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Spatial and temporal scales of variation in bacterioplankton community structure in the NW Mediterranean Sea

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ABSTRACT: An important query in marine microbial ecology is at what temporal and spatial scales variations in the structure of natural bacterial communities occur. Samples of different volumes were collected at different spatial (vertical and horizontal) and temporal (from hours to seasons) scales along a transect between a coastal station (26 m depth) and an offshore Microbial Observatory (1000 m depth) in the NW Mediterranean Sea. The structure of the bacterial communities was determined by capillary electrophoresis–single strand conformation polymorphism (CE–SSCP) fingerprinting of polymerase chain reaction (PCR)-amplified 16S rDNA. This technique is a powerful tool to compare natural microbial assemblages at different spatial or temporal scales. Similar bacterial assemblages were found up to 3.7 km from the coastal station, whereas significant changes were found over greater distances (from 9.3 to 33.3 km). Although the bacterial community structure did not change with depth at coastal and shallow stations, vertical changes were found at deeper stations, most likely due to vertical variations in physico-chemical and biogeochemical parameters. Temporal changes were mainly related to environmental variations that occurred at a seasonal scale and during phytoplankton blooms. Finally, we suggest that long-term studies, at least in the NW Mediterranean Sea, should involve a minimum sampling time scale of 2 wk and a shorter time-scale when environmental changes are detected by the real-time monitoring of a few basic parameters (i.e. fluorescence, temperature, salinity). Sampling strategies should also include different depths depending on the vertical structure of the water column, based on the same basic parameters.

KEY WORDS: Bacteria · CE–SSCP · Community structure · Sampling strategy · Microbial observatory

INTRODUCTION

Micro-organisms represent the most abundant, biogeochemically important organisms in the oceans. At concentrations ranging between 10^4 and 10^6 cells ml^{-1}, an estimated 20 to 50% of marine primary productivity is channelled through bacterioplankton (Azam et al. 1983, Cho & Azam 1990). However, the identity, physiology and ecology of the vast majority of these microbes, as well as the processes they mediate in the environment, remain unknown or poorly understood. Recent advances in molecular biology, genomics, proteomics, bioinformatics, and cultivation technologies herald a new age of exploration of the microbial world. A network of 'Microbial Observatories' has been developed in different habitats and across environmental gradients in order to explore bacterial diversity and understand the role of bacterial community structure in the functioning of ecosystems. Although several microbial observatories are already operational in North America (www.nsf.gov/bio/pubs/awards/mo03.htm), there is an urgent need to define appropriate sampling strategies and analytical tools that should be used for the long-term monitoring of bacterial diversity.

Dynamics of marine pelagic bacterial communities along horizontal transects suggest different trends. Minor differences in community structure have been

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found in surface waters at stations located a few kilometers apart near Anvers Island, Antarctica (Murray et al. 1998), or even between heterotrophic communities separated by 1500 km in the Arabian Sea (Riemann et al. 1999). In contrast, differences have been found between a coastal and an offshore station, 95 km apart, off the Catalan Coast, Spain (Schauer et al. 2000), and also among samples from a transect crossing a salinity gradient in the North Sea (Riemann & Middelboe 2002) or along an estuarine gradient from the Rhone River to the Mediterranean Sea (Troussellier et al. 2002). Variability with depth has been shown to be more marked in marine ecosystems, where changes in community structure are driven by the vertical stratification of waters (Lee & Fuhrman 1991, Murray et al. 1996, 1998, Acinas et al. 1997, Moeseneder et al. 2001). Marine bacterial communities are also influenced by temporal changes that occur at different time scales, from years or seasons (Lee & Furhman 1991, Murray et al. 1998, Riemann et al. 1999, Schauer et al. 2000, 2003) to weeks or days (Acinas et al. 1997, Fandino et al. 1998, 2001). These different trends are generally related to variations in physical, chemical and/or biological parameters.

The volume of sample may also be an important factor for drawing up a general description of the genetic community structure. Although a few studies have suggested that sample size has little importance for the community structure as determined by fingerprinting methods (Kirchman et al. 2001, Long & Azam 2001, Casamayor et al. 2002), this question remains poorly evaluated.

Fingerprinting approaches offer an interesting compromise for the monitoring and comparison of microbial assemblages and for the assessment of temporal and spatial changes that would not have been feasible using time-intensive 16S rDNA sequence analysis. Denaturing gradient gel electrophoresis (DGGE) has become a very popular fingerprinting technique in marine microbial ecology, especially because bands with particular melting behaviour can be excised from the gel and subsequently sequenced to reveal the phylogenetic affiliation of the community members (Muyzer et al. 1998). However, the calibration of the linear gradient of DNA denaturants makes gel-to-gel comparison difficult. This problem has made the DGGE technique impractical for the fine comparison of large number of samples (Moeseneder et al. 1999). The recently developed capillary electrophoresis–single strand conformation polymorphism (CE–SSCP) fingerprinting technique permits high reproducibility for reliable comparison of patterns from a theoretically infinite number of samples (Lee et al. 1996). Because an internal size standard with a different fluorescent label is added to each sample, CE–SSCP and further computing correction solves the problem of gel-to-gel comparison (Zumstein et al. 2000).

This study was undertaken to investigate the temporal and spatial scales at which changes in bacterial community structure occur at a shallow coastal station and at a deep Microbial Observatory both located in the NW Mediterranean. We also evaluated the reliability of the CE–SSCP technique for the long-term analysis of bacterial community structure in terms of both reproducibility and sensitivity (sample volume).

MATERIALS AND METHODS

Sampling strategy. Seawater was sampled from the North Western Mediterranean Sea at the SOLA station (42° 31′ N, 03° 11′ E) located in Banyuls-sur-mer bay from March 2002 to March 2003. Sampling was performed with 5 l Niskin bottles attached to a Sea-Bird conductivity–temperature–depth (CTD) profiler deployed from the oceanographic RV 'Nereis II' or the RV 'Tethys'. Seawater was processed in the laboratory within 2 h of sampling.

Seawater (500 ml) was pre-filtered through 3 µm pore size filters (47 mm, Nucleopore) to remove most eukaryotic organisms and to prevent clogging of the final filter. Bacterial cells were concentrated onto 0.22 µm pore size filters (47 mm, PC Nucleopore) and stored in 2 ml Eppendorf tubes at –20°C until DNA extraction.

Horizontal variability was tested at 20 randomly selected stations between radii of 3 and 200 m around the SOLA station. The variability was also analysed over a larger scale along a linear coast-offshore transect from the coastal Stn SOLA to stations located at 3.7, 9.3, 16.7, 27.8 and 33.3 km from Stn SOLA, named Stns A, B, C, D and MOLA (Microbial Observatory of Laboratoire Arago), respectively (Fig. 1). Vertical variations were investigated by sampling at 3, 5, 10, 15 and 20 m depths at Stn SOLA (maximum depth of 26 m), 3 and 50 m at Stn A (maximum depth of 70 m), 3 and 80 m at Stn B (maximum depth of 90 m), 3, 50 and 80 m at Stn C (maximum depth of 115 m), 3 and 80 m at Stn D (maximum depth of 500 m) and 3, 80, 200 and 800 m at Stn MOLA (maximum depth of 1000 m).

Temporal variations were analysed at different scales (from hours to months) at the SOLA station. Samples were collected monthly from March 2002 to March 2003, weekly from March 2002 to May 2002, daily from 23 to 26 April 2002, and hourly every 6 h from midday 25 April to midday 26 April 2002. All samples were collected in triplicate at 3 and 20 m depths.

A large range of sample volumes (1000, 500, 250, 100, 50 and 10 ml) was collected on 5 March 2002 to investigate the effect of sample size on the community structure determination.

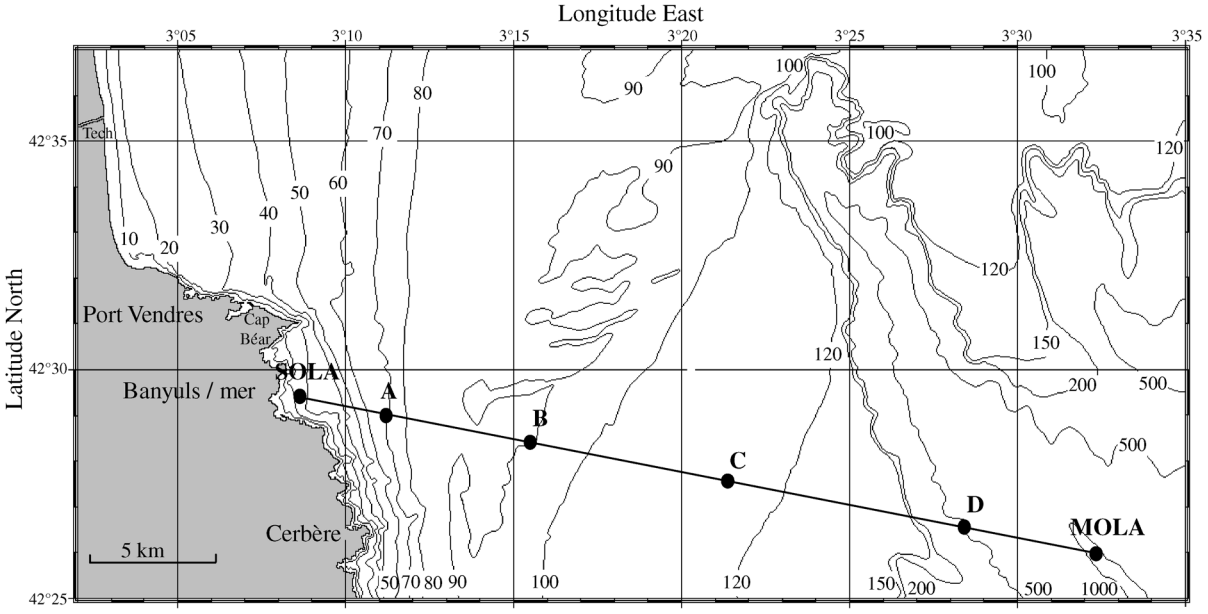


Fig. 1. Location of each sampling station along a linear transect between the coastal Stn SOLA and the offshore microbial observatory (MOLA) in the NW Mediterranean Sea

Bacterial abundances. Total bacterial counts were determined by flow cytometry (Lebaron et al. 1998). Briefly, 2 ml seawater samples were fixed with 2% formaldehyde for at least 1 h at 4°C. A 1 ml sub-sample was incubated with SYBR Green II (final conc. 0.05% [v/v] of the commercial solution) for at least 15 min at 20°C in the dark and analysed with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with an air-cooled argon laser (488 nm, 15 mW). Stained cells were enumerated according to their side-angle-scattered light (SSC) and green cell fluorescence (FL1) collected through a 530 ± 30 nm bandpass filter. Yellow–green fluorescent beads (1 µm diameter, Polysciences) were systematically added to each analysed sample to normalize SSC and FL1 emission. Data acquisition and analysis were done with Cell-Quest software (Becton Dickinson). Acquisition was triggered by FL1. The volume analysed and subsequent estimation of cell concentrations were calculated by measuring the remaining volume and subtracting it from the initial volume. Sheath fluid was seawater filtered through a 0.22 µm pore size membrane.

Nucleic acid extraction. The procedure used for extraction of chromosomal DNA was a modification of that described by Fuhrman et al. (1988). This procedure employs an enzymatic and detergent-based lysis, which is a relatively gentle method that avoids excessive shearing of DNA, produces DNA of suitable quality for PCR and reduces the risk of chimera formation during PCR (Madrid et al. 2001).

The frozen 0.22 µm pore-size filters were cut with sterilized scissors into small strips and vortexed briefly in 840 µl of alkaline lysis buffer (50 mM Tris hydrochloride pH 8.3, 40 mM EDTA, 0.75 M sucrose). Cell lysis was accomplished by an initial incubation for 45 min at 37°C after adding 50 µl of freshly prepared lysosyme solution (20 mg ml^{-1}), and a second incubation at 50°C for 10 min after adding 100 µl of 10% sodium dodecyl sulfate and 10 µl of proteinase K (20 mg ml^{-1}). The lysate was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and with an equal volume of chloroform:isoamyl alcohol (24:1). The nucleic acids were precipitated with 1 volume of isopropanol at –20°C overnight, rinsed with 70% ethanol before resuspending in TE (10 mM Tris, 1 mM EDTA). The molecular size and the purity of the DNA were analysed by agarose gel electrophoresis (1%) and the DNA was quantified either by visual comparison with the molecular weight markers in gels (rough estimate) or by spectrophotometry (GeneQuant II, Pharmacia Biotech.).

PCR–CE–SSCP of 16S rDNA. The primers used were w49 (5′-ACG GTC CAG ACT CCT ACG GG-3′; Delbès et al. 1998) and w34 (5′-TTA CCG CGG CTG CTG GCA C-3′; Lee et al. 1996), which amplify the variable V3 region of the 16S rDNA (*Escherichia coli* positions 329–533; Brosius et al. 1981). The primer w34 was fluorescently labelled at the 5′-end position with phosphoramidite (TET, Applied Biosystems). Both primers were obtained commercially (Eurogentec). Each 50 µl reaction mixture contained 50 µM of each

primer, 1X Pfu reaction buffer, 20 mM dNTPs, 1.0 U of Pfu DNA polymerase (Promega) and 0.1 µg of template DNA. PCR amplification was performed with a Robocycler (Stratagene) under the following conditions: an initial denaturation step of 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 30 s, and extension at 72°C for 30 s, and a final elongation step at 72°C for 10 min. The size (ca 200 bp length) and the amount of PCR products were determined by agarose gel electrophoresis (2%) with a DNA size standard (Low DNA Mass Ladder, GIBCO BRL).

CE–SSCP procedure. The labelled PCR products were purified with a QIAquick PCR purification kit (Qiagen) according to manufacturer's instructions. Purified PCR products were quantified by visualisation in ethidium-bromide-stained agarose gels (2%). Samples were diluted (ranging from 1:2 to 1:30) in sterile TE (10 mM Tris, 1 mM EDTA) in order to obtain 10 ng μl^{-1} of PCR product. From the resulting dilution, 1 µl of PCR product was mixed with 18 µl of formamide (Applera) and 1 µl of an internal size standard GeneScan-400 Rox (Applied Biosystems). The mixture was then denatured for 5 min at 94°C and immediately cooled on ice for at least 5 min. The procedure used for CE–SSCP analysis was a modification of that described by Delbès et al. (2000). Samples were electrokinetically injected (5 s, 12 kV) into a capillary tube (47 cm × 50 µm) filled with a 5.6% GeneScan polymer (Applied Biosystems) gel containing 10% autoclaved glycerol in sterile TBE buffer (90 mM Tris-borate, 2 mM EDTA [pH 8.0]). Electrophoresis was carried out at 15 kV and 30°C for 30 min $sample^{-1}$ and phosphoramidite (TET)-labelled fragments were detected by a laser with a virtual filter C (detection wavelengths 532, 537, and 584 nm). Data were collected with ABI Prism 310 collection software (Applied Biosystems).

Analysis of CE–SSCP fingerprints. Variations in bacterial community composition were determined from the number and intensity of the peaks. In order to normalize mobilities from different runs, all electropherograms were calibrated by fixing the positions of peaks produced by size standard GeneScan-400 Rox (Applied Biosystems) and by using a second-order least-square curve (i.e. linear regression) to provide the best interlane comparison (Genescan analysis software). Peak detection was achieved by computing the first derivative of a polynomial curve fitted to the data within a window that was centred on each data point (Genescan analysis software). Because many overlapping peaks were present in our profiles, we used a high polynomial degree value of 10 in order to increase peak detection sensitivity. The area of each peak was determined by taking into account the full width of the peak measured at half of its height. The relative area of each peak to the total peak area of each pattern was expressed as a percentage to allow inter-sample comparisons. A peak amplitude threshold of 50 was applied for both the Rox and TET dyes. The UPGMA (unweighted-pair-group method with arithmetic averages) Euclidean distances dendrograms were constructed with the software SYSTAT 5.2.1 from a matrix taking into account the presence or absence of individual peaks and the relative contribution of each peak (in percentage) to the total intensity of each pattern.

RESULTS

Methodological considerations

Several tests were performed to investigate the efficiency and reproducibility of the DNA extraction and PCR–CE–SSCP protocols. Cell lysis was monitored several times by microscopy, and in all cases, we did not observe any cells recalcitrant to our lysis procedure. The quality of the DNA (molecular weight and purity) was carefully assessed before proceeding to 16S rDNA gene amplification, and only PCR products showing a single band were used for subsequent analyses. Reproducibility of the PCR–CE–SSCP technique was tested by analysing triplicate samples from the same filtered water that had been collected during 3 successive days at Stn SOLA. Community peak patterns observed between triplicate CE–SSCP analyses were very similar, both in terms of peak number and surface area, suggesting that the different steps from DNA extraction to molecular characterization of the assemblages were very reproducible (Fig. 2).

Effect of sample size

We sampled a wide range of volumes (from 10 to 1000 ml) of 3.0 µm filtered seawater collected at the SOLA station. All samples were treated identically by filtration through 0.2 µm membranes and subsequent DNA extraction. For all samples, total bacterial counts were determined by flow cytometry. A positive correlation was found between bacterial counts and the amount of extracted DNA ($r = 0.51$; $n = 34$; $\alpha = 0.05$). Before carrying out PCR amplification from the DNA extracts and subsequent CE–SSCP analysis of the PCR products, their concentrations were normalized by diluting to 10 µg ml^{-1} for the DNA extracts and 0.5 ng μl^{-1} for the PCR products (denatured products ready for injection). For samples 50, 100, 250, 500 and 1000 ml, CE–SSCP patterns were almost identical with some small differences in peak heights (Fig. 3). For the 10 ml sample, although most major peaks were visible,

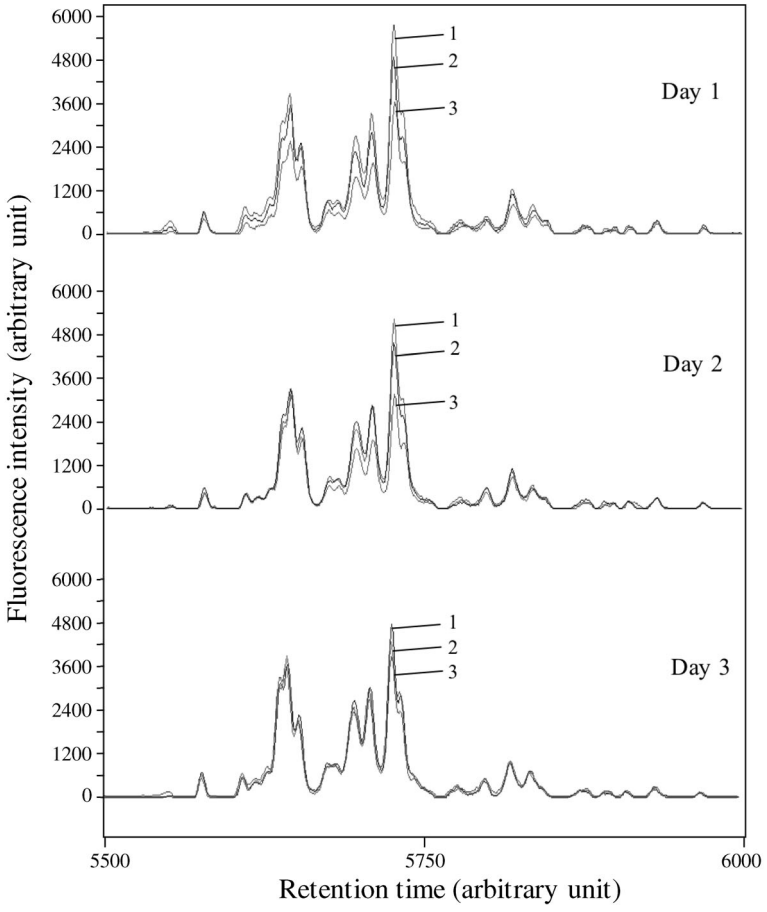


Fig. 2. Reproducibility of CE–SSCP patterns of 16S rDNA PCR products of bacterial communities in triplicate samples (labeled 1, 2, 3) collected on 3 successive days (Days 1, 2 and 3). Retention times are correlated to those for the internal size standard, GeneScan-400 Rox, which were assigned values of 5500 to 6000

some of the smaller peaks were absent, and there were more differences in the heights of the major peaks compared to the other samples (Fig. 3). The richest community peak patterns were obtained for volumes ranging from 50 to 1000 ml of filtered seawater, which corresponded to a minimum of 3×10^7 cells.

Spatial variation scale

Twenty randomly selected samples were collected within a radius of 3 or 200 m around Stn SOLA. Bacterial cell counts (from 3.5 to 4.0×10^5 cells ml^{-1}), as well as the concentration of extracted DNA (from 2.5 to 3.5 µg l^{-1} of filtered seawater), were very similar between samples. CE–SCCP profiles obtained from both radii of 3 and 200 m around Stn SOLA revealed no obvious spatial differences in the composition of bacterial assemblages, and almost no variation in peak area between samples (data not shown).

Differences in the bacterial composition were observed at a larger spatial scale along the linear coast-offshore transect from the coastal Stn SOLA to stations located 3.7, 9.3, 16.7, 27.8 and 33.3 km apart, namely Stns A, B, C, D and MOLA, respectively (Fig. 4). When comparing surface samples collected at all stations, 44 peaks were found and among these peaks, 15 were detected at all stations. In contrast, 5 peaks were found at only 1 sampling site; 4 of these peaks were specific to Stn MOLA and 1 to Stn D. When considering peak intensities as determined from peak area, the 4 specific peaks detected at Stn MOLA were significant, each peak representing from 1 to 4% of the community pattern.

The number of CE–SSCP peaks per sample varied from 25 to 33 and was higher at Stns SOLA, A and D than at Stns B, C and MOLA (Fig. 4a). The community structure at Stns SOLA and A (3.7 km apart) presented the same pattern with only a few differences in the relative intensity of each peak. Differences in microbial community composition were found from 9.3 km (Stn B) off the Stn SOLA. Fig. 4b shows the corresponding UPGMA Euclidean distances dendrogram which takes into account the presence or absence of individual peaks and the relative contribution of each peak (in percentage) to the total intensity of the pattern (including additional samples collected at different depths). Surface samples (3 m) clustered according to their proximity to one another along the transect from the coastal to the offshore stations. Stns SOLA and A formed a distinct cluster from the nearest one, which was composed of Stns B and C. This result indicates that at least in this sampling area, and along this coast–offshore transect, variations in the structure of surface communities can be observed at stations located at least 9.3 km from each other. As expected, surface samples collected at Stn MOLA, located 33.3 km from Stn SOLA, clustered in a distinct branch and therefore were significantly different from samples collected at other stations.

Vertical variations at Stn SOLA were examined in samples taken at 3, 5, 10, 15 and 20 m depths. Bacterial cell and DNA concentrations remained constant with depth, ranging from 5 to 7 $\times 10^5$ cells ml^{-1} and from 4.2 to 4.8 µg l^{-1} of filtered seawater, respectively. Most peaks were found throughout the vertical profile, but one additional peak accounting for 6.6% of the total intensity was only detected at the deepest depth of 20 m (data not shown). Variations

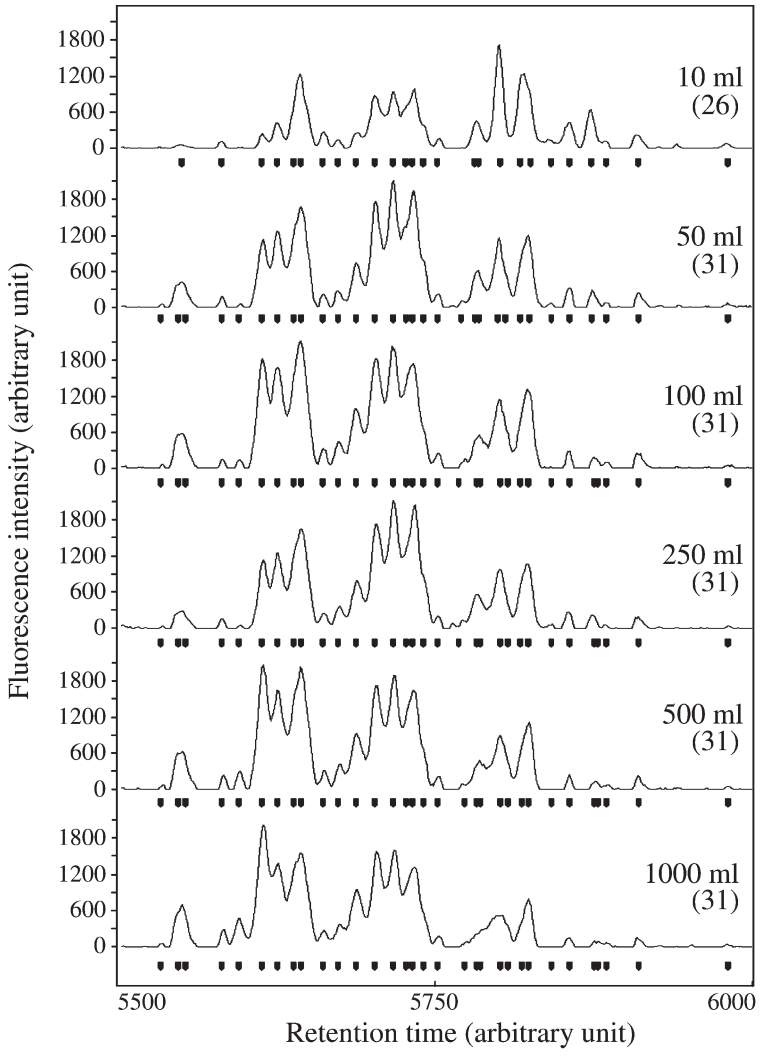


Fig. 3. CE–SSCP patterns of 16S rDNA PCR products of bacterial communities from sample volumes varying from 10 to 1000 ml of seawater collected at 3 m from Stn SOLA. Arrows indicate the peak positions. The total number of estimated peaks per sample are indicated in brackets. Retention times are correlated to those for the internal size standard, GeneScan-400 Rox

with depth were also investigated at other stations (surface and above the bottom for Stns SOLA, A, B, C and MOLA) and including the deep chlorophyll maximum (DCM) at Stns C, D and MOLA (namely C – 50 m, D – 80 m and M – 80 m). The percentage of common peaks at a given station ranged from 89 to 100% at Stns SOLA and A, and from 48 to 77% at deeper Stns B, C and MOLA (data not shown). Clear changes in bacterial community structure were observed between depths at a given station, except for the shallow Stns SOLA and A (Fig. 4b). Samples taken at Stn MOLA clustered together but with relatively low similarity (Fig. 4b).

Temporal variation scale

Variability in bacterial community structure was investigated at different time scales in surface waters (3 m depth) at Stn SOLA. Samples collected every 6 h during 24 h and daily during 4 consecutive days (from 22 to 25 April 2002) showed some variations in both bacterial cell and DNA concentrations (from 5.4 to 7.0 $\times 10^5$ cells ml^{-1} and from 3 to 5 µg l^{-1} of filtered seawater, respectively). However, the number and intensity of the peaks were highly constant at both hourly and daily scales (data not shown).

In contrast, changes were observed at larger time scales. Weekly variations were found during a 3 mo survey in spring 2002 (Fig. 5). A phytoplankton bloom occurred during this period with a maximum chl *a* concentration on 14 May 2002 (see below). Among the 36 peaks found when considering all samples, 26 were persistent as they were present for all sampling dates, and most of these were detected during the course of the bloom. Interestingly, CE–SSCP peak intensities revealed that persistent peaks were not always dominant, since 25% of these peaks were minor peaks (below 1% in relative intensity) (Fig. 5a). The entire community structure remained unchanged during 5 consecutive weeks, from 12 March until 10 April 2002. During the 3 mo tested, all peaks were present in at least 2 consecutive weekly samples (Fig. 5a), suggesting that a given pattern was stable during a minimum of 8 d. During the weekly survey, chl *a* concentration ranged from 0.15 µg l^{-1} in March to a maximum of 3.14 µg l^{-1} for only 1 wk in May (sampling date: 14 May 2002). At this time scale, the maximum concentration of chl *a* was associated with the highest number of bacterial cells (1.2 $\times$ 10^6 cells ml^{-1} during 2 wk) and with the highest number of CE–SSCP peaks observed during this period (33 peaks; Fig. 5a). Five new peaks accounting for 0.8 to 2.7% of the total peak area appeared at that time, and they remained for the next 2 wk, suggesting that the corresponding bacteria were responding to the organic matter input. Cluster analysis of patterns and the relative intensities of the CE–SSCP peaks are presented in Fig. 5b. Samples fell into 3 major clusters separated by small shifts in bacterial abundance, corresponding to maxima of 8.5 $\times 10^5$ and 11.2 $\times 10^5$ cells ml^{-1} on 10 April and 14 May 2002 (mean = 7.6 $\times$ 10^5 cells ml^{-1}, SD = 2.6 $\times$ 10^5 cells ml^{-1} for the 11 wk period), respectively. During this period,

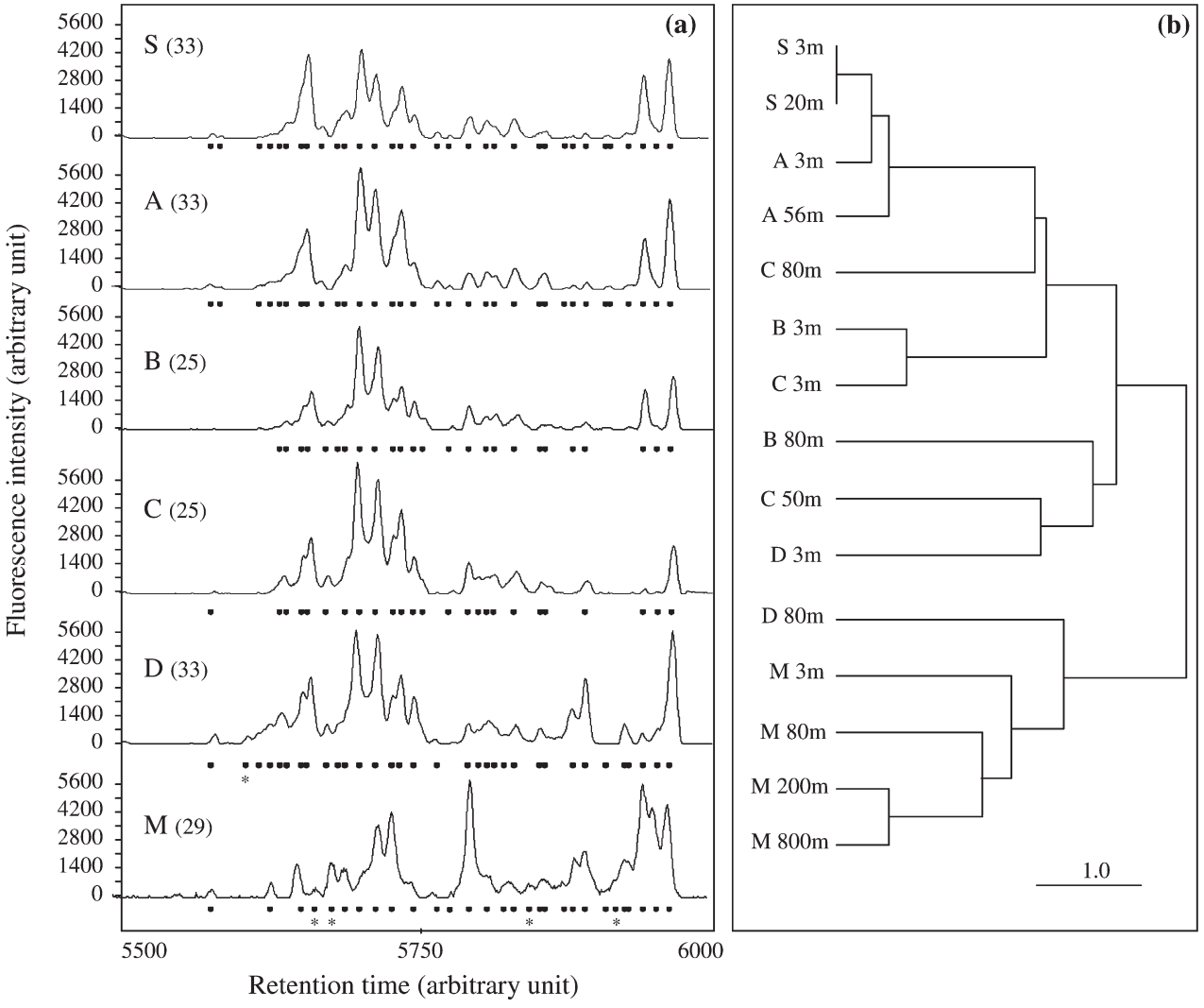


Fig. 4. Community structure of samples from Stns SOLA (S), A, B, C, D and MOLA (M) along a transect from coastal to offshore stations sampled on the same day in July 2003. (a) CE–SSCP patterns of 16S rDNA PCR products of bacterial communities sampled at 3 m. Arrows indicate the peak positions. Retention times are correlated to those for the internal controls. The total number of estimated peaks per sample are indicated in brackets. Peaks detected only at 1 station are marked with an asterisk. (b) UPGMA Euclidean distance dendrogram generated from the CE–SSCP profiles of the transect samples analysed. The different depths are indicated for each station. Scale bar indicates the Euclidean distance

the maximum chl *a* concentration (3.14 µg chl *a* l^{-1}) was observed on 14 May 2002 (mean = 1.21 µg chl *a* l^{-1}, SD = 0.8 µg chl *a* l^{-1}). The UPGMA dendrogram clearly showed that bacterial communities from pre- and post-bloom samples were different (Fig. 5b).

Variability at the monthly scale was investigated during a 13 mo period from March 2002 to March 2003. Fifteen peaks out of a total of 44 peaks identified were found on each sampling date, suggesting that almost one-third of the total number of peaks persisted throughout the year (Fig. 6a). For a given sample, persistent peaks represented between 44 to 60% of the total number of peaks per profile. The mean number of peaks per sample was 30 (SD = 2.9), and the highest numbers were found during the spring (May 2002) and winter (January 2003) phytoplankton blooms. No clear relationship was found between the number of peaks and bacterial abundances. As reported at the weekly scale, only a third of the persistent peaks were also dominant peaks (more than 5% of the relative intensity per sample). During the entire course of the year, bacterial community profiles from different months were always different from each other (Fig. 6a). When subjected to cluster analysis based on Euclidean distance matrix, 3 clusters could be discriminated (Fig. 6b). The UPGMA dendrogram showed that shifts from one cluster to another were strongly related to phytoplankton blooms (March 2002 and January 2003; Fig. 6c).

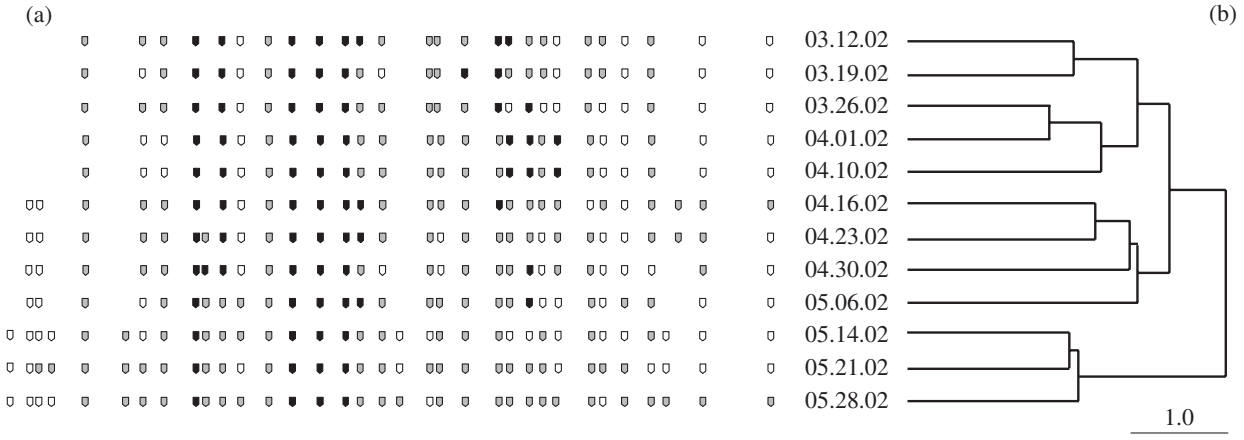
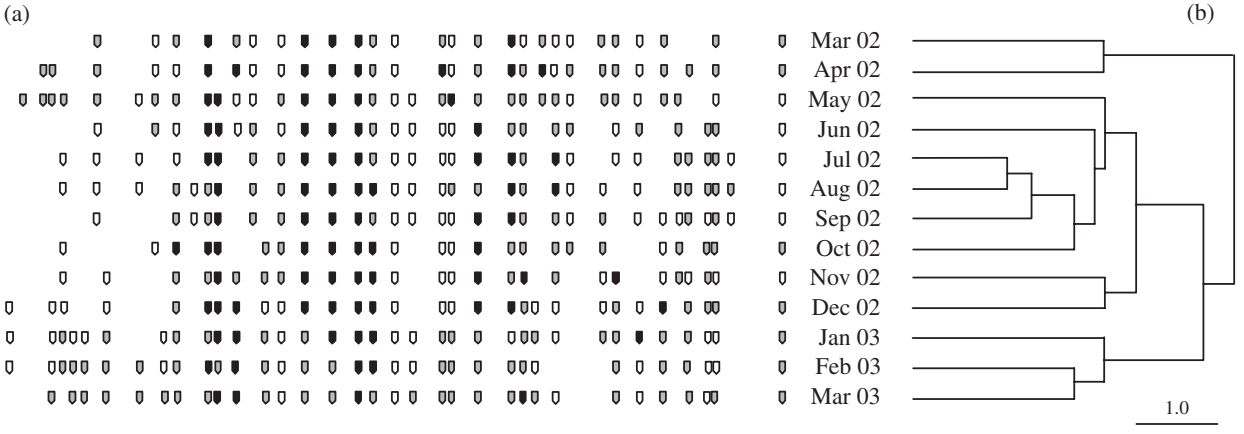


Fig. 5. Community structure of samples taken at Stn SOLA on a weekly time scale. The different sampling dates are indicated for each lane. (a) CE–SSCP patterns of 16S rDNA PCR products were acquired and the identified peaks were drawn in a schematic diagram corresponding to their original retention time and intensity (▮ >5%, ▯ between 1 and 5% and ▯ <1% of the relative intensity per sample). (b) UPGMA Euclidean distance dendrogram generated from the CE–SSCP profiles of the samples analysed. Scale bar indicates the Euclidean distance



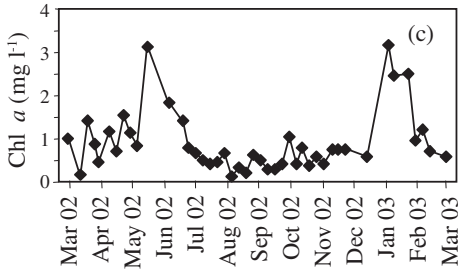


Fig. 6. Community structure and chlorophyll *a* concentration of samples taken at Stn SOLA on a monthly time scale. (a) CE–SSCP patterns of 16S rDNA PCR products were acquired and the identified peaks were drawn in a schematic diagram corresponding to their original retention time and intensity (▮ >5%, ▯ between 1 and 5% and ▯ <1% of the relative intensity per sample). (b) UPGMA Euclidean distance dendrogram generated from the CE–SSCP profiles of the samples analysed. Scale bar indicates the Euclidean distance. (c) Evolution of chlorophyll *a* concentration from March 2002 to March 2003

DISCUSSION

Relevance of CE–SSCP fingerprints

Molecular fingerprinting approaches are commonly used in microbial ecology to compare microbial assemblages and to assess temporal and spatial variability. In this study, CE–SSCP was shown to be a powerful tool to compare a large set of samples from different spatial and temporal origins. The use of an internal standard co-migrating with DNA in each sample allowed us to normalize the peak mobilities and, therefore, to compare samples by computing all electropherograms. This procedure resulted in very good reproducibility of the results in terms of both peak pattern and peak intensities (Fig. 2). Like other PCR-based fingerprinting techniques, CE–SSCP is subject to possible biases introduced by nucleic-acid extraction and PCR proce-

dures (Wintzingerode et al. 1997, Bano et al. 2002, Schauer et al. 2003). Thus, molecular fingerprints may not reflect the exact environmental bacterial diversity and do not allow the consideration of ribotype abundances as absolute values. However, we assume that the relative intensity of detected peaks can be compared between samples because of normalization procedures and the high reproducibility of CE–SSCP. Another limit common to most fingerprinting methods is the potential overlap of different ribotypes and, therefore, the potential underestimation of the diversity of ribotypes. Different bacterial species may have closely related 16S rDNA sequences (Wintzingerode et al. 1997) that could correspond to the same CE–SSCP peak. Similarly, Schauer et al. (2000) mentioned the subjectivity in deciding whether a very weak DGGE band is a real band or a background artefact. In our study, this problem was, at least partly, overcome by taking into account the presence or absence of individual CE–SSCP peaks and the relative contribution of each peak to the total surface area of the pattern. Furthermore, the use of Euclidean distances (UPGMA dendrogram) was very helpful for the final treatment and interpretation of the data. As it was pointed out elsewhere (Riemann et al. 2000, Fandino et al. 2001), we cannot exclude the presence of peaks originating from eukaryotes in the CE–SSCP profiles since the PCR primers used in this study can amplify plastid 16S rRNA genes. However, this should not greatly affect our results, since picoeukaryotes never represented more than 1% (mean = 0.4, SD = 0.4) of total cell counts in the fraction 0.2 to 3 µm (data not shown).

The sample size is of great importance in defining the sampling strategy to provide a representative picture of the bacterial community structure at a given location. The appropriate size may depend on different criteria, including the spatial distribution of bacterial species and the resolution of the methods used to determine the community structure of the dominant species. Our results showed an increasing number of ribotypes per samples up to 50 ml, which then remained constant for greater volumes (26 ribotypes for 10 ml and 31 ribotypes for 50 to 1000 ml, respectively). Other reports based on the DGGE method suggested that the community structure was not affected by the sample size for volumes ranging from milliliters to liters (Kirchman et al. 2001, Casamayor et al. 2002). Similarly, Long & Azam (2001) were able to analyse bacterial communities at the microscale level (from microliter to milliliter) to investigate the role of targeted populations in processes that can appear at a microscale level but are invisible at higher scale. In this study, we were not able to obtain representative patterns using very small volumes. A minimum of 3×10^7 cells (in our case 50 ml of coastal seawater) were necessary to provide representative CE–SSCP patterns (Fig. 3). Differences with other reported studies may be due to the detection sensitivity of each method, but may also be explained by different evenness of the studied communities and/or by a few differences in analytical protocols and reagents. From these results, we determined that further investigations in this area will be undertaken using a minimum of 50 ml.

Horizontal variations in bacterial community structure

Spatial variation in bacterial community structure was also determined at different horizontal scales. Our results showed that bacterial assemblages from surface samples were very similar up to 3.7 km from Stn SOLA, at Stn A, and differences appeared from 9.3 km (Stn B) off Stn SOLA (Fig. 4a,b). Interestingly, the structure of bacterial communities in surface waters changed gradually from coastal to offshore stations. The more striking differences were seen between Stn SOLA and the offshore Microbial Observatory (MOLA), which are 33.3 km apart (Fig. 4b). These changes along the transect may be explained by the local hydrodynamic regime and an increasing influence of offshore waters along the coast–offshore transect. Four ribotypes were detected only at MOLA and may be of great interest for the long-term monitoring at this station. Analyzing temporal fluctuations may help to better understand the ecological properties of these species. Among the 44 ribotypes found in surface waters along the transect, 15 were always present and probably had enough functional plasticity to adapt to both coastal and offshore marine environments. Unfortunately, the CE–SSCP technique used alone does not allow the identification of species by subsequent sequencing. However, the results can be compared with those of clone libraries of 16S rDNA sequences to provide information on which species are present over seasons or along gradients (Delbès et al. 2000). At stations located in the NW Mediterranean Sea but 94.5 km south of the Banyuls-sur-mer bay, Schauer et al. (2000) reported large changes in the bacterial community structure between stations located 70.4 km apart on the Spanish Catalan coast and the open sea. In contrast, almost identical DGGE patterns were obtained from samples taken 1500 km apart along a transect from coastal waters to the open Arabian Sea (Riemann et al. 1999). These opposite results are likely due to variations in the environmental characteristics of these waters and suggest that further investigations will be necessary to determine which environmental factors regulate these changes.

Almost a third of the ribotypes persisted during the year at Stn SOLA (Fig. 6a). This finding suggests that

some bacterial populations can, to some extent, adapt to and proliferate in a changing environment. This is particularly true in coastal ecosystems subject to environmental changes such as sediment resuspension events, and river inputs of freshwater and terrestrial material. Interestingly, we observed that these persistent ribotypes are not always dominant in terms of relative abundance, although they may represent key species in the functioning of these ecosystems and probably play a key role in the resilience of the ecosystems. Again, further investigations should be performed to identify these species by combining CE–SSCP with the analysis of clone libraries.

Vertical variations in community structure

Vertical variations were also analysed at different stations. During the 8 mo monitoring period at Stn SOLA, similar patterns were found for most samples, and the highest recorded differences between the 2 depths (3 and 20 m) were 2 peaks (data not shown). One explanation of the stability of the profiles could be the lack of stratification at this shallow station (values available at http://www.obs-banyuls.fr/Services/sola/). The few differences observed could be due to some sediment resuspending events, which often occur in this very windy part of the Mediterranean coast. UPGMA analysis also showed that surface and deep waters at Stn A (70 m depth) located 3.7 km from Stn SOLA were only slightly different (Fig. 4b), possibly for the same reasons cited above for Stn SOLA. In contrast, differences were found at deeper stations (B, C, D, MOLA) that could be related to a progressive stratification of the water column along the coast–offshore transect, with a clear deep chlorophyll maximum (DCM) found from Stn C to offshore. The different depth samples collected at MOLA clustered together according to their proximity to one another in the water column (Fig. 4b). The offshore Microbial Observatory was chosen because of its special location on top of the Lacaze–Duthiers Canyon, which is responsible for the episodic cascading of dense water that affects the hydrology of the area and plays an important role in the transfer of matter (Bethoux et al. 2002). Similar vertical stratification of the bacterial community structure has commonly been found in a wide diversity of oligotrophic seawaters, including the Pacific and Atlantic Oceans, the Caribbean Sea (Lee & Fuhrman 1991), the Mediterranean Sea (Acinas et al. 1997, Moeseneder et al. 2001) and Antarctic zones (Murray et al. 1998). Therefore, any long-term monitoring should combine different sampling depths according to the physico-chemical and biological properties of the water column. Further investigations are needed to better understand why some species are always present along the vertical profiles and why others are not.

Temporal variations in bacterial community structure

Temporal variations in bacterial community structure were found at Stn SOLA at the largest temporal scales (weeks to seasons) but not at a shorter time scale (hours or days). These changes occurred at a minimum time scale of 2 wk (Fig. 5a). It suggests that bacterial populations in this oligotrophic area did not change very fast in response to environmental changes, since a given assemblage was constant for up to 5 successive weeks. During the year, the most important changes in bacterial community structure were strongly related to the occurrence of phytoplankton blooms (Fig. 6). These events provide rapid and more or less (depending on the bloom) important inputs of organic matter that result in a dynamic response of the bacterial community. This was characterized at Stn SOLA by increases in both cell counts (from 5 to 15×10^5 cells ml^{-1}) and bacterial production (from 0.1 to 0.35×10^{-6} g C l^{-1} h^{-1}). During the spring and winter blooms (Fig. 6c), the maximum concentrations of chlorophyll *a* were associated with the highest number of CE–SSCP peaks observed during the year, with the appearance of new ribotypes that were maintained for at least 2 wk (Figs. 5a & 6a). The UPGMA dendrogram confirmed that bacterial communities from pre- and post-bloom samples were different (Figs. 5b & 6b). Other reports demonstrated that bacterial community successions are commonly associated with the formation and senescence of phytoplankton blooms, likely reflecting the proliferation of bacterial ribotypes specialized in particle colonization and degradation (Van Hannen et al. 1999, Riemann et al. 2000, Fandino et al. 2001, Riemann & Winding 2001, Schäfer et al. 2001). For example, greater species richness was observed in microcosms enriched with diatom detritus (Long & Azam 2001).

We are not aware of other investigations on temporal variations in bacterial community structure from hourly to seasonal scales in marine ecosystems. Because of cruise logistics and meteorological considerations, temporal variations in bacterioplankton assemblages are usually observed at larger time scales, comparing samples collected from year to year (Lee & Fuhrman 1991, Riemann et al. 1999), in different seasons (Murray et al. 1998, Schauer et al. 2000) or daily in a few cases (Acinas et al. 1997, Fandino et al. 1998). By comparing DGGE profiles of 18 different samples taken during 1998, Schauer et al. (2003) showed bacterial

community changes at time scales ranging from weeks to months at a site very close to our sampling area (Blanes Bay, Spain). They suggested that changes in dissolved organic matter supply mediated by different algal populations might be one of the main factors affecting bacterioplankton composition. These results and those presented here suggest that temporal variations should be monitored at different time scales, depending on the questions to be answered. Sampling every month should be probably more than enough for the long-term monitoring of bacterial communities in a given area if the aim is to determine the effect of environmental changes occurring at a large scale (i.e. climate change). In contrast, weekly samplings should be carried out to observe the effects of environmental changes which occur at short time scales (i.e. phytoplankton blooms, freshwater input in coastal areas, local pollution, etc.) if the aim is to further understand the functional role of bacterial biodiversity in the cycling of nutrients.

The long-term monitoring of oceanic regions at Microbial Observatories is of special interest for discovering and characterizing novel micro-organisms, microbial consortia, activities and other novel properties, and to understand the role of microbial community structure in the functioning of ecosystems over time and across environmental gradients. This work underlined the fact that a reliable method should be used, which allows the analysis of a large data set. The CE–SSCP method seems to be a good candidate for this purpose. Then, observations require the use of appropriate temporal and spatial scales, which depends on the question to be answered. At deep stations, variations in bacterial community structure were generally greater on vertical than on regional scales due to higher vertical than regional variation in biotic (primary production, grazer community) and abiotic (nutrients, temperature, pressure) variables. For the same reasons, temporal variations are mainly controlled by changes in biotic and abiotic factors and the most important variations seems to be driven by phytoplankton blooms in the NW Mediterranean Sea.

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