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ABSTRACT: An annual study of the bacterioplankton community structure was carried out at Stn L4 (50° 15′ N, 04° 13′ W) in the western English Channel between August 2003 and July 2004. Bacterioplankton abundance and community structure were assessed using flow cytometry and fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes, respectively. The *Eubacteria* domain dominated over the *Archaea* domain (<15%) at the highest phylogenetic level. The Sphingobacteria-Flavobacteria group of the Bacteroidetes phylum (SFB) numerically dominated in spring and early summer. The α-*Proteobacteria* dominated from late summer to winter. The SAR11 clade represented ~13% of the microbial community throughout the year and accounted for up to 69% of α-*Proteobacteria* in late spring. Annually, γ-*Proteobacteria* were 2 or 3 times less abundant than the other groups and showed no obvious seasonal trend. The SAR86 cluster accounted for up to half of γ-*Proteobacteria* when it peaked in summer. Consequently, we found that community structure at higher taxonomic level did not change dramatically with season but lower level phylogenetic groups showed pronounced seasonal peaks.

KEY WORDS: Bacterioplankton · Seasonal variability · Community structure · English Channel · Fluorescence *in situ* hybridization

INTRODUCTION

Bacterioplankton play key roles in many biogeochemical processes (Ducklow & Carlson 1992, Azam 1998, Ducklow 2000). Understanding which bacterioplankton communities dominate and what they respond to remains a fundamental ecological question. An important first step towards understanding the roles of various prokaryotes in the ocean is determining the numbers and relative abundances of different bacterioplankton groups (Giovannoni & Rappé 2000).

Fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes selectively labels microbial cells with defined phylogenetic affiliations (Amann et al. 1995, 1997). FISH has proved a useful tool for monitoring spatial (Alfreider et al. 1996) and temporal (Pernthaler et al. 1997) dynamics of different phylogenetic groups of the planktonic microbial community in marine and freshwater environments (Glockner et al. 1999, Pernthaler et al. 2002b, 2004).

Few seasonal studies on the dynamics of defined phylogenetic groups of bacteria in natural habitats have been carried out so far (Pernthaler et al. 1998, Glockner et al. 1999, Zwisler et al. 2003). Previous investigations have reported that β-*Proteobacteria* dominate in freshwater systems (Hiorns et al. 1997), while marine microbial communities are usually dominated by bacteria that are phylogenetically affiliated with α-*Proteobacteria* and with members of the SFB group (Glockner et al. 1999). Bacterioplankton com-

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munity biomass, function, structure, and diversity are known to change between winter and summer (Pernthaler et al. 1998, Zaccone et al. 2002). Schauer et al. (2003) showed the overall stability in time of the taxonomic composition of the bacterioplankton in coastal marine system, with gradual changes throughout the year revealing a substitution of closely related phylotypes during the seasonal cycle. Crump et al. (2003) demonstrated that shifts in bacterioplankton community composition were related to seasonal cycles in the source and lability of dissolved organic matter. Similarly, succession in marine bacterioplankton assemblages occurred in response to seasonal shifts in water column stability and water temperature, suggesting that bacterioplankton community composition may demonstrate an annual pattern of variability (Murray et al. 1998). Other studies have demonstrated relationships between bacterioplankton community composition and seasonal dynamics of other members of the aquatic food web (Hofle et al. 1999, Fandino et al. 2001, Hahn & Hofle 2001, Arrieta & Herndl 2002).

A typical seasonal dynamic in temporal coastal waters still remains to be established. Previous mesocosm experiments showed dramatic changes in the composition of the bacterial assemblage on daily to weekly time scales (van Hannen et al. 1999, Schäfer et al. 2001). However, it is not clear whether these changes are frequent in the field, although in some situations, such as during phytoplankton blooms, strong changes in numbers and phylogenetic shifts of the bacterial assemblage have been observed (Fandino et al. 2001, Yager et al. 2001). Only a few studies have addressed temporal changes during a whole seasonal cycle in natural environments in freshwater (Lindström 1998, Pernthaler et al. 1998) or coastal (Murray et al. 1998, Pinhassi & Hagström 2000, Schauer et al. 2003) environments.

The aim of the present study was to examine seasonal variation in phylogenetic composition of the bacterioplankton community at a station off Plymouth in the western English Channel (Stn L4). The site is characterised by a large seasonal variation in physical forcing and exhibits a diverse succession of phytoplankton blooms through the spring, summer and autumn, making it an ideal place to study seasonality of bacterioplankton composition.

MATERIALS AND METHODS

Sampling site. Water samples were collected once a week at 2 m depth at Stn L4 (50° 15′ N, 04° 13′ W, water depth ~55 m) off Plymouth in the English Channel from August 2003 to August 2004. Bacterial abundance in surface waters was also monitored from July 1998 to December 2001.

Flow cytometry. Bacterial abundance in surface waters was monitored from July 1998 to December 2001 and from August 2003 to July 2004 with a FACSort flow cytometer (Becton Dickinson) after DNA staining as described in Marie et al. (1997). Yellow-green beads of 0.5 µm diameter (Fluoesbrite Microparticles; Polysciences) were used as a flow cytometric internal standard in order to normalise samples (Zubkov et al. 2002).

Tyramide signal amplification–fluorescent *in situ* hybridization (TSA–FISH). For TSA–FISH, 50 ml samples were fixed with 1% paraformaldehyde (PFA) and harvested on 0.2 µm (pore-size) polycarbonate filters. TSA–FISH was performed as described in Biegala et al. (2002) with some modifications as described below. Before hybridization, prokaryotic cells were partially lysed by placing the filters for 1 h at 37°C in 1 ml of 100 µg ml^{-1} lysozyme (47 000 U mg^{-1}, Sigma-Aldrich) in 0.1 M Tris-HCl and 0.05 M EDTA (pH 7.7). The enzymatic reaction was stopped by rinsing the filter 3 times in 5 ml of sterile water for 1 min. Filters were then placed for 30 min at 37°C in 1 ml of 60 U Achromopeptidase (2600 U mg^{-1}, Sigma-Aldrich) in 0.1 M Tris-HCl and 0.05 M EDTA (pH 7.7) and the enzyme reaction was stopped by rinsing the filter 3 times in 5 ml of sterile water for 1 min. Filters were then dehydrated in a second ethanol series (50, 80, 100%, 3 min each) and dried.

Oligonucleotide probes. The probe sequences, hybridization conditions, and references are given in Table 1. The probe GAM42a was used with competitor oligonucleotides as described previously (Manz et al. 1992). The probes EUBI (EUB338), EUBII and EUBIII were pooled to maximise targeting the *Eubacteria* (Amann et al. 1990, Daims et al. 1999). A mixture of 2 probes, CREN554 and EURY806 (Massana et al. 1997, J. Pernthaler unpubl. data,), which detect *Crenarchaea* and *Euryarchea* respectively, was used to detect *Archaea.* A mixture of 2 published probes was used to enhance the detection of the SAR11 clade (Morris et al. 2002).

Microscopy counts. The filter sections were inspected and cells were counted under a Zeiss Axioplan II motorised epifluorescence microscope (Carl Zeiss), equipped with a 100× UV Plan Apochromat objective and excitation/emission filters 360/420 for DAPI and 490/515 for FITC and an automated image analysis system KS300 (Image Associates). For each sample and probe, about 1000 cells were counted per sample using automatic exposure. All probe-specific cell counts are presented as the percentage of cells visualized by DAPI.

Statistical analysis. Means and standard deviations for 8 to 10 fields of view, representing about 1000 cells, across the filters were calculated. Reproducibility of the hybridization procedure was also checked using independent filters. A *t*-test was used to statistically compare the means of the data between each month

Table 1. Probes used in the present study. FA: percentage of formamide in *in situ* hybridization buffer

Probe	Specificity	Sequence (5′ – 3′) of probe	FA (%)	Source
EUB338/I	*Eubacteria*	GCTGCCTCCCGTAGGAGT	50	Amann et al. (1990)
EUBII	*Eubacteria*	GCAGCCACCCGTAGGTGT	50	Daims et al. (1999)
EUBIII	*Eubacteria*	GCTGCCACCCGTAGGTGT	50	Daims et al. (1999)
CF319a	SFB	TGGTCCGTGTCTCAGTAC	50	Manz et al. (1996)
Alf968	α-*Proteobacteria*	GGTAAGGTTCTGCGCGTT	50	Glockner et al. (1999)
Gam42a	γ-*Proteobacteria*	GCCTTCCCACATCGTTT	50	Manz et al. (1992)
RSB67	*Roseobacter*	CGCTCCACCCGAAGGTAG	40	Zubkov et al. (2001a)
Sar86/1245	SAR86 cluster	TTAGCGTCCGTCTGTAT	50	Zubkov et al. (2001b)
SAR11-152R	SAR11	ATTAGCACAAGTTTCCYCGTGT	50	Morris et al. (2002)
SAR11-542R	SAR11	TCCGAACTACGCTAGGTC	50	Morris et al. (2002)
Eury806	Some marine *Euryarchaea*	CACAGCGTTTACACCTAG	20	J. Pernthaler (unpubl.)
Cren554	50% of *Crenarchaea*	TTAGGCCCAATAATCMTCCT	20	Massana et al. (1997)

and the following one. An *F*-test was carried out to examine the variance between the bacterial groups and total bacterioplankton abundance.

RESULTS

Bacterioplankton abundance

Total bacterioplankton numbers ranged from 0.2×10^6 to 1.6×10^6 cells ml^{-1} during the studied annual cycle (Fig. 1). Similar trends in bacterioplankton abundance occurred between 1998–2001 and 2003–2004: a general increase from February to September and a decrease from October to January. Therefore the studied annual cycle is typical for these temporal coastal waters.

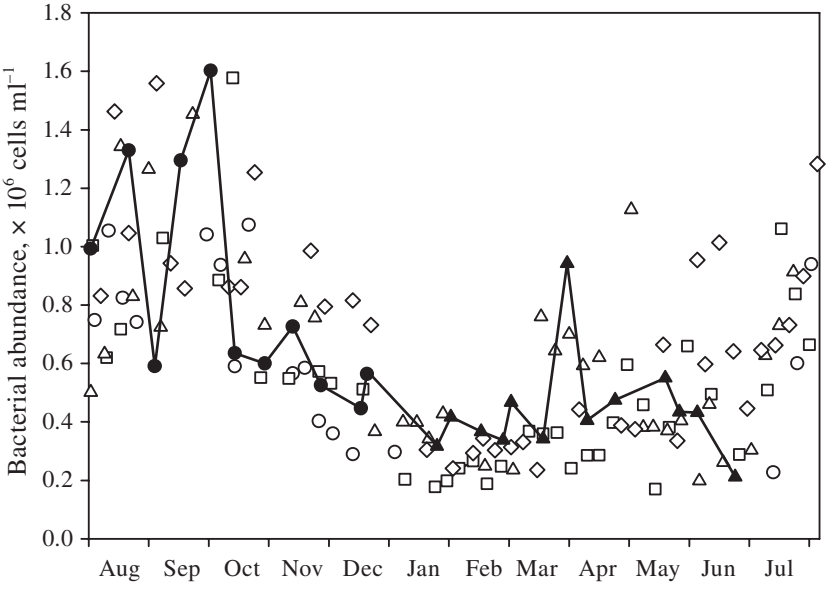

Fig. 1. Seasonal changes of total bacterial abundance measured at Stn L4, Plymouth, UK. ○: 1998; □: 1999; △: 2000; ◇: 2001; ●: 2003; ▲: 2004

Bacterioplankton community structure (FISH)

The *Eubacteria* domain dominated over the *Archaea* domain (Table 2, Fig. 2a). Between 70 and 85% of all DAPI-stained cells (mean: 80%) were visualized with the compilation of *Eubacteria* probes EUBI-III (Table 1), while *Archaea* accounted for less than 6% to a maximum of 13% of all DAPI-stained cells (mean: 8%). Compositional analysis using FISH–TSA revealed that the bacterioplankton at L4 was dominated by the SFB cluster and the α-*Proteobacteria.* On an annual average, members of the SFB group formed 32% (range: 20 to 51%) of of all DAPI-stained cells and 41% (range: 24 to 61% of the cells detected by the *Eubacteria* probes, reaching their maximal abundances in spring and early summer (Table 2, Fig. 2a). α-*Proteobacteria* were the second most abundant bacterial phylogenetic group constituting highest proportions of all bacterioplankton detected in late summer, autumn and winter (August to March). α-*Proteobacteria* accounted for 18 to 36% (mean 28%) of all DAPI-stained cells (Table 2) and, on average, 35% of the EUBI-III counts (range: 23 to 50%). α-*Proteobacteria* were dominated by SAR11 clade and *Roseobacter* genus, representing 8 to 20% and 1 to 11% of all bacterioplankton respectively. SAR11 clade seemed to follow the same pattern as the α subclass, whereas the *Roseobacter* genus significantly increased in early spring and summer with a maximum in May (Table 2, Fig. 2b). The sum of cells targeted by the probes RSB67 specific for *Roseobacter* clade and SAR11 was generally smaller than the number of cells targeted by the general α-*Proteobacteria* probe Alf968. Indeed, the 16S

Table 2. Seasonal changes of bacterioplankton community structure at Stn L4 between August 2003 and July 2004, normalised as percentages of TSA–FISH positive cells compared to total DAPI positive cells. Counts of ~1000 cells from 8 to 10 randomly chosen fields on the filters (mean ± SD). Comparison between 2 consecutive months using *t*-tests, *: 95% significant difference, **: 99% significant difference, no symbol: difference is insignificant

	Eubacteria	*Archea*	SFB	α-*Proteobacteria*	SAR11	*Roseobacter*	γ-*Proteobacteria*	SAR86
Jan	72.1 ± 10.1**	8.8 ± 4.0	19.6 ± 5.1**	29.3 ± 10.0	15.4 ± 5.9	1.5 ± 0.4	8.8 ± 3.9	0.6 ± 0.3**
Feb	84.4 ± 5.5	7.9 ± 2.3	27.8 ± 6.6	35.8 ± 7.3*	18.1 ± 6.3**	2.0 ± 0.8**	8.4 ± 2.0	0.1 ± 0.1*
Mar	79.0 ± 13.2	6.6 ± 3.3	28.0 ± 8.7*	28.3 ± 9.9	19.7 ± 4.9*	4.6 ± 1.2**	11.0 ± 4.1	0.3 ± 0.1**
Apr	81.3 ± 7.9*	6.0 ± 3.9	38.1 ± 10.9	23.1 ± 11.9	12.8 ± 3.2	11.3 ± 4.0	10.1 ± 2.9*	1.8 ± 0.3
May	90.0 ± 8.3	7.8 ± 2.2	45.5 ± 7.8	22.4 ± 6.2	8.9 ± 3.4	9.3 ± 2.6	7.0 ± 2.1	1.5 ± 0.5
Jun	80.0 ± 12.2	10.2 ± 4.9	42.5 ± 9.8	17.6 ± 5.0	9.1 ± 4.5	9.1 ± 3.2	8.2 ± 1.9**	2.0 ± 0.9**
Jul	70.2 ± 15.7*	7.6 ± 3.5	51.3 ± 12.3**	20.0 ± 4.7*	11.1 ± 4.5	8.3 ± 3.6**	13.3 ± 4.2	6.6 ± 1.3
Aug	81.2 ± 8.2	9.4 ± 3.5*	33.5 ± 11.6*	27.6 ± 8.0	12.5 ± 3.6	3.6 ± 0.9	12.5 ± 4.4	5.1 ± 1.5**
Sep	76.9 ± 6.7*	7.0 ± 2.8**	21.8 ± 6.8	35.1 ± 12.2	12.2 ± 3.0	3.5 ± 0.9**	14.5 ± 3.8	2.4 ± 1.2**
Oct	67.3 ± 6.9**	12.5 ± 6.3*	20.1 ± 6.9	34.7 ± 10.4	12.4 ± 2.6	1.2 ± 0.4	13.1 ± 5.0	0.8 ± 0.3
Nov	76.2 ± 4.9	8.8 ± 3.4	22.9 ± 8.0	34.4 ± 6.9	11.4 ± 3.1	1.0 ± 0.3	10.1 ± 2.9	1.2 ± 0.8
Dec	70.0 ± 12.3	7.9 ± 2.1	22.3 ± 6.2	27.6 ± 10.3	13.9 ± 4.1	1.0 ± 0.1**	9.2 ± 3.3	1.0 ± 0.5

database used to generate the probes was not exhaustive and many taxa from the bacterioplankton appeared to correspond to novel lineages not yet represented in the general databases. γ-*Proteobacteria* always comprised the lowest proportions of all bacterial groups (Table 2, Fig. 2c), accounting for 8 to 16% of all DAPI-stained cells (mean 11%) and, on average, 14% of the EUBI-III counts (range: 10 to 19%). The SAR86 cluster of γ-*Proteobacteria* was ~8% of all bacterioplankton in summer and could represent less than 1% in winter (Table 2, Fig. 2c). Moreover, it represented ~50% of the γ-*Proteobacteria* in summer and seems to follow the same seasonal variation as γ-*Proteobacteria*.

An *F*-test was carried out to examine the variance between the bacterial groups and total bacterioplankton abundance (Table 3). The result suggests that there is significantly greater variability in the monthly abundances of *Roseobacter* and SAR86 cluster compared to the total abundance. This supports the hypothesis that the more specific the taxonomic discrimination is, the greater variability in seasonal abundance is observed. However, the high variance in the *Roseobacter* and SAR86 cluster is driven by one or two relatively high peaks whilst throughout much of the year, particularly the winter, their abundance is consistently relatively low.

DISCUSSION

Methodological aspect

Within the last decade, new methods have been described for the study of microbial population dynamics in the marine picoplankton. Denaturing gradient gel electrophoresis (DGGE) has been an attractive approach to study bacterioplankton diversity because it allows a rapid qualitative analysis of the phylogenetic structure of bacterial communities. Its application revealed rather stable seasonal as well as pronounced temporal and spatial variations of bacterial communities in aquatic systems (e.g. Lebaron et al. 1999, Lindström 2000, Riemann & Winding 2001). However, because of biases introduced by PCR, including disproportionately frequent recovery of some sequences and the virtual loss of others (Suzuki & Giovannoni 1996, Cottrell & Kirchman 2000a), quantitative assessment of the contributions of particular groups of bacteria has been problematic (Schauer et al. 2003). Fluorescence *in situ* hybridization (FISH) (e.g. Wagner et al. 1994, Manz 1999) is the other method of choice for looking at bacterial composition and has the advantage of allowing a quantitative determination of the relative abundances of different phylogenetic groups of bacteria (e.g. Glockner et al. 1999, Pernthaler 2002a, 2004). It was also used to determine the factors controlling the bacterial community in freshwater systems (Liu & Leff 2002). In the present study between 85 and 100% of the *Bacteria* were identified by FISH as members of the SFB cluster and α- and γ-*Proteobacteria*, indicating that a substantial fraction of the bacterioplankton community could be phylogenetically characterised. However, compared to the DGGE method that allows identification of unknown populations by sequencing bands, the 16S database used to generate the FISH probes is not exhaustive and bacterioplankton of novel lineages not yet represented in the general databases remained unidentifiable.

Bacterioplankton community structure

Between 85 and 100% of the *Bacteria* were identified as members of the SFB group and α- and γ-*Proteobacteria* which appears to be characteristic for marine

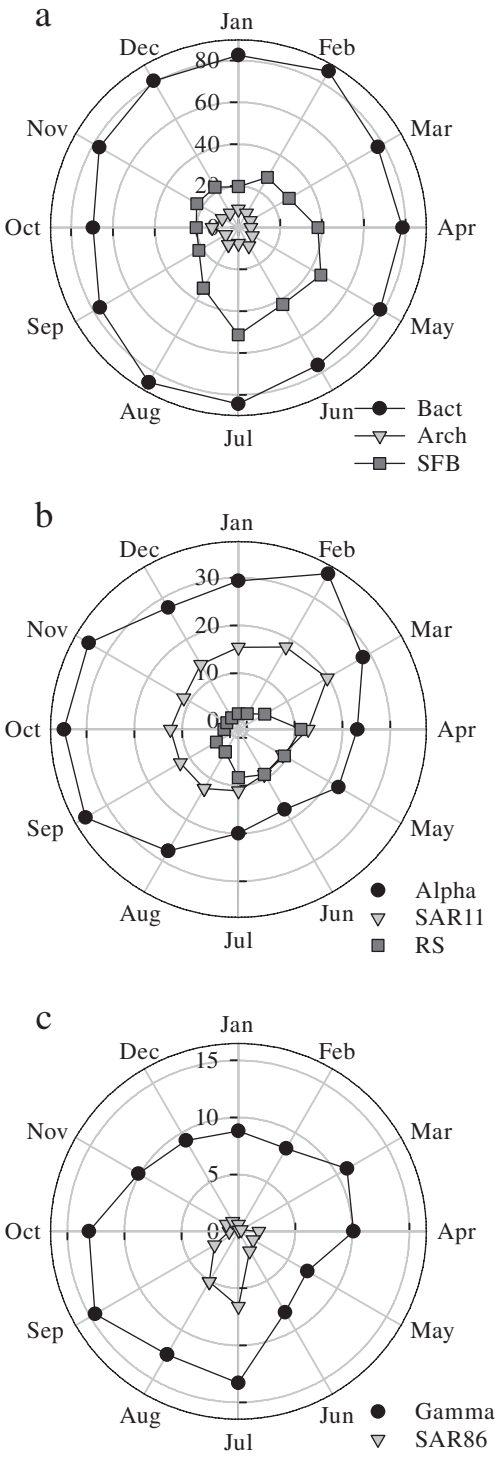

Fig. 2. Seasonal changes in bacterioplankton community structure (relative abundance, %) at L4 between August 2003 and August 2004 studied using TSA–FISH (Tyramide signal amplification–fluorerscent *in situ* hybridisation). (a) General class and group specific probes (Cren554, Eury806, CF319a, Table 1) compared to *Bacteria* (EUBI + EUBII + EUBIII, Table 1); (b) α-*Proteobacteria* at class, clade and genus level (Alf968, RSB67, SAR11-152R + SAR11-542R, Table 1); (c) γ-*Proteobacteria* at class and clade level (Gam42a, SAR86/1245, Table 1)

Table 3. Analysis of the variance between the bacterial groups and total bacterioplankton abundance using the *F*-test. Data have been normalized, so that mean = 0

	Variance	p
Total bacterioplankton	0.34	
Eubacteria	0.35	0.47
Archea	0.36	0.45
SFB	0.68	0.13
α-*Proteobacteria*	0.29	0.41
SAR11clade	0.31	0.44
Roseobacter	1.29	0.02
γ-*Proteobacteria*	0.34	0.49
SAR86 cluster	1.70	0.01

bacterioplankton in general (Glockner et al. 1999, Fuchs et al. 2000). These results are consistent with previous studies that showed that α-*Proteobacteria* and SFB were abundant throughout the year in coastal Mediterranean environments (Schauer et al. 2003), off the Oregon Coast (Suzuki et al. 1997), in Great South Bay, in the North Atlantic (Kelly & Chistoserdov 2001) and from several stations off the California Coast (Cottrell & Kirchman 2000b).

On an annual average, members of the SFB group reached their maximal abundances in spring and early summer (Table 2, Fig. 2a) when labile organic substrates such as amino acids, proteins and carbohydrates are produced in the course of phytoplankton blooms (Simon et al. 1998, Bunte & Simon 1999, Rosenstock & Simon 2001). SFB bacteria were identified as important members of bacterioplankton communities in many aquatic environments including lakes, rivers and marine systems (Pernthaler et al. 1998, Šimek et al. 1999). Their ecological success could be explained by their ability to degrade aerobically at relatively low temperatures (Reichenbach 1992) a large spectrum of organic compounds ranging from various proteins, carbohydrates, to complex macromolecules (Bernardet et al. 1996) abundant in coastal waters.

α-*Proteobacteria* were the second most abundant bacterial phylogenetic group constituting highest proportions of all bacterioplankton detected in late summer, autumn and winter (August to March). There is very limited information available on the occurrence of this subclass of *Proteobacteria*, making it difficult to explain their abundance. There is some evidence that limnic α-*Proteobacteria* comprise bacteria which prefer rather labile organic matter, although more studies need to be carried out to shed more light on the specific properties of members of this subclass of *Proteobacteria* (Zwisler et al. 2003). In marine systems, the relative abundance of α-*Proteobacteria* ranges between 1% in the North Sea and 14% in the Baltic Sea (Glockner et al. 1999).

To better understand the dynamic of this diverse group, more specific phylogenetic affiliations were carried out using probes specific for ecologically important lineages. α-*Proteobacteria* were dominated by 2 lineages: the SAR11 clade which made up to 69% of α-*Proteobacteria* and the *Roseobacter* genus which accounted for up to 11% (Table 2, Fig. 2b, c). The SAR11 clade was among the first groups of marine bacteria to be identified by cultivation-independent approaches and appears to dominate subtropical surface bacterioplankton communities (Morris et al. 2002, Rappé et al. 2002). Although the biogeochemical role of the SAR11 clade remains uncertain, this group has been considered to be among the most successful organisms on Earth (Morris et al. 2002). Recently, Malmstrom et al. (2004) showed that SAR11 bacteria could be responsible for about 50% of amino acid assimilation and 30% of dimethylsulphoniopropionate (DMSP) assimilation in surface waters of the North Atlantic Ocean, suggesting that they are highly active and play a significant role in C, N, and S cycling in the ocean.

The marine *Roseobacter* genus represents the second most abundant lineage of marine bacterioplankton after SAR11 (Allgaier et al. 2003, Selje et al. 2004). This group was found to be the second most abundant subgroup of α-*Proteobacteria* in coastal waters at L4, especially in summer when its abundance increased considerably. The *Roseobacter* genus, was also an abundant bacterioplankton component in the Celtic and North Seas (Eilers et al. 2001, Zubkov et al. 2001a, Wagner-Dobler et al. 2003).

While γ-*Proteobacteria* represented a lower proportion of the bacterioplankton throughout the year, we were also interested in understanding the dynamic of this group at a lower phylogenetic level. Giovannoni & Rappé (2000) hypothesized that the SAR86 clade members, ecologically successful heterotrophs in the surface ocean, are most likely utilizing phytoplankton-derived dissolved organic matter, and that their dominance may be the result of a competitive advantage in procuring limited inorganic nutrients. In the present study, SAR86 cluster was found to account for up to half of γ-*Proteobacteria* in summer. It was shown previously that this clade might be an important contributor to total bacterioplankton activity in coastal North Sea water during periods of low phytoplankton primary production (Pernthaler et al. 2002a) and played a significant role in the DMSP sulphur uptake (Vila et al. 2004).

Seasonal dynamics of the bacterioplankton community

A few studies have addressed temporal changes during a whole seasonal cycle in freshwater (Lindström 1998, Pernthaler et al. 1998) and marine (Murray et al. 1998, Pinhassi & Hagström 2000, Schauer et al. 2003) environments. Here, we found that community structure at higher taxonomic level did not change dramatically with season but lower level phylogenetic groups showed pronounced seasonal peaks (Table 2, Fig. 2). In a previous study, Schauer et al. (2003) also showed the overall stability in time of the taxonomic composition of the bacterioplankton in a Mediterranean coastal system, with gradual changes throughout the year revealing a substitution of closely related phylotypes during the seasonal cycle. The main factors affecting bacterial composition were the change in DOM supply mediated by different algal populations and the different temperature optima of bacterial populations. Similarly, seasonal shifts in bacterioplankton community composition in freshwater systems were related to shifts in the source and lability of DOM (Pernthaler et al. 1998, Crump et al. 2003). Mesocosm experiments have suggested that nutrients and grazing play a key role in shaping the composition as well as the activity of bacterial communities by regulating abundances (Lebaron et al. 2001, Schafer et al. 2001). Especially, very small and large bacteria seemed to be protected from nanoprotozoan grazing whereas active cells within the medium size-class are preferentially consumed (Epstein & Shiaris 1992).

CONCLUSIONS

In the present study, we have shown that bacterioplankton change their abundance seasonally at this coastal site, however, the bacterioplankton community structure at high phylogenetic level changed little (Fig. 2a). At a lower phylogenetic level however, a few bacterial groups showed distinct seasonal abundance patterns, with several groups increasing in relative abundance during particular seasons. What causes the stability in the bacterioplankton communities at a high phylogenetic level, while allowing changes at lower levels, remains to be explained.

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