram obtained from Dice-UPGMA clustering of our DGGE results revealed a clear separation of the samples by seasons (Fig. 1b). Nevertheless, the Pearson product/moment correlation reported a less defined clustering of the sampling seasons. There are controversial opinions on the suitability of either coefficient, but evidence suggests that the Pearson-UPGMA clustering method is more sensitive to differences in background of DNA fingerprint profiles and, to a lesser extent, to variations in relative band intensities (Huys & Swings, 1999). Therefore, the Dice coefficient for fingerprint analysis seems preferable (Schwalbach *et al.*, 2005; Hewson *et al.*, 2006a, b). In relation to our study, the clustering based on the presence/absence of bands using the Dice coefficient showed more coherence, because it was related to the physicochemical characteristics of the samples taken in each season, especially to the corresponding salt concentration (Table 1). Salinity is one of the driving forces of diversity in Rambla Salada as we will discuss below. In June 2006, the salinity was higher than in the other seasons for all the sampling sites. The exception was the upwelling zone (site 3) where salinity was constant at all times.

Distribution of the *Halomonas* populations based on 16S rRNA gene sequence characteristics

The phylogenetic analysis of the bands re-amplified and sequenced showed two phylotypes within the *Halomonas* population at Rambla Salada, with predominance of the denitrifying species in both of them (Fig. 2). Equally, the analyses of band intensity of DGGE also showed that the denitrifier species were also very abundant. The most abundant species among the sequenced population were *H. almeriensis* and two denitrifying species, *H. ilicicola* and *H. ventosae*. *Halomonas ventosae* was the taxon that originated the highest numbers of bands and the third most abundant species in terms of band intensity. The

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bands related to *H. ventosae* showed a high percentage of identity with strains M4-22, M4-15, and M4-38 of this species, which were isolated from the same seasons and sites at Rambla Salada by Luque *et al.* (R. Luque, V. Béjar, E. Quesada, F. Martínez-Checa and I. Llamas, unpublished data). The bands H38, H41, H42, and H43 (*H. almeriensis*) had percentages of identity close or equal to 100% with the strain M3-47 of *H. almeriensis*, also isolated from this habitat by the same authors. *Halomonas almeriensis* and *H. ventosae*, two of the three predominant taxa in Rambla Salada according to our study (Fig. 3), have also been found in this habitat by traditional approaches (R. Luque, V. Béjar, E. Quesada, F. Martínez-Checa and I. Llamas, unpublished data).

Analysis of diversity indexes

The richness and evenness of *Halomonas* in Rambla Salada were significantly different from season to season, the highest being in June 2006, the season with the highest salinity, with an average richness (*Rr*) and Shannon (*H'*) indexes values of 54.43 ± 26.09 and 2.04 ± 0.16, respectively (Table 4). Dominance, calculated from the Simpson Index (*SI'*), was the lowest in June 2006 (0.17 ± 0.04), the highest in November 2007 (0.27 ± 0.03) and somewhat lower again in February 2007 (0.22 ± 0.03; Table 4). These results confirm that dominance decreases concomitantly with an increase in diversity measured with Shannon Indexes (Magurran, 1996). The majority of the samples analyzed in this study showed medium *Fo* values ranging from 40% to 60% (Table 4, Fig. 4), indicating that the community of *Halomonas* is balanced and can therefore potentially deal with changing environmental conditions and preserve its functionality.

Quantitative analysis of *Halomonas*

The samples taken in June 2006 contained the maximum number of cells of *Halomonas* (4.1×10^7 cells mL^{-1}) which is 5.8% of the total number of microbial cells. Nevertheless, the values obtained in February and November 2007 were lower, 3.2×10^7 and 2.9×10^7 cells mL^{-1}, respectively, the total percentage of microbial cells being 5.2% and 4.4%, respectively. This technique confirms the results obtained by DGGE indicating the ubiquity and abundance of the genus *Halomonas* in Rambla Salada.

Relationship between environmental parameters and the composition of the *Halomonas* communities

CCA analysis (Fig. 5) also confirmed that the structure of the *Halomonas* community in Rambla Salada was significantly influenced by salinity and pH. Regarding salinity, there was a clear distinction between the community from the upwelling zone (S7 and S8), strongly related to the salt content and those at the other sites, in all the three seasons studied (Fig. 5b). The same results were observed by Oueriaghli *et al.* (2013), when they studied the archaeal community in Rambla Salada. This could be explained because salinity values were the highest in the upwelling zone (site 3), and they remained almost constant throughout the study (15% w/v salts; Table 1). Bands affiliated to *H. campisalis*, *H. halmophila*, *H. kenyensis*, *H. nitroreducens,* and *H. sabkhae* were mostly collected from the upwelling zone. Therefore, these phylotypes were more strongly correlated with salinity (Fig. 5a). *Halomonas ventosae* constituted 60% of the sequenced bands and the 52.6% of the band intensity in samples taken in June 2006. This species was the most abundant according to these results. As we can see in Fig. 5a, H. *ventosae* was slightly correlated with salinity and negatively with pH, which were the environmental parameters measured in the riverbed zone in June 2006, when *H. ventosae* was mainly detected. Finally, the group B, composed by phylotypes belonging to *H. almeriensis*, *H. ilicicola*, *H. saccharevitans*, *H. salina,* and *H. stenophila* was positively correlated with pH, as expected for samples found in the riverbed and transfer zones in February and November 2007, which had relatively alkaline pH (Fig. 5b).

Specific PCR-DGGE approach to analyze the denitrifying community of *Halomonas*

As mentioned before, our results revealed that *H. ventosae* and *H. ilicicola* were the predominant denitrifying bacteria in Rambla Salada. Moreover, many other denitrifiers were also detected using specific primers for the 16S rRNA gene of *Halomonas*, such as *H. campisalis*, *H. kenyensis*, *H. nitroreducens*, *H. saccharevitans,* and *H. sabkhae*. Therefore, the denitrifying population seems to be the most abundant within the *Halomonas* community of Rambla Salada (62% of this population). To study the functional role of *Halomonas* denitrifying species, we also designed new primer sets targeting denitrification *nir*S and *nos*Z genes. For this aim, we used the *nirS* and *nosZ* gene sequences of other denitrifier species described previously (González-Domenech *et al.*, 2010). Using these primers, we analyzed sediments from the upwelling zone (site 3, sample S8A) collected in June 2006 (see Table 1) where we found the highest percentage of denitrifiers (74%).

The DGGE patterns based on amplified fragments of *nir*S and *nos*Z genes yielded sequences affiliated to three denitrifying strains of *Halomonas*: Strain R53 of *H. cerina*, which was previously isolated from Rambla Salada (González-Domenech *et al.*, 2008), and *H. fontilapidosi*

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and *H. ventosae* type strains (Fig. 6a). Neither *H. cerina* nor *H. fontilapidosi* could be detected in this work when we used the primer for the ribosomal gene of *Halomonas*. In addition, we found, with the primers for *nos*Z, sequences not previously described (Fig. 6b). The sequences assigned to *H. ventosae* were the most abundant among the denitrifying community when we used primers for *nir*S gen. This result is correlated with those obtained in the upwelling zone in June 2006, using primers for 16S rRNA gene of *Halomonas*. Finally, we did not detect the alkaliphilic denitrifying species of *Halomonas*, which would need an *ad hoc* design of specific oligonucleotides because these species may well not share the conservative regions used as probes in the molecular techniques of this study.

Conclusion

Members of the genus *Halomonas* are widely distributed in Rambla Salada with *H. almeriensis* and the denitrifying species *H. ventosae* and *H. ilicicola* being the main representatives. We detected by molecular methods microorganisms previously isolated by culture medium in this habitat. Salinity is the main environmental factor that influences the diversity of *Halomonas* in Rambla Salada along with the pH.

Acknowledgements

We thank Ann-Charlotte Toes for her help with the DGGE technique. We also thank Angela Tate for her critical reading of this manuscript. This research was supported by grants from the Ministry of Technology and Science (CGL2008-02399 and BIO2011-12879-E) and from the Plan Andaluz de Investigación (P06-CVI-01850), Spain.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Morphology and dimensions of the cells hybridized with specific probes of the genus *Halomonas* (left side) in comparison with the total cells stained with DAPI (right side). Scale bars, 10 μm.

Table S1. Summary of CANOCO results.

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