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Dynamics of heterotrophic bacteria in temperate coastal waters: similar net growth but different controls in low and high nucleic acid cells

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ABSTRACT: Heterotrophic bacteria in aquatic environments are almost universally distributed into 2 distinct flow cytometric clusters based on their relative nucleic acid content: low (LNA) and high (HNA). The dynamics and possible regulation processes of both subgroups in southern Bay of Biscay coastal waters were examined by comparing weekly measurements over 2 yr at a shallow environment (L'Arbeyal beach) with monthly samples at 3 continental shelf stations off Xixón (Spain). Similar seasonal variations of temperature and chlorophyll *a* characterized inshore and offshore waters. Total bacterial abundances were also similar in shallow and shelf waters (from 0.22 to 3.72×10^6 cells ml^{-1}), with the most remarkable difference being the higher abundance and larger size of HNA bacteria at L'Arbeyal, probably related to greater substrate availability. There, apparent net growth rates estimated from coherent periods (6 to 26 wk) of sustained increase or decrease (−0.02 to 0.04 d^{-1}) were similar for LNA and HNA cells, although related to different factors. LNA bacteria net growth rates were strongly positively correlated with temperature ($r = 0.95$, $p < 0.01$, $n = 6$) and negatively with chlorophyll *a* ($r = -0.90$, $p = 0.01$, $n = 6$), supporting the hypothesis that they are independent from phytoplankton, which was recently suggested for this and other coastal sites using different approaches. We also show an opposite relationship between cell size and apparent net growth rates of LNA and HNA bacteria, providing further evidence of fundamentally different ecological roles.

KEY WORDS: Bacterioplankton · Nucleic acid content · Net growth rates · Coastal waters · Bay of Biscay

INTRODUCTION

Marine heterotrophic prokaryotes (bacteria hereafter) are responsible for the bulk of nutrient and carbon recycling in pelagic ecosystems (Ducklow 2000). In quantifying bacterial contribution to biogeochemical processes and understanding their specific ecological roles, the opening of what has been termed the 'microbial black box' remains high in the agenda of marine microbial ecology (Gasol et al. 2008). Among other technological progresses, flow cytometry has become a powerful tool for exploring marine bacterial communities. Water samples can be processed quickly for obtaining accurate abundances together with associated single-cell parameters (Gasol & del Giorgio 2000). Across a wide range of aquatic environments, heterotrophic bacteria have been found to aggregate into 2 distinct flow cytometric clusters with characteristic fluorescence and size signatures (Li et al. 1995, Gasol et al. 1999, Lebaron et al. 2001), known as low nucleic acid (LNA) and high nucleic acid (HNA) subgroups. Despite their universal distribution (Bouvier et al. 2007), there is no consensus as to what these 2 flow cytometric populations mean, probably because LNA and HNA clusters are regionally made up of different bacteria at

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the phylogenetic, physiological or ecological level of organization (e.g. Zubkov et al. 2001, Bouvier et al. 2007, Morán et al. 2011).

Initially, HNA and LNA cells were grossly considered as the active and the inactive fractions, respectively, of the bacterial assemblage (Jellett et al. 1996, Gasol et al. 1999, Lebaron et al. 2001). Indeed, HNA cells were usually much more correlated to bacterial production and bulk specific growth rates than LNA cells (Lebaron et al. 2001, Servais et al. 2003, Morán et al. 2011), whereas LNA bacteria were considered as dormant or dead cells (Gasol et al. 1999, Vaqué et al. 2001, Lebaron et al. 2002). This initial picture is rapidly evolving, and LNA bacteria may present growth rates as high as the HNA subgroup (Zubkov et al. 2001, Longnecker et al. 2005, Nishimura et al. 2005) in environments such as the Mediterranean deep chlorophyll maximum (Scharek & Latasa 2007) or stratified waters of the Celtic Sea (Zubkov et al. 2001). LNA bacteria have been also predominantly associated with SAR11 and SAR86 clades, while *Gammaproteobacteria* and *Bacteroidetes* are usually only found as HNA cells (Mary et al. 2006, Teira et al. 2009, Schattenhofer et al. 2011, Vila-Costa et al. 2012), thus suggesting completely different phylogenetic compositions.

Total abundance of heterotrophic bacteria is assumed to vary little across a wide range of aquatic environments (Ducklow 2000, Smith & del Giorgio 2003) from a few hundreds of thousands to a few million cells ml^{-1}. Despite the constancy at ca. 10^6 cells ml^{-1} in surface seawater, natural bacterial communities show fluctuations in response to bottom-up (substrate availability) and top-down (grazing and viral pressure) control mechanisms as well as temperature (e.g. Pernthaler et al. 1996, Gasol & Duarte 2000, Pomeroy & Wiebe 2001, Morán et al. 2010). Recent work has shown that the dynamics of the HNA and LNA subgroups vary across environments and even shift seasonally within the same environment (Scharek & Latasa 2007, Ortega-Retuerta et al. 2008, Morán et al. 2010). Controlled experiments have attempted to isolate bottom-up (Bouvy et al. 2004, Wetz & Wheeler 2004) from top-down controls (Iriarte et al. 2008, Longnecker et al. 2010). However, both types of processes interact in the field as well as with other environmental variables, making it difficult to extrapolate results from laboratory experiments (Sherr et al. 1999, Vaqué et al. 2001, Gasol et al. 2002). Furthermore, by including current knowledge on the phylogenetic affiliation of the 2 subgroups, most experimental evidence points to significantly lower gross growth rates of LNA cells relative to their HNA counterparts (Teira et al. 2009, Ferrera et al. 2011, Morán et al. 2011). If grazing rates are also lower for LNA bacteria, in agreement with studies linking predator preferences to enhanced bacterial activity (del Giorgio et al. 1996), *in situ* variations in cell density should be similar for LNA and HNA cells. By integrating gross growth and loss rates, net growth rates can thus further our understanding of the role of LNA and HNA bacteria in ecosystem functioning (Belzile et al. 2008).

In the central Cantabrian Sea (southern Bay of Biscay) continental shelf, bacterioplankton dynamics have been followed monthly since April 2002 as part of a time series monitoring program. The hydrographic conditions and seasonal patterns in this temperate ecosystem have been described extensively (Calvo-Díaz & Morán 2006, Morán et al. 2007, 2010, Franco-Vidal & Morán 2011). In addition to the shelf stations, the present study analyzes 2 yr of weekly data from a shallow coastal site (L'Arbeyal beach, Xixón, Spain) close to the sampled transect. Other than bacterial abundances and cell properties, environmental conditions (temperature, salinity and total and picoplanktonic chlorophyll *a* [chl *a*]) were explored as proxies for factors regulating LNA and HNA subgroups along the inshore-offshore gradient. The specific aims of the present study were (1) to compare bacterial dynamics and flow cytometric single-cell properties in the beach and the nearby shelf stations and (2) to estimate apparent net growth rates of LNA and HNA bacteria in shallow waters and explore their linkage with environmental properties in order to advance our knowledge of the ecological role of these 2 subgroups.

MATERIALS AND METHODS

Study region and sample collection

L'Arbeyal (LA) is the westernmost beach of Xixón (Asturies, Spain). Beginning at 7.7 km northeast, there is a transect off Xixón sampled by the RADIALES time-series program of the Spanish Institute of Oceanography (comprising 3 stations noted here as SS1, SS2 and SS3; Fig. 1). The beach is located within an urban area and close to an industrial harbour, making limitation by nutrients quite unlikely; surface nitrate concentrations for the 2007 to 2010 period increased on average by 20% when moving from the offshore SS3 to SS2 and an additional 54% from SS2 to SS1.

Surface seawater samples were taken weekly from LA beach from 18 May 2009 to 19 May 2011 and

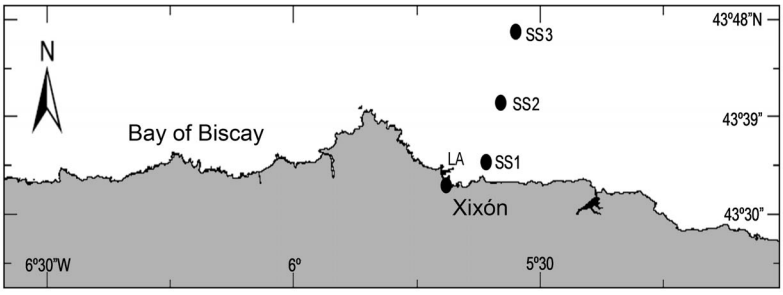

Fig. 1. Location in the southern Bay of Biscay, Spain, of the 3 RADIALES time-series shelf stations (SS1, SS2 and SS3) and the shallow coastal site of L'Arbeyal (LA) beach

monthly from the RADIALES continental shelf stations. Over the 2 yr sampling period, the latter data did not show consistent differences (i.e. increasing or decreasing patterns) along the inshore-offshore gradient in most of the variables measured (e.g. see pooled temperature and chl *a* in Fig. 2). Therefore, unless otherwise indicated, data from the 3 continental shelf stations (SS1, SS2 and SS3) will be considered collectively and referred to as SS. Samples from LA beach were collected with an acid-washed bucket directly from the beach pier usually at the morning high tide (±1 h, ca. 4 m deep during spring tides), transferred to 125 ml dark bottles and processed immediately in the laboratory. Samples from the shelf stations were collected between 10:00 and 13:00 h local time on board of RV 'José de Rioja' with 2.5 l Niskin bottles mounted on a rosette sampler with an attached Seabird 25 CTD (conductivity, temperature and depth) probe. The effect of tide on all 3 shelf stations is negligible compared to monthly changes in physico-chemical and biological variables. Surface water was then transferred to 500 ml dark bottles and kept on board until arrival at the laboratory (2 to 3 h).

Bacterial abundance, single cell properties and biomass

Subsamples of 1.8 to 3.5 ml of unfiltered water were fixed with 1% paraformaldehyde and 0.05% glutaraldehyde (final concentration), left 10 min in the dark and frozen at −80°C until analysis. The abundance and single-cell properties of heterotrophic bacteria were obtained using a FACSCalibur (Becton-Dickinson) benchtop flow cytometer equipped with a laser beam emitting at 488 nm. Samples were acquired with Cell-Quest Pro software, and cytograms were finally analyzed with Paint-A-Gate 3.0.2 (Becton-Dickinson). Upon being thawed at room temperature, 400 µl aliquots were stained with 4 µl of the nucleic acid fluorochrome Syto 13 (Molecular Probes) at 2.5 µmol l^{-1} concentration, and fluorescent beads of 1 µm diameter (Molecular Probes) were added as internal standard. Samples were run at low flow speed (10 to 20 µl min^{-1}). Calibration of bead concentrations and flow rates were always performed prior to analysis. Bead concentrations were compared against TruCount (Becton-Dickinson) beads of known concentration. Flow rate was calibrated by measuring the initial and final volumes of distilled water after 10 min of flow at low speed (Gasol & del Giorgio 2000, Lebaron et al. 2001). Bacterial abundance was calculated from flow rate and time of analysis (Gasol & del Giorgio 2000). The relative contribution of HNA cells to the total bacterial assemblage was expressed as the percentage of HNA cells: %HNA = (HNA cell abundance × 100)/(LNA and HNA cell abundance).

The flow cytometric properties obtained include green fluorescence (FL1) and right angle light scatter or side scatter (SSC). The data used in the present study represent the arithmetic means observed for each subgroup. These 2 variables allowed for the easy recognition in cytograms of the 2 clusters of cells (LNA and HNA) mostly based on FL1 differences. SSC values were converted to cell diameter and volume assuming spherical form as described by Calvo-Díaz & Morán (2006). All 3 size variables (SSC, cell diameter and cell volume) are easily interconvertible, but for the sake of consistency, cell size will be referred to as cell volume (V, µm^3) hereafter. Finally, V was converted into carbon biomass using Norland's (1993) allometric relationship: fg C = $120 \times V^{0.72}$.

Environmental variables

Chl *a* concentrations were obtained from filtered water samples. Sequential filtration of 100 ml through 20, 2 and 0.2 µm pore size Nuclepore polycarbonate filters was done with SS samples, while at LA, we performed 2 separate filtrations of 50 ml each through 2 µm pore size polycarbonate filters and Whatman glass fiber filters (GF/F). We thus obtained total (the sum of the 3 fractions in SS and the amount retained on GF/F filters in LA) and picoplanktonic (<2 µm) chl *a* concentrations. Differences in filter

pore sizes and types are of minor importance for obtaining total chl *a* values (Morán et al. 1999). All filters were frozen at –4°C until analysis. For pigment extraction, filters were submerged in 6 ml of acetone at 90% concentration for 24 h in the dark at 4°C, and the fluorescence was measured with a Perkin Elmer LB-50s spectrofluorometer calibrated with pure chl *a*.

Temperature was measured in LA with a Temp6 digital thermometer (Eutech/Oakton Instruments) from May to December 2009 and additionally with a Seabird 19 CTD from this date on, which enabled us to include salinity measurements. *In situ* temperature and salinity over the continental shelf stations were measured with the Seabird 25 CTD probe.

Apparent net growth rates

Weekly sampling of bacterial abundance in LA allowed us to identify internally coherent periods of abundance increase or decrease for both subgroups. Ordinary least squares linear regression between the natural logarithm of abundance and time in days was applied to each period, and the slope was taken as the net growth rate estimate (d^{-1}). Each period included at least 6 successive dates (weeks), and all regressions were significant at $p < 0.05$.

Data analysis

Ordinary least squares linear regressions, *t*-tests and Pearson correlation matrices were performed using the statistical commercial packages STATISTICA 7.1 (StatSoft) and SPSS 15.0 (SPSS). Figures were plotted with ArcGis 9.2 (ESRI) and Grapher 7 (Golden Software). Unless otherwise noted, all mean data values are presented together with their standard errors (SE).

RESULTS

Environmental variables and LNA and HNA bacterial abundance

Seasonal variations pooled for the present study period are shown in Fig. 2. Surface temperature (Fig. 2A) behaved similarly in both areas. Although slightly lower temperatures were occasionally observed during the coldest months in LA (9.2 to 22.2°C) than in SS (12.6 to 21.8°C), seasonal changes match those previously described (Calvo-Díaz &

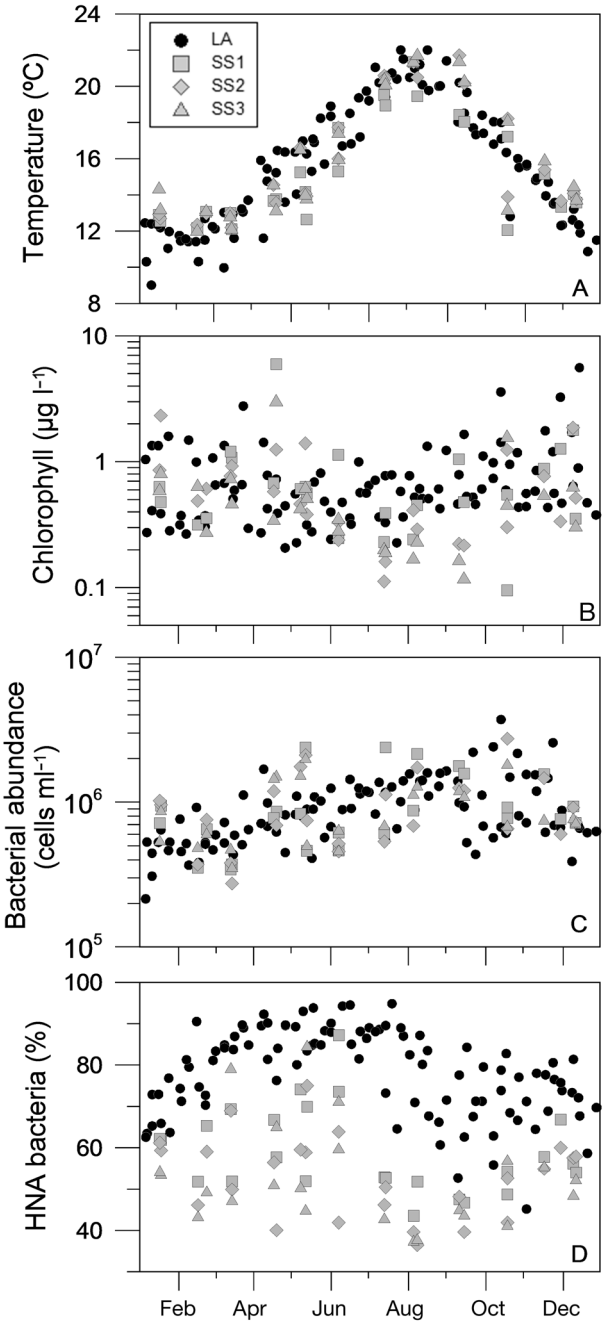

Fig. 2. Temporal variations at L'Arbeyal (LA) beach and the continental shelf stations (SS1, SS2 and SS3) off Xixón, Spain, of (A) temperature, (B) total chlorophyll, (C) total bacterial abundance and (D) contribution of the high nucleic acid (HNA) subgroup to total cell numbers. Note that we pooled data from the 2 years

Morán 2006, Franco-Vidal & Morán 2011). Salinity mean values were higher at SS (35.49 ± 0.03) than at LA (35.14 ± 0.05) due to markedly low values in the latter site from October to March caused by rain events (data not shown). Overall, chl *a* concentra-

tions were similar in both areas and ranged from 0.21 to 5.59 µg l^{-1} in LA and from 0.10 to 5.97 µg l^{-1} in SS (Fig. 2B). The seasonal pattern was more prominent in SS, with 2 maxima in March to April and October, corresponding to transitions between mixing and stratification (Franco-Vidal & Morán 2011).

The total abundance (TA) of heterotrophic bacteria in the region ranged from 2.15×10^5 to 3.72×10^6 cells ml^{-1} with similar mean (±SE) values at the 2 sites (TA_{LA}: $9.52 \pm 0.54 \times 10^5$ cells ml^{-1}; TA_{SS}: $9.72 \pm 0.64 \times 10^5$ cells ml^{-1}). With all data pooled, TA increased progressively in LA from January to September-October and decreased in December, after showing considerable scatter in the autumn months (Fig. 2C). Two relative maxima were observed in SS, in April-May and September-October. In both LA and SS, minimum values were coincident with the lowest temperatures, reflected in positive correlations between TA and temperature (Table 1).

In contrast to total counts, there were marked differences between the abundances of the LNA and HNA groups in each site (Fig. 3; LA, HNA_{LA}: $7.39 \pm 0.41 \times 10^5$ cells ml^{-1} and LNA_{LA}: $2.13 \pm 0.19 \times 10^5$ cells ml^{-1}; SS, HNA_{SS}: $5.16 \pm 0.32 \times 10^5$ cells ml^{-1} and LNA_{SS}: $4.57 \pm 0.3 \times 10^5$ cells ml^{-1}). HNA bacteria were on average more abundant than LNA cells in both LA and SS (Fig. 3A). Consequently, mean %HNA (Fig. 3C) was significantly higher (*t*-test $p < 0.001$; $n_{SS} = 73$; $n_{LA} = 100$) in LA (78.1 ± 1.0%) than in SS (55.0 ± 1.2%), and %HNA values at SS virtually never exceeded those at LA throughout the year (Fig. 2D). At LA, a steady increase was observed from January to April, with constantly high values (>80%) through July followed by largely variable but lower values during the rest of the year. A bimodal distribution of %HNA was observed in SS, with maxima in spring and minima in August to September, with a slight increase in November. Fig. 2D shows also that the periods of relatively high data dispersion differed for the 2 areas, being earlier in the case of the SS. A stronger correlation between HNA and LNA abundances was observed in SS ($r = 0.80$; $p < 0.01$, $n = 73$) than in LA ($r = 0.56$; $p < 0.01$, $n = 100$).

With all data pooled, the LNA and HNA fractions were differently associated with environmental variables in the 2 areas (Table 1). Although weak, we found a significant positive correlation with temperature of the abundance of HNA cells in LA, while in SS, only that of LNA cells was significant. Total and

Table 1. Pearson's correlation coefficients between environmental variables and total and relative bacterial abundances in L'Arbeyal (LA) and the continental shelf stations (SS). *T*: temperature; Chl *a*: total chlorophyll *a*; Chl *a* < 2: chl *a* in the <2 µm filtrate. Significant correlations in **bold** (**p < 0.01; *p < 0.05)

	LA (n = 100)			SS (n = 73)		
	T (°C)	Chl *a* (µg l^{-1})	Chl *a* < 2 (µg l^{-1})	*T* (°C)	Chl *a* (µg l^{-1})	Chl *a* < 2 (µg l^{-1})
LNA (cells ml^{-1})	0.17	0.17	**0.24***	**0.34****	0.02	−0.01
HNA (cells ml^{-1})	**0.43****	0.19	**0.25***	0.09	0.17	0.17
Total (cells ml^{-1})	**0.39****	**0.20***	**0.28****	**0.24***	0.09	0.08
%HNA	0.19	−0.09	−0.09	**−0.42****	0.16	0.10

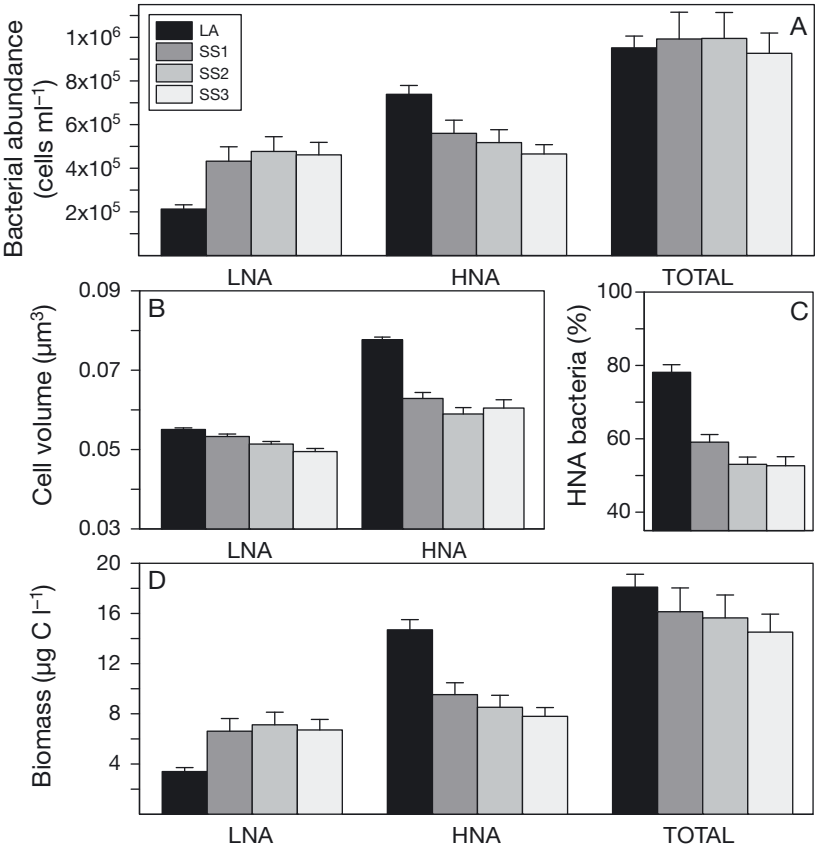

Fig. 3. Mean (±1 SE) values of (A) bacterial abundance, (B) cell volume, (C) %HNA bacteria and (D) biomass at L'Arbeyal (LA) and the 3 shelf stations (SS1, SS2 and SS3) for the 2 yr sampling period

picoplanktonic chl *a* concentrations showed weak correlations with bacterial abundances only in LA, and a negative correlation between %HNA and temperature were found at SS, as previously reported (Morán et al. 2010).

Flow cytometric properties and biomass

Nucleic acid content, estimated as relative green fluorescence (FL1), was essentially the same in LA and SS for each bacterial subgroup (HNA_{LA}: 2.42 ± 0.63 × 10^{-3}; HNA_{SS}: 2.15 ± 0.48 × 10^{-3}; LNA_{LA}: 5.11 ± 0.17 × 10^{-3}; LNA_{SS}: 5.08 ± 0.09 × 10^{-3}), and they also showed the same temporal pattern, with maximum values in spring and minima through summer and autumn (Fig. 4A,B).

In contrast, cell volumes differed markedly in the 2 areas (Fig. 4C,D). HNA cells were larger at the beach than at the shelf stations (*t*-test $p < 0.001$; $n_{SS} = 73$; $n_{LA} = 100$), while LNA cells were more homogeneous (Fig. 3B; mean cell volumes ± SE: HNA_{LA}: 0.077 ±

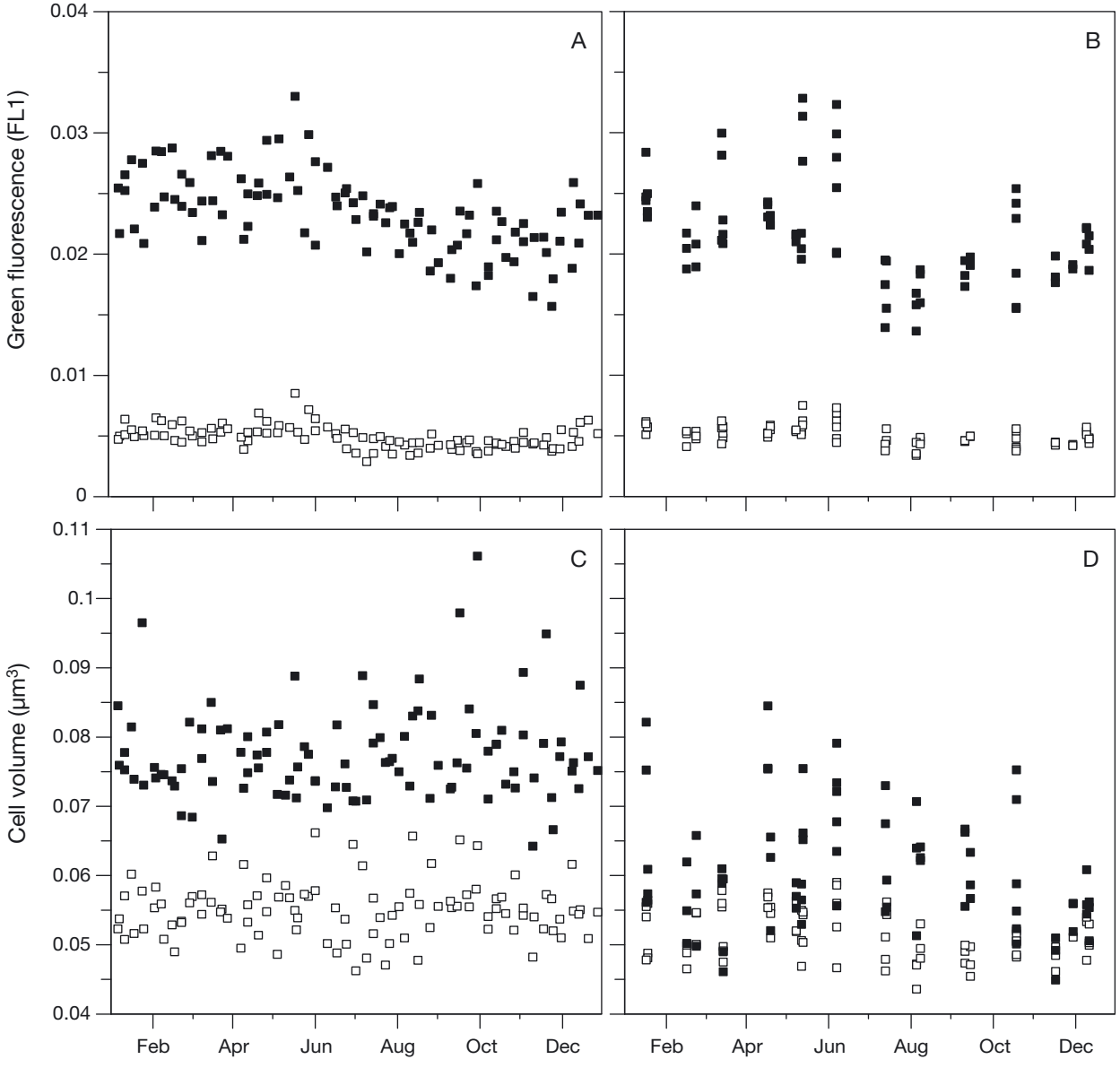

Fig. 4. Temporal variations of the flow cytometric properties relative green fluorescence (a surrogate of nucleic acid content) and cell volume of the HNA (closed symbols) and LNA (open symbols) subgroups at (A,C) L'Arbeyal beach and (B,D) the continental shelf stations

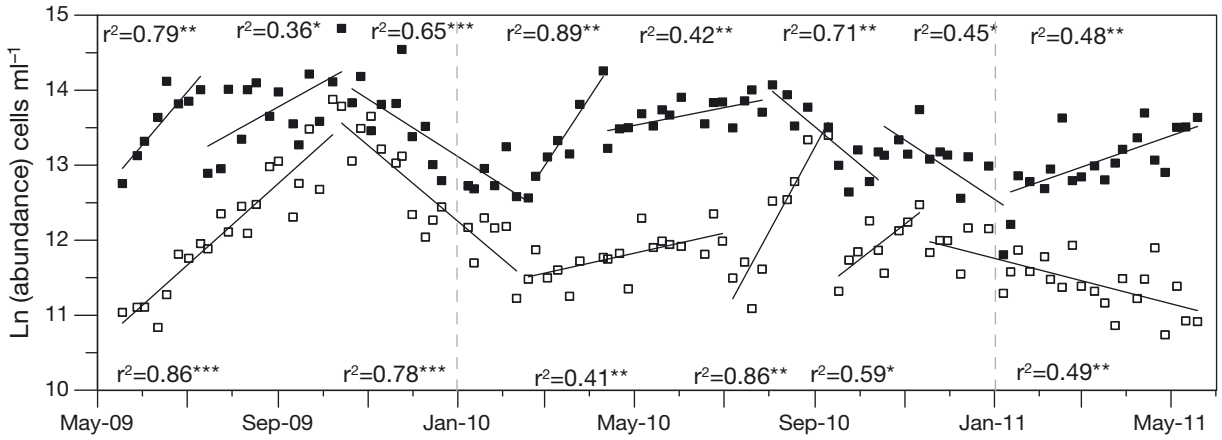

Fig. 5. Time-series of HNA (closed symbols) and LNA (open symbols) bacterial abundances at L'Arbeyal showing the selected periods of net increase or decrease. Ordinary least squares linear regressions fitted to data are shown for each period. The coefficients of determination (r^2) are shown for each respective model, above for HNA cells and below for LNA cells ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$)

0.07 × 10^{-2} µm^3; HNA_{SS}: 0.061 ± 0.1 × 10^{-2} µm^3; LNA_{LA}: 0.055 ± 0.04 × 10^{-2} µm^3; LNA_{SS}: 0.051 ± 0.04 × 10^{-2} µm^3). Generally HNA cells were larger than LNA cells except occasionally during the winter and spring months in SS, when both tended to show volumes around 0.05 to 0.06 µm^3 (Fig. 4D).

Differences between LA and SS bacterial biomass were not significant throughout the year (*t*-test p = 0.44; n_{SS} = 73; n_{LA} = 100). Mean annual contributions of HNA cells to total abundance (Fig. 3C) were mirrored with only slight differences by biomass values (Fig. 3D). HNA cells contributed 82 ± 1% to total biomass in LA in contrast to the 58 ± 1% contribution in pooled SS samples.

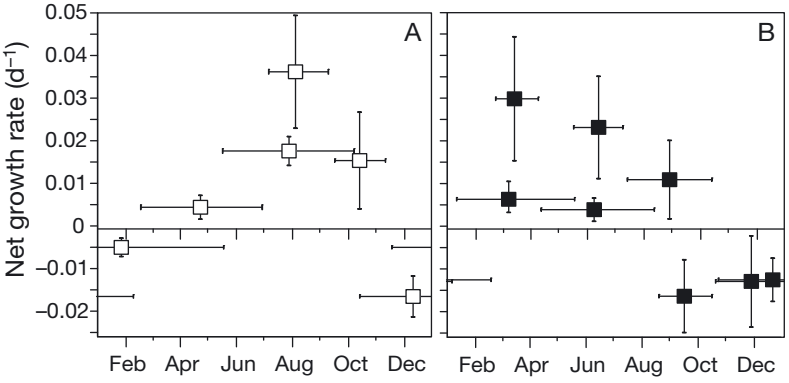

Fig. 6. Seasonal variations in net growth rates of (A) LNA and (B) HNA subgroups with data pooled from the 2 years. Horizontal bars represent the period's length, while vertical bars represent the 95% confidence intervals of the ordinary least squares linear regression slopes (see also Fig. 5 for details)

Apparent net growth rates

Ranges of variation in apparent net growth rates in LA (Fig. 5) were very similar for both subgroups (HNA: –0.016 to 0.030 d^{-1}; LNA: –0.017 to 0.036 d^{-1}). Taking only positive values, the mean annual net growth rate of LNA cells (0.018 ± 0.005 d^{-1}) was slightly higher than corresponding HNA values (0.015 ± 0.003 d^{-1}). On a seasonal basis, net growth rates of LNA and HNA bacteria followed different patterns (Fig. 6). LNA bacteria tended to show positive values from April to November and negative values in winter, with maxima in July to August. Positive net growth rates were found for HNA bacteria from March through August, with negative values for the rest of the year. Seasonal maximum values of HNA cells were more variable than those of LNA cells.

The apparent net growth rates of LNA cells were significantly and positively correlated with temperature (Fig. 7A) and negatively with total chl *a* (Fig. 7C). In contrast, values for HNA bacteria showed no significant correlations with these variables (Fig. 7B,D). Interestingly, we found contrasting associations between cell size and apparent net growth rates for each subgroup (Fig. 7E), increasing in LNA cells and decreasing in HNA cells, although only the latter trend was marginally significant (p = 0.058).

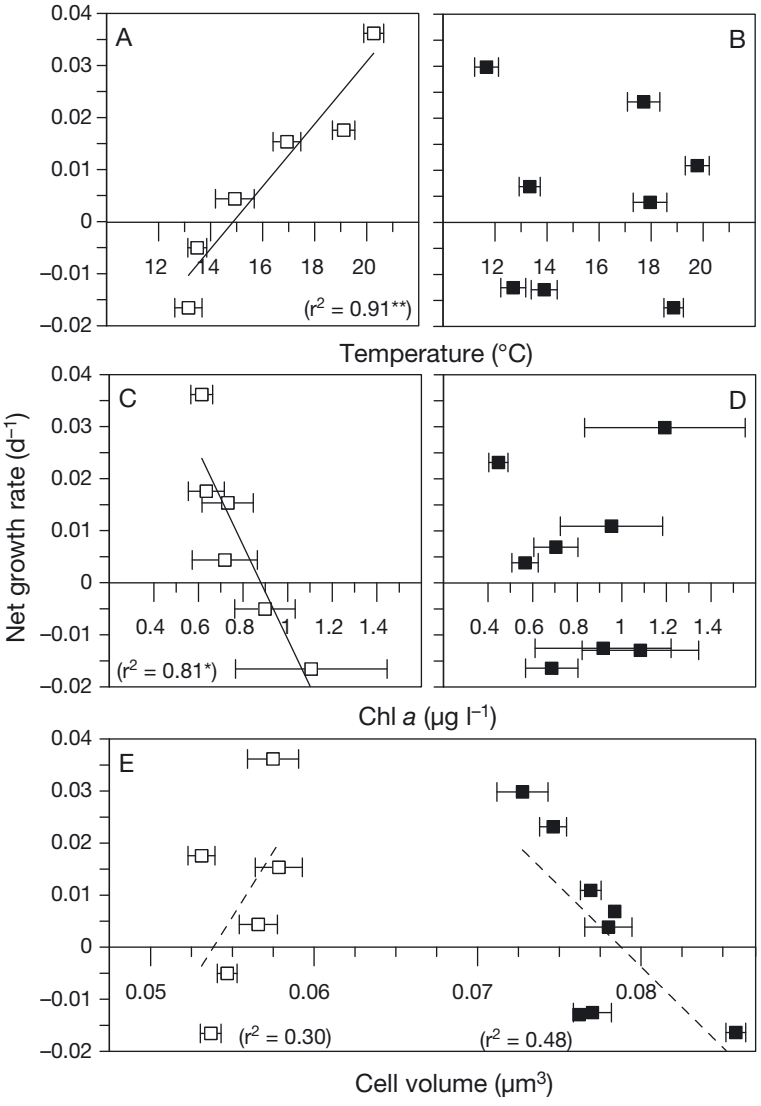

Fig. 7. Relationships of net growth rates of LNA (open symbols) and HNA (closed symbols) cells with mean (A,B) temperature, (C,D) chl *a* and (E) corresponding cell volume during the calculated period. Ordinary least squares linear regressions fitted are shown as solid lines when significant and as dashed lines when not (r^2 values in brackets: *$p < 0.05$; **$p < 0.01$)

DISCUSSION

The common view held in marine ecology that heterotrophic bacterial abundance and biomass remain quite uniform compared with other planktonic components (e.g. Vaqué 1996, Ducklow 1999), both with time and across a wide range of marine regions, was not contradicted by the data analyzed in the present study for the beach and shelf stations (Figs. 1 & 4). In contrast, there were noteworthy differences between shallow and deeper waters off Xixón when we considered the universally distributed clusters of LNA and HNA cells as detected by flow cytometry.

Abundance of LNA and HNA bacteria

Seasonal patterns in temperature, chl *a* and LNA and HNA bacteria match previous time-series observations (Calvo-Díaz & Morán 2006, Franco-Vidal & Morán 2011). However, the recurrent seasonal pattern in %HNA observed from 2002 through 2009 (Calvo-Díaz & Morán 2006, Franco-Vidal & Morán 2011, X. A. G. Morán, L. Á. Suarez & L. Díaz-Pérez unpubl. data), with maxima in April (>75%) and minima in August (<50%), disappeared in 2010, precisely the central year of our sampling period. It partially recovered during 2011 (with an increase in %HNA from 54% in December 2010 to 72% in March 2011), which explains the high %HNA scatter observed from January to April (Fig. 2D). The repeatability of the %HNA peak associated with the phytoplankton spring bloom would agree with the predictable succession of distinct phylogenetic groups with environmental changes found elsewhere (Fuhrman et al. 2006).

In LA, the HNA cluster was far more important than LNA, representing on average 78% of total bacterial counts and 82% of the biomass. The increase in the active fraction with trophic state (del Giorgio & Scarborough 1995, Longnecker et al. 2010) could explain the greater presence of HNA cells at the beach. Most likely, substrate limitation was substantially lower in LA than on the shelf in view of the inshore-offshore gradient in inorganic nutrients found nearby westwards (Llope et al. 2007) and in the Xixón time-series itself (E. Nogueira pers. comm., see also 'Materials and methods'). This is also supported by the fact that HNA_{LA} abundance was generally higher throughout the year and reached maximum abundances in summer coincident with the annual warmest weeks, in contrast to the decrease in HNA_{SS} abundance observed during the same months (Fig. 2D, Table 1).

However, although LNA cell abundances in LA were much lower, they showed higher variability than HNA cells (CV 92 vs. 55%), even reaching or exceeding the latter at some periods. This finding is probably related to different regulating factors acting on each subgroup (see below), as also inferred

by the lower covariance of the abundances of HNA and LNA cells there ($r = 0.54$, $p < 0.05$, $n = 100$) compared to the tighter coupling detected on the shelf.

Flow cytometric properties of LNA and HNA cells

Nucleic acid content did not show great variations between areas, characterized by similar seasonal patterns and mean values for each subgroup (Fig. 4A,B). The distribution of FL1 into the 2 distinct clusters LNA and HNA was related to different replicating stages (n vs. 2n) of the same populations by Bouvier et al. (2007), although most recent evidence points toward differential phylogenetic composition with different genome sizes or copies in the 2 subgroups (Schattenhofer et al. 2011). Particularly, the cultured representative of the SAR11 clade *Pelagibacter ubique* (Rappé et al. 2002), frequently only found within the LNA subgroup, which they dominate (Mary et al. 2006), has the smallest genome among free-living bacteria (Giovannoni et al. 2005).

More relevant were the differences in cell size between and within the HNA and LNA subgroups from LA and SS. Mean cell sizes of HNA bacteria in the shelf stations were smaller than in LA (Fig. 3B), which can be related to differences in trophic state, but it also seems plausible that HNA_{SS} and HNA_{LA} were composed of at least some different species. In contrast, LNA_{SS} and LNA_{LA} cell sizes were much more similar, supporting the contention of a more homogeneous species composition of this subgroup, probably dominated by SAR11 cells (Mary et al. 2006, Teira et al. 2009, Schattenhofer et al. 2011).

We found no obvious seasonal patterns in cell size except for LNA cells in the shelf stations, with maxima in spring and early summer and minima in September to October (Fig. 4D). This pattern was significant for surface waters with all time-series data available through 2011 (X. A. G. Morán & T. M. Huete-Stauffer pers. obs.). During this period, LNA_{SS} cell size even exceeded the corresponding values of the smallest HNA_{SS} found on an annual basis (Fig. 4D), as previously reported (Calvo-Díaz & Morán 2006). This pattern suggests that surface LNA cells from the continental shelf may be more susceptible to the temperature–size rule (Atkinson et al. 2003) than their shallow water counterparts.

LNA and HNA bacterial apparent net growth rates

Although a lack of information on water residence time in LA beach precludes us from assessing the role played by advection, sustained increases or decreases in plankton abundance or biomass have allowed the estimation of apparent growth rates at scales from hours to months (e.g. Pedersen & Borum 1996, Gasol et al. 2005). Monitoring weekly rather than daily changes in abundance probably only slightly affected our estimates of net growth rates. A comparison of weekly based estimates of net growth rates of *Synechococcus* and *Prochlorococcus* cyanobacteria (-0.16 to $0.18\ d^{-1}$) with values obtained from samples taken every 2 to 3 d in LA from 1 August to 18 November 2003 (data not shown) resulted in a strong correlation ($r = 0.98$, $n = 8$) and non-significant differences (paired *t*-test, $p = 0.58$).

Estimated net growth rates in LA were very low (systematically well below the 0.1 to $1\ d^{-1}$ range in specific growth rates frequently accepted for marine bacteria; e.g. Kirchman et al. 1995), reflecting the overall strong losses caused by grazers or viruses hindering abrupt changes in environmental abundance. Even the SS2 specific growth rates estimated from leucine- and thymidine-based bacterial production to biomass ratios in 2006, considerably lower than direct estimates in predator-free incubators (Franco-Vidal & Morán 2011), were on average 7- to 23-fold higher than our values. Net growth rates calculated by Wetz & Wheeler (2004) from changes in cell numbers were higher as well, although their experiments specifically simulated a phytoplankton bloom. Our values match well those observed by Belzile et al. (2008) in the Arctic spring growth season (mean: $0.013\ d^{-1}$ for LNA and $0.018\ d^{-1}$ for HNA cells) but are at the lower end of the ranges reported by Williams et al. (2008) for a Florida shallow estuary (LNA: 0.01 to $0.45\ d^{-1}$; HNA: -0.33 to $0.43\ d^{-1}$). Notwithstanding the different approaches, the apparent net growth rates reported here highlight differences between the 2 bacterial subgroups.

In LA, LNA cells reached and even exceeded the net growth rates of HNA cells (Fig. 6), as previously reported by Williams et al. (2008). However, recent work indicates that gross growth rates of HNA cells are frequently 1 order of magnitude higher than those of LNA cells: differences of 3- to 5-fold higher values were reported in direct comparisons of HNA versus LNA bacteria (Morán et al. 2011) and of phylogenetic groups ascribed to one or the other flow cytometric clusters (Teira et al. 2009, Ferrera et al. 2011). Therefore, essentially equal net growth rates

of both subgroups mean that predation pressure must have been notably higher on HNA cells than on LNA cells (see 'Bottom-up and top-down controls').

With a very limited set of environmental variables measured, the different responses to temperature and chl *a* also point to different regulation processes for each subgroup. Neither temperature nor chl *a* had significant effects on HNA_{LA} net growth rates (Fig. 7). In contrast, the principal variable regulating LNA_{LA} growth seems to be temperature, with a clear seasonal pattern emerging for LNA cells. Periods of increasing or decreasing abundance were more stable, longer and easier to identify for the LNA subgroup (Fig. 5), suggesting that regulation of LNA bacteria was subjected to fewer control processes than that of HNA bacteria. The observed high positive correlation between LNA_{LA} apparent net growth rates and temperature (Fig. 7A) could be related to the physiological activation of LNA_{LA} cells above the 15°C threshold (Fig. 7A). Interestingly, compared with HNA bacteria and other physiological fractions, such as membrane-intact or actively respiring (CTC-positive) cells, the specific growth rates of LNA bacteria also showed the strongest response to temperature in a temperate estuary (Morán et al. 2011). This response was also detected offshore as a continuous increase in LNA cell abundance with temperature (Table 1; see also Morán et al. 2010), indicating that this subgroup probably performed better than HNA cells against grazing and other mortality processes (Longnecker et al. 2010) with low, temperature-controlled metabolic rates.

Controversy about the identity, physiological status and ecosystem role of LNA bacteria has followed the widespread earlier consideration that they were inactive cells. Clear phylogenetic differences between both subgroups have been described (Mary et al. 2006, Teira et al. 2009, Schattenhofer et al. 2011, but see Servais et al. 2003), although species composition and relative contribution may vary with trophic state and abiotic factors, such as temperature and salinity (Alonso-Sáez et al. 2007). HNA and LNA cells may not have the same ecological meaning in different systems, but clearly the dichotomous classification of active versus inactive cells is too rough to prove useful (Morán et al. 2010). A growing body of literature rejects the consideration of LNA as inactive or dead cells (Zubkov et al. 2001, Jochem et al. 2004, Longnecker et al. 2005), and we demonstrate that this fraction was able to actively grow. In our particular case, the observation of both coupled and uncoupled phases of HNA_{LA} and LNA_{LA} net growth (Fig. 5) would argue against a sustained, completely different regulation of both subgroups, suggesting that there may be shared periods with common responses to factors out of the scope of the present study. However, the fact that there was virtually no size or fluorescence overlap between the 2 fractions in LA over time (Fig. 4A,C) makes it hard to imagine a continuum of metabolic stages within the same phylogenetic groups (Bouvier et al. 2007). It seems more likely that each cell cluster comprised distinct taxa with generally separate dynamics at this shallow site.

Bottom-up and top-down controls

Bearing in mind that net growth rates are the balance between bottom-up (substrate) and top-down (mortality) controls, we can investigate the effect of the former based on apparent bacterial responses to chl levels. Positive associations of bulk bacterial production and specific growth rates with chl *a* have been reported for a wide range of ecosystems (Cole et al. 1988, Gasol & Duarte 2000, Barbosa et al. 2001, Scharek & Latasa 2007, Ortega-Retuerta et al. 2008), including previous studies in the same region (Morán et al. 2010, Franco-Vidal & Morán 2011). However, phytoplankton bottom-up control was weak throughout the year for the present study period, in contrast with a previous study, suggesting a higher bacterial dependence on algal products in winter and spring than in summer at SS2 (Morán et al. 2010). The absence of positive relationships between bacterial net growth rates and chl *a* (negative correlation in the case of LNA cells; Fig. 7C), arguing against a direct trophic coupling between phytoplankton and bacteria in LA, could also be interpreted as evidence either that phytoplankton were not limiting for HNA and LNA bacterial growth in LA or that non-phytoplanktonic dissolved organic carbon (DOC) sources were also available, consistent with the weak correlations found (Table 1).

Phytoplankton–bacterioplankton coupling has been evidenced particularly by the HNA subgroup (Gasol & Duarte 2000, Wetz & Wheeler 2004, Morán et al. 2007), although it has also been attributed sporadically to LNA cells (Scharek & Latasa 2007, Ortega-Retuerta et al. 2008). The recently formulated hypothesis that HNA bacteria depend more on phytoplankton-derived products than LNA cells (Morán et al. 2011), probably linked to their higher activity and substrate needs (Cuevas et al. 2011), could not be demonstrated in the present study other than the discovery that LNA cells were apparently even more independent of phytoplankton substrates than HNA

cells (cf. Fig. 7C,D). The absence of data of inorganic and organic nutrient concentrations preclude us to conclude whether these bacterial communities rely on sources of DOC other than that freshly produced by phytoplankton (including inland inputs or the result of particulate organic matter solubilization) or whether the true relationships of bacteria and phytoplankton were obscured in a meso- to eutrophic site (Findlay et al. 1991) by a low temporal resolution or grazing processes.

Bacterial size is a key factor determining top-down regulation (González et al. 1990). The cell volumes measured in the present study may have been related to internal cell cycles (Gasol & del Giorgio 2000, Bouvier et al. 2007), but Fig. 7 strongly suggests that they are also the result of protistan grazing. At higher growth rates, HNA_{LA} bacteria tended to show smaller sizes (Fig. 7E). This finding, together with the significant (although low) negative correlation between HNA_{LA} abundance and size ($r = -0.20$; $p < 0.05$; $n = 100$) is consistent with the size-selective predation theory (González et al. 1990, Jürgens & Güde 1994, Longnecker et al. 2010), roughly stating that more active and bigger cells are preferentially preyed upon (del Giorgio et al. 1996), with a subsequent decrease in the mean size of the remaining cells. LNA_{LA} bacteria showed the opposite trend, with higher growth rates of larger cells (Fig. 7E). According to this hypothesis, LNA_{LA} cells may have escaped systematically high predation pressures due to the small size they reached even at maximum values, also consistent with the development of seasonal patterns more closely related to temperature (Figs. 6A & 7A). Without being too speculative, different predation pressures on LNA and HNA bacteria were surely responsible for the uniformly low apparent net growth rates (Fig. 6) derived from the observed changes in abundance (Fig. 5). Clearly, our data are insufficient to fully explain the complex interactions of bacterial assemblages with other environmental variables, but the estimated net growth rates suggest distinct responses of the HNA and LNA subgroups to temperature and phytoplankton in a shallow ecosystem. Ongoing experimental work seeks to demonstrate an essentially different behaviour of these universally distributed flow cytometric subgroups of heterotrophic bacterioplankton.

CONCLUSION

Bacterial flow cytometric subgroups according to nucleic acid content differed in their dynamics in shallow and deeper waters over the southern Bay of Biscay continental shelf. We suggest that the HNA cluster from LA beach may differ in at least some important species from the HNA cluster of the shelf stations, as reflected in different dynamics and size distributions, while LNA cells were probably more homogeneous over the 2 areas. LNA bacteria were physiologically active, showing temporal changes in abundance and size, and could grow at apparent net rates equal to those of HNA cells. We found no evidence of bacteria being dependent on phytoplankton at the beach. Our results support the view that LNA and HNA are phylogenetically distinct fractions regulated by different factors. Specifically, temperature was the most relevant factor controlling LNA bacteria, while HNA cells were probably more preferred by grazers due to their larger size.

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