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PII: S0967-0637(14)00211-8
DOI: http://dx.doi.org/10.1016/j.dsr.2014.11.007
Reference: DSRI2424

To appear in: *Deep-Sea Research I*

Received date: 7 February 2014
Revised date: 19 October 2014
Accepted date: 23 November 2014

Cite this article as: Ana Gomes, Josep M. Gasol, Marta Estrada, Leticia Franco-Vidal, Laura Díaz-Pérez, Isabel Ferrera, Xosé Anxelu G. Morán, Heterotrophic bacterial responses to the winter-spring phytoplankton bloom in open waters of the NW Mediterranean, *Deep-Sea Research I,* http://dx.doi.org/10.1016/j.dsr.2014.11.007

Heterotrophic bacterial responses to the winter-spring phytoplankton bloom in open waters of the NW Mediterranean

Ana Gomes[1], Josep M. Gasol[1], Marta Estrada[1], Leticia Franco-Vidal[2], Laura Díaz-Pérez[2], Isabel Ferrera[1] and Xosé Anxelu G. Morán[2,3] *

[1] Institut de Ciències del Mar, CSIC, Pg. Marítim de la Barceloneta 37-49, 08003 Barcelona, Catalunya, Spain

[2] Centro Oceanográfico de Xixón, Instituto Español de Oceanografia, Camín de L'Arbeyal, s/n, 33212 Xixón, Asturies, Spain

[3] King Abdullah University of Science and Technology, 23955-6900 Thuwal, Saudi Arabia

* corresponding author: xelu.moran@kaust.edu.sa

ABSTRACT

The response of planktonic heterotrophic prokaryotes to the NW Mediterranean winter-spring offshore phytoplankton bloom was assessed in 3 cruises conducted in March, April-May and September 2009. Bulk measurements of phytoplankton and bacterioplankton biomass and production were complemented with an insight into bacterial physiological structure by single-cell analysis of nucleic acid content [low (LNA) vs. high (HNA)] and membrane integrity ("Live" vs. "Dead" cells). Bacterial production empirical conversion factors (0.82 ± 0.25 SE kg C mol leucine^{-1}) were almost always well below the theoretical value. Major differences in most microbial variables were found among the 3 periods, which varied from extremely high phytoplankton biomass and production during the bloom in March (>1 g C m^{-2} d^{-1} primary production) to typically oligotrophic conditions during September stratification (<200 mg C m^{-2} d^{-1}). In both these periods bacterial production was ~30 mg C m^{-2} d^{-1} while very large bacterial production (mean 228, with some stations exceeding 500 mg C m^{-2} d^{-1}) but low biomass was observed during the April-May post-bloom phase. The contribution of HNA (30-67%) and "Live" cells (47-97%) were temporally opposite in the study periods, with maxima in March and September, respectively. Different relationships were found between physiological structure and bottom-up variables, with HNA bacteria apparently more responsive to phytoplankton only during the bloom, coinciding with larger average cell sizes of LNA bacteria. Moderate phytoplankton-bacterioplankton coupling of biomass and activity was only observed in the bloom and post-bloom phases, while relationships between both compartments were not significant under stratification. With all data pooled, bacteria were only weakly bottom-up controlled. Our analyses show that the biomass and

production of planktonic algae and bacteria followed opposite paths in the transition from bloom to oligotrophic conditions.

Keywords: Bacteria; Phytoplankton; Bacterial production; Primary production; Phytoplanktonbacterioplankton coupling; Winter-spring bloom; Mediterranean

INTRODUCTION

Heterotrophic prokaryotes (mostly bacteria, a term used as equivalent hereinafter) comprise the largest fraction of the living planktonic biomass in most aquatic systems. Their impact on marine food webs increases with increasing oligotrophy (Kirchman, 2008), with heterotrophic bacterial biomass equaling or even exceeding that of phytoplankton (Furhman et al., 1989; Cho & Azam, 1990; Gasol et al., 1997; Duarte et al., 2000). Heterotrophic bacteria and phytoplankton communities interact in very complex ways (Azam & Malfatti, 2007), but both the biomass and production of heterotrophic bacteria usually covary with those of phytoplankton (Bird & Kalff, 1984; Cole et al, 1988; Simon et al, 1992; Gasol & Duarte, 2000). Initially taken as evidence of the role played by phytoplankton in supplying the substrates needed for bacterial growth (Morán et al., 2002; Sarmento & Gasol, 2012), this trophic dependence of bacterioplankton on phytoplankton has recently been challenged, particularly in oligotrophic ecosystems (Gasol et al., 2009; Fouilland & Mostajir, 2010; Morán & Alonso-Sáez, 2011). Although information on carbon fluxes in oligotrophic planktonic ecosystems has grown over the last decades (del Giorgio & Cole, 1998; Cotner et al., 2001; Stock et al., 2014; del Giorgio et al., 2011), there are still serious gaps in our knowledge compared with that from the usually richer coastal environments.

The Mediterranean Sea has traditionally been considered as a suitable oligotrophic model ecosystem for its similarities to true oceanic open waters (Turley, 1999; Robinson et al., 2001). Continuous inputs of Atlantic waters, impoverished in inorganic nutrients but characterized by moderate primary production levels, can be traced in this quasi-enclosed sea (Morel & Antoine, 1996; Bosc et al., 2004). Recent field studies and satellite observations revealed that the northwestern sub-basin around the Gulf of Lion is a key area of great biogeochemical importance within the Mediterranean (Bosc et al., 2004; Somot et al., 2006). There, wind driven upwelling (Morales Blake et al., 2006) accounts for 15% of primary production of the entire basin, with the annual winter-spring bloom as the main contributor to these high primary production values (Antoine et al., 1996; Bosc et al., 2004; Estrada, 1996; Morán & Estrada, 2005). Similar to phytoplankton blooms occurring in richer regions, noticeable blooms are observed when high nutrient concentrations from winter mixing coincide with increased solar irradiance at the onset of stratification in the NW Mediterranean (Zingone et al., 2010). The singularity of these large blooms is that they recurrently occur almost exclusively in open waters throughout late winter and spring, thus lasting for more than 3 months with biomass values up to 6-fold higher than in surrounding waters (D'Ortenzio & Ribera d'Alcalà, 2008). During the rest of the year, resemblance to true oligotrophy is more evident. A full seasonal characterization of the effects of these short-lived but extensive phytoplankton blooms on ecosystem functioning, and specifically on carbon flowing through heterotrophic bacteria is lacking, with partial studies focusing on smaller temporal scales (e.g. Pedrós-Alió et al., 1999; Van Wambeke et al., 2001; Van Wambeke et al., 2002).

With the objective of assessing the physiological and ecological responses of heterotrophic bacterial assemblages to algal seasonal growth, we conducted oceanographic cruises at 3 different stages of a phytoplankton bloom (late bloom, post-bloom and stratification) over a total area of 12380 km^2. Assuming an effect of the availability of dissolved organic matter released by phytoplankton and the existence of a lag phase between phytoplankton and bacterial growth (Smith et al., 1995), we expected the strength of phytoplankton-bacterioplankton coupling (Morán et al., 2002) to change in the different phases of the algal bloom progression. We also hypothesized that bacterial trophic dependence on phytoplankton should translate into changes in the physiological structure of bacterial assemblages as defined by del Giorgio & Gasol (2008), and thus could be better detected at the single-cell rather than at the bulk level. Here we combined flow cytometric single-cell analysis of the abundance and individual size of low (LNA) and high (HNA) nucleic acid content bacteria (Gasol et al., 1999) and the abundance of "Live" and "Dead" cells (i.e. cells with intact or damaged membranes, Grégori et al., 2001) with bulk measurements of bacterioplankton and phytoplankton biomass and production in order to advance in our understanding of bacterioplankton bottom-up control mediated by phytoplanktonic carbon supply in open waters of the NW Mediterranean.

MATERIALS AND METHODS

Three sets of stations were sampled in epipelagic NW Mediterranean open waters of the Catalano-Balearic and Algero-Provençal basins around the Gulf of Lion as part of the FAMOSO cruises on board the RV *Sarmiento de Gamboa* conducted in March (6-23), April-May (29-14, May hereinafter) and September (14-22) 2009, designated as F1, F2 and F3, respectively. Throughout the text these sampling periods

will also be referred to as bloom, post-bloom and stratification stages (see below) as we intended to follow the progression of the phytoplankton bloom.

CTD data, chlorophyll *a* and primary production

Vertical profiles of physico-chemical properties were obtained with a Sea-Bird 911 Plus CTD equipped with a fluorometer. Samples were taken at selected depths with Niskin bottles mounted on a rosette attached to the CTD probe.

Chlorophyll *a* (Chl) seawater samples of 50-200 mL were filtered through Whatman GF/F filters, and immediately frozen at -20 ºC. After 4-6 hours, the filters were placed in vials with 90% acetone for extraction in the dark, at 4ºC, during 20 hours. Concentration was determined by measuring the fluorescence in acetone extracts with a Turner Designs Fluorometer. Phytoplankton biomass was roughly estimated by assuming a C:Chl ratio of 50, close to the average estimated for the production of new biomass in the study area in late spring (Latasa et al., 2005).

Primary production was estimated as described in Morán & Estrada (2005) with slight modifications (Estrada et al., 2014). Water was taken from the surface and deeper in the water column, at the depth of the subsurface chlorophyll maximum. At each depth, one dark and 13 light polycarbonate bottles were filled with 70 mL of sample, inoculated with 3.7 10^5 Bq of ^{14}C-bicarbonate and incubated for 2 h in photosynthesis-irradiance (*P-E*) incubators. Samples were then filtered using Whatman GF/F filters (25 mm diameter). After being exposed overnight to HCl fumes to remove inorganic ^{14}C, filters were placed in 6 mL vials with Beckman Coulter Ready Safe cocktail, followed by determination of disintegration per minute (dpm) in the lab using a Beckman LS6500 liquid scintillation counter. Dpm

conversion to carbon units was performed assuming a conversion factor of 25000 mg C m^{-3}. *P-E* parameters (light-limited slope, maximum photosynthetic rate and photoinhibition if present) were estimated with the models detailed in Morán & Estrada (2005). Surface and deep *P-E* parameters, together with the vertical profiles of photosynthetically available radiation (PAR) and Chl, were used to estimate daily rates of primary production at different depths of the water column.

Flow cytometry single-cell analyses

All flow cytometry analyses of "Live" and "Dead" prokaryotic cells and most picophytoplankton abundances were performed on board using a FACSAria (BD Biosciences) flow cytometer equipped with a laser emitting at 488 nm set at 20 mW. Total abundance of heterotrophic prokaryotes was determined on land after each cruise with a FACSCalibur (Becton-Dickinson) flow cytometer with a laser of the same characteristics. Flow rate calibration was performed daily for abundance estimations, and 1-μm fluorescent latex beads (Molecular Probes) were used as internal standards.

Seawater samples (1.8 mL) were preserved with 1% paraformaldehyde + 0.05 mL glutaraldehyde (final concentration), kept for 10 min at 4ºC in the dark, deep frozen in liquid nitrogen, and stored at -80ºC until further analysis. Prior to analyses, the samples were thawed and stained with a 10x SybrGreen I (Molecular Probes) solution (final dilution, 1:1000 (v:v)), kept in the dark for 15 - 20 minutes, and run at low speed (~17 μL min^{-1}) for 90 seconds. Determination of populations was done discriminating high (HNA) and low (LNA) nucleic acid content bacterial groups on side scatter vs. green fluorescence (FL1) and red (FL3) versus green (FL1)

fluorescence plots (Gasol & del Giorgio, 2000). To discriminate heterotrophic from picoautotrophic abundance, a volume of 0.6 mL of unfixed sample was run at high speed (~ 74 μL min^{-1}) for 280 s, on orange (FL2) vs. red fluorescence (FL3), and side scatter (SSC) vs. red (FL3) fluorescence plots. Bacterial cell volume was estimated via an empirical calibration between SSC and cell diameter (cell diameter = 0.908 + 0.34 log SSC) proposed by Calvo-Díaz & Morán (2006). This estimation assumes bacterial cells to have a spherical shape. Heterotrophic bacterial biomass (BB) was then estimated from cell size (μm^3) and abundance using the relationship by Norland (1993): fg C $cell^{-1}$ = 0.12 × cell $size^{0.72}$.

We differentiated between membrane-intact ("Live") and membrane-damaged ("Dead") prokaryotic cells with the nucleic acid double staining (NADS) protocol (Falcioni et al., 2008; Grégori et al., 2001). Briefly, live samples were stained simultaneously with 1:10000 (v:v) SybrGreen I (Molecular Probes, Invitrogen) and 10 μg mL^{-1} propidium iodide (PI, Sigma). Both dyes stain nucleic acids, but PI can only penetrate compromised cell membranes. Samples were incubated in the dark at room temperature for 15-20 min and analysed flow cytometrically at low speed (~ 21 μL min^{-1}). Detection of both subpopulations was done in a green (FL1) vs red fluorescence (FL3) plot as explained elsewhere (Falcioni et al., 2008).

Bulk bacterial activity and growth rates

Bacterial heterotrophic activity and production (BP) was estimated from the incorporation of ^{3}H-leucine (Kirchman et al., 1985, 40 nM final concentration) by the centrifugation method (Smith & Azam, 1992). Succinctly, four 1 mL aliquot samples were incubated with [^{3}H] Leu, one of them being a trichloroacetic acid (TCA) killed

control. Incubation was carried out in the dark at in situ temperature (± 1ºC) for 1.5 – 2 h, stopped by 50% TCA addition (100 µL) and stored at -20ºC until analysis. Samples were then thawed and centrifuged twice, with 5% TCA washing in between, and placed in Optiphase Highsafe II liquid scintillation cocktail. Radioactivity in the samples was measured on a Packard 2500 TR liquid scintillation counter.

Leucine-to-carbon empirical conversion factors (CFs) were calculated by dilution experiments, following the cumulative method that uses the slope of bacterial biomass against cumulative leucine incorporation at different time intervals, as detailed in Calvo-Díaz & Morán (2009). Briefly, 300 mL water samples from two depths – 5 m and DCM – were diluted (1:5) with 0.2-µm-filtered seawater and kept in 1.5-liter acid-cleaned polycarbonate bottles in the dark at in situ temperature (± 1ºC). Subsamples were then taken for Leu-incorporation and bacterial biomass (as described above) at intervals of 8-20 h. Incubations usually lasted up to 4 days or until bacteria reached a stationary growth phase. A minimum of four data points were used for each estimation. CFs varied considerably, but except for one station in May, values did not exceed 1.5 kg C mol Leu^{-1} (Table 1) with higher values usually corresponding to surface samples. In order to estimate bacterial production, empirical CFs were then applied to each station and depth according to the horizontal and vertical distributions of physico-chemical properties.

Finally, bacterioplankton bulk specific growth rates (SGR, d^{-1}) were estimated as follows:

$$SGR = \ln (1+(BP / BB))$$

with BP measured in µg C L^{-1} d^{-1} and BB in µg C L^{-1}.

Integration and statistical analyses

Integration of phytoplankton and heterotrophic prokaryote standing stocks and production rates was made from the surface down to 80 m depth using the trapezoidal method. Except for percentages, data were usually log-transformed in order to normalize and homogenize variances for further analysis. The assessment of the responses of heterotrophic prokaryotes to phytoplankton used the ordinary least squares (Model I) linear regression slopes rather than the more appropriate Model II or the correlation coefficients (usually strongly correlated with the slope values) in order to be comparable with previous studies (e.g. Wright & Coffin, 1984; Ducklow, 1992; Gasol and Duarte, 2000). All statistical analyses were done with JMP 7 software (SAS Institute).

RESULTS

Oceanographic characteristics significantly differed from March through September. While temperature increased as expected salinity showed little variation (data not shown). Consequently, stratification proceeded with mixed conditions in March, slightly stratified in May and strongly stratified in September. ANOVA tests for temperature and salinity of all depths <200 m (with Tukey HSD as post-hoc tests, all $P<0.001$, data not shown) indicated that the different stations sampled were more similar within than between cruises.

Phytoplankton and bacterioplankton biomass and production

Total Chl decreased with time (Table 2), with volumetric maxima of 2.98, 1.12 and 0.77 μg L^{-1} in March, May and September, respectively (Fig. 1A-C). Changes in Chl were caused by changes in the relative contribution and composition

of the different size-classes, including picophytoplankton (Mouriño-Carballido et al. submitted). While in winter and spring Chl peaked towards the surface (less marked in May), in September a distinct DCM could be observed at 60-80 m (Fig. 1 A,B,C).

Heterotrophic prokaryotic abundance (Fig. 1D-F) tended to peak at the surface in March, with a maximum value of 9.49×10^5 cells mL^{-1}. In contrast, maximum abundances in May and September (7.19×10^5 cells mL^{-1} and 7.01×10^5 cells mL^{-1}, respectively) were found deeper in the water column.

Bacterioplankton mean cell size increased during the sampled period (Table 2). Differences became more apparent when analyzing LNA and HNA cells separately (Fig. 1G, H, I). The usually larger HNA cells, as found in April/May and September, were significantly smaller (paired *t*-test, $p<0.001$, $n=57$) than LNA cells in March. There was an increase in HNA cell size from March through September in all layers, particularly conspicuous at the surface, whereas LNA cell volume decreased (Fig. 1 G, H, I, Table 2). The vertical decrease in HNA cell size was especially marked in the last cruise (Fig. 1 I).

The vertical distribution of the percent contribution of "Live" and HNA bacteria is shown in Fig. 1J-L. While %"Live" increased gradually along the year, from ca. 70% in March to ca 90% in September, %HNA values were approximately opposite to %"Live" ones (Table 2), with highest values in March (mean 57%) and similarly low values in May (46%) and September (48%). %HNA distribution in the water column was rather constant in March, increased with depth in May and decreased in September.

Following phytoplankton biomass, primary production rates (PP, Fig. 1M-O) peaked in March especially at the stations sampled later within the cruise, with

maxima reaching 100 μg C L^{-1} d^{-1}, and decreased gradually in the other two cruises (Table 2), down to values <5 μg C L^{-1} d^{-1} in September. Vertical decreases were especially marked in March (Fig. 1M) due to large biomass accumulation and enhanced light extinction (Estrada et al., in press/2014).

Leucine incorporation rates (1.6 – 480.8 pmol Leu L^{-1} h^{-1}) were in general higher earlier in the year (Table 2). However, variability in leucine-to-carbon CFs (Table 1) resulted in higher BP rates in May relative to the other two cruises (Table 2, Fig. 1M, N, O), reaching maxima >10 μg C L^{-1} d^{-1}. The lowest BP (0.07 μg C L^{-1} d^{-1}) was measured at the bottom layer in March (Fig. 1M). Consequently, bulk specific growth rates (SGR, mean 0.15 d^{-1}, range 0.003-1.52 d^{-1}) largely reflected these differences (Table 2). Expectedly, SGR scaled to "Live" cells (mean 0.24 d^{-1}, 0.007-1.68 d^{-1}) were substantially higher (2.3-fold on average) than total bacteria SGR.

Empirical assessment of the linkage between bacteria and primary producers

To approach the degree of coupling between bacterial growth and phytoplankton-derived resource supply we firstly looked into the relationships with volumetric units (Tables 3 and 4) and secondly addressed integrated data (Table 5).

Regarding heterotrophic and autotrophic standing stocks (Table 3), total prokaryotic abundances were significantly related to Chl in all cruises, with a pooled log-log slope of 0.37. The slope was 2-fold significantly higher in May than in September (Table 3, *t*-test, p=0.03), indicating a stronger relationship between bacteria and phytoplankton in post-bloom conditions. As expected, a very similar pattern with slightly higher slopes was obtained with bacterial biomass. Differences in

slopes among cruises became larger when we considered "Live" rather than total bacteria (Fig.2). In May, the linear regression of "Live" cell abundance to Chl had a slope of nearly 1, significantly higher than in March (*t*-test, p=0.0006), indicating a very strong response of bacteria to changes in phytoplankton standing stocks, whilst in September this relationship was not significant. Although production rates are analyzed in detail below, the relationship of volumetric BP to Chl had high slopes in March and May (Table 3): 1.28 and 1.43, respectively explaining 73% and 42% of total variance. No relationship was found in September, hence the pooled data regression explained only 11% of variance with a slope of 0.48.

Table 4 focuses on the relationships involving bacterial production and specific growth rates. When the BP and PP rates were compared (approach #1), the slopes were very similar for the first two cruises and the entire dataset. However, the percentages of explained variance differed largely, from 66% in March to 23% in May, with 37% for the overall regression (slope 0.35). The relationship between bacterial production (as a proxy for substrate supply rate) and bacterial biomass, suggested by Billen et al. (1990), and afterwards used by others (Ducklow, 1992; Gasol et al., 2002; Morán et al., 2010) as an indication of bottom-up control, was assessed both for total bacteria (approach #2) and for the "Live" fraction (approach #3). An overall slope of 0.15 would indicate almost inexistent bottom-up control. Nevertheless, the relationships improved in March and May, with higher slopes and percentages of variance explained (Table 4). However, all BB vs. BP slopes were substantially higher when considering only "Live" bacteria, especially in May and globally (0.48 and 0.32, respectively). These values would indicate a release of top-down control in the post-bloom (i.e. the smaller the slope, the more top-down control) particularly relevant for the "Live" bacteria. A different approach, based on the work

of Wright & Coffin (1984) as explained in Gasol et al. (2002), consists in exploring the relationship between bacterial specific growth rates and abundances (approach #4). Although the dependent variable includes the independent in the denominator and thus a spurious, negative relationship such as that found with all data pooled is expected, the relationship was positive in March (i.e. communities with more bacteria grew faster) and not significant in May and September (Table 4).

Mean integrated phytoplankton and bacterioplankton biomasses and production rates for the three periods are shown in Table 5. Temporal changes in phytoplankton biomass (PB) were usually accompanied by changes in PP (Fig. 3). BP values were in turn 7-fold higher in May than in the other two cruises although integrated BB was lowest in the post-bloom phase (Fig. 3). The percentage of integrated BB to PB increased with the progression of the cruises, from mean values of 17% (13-27%) in March, 47% (20-76%) in May and 65% (51-93%) in September. PB and BB were significantly correlated only in F2 ($r=0.71$, $p=0.023$, $n=10$). Lower values characterized the percentage of BP to PP and its temporal evolution also differed): mean integrated BP was 4% (2-11%) of integrated PP in March, it increased to 46% (9-101%) in May, and decreased to 23% (13-39) in September. Integrated bacterial and primary production rates were not significantly correlated in any cruise. Using the graphic approach of Billen & Becquevort (1991), we summarize the relationships for each cruise between heterotrophic prokaryotes and phytoplankton standing stocks (Fig. 4A) and production rates (Fig. 4B). In Fig. 4A, together with total values, we additionally included the abundance of HNA and "Live" cells. Finally, Fig. 5 illustrates the integrated production vs. integrated biomass relationships for both planktonic compartments in the bloom, post-bloom and stratification periods.

DISCUSSION

We aimed with this study to shed light on the role of heterotrophic prokaryotes in the fate of the annual yet undersampled winter-spring blooms in NW Mediterranean open waters. Variability in bulk phytoplankton and bacterioplankton standing stocks and production rates in the three periods starting with the 2009 algal bloom was similar to previous surveys conducted in the region's coastal and open waters (Pedrós-Alió et al., 1999; Lemée et al., 2002; Pulido-Villena et al., 2012; Van Wambeke et al., 2001), although primary production values as high as those reported here (>2 g C m^{-2} d^{-1}) have seldom been observed (Morán & Estrada, 2005; Siokou-Frangou et al., 2010). Analogous to phytoplankton evolution, the highest heterotrophic prokaryote abundance was registered during the peak of the bloom in surface waters (Figs. 1 and 3), exceeding 1.5 10^6 cells mL^{-1}, a high value for offshore waters. Despite high prokaryotic abundances, both BP (Fig. 1) and SGRs were relatively low during the bloom as compared to the post-bloom, with values quite similar to those observed in the stratification phase (Table 2).

This study allowed us also to assess the vertical patterns of bacterial assemblages (Fig. 1). Bacterial production showed ranges similar to previous reports (Christaki et al., 1996; Christaki et al., 1998; Pedrós-Alió et al., 1999; Vaqué et al., 2001; Lemée et al., 2002; Pulido-Villena et al., 2012). Similarly to PP, BP tended to peak in the upper layers, although consistently steeper vertical declines were found for PP (Fig. 1 M,N,O). Decreases in bacterial abundance with depth were different however for the single-cell physiological fractions of LNA, HNA and "Live" cells. While %"Live" cells generally decreased, at least within the upper 40 m, as also observed annually in the S Bay of Biscay (Morán & Calvo-Díaz 2009), %HNA

vertical distributions differed largely among cruises (Fig. 1J,K,L). The strong summer stratification resulted in an initial %HNA decrease with depth rather than the consistent increase observed here in May and elsewhere (Morán & Calvo-Díaz, 2009; La Ferla et al., 2012).

Contrary to most reports (Bouvier et al., 2007), in our study LNA cells were uniformly larger than HNA cells (20% on average) during the bloom (Fig. 1G). This finding is not entirely novel (e.g., Calvo-Díaz & Morán, 2006; Bouvier et al., 2007) but had never been shown so clearly. The possibility that a strong preference of grazers (Gasol et al., 1999; Vaqué et al. 2001) or viruses (Bonilla-Findji et al. 2008) for HNA cells during the bloom decreased their mean size to such very low values cannot be discarded. However, this pressure should also be reflected in HNA cell abundance and yet %HNA values peaked during this period as previously found (Alonso-Sáez et al., 2008; Van Wambeke et al., 2011). In the post-bloom and stratification phases the situation reversed (Fig. 1H and I), with HNA cells being significantly larger than LNA cells and predominance of LNA cells (%HNA<50%) in the upper 60 m (Fig. 1K and L). One possibility is that in March we may have sampled a short-lived phenomenon (only seldom were LNA cells larger than HNA cells in spring in a decadal NE Atlantic time-series, Morán et al. submitted). On the other hand, the overall decrease from late winter through late summer in the cell size of LNA bacteria and the corresponding increase of HNA bacterial values agrees reasonably well with previous observations in the NW Mediterranean continental shelf (Vila-Costa et al. 2012) and with the seasonal cycle in temperate coastal waters (Morán et al., submitted). Accurate determinations of cell size are essential when converting abundance to biomass. The corresponding cellular carbon contents of LNA and HNA cells using Norland's (1993) equation ranged between 12.3 and 16.6, and

11.8 and 21.5 fg C $cell^{-1}$, respectively, resulting in total bacterial cellular carbon content of 12.6 to 18.7 fg C $cell^{-1}$ (mean 14.6 fg C $cell^{-1}$), similar to previous studies in the Mediterranean and elsewhere (cf. Table 5 in La Ferla et al., 2012), but much lower than the recent estimates provided by these authors for epipelagic waters (46 fg C $cell^{-1}$) after image analysis of DAPI-stained samples (La Ferla et al., 2012). This discrepancy may be partially attributable to our assumption that all cells had spherical forms.

Early claims that HNA cells were more active than LNA cells (Gasol et al., 1999; Lebaron et al., 2001; Lebaron et al., 2002; Morán et al., 2007) have been substantiated by recent work supporting that each group is made up of different phylotypes (Schattenhofer et al., 2009; Vila-Costa et al., 2012). Vila-Costa et al. (2012) showed that in surface waters of the NW Mediterranean HNA bacteria were mainly composed by Rhodobacterales, SAR116 and Bacteroidetes, groups generally associated with blooms (Wietz et al., 2010). Other authors have found close associations of HNA cells with phytoplankton extracellular production (Morán et al., 2011). These findings, and the fact that in temperate waters HNA cells prevail in the colder season (Calvo-Díaz and Morán, 2006) as also found in this study (cf. Fig. 1J with 1K, 1L) , may explain the higher association of this group with Chl during the March bloom although the slopes of LNA and HNA abundance vs. chlorophyll (Table 3) were not significantly different.at any period.. That HNA cells were not consistently associated with phytoplankton nor were necessarily the most active fraction of the community leaves “Live” cells as a possibly better indicator of activity. The general relationships shown in Tables 3 and 4 clearly improved when “Live” cells were chosen rather than total, LNA or HNA bacteria.

Compared with the Bird & Kalff's (1984) relationship between heterotrophic prokaryotes abundance and chlorophyll for all aquatic systems, total values in 2009 tended to lie above their line in all 3 stages except for March highest values (Fig. 2A), whilst "Live" heterotrophic prokaryotes in that month appeared below the line (Fig. 2B). A closer look at the relationship with chlorophyll in the post-bloom and stratification periods also indicates the switch in the relative importance of bacteria versus algae in the microbial food web of open NW Mediterranean waters at different phases of the annual cycle. Gasol & Duarte (2000) synthesized the relationships between bacterial abundance and chlorophyll in aquatic ecosystems, resulting in an average log-log slope of 0.47. In our study case, the average slope of the entire dataset was 0.37 (Table 3), thus suggesting that heterotrophic prokaryotes and phytoplankton were loosely coupled on a whole annual basis, with the only exception of the post-bloom phase. Still, the slopes of BP vs. Chl were similarly high during both the bloom and the post-bloom phases. According to White et al. (1991), our slopes of 1.28 and 1.43 (Table 3) would be more similar to estuaries and coastal waters (1.23) than to open ocean waters in those periods. Due to the absence of a significant relationship during stratification the overall slope was 0.48, a value closer to the marine environments average (0.50). This shows a singularly marked seasonal response of heterotrophic prokaryotes production to phytoplanktonic blooms that is obscured when analyzed at larger temporal scales.

As the relationships between bacterioplankton and phytoplankton biomass and production were not constant but changed seasonally (Table 5, Figs. 3-5), a better estimate of the trophic dependence between both planktonic groups should be made with production rather than with standing stocks. First of all, the choice of the conversion factors is key in order to obtain realistic estimates of bacterial biomass

production from leucine incorporation rates (Simon and Azam, 1989; Kirchman and Ducklow, 1993; Alonso-Sáez et al., 2007; Calvo-Díaz and Morán, 2009). The use of the theoretical CF of 1.5 Kg C mol Leu^{-1} would have resulted in seriously overestimated production values. Secondly, it is important to ensure that all primary production (particulate plus dissolved) has been measured. Unfortunately, the latter fraction, with variable contributions usually below 30% in the NW Mediterranean (Morán et al., 2002; López-Sandoval et al., 2011) was not included here. Yet, we assessed the bacterial bottom-up control in NW Mediterranean offshore waters using four different empirical approaches (Table 4), not yet tried simultaneously to the best of our knowledge

In the first approach, the pooled dataset slope of 0.35 in the response of BP to PP (Table 4, #1) was significantly lower than the 0.81 value found for marine systems by Cole et al. (1988) (*t*-test, $p<0.001$), and also than the mean slope of 0.67 compiled by Gasol & Duarte (2000) for all aquatic systems, indicating an apparently weak response of bacterial production to the organic carbon released by phytoplankton. Very similar slopes characterized the bloom and post-bloom cruises separately, although the highest apparent association between PP and BP was found in March, explaining an appreciable 66% of total variance. Altogether, these results suggest that the low dependence of bacteria on phytoplankton in NW Mediterranean offshore waters can only be expected earlier in the growth season. Furthermore, the percentage of integrated bacterial to algal biomass, ranging from 18% in March to 65% in September, confirmed the well-known trend of increasing heterotrophy within the plankton community as summer stratification develops and nutrients become scarce causing PP declines (Gasol et al., 1997; Duarte et al., 2000). Previous work in the NW

Mediterranean found a summer value of 69% at the deepest, most oligotrophic station studied (Pedrós-Alió et al., 1999), a value very close to that observed in September.

In the second and third approaches, the response of bacterial biomass to the available substrate supply, using BP as a proxy of the latter variable (Table 4), indicates the degree of total bottom-up control of bacteria, including that mediated by phytoplankton (Morán et al., 2010) discussed in the previous paragraph. The strongest relationship with total BB (#2) was detected in May with a slope of 0.32, although this value was statistically indistinguishable from the March one and virtually the same found by Pulido-Villena et al. (2012) in a Mediterranean data set compilation. The pooled data slope was roughly half these values (0.15). Following Ducklow's (1992) argumentation, slope values between 0.2 and 0.4 would indicate weak bottom-up control and thus noticeable control of bacteria by predators. Concerning the "Live" cells biomass vs. BP relationship (Table 4, #3), the overall slope was twice higher than that of total BB. During the stratification in F3, however, neither total nor "Live" cells biomass showed linkages with production, which was not correlated either to the activity primary producers (Table 3).

Differences in response among cruises were most clear in the fourth approach relating SGRs to total prokaryotic abundance. The positive response with a slope statistically indistinguishable of 1 during the bloom (Table 4, #4) indicates equilibrium between regulation by predators and nutrient availability since SGR was directly proportional to changes in abundance. Conversely, the negative although marginally significant ($p=0.07$) response in the stratification period indicates that bacteria were under strong resource limitation: more bacteria resulted in lower growth rates. The negative response of the entire dataset would indicate that epipelagic

bacteria in NW Mediterranean open waters were largely predator-limited, as postulated earlier (Gasol et al., 2002; Pulido-Villena et al., 2012).

The four approaches used can be summarized as follows: at the onset of the winter-spring bloom phytoplankton start supplying the DOC needed for the growth of the heterotrophic prokaryote phylotypes most capable to respond (Sarmento and Gasol, 2012), albeit only in the post-bloom phase this is reflected in bulk activity increase. While the estimated relative strength of total and phytoplankton mediated bottom-up controls versus top-down might change depending on the approach used, the bloom and post-bloom phases were in any case much more similar to each other than the stratification period, in which bacteria were clearly not bottom-up controlled and totally independent of extant planktonic algae.

Our findings reveal that bottom-up control of bacterial communities is not general in open waters of the NW Mediterranean. With the limitations previously expressed, the strength of phytoplankton-bacterioplankton coupling changed throughout the year simultaneously to changes in the physiological structure of heterotrophic prokaryotes. The responses of BB and BP to the phytoplankton bloom were contrasting, noticeably the faster response of BB, resulting in a very different situation of both variables in the post-bloom period. Fig. 4 shows how the dynamics of phytoplankton and bacterioplankton starting with the winter bloom described a flat clockwise trajectory for total standing stocks and a counterclockwise trajectory for production. Fig. 4A also shows that the strong variability in mean integrated phytoplankton biomass (characterized by a CV of 81%, n=3) were only slightly mirrored by bulk bacterioplankton values (CV 11%), whereas the much more similar ranges of variability in mean carbon production (CV 83% and 117% for phytoplankton and bacterioplankton, respectively) were apparently temporally

uncoupled by ca. 1.5 months lag (Fig. 3B). Interestingly, in Fig. 4A the trajectories of HNA and "Live" cells were opposed, again suggesting that "Live" cells better reflected changes in activity than HNA or LNA groups (the latter with virtually no variation in mean abundance among cruises, data not shown). While changes in mean integrated biomass and production rates tended to covary positively for phytoplankton over the entire period (Fig. 5), only during the post-bloom period bacteria showed integrated production to biomass ratios comparable to those of phytoplankton (0.2-0.3 d^{-1}). This study suggests that offshore Mediterranean heterotrophic prokaryotes are only bottom-up controlled by phytoplankton early in the growth season, with different timing for biomass and production, whereas they become totally independent of phytoplankton during late-summer stratification.

Acknowledgements: We thank Mikel Latasa, the FAMOSO project and cruises leader, and the rest of the scientists and crew on board of RV "Sarmiento de Gamboa". This work was financially supported by grant FAMOSO (CTM2008-06261-C03/MAR) and, partially, also by grant HotMix (CTM2011-30010/MAR) and the Grup de Recerca de la Generalitat de Catalunya (2009SGR/1177). A.G. had a fellowship from the Portuguese FCT and L. F.-V. was supported by an IEO fellowship.

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Table 1. Empirical Leu-to-C conversion factors (CF, kg C mol Leu^{-1}) and corresponding *in situ* heterotrophic prokaryotic abundance (Prok, x 10^5 cells mL^{-1}) and leucine incorporation rates (LIR, pmol Leu L^{-1} d^{-1}). Also shown is the time span used for the estimations. SE, standard error of the estimate.

Cruise	Stn	Time span (d)	Depth (m)	Prok	LIR	CF ± SE
F1	11	2.93	5	-		1.49 ± 0.66
	11	2.93	65	-		0.43 ± 0.11
	21	2.89	25	6.57	77.3	0.19 ± 0.05
	21	2.89	65	2.96	25.4	0.11 ± 0.07
F2	6	2.12	5	7.04	125.0	1.25 ± 0.56
	6	2.12	45	1.04	63.9	0.70 ± 0.48
	15	2.26	30	7.84	178.9	0.47 ± 0.16
	15	2.26	80	3.51	24.2	0.54 ± 0.20
	30	1.85	5	2.11	40.2	0.34 ± 0.09
	30	1.85	60	3.12	39.4	0.92 ± 0.20
	36	2.00	5	2.00	126.8	3.73 ± 0.64
F3	5	1.24	5	8.80	27.4	0.57 ± 0.09
	12	1.22	5	5.24	14.5	0.53 ± 1.79
	20	1.26	5	4.70	53.5	0.24 ± 0.01

Table 2. Results of ANOVA tests for significant differences between the cruises, and post-hoc Tukey-Kramer HSD tests indicating which cruises have significantly different values (indicated with a > or < sign), or not significantly different values (=). All depth resolved data included. NS, not significant.

Variable	P-value	Cruise differences
Chlorophyll	<0.0001	F1 > F2 = F3
Primary production	0.0016	F1 > F2 = F3
LNA bacterial abundance	NS	
HNA bacterial abundance	<0.0001	F1 > F2 = F3
All bacterial abundance	0.0016	F1 > F2 = F3
%HNA	<0.0001	F1 > F2 = F3
"Live" cell abundance	0.048	F1 = F2 > F3
"Dead" cell abundance	<0.0001	F1 > F2 > F3
%"Live"	<0.0001	F3 > F2 > F1
LNA cell size	<0.0001	F1 > F2 = F3
HNA cell size	<0.0001	F2 = F3 > F1
All bacteria cell size	0.0033	F2 = F3 > F1
Bacterial biomass	NS	
"Live" bacterial biomass	0.01	F1 = F2 > F3
Leucine incorporation rate	0.0004	F1 > F2 = F3
Empirical conversion factor	<0.0001	F2 > F1 = F3
Bacterial production	<0.0001	F2 > F1 = F3
Specific growth rate	<0.0001	F2 > F1 = F3

Table 3. Log-log relationship (slope ± SE) between volumetric values of chlorophyll and total heterotrophic prokaryotic abundance (Prok), HNA and LNA bacteria, "Live" bacteria, , bacterial biomass (BB) and bacterial production (BP) and primary production (PP) for all data pooled. n, number of samples. Asterisks denote level of significance: -, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. r^2, coefficient of determination.

Cruise		Prok		HNA		LNA		"Live"		BB	
	n	slope	r^2	slope	r^2	slope	r^2	slope	r^2	slope	r^2
F1	40	0.43 ± 0.07***	0.51	0.47 ± 0.07***	0.53	0.36 ± 0.07***	0.43	0.43 ± 0.07***	0.52	0.46 ± 0.08***	0.47
F2	60	0.62 ± 0.13***	0.29	0.45 ± 0.13**	0.17	0.76 ± 0.13***	0.36	0.92 ± 0.12***	0.50	0.70 ± 0.12***	0.36
F3	24	0.29 ± 0.08**	0.39	0.20 ± 0.08*	0.20	0.37 ± 0.07***	0.53	-	-	0.25 ± 0.09*	0.26
All	124	0.37 ± 0.05***	0.31	0.39 ± 0.05***	0.33	0.33 ± 0.05***	0.24	0.46 ± 0.05***	0.38	0.38 ± 0.05***	0.31

	BP		
	N	slope	r^2
F1	19	1.28 ± 0.19***	0.73
F2	32	1.43 ± 0.30***	0.42
F3	24	-	-
All	75	0.48 ± 0.16**	0.11

Table 4. Different empirical assessments of the linkage between heterotrophic prokaryotes and primary producers (Model I linear regressions between Y and X variables as indicated). All values in logarithmic scale. n, number of samples (values for #2 also applicable to #3 and #4). Asterisks denote level of significance: -, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. r^2, coefficient of determination.

	#1			# 2			#3			#4	
Y	Bacterial production			Total bacterial biomass			"Live" bacterial biomass			Bacterial specific growth rate	
X	Primary production			Bacterial production			Bacterial production			Prokaryotic abundance	
Cruise	n	slope	r^2	n	slope	r^2	slope		r^2	slope	r^2
F1	19	0.37 ± 0.06***	0.66	21	0.25 ± 0.06***	0.49	0.29 ± 0.06***		0.59	1.20 ± 0.38**	0.35
F2	32	0.35 ± 0.11**	0.23	50	0.32 ± 0.07***	0.33	0.48 ± 0.07***		0.52	-	-
F3	23	-	-	25	-	-	-		-	-	-
All	73	0.35 ± 0.05***	0.37	96	0.15 ± 0.0**	0.09	0.32 ± 0.05	< 0.001	0.29	-0.38 ± 0.18*	0.04

Table 5. Integrated values (down to 80 m) of total phytoplankton biomass (PB, mg C m^{-2}), primary production (PP, mg C m^{-2} d^{-1}), bacterial biomass (BB, mg C m^{-2}) and bacterial production (BP, mg C m^{-2} d^{-1}). Also shown the averages for each cruise.

Cruise	Station	Month	PB	PP	BP	BB
F1	13	March	2558	465	52	49
	16		4631	646	11	662
	19		-	-	-	761
	21		4874	673	12	437
	24		5595	1133	67	852
	27		4232	1411	41	1131
	31		7979	1817	29	1350
	Mean ± SE		4978 ± 783	1024 ± 214	35 ± 9	812 ± 125
F2	5	April/May	1392	191	166	722
	10		1062	281	98	805
	15		1226	349	114	726
	20		1030	505	52	604
	26		883	336	47	406
	30		1021	294	41	363
	32		1049	423	40	222
	36		1353	643	652	277
	40		1844	581	577	958
	44		3234	881	493	1650
	Mean ± SE		1410 ± 221	448 ± 66	228 ± 77	673 ± 133
F3.1	5	September	1355	106	34	973
	8		999	95	37	931
	12		1101	184	27	607
F3.2	15		1251	134	19	649
	20		1357	186	24	699
	Mean ± SE		1213 ± 71	141 ±19	28 ± 3	772 ± 75

Legends to figures

Figure 1. Pooled vertical profiles in the three FAMOSO cruises (left, F1; middle, F2; right, F3) of chlorophyll concentration (μg L^{-1}, A-C), total heterotrophic prokaryotes abundance (cells mL^{-1}, D-F), cell size of LNA (grey dots) and HNA (black dots) bacteria (μm^3, G-I), percent contribution of HNA and "Live" cells (black and white dots, respectively, J-L) and production rates of heterotrophic bacteria and phytoplankton (black and grey dots, respectively, μg C L^{-1} d^{-1}, M-O). White squares in A-F represent mean values for 0-25, 25-50, 50-75 and 75-100 depth intervals.

Figure 2. Log-log relationships between the abundance of total heterotrophic prokaryotes (A) and "Live" cells (B) with chlorophyll. Fitted lines represent significant regressions detailed in Table 3. The long-dashed line in (A) is Bird and Kalff's (1984) overall equation for fresh and marine waters.

Figure 3. Temporal variability of mean integrated values of phytoplankton and heterotrophic prokaryotes biomass (A) and production rates (B) in 2009 in offshore NW Mediterranean waters. Grey and black bars represent phytoplankton and heterotrophic prokaryotes, respectively. Dates shown represent the average of all dates within cruises with microbial plankton data available.

Figure 4. Schematic temporal evolution of phytoplankton and bacteria in the NW Mediterranean according to Billen & Becquevork (1991), showing mean integrated values (± SE) of standing stocks (A) and production rates (B) at each stage. Black dots in (A) are total values while white and grey dots represent abundances of HNA and "Live" bacteria, respectively. B, bloom; P-B, post-bloom; S, stratification.

Figure 5. Relationship between the integrated values (mean ± SE) of production versus phytoplankton biomass (black dots) and of bacterial production versus bacterioplankton biomass (grey dots) for the 3 periods. Codes as in Fig. 4

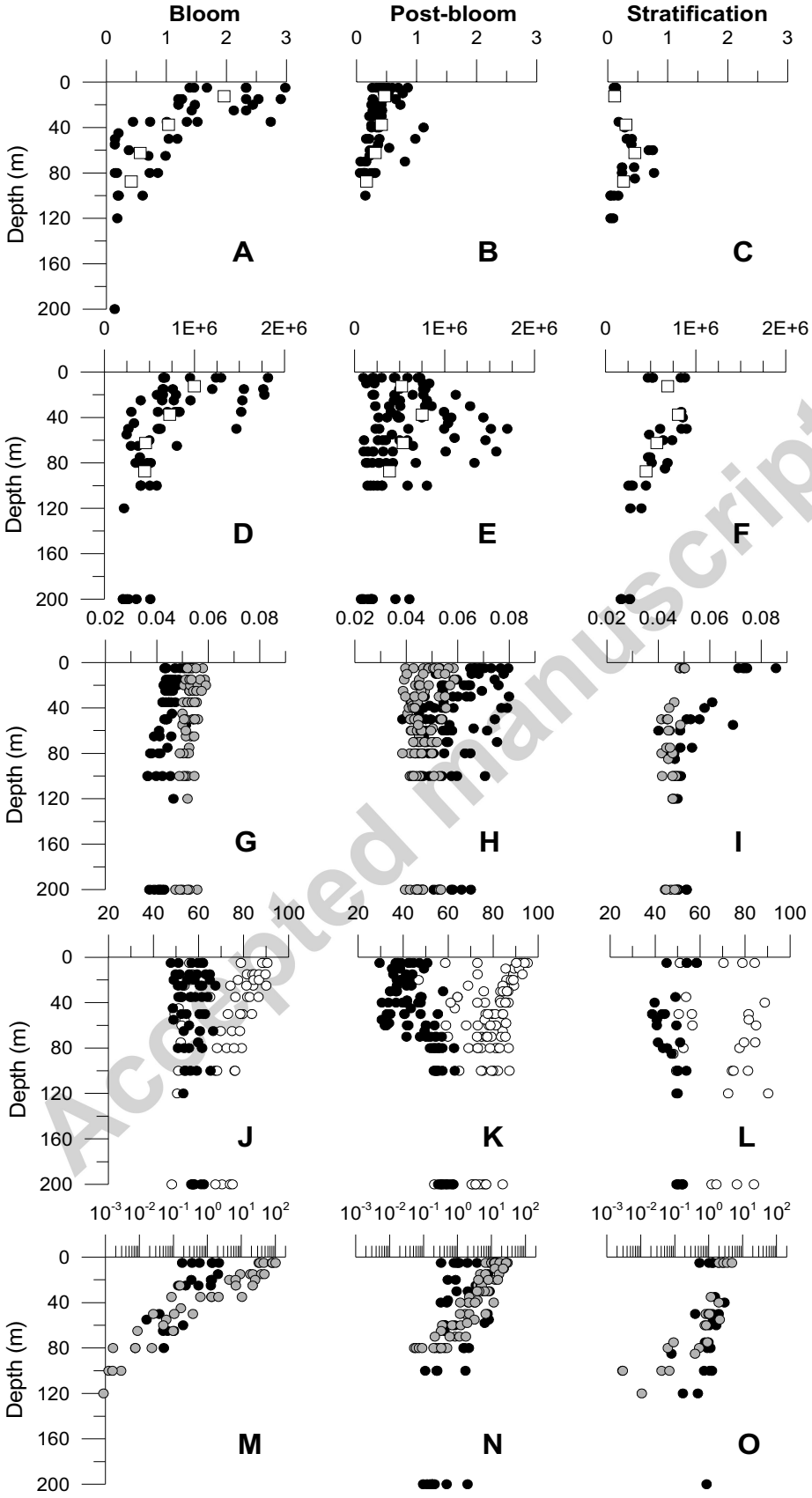

Fig. 1

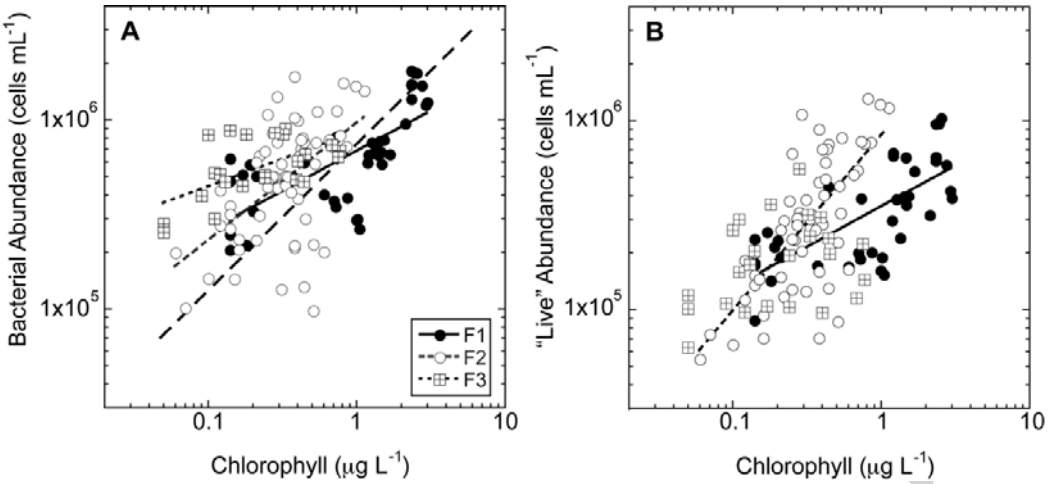

Fig. 2

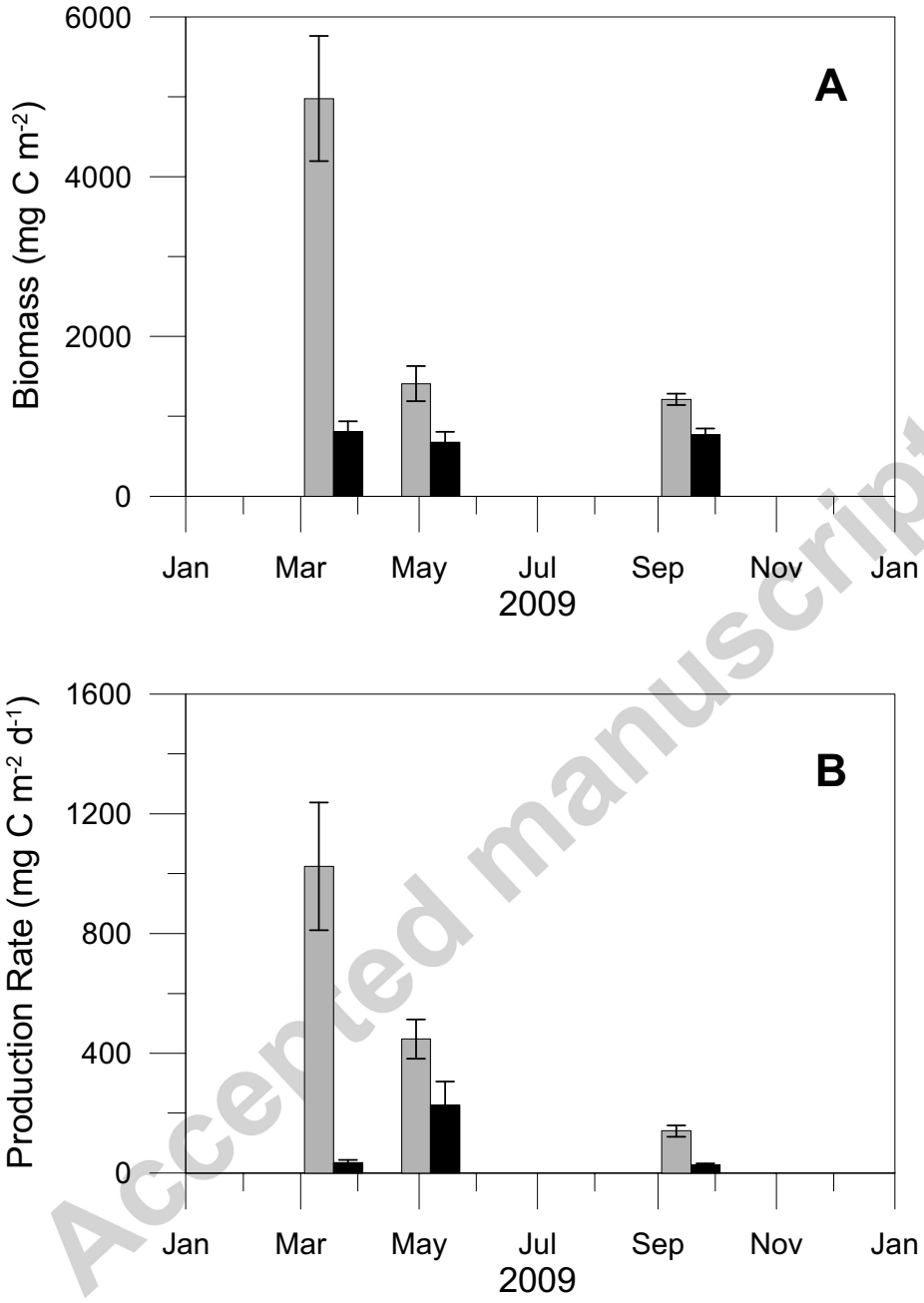

Fig. 3

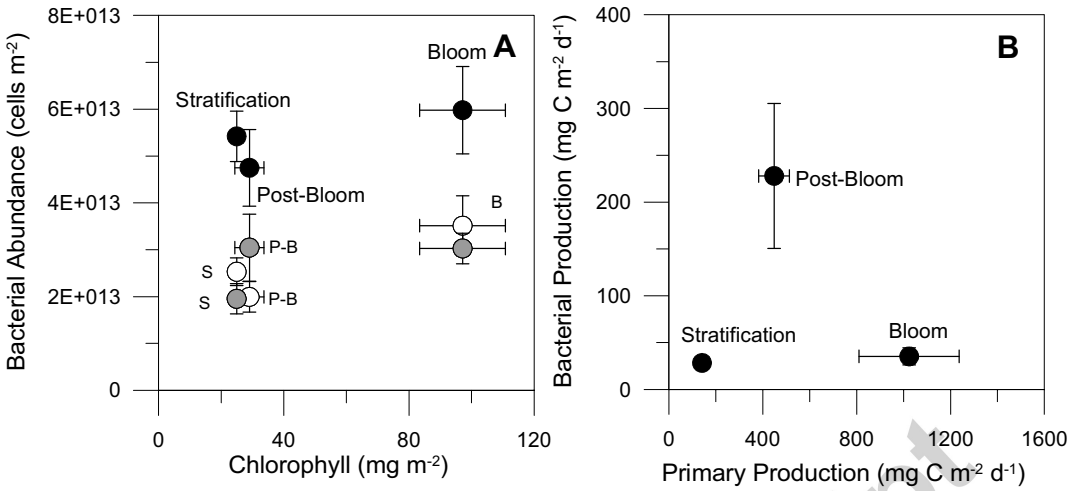

Fig. 4

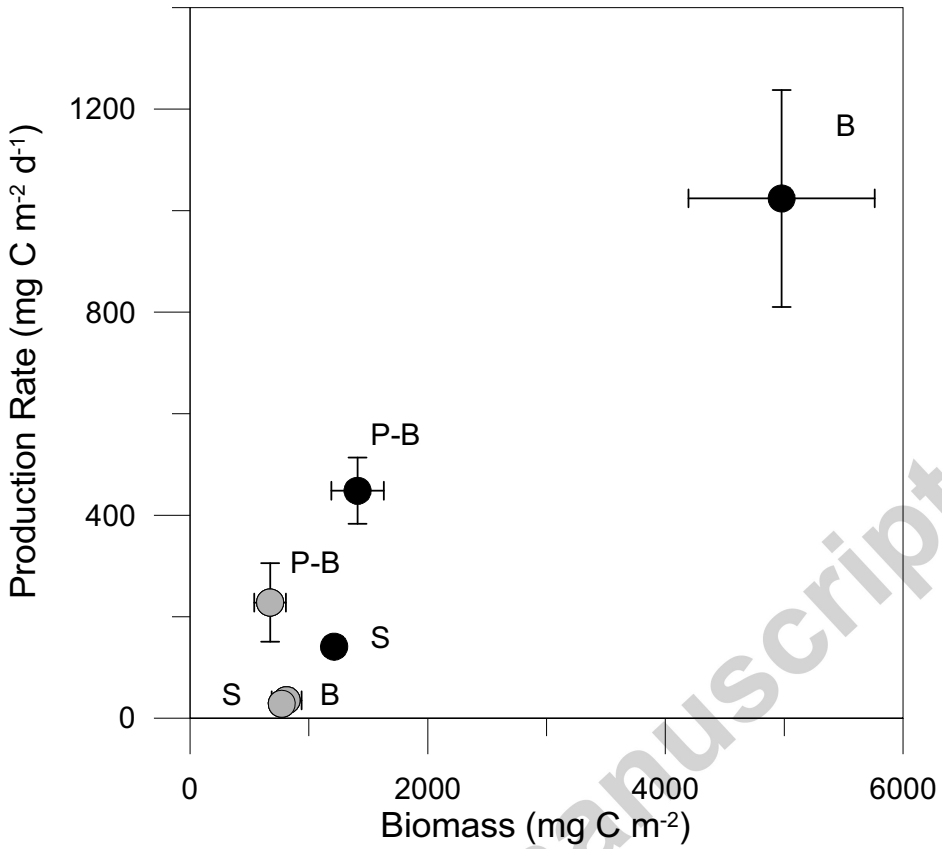

Fig. 5

HIGHLIGHTS

- Heterotrophic prokaryotes and phytoplankton were studied in the open NW Mediterranean
- Primary production and bacterial production peaks were lagged by ca. 1.5 months
- Bacterial single-cell properties correlated better with phytoplankton than bulk data
- Bacteria were bottom-up controlled by algal DOM only in the bloom and post-bloom