Seasonality in bacterial diversity in north-west Mediterranean coastal waters: assessment through clone libraries, fingerprinting and FISH

Laura Alonso-Sáez[1], Vanessa Balagué[1], Elisabet L. Sà[1], Olga Sánchez[2], José M. González[3], Jarone Pinhassi[4], Ramon Massana[1], Jakob Pernthaler[5], Carlos Pedrós-Alió[1] & Josep M. Gasol[1]

[1]Departament de Biologia Marina i Oceanografia, Institut de Ciències del Mar CSIC, Barcelona, Catalunya, Spain; [2]Departament de Genètica i Microbiologia. Universitat Autonoma de Barcelona, Bellaterra, Catalunya, Spain; [3]Departamento de Microbiología, Universidad de La Laguna, La Laguna, Tenerife, Spain; [4]Marine Microbiology, Department of Biology and Environmental Sciences, University of Kalmar, Kalmar, Sweden; and [5]Limnological Station, Institute of Plant Biology, University of Zurich, Switzerland

Correspondence: Laura Alonso-Sáez, Departament de Biologia Marina i Oceanografia, Institut de Ciències del Mar CMIMA, CSIC, 08003-Barcelona, Catalunya, Spain. Tel.: +34 932309611; fax: +34 932309555; e-mail: lalonso@icm.csic.es

Received 15 June 2006; revised 2 November 2006; accepted 10 November 2006.
First published online 24 January 2007.

DOI:10.1111/j.1574-6941.2006.00276.x

Editor: Michael Wagner

Keywords
bacterial diversity; marine; SAR11; *Roseobacter*; seasonality.

Abstract

We combined denaturing gradient gel electrophoresis (DGGE), catalysed reporter deposition-FISH (CARD-FISH) and clone libraries to investigate the seasonality of the bacterial assemblage composition in north-west Mediterranean coastal waters. DGGE analysis indicated that bacterial diversity changed gradually throughout the year, although with a clear distinction of the summer period. *Alphaproteobacteria* were the dominant group on an annual basis [29% of the DAPI (4′,6-diamidino-2-phenylindole) counts by CARD-FISH, and 70% of the bacterial clones]. The SAR11 clade was most abundant during spring and summer (> 20% of DAPI counts), while the *Roseobacter* clade was abundant primarily in winter and spring (up to 7% of DAPI counts). The phylum *Bacteroidetes* constituted the second most important group and was quantitatively uniform throughout the year (average 11% of the DAPI counts). *Gammaproteobacteria* showed a peak during summer (8% of DAPI counts), when most of them belonged to the NOR5 cluster. Clone libraries and CARD-FISH showed reasonable agreement in the quantitative proportions of *Bacteroidetes* and *Gammaproteobacteria*, but *Alphaproteobacteria* were overrepresented in clone libraries. Sequencing of the most predominant DGGE bands failed to detect the SAR11 group despite their high abundance. The combination of the three molecular approaches allowed a comprehensive assessment of seasonal changes in bacterial diversity.

Introduction

The application of molecular techniques by cloning and sequencing of 16 rRNA genes extracted from marine samples has allowed the identification of abundant bacterial taxa in the sea (Giovannoni *et al*., 1990; Giovanonni & Rappé, 2000). However, given that clone libraries are relatively expensive and time-consuming, most studies are based on single sampling points, which seriously limits our understanding of spatiotemporal variations in bacterial diversity. The development of fingerprinting techniques [such as denaturing gradient gel electrophoresis (DGGE) or terminal restriction fragment length polymorphism (T-RFLP)] and FISH with specific probes has overcome some of the problems associated with the use of clone libraries.

Fingerprinting methods allow a reasonably straightforward comparison of the phylogenetic composition of a large number of samples along spatial and temporal gradients (e.g. Schauer *et al*., 2000; Schauer *et al*., 2003; Ghiglione *et al*., 2005). Some of them, such as DGGE, further allow the assessment of the diversity of the assemblage by subsequent sequencing, although not to the same level of detail as permitted by clone libraries. By contrast, FISH allows the appropriate quantification of distinct bacterial groups, independently of the biases (Wintzingerode *et al*., 1997) associated with PCR amplification. However, this technique is limited by the number of probes that, in practice, can be used, and can only account for previously known lineages. Although some comparisons between the various molecular approaches have been carried out (Cottrell & Kirchman, 2000; Castle & Kirchman, 2004), to what extent the different techniques can be quantitatively compared is still unknown.

Relatively few studies have assessed seasonal bacterial diversity in marine waters, applying different molecular

approaches (Schauer *et al.*, 2003; Brown *et al.*, 2005; Ghiglione *et al.*, 2005). Changes in environmental factors such as temperature and inorganic nutrients are known to control the appearance of specific phytoplankton populations, but much less is known about phylogenetic groups of bacteria. The same environmental factors as well as others, such as availability of organic substrates (e.g. during bloom periods), could promote the appearance of specific bacterial taxa. Pinhassi & Hagström (2000) used whole-genome DNA hybridization in the Baltic Sea and found a clear differentiation between the bacterial assemblages during spring, dominated by *Bacteroidetes*, and summer, dominated by *Alphaproteobacteria*. Eilers *et al.* (2001) studied the seasonality of marine bacterial groups by FISH in the North Sea, and found that *Bacteroidetes* dominated during spring and early summer, while a group of *Gammaproteobacteria* (i.e. NOR5) was abundant during summer. Mary *et al.* (2006) also found dominance of *Bacteroidetes* in spring and early summer in the English Channel by catalysed reporter deposition (CARD)-FISH, and reported a dominance of *Alphaproteobacteria* from late summer to winter. The seasonality of specific groups such as SAR11, SAR86 and SAR116 has also been studied in more oceanic samples (Bermuda Atlantic Time Series station). These groups exhibited the strongest increases during summer periods, as shown by T-RFLP and bulk nucleic acid hybridization (Morris *et al.*, 2005).

Schauer *et al.* (2003) provided a first approach to the seasonality of bacterial assemblages of Blanes Bay (Catalan Sea, north-west Mediterranean) using the DGGE technique. In the present study we constructed five clone libraries and obtained monthly DGGE and CARD-FISH data throughout 1 year, with two main objectives: (1) to obtain a detailed picture of the seasonal changes in bacterial diversity in Mediterranean coastal waters, and (2) to compare the results obtained by the three different methods, in order to test the strong and weak points of each approach, and how they affect the overall image of bacterioplankton diversity generated by each technique. To our knowledge, this is the first study that compares results of the three different approaches simultaneously on the same set of marine samples.

Materials and methods

Location and sampling

We carried out a monthly study in Blanes Bay (The Blanes Bay Microbial Observatory) from 4 March 2003 to 22 March 2004 (14 samples). Surface waters were monthly sampled at about 1 km offshore (41°40′N, 2°48′E), filtered through a 200-μm-mesh net and transported to the laboratory under dim light (within 1.5 h) in 25-L polycarbonate carboys. For convenience, the sampling on 4 March 2003 will be referred to as 'February 2003', to avoid confusion with the sampling on 25 March 2003 (i.e. March 2003). Samples were filtered (for DGGE and clone libraries) or fixed (for CARD-FISH) immediately upon arrival in the laboratory.

Basic data

Surface water temperature was measured *in situ* with a mercury thermometer. For determination of Chlorophyll *a* (Chl *a*) concentration, 150 mL of seawater was filtered on GF/F filters (Whatman) and subsequently extracted in acetone (90%, v/v) in the dark at 4 °C for 24 h. Fluorescence was measured with a Turner Designs fluorometer. Hourly rain values (mm) were obtained from the automatic meteorological station located at Malgrat de Mar (6 km away from Blanes Bay), run by the SMC (Servei Meteorològic de Catalunya) and integrated for the 7 days prior to each sampling date.

Abundance of prokaryotes and photosynthetic picoplankton

Synechococcus, *Prochlorococcus* and photosynthetic picoeukaryotes were enumerated by flow cytometry and distinguished by their different size and pigment properties in unstained samples following common procedures (i.e. Marie *et al.*, 1997). Heterotrophic prokaryotes were also counted by flow cytometry (Gasol & del Giorgio, 2000) after staining with Syto13.

DNA extraction

Surface microbial biomass was collected by sequentially filtering around 8 L of seawater through a 3-μm pore-size polycarbonate filter (Poretics) and a 0.2-μm Sterivex filter (Durapore, Millipore), using a peristaltic pump. The Sterivex units were filled with 1.8 mL of lysis buffer (50 mM Tris-HCl pH 8.3, 40 mM EDTA pH 8.0, 0.75 M sucrose) and kept at −80 °C. Microbial biomass was treated with lysozyme, proteinase K and sodium dodecyl sulfate, and the nucleic acids were extracted with phenol and concentrated in a Centricon-100 (Millipore), as described in Massana *et al.* (1997).

DGGE, band sequencing and phylogenetic analysis

DGGE and band sequencing were performed as previously described (Schauer *et al.*, 2003). Briefly, 16S rRNA gene fragments were amplified by PCR using the universal primers 907rM and 358f with a GC clamp. PCR products were loaded on a 6% polyacrylamide gel with a DNA-denaturant gradient ranging from 40% to 80%. The gel was run at 100 V for 16 h at 60 °C in 1 × Tris-acetate-EDTA (TAE) running buffer using a DGGE-2000 system (CBS

Scientific company). These conditions had been previously optimized for the Blanes samples. DGGE gel images were analysed using the CHEMIDOC software (Bio-Rad). A matrix was constructed for all lanes taking into account the relative contribution of each band (%) to the total intensity of the lane. Based on this matrix, we obtained a dendrogram based on UPGMA clustering (Euclidean distances, Statistica 6.0). DGGE bands were excised and reamplified with the original primer set. The position of the bands was confirmed in another DGGE gel. Bands were purified and subsequently sequenced using the primer 358f without the clamp, with the Big Dye Terminator Cycle Sequencing Kit v3.1 (PE Biosystems) and an ABI 3100 (Applied Biosystems) automated sequencer. The sequences obtained were compared with public database DNA sequences using BLAST (Altschul *et al.*, 1997) to determine their phylogenetic affiliation. Seven bands [i.e. operational taxonomic units (OTUs)] were excised at their corresponding position in several lanes in order to analyse the similarity within each band or OTU. Excluding one case (out of 20), the average similarity of sequences within the same band position was 99.1%.

Clone libraries, RFLP analysis and sequencing

Cloning and RFLP analysis were performed as previously described (Ferrera *et al.*, 2004). 16S rRNA genes were amplified by PCR with the universal primers 27f and 1492r. Products from three individual PCR reactions were pooled and cleaned with the Qiagen PCR purification kit and cloned using the TOPO-TA cloning kit (Invitrogen). PCR amplifications were digested with the restriction enzyme HaeIII (Invitrogen), and the RFLP patterns of the clones were compared. Clones showing the same RFLP pattern (DNA fragments of equal size) were grouped together and considered to belong to the same OTU. We analysed the similarity between clones from the same OTU (a total of 58 clones within 17 different OTUs); the average was 98.8%. One clone for each OTU was partially sequenced with the internal primer 358f. Chimeric sequences were identified using the CHECK_CHIMERA (Maidak *et al.*, 2001) and by BLAST search with different sequence regions. Sequences were aligned using CLUSTALW 1.82 (Thompson *et al.*, 1997) and highly variable regions of the alignment were automatically removed with Gblocks (Castresana, 2000). Maximum-likelihood analysis was carried out with PAUP 4.0b10 (Swofford, 2002), using the model of evolution and the parameters estimated by ModelTest 3.7 (Posada & Crandall, 1998). Gene sequences were deposited in Genbank under accession numbers DQ778132–DQ778298.

CARD-FISH

Samples were fixed with paraformaldehyde (2% final concentration, overnight at 4 °C) for determination of the *in situ* abundance of different bacterial populations by CARD-FISH (Pernthaler *et al.*, 2004). Filters were permeabilized with lysozyme (37 °C, 1 h) and hybridization was performed at 35 °C for a minimum of 2 h. Horseradish peroxidase (HRP)-labeled probes (50 ng μL^{-1}) were added to the hybridization buffer (HB, 1 : 300) containing the following concentrations of formamide: 20% for probe Eury806 (Teira *et al.*, 2004) and Cren554 (Massana *et al.*, 1997), 50% for NOR5-730 (Eilers *et al.*, 2000), 60% for Alt1413 (Eilers *et al.*, 2000), and 55% for Eub338-II-III (Amann *et al.*, 1990; Daims *et al.*, 1999), Ros537 (Eilers *et al.*, 2001), SAR86/1245 (Zubkov *et al.*, 2001), Gam42a and CF319a (Amann *et al.*, 1990). We used higher concentrations of probes in the HB (1 : 100%, 45% formamide) and overnight hybridization to detect the cells with probes SAR11-441R (Morris *et al.*, 2002) and Alf968 (Neef, 1997). The Eub antisense probe Non338 (Wallner *et al.*, 1993) was used as negative control. For amplification, we used tyramide labeled with Alexa 488. Counterstaining of CARD-FISH preparations was done with 4′,6-diamidino-2-phenylindole (DAPI, final concentration 1 μg mL^{-1}). DAPI- and FISH-stained cells were counted by automated image analysis (Pernthaler *et al.*, 2003).

Diversity estimates

The relative distribution of OTUs in each library was used to calculate coverage values (Good, 1953) and the nonparametric S_{Chao1} estimator (Chao, 1984) using the software tool provided by Kemp & Aller (2004). Good's coverage is a nonparametric estimator of the proportion of phylotypes in a library of infinite size that would be represented in a smaller library. As defined by Good (1953), coverage is calculated as: $C = 1 - n_1/N$ where n_1 is the number of phylotypes appearing only once in a library and N is the library size. The S_{Chao1} nonparametric estimator yields an estimate of the probable total number of phylotypes present in the source assemblage (Chao *et al.*, 1993; Lee & Chao, 1994). When it reaches a plateau, the library could be considered 'large enough' to provide an unbiased estimate of OTU richness (Kemp & Aller, 2004).

Results

The summer period (June–September) was characterized by high temperatures (23–25 °C) and low concentrations of Chl *a* (around 0.2 μg L^{-1}, Fig. 1a). Water transparency was also higher in summer, and nutrient concentrations were substantially lower from May to October (data not shown). Higher concentrations of Chl *a* were found in winter (from December to the end of March), generally over 1 μg L^{-1}. Abundance of heterotrophic prokaryotes followed roughly the pattern of Chl *a*, with two peaks at the end of March (2003) and December (Fig. 1b). There was a succession of

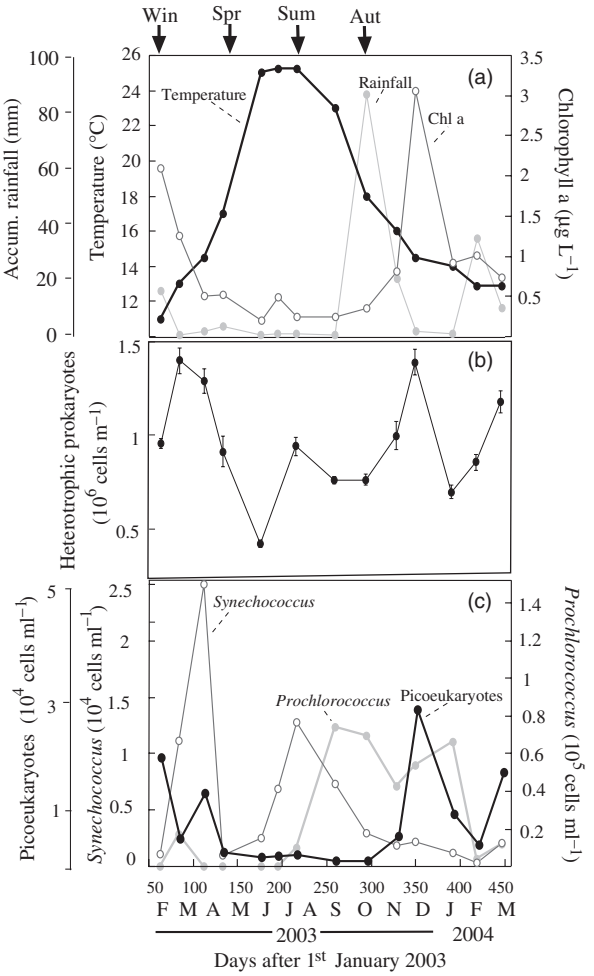

Fig. 1. (a) Seasonal changes in chlorophyll *a* concentration, temperature and accumulated rain (during the week prior to each sampling); (b) abundance of heterotrophic prokaryotes; and (c) abundance of photosynthetic picoeukaryotes, *Synechococcus* and *Prochlorococcus* in Blanes Bay. Arrows indicate the samples from which seasonal clone libraries were established.

picophytoplankton populations with picoeukaryotes (peak during the winter), *Synechococcus* (peaks in spring and summer) and *Prochlorococcus* (higher abundances from September to January, Fig. 1c).

Seasonal pattern of bacterial assemblage structure as revealed by DGGE

The DGGE analysis yielded a total of 73 different band positions or OTUs (Fig. 2), and showed that the bacterial assemblage changed gradually throughout the year, indicating seasonal succession. The dendrogram based on the DGGE banding pattern separated the samples taken in the summer period (June–September) from the samples taken in other seasons (Fig. 3). Within the nonsummer cluster, spring samples (end of March–May) were separated from winter and autumn samples, which also formed different clusters. The number of bands per sample was significantly lower (*t*-test, $P < 0.0001$) in the spring–summer period (end of March to September; range 21–32) than in the autumn–winter period (October to beginning of March; range 35–41).

A total of 30 band positions were excised in order to determine their phylogenetic affiliation, and informative sequences were obtained from 23 bands (Table 1). These bands accounted for 35–80% of the total band intensity in each sample and most of them showed high similarity (> 97%) with sequences from uncultured clones by BLAST search. Only three bands of bacterial origin persisted throughout the year. These bands were affiliated with two different *Roseobacter* (bands 35 and 43, Table 1) and SAR116 (band 33, Table 1), although this last band was always present at low intensities (lower than 3% of total intensity per lane). By contrast, nine bands (12% of the OTUs) were exclusively detected at only one sampling date.

Three bands were affiliated with *Synechococcus* (Table 1), and the relative intensity of these bands was significantly correlated with the relative abundance of this population (over total prokaryotic abundance) determined by flow cytometry (arcsine-transformed, Pearson's $r = 0.64$, $n = 14$, $P = 0.01$). Similarly, the relative abundance of band 56, corresponding to *Prochlorococcus marinus*, was highly correlated with the relative abundance of *Prochlorococcus* determined by flow cytometry (arcsine-transformed, $r = 0.74$, $n = 14$, $P = 0.0025$). The intensity of bands of eukaryotic plastidial origin was not included in total band intensity for these and the following calculations.

Bands affiliated with *Alphaproteobacteria* represented a rather constant percentage of total band intensity (average 32%). Five of these bands were affiliated with the *Roseobacter* cluster (21% of total band intensity, Table 1), showing seasonal substitution of OTUs. Bands 35 and 37 (very similar to NAC11-7 and NAC11-6 clones, respectively) were found at higher intensities in winter and spring, while band 46 was mainly found during the summer. Band 48, affiliated with the genus *Erythrobacter*, was mainly found during the summer–autumn period.

Seven bands were affiliated with the *Bacteroidetes* (Table 1), with higher contributions to total band intensity in the autumn–winter (average of 19%) than in the spring–summer period (only 3% on average). None of the sequenced bands was affiliated with the *Gammaproteobacteria* or with the SAR11 group. In order to observe the band positions that corresponded to SAR11 sequences, we amplified and ran several SAR11 clones from our clone libraries in a DGGE gel (Fig. 2). These clones migrated within a quite narrow region of the gel, where several faint bands were visible in the environmental samples. Several attempts to obtain clean sequences from these bands of the gel were unsuccessful.

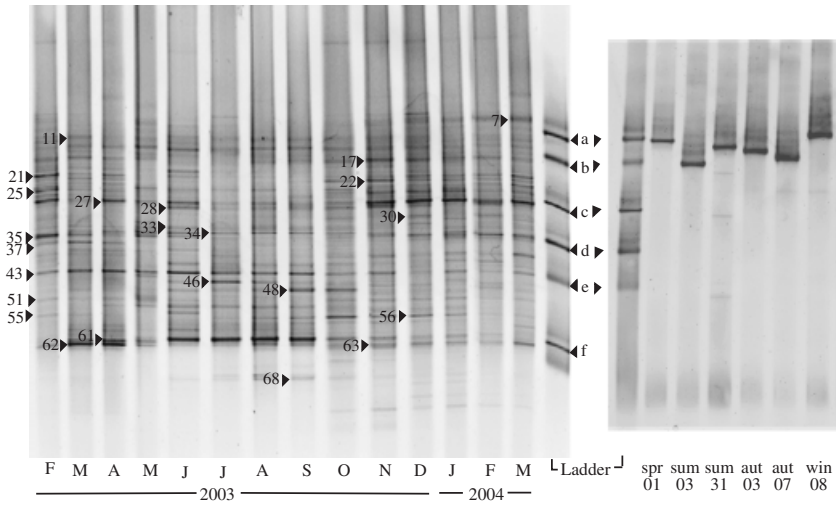

Fig. 2. DGGE gels of bacterial 16S rRNA gene fragments from the seasonal samples, indicating the band positions which were successfully sequenced (presented in Table 1). On the right, DGGE gels of clones affiliated with the SAR11 cluster retrieved from the spring (clone BL03-SPR01), summer (BL03-SUM03 and SUM31), autumn (BL03-AUT03 and BL03-AUT07) and winter (BL03-WIN08) clone libraries. A ladder including different environmental clones from Blanes Bay (a, BL03-AUT19-Bacteroidetes; b, BL03-SPR52-SAR11; c, BL03-SPR25-Plastid of *Dinophysis norvegica*; d, BL03-SPR09-*Roseobacter* NAC11-7, e, BL03-JUL18-*Gammaproteobacteria*; and f, BL03-JUL23-*Synechococcus*) was included in both DGGE gels in order to compare the band positions.

Quantitative analysis of microbial assemblage composition by CARD-FISH

On average ($\pm$SD), 73 $\pm$ 10% of DAPI-stained cells were detected with the universal set of probes for *Bacteria* (Eub+ cells). Most Eub+ cells were identified with probes for broad phylogenetic groups (*Bacteroidetes*, *Alphaproteobacteria* and *Gammaproteobacteria*; average 75%), with a lower proportion of identified Eub+ cells in autumn–winter (63%) than in the spring–summer period (87%). We found that *Euryarchaea* reached higher proportions in winter (up to 6% of DAPI cells) and remained below detection levels in summer (Table 2). Several counts of *Crenarchaea* (probe Cren 554) in different months indicated that this group was below 3% of DAPI counts throughout the year (data not shown).

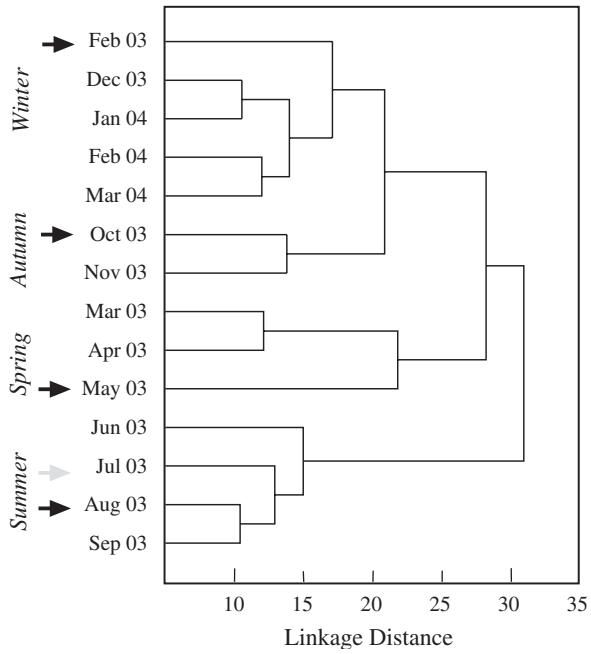

Fig. 3. Dendrogram of the DGGE banding pattern constructed from the intensity matrix, grouping the Blanes Bay samples. Arrows indicate the samples from which seasonal clone libraries were established.

Alphaproteobacteria were the dominant bacterial group (30 $\pm$ 7% of DAPI counts), with the exception of the sample taken in July 2003. At this time, the bacterial assemblage structure showed a drastic change with an unusual burst of *Gammaproteobacteria* (50% of DAPI counts), and more specifically *Alteromonas* (probe Alt1413; 30% of DAPI counts, Table 2). This unusual event was not considered as a seasonal feature, and for this reason data from the July sampling are presented as outliers in Fig. 4. Within the *Alphaproteobacteria*, the SAR11 cluster showed very high proportions in the spring–summer period (27 $\pm$ 8% of DAPI counts), and lower proportions during the autumn–winter period (18 $\pm$ 8% of DAPI counts; Fig. 4b). *Roseobacter* were generally found in significantly lower proportions than SAR11, and showed an opposite seasonal trend to this group. After a peak in May, *Roseobacter* were almost absent during the summer (1% of DAPI counts) but showed higher contributions to the bacterial assemblage in the autumn–winter period (up to 7%, Fig. 4b).

Bacteroidetes were the second most abundant broad phylogenetic group, with a rather constant contribution to

Table 1. Phylogenetic affiliation of sequences obtained from DGGE bands, closest uncultured and cultured matches, and the presence and average relative intensity of the band in different samples. Number of bases used to calculate the sequence similarity is shown in parentheses in the third column

Band	Closest match (environmental or culture)	Sequence similarity (%) (number of bases)	Taxonomic group	GenBank accession number	Closest culture match (% similarity)	Presence (number of samples)	Average intensity (%)
BL03-7	Clone OUT_A	100 (374)	*Bacteroidetes*	AF207850	*Tenacibaculum mesophilum* (98)	6	5.2
BL03-11	S1-005-F-B-Nr3	95.9 (439)	*Bacteroidetes*	AJ508420	*Owenweeksia hongkongensis (87)*	9	1.8
BL03-17	SCF-4969	98.1 (263)	*Bacteroidetes*	AJ630719	*Bizionia paragorgiae* (93)	9	4.0
BL03-21	Clone OM5	100 (496)	Prasinophyte chloroplast	U70715	*Ostreococcus* sp. RCC393 (95)	13	2.4
BL03-22	SPOTSAPR01_5m159	99.2 (266)	*Bacteroidetes*	DQ009089	*Owenweeksia hongkongensis* (87)	11	2.3
BL03-25	*T. amphioxeia*	99.8 (491)	Cryptophyte chloroplast	AY453067	*Teleaulax amphioxeia* chlorop (99.8)	9	3.1
BL03-27	*Micromonas* RCC434	98.8 (430)	Prasinophyte chloroplast	AY702163	*Micromonas* sp (98.8)	14	7.3
BL03-28	CONP48	98.8 (342)	*Bacteroidetes*	AY828419	*Gelidibacter algens* (92)	7	3.1
BL03-30	Clone SBI04_177	97.5 (324)	*Bacteroidetes*	DQ186969	*Chryseobacterium* (95)	7	2.2
BL03-33	NAC11-16	99.5 (402)	*Alphaproteobacteria*/ SAR116	AF245641	*Ahrensia kielensis* (90)	14	1.7
BL03-34	*M. methylotropha*	97.3 (299)	*Alphaproteobacteria*/ *Roseobacter*	U62894	*Marinosulfonomonas methylotropha* (97.3)	10	4.3
BL03-35	NAC11-7	99.8 (499)	*Alphaproteobacteria*/ *Roseobacter*	AF245635	*Ophiopholis aculeata* symbiont (99)	14	6.0
BL03-37	NAC11-6	99.3 (437)	*Alphaproteobacteria*/ *Roseobacter*	AF245634	*Roseobacter* sp. 3008 (98)	6	2.4
BL03-43	EF100-65C12	100 (499)	*Alphaproteobacteria*/ *Roseobacter*	AY627371	*Roseobacter* sp. LA7 (98)	14	6.3
BL03-46	DGGE band DI-12	99.5 (434)	*Alphaproteobacteria*/ *Roseobacter*	AY919600	*Roseobacter* sp. (97)	10	2.7
BL03-48	*Erythrob*. 12IX/A01/ 170	100 (430)	*Alphaproteobacteria*/ *Erythrobacter*	AY612770	*Erythrobacter citreus* (97)	10	3.4
BL03-51	Clone 185	97.6 (411)	*Alphaproteobacteria*	DQ187755	*Rhodothalassium salexigens* (91)	12	2.3
BL03-55	SPOTSAPR01_5m244	99.1 (424)	*Alphaproteobacteria*/ *Rhodobacteraceae*	DQ009316	*Antarctobacter* sp. (91)	3	1.5
BL03-56	*P. marinus* MED4	99.2 (381)	*Cyanobacteria*/ *Prochlorococcus*	BX572090	*Prochlorococcus marinus* (99)	9	3.2
BL03-61	*Synechococcus* CC9605	100 (471)	*Cyanobacteria*/ *Synechococcus*	CP000110	*Synechococcus* sp (100)	4	10.2
BL03-62	*Synechococcus* Almo3	99.8 (457)	*Cyanobacteria*/ *Synechococcus*	AY172800	*Synechococcus* sp (99.8)	12	9.8
BL03-63	SPOTSAUG01_5m57	100 (256)	*Bacteroidetes*	DQ009288	*Aquaspirillum peregrinum* (91)	2	6.3
BL03-68	*Synechococcus* CC9605	99.8 (409)	*Cyanobacteria*/ *Synechococcus*	CP000110	*Synechococcus* sp. (99.8)	10	2.5

the bacterial assemblage throughout the year ($11 \pm 3\%$ of DAPI counts, Fig. 4a). *Gammaproteobacteria* were not abundant ($4 \pm 2\%$ of DAPI counts, with the exception of the sample from July), but increased during the summer period with a peak in August (8% of DAPI counts, Fig. 4c). Most identified *Gammaproteobacteria* hybridized with the NOR5-730 probe at this time, with very similar dynamics of both groups through the year except in spring (Fig. 4c). Other tested groups within the *Gammaproteobacteria* (*Alteromonas* and SAR86) were almost undetectable for most of

Table 2. Percentage of total DAPI counts (±SD of replicate filters) detected with the group-specific HRP-probes Eury806 (*Euryarchaea)*, Eub338-II-III (*Eubacteria*), CF319a (*Bacteroidetes)*, Gam42a (*Gammaproteobacteria*), Alf968 (*Alphaproteobacteria*), Ros537 (*Roseobacter*), SAR11-441R (SAR11 clade), Alt1413 (*Alteromonas*), NOR5-730 (NOR5 cluster) and SAR 86/1245 (SAR86 cluster)

Date	Eury806	Eub338-II-III	CF319a	Alf968	Gam42a	SAR11	ROS537	NOR5-730	Alt1413	SAR86
4 Mar. 03	4±3	63±0	12±2	22±2	2±2	15±7	4±0	2±2	<1	3±3
25 Mar. 03	<1	63±4	7±3	42±4	4±2	28±2	2±3	3±3	2±3	<1
22 Apr. 03	3±1	69±4	8±4	30±4	4±4	23±2	3±2	2±2	1±2	<1
12 May 03	3±1	75±6	16±1	37±2	5±0	33±1	7±0	1±1	1±0	<1
25 Jun. 03	<1	67±1	11±5	39±8	7±0	37±6	1±1	1±3	<1	<1
14 Jul. 03	1±0	91±0	8±3	33±3	50±1	16±5	4±1	6±3	30±1	<1
4 Aug. 03	1±1	74±2	12±5	38±4	8±1	20±2	1±1	5±4	<1	<1
16 Sep. 03	<1	59±5	12±6	35±3	3±4	33±1	1±1	2±1	<1	<1
21 Oct. 03	1±1	63±1	6±1	19±2	1±2	6±0	<1	<1	<1	<1
25 Nov. 03	3±3	78±5	11±3	34±3	4±0	18±2	3±2	1±0	<1	<1
16 Dec. 03	5±3	85±3	12±2	27±3	3±1	15±1	5±1	1±0	1±0	<1
26 Jan. 04	6±4	73±7	14±0	24±2	2±1	22±1	3±2	<1	<1	<1
23 Feb. 04	3±2	84±1	14±3	21±1	4±1	15±3	7±1	<1	<1	<1
22 Mar. 04	5±3	84±0	13±3	24±2	4±2	29±6	5±2	1±0	<1	<1

the year (Table 2), again with the exception of the July sample).

Identification and seasonality of taxonomic groups of bacteria by clone libraries

We analysed between 91 and 107 clones in each of four clone libraries constructed with samples from winter (3 March 2003), spring (14 May 2003), summer (4 August 2003) and autumn (21 October 2003). We obtained informative sequences from 78–93 clones in each of the four seasonal clone libraries (corresponding to 28–52 OTUs per library after screening with RFLP). The number of clones analysed was sufficiently large to describe appropriately the diversity of the bacterial assemblage in spring and autumn, as revealed by the asymptotic behavior of the S_{Chao} index (Fig. 5). By contrast, a linear behavior of S_{Chao} vs. library subsample size was found for the winter and summer clone libraries (Fig. 5). Good's coverage values ranged from 40% in winter to 80% in spring, with an average of 62%.

Most of the clones showed >97% similarity to sequences of uncultured marine bacteria deposited in GenBank. However, in the autumn clone library we found a relatively high percentage of sequences (10/48, representing 12% of total clones in the library) with similarities below 97% to published sequences. In this library, four highly related clones (similarity among them of 99%) affiliated to the phylum *Verrucomicrobia* (BL03-AUT08, AUT37, AUT66 and AUT91) showed very low similarities (below 92%) to other published sequences, and could form a novel cluster.

We recovered a high proportion of plastids in winter (61% of the clones, mostly from cryptophytes and prasinophytes) and spring (19% of the clones, mostly from prymnesiophytes and prasinophytes). For the comparison among different clone libraries, we only considered the bacterial (not plastidial) origin clones (Fig. 6). In this figure, we show the results of the clone libraries together with the count results obtained by CARD-FISH (as discussed below).

There were pronounced differences between the clone libraries collected during different seasons (Fig. 6, 'LIB' columns). A remarkably low diversity was found in the spring clone library, in which 65% of the clones were affiliated with the SAR11 cluster, and 26% with the *Roseobacter* group. Other groups, such as *Bacteroidetes* and *Gammaproteobacteria*, represented less than 4% of the clones each (Fig. 6).

The SAR11 cluster also dominated in the other clone libraries (around 40% of the clones, Fig. 6). Some *Alphaproteobacteria* clones appearing in summer and autumn (14% and 3% of the clones, respectively, included in 'other *Alphaproteobacteria*' in Fig. 6) were closely related to a group of clones found in the Aegean Sea, which clustered with SAR11 (AEGEAN_233, 169 and 112, Fig. 7a). *Roseobacter* clones were less abundant, showing higher proportions in winter (13% of clones) than in summer and autumn (4% and 1% of clones, respectively). *Bacteroidetes* and SAR86 groups were rather constant (around 10% and 7% of the clones, respectively), while other groups, such as the *Verrucomicrobia* and *Actinobacteria*, only appeared in substantial proportions in the autumn clone library (Fig. 6).

Clones affiliated with the SAR11 cluster were distributed into two separate clusters (sequence similarity between clusters of *c.* 91–94%) without a marked seasonality, indicating that a diverse set of SAR11 clones were present throughout the year (Fig. 7a). One of the clusters, which includes the sequence of *Pelagibacter ubique*, contained most of the clones, with a sequence similarity of around 98% to this recently isolated bacterium or to the original SAR11 clone. A group of seven clones (which did not include any winter sequences) clustered with the second group of SAR11

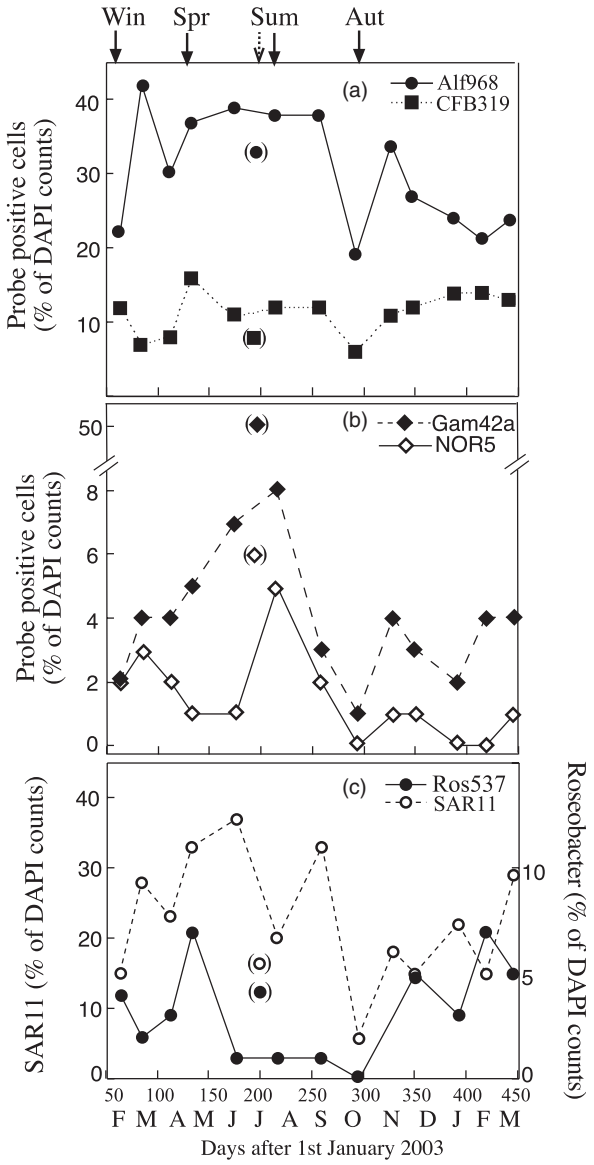

Fig. 4. Proportions of bacterial groups detected by CARD-FISH with HRP-probes specific for: (a) *Alphaproteobacteria* (Alf968) and *Bacteroidetes* (CF319), (b) *Gammaproteobacteria* (Gam42) and the Nor5 cluster (NOR5-730), and (c) *Roseobacter* (Ros537) and SAR11 (SAR11-441R). Samples taken in July, when a drastic change in the bacterial assemblage structure was found, are presented as outliers. The arrows indicate the samples from which seasonal clone libraries were established.

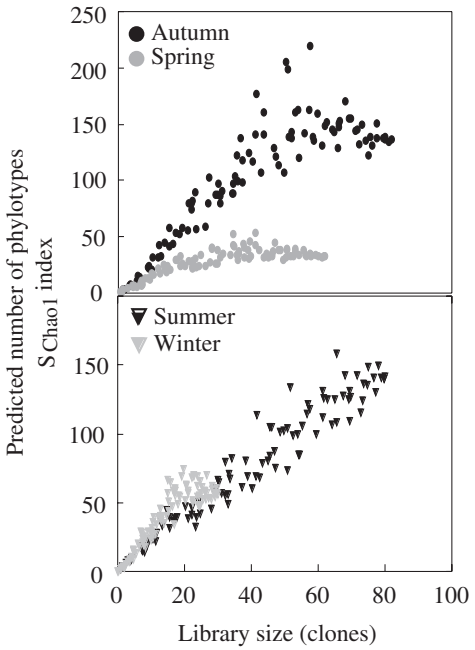

Fig. 5. Predicted number of phylotypes based on the S_{Chao1} index vs. size of subsamples of the four clone seasonal libraries from Blanes Bay. Each point is the mean of 10 replicate subsamples of the library. When the estimated phylotype richness reaches an asymptote, the library can be considered large enough to yield a stable estimate of phylotype richness (Kemp & Aller, 2004).

clones, which includes the SAR211, ZD0410 or MB11D08 clones, previously found in the Sargasso Sea, North Sea and the Pacific Ocean, respectively.

Clones affiliated with *Roseobacter* were distributed in several different positions in the *Roseobacter* phylogenetic tree (Fig. 7b). Some spring clones clustered with cultivated strains of *Roseobacter* such as *Octadecabacter antarcticus* (BL03-SPR10) and *Nereida ignava* (BL03-SPR23, BL03-SPR21 and BL03-SPR19), the latter of which were isolated from Mediterranean seawaters. The other clones were included in clusters mostly represented by clone sequences (i.e. CHAB-I-5, NAC11-7).

A separate clone library constructed with a smaller number of clones (48 clones, data not shown) confirmed the unusual bacterial assemblage composition obtained by CARD-FISH in July (i.e. outlier points in Fig. 4). As with to CARD-FISH results, *Gammaproteobacteria* dominated the bacterioplankton assemblage (57% of the clones) in July, with a high abundance of a phylotype belonging to the *Alteromonadaceae* (*Glaciecola*, 38% of the clones).

Quantitative comparison between the three molecular approaches (DGGE, CARD-FISH and clone libraries)

The analysis of DGGE bands showed a remarkably different picture of the seasonality of bacterial assemblage structure compared with CARD-FISH and clone libraries. Although most bands were affiliated with the *Alphaproteobacteria*, their quantitatively most important group (i.e. the SAR11

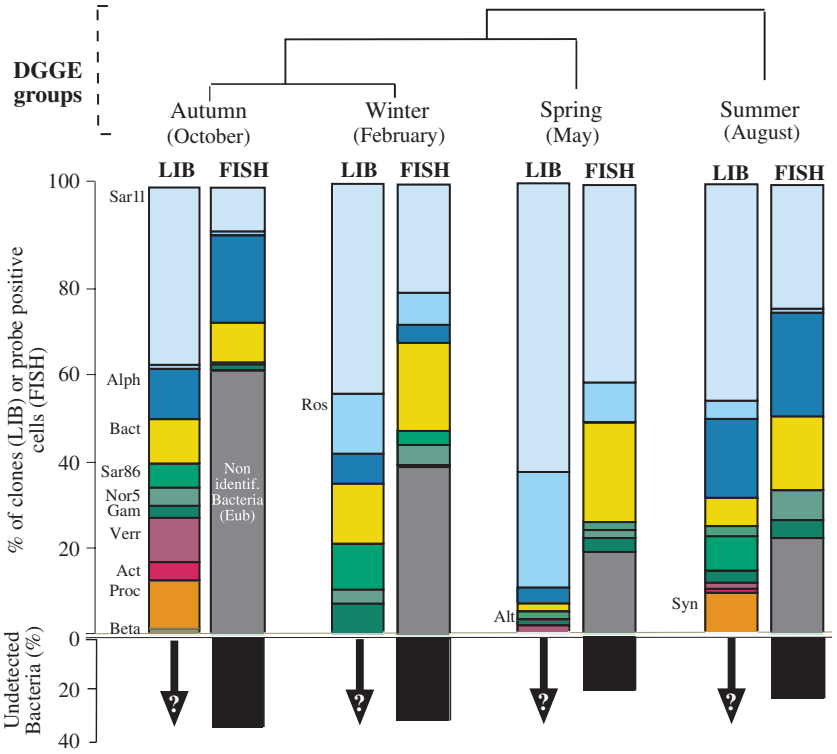

Fig. 6. Proportions of clones of Eub+ cells affiliated with different phylogenetic groups [SAR11, *Roseobacter* (Ros), other *Alphaproteobacteria* (Alpha), *Bacteroidetes* (Bact), SAR86, NOR5, *Alteromonas* (Alt), other *Gammaprotebacteria* (Gam), *Betaproteobacteria* (Beta), *Verucomicrobia* (Verr), *Actinobacteria* (Act), *Prochlorococcus* (Proch), and *Synechococcus* (Syn)] found by cloning and sequencing in the four seasonal clone libraries (LIB columns), or by CARD-FISH (FISH columns), respectively. The samples from winter (February 2003), spring (May 2003), summer (August 2003) and autumn (October 2003) were chosen as representative of the groups found in the DGGE dendogram. The proportion of undetectable *Bacteria* in 'FISH' columns refers to the percentage of DAPI cells (excluding *Archaea*) that did not hybridize with the set of probes Eub338-II-III. The arrows with question marks indicate that an unknown proportion of phylogenetic groups cannot be retrieved by the set of primers used in the clone libraries.

cluster) was not detected by DGGE. *Roseobacter* and *Bacteroidetes* were detected in higher proportions by DGGE compared with clone libraries or CARD-FISH (Tables 1 and 2, Fig. 6). Likewise, the *Gammaproteobacteria* group was not detected in DGGE bands, even during July, when the proportion of *Gammaproteobacteria* increased to around 50% of clones and DAPI counts. However, we found that changes in the relative intensity of some species agreed with their proportions obtained from flow cytometric counts (*Synechococcus* and *Prochlorococcus*) and from clone libraries, such as the appearance of *Erythrobacter* in September and October in the DGGE (band 48; Fig. 2), as well as in the clone library constructed in October (autumn clone library, clones BL03-AUT04 and AUT12).

We compared the proportion of clones (bacterial origin) of different phylogenetic groups with the proportion of cells with the same phylogenetic affiliations analysed by CARD-FISH with specific probes (Fig. 8). This figure shows the ratio of the two percentages, including only those samples in which the group was present in significant percentages ($\geq$3%) in either clone libraries or CARD-FISH. Most of the cases where groups were detected by FISH but not in clone libraries (ratio equal to 0) belonged to the July clone library (open circles), which was constructed with a significant lower number of clones. Remarkably, in this clone library the percentages of *Alphaproteobacteria*, SAR11, *Gammaproteobacteria* and *Alteromonas* were similar by both methods. *Alphaproteobacteria*, and specifically the SAR11 and *Roseobacter* clusters, were generally overrepresented in clone libraries as compared with CARD-FISH counts (Fig. 8). *Bacteroidetes*, by contrast, were generally underrepresented in clone libraries. The detection of *Gammaproteobacteria* was more proportionate by both methods (Fig. 8), but SAR86 was always overrepresented in clone libraries. The NOR5 group showed variable results, being under- or overrepresented in clone libraries in different samplings.

Discussion

Seasonal changes in bacterial diversity should be relevant to understand the year-round variability in important bacterially mediated processes in the ocean, such as carbon metabolism. However, there are still relatively few studies that have assessed this topic in marine waters. Although different approaches have been used, including whole-genome hybridization (Pinhassi & Hagström, 2000) and FISH (Eilers *et al.*, 2001; Mary *et al.*, 2006), most studies have relied on PCR-based techniques (Schauer *et al.*, 2003; Ghiglione *et al.*, 2005; Morris *et al.*, 2005), which may not provide a reliable quantification of the abundance of different bacterial groups. In our study, the objective was to obtain a detailed picture of the seasonality of bacterial assemblages in a coastal oligotrophic site, combining and comparing different molecular approaches (DGGE, CARD-FISH and clone libraries).

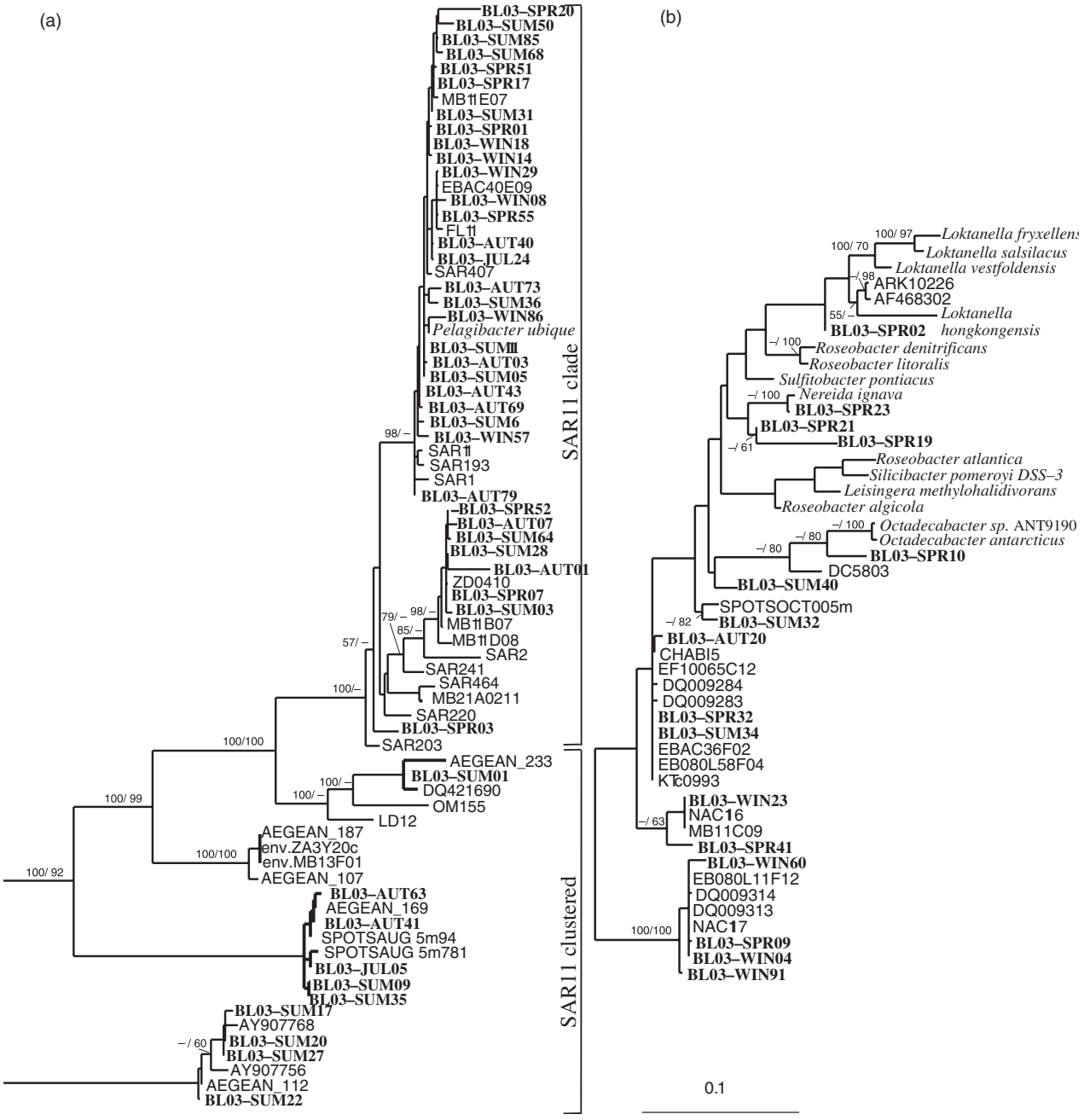

Fig. 7. Maximum-likelihood phylogenetic trees of environmental clones affiliated with (a) SAR11 and (b) *Roseobacter* in the four seasonal clone libraries: clones from the winter (WIN), spring (SPR), summer (SUM) and autumn (AUT) clone libraries are shown in bold type. The SAR11 phylogenetic tree includes several clones isolated in the summer and autumn clone libraries related to the SAR11 cluster, and with high similarity to a group of clones from the Aegean Sea (AEGEAN clones; Moeseneder *et al*., 2005). Neighbor-joining bootstrap values (> 50%) and Bayesian posterior probabilities are shown (in that order) on the left of the relevant nodes. The trees were rooted with the sequence of *Sinorhizobium melitoti* (not shown). Scale bar denotes 0.1 substitutions per nucleotide position.

Seasonal changes in bacterial assemblage structure in Blanes Bay

We carried out a monthly sampling, which had been previously shown to be an adequate time scale to detect seasonal changes in bacterial assemblage composition in our sampling area (Schauer *et al*., 2003). These authors hypothesized that the gradual change in bacterial assemblage composition was due to the complex control by bottom-up or

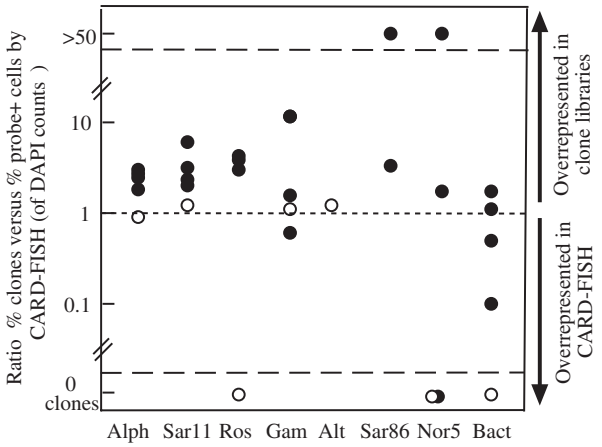

Fig. 8. Ratio of the percentage of clones (scaled to clones of bacterial origin) of the different phylogenetic groups to the percentage of bacteria (scaled to total DAPI counts) detected by CARD-FISH with specific HRP-probes for the same groups. Data points above or below the line (ratio = 1) indicate samples in which the groups were overrepresented in clone libraries or CARD-FISH, respectively. If percentages of bacteria scaled to Eub338-II-III probes (instead of DAPI) were used, the general picture would be very similar. Open circles refer to the clone library constructed in July, and closed circles to the other clone libraries. Alph, *Alphaproteobacteria*; Ros, *Roseobacter*; Gam, *Gammaproteobacteria*; Alt, *Alteromonas*; Bact, *Bacteroidetes*.

environmental factors (such as temperature or substrate availability), as top-down factors seem to vary at much shorter time scales. During the July sampling, *Gammaproteobacteria*, and particularly the phylotype *Glaciecola* (*Alteromonadaceae*), increased their proportions drastically, reaching 50% of the bacterial counts (by CARD-FISH). This burst of growth is striking, as the abundances of these organisms are typically $< 1\%$ over the year in Blanes Bay, as well as in other marine environments (Eilers *et al.*, 2000). These results suggest that episodic events can promote the blooming of specific populations (Pernthaler & Amann, 2005), superimposed on the smooth seasonal changes generally captured by the monthly sampling.

Setting aside the July event, the change in the bacterial assemblage was as gradual as commonly observed in other studies (Schauer *et al.*, 2003). *Alphaproteobacteria*, and more specifically the SAR11 cluster, dominated year-round, in agreement with previous studies that suggest that SAR11 could be the most abundant bacterial group in the ocean (Giovannoni & Rappé, 2000; Morris *et al.*, 2002). The proportion of SAR11 clones was higher at our sampling site than in other coastal systems (12% of the clones on the Oregon coast and Cape Hatteras; Rappé *et al.*, 1997, 2000), but was similar to open waters from the Mediterranean (35% of clones; Acinas *et al.*, 1999) and other marine regions (Giovannoni & Rappé, 2000). This could be related to the open ocean influence in Blanes Bay by intrusions of offshore waters through a nearby submarine canyon (Masó & Tintoré, 1991), which seems to affect the bacterial assemblage composition (Schauer *et al.*, 2000).

The marked seasonality in the proportions of the SAR11 group, with increasing values in spring and maximal values during the summer, is also in agreement with the results of Morris *et al.* (2002, 2005) from the Atlantic Ocean. Lower proportions of SAR11 cells have been found during the summer in the English Channel (Mary *et al.*, 2006). However, these authors apparently did not adapt the protocol for overnight hybridization, and we found that this can be crucial for the successful hybridization of SAR11 cells (details not shown). The high capacity of these bacteria to grow in nutrient-limited waters (Rappé *et al.*, 2002) could be related to the importance of SAR11 in Blanes Bay during summer, the season during which phosphorus limitation of bacterial activity is maximal (Pinhassi *et al.*, 2006).

Another group of *Alphaproteobacteria* clones that clustered with the SAR11 group appeared in summer and autumn in Blanes Bay (Fig. 7a). These clones showed high similarities with some phylotypes retrieved from the Aegean Sea (AEGEAN_233, 169 and 112; Moeseneder *et al.*, 2005), and with some clones recently isolated from the San Pedro Channel (Brown *et al.*, 2005) and Arabian Sea (Fuchs *et al.*, 2005). Some of the phylotypes (clone AEGEAN 233; Moeseneder *et al.*, 2005) appeared in a RNA-based clone library, suggesting that members of this group can be metabolically active.

Remarkably, a seasonal pattern opposite to that observed for SAR11, with higher proportions in winter compared with summer, was found for the other relevant group of *Alphaproteobacteria*, *Roseobacter*. This suggests that this group is favored by nutrient-rich conditions, in agreement with its common association with phytoplankton blooms (González *et al.*, 2000; Suzuki *et al.*, 2001). Most of the clones during the Chl *a*-rich season (winter) were highly similar ($> 97\%$) to the NAC11-7 phylotype, which is primarily represented by clone sequences, some of them associated with algae and algal blooms. Sequences related to this phylotype were also found in higher proportions by DGGE during the winter in Blanes Bay in our study (band 35, Fig. 2), as well as in the study conducted by Schauer *et al.* (2003) in 1998. Another peak of abundance of *Roseobacter* was found in spring, coincident with a high diversity of clones within this cluster. Higher abundances of *Roseobacter* in spring, associated to higher nutrient availability, have been reported from offshore California (Brown *et al.*, 2005).

Bacteroidetes were remarkably constant over the year as revealed by FISH, in contrast with other studies where the abundance of this group increased with higher levels of chlorophyll (Pinhassi & Hagström, 2000). *Gammaproteobacteria* showed higher abundances (by CARD-FISH) during the summer, in agreement with the results presented by

Mary *et al.* (2006) for the English Channel. In Blanes Bay, most of the clones in this group were affiliated with the SAR86, NOR5 and *Alteromonas* lineages, although only NOR5 could be detected in substantial proportions by CARD-FISH year-round. Specifically, three clones affiliated with SAR86 (BL03-AUT16, BL03-SUM93 and BL03-JUL28) showed 97–99% similarity to a clone retrieved from the Aegean Sea (AEGEAN_234; Moeseneder *et al.*, 2005). This clone was found in much higher proportions in an RNA- compared with a DNA-based clone library, indicative of its high metabolic activity. However, it is remarkable that we usually could not hybridize SAR86 cells by CARD-FISH in Blanes Bay, a problem that might be due to their low ribosomal content (Pernthaler *et al.*, 2002).

The NOR5 cluster has been retrieved in several clone libraries from the Mediterranean Sea (Schäfer *et al.*, 2001), Atlantic Ocean (Rappé *et al.*, 1997) and North Sea (Eilers *et al.*, 2000), and a strain of this group was isolated from open waters in the German Bight (Eilers *et al.*, 2001). Similar dynamics were found in Blanes Bay and in the German Bight for the NOR5 group, with higher proportions in the summer months (peaks of 5% and 8% of DAPI counts in Blanes Bay and the German Bight, respectively) and lower proportions in autumn and winter. The contribution of this group to total *Gammaproteobacteria* was also similar (around 60%) in both studies. Interestingly, Eilers *et al.* (2001) found that the peaks of NOR5 coincided with biomass peaks of a diatom (*Lauderia* sp.). In our study, the dynamics of this group (measured by CARD-FISH) was rather similar to that of *Synechococcus*, suggesting a possible association of this lineage with some phytoplankton populations.

In summary, *Alphaproteobacteria* dominated the bacterial assemblage throughout the year, but a succession between the SAR11 group (which peaked in the nutrient-limited season, i.e. summer) and *Roseobacter* (which peaked in the nutrient-rich season, i.e. winter) was observed. *Bacteroidetes* were the second most important group in Blanes Bay, and *Gammaproteobacteria* showed low abundances, with a predominance of the NOR5 group. It is noticeable that after a stormy period (October sampling, Fig. 1), the proportion of the three main broad phylogenetic groups (*Bacteroidetes*, *Alphaproteobacteria* and *Gammaproteobacteria*) decreased concomitant with an increase of unusual groups such as *Actinobacteria*, *Verrucomicrobia* and *Betaproteobacteria*. The last two groups are commonly found in freshwater environments (Zwart *et al.*, 2002). Thus, their appearance could be related to the increase in the riverine discharge and coastal runoff to the bay after such stormy events (see Fig. 1). Additionally, a relatively high percentage of clones showed low similarities with published sequences (90–96% similarities) during this sampling period, suggesting that a substantial amount of additional diversity can be found in water samples in which drastic environmental changes occur.

Shortcomings and biases of clone libraries, DGGE and CARD-FISH methods in environmental studies

The comparison between clone libraries, DGGE and FISH results is not straightforward because of the different levels of phylogenetic resolution of each technique. There is general agreement regarding the limitations of each technique (Amann *et al.*, 1995; Wintzingerode *et al.*, 1997), but few studies have compared the results of different techniques in marine waters (Cottrell & Kirchman, 2000; Díez *et al.*, 2001), and none has compared them through a complete annual cycle. Figure 6 presents a snapshot of the kind of information and results that can be obtained by these three techniques.

DGGE allowed an assessment of the changes in the composition of the bacterial assemblage through the seasons with sufficient time resolution. However, a failure to obtain sequences from faint bands (Schauer *et al.*, 2003) prevents the use of DGGE for describing bacterial diversity accurately. Because not all bands were sequenced, we cannot discard the possibility that differences between the assemblage composition shown by DGGE and those by other techniques are due to insufficient sequencing. However, it is noticeable that the dominant bacterial group, SAR11, was not retrieved in any of the sequenced DGGE bands, unlike in other studies, such as those of Selje & Simon (2003) and Balagué *et al.* (unpublished data). Castle & Kirchman (2004) carried out a comparative study between DGGE and FISH, and also showed that DGGE failed to detect the most abundant phylogenetic group detected by FISH in some samples. Besides primer specificity, these authors argued that high richness within groups could lead to an underestimation as compared with FISH, because different sequences would appear as different faint bands, which could be difficult to excise from the gels for sequencing. Our results support this hypothesis, given the high microdiversity found in the SAR11 clones in this and in previous studies (Fig. 7; García-Martínez & Rodríguez-Valera, 2000; Brown & Fuhrman, 2005). Indeed, the SAR11 bands appeared in close proximity in the gel (Fig. 2), and this makes excision and DNA extraction difficult.

Clone libraries provided the highest phylogenetic resolution and a detailed picture of the species within each phylogenetic group. However, PCR bias produced over or underestimations of specific groups compared with the direct quantification obtained by CARD-FISH. The picture of the bacterial assemblage composition provided by this last technique, in turn, was severely limited by the number and phylogenetic resolution of the probes. Substantial proportions of the cells remained unidentified by the general probes used (Alf968, CF319a, Gam42a) or undetectable with the Eub338-II-III probe.

In agreement with the results obtained by Cottrell & Kirchman (2000), *Alphaproteobacteria* and *Bacteroidetes* were generally over and underestimated in clone libraries, respectively. *Gammaproteobacteria*, however, were found either on the 1 : 1 line or were overrepresented in clone libraries (Fig. 8). The dominance of *Alphaproteobacteria* and more specifically of SAR11 is a common feature in clone libraries constructed with marine samples (Giovannoni & Rappé, 2000). Even if this group has been shown to be numerically dominant (Morris *et al.*, 2002), clone libraries still seem to overestimate their proportion (up to six-fold) as compared with CARD-FISH counts (Fig. 8). Such overestimation could be explained by different amplification efficiencies based, for example, on G+C content (Dutton *et al.*, 1993). The genomic analysis of a member of the SAR11 clade showed that this group has a low G+C content (Giovannoni *et al.*, 2005), which, in principle, could increase their amplification efficiency by PCR.

Alternatively, differences between clone libraries and FISH results could be due to possible mismatches of the specific probes, which would lead to the underestimation of this group by FISH. We analysed the specificity of the FISH probe used in this study (SAR11-441R) using the ARB software (Ludwig *et al.*, 1998), and found that this probe matched most (91%) of our clones. This points to PCR bias as the most probable explanation for the overestimation of SAR11 in clone libraries.

A remarkable result in this respect was that the Alf968 probe had mismatches with all our SAR11 clones, which were mostly located in the last nucleotide and thus were possibly not critically affecting the hybridization. The probe also had a mismatch with the group of *Alphaproteobacteria* clones related to those retrieved by Moeseneder *et al.* (2005) located in the center of the oligonucleotide, and thus probably critically affected the detection of these cells by FISH. This suggests that Alf968 can in some cases severely underestimate the real proportions of groups of *Alphaproteobacteria*.

Another group overrepresented in clone libraries was SAR86, in agreement with previous studies (Eilers *et al.*, 2000). This group was abundant in numerous clone libraries in both coastal (up to 29% of bacterial clones; Rappé *et al.*, 1997) and open waters (Giovanonni & Rappé, 2000), but its optimal detection by *in situ* hybridization required the development of the CARD-FISH methodology (Pernthaler *et al.*, 2002). Although we used this optimized protocol in our study, we could only detect this group in one out of the 14 months sampling (Table 1). Even if SAR86 has been detected in important proportions by *in situ* hybridization in relatively eutrophic marine areas such as the North Sea (Eilers *et al.*, 2000; Pernthaler *et al.*, 2002) or the English Channel (Mary *et al.*, 2006), whether this widely distributed group of uncultivated *Gammaproteobacteria* is quantitatively abundant in more oligotrophic oceanic waters remains to be determined.

In summary, the combination of the three approaches was very useful for assessing changes in bacterial diversity, but no single technique alone can be trusted to cover all aspects of an acceptable description of the bacterial phylogenetic diversity or population dynamics. A great part of the disagreement between PCR-based and direct methods such as FISH can be due to mismatches in the commonly used probes (such as that for Alf968). This indicates that more effort should be devoted to the design of new probes and the specificity of old probes should always be checked against the growing public databases.

Acknowledgements

This work was supported by the Spanish projects MicroDiff (REN2001-2110/MAR) and MODIVUS (CTM2005-04975/MAR), and the EU project BASICS (EVK3-CT-2002-00078) to J.M.G., GENμMAR (CTM2004-02586/MAR) to C.P.A. and ESTRAMAR to R.M. (CTM2004-12631/MAR). This is also a contribution to the NoE MARBEF. Financial support was provided by a PhD fellowship from the Spanish government to L.A.S. We thank F. Unrein and I. Forn for their help during the monthly sampling, and C. Alonso for her help during the processing of CARD-FISH samples in Bremen.

References

Acinas SG, Antón J & Rodríguez-Valera F (1999) Diversity of free-living and attached bacteria in offshore western Mediterranean waters as depicted by analysis of genes encoding 16S rRNA. *Appl Env Microbiol* **65**: 514–522.

Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W & Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389–3402.

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R & Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotice probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**: 1919–1925.

Amann RI, Ludwig W & Schleifer K (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* **59**: 143–169.

Brown MV & Fuhrman JA (2005) Marine bacterial microdiversity as revealed by internal transcribed spacer analysis. *Aquat Microb Ecol* **41**: 15–23.

Brown MV, Schwalbach MS, Hewson I & Fuhrman JA (2005) Coupling 16S-ITS rDNA clone libraries and automated ribosomal intergenic spacer analysis to show marine microbial diversity: development and application to a time series. *Environ Microbiol* **7**: 1466–1479.

Castle D & Kirchman DL (2004) Composition of estuarine bacterial communities assessed by denaturing gradient gel electrophoresis and fluorescence in situ hybridization. *Limnol Oceanogr: Methods* **2**: 303–314.

Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molec Biol Evol* **17**: 540–552.

Chao A (1984) Non-parametric estimation of the number of classes in a population. *Scan J Statistics* **11**: 265–270.

Chao A, Ma M-C & Yang MCK (1993) Stopping rules and estimation for recapture debugging with unequal failure rates. *Biometrika* **80**: 193–201.

Cottrell MT & Kirchman DL (2000) Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence in situ hybridization. *Appl Environ Mcrobiol* **66**: 5116–5122.

Daims H, Bruhl A, Amann R, Schleifer KH & Wagner M (1999) The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* **22**: 434–444.

Díez B, Pedrós-Alió C, Marsh TL & Massana R (2001) Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques. *Appl Env Microbiol* **67**: 2942–2951.

Dutton CM, Paynton C & Sommer S (1993) General method for amplifying regions of very high G + C content. *Nucleic Acids Res* **21**: 2953–2954.

Eilers H, Pernthaler J, Glöckner FO & Amann R (2000) Culturability and in situ abundance of pelagic bacteria from the North Sea. *Appl Environ Microbiol* **66**: 3044–3051.

Eilers H, Pernthaler J, Peplies P, Glöckner FO, Gerdts G & Amann R (2001) Isolation of novel pelagic bacteria from the German Bight and their seasonal contribution to surface picoplankton. *Appl Env Microbiol* **67**: 5134–5142.

Ferrera I, Massana R, Casamayor EO, Balagué V, Sánchez O, Pedrós-Alió C & Mas J (2004) High-diversity biofilm for the oxidation of sulfide-containing effluents. *Appl Microbiol Biotechnol* **64**: 726–734.

Fuchs BM, Woebken D, Zubkov MV, Burkill P & Amann R (2005) Molecular identification of picoplankton populations in contrasting waters of the Arabian Sea. *Aquat Microb Ecol* **39**: 145–157.

García-Martínez J & Rodríguez-Valera F (2000) Microdiversity of uncultured marine prokaryotes: the SAR11 cluster and the marine Archaea of Group I. *Molec Ecol* **9**: 935–948.

Gasol JM & del Giorgio PA (2000) Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Sci Mar* **64**: 197–224.

Ghiglione J, Larcher M & Lebaron P (2005) Spatial and temporal scales of variation in bacterioplankton community structure in the NW Mediterranean Sea. *Aquat Microb Ecol* **40**: 229–240.

Giovannoni SJ, Britschgi TB, Moyer CL & Field KJ (1990) Genetic diversity in Sargasso Sea bacterioplankton. *Nature* **345**: 60–63.

Giovannoni SJ & Rappé M (2000) Evolution, diversity and molecular ecology of marine prokaryotes. *Microbial Ecology of the Oceans* (Kirchman DL, ed), pp. 47–84. Wiley-Liss, New York.

Giovannoni SJ, Tripp HJ, Givan S *et al*. (2005) Genome streamlining in a cosmopolitan oceanic bacterium. *Science* **309**: 1242–1245.

Good DL (1953) The population frequencies of species and the estimation of population parameters. *Biometrika* **40**: 237–264.

González JM, Simó R, Massana R, Covert JS, Casamayor EO, Pedrós-Alió C & Moran MA (2000) Bacterial community structure associated with a dimethylsulfoniopropionate-producing north atlantic algal bloom. *Appl Environ Microbiol* **66**: 4237–4246.

Kemp PF & Aller JY (2004) Estimating prokaryotic diversity: when are 16S rDNA libraries large enough? *Limnol Oceanogr Methods* **2**: 114–125.

Lee SM & Chao A (1994) Estimating population size via sample coverage for closed capture-recapture models. *Biometrics* **50**: 88–97.

Ludwig W, Strunk O, Klugbauer S, Klugbaur N, Weizeneger M, Neumaier J, Bachleitner M & Schleifer KH (1998) Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* **19**: 554–568.

Maidak BL, Cole JR, Lilburn TG, Parker CT, Saxman PR, Farris RJ, Garrity GM, Olsen GJ, Schmidt TM & Tiedje JM (2001) The RDP-ii (ribosomal database project). *Nucleic Acid Res* **29**: 173–174.

Marie D, Partensky F, Jacquet S & Vaulot D (1997) Enumeration and cell cycle analysis of natural populations of marine picoplankton by flow cytometry using the nucleic acid stain SYBR Green I. *Appl Environ Microbiol* **63**: 186–193.

Mary I, Cummings DG, Biegala IC, Burkill PH, Archer SD & Zubkov MV (2006) Seasonal dynamics of bacterioplankton community structure at coastal station in the western English Channel. *Aquat Microb Ecol* **42**: 119–126.

Masó M & Tintoré J (1991) Variability of the shelf water off the Northeast Spanish coast. *J Man Sys* 1: 441–450.

Massana R, Murray AE, Preston CM & DeLong EF (1997) Vertical distribution and phylogenetic characterization of marine planktonic Archaea in the Santa Barbara Channel. *Appl Env Microbiol* **63**: 50–56.

Moeseneder MM, Arrieta JM & Herndl GJ (2005) A comparison of DNA- and RNA-based clone libraries from the same marine bacterioplankton community. *FEMS Microbiol Ecol* **51**: 341–352.

Morris RM, Rappé MS, Connon SA, Vergin KL, Slebold WA, Carlson CA & Giovannoni SJ (2002) SAR11 clade dominates ocean surface bacterioplankton communities. *Nature* **420**: 806–810.

Morris RM, Vergin KL, Cho J, Rappé MS, Carlson CA & Giovannoni SJ (2005) Temporal and spatial response of bacterioplankton lineages to annual convective overturn at the

Bermuda Atlantic Time-series study site. *Limnol Oceanogr* **50**: 1687–1696.

Neef A (1997) *Anwendung der in situ-Einzelzell-Identifizierung von Bakterien zur Populationsanlayse in komplexen mikrobiellen biozönosen*, PhD Thesis,). Technische Universität München, Munich, Germany.

Pernthaler J & Amann R (2005) Fate of heterotrophic microbes in pelagic habitats: focus on populations. *Appl Env Microbiol* **69**: 440–461.

Pernthaler A, Pernthaler J & Amann R (2002) Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. *Appl Env Microbiol* **68**: 3094–3101.

Pernthaler J, Pernthaler A & Amann R (2003) Automated enumeration of groups of marine picoplankton after fluorescence in situ hybridization. *Appl Environ Microbiol* **69**: 2631–2637.

Pernthaler A, Pernthaler J & Amann R (2004) Sensitive multicolor fluorescence in situ hybridization for the identification of environmental microorganisms. *Molecular Microbial Ecology Manual*, *Vol. 3*, 2nd edn., pp. 711–726. Kluwer Academic Publishers, the Netherlands.

Pinhassi J & Hagström Å (2000) Seasonal succession in marine bacterioplankton. *Aquat Microb Ecol* **21**: 245–256.

Pinhassi J, Gómez-Consarnau L, Alonso-Sáez L, Sala MM, Vidal M, Pedrós-Alió C & Gasol JM (2006) Seasonal changes in bacterioplankton nutrient limitation and their effects on bacterial community composition in the NW Mediterranean Sea. *Aquat Microb Ecol* **44**: 241–252.

Posada D & Crandall KA (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817–818.

Rappé MS, Kemp PF & Giovannoni SJ (1997) Phylogenetic diversity of marine picoplankton 16S rRNA genes cloned from the continental shelf off Cape Hatteras, North Carolina. *Limnol Oceanogr* **42**: 811–826.

Rappé MS, Vergin K & Giovannoni S (2000) Phylogenetic comparisons of a coastal bacterioplankton community with its counterparts in open ocean and freshwater systems. *FEMS Microbiol Ecol* **33**: 219–232.

Rappé MS, Connon SA, Vergin KL & Giovannoni SJ (2002) Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature* **418**: 630–633.

Schäfer H, Bernard L, Courties C *et al.* (2001) Microbial community dynamics in Mediterranean nutrient-enriched seawater. *FEMS Microbiol Ecol* **34**: 243–253.

Schauer M, Massana R & Pedrós-Alió C (2000) Spatial differences in bacterioplankton composition along the Catalan coast (NW Mediterranean) assessed by molecular fingerprinting. *FEMS Microbiol Ecol* **33**: 51–59.

Schauer M, Balagué V, Pedrós-Alió C & Massana R (2003) Seasonal changes in the taxonomic composition of bacterioplankton in a coastal oligotrophic system. *Aquat Microb Ecol* **31**: 163–174.

Selje N & Simon M (2003) Composition and dynamics of particle-associated and free-living bacterial communities in the Weser estuary, Germany. *Aquat Microb Ecol* **30**: 221–237.

Suzuki MT, Preston CM, Chavez FP & DeLong EF (2001) Quantitative mapping of bacterioplankton populations in seawater: field tests across an upwelling plume in Monterey Bay. *Aquat Microb Ecol* **24**: 117–127.

Swofford DL (2002) *PAUP*: phylogenetic analysis using parsimony (*and other methods)*, *Version 4*. Sinauer Associates, Sunderland, Mass.

Teira E, Reinthaler T, Pernthaler A, Pernthaler J & Herndl G (2004) Combining catalyzed reported deposition-fluorescence in situ hybridization and microautoradiography to detect substrate utilization by Bacteria and Archaea in the deep ocean. *App Env Microbiol* **70**: 4411–4414.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F & Higgins DG (1997) The clustalx windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acid Res* **25**: 4876–4882.

Wallner G, Amann R & Beisker W (1993) Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* **14**: 136–143.

Wintzingerode F, Göbel UB & Stackebrandt E (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microb Rev* **21**: 213–229.

Zubkov MV, Fuchs B, Burkill PH & Amann R (2001) Comparison of cellular and biomass specific activities of dominant bacterioplankton groups in stratified waters of the Celtic Sea. *Appl Environ Microbiol* **67**: 5210–5218.

Zwart G, Crump BC, Kamst-van Agterveld MP, Hagen F & Han S (2002) Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. *Aquat Microb Ecol* **28**: 141–155.