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Heterotrophic bacterial growth and substrate utilization in the oligotrophic Eastern Mediterranean (Aegean Sea)

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Abstract

Heterotrophic bacterial growth and substrate utilization were studied in March and September of 1997 in the oligotrophic Aegean Sea. Maximum velocities of ectoproteolytic activity (ectoaminopeptidase activity, EAP), as well as amino acid assimilation and respiration rates (AA-A, AA-R) were measured along with bacterial production (protein synthesis). At the northern stations which are influenced by the input of the Black Sea waters, rates at 5 m depth of EAP, AA-A and bacterial production were 2 to 3 times higher than at southern stations. Influenced by the Black Sea water, mean bacterial numbers in the 0-100 m layer showed typical oceanic concentrations averaging 0.7 x 10^6 cells ml^{-1}. These values, along with low bacterial production rates (30 ng C l^{-1} h^{-1}) implied slow growth for bacteria and/or that a large number among them were inactive. Neither bacterial abundance nor production were correlated with primary production. The percentage of amino acids respired was higher in September compared to March, particularly in the northern Aegean (mean 69 %). The enzyme kinetic analysis showed a biphasic model, the transition between the high and low affinity enzymes being obtained at 50 μM. Ectoaminopeptidase activity was weakly correlated with bacterial production ($p < 0.05$), but strongly correlated with respiration rates of amino acids ($p < 0.001$), suggesting that the substrate used was devoted to maintain energy requirements.

Keywords: Heterotrophic bacteria, Production, Ectoaminopeptidase activity, Amino acid assimilation, Eastern Mediterranean

Introduction

The eastern Mediterranean Sea is one of the most oligotrophic seas, where mineral nutrients and phosphorous in particular are limited in this region (SALIHOGLU *et al.* 1990, KROM *et al.* 1991, SIOKOU *et al.* 2002, VAN WAMBEKE *et al.* 2002). Phosphate limitation in the eastern Mediterranean is reflected by low primary productivity levels (IGNATIADES 1998, IGNATIADES *et al*. 2002, MOUTIN & RAIMBAULT 2002) and dominance of smaller

phototrophs in the phytoplankton (< 10 m, Li *et al.* 1993, IGNATIADES *et al.* 2002). The importance of microheterotrophs in the eastern Mediterranean can be appreciated when primary and bacterial production are compared. For example, in reports regarding the Levantine Basin, bacterial production averaged 24 mg C m^{-2} d^{-1} (ROBARTS *et al.* 1996) compared to reported primary production values ranging between 40 and 50 mg C m^{-2} d^{-1} (BEMAN *et al.* 1984). In the southern Aegean Sea, bacterial production ranged from 8 to 130 mg C m^{-2} d^{-1} whereas primary production ranged from 39 to 243 mg C m^{-2} d^{-1} (TURLEY *et al.* 2000, IGNATIADES *et al.* 2002, VAN WAMBEKE *et al.* 2002). It appears that microheterotrophic activity is a dominant energy pathway in the planktonic food web in the eastern Mediterranean, acting as an energy sink (TURLEY *et al.* 2000, CHRISTAKI *et al.* 2001). Previous studies on heterotrophic bacterial dynamics in the eastern Mediterranean Sea focused on measurements of bacterial production and consumption by nanoflagellates (e.g. ROBARTS *et al.* 1996, CHRISTAKI *et al.* 1999, 2001 VAN WAMBEKE *et al.* 2002). However, bacterial activity can modify the composition of organic matter without necessarily involving large bacterial production rates. This is only one of the final steps in the transformation of dissolved organic matter (DOM) by heterotrophic bacteria. Knowledge of production rates alone is insufficient in understanding the consequences of bacterial metabolism at the ecosystem-level (AZAM, 1998). Organic matter >500–1000 Dalton requires enzymatic breakdown before it can incorporate the resulting low molecular weight compounds into the bacterial cell for metabolism and growth (e.g. CHROST 1991, UNANUE *et al.* 1999). Due to its potentiality to be inducible, ectoaminopeptidase activity generally occurs in substrate-limiting conditions or in the presence of large amounts of polymeric material (CHROST & RAI, 1993). Therefore, the study of bacterial ectoenzymatic activity and the uptake of hydrolysis products are important in understanding carbon flux in the ocean. Actually, there is still very little data available relating to ectoenzymatic activity and monomer uptake in oligotrophic marine environments (WILLIAMS, 1970; JONAS *et al.* 1988; UNANUE *et al.* 1999) and such data is completely lacking for the eastern Mediterranean. Moreover, studies relating substrate hydrolysis to substrate uptake with bacterial production are rare and have been mostly performed in the laboratory or in highly eutrophic environments (CHRISTAKI, 1995; UNANUE *et al.* 1998; HOPPE *et al.* 1998; VAN WAMBEKE *et al.* , in press).

The goal of the present study was to describe heterotrophic bacterial dynamics in the Aegean Sea as a means for understanding bacterioplankton strategies and adaptations in an oligotrophic system. In order to do this we measured bacterial production, hydrolysis of macromolecules, and uptake of resulting low molecular weight compounds (including assimilation and respiration). Special attention was also given to the different methodologies that we employed.

Material and Methods

Study site, sampling strategy

This study was conducted within the framework of the European Community MATER program (Mass Transfer and Ecosystem Response). The overall goal of MATER was to collect data on the dynamics of water masses and the behavior of dissolved and particulate matter in the Mediterranean (MONACO & PERUZZI, 2002). This work was carried out aboard the research vessel *Aegaio* during two distinct seasons. During the spring (6 March - 3 April 1997), stations S1, S2, S6 and S7 (depth 1300-2000 m) and N1 to N7 (depth 100-1200 m) were sampled in the southern and the northern Aegean Sea, respectively (Fig. 1). The second cruise was conducted during the end of the summer (10

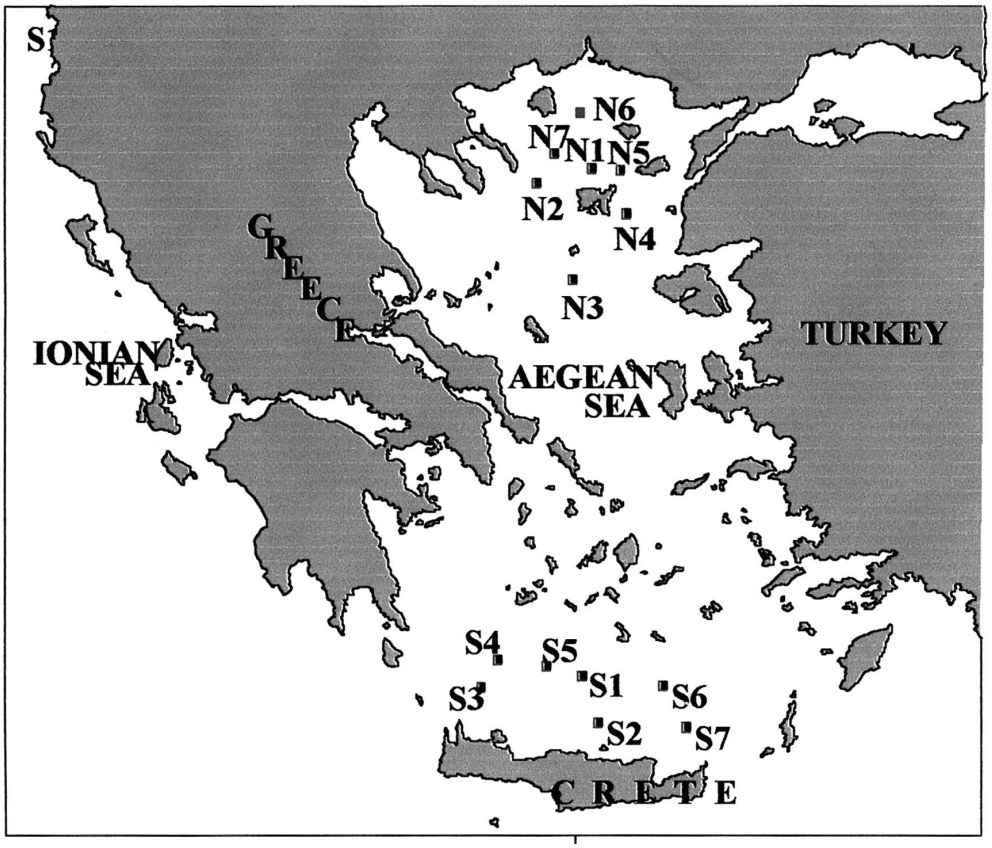

Fig.1: Map of the Aegean Sea, showing the positions of the stations studied.

- 24 September 97). Six stations were sampled in the southern (S1, S2, S3, S4, S6 and S7, depth 900 - 2200 m) and seven in the northern Aegean Sea (N1 to N7, depth 100 - 1200 m). Sampling was always conducted at 10 - 11 am with Niskin bottles on a CTD-rosette.

The study area (Aegean Sea, Fig. 1) is oligotrophic but in the northern part there are regions with higher productivity due to input from the Black Sea and adjacent rivers (LIKOUSIS *et al.* 2002). Daily primary production in the northern Aegean (81 mg C m^{-2} d^{-1}) was higher than that in the southern Aegean (39 mg C m^{-2} d^{-1}, IGNATIADES *et al.* 2002). Due to the influence of Black Sea waters, primary production ranged from 70 to 570 mg C m^{-2} d^{-1} and peaked around 1000 to 1500 mg C m^{-2} d^{-1} at some stations (N4 and N5 in spring, this range is calculated from IGNATIADES *et al.* 2002 hourly values using the model developed by MOUTIN *et al.* 1999). In September, the water column was stratified. In the northern Aegean Sea, low temperature and salinity in surface waters are caused by the entry of Black Sea waters (BSW, Fig. 2). Phosphates in the euphotic zone ranged from undetectable to 0.08 μM, N/P ratios of nutrients averaging 21 (SIOKOU *et al.* 2002).

Bacterial production and abundance

Samples for bacterial enumeration were fixed with 0.2 μm filtered and buffered formalin (final concentration 2 % v/v), filtered within two hours after fixation on black 0.2 μm Nuclepore filters stained with DAPI (Porter

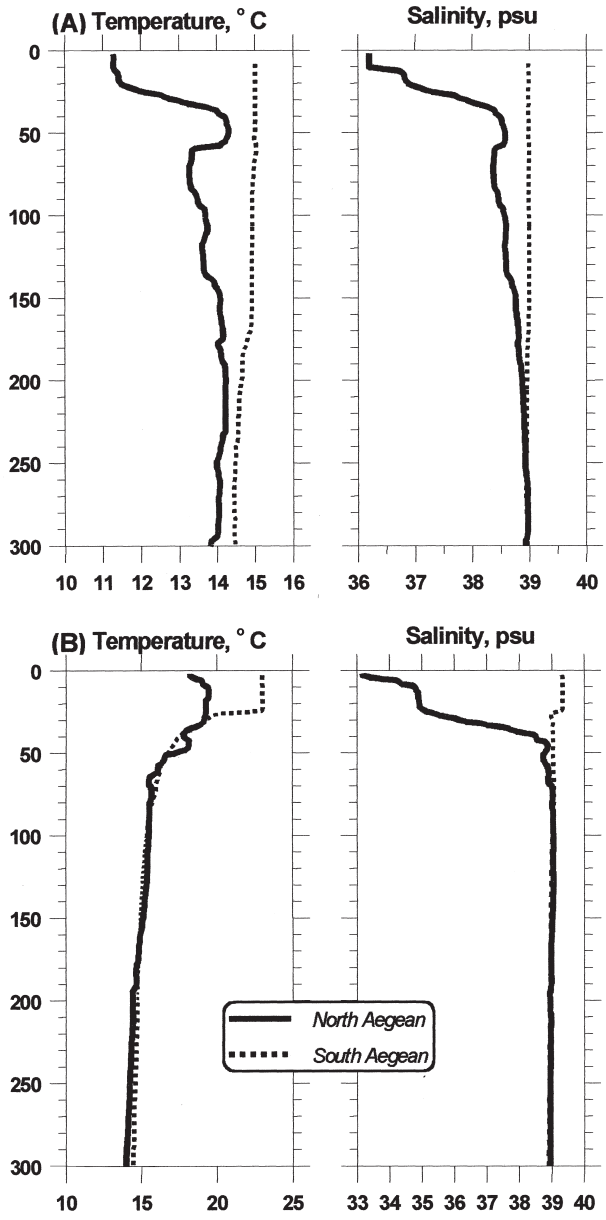

Fig. 2: Typical vertical profiles of temperature (°C) and salinity (psu) in the Aegean Sea. (A) March 1997, (B) September 1997 (CTD data provided by GEORGOPOULOS *et al.* & THEOHARIS *et al.*) Note different x-scales.

& Feig 1980) and stored at -20°C until counting by epifluorescence microscopy. Bacterial production was estimated by the 3H-leucine method (Kirchman 1993). At each depth, duplicate samples and a control were incubated with 1 - 2 nM L-[4,5 -3H] leucine (specific activity 126 Ci mmol^{-1} in March, 136 Ci mmol^{-1} in September) + 19 nM non-radioactive leucine. Appropriate incubation times were checked with time series experiments on 4 occasions.

Samples were incubated anytime between 2 to 9 hours, and with 20 to 40 ml water samples according to expected activities, and were kept in the dark and at in situ temperature. Bacterial production (BP) was calculated according to Kirchman (1993):

$$BP = Leu \times 131.2 \times (\%\ leu)^{-1} \times (C/protein) \times ID \times (hot + cold) / hot \quad (1)$$

Where Leu = rate of leucine incorporation (mol l^{-1} h^{-1}), 131.2 = formula weight of leucine, % leu = fraction of leucine in protein (0.073), C/protein = ratio of cellular carbon to protein (0.86), 'hot' and 'cold' = respective concentrations of labeled and unlabelled leucine and ID = the isotope dilution.

In order to calculate the ID, concentration kinetics were performed on 7 occasions (POLLARD & MORIARTY, 1984). The results showed that the degree of participation (DP) of 20 nM of leucine routinely used for the BP production measurements was always more than 90%, and thus the isotopic dilution was negligible (ID = 1). During both the both March and September cruises, samples for estimating bacterial production and abundance were taken throughout the whole water column (5 - 2000 m depending on the depth of the station).

Ectoaminopeptidase activity

The ectoaminopeptidase activity (EAP, nmol leu hydrolyzed l^{-1} h^{-1}) was estimated by measuring the hydrolysis rate of L-leucine-7-amido-4-methylcoumarin (leu-MCA, Sigma) which is used as an analog model substrate for natural peptides (HOPPE 1993, HOPPE *et al.* 1993). Stock solutions of 10 mM leu-MCA were prepared in methylcellosolve and kept at -20°C. Routinely, activities were measured at 200 μM leu-MCA in 40 ml samples. Leu-MCA contains the artificial fluorescent molecule MCA, linked by a peptide binding with the natural molecule of leucine. The increase of fluorescence, observed after enzymatic splitting of the molecule, was measured with a Hoefer TKO 100 spectrofluorometer (excitation 365 nm, emission 460 nm) in March. During the September cruise, a Hitachi F-2000 spectrofluorometer (excitation 380 nm, emission 440 nm) was used. Incubation was performed at *in situ* temperature, in the dark. The fluorescence increased linearly with time during 12 h incubations in duplicate samples. In order to convert fluorescence units into nM hydrolyzed h^{-1}, relative fluorescence units were calibrated with 7-amido-4-methylcoumarin standards (MCA) from 5 to 400 nM on each spectrofluorometer. Blanks were run adding the leu-MCA in 0.2 μm filtered and boiled seawater during 15 min and were shown to be insignificant.

Enzyme kinetic assays were performed on 4 occasions with different leu-MCA concentrations (0.5, 1, 10, 25, 50, 100, 200, 400, 1000 μM). Leu-MCA kinetic parameters (Vm and Km) were calculated directly by non-linear least square analysis of equation:

$$V = Vm \times S / (Km + S) \quad (2)$$

where Vm = the maximum velocity of enzyme reaction, S = the concentration of leu-MCA added, and Km = the Michaelis - Menten constant that indicates the affinity of the enzyme for the substrate.

In the four concentration kinetics assays conducted during the two cruises, the ectoaminopeptidase activity increased with increasing substrate concentration. This is indicative of multiple proteolytic mechanisms, each with different Vm and Km values. This was also confirmed by the non-linearity of 1/V against 1/S plot (Lineweaver - Burk plots, figure not shown). Thus, Vm and Km values were calculated considering low and high concentration of leu-MCA, each of which follows simple Michaelis-Menten kinetics (Table 1). Simple kinetic parameters considering the whole concentration range of leu-MCA (Table 1) were also calculated for eventual comparison with previously published

Table 1
Kinetic parameters (mean ± sd) obtained from non linear least square regression of leu-MCA hydrolysis rates related to added concentrations of leu-MCA.

	station	depth	a	Conc range µM	Vm nM h^{-1}	Km µM	n
March	S6	5m	all data	0.5 - 1000	4.7 ± 0.7	241 ± 94	8
			low substrate	0.5 - 50	1.2 ± 0.2	3.2 ± 2.6	4
			high substrate	100-1000	5.3 ± 0.8	349 ± 135	4
September	N2	5m	all data	0.5-400	27 ± 3	102 ± 27	8
			low substrate	0.5-25	6.2 ± 1.4	2.7 ± 2.5	4
			high substrate	50-400	31 ± 8	303 ±145	4
September	N4	5m	all data	0.5-400	22 ± 6	154 ± 98	8
			low substrate	0.5-25	uns	uns	4
			high substrate	50-400	31 ± 2	147 ± 28	4
September	N6	5m	all data	0.5-400	20 ± 3	97 ± 34	8
			low substrate	0.5-25	9 ± 4	15 ± 13	4
			high substrate	50-400	24 ± 3	163 ± 41	4

a: results calculated from the whole range of leu-MCA concentration (all data), low concentration range (low substrate) or high substrate concentration range (high substrate)

uns, insignificant fit.

Table 2
Literature data on kinetic parameters of ectoaminopeptidase activities (Vm, Km).

location	substrate	conc range µM	V or Vm nM h^{-1}	Km µM	Reference
eutrophic (rivers, North Sea)	LLßN	1000 *	120-1200	100	Somville & Billen 1983
Kiel fjord	leu-MCA	5-50	22-80	-	Hoppe 1983
Kiel fjord	leu-MCA	0.1-40	4-311	-	Hoppe 1984
Eutrophic lake	leu-NTA	10-100	0.4-6.4	9-63	Halemejko & Chrost 1986
Santa Monica Basin	LLßN	1000 *	1.4-230	-	Rosso & Azam 1987
Coral Reef (Philippines)	leu-MCA	0.1-250	1.5-22	-	Hoppe et al 1988
Impoverished mesocosms	leu-MCA	10-500	51-126	109-115	Chrost & Rai 1993
Coral reef (Gulf of Mexico)	leu-MCA	2.5 (0.1-25) **	246-305	-	Rath et al. 1993
Coastal Mediterranean Sea	leu-MCA	50 *	16-63	-	Karner & Rassoulzadegan 1995
Eutrophic rivers	leu-MCA	50 (0.5-100) **	99-2217	-	Chappel & Goulder 1995
Pacific subtropical and equatorial	LLßN	1000 *	1.5-27	-	Christian & Karl 1995
Magellan Strait	leu-MCA	200 *	4.6-15.6	-	Talbot *et al.* 1997
Antarctic Peninsula	LLßN	30-110	-	48-218	Christian & Karl 1998
marine pond	leu-MCA	7.8-1000	400-4450	94 -183	Crottereau & Delmas 1998
Aegean Sea	leu-MCA	200 (0.5-1000) **	2.9-29.5	97-241	this study

Conc Range, range of concentration used for the kinetics,
leu-MCA, L-leucine-4-methyl-7-coumarin,
LLßN: L-leucine ß-naphthylamide,
leu-NTA: L-leucine-p-nitroanilide,
* one single concentration is added and assumed to satisfy saturating conditions.
** for routine measurements one single concentration was used, but saturating conditions were also verified, (in parenthesis the concentration range tested).

data (Table 2). EAP was measured at 5 m depth, except in March 97, in southern Aegean Sea when profiles of ectoaminopeptidase activity were performed (5, 50, 75, 300, 500 m and max. depth).

Amino acid assimilation and respiration

Amino acid assimilation and respiration rates (AA-A and AA-R, nM h^{-1}) were measured adding ^{14}C-amino acids (^{14}C-amino acids mixture, Amersham, 52 mCi mat C^{-1}) at a final concentration of 245 nM. On 8 occasions, we checked the validity of concentrations used. The concentration of 245 nM was always satisfying saturating conditions. No significant increase was found for the amino acid uptake rates from 100 to 400 nM (F-test on the slope, Fcalc < F0.05), thus, we can consider the rates measured here as representative of the maximal activity of bacteria.

The Amersham ^{14}C-AA mixture included 16 amino acids, and their individual proportion (by activity) ranged from 0.6 (methionine) to 13 % (leucine). The radiolabelled amino acids were added in 20 ml duplicates and a formalin killed control. Samples were incubated during 2 hours at *in situ* temperature in the dark and incubation was stopped by addition of formalin buffered solution with saturating sodium tetraborate (1% final concentration). After fixation, samples were filtered on 0.2 μm polycarbonate Nuclepore filters which were rinsed 3 times with 0.2 μm filtered seawater; following the method described by SIMON (1993) and WEISS & SIMON (1999). The filtrates of the ^{14}C-AA samples were collected and kept at +4°C. In the laboratory, the filtrate was acidified (200 μl HCl, 6 N) and released ^{14}C-CO_2 was flushed using N_2 blowing (100 ml min-1) for 10 min. CO_2 was trapped in two successive scintillation vials containing each 9 ml of a cocktail of ethanolamine-methanol-PCS scintillation liquid in a ratio 1:1:7 v/v (GARABETIAN, 1991) and counted on a Packard 1600 TR scintillation counter. In March 97, assimilation and respiration rates of amino acids were measured throughout the whole water column. In September A-AA and AA-R were measured at 5 m depth.

Table 3
Mean ± sd and ranges of surface values (5 m) of heterotrophic bacterial parameters in the South and the North Aegean Sea in March and September 1997.

	BN 105 ml^{-1}	BP ng C l^{-1} h^{-1}	EAP nM h^{-1}	AA-A nM h^{-1}	AA-R nM h^{-1}	% AA-R %
South Aegean						
March 97	7.3 ± 0.2	31.2 ± 6.9	3.5 ± 0.5	0.049 ± 0.012	0.027 ± 0.015	35 ± 18
n=4	7.1 - 7.5	22.6 - 38.9	2.9 - 3.7	0.033 - 0.061	0.007 - 0.040	10 - 52
September 97	6.8 ± 2.8	24.6 ± 3.9	3.9 ± 0.6	0.035 ± 0.010	0.045 ± 0.012	56 ± 10
n=6	3.3 - 10.8	19.2 - 30.2	3.0 - 4.7	0.022 - 0.051	0.033 - 0.060	45 - 67
North Aegean						
March 97	10.9 ± 4.1	53.4 ± 40.9	nd	0.12 ± 0.07	0.073 ± 0.057	38 ± 13
n=7	5.2 - 15.9	9.4 - 103		0.05 - 0.21	0.020 - 0.16	10 - 54
September 97	9.6 ± 1.9	63.6 ± 34.6	14.3 ± 7.8	0.059 ± 0.031	0.15 ± 0.05	69 ± 11
n=7	8.3 - 13.6	25.6 - 12	4.4 - 29.5	0.018 - 0.11	0.09 - 0.23	56 - 88

BP, bacterial production (^{3}H-leucine, 20 nM),
BN, bacterial number (DAPI counts),
EAP, ectoaminopeptidase activity (leu-MCA, 200 μM),
AA-A: assimilation rates of amino acids (^{14}C-aminoacids, 196 nM in March, 245 nM in September),
AA-R : respiration rates of amino acids
% AA-R : percentage of respired amino acids.

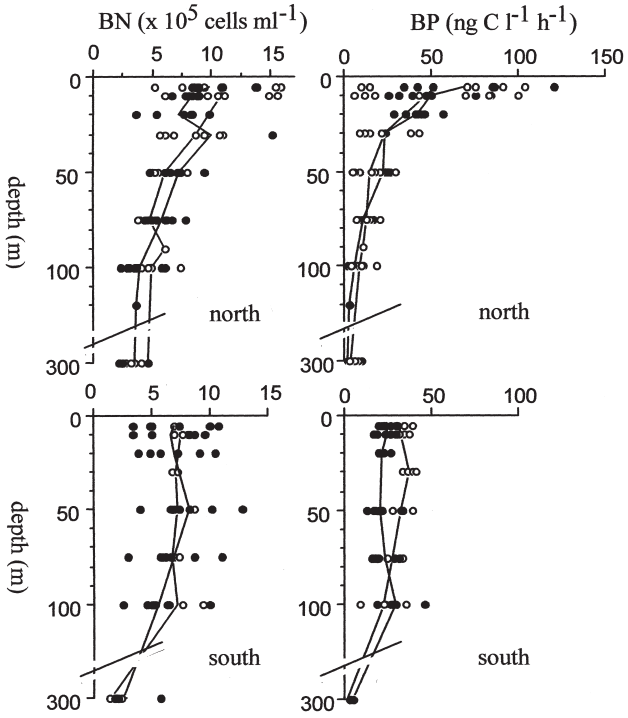

Fig. 3: Bacterial abundance (BN, 10^5 cells ml^{-1}), bacterial production (BP, ng C l^{-1} h^{-1}) in the 0-300 m layer in north and the south Aegean Sea. Solid lines represent mean values for the given season. Open symbols: March 1997, closed symbols: September 1997. Number of profiles as follows: North March 7, North September 7, South March 4, South September 6.

Results

Bacterial parameters, vertical and spatial distribution

Bacterial numbers were in most cases lower than 10 x 10^5 cells ml^{-1} (Table 3). In the north, the highest bacterial numbers were recorded in the 0-30 m layer (max. 16 x 10^5 cells ml^{-1}). Below 30 m, bacterial numbers decreased reaching *ca* 4 x 10^5 cells ml^{-1} at 300 m depth (Fig. 3). In the south, the bacterial distribution was quite homogenous in the 0-100 m layer during both seasons (4 x 10^5 cells ml^{-1}). Bacterial concentration at 300 m was ca 2.5 x 10^5 cells ml^{-1} (Fig. 3).There was no significant difference (t-test on the means, $p > 0.05$) of average bacterial numbers in surface layers between seasons or between northern and southern areas.

The highest bacterial production values were recorded in the surface waters of the stations influenced by BSW (Black Sea Water). Upon leaving the Dardanelles, the BSW tongue is generally oriented towards the northwest. Thus, stations N4 and N5 were always highly influenced, N1, N6 and N7 moderately influenced, and N3 unaffected (see Fig. 1). At stations influenced by BSW, the drastic drop of bacterial parameter values in the water column is related to the sharp halocline. Salinity increases from < 37 psu in surface waters to > 38 psu at 50 m (Fig. 2). Vertically-integrated values of bacterial production (0-100 m) ranged from 46 (St N3) to 84 (St N5) mg C m^{-2} d^{-1} in March, and 17 (St N7) to 164 (St N5) mg C m^{-2} d^{-1} in September.

Mean vertical profiles at both seasons were similar. Although no differences were detected in means ($p > 0.05$) in surface layers between both seasons (Fig. 3, Table 3), a higher variability of bacterial production was observed in March. In the north, the bacterial production in the surface layer (0-10 m) reached 120 ng C l^{-1} h^{-1} (Fig. 3). Below 20 m the bacterial production showed a decrease during both seasons with no noticeable subsurface peak, ranging from 1.5 to 57 ng C l^{-1} h^{-1} in the 20 - 100 m layer. In the south, the bacterial production was generally lower and homogeneously distributed in the 0 - 100 m layer. Bacterial production values ranged from 9.2 to 46.5 ng C l^{-1} h^{-1}. In deeper layers (300 m – bottom), bacterial production averaged 2.5 ± 2.8 and 1.5 ± 1.9 ng C l^{-1} h^{-1} in the north in March and September, respectively; and 0.54 ± 0.31 and 0.47 ± 0.45 ng C l^{-1} h^{-1} in the south (where stations were deeper) in March and September, respectively. In the south, integrated bacterial production ranged 63 – 82 mg C m^{-2} d^{-1} in March, and 46 - 75 mg C m^{-2} d^{-1} in September.

The relative importance of bottom-up (resource supply) to top down (predation) control of bacterial biomass was examined using the approach suggested by BILLEN *et al.* (1990). Log-log linear regression of our data set was always significant (Table 4). The variation of log-transformed bacterial production explained 58 to 92 % of the variations of log-transformed-bacterial biomass. The regression slopes ranged 0.27 - 0.37 (Fig. 4, Table 4).

Table 4
Data range and Statistics for log-log linear regressions between bacterial biomass and bacterial production.

Area	range BB µg C l^{-1}	range BP ng C l^{-1} h^{-1}	Slope ± se[a]	Y int ± se[a]	N	R^2	Reference
South Aegean. March 97	1.8 - 19	0.2 - 41	0.35 ± 0.01	1.69 ± 0.04	45	0.92	this study
South Aegean. September 97	1.4 - 25	0.1 - 46	0.27 ± 0.03	1.54 ± 0.07	61	0.58	this study
North Aegean. March 97	2.8 - 32	0.4 - 215	0.37 ± 0.03	1.78 ± 0.06	57	0.75	this study
North Aegan. September 97	2.3 - 30	0.1 - 121	0.35 ± 0.02	1.68 ± 0.04	60	0.83	this study
all data 97	1.4 - 32	0.1 - 215	0.33 ± 0.01	1.66 ± 0.03	223	0.75	this study
Drinking to river waters	0.2 - 4000	1 - 50000	0.7	1.67	288	0.83	Billen et al. 1990
Open Ocean	0.2 - 100	0.1 - 1500	0.28	1.5	1296	0.32	Ducklow 1992
Chesapeake Bay	na	na	0.51	na	1228	0.48	Ducklow 1992
Gulf Stream rings	0.2 - 40	10 - 1500	0.39	1.26	194	0.35	Ducklow 1992
Indian Ocean	0.2 - 100	0.2 - 800	0.39	1.86	160	0.72	Ducklow 1992
Subartic N. Pacific	na	na	0.32	na	730	0.36	Ducklow 1992
Atlantic Ocean Torréton 1996	0.2 - 50	0.02 - 1000	0.47 ± 0.01	1.84 ± 0.03	68	0.90	Dufour &
South Aegean. Feb 95	3.3 - 18	0.1 - 83	0.26 ±0.02	1.53 ± 0.05	29	0.83	Van Wambeke *et al.* 2000
South Aegean. Sep 95	2.5 - 16	0.8 - 55	ns	ns	42	0.03	Van Wambeke *et al.* 2000

na, data not available
ns, regression not significant
[a] standard error not always cited

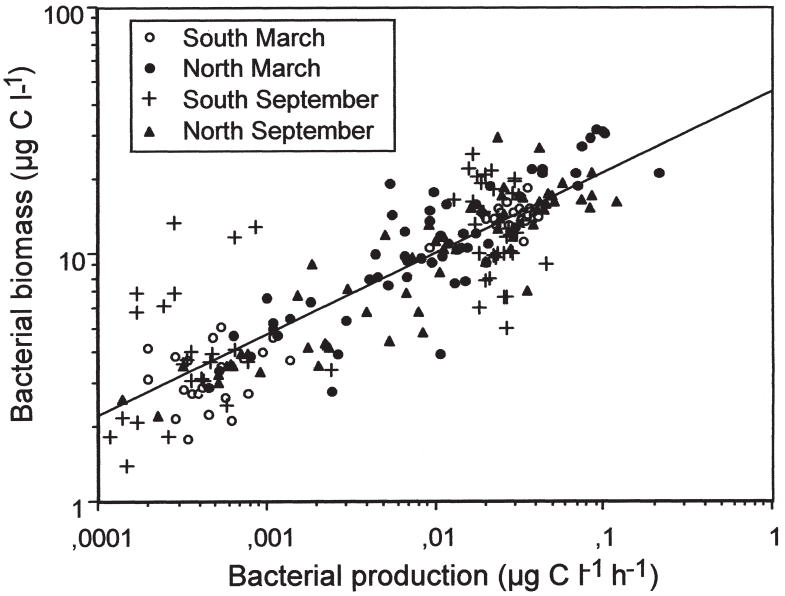

Fig. 4: Log-log linear regression between bacterial biomass and bacterial production for the full data set. Regression equations and statistical parameters in Table 4.

Similar to bacterial production, surface (5 m depth) amino acid uptake rates (AA-A) and ectoaminopeptidase activities (EAP) values were 2 to 3 times higher in the north where they also showed a wider range and variability than in the southern stations (Table 3). EAP ranged from 3.0 to 4.7 nM in the south, where no difference in means between seasons was noticed (t-test on the means, $p > 0.05$) and from 4.4 to 29.5 nM h^{-1} in the north during September cruise. At 50 and 75 m depth in March in the southern Aegean, EAP showed higher variability than production or amino acid assimilation rates (Figs. 3 and 5).

The enzyme kinetics analysis showed a biphasic model with one exception (station N4, September 97, Table 1). The change between the high affinity hydrolysis systems (low Vm and low Km) and the low affinity systems (high Vm and high Km) occurred in all cases at 50 µM. From the set of parameters calculated in this study (Table 1) the high affinity - low Vm system was saturated by the 200 µM leu-MCA routinely used for measurements (Table 3). The concentration of 200 µM leu-MCA was in the range of the Km corresponding to the Km of the low affinity - high Vm system (147 - 349 µM, Table 1), thus, saturating conditions were not satisfied for this system.

Amino acid assimilation rates were measured at saturating substrate concentrations (see methods). Means were statistically higher in the surface waters of the northern Aegean compared to the south in March, but they did not change significantly between seasons both in northern and southern Aegean. On the contrary, amino acid respiration rates increased significantly ($p < 0.05$) between March and September in the north (from 0.045 to 0.15 nM h-1, Table 3). Mean amino acid respiration percentage (% AA-R) was relatively high (50 ± 18 %) in both basins and varied in a wide range (10 - 88 % of the amino acids taken up by bacteria were respired, Table 3).

Relations between bacterial parameters

Amino acid assimilation rates increased with increasing bacterial production ($r = 0.73$, $p < 0.001$, Table 5). However, the correlation of EAP with bacterial production was weak ($r = 0.65$, $p < 0.05$, Table 5). The correlation

coefficient between the respiration rate of amino acids and EAP was higher than with bacterial production (0.63 and 0.83, $p < 0.01$ respectively, Table 5).

In order to examine the potential sources of substrates involved in ectoaminopeptidase activity, amino acid assimilation or respiration, relationships between bacterial activities and other biological and environmental parameters were also tested (Table 6). As was already mentioned above, the highest values of microbial parameters were recorded in the stations influenced from the Black Sea water (BSW). Salinity showed a significant negative correlation with all microbial parameters (Table 6) underlying the higher activity in the BSW (Figs. 3 and 5). TOC was correlated with BP and EAP (Table 6) whereas POC correlated significantly only with assimilation rates of amino acids, which significantly increased with increasing POC ($r = 0.54$, $p < 0.01$). Although chlorophyll *a* (chl *a*), primary production and bacterial activities showed highest values in the northern and lowest in the southern stations (Table 3), correlations were weak or absent. Chl a showed weak correlation ($r = 0.43$, $p < 0.05$) with bacterial production and assimilation rates of amino acids whereas primary production was the most poorly correlated parameter (Table 6).

Table 5
Spearman's rank correlation between different parameters at 5 m depth (March and September 1997, South and North Aegean Sea). Correlation coefficients (Spearman's rhos) shown, with n=24 except EAP where n=16.

	BP	BN	EAP	AA-A
BN	0.55**			
EAP	0.65*	0.68**		
AA-A	0.73***	0.61**	0.41	
AA-R	0.63**	0.61**	0.83**	0.35

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.
BP, bacterial production, BN, bacterial number,
EAP, ectoaminopeptidase activity,
AA-A, assimilation rates of aminoacids,
AA-R, respiration rates of aminoacids.

Discussion

According to the model of BILLEN *et al.* (1990), our BB - BP data showed that the variation of LOG transformed - BP explained most of the variation of log - transformed BB, suggesting that bacteria were controlled by resources (Fig. 4, Table 4). The value of the regression slope indicates the degree of bottom–up *versus* top-down control, going from extreme resource control (high correlation + high slope) to complete grazing control (no correlation at all). Our slope values were relatively shallow, as typically obtained in the upper ocean (DUCKLOW 1992),

Table 6
Spearman's rank correlation between bacterial parameters and other environmental and biological variables at 5 m depth (March and September 1997, South and North Aegean). Correlation coefficients (Spearman's rhos) shown, with n=24 except PP where n=19 and EAP where n=16.

	BP	BN	EAP	AA-A	AA-R
Sal	-0.68**	-0.55**	-0.74**	-0.62**	-0.64**
TOC	0.65**	0.28	0.63*	0.40	0.40
POC	0.26	0.21	-0.16	0.54**	-0.27
Chl *a*	0.43*	0.34	0.18	0.50*	0.16
PP	0.33	0.37	0.18	0.55*	0.05

Bacterial parameters as in Table 5, Sal, Salinity
TOC, total organic carbon (Sempéré *et al*. 2002)
POC, particulate organic carbon, (Likousis *et al*. 2002)
Chl *a*: Chlorophyll *a* , Ignatiades *et al*. (Ignatiades *et al*. 2002)
PP, Primary production, Ignatiates *et al*. (Ignatiades *et al*. 2002)

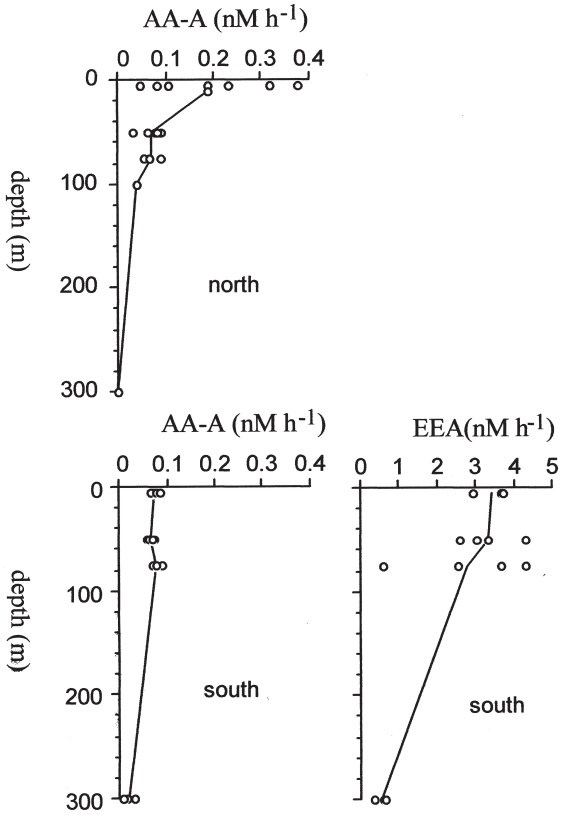

Fig. 5: Vertical profiles of assimilation rates of amino acids (AA-A, north and south Aegean) and ectoaminopeptidase activity (EAP, south Aegean only) in March 1997. Solid lines represent mean values. Number of profiles as follows: North 7, South 4.

indicating moderate resource control. During the course of our study, nanoflagellate bacterivory was important and accounted for 40 to over 100 % of the daily bacterial production (CHRISTAKI *et al.* 1999). This introduces certain contradictions in the interpretation of the BB-BP relationship. In the model initially developed by BILLEN *et al.* (1990), BP is an indicator of the flux of input of biodegradable organic matter, essentially of phytoplankton or allochtonous inputs origin. In situ and experimental data indicate that the Mediterranean can be effectively P- limited, and that phosphorus limits both phytoplankton and heterotrophic bacteria (KROM *et al.* 1991, THINGSTAD & RASSOULZADEGAN 1995, ZOHARY & ROBARTS 1998). Results from an enrichment experiment conducted during the March 1997 cruise (CHRISTAKI *et al.* 1999) and results obtained during two longitudinal cruises in the Mediterranean in 1999 (VAN WAMBEKE *et al.* 2002) clearly supported the view of phosphorous-limited bacteria. In these conditions, it is expected that predators exert simultaneously a role in bottom-up and top-down controls, through nutrient regeneration and sloppy feeding (THINGSTAD, 1998).

The question is, how do bacteria adapt their *in situ* metabolism in such conditions? Except for some isolated high values recorded in the northern Aegean surface waters influenced by Black Sea waters, bacterial parameters exhibited low activities. Bacterial production rates were similar to previous measurements in the southeastern Mediterranean (CHRISTAKI *et al.* 2001 ; VAN WAMBEKE *et*

al. 2002). Considering a mean bacterial production of 30 ng C l^{-1} h^{-1} and a mean bacterial biomass of 10.8 μg C l^{-1} (calculated with a conversion factor of 15 fg C $cell^{-1}$, ROBARTS *et al.* 1996); mean bacterial generation time (defined as ln2 x BB / BP) in the euphotic zone would be as long as 10.5 days. We suggest that long generation times calculated this way (ROBARTS *et al.* 1996) potentially include a large number of bacteria present in the seawater which were not actively growing.

As expected, amino acid assimilation rates (AA-A) increased with increasing bacterial production (Table 5). It should be noted that AA concentration used in this study was saturating (see methods), thus, AA-A and AA-R measured here can be considered as maximal. In this study we obtained high values of amino acid respiration percentages, particularly in September (56 and 69 % on average, table 3). The % of respired amino acids depends on the nature of the amino acid and the environmental conditions. For example, respiration rates recorded for amino acids such as glutamic acid, are higher than those measured for leucine, lysine or alanine (e.g. JØRGENSEN, 1987). However, the ^{14}C-amino acid mixture used was not particularly rich in glutamic acid (9 % by activity, compared to 13 % for leucine). In the few existing studies from oligotrophic environments, the percentage of amino acid respiration varies greatly. With a ^{14}C amino acid mixture, values from 14 to 47 % have been obtained by UNANUE *et al.* (1999) in western Mediterranean Sea. With ^{3}H substrates allowing lower concentrations to be used, this percentage ranged from 33 to 41 % in southern California (CARLUCCI *et al.* 1984) and was 64 % in Sargasso Sea (JONAS *et al.* 1988). The high % respiration rates measured in the eastern Mediterranean may also reflect an important consequence of P-limitation on bacterial metabolism suggesting that bacteria used most of the available carbon for energy requirements rather than for biosynthesis. Around the Antarctic Peninsula a substantial fraction of amino acids were respired because bacteria were assumed to be auxotrophic for some lacking amino acids (CHRISTIAN & KARL, 1998), thus being also deficient in some compound for growth.

EAP values measured in this work were close to the values reported by TALBOT *et al.* (1997) from the Strait of Magellan (EAP 4.6 nM h^{-1} with 200 μM leu-MCA added and BP 32 ng C l^{-1} h^{-1}) which were measured at an ambient temperature (8 ± 1 °C) considerably lower than in our study. They also fell within the range of values obtained from the subtropical and equatorial Pacific with an addition of 1000 μM of another analog substrate for aminopeptidase (LLßN, CHRISTIAN & KARL 1995, Table 2). However, comparison with previous data is particularly difficult considering the unique or narrow range of substrate concentrations usually tested which could mask indications of multiple kinetics as those found in the present study. Multiple kinetics have been reported in eutrophic fjords (HOPPE, 1983), in the Mediterranean Sea (UNANUE *et al.* 1999) as well as in sediments and near-bottom water layers (TALBOT *et al.* 1997, THOLOSAN *et al.* 1999). In a previous laboratory study, we observed that the response of bacterial ectoaminopeptidase activity to the increase of polymers of phytoplanktonic origin was not uniform in different microcosms and depended both on the composition of the planktonic food web and on the way the phytoplanktonic polymers were released (autolysis, copepod or ciliate grazing, CHRISTAKI 1995, CHRISTAKI & VAN WAMBEKE 1995). Under oligotrophic conditions bacteria should be adapted to use the high affinity systems operating at low substrate concentrations. However, in spite of the limitations of the experimental approach, biphasic kinetics observed here show that bacteria could efficiently hydrolyze substrates introduced in the environment at high concentrations. The ability of bacteria to adapt ectoenzymatic activity to high concentrations

of polymers discontinuously distributed in the seawater, is probably representative of bacteria living on 'hot spots' (e.g. micro aggregates, SMITH *et al.* 1992, UNANUE *et al.* 1999) and may involve different enzymatic systems (AGIS *et al.* 1998).

In contrast with previous studies (e.g. SOMVILLE & BILLEN 1983, ROSSO & AZAM 1987, TALBOT *et al.* 1997), EAP in our study, demonstrated a weak correlation with bacterial production ($p < 0.05$, Table 5). In a recent study, SALAT *et al.* (2001) stated that correlations of ectoaminopeptidase activity with bacterial production or abundance are not relevant as regulatory mechanisms and organisms responsible for the expression of the hydrolytic activity cannot be derived directly from in situ observation. EAP showed strong correlation with respiration rates of amino acids. MIDDELBOE & SONDERGAARD (1993) reported diel cycle studies where bacterial growth efficiency was inversely correlated with glucosidase activity. These results, along with the relatively high % of respiration measured in the present study implied that bacteria used the amino acids added in the samples as an energy source and/or ectoenzyme synthesis.

Bacteria in the Aegean Sea showed slow growth and low activity. The relatively high % of respiration of amino acids measured in the present study, as well as good correlations between respiration rates of amino acids and ectoaminopeptidase activity implied that bacteria used the amino acids to maintain energy requirements and ectoenzyme synthesis.

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