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Changes in archaeal, bacterial and eukaryal assemblages along a salinity gradient by comparison of genetic fingerprinting methods in a multipond solar saltern

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Summary

Microbial communities inhabiting a multipond solar saltern were analysed and compared using SSU rRNA polymerase chain reaction (PCR)-based fingerprintings carried out in parallel by four laboratories. A salinity gradient from seawater (3.7%) to NaCl precipitation (37%) was studied for Bacteria, Archaea and Eukarya, and laboratories applied their own techniques and protocols on the same set of samples. Members of all three domains were retrieved from all salt concentrations. Three fingerprinting techniques were used: denaturing gradient gel electrophoresis (DGGE), ribosomal internal spacer analysis (RISA), and terminal-restriction fragments length polymorphism (T-RFLP). In addition, each laboratory used its own biomass collection method and DNA extraction protocols. Prokaryotes were addressed using DGGE and RISA with different 'domain-specific' primers sets. Eukaryotes were analysed by one laboratory using DGGE and T-RFLP, but targeting the same 18S rDNA site. Fingerprints were compared through cluster analysis and non-metric multidimensional scaling plots. This exercise allowed fast comparison of microbial assemblages and determined to what extent the picture provided by each laboratory was similar to those of others. Formation of two main, salinity-based groups of samples in prokaryotes (4–15% and 22–37% salinity) was consistent for all the laboratories. When other clusters appeared, this was a result of the particular technique and the protocol used in each case, but more affected by the primers set used. Eukaryotic microorganisms changed more from pond to pond; 4–5% and 8–37% salinity were but the two main groups detected. Archaea showed the lowest number of bands whereas Eukarya showed the highest number of operational taxonomic units (OTUs) in the initial ponds. Artefacts appeared in the DGGE from ponds with extremely low microbial richness. On the other hand, different 16S rDNA fragments with the same restriction or internal transcribed spacer (ITS) length were the main limitations for T-RFLP and RISA analyses, respectively, in ponds with the highest OTUs richness. However, although the particular taxonomic composition could vary among protocols, the general structure of the microbial assemblages was maintained.

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Introduction

The study of microbial diversity (both species richness and species evenness) in natural environments has advanced significantly after the introduction of SSU rDNA-based molecular techniques (for example, see reviews in Amann et al., 1995; Muyzer, 1998). Different molecular methods are available, and the choice of the right method would be dependent on the questions to be answered and on the amount of samples to be processed within a reasonable period of time. To compare microbial assemblages and assess temporal and spatial changes, fingerprinting techniques offer the best compromise. Polymerase chain reaction (PCR)-fingerprinting methods generate band patterns that, in the best case, relate each band to a single taxon. These methods are widely used in environmental microbiology and several lines of evidence indicate that fingerprints reflect the composition of

the predominant PCR-targeted members of the community (e.g. Felske et al., 1998; Nübel et al., 1999; Riemann et al., 1999; Casamayor et al., 2000a). According to PCR fingerprints, microbial assemblages are generally dominated by a few taxons (between three and 35 bands are commonly observed), although many more are likely to be present in low abundance (e.g. Casamayor et al., 2000a). It is possible that fingerprints provide a biased view of microbial assemblages. Most fingerprinting methods rely on PCR, a technique that may introduce different biases and therefore alter the natural abundance of sequences (for a review, see Wintzingerode *et al*., 1997). Other problems are the presence of heteroduplexes (Muyzer *et al*., 1998), different sequences that might stop at the same position in a gradient gel or have the same restriction length and, finally, different bands belonging to different operons of the same organism (Nübel *et al*., 1996). Furthermore, some 16S rDNA-defined microdiverse populations can be lumped together or separated depending on the resolution of the methods (e.g. Casamayor *et al*., 2002). Another source of problems arises from nucleic acid extraction procedures, because it is not clear whether microbial cells in nature exhibit different degrees of resistance to cell breakage and extraction efficiency might be dependent on assemblage composition. Next, the influence of the sampling size has been poorly explored for microorganisms. Finally, the so called 'universal' primers used for PCR amplification might overlook some of the potentially targeted microorganisms (Schmalenberger *et al*., 2001). A good comparison between the different protocols has rarely been made and each research laboratory tends to use its own procedure. Thus, different biomass collection steps, DNA extraction methods, different 'universal' primers sets for the PCR, and different fingerprinting techniques are widely used. To what extent does this methodological variability alter the picture of a natural assemblage is, however, a matter of debate. In the present work, we have used a solar saltern as a model environment to address an interlaboratory comparison. Thus, four different research groups met in Bras del Port salterns in Santa Pola (Alicante, Spain). Each group applied its own protocol on the same set of original samples and final results were compared. Protocols included different DNA extraction methods and different sets of 'domain-specific' primers. Solar salterns are semi-artificial systems to harvest NaCl from seawater, and we have selected this environment because it provides a very useful tool for microbial diversity studies (Pedrós-Alió *et al*., 2000). Seawater is pumped along a series of ponds, water evaporates and salts concentrate, changing the physicochemical properties. Sequential precipitation of $CaCO_3$ and $CaSO_4$ occurs during the first stages and, finally, when seawater has evaporated to about one tenth of the original volume, NaCl precipitates in ponds called crystallizers. Thus, a wide range of environments can be sampled within walking distance and completely different communities can be simultaneously studied. Fingerprintings should reflect this trend. It is widely accepted that diversity decreases with increasing salinity (Rodríguez-Valera *et al*., 1985; Pedrós-Alió *et al*., 2000), providing a working hypothesis about which environments are high or low in diversity. Mostly, three extreme-halophilic microorganisms (one archaeon (Benlloch *et al*., 1995; Antón *et al*., 1999), one bacterium (Antón *et al*., 2000) and the alga *Dunaliella salina*) thrive in crystallizers (35–37% salinity). At lower salinity ponds, on the contrary, a rather diverse assemblage of halotolerant and halophilic microorganisms is found (Rodríguez-Valera *et al*., 1985; Benlloch *et al*., 2002). Again, fingerprints should show this trend. In the work we present here, different fingerprints are analysed to check to what extent the picture provided by each laboratory was similar to those of others along the gradient. The 16S rDNA sequences obtained from fingerprints and clone libraries are discussed in a separate paper (Benlloch *et al*., 2002).

Results

Methodological considerations

The aim of the present work was not to carry out a detailed, step-by-step comparison of methods and protocols but to compare the final results obtained by four independent approaches. Thus, each laboratory was free to use its own protocol on the same set of water samples (Table 1). All four laboratories targeted Bacteria but only two groups targeted Archaea. Eukarya were analysed by one laboratory but using two fingerprinting techniques. In total, four biomass collection procedures, four DNA isolation methods, seven sets of primers and three fingerprinting techniques were utilized. For Bacteria, three different 'domain-specific' primers sets were used (Fig. 1).

DNA was extracted and PCR-amplified with all the methods applied. Differences were seen, however, for the long-term performance of DNA extracts. DNA collected with microconcentrators and washed several times (ICM protocol, see *Experimental procedures*) yielded PCR amplification products even after several months of being kept at –70°C, but that which was DNA-precipitated and washed once with ethanol did not. On the other hand, Archaea from ponds of up to 8% salinity yielded very little PCR product with all protocols. Yield improved after a nested PCR step (data not shown, see Benlloch *et al*., 2002). Therefore, the low amplification yield was probably due to a very low original concentration of archaeal DNA (i.e. very low archaeal abundance).

To determine the extent of the variability introduced

Table 1. General conditions used by the groups of Barcelona (ICM), Alicante (UMH), Plymouth (PLM) and Bergen (UiB).

Laboratory	Method	Target	Volume (ml)	DNA extraction	PCR conditions	Primer set
ICM	DGGE	Bacteria	600–20	Lys + PrK + SDS + phenol	55°C, 30 cycles	341fGC-907r
	DGGE	Archaea	600–20	Lys + PrK + SDS + phenol	61°C, 30 cycles	344fGC-915r
	DGGE	Eukarya	600–20	Lys + PrK + SDS + phenol	56°C, 35 cycles	1Af-516GCr
	T-RFLP	Eukarya	600–20	Lys + PrK + SDS + phenol	56°C, 35 cycles	1Af-516r
UMH	RISA	Bacteria	100–15	PrK + SDS + phenol	55°C, 35 cycles	1055f-38r[a]
	RISA	Archaea	100–15	PrK + SDS + phenol	55°C, 35 cycles	915f-71r[a]
PLM	DGGE	Bacteria	100–10	CTAB + phenol	52°C, 35 cycles	1055f-1392rGC
UiB	DGGE	Bacteria	1.5	Direct into PCR	55°C, 30 cycles	8fGC-518r

Volume indicates the amount of sample collected. DNA extraction involved a combination of lysozyme (Lys), proteinase K (PrK), SDS or CTAB treatment and phenol extraction, except for the UiB laboratory at which cells were directly introduced in the PCR tube. For PCR conditions, the annealing temperature and total number of cycles are given. Sequences of the primers used are in Table 2.
a. 23S rDNA targeted site.

by the different biomass collection protocols, volumes processed, and DNA extraction methods used by each laboratory, one denaturing gradient gel electrophoresis (DGGE) and one ribosomal internal spacer analysis (RISA) were run with nucleic acids extracted by all methods. DGGE for Bacteria of two selected ponds is shown in Fig. 2. The Plymouth Marine Laboratory (PLM) DGGE protocol was used. Despite the facts that the total amount of PCR product in each lane was not the same and that some difficulties may arise with detection limits, fingerprints showed a very similar banding pattern. RISA fingerprints (Universidad Miguel Hernández (UMH) protocol) did not show differences among methods either (data not shown).

Results from all four laboratories have been used in the present paper in different combinations as needed. First, we used the DGGE fingerprints carried out at the Institut de Ciències del Mar (ICM) laboratory in Barcelona (i.e. DGBAC-ICM, DGARC-ICM and DGEUK-ICM) to compare the microbial structure (Bacteria, Archaea and Eukarya respectively) along the salinity gradient. Second, we tested whether conclusions were reproducible with results from two other fingerprinting techniques [RISA and terminal-restriction fragments length polymorphism (T-RFLP)]. Finally, we tested different bacterial ‘domain-specific’ targeted 16S rRNA sites, running different protocols for Bacteria (DGGEs in Table 2).

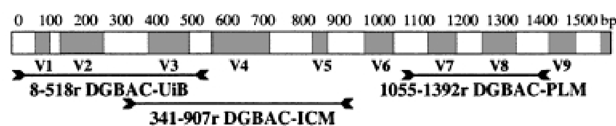

Fig. 1. Regions of the bacterial 16S rRNA gene amplified and run in DGGE by three laboratories: Bergen (UiB), Barcelona (ICM) and Plymouth (PML). The bacterial domain-specific primers used are indicated by arrows. Shaded areas represent hypervariable regions.

Comparison of phylogenetic groups along the gradient by DGGE

DGBAC-ICM, DGARC-ICM and DGEUK-ICM gels shown in Fig. 3 were carried out with the same batch of DNA (ICM protocol). In DGBAC-ICM between 9 and 15 operational taxonomic units (OTUs) per pond were found. Identical fingerprints at 32% and 37% salinity were observed (data not shown in Fig. 3), and we did not see a decrease in the number of DGGE bands as salinity increased (Fig. 4, upper panel). Comparison of bacterial assemblages through the combined representation non-metric multidimensional scaling plots (NMDS) + dendrogram (see *Experimental procedures* for explanation) produced

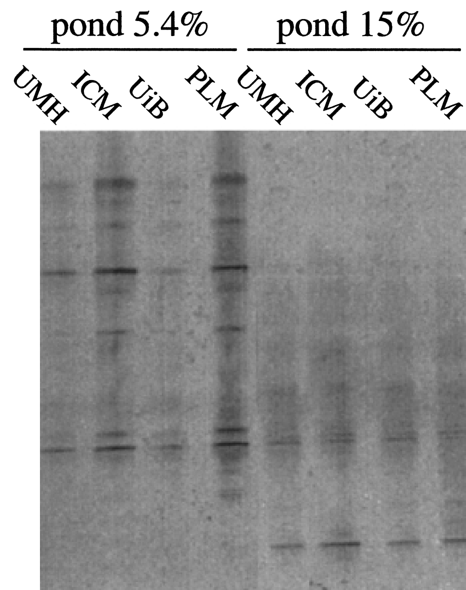

Fig. 2. Negative image of a DGGE gel containing PCR-amplified fragments of bacterial 16S rRNA genes obtained from different biomass collection and DNA extraction protocols.

Table 2. Primers used in the present work for DGGE (DG), RISA (RI) and T-RFLP (TR) techniques by the laboratories of Barcelona (ICM), Bergen (UiB), Plymouth (PLM) and Alicante (UMH).

Target	Protocol	Primer[a]	Sequence (5′⊂3′)
Bacteria	DGBAC-ICM	341f-GC	CCTACGGGAGGCAGCAG
		907r	CCGTCAATTCCTTTGAGTTT
	DGBAC-UiB	8f-GC	AGAGTTTGATCCTGGCTCAG
		518r	ATTACCGCGGCTGCTGG
	DGBAC-PLM	1055f	AATGGCTGTCGTCAGCTCGT
		1392r-GC	GYACACACCTCCCGT
	RIBAC-UMH	1055f	AATGGCTGTCGTCAGCTCGT
		38r[b]	AGGTGGGTTTCCCCATTC
Archaea	DGARC-ICM	344f-GC	ACGGGGYGCAGCAGGCGCGA
		915r	GTGCTCCCCCGCCAATTCCT
	RIARC-UMH	915f	AAAGGAATTGGCGGGGGAGCAC
		71r[b]	TCGGYGCCCGAGCCGAGCCATCC
Eukarya	DGEUK-ICM	1Af	CTGGTTGATCCTGCCAG
		516r-GC	ACCAGACTTGCCCTCC
	TREUK-ICM	1Af-hex	CTGGTTGATCCTGCCAG
		516r	ACCAGACTTGCCCTCC

Protocol code indicates the technique used, the microbial domain targeted and the laboratory respectively. GC, 40 bp-rich GC clamp attached to the 5′-end (see references in the text).
a. *Escherichia coli* numbering.
b. 23S rDNA targeted site.

two main clusters (DGBAC-ICM in Fig. 5). One contained the ponds with 22%, 32% and 37% salinity (22% being separated from the other two), and the other included the ponds of up to 15% salinity with the 4% pond separated from the rest.

In the archaeal DGGE, between two and 12 bands per pond were detected (Figs 3 and 4, upper panel). Only two weak archaeal bands were detected in the 4% pond because of a detection problem. A nested PCR on the same samples showed about 10 bands present in the 4%, 5% and 8% ponds and very similar fingerprints among them (Benlloch *et al*., 2002). In addition, uncultured Archaea related to Thermoplasmales were found in the lower salinity ponds after cloning and sequencing (Benlloch *et al*., 2002). To get more information about such uncultured microorganisms, we compared the fingerprints obtained from the whole microbial community (W sample; size range 52–0.2 µm) with a GF/F prefiltered sample (F sample; size range 1.6–0.2 µm). Identical fingerprints in both cases (DGARC-ICM, Fig. 3) indicated that we were dealing with a free-living population and, thus, we rejected the hypothesis that unclassified branches related to Thermoplasmales were endosymbionts of larger organisms such as ciliates. Up to seven

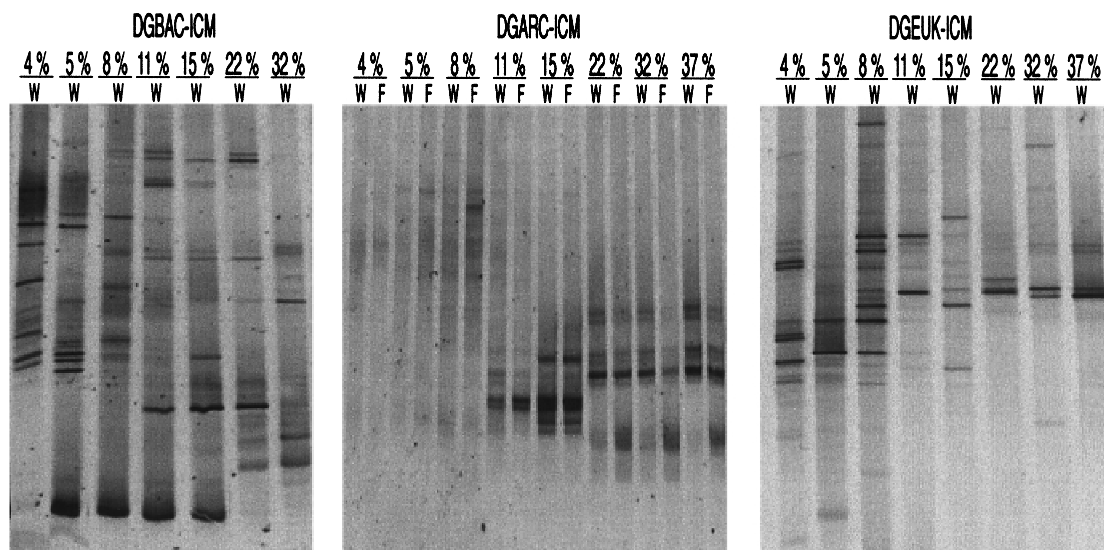

Fig. 3. Negative images of DGGE gels containing PCR-amplified fragments of bacterial 16S rRNA genes (left panel), archaeal 16S rRNA genes (centre panel) and eukaryal 18S rRNA genes (right panel) along the salinity gradient. In the case of Archaea, both the whole community (W) and filtered samples (F < 1.6 µm) were analysed (see text for explanation).

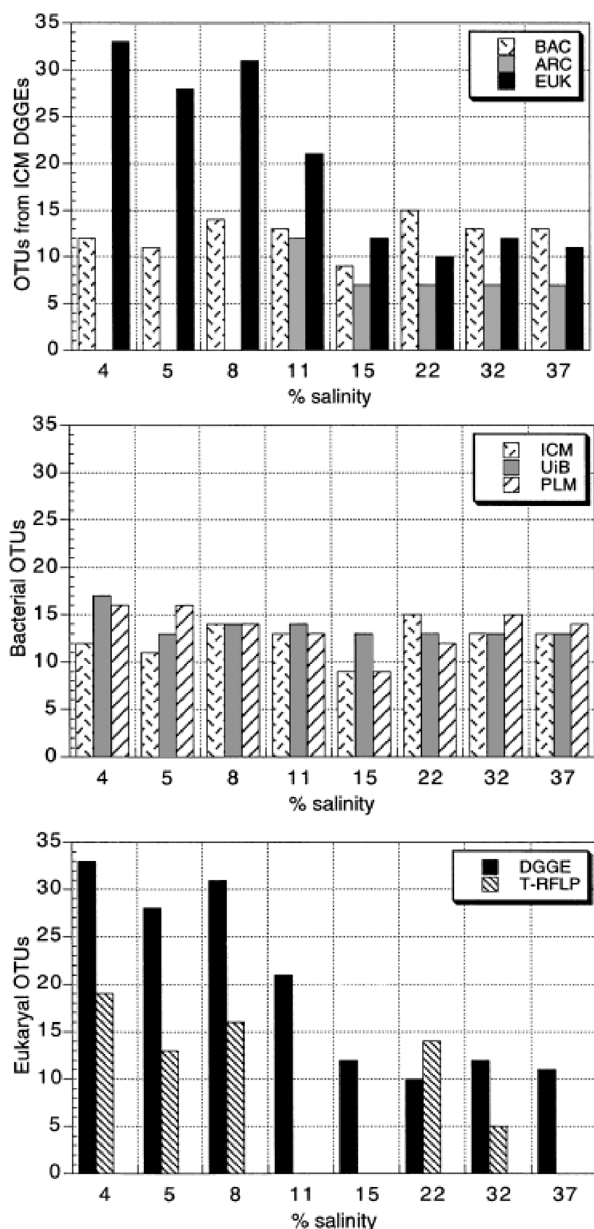

Fig. 4. Upper graph: evolution of the number of DGGE bands along the salinity gradient for Bacteria (BAC), Archaea (ARC) and Eukarya (EUK) according to ICM protocol. Middle graph: evolution of the number of DGGE bands along the salinity gradient for Bacteria using three different domain-specific primers sets at the ICM, UiB and PLM laboratories. Lower graph: evolution of the number of eukaryal OTUs along the salinity gradient according to ICM protocol. Original data were obtained from the gels shown in Figs 3 and 6B and Benlloch *et al*. (2002). Some bands at the high salinity ponds may be artificial (see *Discussion*).

archaeal bands appeared at 37% salinity and, again, we did not see a decrease in the number of DGGE bands as salinity increased (Fig. 4, upper panel). The combined plot produced the same two main salinity groupings found for Bacteria: ponds up to 15% salinity and ponds higher than 15%. The 22%, 32% and 37% ponds showed identical band patterns, whereas the 4%, 5% and 8% ponds grouped separately from the other two ponds (DGARC-ICM in Fig. 5). Finally, Eukarya yielded between 10 and 32 DGGE bands and each fingerprint was quite different in each pond (Fig. 3). The number of bands decreased in the 4% to 15% ponds, but it did not in the ponds with salinity higher than 15% (Fig. 4, upper panel). Grouping of ponds was different from those obtained for Bacteria and for Archaea: here the 4% and 5% ponds were separated from the rest (DGEUK-ICM in Fig. 5).

Comparison of DGGE results with other fingerprinting techniques and with the amplification of other 16S rRNA-targeted sites

Two alternative fingerprinting techniques (RISA and T-RFLP) were compared with DGGE. RISA for both Bacteria and Archaea offered low discrimination power with only a very few OTUs in the gel (data not shown). Therefore, RISA fragments were further digested with restriction enzymes (RISA + RFLP); the pattern was enriched and offered a better fingerprint (see example in Fig. 6A). With this modification, however, the number of RISA + RFLP bands cannot be directly compared with the number of DGGE bands. Despite this inherent limitation, we found a slightly larger number of bands in Bacteria (67 RISA + RFLP bands for all ponds) than in Archaea (61 bands), in agreement with the trend showed by DGGE data. The grouping of ponds indicated also two main clusters, i.e. one above and the other below 15% salinity for both Bacteria (RIBAC-UMH in Fig. 5) and Archaea (RIARC-UMH, Fig. 5). Some differences were seen, however, in the subclusters obtained as compared with DGGE.

Terminal-restriction fragments length polymorphism (T-RFLP) of eukaryal assemblages was carried out with two restriction enzymes, *Msp*I and *Hha*I. Only five ponds were analysed (Fig. 4, lower panel). Digestion with *Msp*I generated a number of terminal restriction fragments (TRFs, i.e. OTUs) per pond (mean 13.6 TRFs) and a number of different OTUs for all the ponds (47 TRFs) quite similar to those obtained with *Hha*I (13.8 and 45 respectively). Therefore, both enzymes yielded similar data. We retrieved less OTUs using T-RFLP than with DGGE in both extremes of the salt gradient (Fig. 4, lower panel) despite the fact that the same primer set was used in both cases (Table 2). The grouping by T-RFLP (TREUK-ICM in Fig. 5, built using the combined digestion pattern generated with both enzymes) showed, however, the same pattern as DGGE.

Finally, three domain-specific 16S rDNA regions for Bacteria were targeted using DGGE by three laboratories (Fig. 1 and Table 2): DGBAC-UiB (UiB, University of Bergen) (gel shown in Benlloch *et al*., 2002), DGBAC-

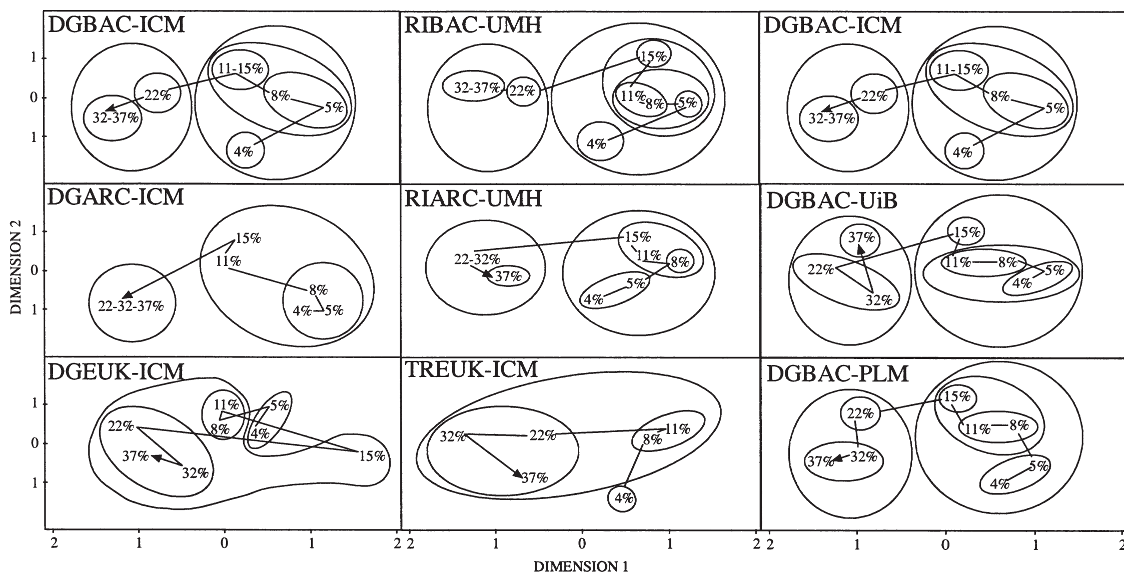

Fig. 5. Combined representation of non-metric multidimensional scaling plots and cluster analysis along the salinity gradient for the different protocols used. Left panels: DGGEs carried out at ICM in Barcelona for Bacteria, Archaea and Eukarya. Central panels: RISA (+RFLP) gels carried out at UMH in Alicante for Bacteria and Archaea; last panel shows T-RFLP for Eukarya carried out at ICM. Right panels: DGGEs for Bacteria carried out using three 'domain-specific' primers sets in Barcelona (ICM), Bergen (UiB) and Plymouth (PML).

PML (Fig. 6B), and DGBAC-ICM (Fig. 3) respectively. The three protocols identified the same two main salinity groups, whereas some different groupings were found within each cluster (Fig. 5, right panels). The number of bands recovered from each pond and, thus the trend shown by all data (Fig. 4, middle panel), was quite similar.

Altogether, in the case of prokaryotes, different protocols showed one major and consistent discontinuity between 15% and 22% salinity. Below 15% and above 22%, changes from one pond to the next were progressive but the grouping here was rather a result of the particular protocol used in each case. Similar prokaryotic richness corresponded with different fingerprints in the lower salinity ponds and with very similar fingerprints in the highest salinity. Changes in taxonomic composition without parallel changes in OTUs' richness were confirmed after sequencing (Benlloch *et al*., 2002). For Eukarya, different protocols showed the major and consistent discontinuity to be between 5% and 8% salinity.

Discussion

Several difficulties and uncertainties are found along the pathway from natural microbial communities to the molecular characterization of the assemblages. Artefacts in genetic fingerprints could potentially be introduced during biomass collection and nucleic acid extraction, during PCR amplification, or during gel casting or electrophoresis. Some of these steps were analysed in detail previously by one of the laboratories participating in this work (Schauer *et al*., 2000). These authors and others (e.g. Eichner *et al*., 1999) concluded that variability obtained using the same protocol on the same sample, or duplicate sets of samples, was minimal. In the present work, we have found that results were independent of the volume of sample processed and of the DNA extraction method used. This was rather surprising. In agreement with this, sample size from microlitre to the litre scale has not had qualitative effect on DGGE band patterns recently (Kirchman *et al*., 2001; Long and Azam, 2001). Omission of a dedicated DNA extraction method (UiB method, which used the biomass directly for PCR and avoided the laborious phenol extraction procedure) did not influence DGGE band patterns either. This has recently been reported by others in coastal seawater (Kirchman *et al*., 2001). Probably, more laborious extraction methods are needed for lysis of certain cyanobacteria and Gram-positive bacteria, but such groups are probably minor components of our samples.

Different resolution levels of each fingerprinting technique can also be a source of variability. RISA carried out in the salterns needed further RFLP steps for better discrimination, probably because different populations had the same internal transcribed spacer (ITS) length. In T-RFLP, different populations may have the same restriction length and escape detection. Thus, both techniques would lump together sets of closely related sequences and yield a lower number of bands in the fingerprint. DGGE, on the other hand, can resolve closely related environmental sequences (Casamayor *et al*., 2002), but it is more affected by the formation of heteroduplexes and the presence of multiple heterogeneous rRNA operons

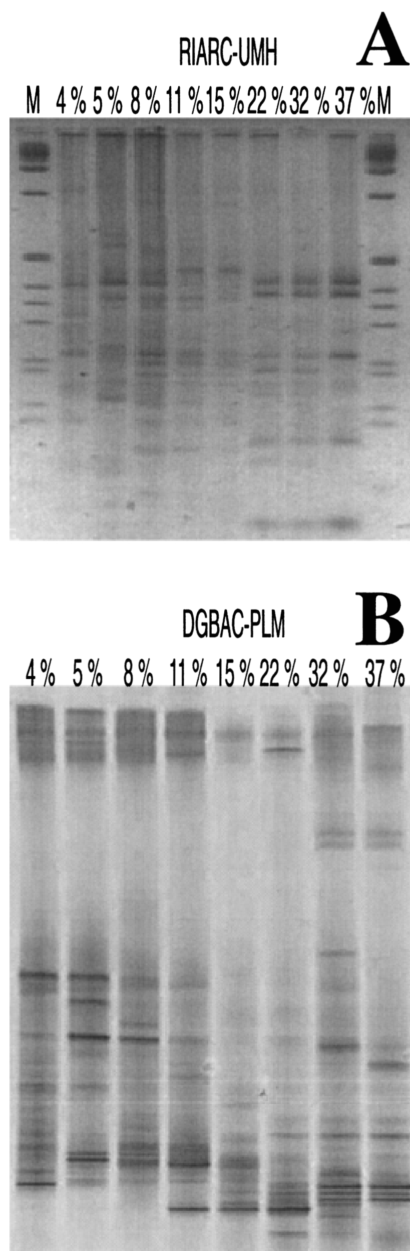

Fig. 6. Example of a RISA (+RFLP) gel for Archaea (A) and the DGGE gel obtained from PML protocol (B) along the salinity gradient. PCR products run in RISA were digested with the restriction enzyme *Hin*fI; M, 1 kb ladder.

(Muyzer *et al*., 1998), which tend to increase the number of bands in the fingerprint artificially. Therefore, several alternative explanations can be found for the different number of OTUs recovered by such techniques.

According to general ecological principles (Frontier, 1985), a more extreme environment is expected to be less diverse. In the solar salterns, several lines of evidence based on microscopy, cultures, clone libraries, FISH and 5S rRNA fingerprints, indicated this trend (Rodríguez-Valera *et al*., 1985; Oren, 1994; Benlloch *et al*., 1995; Antón *et al*., 1999; 2000; Casamayor *et al*., 2000b; Pedrós-Alió *et al*., 2000; Benlloch *et al*., 2002). In summary, and regardless of their changes in abundance, both Bacteria and Archaea showed the same pattern: as salinity increased, the number of different groups decreased, and only one group became dominant but with a considerable degree of microdiversity (Benlloch *et al*., 2002). In the present study, we found that DGGE band richness did not decrease above 15% salinity. This fact occurred in DGGEs from different laboratories, using different primer combinations. Therefore, some of the bands in DGGE could belong to such closely related sequences that might have been overlooked and lumped by methods with lower resolution (e.g. 5S rRNA or FISH with group-specific probes). Furthermore, because simultaneous presence of several closely related sequences may easily result in heteroduplex formation (Espejo *et al*., 1998), some other bands might be artefacts. To check this, DGGE bands from the crystallizer were excised and sequenced. We observed that half of the total number of DGGE bands produced the same sequence (heteroduplexes) and the other half produced very similar sequences (microdiversity). Thus, a redundancy in sequences was obtained and only two types of organisms were recovered: one extremely halophilic bacterium (*Salinibacter ruber*) and one haloarchaeon not closely related to any cultured strain (SPhT phylotype). Whether this microdiversity belongs to ecologically distinct populations (Casamayor *et al*., 2002) or to multioperons is a question we have not yet solved. In other microbial communities such as microbial mats (also with only a few populations reaching high abundance and microheterogeneities in the 16S rDNA-defined populations), heteroduplex formation seems to be a significant problem (Ferris and Ward, 1997). Limitations have been reported for the use of DGGE in very diverse microbial assemblages, such as soils, because they presented so many bands that they could not be discriminated by DGGE (Muyzer *et al*., 1998). In extremely low diversity assemblages, such as crystallizers, DGGE fingerprints require careful interpretation because the number of OTUs detected can overestimate the actual prokaryote richness.

In all the ponds that we examined, the total number of different OTUs detected were 57 for Eukarya, 37 for Bacteria, and 20 for Archaea. Furthermore, in the less saline ponds (where heteroduplexes are probably not a problem) the number of eukaryotic OTUs retrieved was more than twice that of bacterial OTUs. Altogether, eukaryotes seemed to show a surprisingly larger genetic richness than prokaryotes in solar salterns. Recently, large genetic diversity has been revealed in picoeukaryotic microorganisms in several marine environments (Díez *et al*., 2001b; López-García *et al*., 2001; Moon-van der Staay *et al*., 2001) and most probably what we have

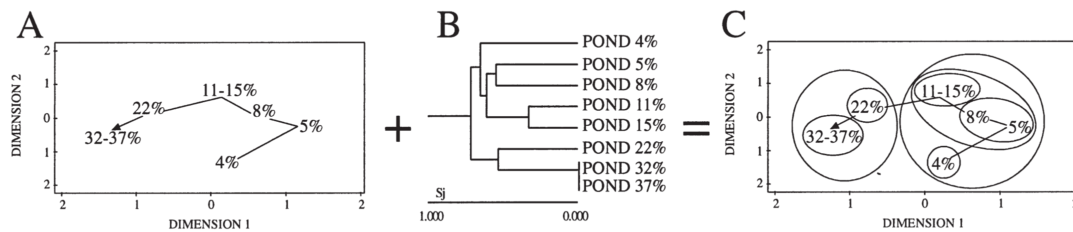

Fig. 7. Combined representation (C) using Euclidean distances in non-metric multidimensional scaling plots (A) and Jaccard similarity values (Sj) in cluster analysis (B).

found in solar salterns is not an artefact. As an example, halophilic black yeast have recently been reported inhabiting hypersaline waters (Gunde-Cimerman *et al.*, 2000). Characterization of microbial eukaryotes living in Santa Pola salterns through 18S rRNA gene sequencing is now in process.

Overall, despite the substantial technical differences among the different approaches carried out by the four laboratories, the final results were reasonably similar. When the same primer set (DGEUK/TREUK) or very similar primer sets (DGARC/RIARC and DGBAC-PLM/RIBAC) were used with different fingerprinting techniques, the results were quite satisfactory. When different primer sets were used with the same fingerprinting technique, variability was higher. Potentially, different targeted SSU rDNA sequences might not be PCR-amplified with equal efficiency by the different primers, and variability within the amplified region may influence the results (Wintzingerode *et al.*, 1997; Schmalenberger *et al.*, 2001). The estimated coverage of the primers on all the sequences deposited in database varied from 53% by primer set 8f-518r to more than 84% by primers 341f-907r (Liu *et al.*, 1997). This may also explain differences found among primer sets. Despite the inherent limitations, the three primer sets used for Bacteria recovered a similar number of OTUs in all three cases. Thus, whatever the PCR biases, they appeared to be consistent in all the samples along the gradient. Unfortunately, from the three band patterns alone, without sequencing of excised bands, it remains impossible to determine whether these bands belonged to the same organisms or not. However, although the particular taxonomic composition could vary among protocols, the general structure of the bacterial assemblages was maintained.

Experimental procedures

Environment and general procedure

The Bras del Port solar salterns constitute a multipond hypersaline environment located in Santa Pola, Alicante (Spain) (38°12′N, 0°36′W). A survey for microbial assemblages along the salt gradient was performed during MIDAS workshop II, on 18 May 1999. Sampling was carried out in eight different ponds with a bucket at the end of a pole, avoiding corners and dead-ends of ponds, as reported elsewhere (Pedrós-Alió *et al.*, 2000). Ponds were sampled a few centimetres below water surface. Because of the long residence time and relatively small size, water in such shallow ponds is well mixed by wind. Salinity was measured with a hand refractometer. The waters with the highest salinity were diluted before measurements could be taken. A wide salinity range was covered (4%, 5%, 8%, 11%, 15%, 22%, 32% and 37% salinity) and mean temperatures ranged between 23°C in the 4% pond and 28°C in the 37% pond. For DNA analysis, water was kept in 25 l plastic carboys for 1–3 h until processing in the laboratory. Once the samples arrived at the laboratory, each group started its own protocol (Table 1). Different ‘domain-specific’ primers sets were used for polymerase chain reaction (PCR) (Table 2) and three fingerprinting techniques were carried out: (i) denaturing gradient gel electrophoresis (DGGE) was used by three laboratories, and DNA fragments with the same size but different sequence were resolved using denaturant conditions in a polyacrylamide gel (Muyzer *et al.*, 1998); (ii) ribosomal internal spacer analysis (RISA) is based on the amplification of the different sized ITS (internal transcribed spacer) located between the 16S and 23S ribosomal genes (Gürtler and Stanisich, 1996), and was used by one laboratory; and (iii) terminal restriction fragments length polymorphism (T-RFLP) fluorescently labelled, enzymatically digested, PCR products generated different sized bands (Marsh, 1999), and was used by one laboratory. The fingerprints were compared using statistical analysis and by the number of operational taxonomic units (OTUs) detected.

ICM protocol

This protocol was carried out by the group from Institut de Ciències del Mar (ICM) of Barcelona and was applied to DGGE for Bacteria (DGBAC-ICM), Archaea (DGARC-ICM) and Eukarya (DGEUK-ICM), and to T-RFLP for Eukarya (TREUK-ICM). Microbial biomass was collected using a peristaltic pump in 0.2 μm Sterivex filters (Millipore). The Sterivex units were filled with 1.8 ml of lysis buffer (40 mM EDTA, 50 mM Tris-HCl, 0.75 M sucrose) and stored at −70°C. Only small volumes of water could be filtered because of the combination of high biomass and high viscosity owing to salinity. Thus, we could only filter about 600 ml from the 4% pond and about 20 ml from the 37% pond (Table 1). Extracted DNA was concentrated by centrifugation on a microconcentrator (Centricon 100, Amicon; 100 000 MW cut-off) and washed

with MQ water several times. This system allowed several rinsing steps to eliminate impurities. Nucleic acid extracts were stored at −70°C. Fingerprinting analyses at the ICM were carried out as follows:

Bacterial DGGE (DGBAC-ICM) was carried out using the DGGE-2000 system (CBS Scientific Company) in a 6% (w/v) polyacrylamide gel and with a gradient of DNA-denaturant agents from 40% to 80% (100% denaturant is 7 M urea and 40% deionized formamide) (Schauer *et al*., 2000). Then, 600 ng of PCR product was loaded in each lane and the gel was run at 100 V for 16 h at 60°C. Archaeal DGGE (DGARC-ICM) was carried out as described previously (Casamayor *et al*., 2001), with some modifications. DGGE was run at 250 V for 5 h under the same conditions described for DGBAC-ICM. Finally, Eukaryal DGGE (DGEUK-ICM) was carried out as described previously (Díez *et al*., 2001a) in a denaturant gradient from 40% to 65%. DGGE gels were stained with a solution of GelStar (1:5000 dilution; FMC BioProducts) and visualized under UV radiation with the Fluor-S MultiImager (Bio-Rad) and the MULTI-ANALYST software (Bio-Rad).

For T-RFLP analysis (TREUK-ICM), we used the same primer set and PCR conditions described for DGEUK-ICM, except for the forward primer that was 5′-labelled with hexachlorofluorescein (HEX, Operon Technologies) and for the reverse primer that lacked GC-clamp. Fluorescently labelled PCR products were purified with Wizard PCR purification columns (Promega) and 6 μl aliquots of purified PCR products were separately digested with 20 U of restriction enzymes *Hha*I, and *Msp*I (Boehringer Mannheim Biochemicals) according to the manufacturer's instructions. Terminal restriction fragments (TRFs) were resolved by electrophoresis at 3000 V for 14 h in a 6% denaturing acrylamide gel (acrylamide-NN-methylenebisacrylamide, 19:1) in an ABI PRISM model 373 Automated Sequencer. TRFs were sized with the software GENESCAN 2.1 up to a one basepair resolution using the size standard TAMRA-2500 (ABI).

UMH protocol

This protocol was carried out by the group from the Universidad Miguel Hernández (UMH) of Alicante and was applied to RISA for both Bacteria (RIBAC-UMH) and Archaea (RIARC-UMH). Microbial biomass was collected by filtration on 9-cm-diameter, 0.22-μm-pore-size, Durapore filters (Millipore). Water was pumped until filter clogging decreased flow rate to a few ml per min. Filters were stored at −70°C until DNA extraction. Filters were cut in small pieces and were vigorously mixed with 2 ml of MQ water using 50 ml conical bottom polypropylene centrifuge tubes (Benlloch *et al*., 2001). The supernatant was transferred to a fresh tube, containing SDS and proteinase K, incubated at 55°C for 2 h and transferred to a boiling water-bath for 2 min. Lysates were phenol-extracted and nucleic acids were concentrated by ethanol precipitation. For RISA analysis, fragments comprising the end of the 16S ribosomal gene, the entire 16S-23S spacer and some 50 bp of the 5′-end of the 23S gene were amplified (Acinas *et al*., 1999; Benlloch *et al*., 2001). Then, 15 μl of PCR product was digested with 10 U of restriction enzyme *Hin*fI, to increase discrimination power of the banding pattern, and were loaded in a 2% Nusieve gel with a 1 kb ladder (Life Technologies). The gel was stained with ethidium bromide, photographed under UV radiation, scanned, and kept as a computer file.

PML protocol

This protocol was carried out by the group from the Plymouth Marine Laboratory and was applied to DGGE for Bacteria (DGBAC-PLM). Microbial biomass was collected by filtration and filters were digested with a procedure based on the CTAB method and phenol extraction (Goddard *et al*., 2001). PCR product (500 ng) was loaded in the gel and DGGE ran in a 6% polyacrylamide gel, with a 30–50% denaturant gradient, 200 V for 3.5 h at 60°C (Ferris and Ward, 1997). A Bio-Rad DCode system was used. Gels were stained with Sybr Gold (1:10 000 dilution; Molecular Probes) and viewed under UV.

UiB protocol

This protocol was carried out by the group from University of Bergen and was applied to DGGE for Bacteria (DGBAC-UiB). Microbial biomass was collected by direct centrifugation of a small volume of sample (i.e. 2 × 1.5 ml sample; 14 000 r.p.m. in a microfuge). The cell pellets were washed with 70% ethanol, centrifuged once more, and were then dried and stored at −70°C. No DNA extraction or purification steps were performed, and the pellets were thawed and resuspended in 50 μl MQ water. Then, 5 μl of suspension was used directly as template for PCR. DGBAC-UiB was as described previously (Øvreås *et al*., 1997). DGGE was performed with a Hoefer Scientific SE600 vertical dual-cooler system using a linear gradient of urea and formamide from 20% to 40% denaturant. Then, 400 ng of PCR samples was loaded onto 8% (w/v) polyacrylamide gels. The electrophoresis was run at 60°C, first for 10 min at 20 V and subsequently for 20 h at 60 V. After electrophoresis, gels were stained for 45 min with Sybr Gold, rinsed in distilled water and photographed with a Polaroid camera. Photographs were scanned, and digitized images were kept as computer files.

Processing band patterns and cluster analysis

High-resolution digitized images were processed with Diversity Database (Bio-Rad) and NIH Image (National Institute of Health, Bethesda, Maryland) software. The software carried out a density profile through each lane, detected the bands, and calculated the relative contribution of each band to the total band signal in the lane after applying a rolling disk as background subtraction (rolling ball radius = 50). A band was defined as a stain signal whose intensity was more than 0.2% of the total intensity for each lane (Schauer *et al*., 2000). Several of the weaker bands could not be distinguished in the figures presented here, and were only detectable on the computer screen after zooming the image. Bands occupying the same position in different lanes were identified using software facilities and one matrix with presence or absence data was built. Binary data set was used to calculate a similarity matrix using the Jaccard's coefficient with the software SYSTAT 5.2.1 for Macintosh, and a dendrogram was obtained using UPGMA (Unweighted Pair-Group Method with Arithmetic

averages) in cluster analysis (SYSTAT). Non-metric multidimensional scaling plots (NMDS) were also constructed from the binary matrix. NMDS plots present data in an Euclidean plane (with dimensions of no special significance) in which similar measurements appear close together. Each band pattern is a point in the plane and, by connecting consecutive points (i.e. consecutive ponds of increasing salinity), changes in community structure could be visualized and interpreted (see van Hannen *et al*., 1999). Dendrograms and NMDS plots are a robust and widely used way for comparison of microbial assemblages (e.g. Eichner *et al*., 1999; El Fantroussi *et al*., 1999; van Hannen *et al*., 1999; Schauer *et al*., 2000; Díez *et al*., 2001a). For statistical comparison of data sets, we combined here the information of NMDS and of similarity dendrograms (see Fig. 7 for an example). This combined representation summarizes in a single plot how consecutive ponds (connected by lines) were related to each other according to Euclidean distances and Jaccard's values. The number of operational taxonomic units (OTUs) in each sample was defined as the number of DGGE bands or terminal restriction fragments in the T-RFLP gels.

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