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Seasonal changes in bacterioplankton nutrient limitation and their effects on bacterial community composition in the NW Mediterranean Sea

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ABSTRACT: Bacterioplankton growth is expected to depend on the availability of organic and inorganic nutrients. Still, no studies have investigated how the magnitude and type of nutrient limitation experienced by marine bacteria change on a temporal scale. We carried out a series of nutrient enrichment experiments to examine the variability in nutrient limitation of bacteria in the NW Mediterranean Sea, at monthly intervals, over an 18 mo period. Short-term enrichment bioassays (24 h incubation) showed that bacterial P limitation could occur throughout the year, but was most pronounced during spring and summer, coinciding with very low concentrations of dissolved inorganic phosphorus and chlorophyll *a*, and higher N:P ratios. During the non-stratified period in autumn and winter, bacteria were at times strongly C limited. Inorganic nitrogen limitation was not detected at any time. Long-term bioassays with and without enrichment, where growth was monitored until stationary phase using the seawater dilution culture approach, largely confirmed the results from the short-term bioassays. Analysis of the bacterial assemblages in these cultures, using denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene sequencing, suggested that the growth of some central components of the native bacterioplankton assemblage (i.e. specific *Roseobacter* and Flavobacteria phylotypes) was restricted due to the limited availability of P in spring and summer. We conclude that seasonal variability in the type and severity of nutrient limitation can substantially contribute to the regulation of bacterioplankton growth and community composition, and thereby affect the turnover of dissolved organic matter and inorganic nutrients in the sea.

KEY WORDS: Marine bacteria · Nutrients · Bioassays · Seasonal variability · Diversity

INTRODUCTION

Marine microbes experience seasonally changing growth conditions that eventually lead to differences in the structuring of community composition over seasonal scales. Changes in inorganic nutrient availability are of decisive importance for defining ocean primary productivity and for regulating phytoplankton community composition and succession. The concentrations of dissolved inorganic nutrients in the photic zone of the sea are mainly regulated through physical mixing with deep water, e.g. autumn and winter overturn or upwelling, leading to increased concentrations and biological consumption by microorganisms that leads to their depletion. The variation in nutrient regimes affects phytoplankton production, diversity and succession (Smayda & Reynolds 2001, 2003), but an understanding of how seasonal changes in nutrient

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availability affect bacterioplankton heterotrophic production and community composition is still lacking.

The primary limiting nutrient for bacterial growth differs between sea areas. It is generally assumed that growth of bacterioplankton in the Mediterranean Sea is limited by the availability of inorganic P (Zweifel et al. 1993, Thingstad et al. 1998, 2005, Zohary & Robarts 1998, Sala et al. 2002, Tanaka et al. 2004, Pitta et al. 2005), while, for example, the availability of organic C or inorganic P or Fe can limit the growth of bacteria in the subarctic Pacific, the Sargasso Sea or Antarctica (Kirchman 1990, Pakulski et al. 1996, Cotner et al. 1997, Rivkin & Anderson 1997). Considering the often large seasonal fluctuations in nutrient concentrations in the sea, variability in the type of bacterial nutrient limitation can be expected (Sala et al. 2002). Indeed, it has been speculated that inconsistent findings of C or P limitation in the Sargasso Sea may be explained by temporal variability (Cotner et al. 1997, Carlson et al. 2002, 2004). Studies in freshwater environments have shown substantial seasonal variability in bacterioplankton nutrient limitation in relation to nutrient availability, with bacterial growth responding primarily to P or combined P and C enrichments (Morris & Lewis 1992, Carlsson & Caron 2001). However, in the sea, little is known about the temporal variability in the kind and extent of nutrient limitation experienced by bacterioplankton and its consequences in terms of regulating bacterial growth.

Because heterotrophic bacteria are the major consumers of dissolved organic matter in the aquatic environment, limitation of bacterial growth by organic or inorganic nutrients can have important consequences in terms of biogeochemical C cycling. For example, modeling, experimental, and *in situ* results all show that labile DOC can accumulate when bacteria are limited by inorganic nutrients (Zweifel et al. 1995, Thingstad et al. 1997, 1999), which may occur for example in the P limited Baltic Sea in summer (Zweifel et al. 1995). In the Sargasso Sea, DOC also accumulates in summer (Carlson et al. 1994, 2002). In this sea, however, bacteria are limited by the availability of organic C in summer, and labile DOC is continuously taken up by bacteria while semilabile DOC resistant to rapid degradation by bacteria accumulates (Carlson et al. 2002, 2004). Thus, bacterial nutrient limitation may define whether DOC accumulated in surface waters is labile or not. This has important consequences in the long term for defining the quality of DOC that is exported to deep waters by convective processes (Murray et al. 1994) and in the short term for determining the ability of bacteria to take advantage of pulses of inorganic nutrients (Hagström et al. 2001).

Bacterioplankton community structure changes relatively fast in response to the growth and decay of different phytoplankton, indicating that dissolved organic matter from different algae select for different bacteria (Pinhassi et al. 2004, Abell & Bowman 2005, Grossart et al. 2005). Further, experiments with estuarine bacterioplankton show that different complex dissolved organic carbon sources selectively stimulate the growth of specific phylotypes (Covert & Moran 2001, Kisand & Wikner 2003). In North Sea and east Mediterranean seawater dilution cultures, enrichment with easily degradable carbon sources (e.g. glucose) favored the growth of *Vibrio* species (Eilers et al. 2000, Pinhassi & Berman 2003). Also, the growth of Gammaproteobacteria was preferentially stimulated by Fe enrichment in different seas where bacterial growth was limited by Fe availability (Hutchins et al. 2001). These studies focused mainly on the selective role of organic matter on the growth of specific bacteria, but also considered aspects of inorganic nutrient availability. Taken together, they suggest that changes in the availability of both organic and inorganic nutrients could be important for defining the composition of bacterial communities in the sea.

In the present study, we carried out a series of nutrient enrichment experiments to examine the extent of organic and/or inorganic nutrient limitation of marine bacterioplankton in a defined coastal marine area over an 18 mo period. To estimate nutrient limitation, we used 2 complementary bioassay approaches that are frequently used to investigate bacterial growth characteristics (see Pinhassi & Berman 2003 for details of methodologies). Short-term enrichment bioassays (24 h incubation) were used to estimate the immediate growth response of the bacteria. Long-term bioassays, whereby growth was monitored until stationary phase using the seawater dilution culture approach, allowed us to evaluate how differences in nutrient availability affected particular members of the bacterioplankton assemblage. It was hypothesized that differences in the composition of the bacterial assemblage developing in seawater cultures with and without nutrient enrichment would depend on the type and extent of *in situ* nutrient limitation.

MATERIALS AND METHODS

***In situ* sampling.** NW Mediterranean Sea water from 0.5 m depth was collected at monthly intervals between 28 January 2003 and 19 July 2004 (see Table 1), 1 km off the coast in the Bay of Blanes (The Blanes Bay Microbial Observatory, 41° 40′ N, 2° 48′ E, 60 km north of Barcelona, Spain). Water was filtered through a 200 µm mesh net, collected in a 25 l polycarbonate bottle (Nalgene), and brought to the laboratory for processing within 3 h. For convenience, the sampling on 4 March 2003 is referred to as 'February

2003', to avoid confusion with the sampling on 25 March 2003 (i.e. 'March 2003').

Chlorophyll *a* (chl *a*). Chl *a* was measured fluorometrically, from 200 ml samples filtered onto GF/F filters (Whatman). The filters were ground in 90% acetone and left in the dark at room temperature for at least 2 h. The fluorescence of the extract was measured with a Turner Designs fluorometer.

Chemical analyses. For analyses of dissolved nutrient concentrations, seawater samples were filtered through 0.2 µm pore size, 47 mm diameter, polycarbonate filters (Supor-200; Gelman Sciences) using a polycarbonate filtration device (Millipore). All utensils in contact with the samples (filters, filter holder, tubes) were acid rinsed with 1 M HCl and extensively washed with MilliQ water prior to use.

Total dissolved nitrogen and phosphorus (TDN and TDP) were determined following the wet oxidation of additional filtered samples in alkaline and acidic persulfate, respectively, and the subsequent analysis of dissolved nitrate and phosphate (Grasshoff et al. 1983).

Dissolved inorganic nitrogen (DIN; NO_3^- + NO_2^- + NH_4^+) and dissolved inorganic phosphorus (DIP; PO_4^{3-}) were measured spectrophotometrically with an Alliance Evolution II autoanalyzer following standard procedures (Grasshoff et al. 1983). Phosphate concentrations were also determined manually, using a 10 cm cuvette to increase the detection limit.

For dissolved organic carbon (DOC), 20 ml samples were acidified with 16 mM HCl (final concentration) in polypropylene tubes, and stored at 4°C until analysis. DOC was measured with a high temperature carbon analyzer (Shimadzu TOC 5000) at the intercalibration facilities at Umeå Marine Research Station (UMF), Sweden.

Short-term nutrient limitation bioassays. The effect of nutrient additions on the growth of heterotrophic bacteria was examined in short-term bioassays. Seawater was transferred to acid-rinsed 250 ml polycarbonate bottles (Nalgene), and thoroughly rinsed with MilliQ-water and sample water. Nutrients were added to final concentrations of 24 µM C (glucose), 2 µM N (NH_4Cl), and 0.6 µM P (Na_2HPO_4), singly and in all different combinations in duplicate. Control bottles received no nutrients. After dark incubation for 24 h at *in situ* temperature, bacterial production and abundance were determined. In general, leucine incorporation rates in the unamended controls had increased 20 to 90% after 24 h incubation, compared to the rates measured upon sampling *in situ* (data not shown). Since the bacterial growth response determined by leucine incorporation was up to 10 times higher in the enriched treatments compared to the controls, while only minor changes (<50%) were observed in bacterial abundance, we focused the subsequent analyses on the leucine incorporation data. Such delays in increase in bacterial abundance compared to leucine incorporation are most likely to be due to the longer time needed for biosynthesis before cell division compared to nutrient or tracer uptake.

Long-term nutrient limitation bioassays. For each seawater culture, approximately 1900 ml of sampled water to be used as growth medium was filtered through a 0.2 µm pore-size Sterivex filter capsule (Millipore) using a peristaltic pump. A total of 100 ml inoculum was added to each culture to give a 20-fold dilution of bacterial abundance. The inoculum was prepared by gravity filtration of sampled water through a 47 mm diameter 0.8 µm pore size polycarbonate filter (Nuclepore). These filtration procedures resulted in cultures that were essentially free from heterotrophic flagellates grazing on bacteria, at least within the time frame of the experiments. The bioassays were done in 2 l polycarbonate bottles (Nalgene). All utensils in contact with the samples were acid rinsed with 1 M HCl and extensively washed with MilliQ-water prior to use. Duplicate nutrient enriched cultures received a final concentration of 2.0 µM N (NH_4Cl) and 0.6 µM P (Na_2HPO_4). Duplicate control cultures received no addition of nutrients. Seawater cultures were maintained at *in situ* temperatures in the dark for 68 to 85 h (45 to 55 h in the June to August 2003 experiments), after which samples for community DNA were collected. Near stationary phase bacterial abundance was reached after approximately 24 h (May to September 2003) or after 48 to 55 h in the remaining experiments.

Bacterial abundance and production. Samples for enumeration of bacteria by flow cytometry were preserved with 1% paraformaldehyde and 0.05% glutaraldehyde (final concentrations), and stored frozen at −70°C. Cell counts were performed with a FACSCalibur flow cytometer after staining with Syto13 (Gasol & del Giorgio 2000).

Bacterial production was measured using the [^{3}H]-leucine incorporation method (Kirchman et al. 1985), as subsequently modified by Smith & Azam (1992). For each sample, triplicate aliquots (1.2 ml) and a trichloroacetic acid killed control were incubated with 40 nM [^{3}H]-leucine (final concentration) for 1 to 1.5 h, at *in situ* temperature in darkness. Previous studies in the studied sea area have shown that leucine incorporation rates are saturated at concentrations of 20 to 40 nM (Gasol et al. 1998, Pedrós-Alió et al. 1999). Thus, even at the highest incorporation rates found in the present study (approximately 11 nmol leu l^{-1} h^{-1}), the leucine uptake should have been saturated; even if this were not the case, our measurements should represent a slight underestimation, and therefore a conservative estimate, of leucine incorporation rates in the P

enriched bottles. The variability in leucine incorporation rates between replicate bottles using the short-term nutrient limitation bioassay approach is frequently <10%.

Collection of microbial community DNA. A 1 l aliquot of the long-term bioassays was filtered through a 0.2 µm pore-size polycarbonate filter at <200 mm Hg to collect bacterial cells. Filters were immediately frozen at –70°C in sucrose buffer until further processing; community DNA was obtained using a standard phenol-extraction protocol (Schauer et al. 2003).

Denaturing gradient gel electrophoresis (DGGE). DGGE and gel analysis were performed as previously described (Schauer et al. 2003). Briefly, 16S rRNA gene fragments (around 550 bp in length) were amplified by PCR, using the universal primer 907rm complimentary to positions 927 to 907 (5′-CCGTCAATTCA/CTTTGAGTTT) and the bacterial specific primer 358f complementary to positions 341 to 358, with a GC clamp (underlined) (5′-CGCCCGCCGCGCGCG-GCGGGCGGGGCGGGGG-CACGGGGGGCCTACG GGAGGCAGCAG). 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× TAE (40 mM Tris [pH = 7.4], 20 mM sodium acetate, 1 mM EDTA) running buffer. DGGE analysis of samples from the duplicate N+P and control cultures from each experiment consistently yielded identical number of bands and band intensities of the duplicates (e.g. see Fig. 4). DGGE bands were excised, reamplified, and verified by a second DGGE. Bands were sequenced using Primer 358f without the GC-clamp, with the BigDye terminator cycle-sequencing kit (Perkin Elmer) and an ABI PRISM Model 377 (v3.3) automated sequencer. Our 16S rRNA gene sequences were compared to sequences in GenBank using BLAST. (For GenBank Accession Nos. of sequences determined in the present study see Table 2).

RESULTS

Chlorophyll *a* and nutrients

The concentrations of chl *a* were between 1.0 and 2.0 µg l^{-1} from January to March 2003, and thereafter fell to values below 0.5 µg l^{-1} from April to October (Fig. 1A). Following a period of stormy weather at the beginning of October, chl *a* increased to a peak at 4.0 µg l^{-1} in December, and settled to around 1.2 µg l^{-1} from January to April 2004. Chl *a* decreased again to low summer values (<0.5 µg l^{-1}) from May onwards.

Dissolved inorganic nitrogen (DIN) concentrations from January to April 2003 were between 1.5 and 4.2 µM, and thereafter fell below 0.9 µM until September (Fig. 1B). In October 2003 a pronounced peak at 5.7 µM was observed after the storm. Thereafter, concentrations were mostly above 1.7 µM. Concentrations of dissolved inorganic PO_4 (DIP) were mostly below 0.05 µM from March to November 2003. However, a peak at 0.22 µM P was reached in October (Fig. 1B). Values remained between 0.07 and 0.15 µM during winter and spring, and fell below 0.05 µM from May 2004 onwards. There was a strong positive correlation between DIN and DIP (DIN = 27.4 DIP + 0.19, $p < 0.01$, $R^2 = 0.81$), possibly indicating the importance of nutrient input through water mixing with nutrient rich deep water from the coastal shelf moving through the nearby Blanes canyon.

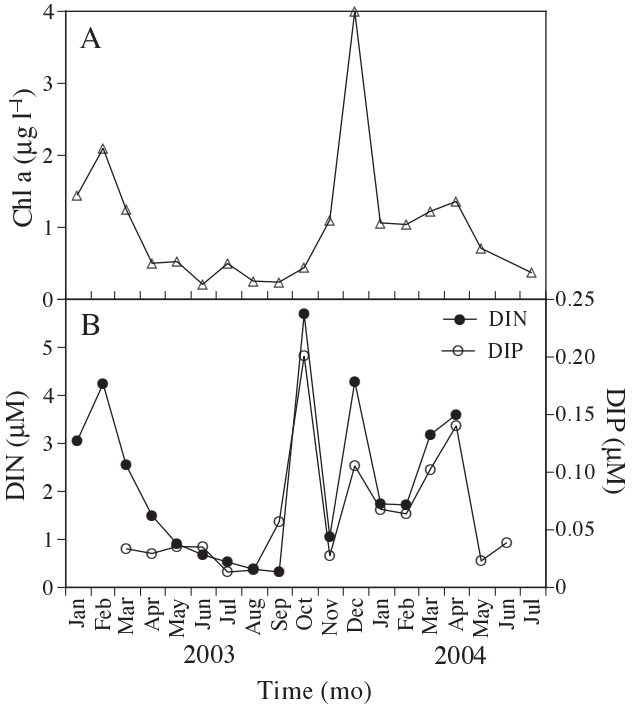

Fig. 1. Seasonal dynamics of (A) chl *a*, and (B) dissolved inorganic nitrogen (DIN) and phosphorus (DIP) concentrations in the NW Mediterranean Sea

The concentration of total dissolved nitrogen (TDN) varied between 7 and 12 µM during the sampling period, while total dissolved phosphorus (TDP) was slightly lower in spring and summer compared to winter (Table 1). The TDN:TDP ratio was between 102 and 207 during spring and summer, and below 100 during late autumn and winter.

During the investigated period the concentrations of DOC ranged from 83 to 177 µM. During winter 2003 concentrations were around 170 µM, and decreased to values between 85 to 105 µM from May to August (Table 1). A second peak was reached in September 2003 at 117 µM, after which concentrations declined to a minimum of 83 µM in February 2004.

Table 1. Environmental variables and indicators of bacterial nutrient limitation in Bay of Blanes from 28 January 2003 to 19 July 2004. nd: not determined. TDP: concentrations in boldface represent values <0.10 µM; TDN:TDP: ratios in boldface represent values >100; C + P: –, +, ++ indicate stimulation <20, 20–100 and >100%, respectively, compared to treatment with P only; C, P, N + P: –, +, ++ indicate stimulation <20, 20–100% and >100%, respectively, compared to unamended controls

Expt	Date	Temp (°C)	DOC (µM, mean±SD)	TDN (µM)	TDP (µM)	TDN:TDP	Growth response in bioassays			
							Short-term			Long-term
							C + P	C	P	N + P
Jan 03	28 Jan	14.0	nd	nd	nd	nd	–	–	+	–
Feb 03	4 Mar	11.0	176.9 ± 4.4	nd	nd	nd	+	+	–	–
Mar 03	25 Mar	13.0	164.6 ± 2.2	9.42	**0.07**	**135**	++	–	++	+
Apr 03	22 Apr	14.5	112.5 ± 1.2	9.26	**0.07**	**132**	++	–	++	++
May 03	13 May	17.0	92.9 ± 0.6	7.79	**0.06**	**130**	++	–	++	++
Jun 03	25 Jun	25.0	102.5 ± 10.6	10.33	**0.05**	**207**	–	–	++	++
Jul 03	14 Jul	25.0	85.0 ± 7.1	8.05	**0.05**	**161**	++	–	++	++
Aug 03	4 Aug	25.2	88.1 ± 3.6	7.41	**0.04**	**185**	++	–	++	++
Sep 03	16 Sep	23.0	117.1 ± 4.1	9.46	**0.07**	**135**	–	–	++	+
Oct 03	21 Oct	18.0	112.9 ± 0.6	11.68	0.17	69	++	++	–	–
Nov 03	25 Nov	16.0	91.7 ± 1.2	7.14	**0.08**	89	++	–	+	++
Dec 03	16 Dec	14.5	102.1 ± 5.3	12.08	0.13	93	–	–	+	–
Jan 04	26 Jan	14.0	103.5 ± 6.2	9.50	0.12	79	++	+	–	–
Feb 04	23 Feb	12.9	82.8 ± 4.3	8.61	0.10	86	+	+	–	–
Mar 04	22 Mar	12.9	98.8 ± 4.1	11.50	0.17	68	+	–	++	(++)[a]
Apr 04	19 Apr	12.6	116.3 ± 1.8	11.30	**0.09**	**126**	+	+	–	nd
May 04	25 May	17.0	122.5 ± 2.4	10.82	**0.08**	**135**	+	–	++	nd
Jun 04	28 Jun	21.1	113.3 ± 0.1	11.21	0.11	**102**	+	–	+	nd
Jul 04	19 Jul	24.0	126.3 ± 4.1	nd	nd	nd	++	–	++	nd

[a]Data referring to strong stimulation of leucine incorporation (>100% compared to control)

Short-term nutrient limitation bioassays

These bioassays were done to determine the bacterioplankton growth response to nutrient enrichment in 24 h incubations with water filtered through 200 µm mesh (i.e. in the presence of flagellate grazing). Leucine incorporation in the controls without nutrient addition remained well below 1 nmol l^{-1} h^{-1} throughout the sampling period (average ± SD: 0.39 ± 0.39, n = 19, Fig. 2). Leucine incorporation rates in the treatments that received additions of only N did not differ significantly from those in the control.

Leucine incorporation rates in the treatment with only C increased 20 to 50% compared to the control in 4 out of 19 experiments (February 2003, January, February and April 2004), i.e. primarily during winter, Fig. 2 & Table 1). In a fifth experiment (October 2003), the addition of only C resulted in a 5-fold stimulation compared to the control, reaching 6 nmol l^{-1} h^{-1}, which was the highest value recorded for any single nutrient addition during the study (Fig. 2).

The addition of only P stimulated bacterial production on 14 of the 19 sampling occasions (Fig. 2). Very little or no stimulation by P addition (<50% compared to the control) was observed from January to April 2003, and from October 2003 to April 2004. However, from May to September 2003, and from May 2004 onwards, bacterial activity was strongly stimulated by P addition (i.e. >100% increase compared to unamended controls; Table 1). During these summer periods leucine incorporation rates in the P treatments generally ranged from 1.3 to 2.4 nmol leu l^{-1} h^{-1}, with a pronounced peak at 5.9 nmol leu l^{-1} h^{-1} in September 2003 (Fig. 2).

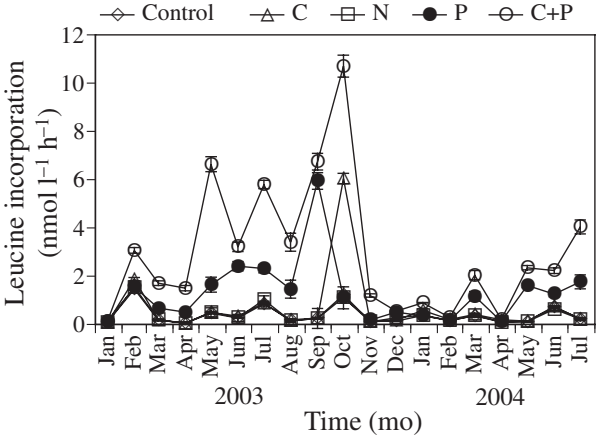

Fig. 2. Nutrient limitation of bacterioplankton during sampling period. Stimulation of bacterial production after addition of glucose (C), ammonium (N) and phosphate (P), and after combined additions of C and P (C + P), compared to control. Leucine incorporation rates were measured 24 h after addition of nutrients. Error bars represent standard deviations for pooled measurements of triplicate subsamples from each duplicate treatment (n = 6)

Combined additions of glucose (C) and P caused nearly a doubling or more of leucine incorporation compared to any of the single nutrient additions on 15 of the 19 sampling occasions (exceptions being January, June, September, and December 2003, Fig. 2). Leucine incorporation rates in the combined C + P treatments ranged from 0.1 to 3.1 nmol leu l^{-1} h^{-1} during winter (November to April), and from 2.2 to 10.7 nmol leu l^{-1} h^{-1} during summer (May to October). Combined additions of C + P in October 2003 caused the largest stimulation of leucine incorporation observed during the study (10.7 nmol leu l^{-1} h^{-1}).

Long-term nutrient limitation bioassays

The bacterial response to nutrient enrichment in 24 h experiments could possibly be suppressed during periods of lower temperatures, potentially underestimating nutrient limitation. To reduce this problem, and to avoid potentially confounding impacts of flagellate grazing on bacteria, the growth of bacteria was also monitored in seawater dilution cultures with and without N + P enrichment to evaluate the bacterioplankton growth potential over several days (until stationary phase). The yield of bacteria in the seawater cultures varied substantially during the sampling period, ranging from 0.2 to 5.0 × 10^6 cells ml^{-1} (Fig. 3A), although most yields were within 0.5 to 1.5 × 10^6 cells ml^{-1}, which was within the range of bacterial abundances *in situ* (L. Alonso-Sáez et al. unpubl.). Bacterial abundance in the enriched and control seawater cultures reached similar levels (<15% difference) on 6 sampling occasions, coinciding with the times when no or only slight P limitation was detected in the short-term bioassays (i.e. during winter, Table 1 & Fig. 3A). On 9 of the sampling occasions bacterial growth was significantly (20 to 250%) higher in the N + P cultures compared to the controls, most notably so during summer when strong P limitation was also found in the short-term bioassays (i.e. April to September 2003, Table 1). Bacterial growth rates in the unamended controls and the N + P enriched cultures ranged from 1.1 to 4.7 and 1.7 to 7.2 d^{-1}, respectively (Fig. 3B). In general, the largest differences between treatments were found at times when differences were also observed in bacterial yields (i.e. May to September and November 2003).

Bacterioplankton composition in the long-term bioassays

The seawater cultures allowed monitoring of changes in the composition of bacterioplankton assemblages in the enriched seawater cultures as compared to the controls; that is, to test whether differences in growth yields were associated with changes in the composition of the bacterial assemblage. Furthermore, identification of specific bacterial phylotypes would indicate whether the observed growth responses were due only to fast-growing bacterial 'weeds' or whether bacteria representative of the native bacterial assemblage were present among those dominating the seawater cultures.

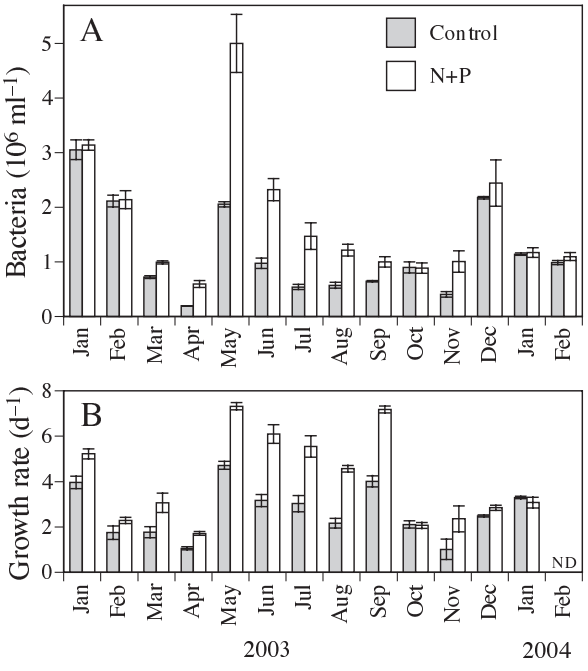

Fig. 3. (A) Bacterial yields and (B) growth rates in seawater cultures enriched with N + P compared to unenriched controls. Yields were determined after entry into stationary phase. Error bars denote standard deviations for duplicate seawater cultures. ND: not determined

Fig. 4 shows the denaturing gradient gel electrophoresis (DGGE) 'fingerprints' of the bacterial assemblages in the duplicate N + P cultures compared to the unamended controls and to the initial sample from July 2003, which was the experiment in which differences in bacterioplankton composition between treatments were most pronounced. From January to May 2003 and from October 2003 to March 2004 (10 experiments) similar pair-wise comparisons resulted in bacterial assemblages in the N + P cultures and controls that were identical, or with only slight differences in band intensity of shared bands (data not shown). However, from June to September 2003 a number of phylotypes thrived specifically in the N + P cultures, in addition to the phylotypes found in both treatments (Fig. 5). Phylotypes specific to the N + P cultures during summer included 3 Bacteroidetes (B218, B221 and B219) and 3 Alphaproteobacteria phylotypes (B243,

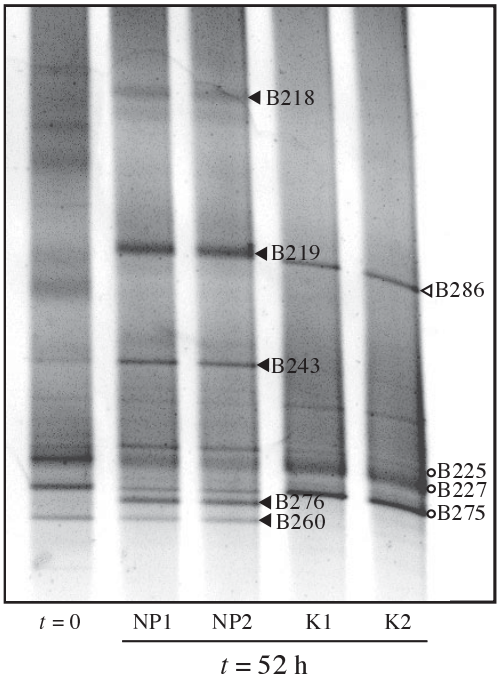

Fig. 4. DGGE fingerprints of bacterial assemblage in duplicate N + P cultures (NP1, NP2) compared to unamended controls (K1, K2) and initial sample (t = 0) from July 2003, determined by DGGE of PCR-amplified partial 16S rRNA genes. Excized and sequenced bands are indicated by their codes. ○: phylotypes present in both enriched and control cultures; black arrowheads: bands present in enriched cultures only; open arrowhead: phylotype found in control culture only. Identities of sequenced bands in Table 2

B276 and B260) (Table 2). In addition, 1 phylotype (B286; Bacteroidetes) was found only in the controls in June and July 2003.

The composition of the bacterioplankton assemblage in the N + P enriched seawater cultures changed substantially during the sampling period (Fig. 5). Flavobacteria phylotype B218 (*Tenacibaculum* sp.) was found in low relative abundance during most of the sampling period, but became the single most abundant phylotype during November and December 2003 both in the control and N + P treatments, coinciding with the rapid increase in nutrients and chl *a* following the storm in October (Fig. 1). In summer 2003, phylotype B218 was restricted to the N + P cultures where it occurred together with *Bacteroidetes* phylotype B219 (*Microscilla* cluster, cf. Kirchman et al. 2003). Another *Bacteroidetes* phylotype, B286 (novel *Bacteroidetes* lineage), became abundant in the control cultures in June and July 2003. Flavobacteria phylotype B240 (*Polaribacter* sp.) was the most abundant phylotype in the cultures from March to May 2003 and was also abundant from January to March 2004, but was not detected between the spring periods (i.e. from June to December 2003).

The *Roseobacter* phylotype B243 was found in the seawater cultures only during spring and early summer; it occured in both the N + P cultures and controls from March to May, but thereafter was found only in the N + P cultures in June and July 2003. A total of 4 identified *Roseobacter* phylotypes occurred mainly

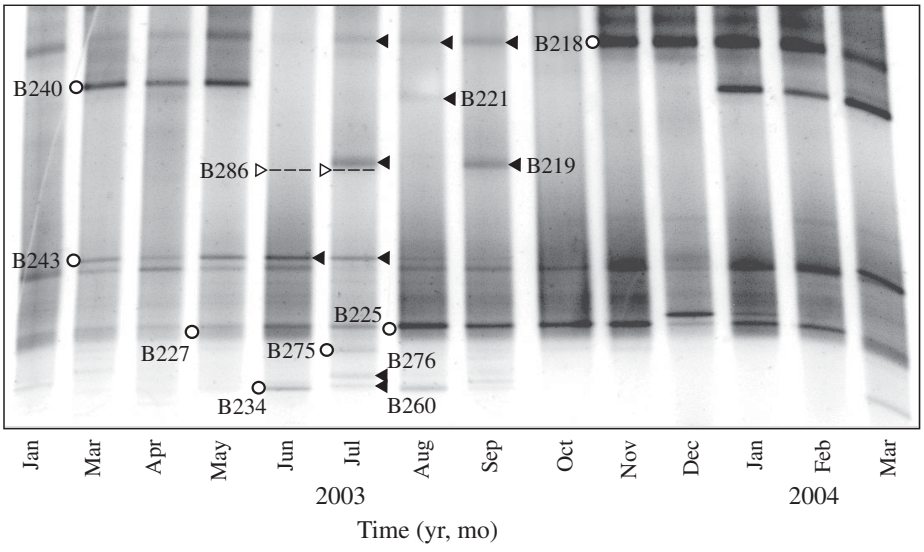

Fig. 5. DGGE fingerprints of bacterial assemblages in N + P enriched seawater culture experiments visualized by DGGE of PCR-amplified partial 16S rRNA genes. Samples for community DNA were collected after 2 to 4 d incubation. ○ and unlabelled bands (since the latter were not sequenced): phylotypes present in both enriched and control cultures; black arrowheads: bands present in enriched cultures only. Open arrowheads: position of phylotype B286, which was found in control cultures only (e.g. see Fig. 4) and not in samples shown here. The stippled bands of this phylotype have been drawn on top of the image. No sample is available for February 2003

Table 2. Phylogenetic affiliation of 16S rRNA gene sequences from excised DGGE bands obtained during long-term bioassays. For each phylotype, closest relative in GenBank and closest cultured relative are shown, together with their accession numbers and sequence similarity. Uncult.: uncultured; CFB: *Cytophaga–Flavobacterium–Bacteroides* or Bacteroidetes phylum; Alpha: Alphaproteobacteria; Gamma: Gammaproteobacteria

Phylotype	Accession number	Relatives in GenBank; Accession no.	Similarity (%)	Family	Taxon
B218	DQ473559	Uncult. marine bacterium BY-65, AJ298376	100.0		
		Tenacibaculum mesophilum, AB032504	98.1	Flavobacteriaceae	CFB
B219	DQ473560	Uncult. Bacteroidetes Clone CF96, AY274863	97.0		
		Owenweeksia hongkongensis, AB125062	91.2	Cryomorphaceae	CFB
B221	DQ473561	Uncult. Bacteroidetes DGGE Band S-1, AY573520	100.0		
		Cellulophaga fucicola, AJ005973	92.4	Flavobacteriaceae	CFB
B225[a]		Uncult. Alphaproteobacterium, Clone PI_4a9f, AY580451	>97		
		Roseovarius mucosus, AJ534215	>96	Rhodobacteraceae	Alpha
B227	DQ473562	Uncult. *Glaciecola* sp. Clone F2C105, AY794109	99.0		
		Alteromonas addita, AY682202	96.5	Alteromonadaceae	Gamma
B234	DQ473563	Uncult. bacterium Clone ELB19-099, DQ015817	97.9		
		Loktanella koreensis Strain GA2-M3, DQ344498	97.5	Rhodobacteraceae	Alpha
B240	DQ473564	Uncult. Bacteroidetes Clone CF10, AY274847	100		
		Polaribacter dokdonensis Strain DSW-5, DQ004686	97.2	Flavobacteriaceae	CFB
B243	DQ473565	Alphaproteobacterium 9IX/A01/152, AY612764	100.0		
		Nereida ignava, AJ748748	99.6	Rhodobacteraceae	Alpha
B260	DQ473566	Uncult. *Roseobacter* DGGE Band ST-13, AY573530	100.0		
		Phaeobacter inhibens, AY177712	97.7	Rhodobacteraceae	Alpha
B275	DQ473567	Uncult. Rhodobacteraceae Clone DS023, DQ234107	100.0		
		Roseobacter algocolus, X78315	97.5	Rhodobacteraceae	Alpha
B276[a]		Uncult. *Roseobacter* DGGE Band ST-13, AY573530	>96		
		R. algocolus (ATCC 51440 T-FF3), X78315	>95	Rhodobacteraceae	Alpha
B286	DQ473568	Uncult. marine bacterium BY-71, AJ298380	91.7		
		Owenweeksia hongkongensis, AB125062	89.9	Cryomorphaceae	CFB

[a]Sequence of low quality, not submitted to GenBank

from June to September, of which 2 (phylotypes B260 and B276) were restricted to the N+P cultures. *Roseobacter* phylotype B225 and *Alteromonas* phylotype B227 were detected in both treatments throughout the sampling period. Phylotype B225 achieved a particularly high relative abundance in the seawater cultures from August to November 2003.

DISCUSSION

Chl *a* concentrations in Blanes Bay followed the seasonal dynamics regularly observed in the coastal NW Mediterranean Sea, with low values during summer and maximum values in winter (Duarte et al. 1998, Schauer et al. 2003). Phytoplankton growth in the Mediterranean Sea is primarily P limited (Berland et al. 1980, Krom et al. 1991); thus, the low concentrations of chl *a* during summer are consistent with the low concentrations of DIP observed during this period. The elevated concentrations of chl *a* during late autumn and winter are consistent with increased availability of P and other mineral nutrients at these times. As a consequence of the seasonal changes in growth conditions there is a typical seasonal succession of phytoplankton in the NW Mediterranean Sea, from a winter bloom dominated by diatoms, followed by *Synechococcus* spp. in summer and a minor autumn bloom of *Prochlorococcus* spp. (Schauer et al. 2003). Considering the strong effect of nutrients on phytoplankton growth and community composition in the NW Mediterranean Sea and elsewhere (Agawin et al. 2000, Duarte et al. 2000, Olsen 2006), we investigated the extent to which bacterioplankton was subjected to similar forcing by nutrient availability.

Both short- and long-term nutrient enrichment experiments showed that bacterial growth was strongly limited by the availability of P during the period following the winter phytoplankton bloom until the breakdown of water column stratification in autumn (Table 1). Although P limitation could occur all through the year, its severity was most pronounced during spring and summer, coinciding with (for example) very low concentrations of DIP and chl *a* and higher N:P ratios. These findings are in agreement with previous data showing that bacterial growth in the Mediterranean Sea is strongly P limited in summer (Zweifel et al. 1993, Thingstad et al. 1998, Sala et al. 2002, Tanaka et al. 2004). However, Thingstad et al. (1998) suspected that P limitation would primarily be

restricted to the summer period, while during other seasons mixing of surface and deep water would alleviate P limitation and possibly lead to C limitation. Similarly, Zohary & Robarts (1998) suggested that P limitation in the eastern Mediterranean Sea may be less pronounced during periods when water column stratification is weakened. Indeed, subsequent studies indicated that the microbial turnover of P in the NW Mediterranean is significantly slower during non-summer seasons, suggesting that P limitation may be less pronounced at these times (Tanaka et al. 2003, 2004). No incidence of inorganic N limitation was detected during our study, and observations of inorganic N limitation of bacterial growth in marine surface waters seem to be relatively uncommon (but see Sala et al. 2002). In the present study, C limitation of bacterial growth was observed on several occasions from October to April, thus providing experimental evidence for the hypothesis of Thingstad et al. (1998). It is thus concluded that bacteria in the NW Mediterranean are strongly P limited during stratification periods (i.e. summer), while during periods of mixing, bacteria experience shifts in the primary limiting nutrient (P versus C) and in the severity of limitation. Considering that, for example, DOC sequestration is significantly enhanced when bacteria are C limited (Carlson et al. 2002) while accumulation of labile DOC may take place when bacterial growth is limited by inorganic nutrients (Zweifel et al. 1995), our findings suggest that frequent shifts in the type of nutrient limitation could have large consequences for biogeochemical processes driven by bacterioplankton.

On two occasions the single addition of nutrients evoked particularly large responses in leucine incorporation in the short-term bioassays. These occurred in September and October 2003, when very strong responses to enrichment with P and C were observed, respectively. In both experiments, the stimulation of leucine incorporation was as large (approximately 6 nmol leu l^{-1} h^{-1}) as that induced by combined additions of C + P during the summer months (when P was the primary limiting nutrient). Incidentally, DOC concentrations in the Bay of Blanes had increased by nearly 30 µM in September compared to the previous months (Table 1). Thus, it is possible that the strong response (as strong as the response in the combined C + P treatment), was due to a recent buildup of the labile organic carbon pool (i.e. the P enriched treatment essentially became a natural C + P treatment). Conversely, the large stimulation of bacterial leucine incorporation by the addition of C in October coincided with an *in situ* peak in inorganic PO_4 concentration of 0.22 µM. This peak followed a period of stormy weather, which could have caused increased P availability through mixing with nutrient rich deep water from off the coast. These 2 'extreme' situations, separated by only 1 mo, are evidence of how rapidly and with what magnitude bacterioplankton nutrient limitation can potentially change in any specific marine area. We suspect that given a higher frequency of sampling, such changes could even be detected within days to weeks.

Knowledge of microbial nutrient limitation in different ocean provinces derives from experiments ranging from the kilometer scale of *in situ* enrichments (Martin et al. 1994, Boyd et al. 2000, Krom et al. 2005) down to the centimeter scale in bottle incubations (Kirchman 1990, Zweifel et al. 1993). Irrespective of the experimental scale, detection of limitation primarily relies on observation of changes in microbial production and abundance (of phyto- or bacterioplankton, or both). It is reasonable to assume that experimental scale as well as bioassay approach (e.g. the use of filtered or unfiltered samples) will affect the details of how microbes respond. Nevertheless, the general conclusions derived from different experimental approaches are likely to be similar. For heterotrophic bacteria, short-term bioassays (typically 24 h) with unfiltered water provide straightforward indications of nutrient limitation (Thingstad et al. 1998), but relatively limited additional information. Long-term bioassays, such as seawater dilution cultures, on the other hand, provide not only direct estimates of limitation and substrate utilization, but also indications of growth kinetics and diversity of bacteria responding to the enrichment. In particular, this approach has been rewarding in yielding information on the preferences for growth of diverse representatives of Alpha- and Gammaproteobacteria and Bacteroidetes (Eilers et al. 2000, Covert & Moran 2001, Kisand & Wikner 2003, Pinhassi & Berman 2003).

Two findings concerning the changes in composition of the bacterioplankton assemblages in our seawater cultures were most conspicuous. First, the composition in the control and enriched cultures were similar at those times when little or no P limitation was detected (i.e. during non-summer seasons). However, when bacterioplankton growth was strongly P limited (i.e. June to September), large differences between the enriched and the unenriched treatments were found (with the exception of the experiments in May and November 2003). In the experiments during summer, 6 phylotypes reached a high relative abundance exclusively in the N + P enriched seawater cultures. Since there were no indications that N ever limited the growth of bacteria in the Bay of Blanes, the growth response of these bacteria was attributed to the increased availability of P. The responses of *Roseobacter* B243 and *Tenacibaculum* B218 were particularly interesting. *Roseobacter* B243 was abundant both in the enriched cultures and the controls, from March to

May. In June and July, however, when P limitation was most pronounced, this phylotype was encountered only in the enriched cultures. Similarly, *Tenacibaculum* B218 was found in both enriched and control cultures during non-summer seasons, but in July and September in the enriched cultures only. Both these phylotypes have been previously detected *in situ*, in spring, before the onset of summer stratification, by culture-independent techniques (see below). The differences in composition of the bacterial assemblages in the seawater cultures during summer (i.e. during times of strong P limitation) imply that during this period the availability of P has the potential to directly affect bacterioplankton composition.

Second, the composition in both the control and enriched cultures changed substantially during the sampling period, with pronounced changes between seasons. Seasonal changes in bacterioplankton composition are a common feature in temperate seas (Pinhassi & Hagström 2000, Schauer et al. 2003, Ghiglione et al. 2005, Morris et al. 2005). At the same study site as in the present study, Schauer et al. (2003) showed that bacterioplankton composition was relatively stable with respect to the broad phylogenetic groups present (e.g. Alphaproteobacteria and Bacteroidetes), but that changes occurred in the abundance of specific phylotypes within these groups over a 1 yr period. Specifically, shifts in bacterioplankton composition were observed from winter to spring and from spring to summer (Schauer et al. 2003). Several sequences from the seawater culture DGGE analyses were identical to DGGE phylotypes or 16S rRNA gene clone library sequences obtained from *in situ* samples from the Bay of Blanes, suggesting that their occurrence in the cultures was related to their occurrence *in situ*. For example, *Tenacibaculum* phylotype B218 (Flavobacteriaceae), which was dominant in the seawater cultures during the phytoplankton bloom period November 2003 to March 2004, was identical to *in situ* DGGE phylotype BL98-5. BL98-5 was one of the dominant phylotypes during the phytoplankton bloom in the Bay of Blanes from February to April 1998 (Schauer et al. 2003). *Roseobacter* phylotype B243, which was found to be an important member of the N + P seawater cultures from March to July 2003, was identical to 16S rRNA gene Clone SPR23 found in a clone library from May 2003 (L. Alonso-Sáez et al. unpubl.). *Roseobacter* phylotype B275, which was found in the seawater cultures only during summer, was identical to phylotype BL98-35. BL98-35 was an important member of the bacterioplankton assemblage in Bay of Blanes in summer 1998 (Schauer et al. 2003), and it was nearly identical to 16S rRNA gene Clone SUM30 found in August 2003 (L. Alonso-Sáez et al. unpubl.). Furthermore, some bands observed to develop in the seawater cultures corresponded to bands present also in the *in situ* samples (e.g. bands B243, B275 and B260 in July 2003, Fig. 4). Members of the SAR11 clade are an integral component of Mediterranean Sea microbial communities (Zaballos et al. 2006), yet were not detected in our seawater dilution cultures. This was probably due to the slow growth rates of SAR11 bacteria (approximately 0.4 to 0.6 d^{-1}; Rappé et al. 2002) compared to the bacteria that thrived in the cultures (approximately 1.0 to 7.0 d^{-1}). Langenheder et al. (2006) recently investigated the composition of bacterial assemblages that developed in lakewater cultures with inocula from different lakes. They concluded that the original composition of the inoculum was a central factor in determining the final structure of the bacterial assemblages in their experiments. This would lend support to the suggestion that the seasonal changes observed in our experiments occurred largely in response to *in situ* changes in bacterioplankton composition in combination with changes in the nutrient regime.

The concentration of organic and inorganic nutrients in most seas typically changes during the year. Our present data suggest that seasonal variability in nutrient availability has strong direct effects on the activity and composition of marine bacterial assemblages. Furthermore, the availability of nutrients directly affects phytoplankton growth and composition. Recent findings indicate that qualitative and quantitative differences in phytoplankton community composition are important for structuring the composition of the bacterial assemblage (Pinhassi et al. 2004, Abell & Bowman 2005), possibly due to differences in the stoichiometry of organic matter produced by different algae. Thus, changing nutrient concentrations are likely to have both direct and indirect effects on bacterioplankton growth and composition.

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