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France Van Wambeke [a,*], Urania Christaki [b], Micheline Bianchi [a], Stella Psarra [c], Anastasios Tselepides [c]

[a] *Microbiologie Marine, C.N.R.S., EP 2032, Campus de Luminy, Case 907, 13288 Marseille Cedex 9, France*

[b] *National Centre for Marine Research, Aghios Kosmas Hellinikon, 166 04 Athens, Greece*

[c] *Institute of Marine Biology of Crete, P.O. Box 2214, Heraklion, Crete, Greece*

Abstract

In March and September 1995, bacterial production was measured by the ^{3}H-leucine method in the oligotrophic Cretan Sea (Aegean Sea, Eastern Mediterranean) in the framework of the CINCS/MTP program. Samples were obtained from four stations (a coastal, a continental shelf and 2 open-sea stations) for the construction of vertical profiles of bacterial abundance and production. Bacterial production ranged from 0.1 μg C m^{-3} h^{-1} at 1500 m depth, to 82 μg C m^{-3} h^{-1} in March at 50 m at the coastal station. Higher bacterial integrated production was observed in March at the coastal station (131 mg C m^{-2} d^{-1} for the 0–100 m layer). Bacterial production, integrated through the water-column, was similar in March and September for the open-sea stations (60–70 mg C m^{-2} d^{-1}). Relative to production, bacterial concentrations varied little between stations and seasons ranging from 9×10^{5} ml^{-1} to 3×10^{5} ml^{-1}. Relationships between bacterial biomass and bacterial production indicated seasonal differences, likely reflecting resource limitation of bacterial biomass in March (bloom situation), and predator limitation of bacterial biomass in September (post-bloom situation).

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* Corresponding author.

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1. Introduction

Microbial food webs have been the focus of many studies over the last two decades, following the seminal paper of Pomeroy (1974). In general terms, Dortch and Packard (1989) proposed that food webs in eutrophic waters are dominated by the biomass of primary producers while food webs in oligotrophic waters are dominated by the biomass of decomposers. In oligotrophic oceans, bacterial biomass and activities can be the major factor in food web structure, controlling nutrient cycling pathways, and biochemical dynamics (Cho & Azam, 1990).

Of all oligotrophic marine environments, the Eastern Mediterranean is perhaps the system for which the least amount of data exist. Apart from the studies of Zohary and Robarts (1992) and Robarts, Zohary, Waiser and Yacobi (1996) in the Levantine Basin, there are no other published data, to our knowledge, dealing with bacterial production in the area. Given the absence of any bacterial production measurements in the extremely oligotrophic Southern Aegean Sea, the main objective of our study was to quantify this parameter and to assess the fluxes of carbon cycling through the microbial food web within the pelagic zone. The results are to be integrated in the framework of CINCS/MTP program, whose main topic was the benthic-pelagic coupling in the Cretan Sea (South Aegean Sea, NE Mediterranean).

In pelagic processes, the proportion of primary production cycling through the microbial loop can be used as an index of the coupling between heterotrophic and autotrophic processes in the water column (Cole, Findlay & Pace, 1988; Hoch & Kirchman, 1993), i.e. as an indicator of the possible fate of phytoplankton-derived material in the Cretan Sea. To facilitate flux comparisons in terms of carbon, special attention was given to the method used (incorporation of ^{3}H-leucine) which can be directly converted into carbon units. Time and concentration series experiments were carried out to measure bacterial production while testing the possibility of unspecific labeling. Bacterial production and biomass were investigated during March (spring situation) and September 1995 (post-bloom situation), along the transect lying northwards of the city of Heraklion (Crete, Greece).

2. Materials and methods

Bacterial production was studied during two cruises in March (February 28–March 2) and September (5–7) 1995, aboard the R/V *Philia*. Samples were collected along

a transect along 25°06′ E at station D2 (coastal station, 100 m max. depth 35°23′ N) and D4 (continental slope station, 540 m max. depth, 35°30′ N), where primary production was simultaneously measured, also at two open-sea stations D6 (940 m max. depth, 35°40′ N) and D7 (1570 m max. depth, 35°45′ N). Water samples were taken around midday in 5 l Niskin bottles wherever possible from standard depths of (5, 10, 20, 30, 50, 75, 100, 200, 300, 500, 1000 and 1500 m). These were processed immediately onboard.

Seawater samples for bacteria enumeration were preserved in buffered formalin (2% w/v final concentration) and stored at 4°C. Slides for microscopical counts were prepared (including filtration and DAPI staining, 2500 μg l^{-1} final concentration) within 24 h of sampling. Slides were stored frozen until examination with an epifluorescence microscope equipped with an image analysis system (Van Wambeke, 1995). Cell numbers were converted to biomass, assuming each cell contained 20 fg C (Lee & Fuhrman, 1987).

Bacterial production was estimated by the ^{3}H-leucine approach (Kirchman, Newell & Hodson, 1986). For each depth, duplicate samples and a control were incubated with 1 nM L-[4,5 ^{3}H]-leucine (specific activity 128 Ci $mmol^{-1}$ in March, 158 Ci $mmol^{-1}$ in September) +18 nM non radioactive leucine. All subsamples were incubated in the dark, at sea surface temperatures (around 15°C in March and 26°C in September) in a flow-through on-deck incubator flushed with seawater drawn from 3 m depth. The volume and the incubation time varied, according to the sampling depth and the expected production rates: 20–150 ml and 2–12 hours, respectively. At the end of incubation samples were killed with formalin (2% final concentration).

The duplicates and the control were filtered through 0.2 μm Millipore filters (25 mm diameter, Type GS). When the vacuum was turned off, the funnels were filled with 10 ml ice-cold 5% trichloroacetic acid (TCA), held for 10 min and refiltered. The filters were then washed 3 times with ice-cold 5% TCA. Various authors have shown that there are no significant differences in results obtained after the use of either cold or hot TCA for protein precipitation (Servais, 1995; Chin-Leo & Kirchman, 1988; Riemann & Azam, 1992). We therefore used the cold-TCA procedure instead of hot-TCA as originally described with the leucine method (Kirchman et al., 1986), because it was faster and easier to use routinely at sea. Ethyl acetate (1 ml) was added to the vials to dissolve the filters and the samples were radioassayed with BECKMAN LS 1800 Scintillation Counter in 10 ml of fluor (PCS, Amersham).

Bacterial production was calculated according to Kirchman (1993) from ^{3}H-leucine incorporation rates. Determination of the isotopic dilution factor is described below. Because all samples were incubated at surface water temperature in a water bath set up on board, leucine incorporation rates were corrected for temperature using an Arrhenuis equation experimentally determined using bacterial production data from the Atlantic ocean (Q_{10}=2.6).

On each cruise, time series experiments were carried out on surface waters where activity was likely to be high and in deeper waters where activity was likely to be lower. The aim was to determine the appropriate incubation time for different levels of activity. Concentration kinetics were also examined in order to verify that the concentration of leucine added (19 nM) was sufficient to saturate incorporation and

to check whether or not isotopic dilution was negligible. For this purpose, constant concentrations of labeled leucine (1 nM) and five different concentrations of non-radioactive leucine (1.8–37 nM) were added to different subsamples. The plot of *T*/*f* (radioactive leucine incorporated per unit time) against leucine concentration has permitted the calculation of an index of isotopic dilution (*X* intercept) and the *V*max (inverse of the slope, Pollard & Moriarty, 1984; Kirchman et al., 1986). During September 1995, the possibility of incorporation of the label in the lipid fraction was examined. At some depths, the radioactivity retained on two sets of filters was compared. One set was rinsed with TCA only while the second set was further rinsed with ice-cold 80% ethanol (Wicks & Robarts, 1988).

3. Results and discussion

3.1. Assessment of optimal protocol

During both cruises, ^{3}H-leucine incorporation into bacterial protein was linear over the first 4 h of incubation in the surface waters (5 m and 50 m, stations D2 and D4). For the deeper layers, incorporation was linear up to 12 h in March (300 m, D4) and September (300 m D4, 1500 m D7). The concentration kinetics followed a Michaelis–Menten model and the transformed data showed the expected linear regression in March (5, 50 and 300 m stations D2 and D4). However, a continuous increase of leucine incorporation rate with increased concentration of leucine was observed in September in the deeper samples. This artifact has already been described in estuarine samples, where leucine was not saturated even up to 333 nM concentration (Hollibaugh & Wong, 1992). This can be explained by the presence of bacteria (such as attached bacteria) adapted to high concentrations of leucine or by leucine assimilation by other micro-organisms (Logan & Fleury, 1993). In all other interpretable cases, results showed that, even with a concentration of 18 nM of leucine added, more than 90% of the *V*max was reached, indicating conditions of saturation. The degree of participation of the leucine added to the leucine incorporation rate in proteins was always more than 90% for the upper layers (Table 1), showing negligible isotopic dilution by de novo synthesis and/or natural leucine concentration. In con-

Table 1
Kinetic parameters calculated from the isotopic dilution plots. DP is the degree of participation of added leucine to the leucine incorporation rate and ID is the isotopic dilution factor

	*V*max (pmole l^{-1} h^{-1})	*X* intercept (nM)	DP (%)	ID
March, D4, 5 m	9.1	1.14	94	1.06
September, D2, 5 m	3.7	1.64	92	1.08
September, D2, 50 m	9.3	2.24	89	1.12
March, D4, 300 m	0.55	11.1	62	1.61

trast, the degree of participation (DP) value was about 62% for deeper layers. Thus, we applied an isotopic dilution of 1 in both seasons for samples down to 200 m, and a factor 1.6 for deeper layers.

The lipid labeling was checked during the second cruise, in September at station D7. The results showed good agreement of leucine incorporation rates at all depths tested. Activities were not statistically different with or without an additional ethanol rinse (Wilcoxon test, 5 pairs of data, p=0.89) implying no lipid incorporation of radioactive leucine.

3.2. Vertical profiles

Bacterial production in the surface layer varied considerably (Fig. 1). Generally, peaks of bacterial production and bacterial abundance coincided with primary production peaks (Psarra, Tselepides & Ignatiades, 2000). The maximum value of bacterial production was observed at the coastal station D2, where it reached 82 μg C m^{-3} h^{-1} at 50 m in March, and showed no decrease with depth (Fig. 1). Overall, bacterial production in the surface layers of the coastal and continental shelf stations

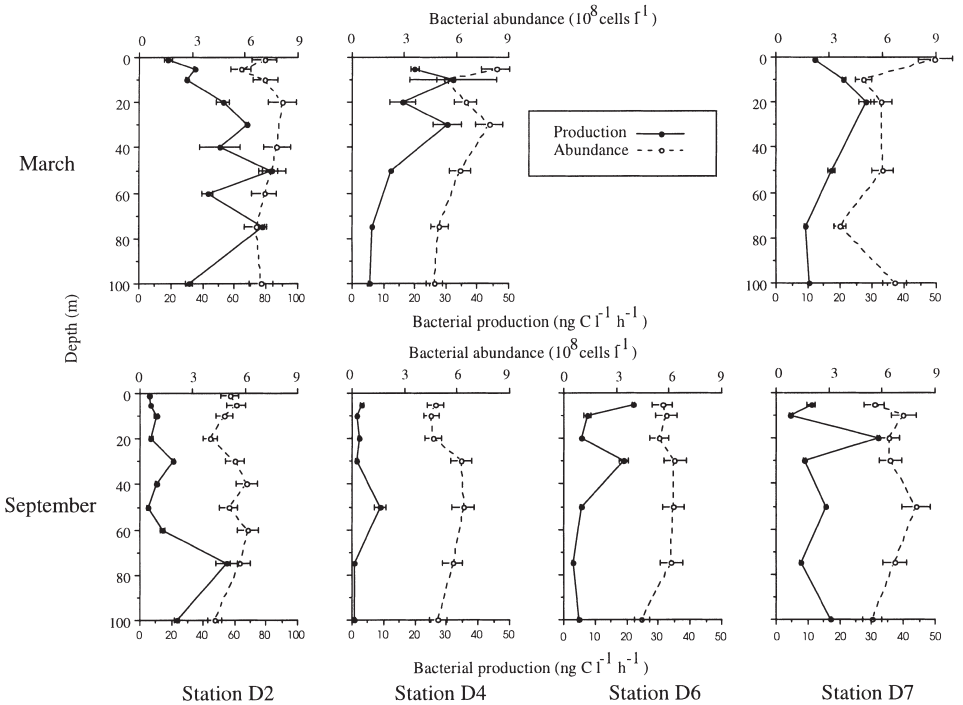

Fig. 1. Vertical profiles of bacterial production (solid line) and bacterial abundance (dotted line) in the upper 100 m layers at stations D2, D4, D6 and D7. Error bars represent difference between duplicates for bacterial production, and standard deviation for bacterial abundance.

was about half that reported in the Western Mediterranean (Christaki, Van Wambeke, Christou, Conan & Gaudy, 1996; Fernandez, Bianchi & Van Wambeke, 1994).

In the deep water stations, maximum bacterial production always occurred in the surface layers, although in some cases high values were also recorded deeper in the water column (e.g. 200 m D4, 500 and 1500 m D7, September, Table 2). At these stations, production in terms of cells per h, calculated assuming 20 fg C $cell^{-1}$ (Lee & Fuhrman, 1987), was low (0.1–1.0 10^9 cells m^{-3} h^{-1}) and similar to those reported by Zohary and Robarts (1992): 0.1–0.5 10^9 cells m^{-3} h^{-1}). Bacterial generation times, calculated from standing stocks and production data in terms of cells h^{-1}, were quite long, especially in the deeper layers where generation times may exceed 100 days. Similar results were also reported by Zohary and Robarts (1992), who assumed that this was an artefact originating from the use of a theoretical conversion factor for thymidine incorporation. While the leucine method allows for calculation of bacterial production directly from leucine incorporation rates independent of the cell biovolume (Simon & Azam, 1989), our calculations are sensitive to the conversion factor employed for cell carbon.

The long turn-over times of bacterial carbon may be explained in part by the possibility that a high percentage of the bacteria present in the deeper waters are inactive (Bianchi & Giuliano, 1996). Another possible source of error in surface samples of oligotrophic waters may be that the heterotrophic bacterial biomass may be overestimated because *Prochlorococcus* nuclei may be included in the counts of heterotrophic bacteria. For example, in central North Pacific Ocean *Prochlorococcus* contribution to total bacterial counts was 31% (Campbell, Nolla & Vaulot, 1994) and 15–20% in Central Equatorial Pacific (Landry, Kirshtein & Constantinou, 1996). Pigments were analyzed by HPLC during METEOR cruise in Cretan Sea (Vidussi, personal communication). Two stations were sampled at 35.8°N 24.1°E, and 35.8°N 24.2°E in January 1995. Concentrations of divynylchlorophyll *a* ranged from 11 to 22 ng l^{-1} at depths of 5–100 m, corresponding to 7.2–17×10^3 prochlorophytes cells ml^{-1} (assuming a minimum conversion factor of 1.5 fg div-chl a per cell (Caillau, Claustre, Vidussi, Marie & Vaulot, 1996)). For the eastern Mediterranean, the little data available on concentrations of *Prochlorococcus* suggest that peak concentrations

Table 2
Bacterial production (μg C m^{-3} h^{-1}) in deep layers (100 m to the bottom). Mean ± half of the difference between duplicates. M: March, S: September

Depth (m)	D4 M	D7 M	D4 S	D6 S	D7 S
100	5.5±0.1	10.4±0.1	0.8±0.3	4.7±0.1	17.3±0.2
200	5.5±0.2	0.64±0.3	16.6±0.5	1.4±0.1	3.9±0.5
300	2.1±0.3	1.4±0.1	3.1±0.7	2.4±0.4	18.3±2.3
500	0.26±0.05	0.82±0.05		2.1±0.8	25.8±1.6
900				3.8±0.6	
1000		0.35±0.05			4.2±0.8
1500		0.12±0.05			11.7±3.4

of 10^4 cells ml^{-1} occur at depths of about 100 m (Li, Zohary, Yacobi & Wood, 1993; Vaulot, Partensky & Marie, 1997).

Based on *Prochlorococcus* abundances of 10^4 ml^{-1}, it appears unlikely that our estimates of heterotrophic bacterial abundances would be over by more than 15%. Nonetheless, while the leucine incorporation method provided estimates of bacterial production in the study area, the existing abundance data do not permit accurate calculation of specific bacterial growth and doubling times.

During the September '95 experiment, there was a considerable difference between in situ and incubation temperature for the deeper layers. In spite of the temperature correction (Q_{10}=2.6, see methods), some peaks of bacterial production were obtained at station D7 (500 m, 1500 m). Nevertheless, measured bacterial production in the deep layers of station D6 was low (Table 2, D6 S). Thus, these peaks were not systematically observed. Some of the high values observed in the deep water column during September corresponded to maxima in the concentrations of particulate organic carbon (118 and 110 μg C l^{-1} at 500 m D7 and 200 m D4, respectively (Tselepides, Zarvakis, Polychronaki, Danovaro & Chronis, 2000)). This suggests that relatively high values of bacterial production in deeper samples can also be attributed to micro-niches (particles and/or colloidal material colonized by productive bacteria (Azam, Smith, Steward & Hagström, 1993; Turley, Lochte & Lampitt, 1995)), and/or lateral intrusion of different water masses, such as the Transition Mediterranean Waters (TMW (Georgopoulos, Chronis, Zervakis, Likousis, Poulos & Iona, 2000)).

3.3. Integrated fluxes and stocks: relationships with primary producers

One characteristic feature of this oligotrophic system was the dominance of bacterial biomass over phytoplankton biomass. Except for the coastal station in March, calculated bacterial biomass was consistently higher than calculated autotrophic biomass (Fig. 2); this observation is unaffected even if the maximum allowance is made for the potential overestimate of bacterial biomass that may result from including *Prochlorococcus*. Robarts et al. (1996) found an opposite relationship in the Levantine basin, while using integrated data and the same chlorophyll/carbon ratios we employed. Their bacterial counts never exceeded $3.9.10^8$ l^{-1}, and 83% of their bacterial population was assumed to be cocciform containing only 15 fg C. The controversy about the significance of this ratio is important. Bacterial biomass is controlled by shifts between bottom-up and top-down control. So bacteria, which do not sink, constitute crucial reservoirs for both particulate nitrogen (Danovaro et al., 2000) and phosphorus being maintained in the euphotic zone. Moreover, DOC may accumulate (Thingstad, Hagstron & Rassoulzadegan, 1997). Maintaining a continuum of physiological states and a high bacterial diversity in a damped biomass stock could benefit bacteria in oligotrophic systems in which labile nutrients appear during small-scale rapid events.

There are many problems in making such calculations, for example: i) the high variability of the chlorophyll/carbon ratio in oligotrophic and stratified conditions (Claustre & Marty, 1995); ii) the alternative ways to calculate carbon per bacteria conversion factor (Norland, 1993); and iii) the recent debate on the significance of

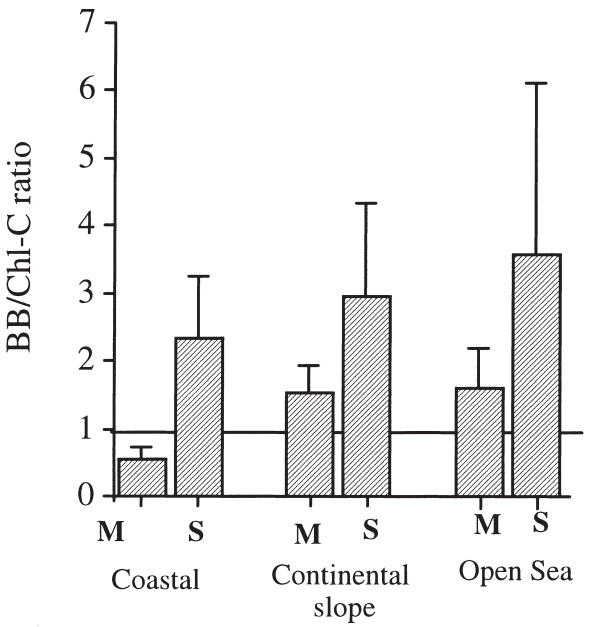

Fig. 2. Average BB/C-Chl a ratios in March (M) and September (S) BB: Bacterial biomass, C-Chl *a*: carbon equivalent of chlorophyll *a*. Conversion factors were: 20 fg C per bacteria and C/Chl=50. Data taken into account corresponded to layers where Chl *a* was higher than 0.03 μg Chl l^{-1}, i.e. *n*=2–10 depending on the station.

bacterial epifluorescence counts concerning active–inactive bacteria and counting of prochlorophytes as heterotrophic bacteria (Campbell et al., 1994; Zweifel & Hagström, 1995; Sieracki, Haugen & Cucci, 1995; Landry et al., 1996).

In the euphotic zone, a higher value of integrated bacterial production was recorded at the coastal station during the bloom (March), and a lower value in September, during a post-bloom situation (Table 3). In open sea stations, the magnitude of integrated bacterial production was characteristic of highly oligotrophic conditions. In the Levantine Basin, very low bacterial production, integrated through 200 m, has been noted previously 8–43 mg C m^{-2} d^{-1} (Zohary & Robarts, 1992; Robarts et al., 1996).

Table 3
Integrated bacterial production (mg C m^{-2} d^{-1}) over the upper layer (0–100 m) and the whole water column. M: March, S: September

Stations	D2		D4		D6	D7	
Date	M	S	M	S	S	M	S
0–100 m	131	51.1	36.5	7.4	19.5	40.1	32.1
Whole water column	131	51.1	64.5	61.9	70.5	70.9	465
Depth of integrated value	100 m	100 m	500 m	500 m	900 m	1500 m	1500 m

Differences between the two seasons were marked at the coastal station but less extreme at the continental slope station D4. At D4, the peak of bacterial production observed in September had deepened consistently with the chlorophyll *a* maximum (Psarra et al., 2000). Integrated bacterial production in the whole water-column was almost equal for stations D4, D6 and D7 at both seasons, except for the high value observed during September in D7, due to high values measured in the deeper layer (Table 2).

The relative importance of food supply or grazing as factors controlling bacteria were examined using the approach outlined by Billen, Servais and Beckevort (1990). The method employs bacterial production as an index of the input flux of dissolved organic matter, but only the data from the March cruise yielded a significant regression (Fig. 3). The slope of the regression (0.26) was close to the value cited for a large data set for the open ocean (0.28 (Ducklow, 1992)) suggesting bottom-up control is weak. Our data was obtained over a small spatial and temporal scale, the extent of which has to be taken into account in the control of bacterial biomass (Billen et al., 1990; Ducklow, 1992; Dufour & Torreton, 1996). However, our data range over three orders of magnitude for bacterial production and two orders of magnitude for bacterial biomass. The absence of correlation in September may indicate, as suggested by Ducklow (1992), a seasonal progression from a resource limi-

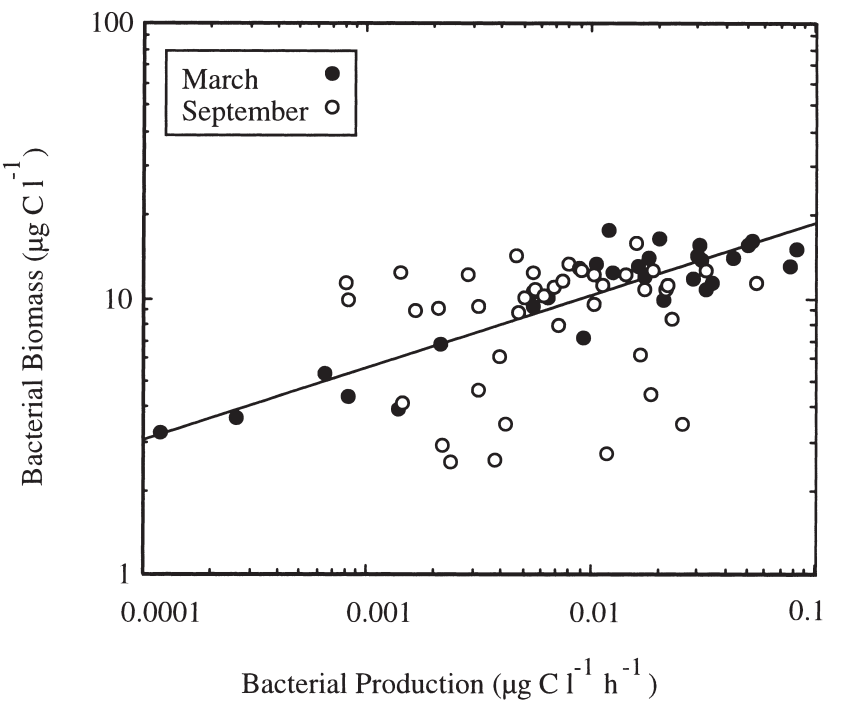

Fig. 3. Regression between bacterial biomass and bacterial production. The line is the least-squares fit to the log-transformed March data; log (biomass)=1.53+0.26 log (production); r=0.9; n=29.

tation of bacterial biomass in March (bloom situation) to predator limitation in September.

An index of the fraction of the photosynthetically produced carbon that enters the microbial food web in the euphotic zone (0–100 m) can be estimated by the ratio BCD/PP, where bacterial carbon demand (BCD) is calculated from integrated bacterial production, assuming 50% as bacterial growth efficiency, and PP is the integrated primary production (Psarra et al., 2000). At the coastal station, regardless of the season, bacterial carbon demand was very close to phytoplankton production (BCD/PP=1.03 in March, 0.83 in September). A ratio of BCD to PP equal to 1 does not necessarily imply that all primary production is dissipated by the microbial loop. BCD and PP were not necessarily coupled on the time scale of our study and bacterial growth could also be sustained by other carbon sources (Azam et al., 1993). In contrast, at the continental slope station D4, this ratio was only 0.35 during March and 0.37 during September. According to some recent studies on seawater cultures, bacterial growth efficiencies in an oligotrophic sea should be lower than 0.5, reaching 0.1–0.3 (Kirchman, Suzuki, Garside & Ducklow, 1991; Carlson & Ducklow, 1996); this suggests that, more than half of the primary production may have been dissipated by mineralization in the upper layers of the open Cretan Sea during the two study periods. Future research in this area should focus on the seasonal switch of the main parameters controlling bacterial production and phytoplankton–bacteria coupling among seasons, i.e. food resources (mineral and organic) and predation.

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