

## Morphology and LPS content for the estimation of marine bacterioplankton biomass in the Ionian Sea\*

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**SUMMARY:** The abundance, morphotypes and biomass of the bacterial assemblages were investigated in the Ionian Sea by using two different methods: the epifluorescent microscopy technique for enumerating and sizing bacterial cells, and the determination of bacterial lipopolysaccharides (LPS). Five bacterial morphotypes were distinguished: cocci, rods, coccobacilli, vibrios and spirillae. The proportions of cocci were higher than those of other morphotypes at every depth, ranging from 39% to 73%. Both rod-shaped bacteria and coccobacilli were homogeneously distributed in the water column, while the proportions of vibrios were rather variable. Spirillae occurred only in surface samples and disappeared below 100 m. The two methodologies were compared: LPS concentrations showed a highly significant correlation with the bacterial numbers ( $P < 0.01$ ;  $n = 88$ ;  $r = 0.68$ ), but not with biovolumes, and different ratios between LPS concentrations and bacterial volumes were recorded for the photic and aphotic zones ( $3.11 \pm 1.35$  and  $0.96 \pm 0.37$  ng LPS per  $\mu\text{m}^3$  respectively). LPS-derived cell carbon content on average was  $23 \text{ fg C cell}^{-1}$ , similar to the C amount derived by mean cell biovolume ( $19 \text{ fg C cell}^{-1}$ ) and the biomass from two highly correlated methods ( $P < 0.01$ ;  $n = 95$ ;  $r = 0.59$ ). Our results confirm that the widely used factor of  $20 \text{ fg C cell}^{-1}$  (Lee and Furhman, 1987) should be plausible for studying the biomass of the natural microbial populations in the study area. Nevertheless, the wide variability of the cell size classes, also along the whole water columns, questions the applicability of a constant conversion factor for all the marine ecosystems. Consequently, locally derived biomass estimates of bacteria are essential in order to obtain an accurate evaluation of the bacterial role in biogeochemical cycles.

**Key words:** marine bacterioplankton, biomass estimation, lipopolysaccharides, biovolume, Ionian Sea.

**RESUMEN:** DETERMINACIÓN DE LA BIOMASA BACTERIANA EN EL MAR JÓNICO MEDIANTE EL ANÁLISIS DE LA MORFOLOGÍA CELULAR Y EL CONTENIDO EN LPS. – Fue estudiada la comunidad bacteriana del Mar Jónico mediante el análisis directo en epifluorescencia (recuentos, morfometría y biovolumen celular) y la determinación del contenido de lipopolisacáridos (LPS). Los microorganismos fueron agrupados en cinco morfotipos: cocos, bacilos, cocobacilos, vibriones y espirilos. La columna de agua se encontró dominada por formas cocoideas (39-73%); las formas bacilares y cocobacilares representaron morfotipos uniformemente distribuidos mientras la presencia de vibriones resultó ser muy variable y los espirilos prácticamente ausentes por debajo de los 100 m de profundidad. Los contenidos de LPS fueron encontrados estrechamente correlacionados con los recuentos celulares ( $P < 0,01$ ;  $n=88$ ;  $r=0,68$ ) y sin relación significativa alguna con los biovolúmenes totales de las muestras. El contenido medio de LPS por  $\mu\text{m}^3$  celular resultó ser de  $3,11 (\pm 1,35)$  ng en las muestras provenientes de la zona fótica y de  $0,96 (\pm 0,37)$  ng en las muestras provenientes de la zona afótica. El contenido medio de C por célula calculado a partir del contenido medio de LPS resultó ser de  $23 \text{ fg C célula}^{-1}$  siendo similar al calculado a partir del volumen celular ( $19 \text{ fg C célula}^{-1}$ ); las biomasa celular calculadas a partir de ambos parámetros (LPS y volumen celular) resultaron estar significativamente correlacionadas ( $P < 0,01$ ;  $n=95$ ;  $r=0,59$ ). Del análisis de nuestros resultados surge que el contenido medio de  $20 \text{ fg C célula}^{-1}$  (Lee and Furhman, 1987), utilizado ampliamente en la determinación de la biomasa bacteriana marina, podría ser utilizado en línea de máxima también en el Mar Jónico. No obstante, la amplia variabilidad morfológica-dimensional de las células bacterianas observadas, frecuentemente en una misma columna de agua, cuestionan firmemente el empleo de un único factor de conversión en el estudio de todos los ecosistemas marinos. En consecuencia, resulta esencial el análisis de la biomasa bacteriana en cada ambiente en particular para lograr una visión más precisa de los flujos de los principales bioelementos en el ecosistema.

**Palabras clave:** bacterioplancton marino, biomasa bacteriana, lipopolisacáridos, biovolumen, Mar Jónico.

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## INTRODUCTION

The biomass of bacterial populations is perhaps the most basic parameter that may be considered in microbial ecology (Bratbak, 1993). Its estimation is an essential step in understanding the food webs and the biogeochemical fluxes (Fukuda *et al.*, 1998; Nagata, 1986). A great number of methods have been proposed for biomass estimation, such as quantification of macromolecular components (i.e. muramic acid, ATP, specific lipids, DNA or proteins) (Jochem, 2001; Bratbak, 1993; Simon and Azam, 1989).

Among the indirect methods, the measurement of lipopolysaccharides (LPS) has been suggested as a good indicator of the bacterioplankton biomass in marine waters (Herbert, 1990; Maeda *et al.*, 1983; Maeda and Taga, 1979), also taking into account bacterial numbers by epifluorescence microscopy (La Ferla *et al.*, 2001; Crisafi *et al.*, 2000; Bruni *et al.*, 1995; La Ferla *et al.*, 1995; Naganuma *et al.*, 1990; Watson *et al.*, 1977). The recent application of flow cytometry to aquatic microbial ecology helps to quantify heterotrophic bacteria and distinguishes subpopulations with a different apparent DNA content (Gasol and del Giorgio, 2000).

Anyhow, fluorescent staining and counting under epifluorescence microscopy remain the standard method for enumerating bacteria in aquatic environments. This direct method is irreplaceable as a ground truth baseline on which a microbiological interpretation can be developed (Ducklow, 2000). The total cell count in combination with cell volume measurements is considered a key parameter that provides ecologically important information about the bacterial assemblage (Posch *et al.*, 2001; Massana *et al.*, 1997; Robarts *et al.*, 1996) and the amount of bacterial carbon is routinely estimated from cell concentrations or biovolumes by applying conversion factors. The choice of the conversion factor directly influences the results of bacterial biomass determination and, for this reason, an appropriate factor is needed.

Carbon contents change from one bacterial community to another because of the fluctuation of natural conditions and the physiological state of bacterial assemblages. Thus, the use of a universal conversion factor leads to large errors of bacterial biomass estimates. The assumption that each marine bacterium contains 20 fg of carbon has been assessed by Lee and Fuhrman (1987) and con-

firmed by Ducklow and Carlson (1992). Nevertheless, Christian and Karl (1994) and Caron *et al.* (1995) calculated a conversion factor of 10-15 fg C cell<sup>-1</sup>, while Fukuda *et al.* (1998) reported a mean carbon content of 12 ± 6 and 30 ± 12 fg C cell<sup>-1</sup> for oceanic and coastal locations respectively. Gundersen *et al.* (2002) and Zubkov *et al.* (2000) calculated an average elemental cell content of 4-9 and 7 fg C cell<sup>-1</sup> respectively. Vrede *et al.* (2002) reported the C content per cell varying between 3 and 276 fg C cell<sup>-1</sup> for exponentially growing and nutrient-limited bacterioplankton.

In this paper, we derive from biovolume and LPS quantification the conversion factor to estimate the biomass of natural marine bacteria inhabiting the Ionian marine pelagic system. In the eastern Mediterranean, the main water masses are roughly constituted by superficial (MAW, Modified Atlantic Waters; ISW, Ionian Surface Waters), intermediate (LIW, Levantine Intermediate Water; CIW, Cretan Intermediate Water) and deep waters (EMDW, Eastern Mediterranean Deep Water; CSOW, Cretan Sea Overflow Water). These waters, characterised by different TS properties and densities (Malanotte-Rizzoli *et al.*, 1997), spread from their sites of formation towards the Ionian Sea, transporting the preformed dissolved oxygen and organic matter to the sea interior (Klein *et al.*, 1999) as well as sustaining the distribution patterns of metabolic activities at different depths (La Ferla *et al.*, in press; Zaccone *et al.*, in press).

This study focuses on the estimation of microbial biomass in a pelagic area of the Mediterranean Sea. Novel aspects of this research in the microbial ecology literature include both the analysis of LPS and the study of deep marine bacteria.

## MATERIALS AND METHODS

Seawater samples were collected in Spring 2002 during two oceanographic surveys (TALASTRO-4: 19-25 March; SINAPSI-3: 26 March- 09 April) on board the R/V Urania of the Italian CNR at nine stations located in the Ionian Sea (Fig. 1). We took vertical sampling profiles from 5 m to the bottom depth (max 4000 m). Subsamples for microscopy determinations were fixed with formaldehyde (final concentration 2%) and stored in the dark at 4°C. LPS subsamples were stored at -20°C in pyrogen-free vials. Lab treatments of both subsamples were performed within a month.

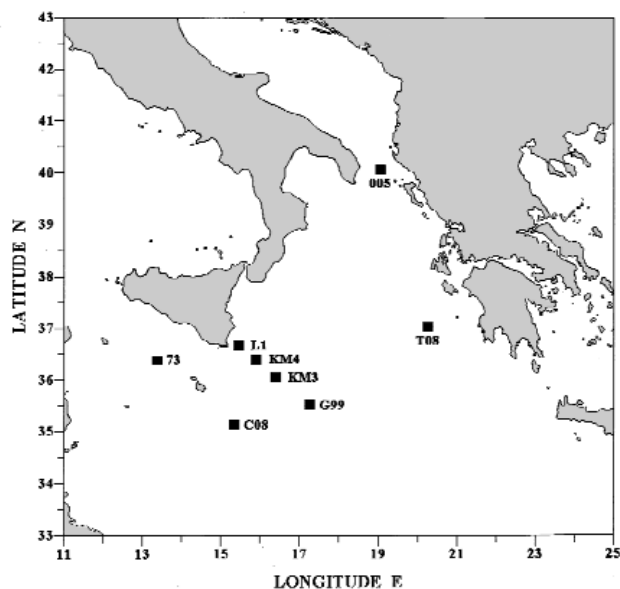


FIG. 1. – Map of the Ionian Sea showing the stations sampled from surface to the bottom depth (maximum 4000 m) during the TALASTRO-4 and SINAPSI-3 surveys.

## Direct counts

The epifluorescence direct count technique was applied to estimate total bacterial number, including cyanobacteria, according to Porter and Feig (1980). Slides were examined by epifluorescence with the AXIOPLAN 2 *Imaging* microscope (Zeiss). All cells in a minimum of twenty eyefields per sample were counted.

## Morphometric analysis

The microscope was equipped with the digital camera AxioCam (Zeiss). The images were captured and digitised on a personal computer using the AXIOVISION 3.1 software for the subsequent morphometric analysis. The standard resolution of 1300 x 1030 pixels was used for the image acquisition. The pixel size in the resulting image was 0.067  $\mu\text{m}$ .

The length (L) of bacterial cells was measured down the central axis and the width (W) at right angle to the length. The measurements of the central length of curved bacteria were more difficult but reasonable estimates can equally be made (Fry, 1990). Bacteria were sized by using the measurement functions of the Axiovision 3.1 software, classifying them into five morphotypes: cocci (spherical cells), coccobacilli, rods (elongated cells) and curved bacteria (C-shaped and S-shaped cells, i.e. vibrios and spirillae respectively). Cells were operationally

defined as cocci if their length and width differed by less than 0.10  $\mu\text{m}$ , coccobacilli if their length and width differed by more than 0.10  $\mu\text{m}$ , and rods if their length was at least double their width. According to Lee and Fuhrman (1987), the pixels which constituted the fluorescent “halo” around the bacterial cells were not measured. Bacterial biovolume (V;  $\mu\text{m}^3$ ) was derived from the two-dimensional parameters (W and L) obtained by image analysis, assuming that bacteria are cylindrical straight rods with hemispherical or, in the case of coccoid forms, spherical caps (Massana *et al.*, 1997). At least one hundred bacterial cells were measured in each sample to obtain adequate mean volumes (Bratbak, 1993).

The volume of a single cell was calculated according to Bratbak (1985):

$$V (\mu\text{m}^3) = (\pi/4)W^2(L-W/3).$$

For coccoid forms,  $W=L$ .

Cellular carbon content in fg C cell<sup>-1</sup> (C) was derived from the estimated biovolumes by using the allometric relation  $C = 218 V^{0.86}$  (Loferer-Kröbächer *et al.*, 1998) as proposed by Posch *et al.* (2001) for DAPI stained bacteria.

## Lipopolysaccharide concentrations

The Quantitative Chromogenic LAL test (QCL-1000, Bio Whittaker, Inc.) was used for the quantitative analysis of the LPS present in the outer membrane of gram-negative bacteria. Each seawater sample was mixed gently with the *Limulus* amoebocytes lysate (LAL) and incubated at 37°C for 10 minutes. The subsequent addition of the chromogenic reagent was followed by a further period of incubation at 37°C for 6 minutes. The reaction was stopped by adding 100  $\mu\text{l}$  acetic acid (final concentration 25%). The change in the colour of the solution from white to yellow indicated the concentration of LPS in the sample. The absorbance of the solution was read at 405 nm using a MICROTITER ELX-808 spectrophotometer (Bio Whittaker, Inc.) equipped with an Automatic Microplate Reader (for 96 microtiter) controlled by the specific software WIN KQCL (Bio Whittaker, Inc.) for the data processing. The LPS concentration in each sample, expressed as ng LPS ml<sup>-1</sup>, was converted into C using the conversion factor 6.35 proposed by Watson and Hobbie (1979). The obtained carbon content per volume was divided by the corresponding bacterial number in order to obtain the cell quantity of carbon as fg C cell<sup>-1</sup>.

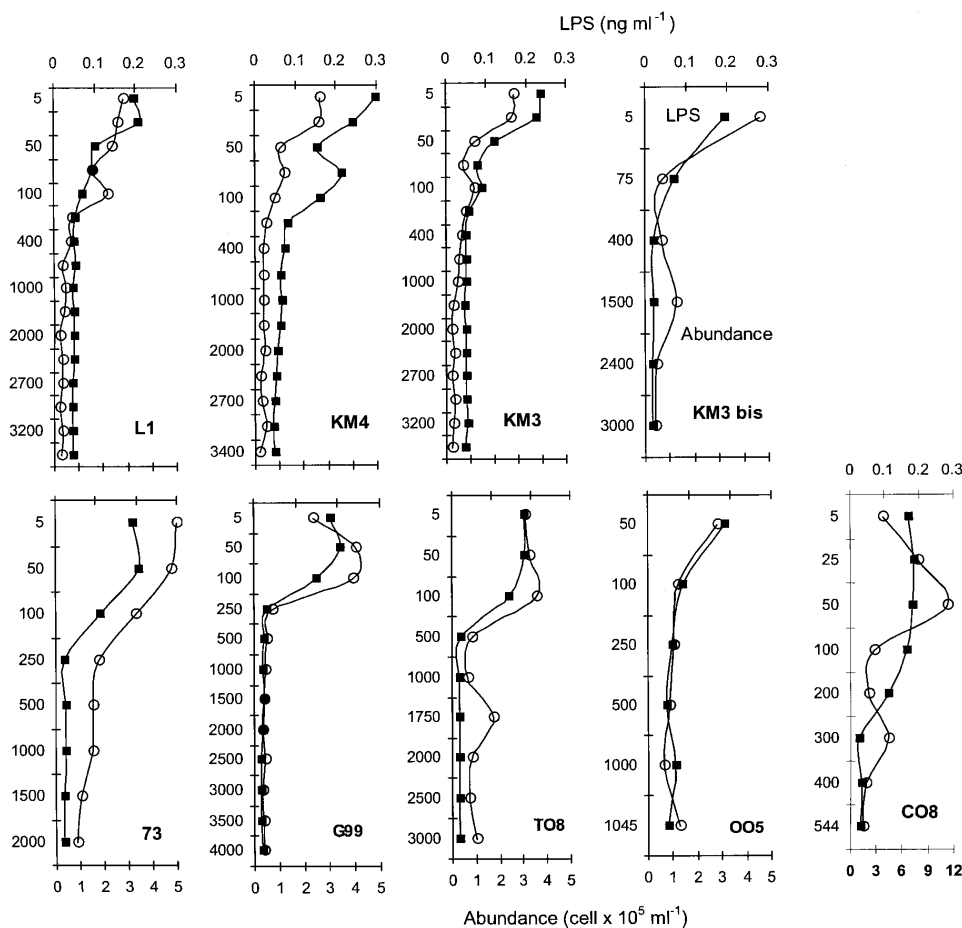


FIG. 2. – Variability in the vertical distribution of bacterioplankton abundances ( $10^5$  cell  $\text{ml}^{-1}$ ) and LPS concentrations ( $\text{ng LPS ml}^{-1}$ ) at the nine sampling stations. Note that the stations have different depth scales depending on each bottom depth; the profile of station C08 has a different scale for cell abundance.

## RESULTS

### Bacterial abundance and LPS quantification

Bacteria and LPS quantifications are reported in Figure 2. Cell abundance was generally highest in the upper water column (upper 200 m) and decreased with depth. In the upper 200 m bacterial abundance ranged from  $0.45$  to  $11.4 \times 10^5$  cell  $\text{ml}^{-1}$ . The highest values were registered at station C08 (note the different scale), which was characterised by anomalous abundances throughout the examined water column. Below 200 m depth bacterial abundance was generally lower, ranging from  $0.18$  to  $4.49 \times 10^5$  cell  $\text{ml}^{-1}$  at the different stations. Rare peaks locally occurred at 100, 1500 and 1700 m depths.

A total of 95 seawater samples were assayed by the LAL test. LPS concentrations ranged from 0.02 to  $0.30 \text{ ng ml}^{-1}$ , with a majority of samples containing  $<0.1 \text{ ng ml}^{-1}$ . The average concentration of LPS

was  $3.66 \pm 2.37 \text{ fg LPS cell}^{-1}$ . A highly significant correlation ( $P < 0.01$ ;  $n = 88$ ;  $r = 0.68$ ;  $y = 1\text{E}+06x + 24993$ ;  $R^2 = 0.4678$ ) was found between bacterial abundances and LPS concentrations (samples from the C08 station were excluded from the regression due to the different behaviour compared to other stations).

### Biomass estimation

Morphometric data of bacteria are summarised in Table 1. The averaged biovolumes of the bacteria in this study ranged from  $0.003$  to  $0.927 \mu\text{m}^3$ , with a mean value of  $0.0593 \mu\text{m}^3$ . The mean cell volumes varied in a similar range in both the euphotic and aphotic zones ( $0.035$  to  $0.073 \mu\text{m}^3$  and  $0.039$  to  $0.075 \mu\text{m}^3$  respectively). The high variability observed and the morphometric analysis of bacterial community in relation to depth at each sampling station are shown in Figure 3. The highest value was recorded at the KM3 station in superficial samples (where

TABLE 1. – Morphometric data set: average cell measures on total cells and per bacterial type ( $n = 95$ ; SD= standard deviation).

	Length ( $\mu\text{m}$ )			Width ( $\mu\text{m}$ )			Biovolume ( $\mu\text{m}^3$ )		
	Mean $\pm$ SD	Min	Max	Mean $\pm$ SD	Min	Max	Mean $\pm$ SD	Min	Max
Total cells	$0.65 \pm 0.52$	0.23	3.75	$0.37 \pm 0.16$	0.10	1.21	$0.059 \pm 0.076$	0.003	0.927
cocci	$0.44 \pm 0.13$	0.23	1.21	$0.44 \pm 0.13$	0.23	1.21	$0.055 \pm 0.080$	0.006	0.927
rods	$0.71 \pm 0.31$	0.30	2.70	$0.29 \pm 0.08$	0.10	0.53	$0.046 \pm 0.037$	0.003	0.196
coccobacilli	$0.54 \pm 0.17$	0.23	1.48	$0.35 \pm 0.12$	0.15	1.15	$0.057 \pm 0.108$	0.003	1.138
vibrios	$1.74 \pm 0.58$	0.64	3.32	$0.26 \pm 0.07$	0.13	0.47	$0.102 \pm 0.080$	0.012	0.401
spirillae	$2.57 \pm 0.81$	0.93	3.75	$0.31 \pm 0.08$	0.19	0.47	$0.201 \pm 0.125$	0.027	0.470

TABLE 2. – Means and ranges of cell abundance, C-content and biomass derived from biovolume data, C-content and biomass derived from LPS data ( $n = 95$ ; SD= standard deviation).

	Mean $\pm$ SD	Minimum	Maximum
Abundance (cells $\times 10^5 \text{ ml}^{-1}$ )	$1.4 \pm 1.7$	0.18	11.4
Cell C-content (fg) from biovolume data	$19.19 \pm 23.84$	1.36	240.26
Biomass ( $\mu\text{g C l}^{-1}$ )	$2.79 \pm 3.31$	0.34	21.89
Cell C-content (fg) from LPS data	$23.24 \pm 15.03$	6.41	63.05
Biomass ( $\mu\text{g C l}^{-1}$ )	$3.70 \pm 4.50$	0.27	15.29

abundant vibrios were observed) and volumes decreased with increasing depth. In KM3bis, 73, C08, G99, T08 and 005 stations, high volumes were registered at superficial layers but smaller peaks occurred with depth. The widest variability in cell size along the whole water column and the highest

volumes at great depths characterised the KM4 and L1 stations, where large cocci occurred. However, at almost all stations, peaks were observed at about 1500, 2500 and 3250 m.

The regression analysis between biovolumes and LPS concentrations did not show any correlations. Different ratios between LPS concentrations and biovolumes were computed for the superficial and deep layers ( $3.12 \pm 1.35$  and  $0.95 \pm 0.37 \text{ ng LPS per } \mu\text{m}^3$  respectively).

The data obtained from the morphometric analysis and the LPS quantification were converted to carbon content. In the first case, the biovolumes were applied to the allometric equation proposed by [Loferer-Kröbächer et al. \(1998\)](#) and the resulting bacterial carbon content was on average  $19.19 \pm 23.84 \text{ fg C cell}^{-1}$ . In order to convert the LPS values to bacterial biomass, the conversion factor 6.35 was used, as suggested by [Watson and Hobbie \(1979\)](#). The mean bacterial carbon content was  $23.24 \pm 15.03 \text{ fg C cell}^{-1}$ . Nevertheless, analysing the data per cell resulted in very wide ranges of variability from 1 to 240 (from biovolume data) and 6 to 63  $\text{fg C cell}^{-1}$  (from LPS data).

Multiplying the cell abundance for the cell carbon contents, both separately derived from biovolume and LPS data, we obtained the mean biomass of 2.8 and  $3.7 \mu\text{g C l}^{-1}$  respectively (Table 2). The biomass derived from biovolumes showed a high significant correlation with the biomass derived from LPS ( $P < 0.01$ ;  $n = 95$ ;  $r = 0.59$ ;  $y = 4.4476x + 0.4839$ ;  $R^2 = 0.3426$ ).

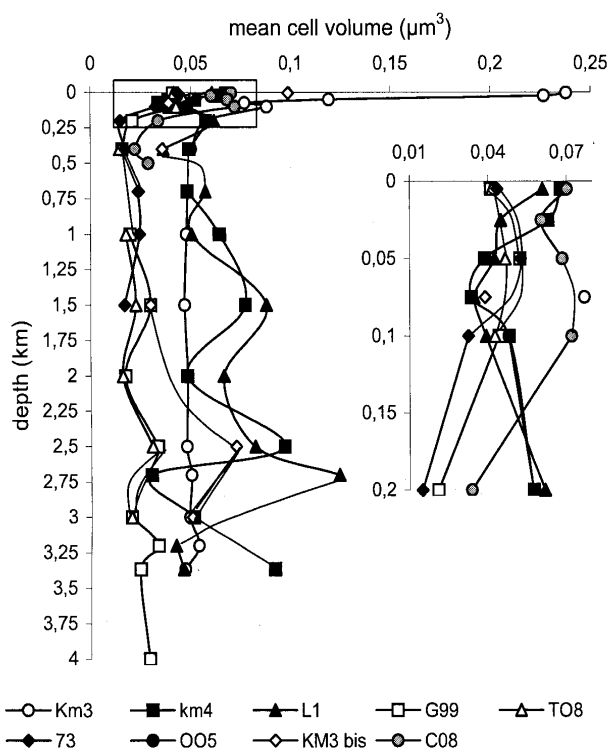


FIG. 3. – Mean cell volumes of the cells from the nine sampling stations. The distribution of cell volumes in the upper 200 m, enclosed on the left within the box, is enlarged on the right side of the figure.

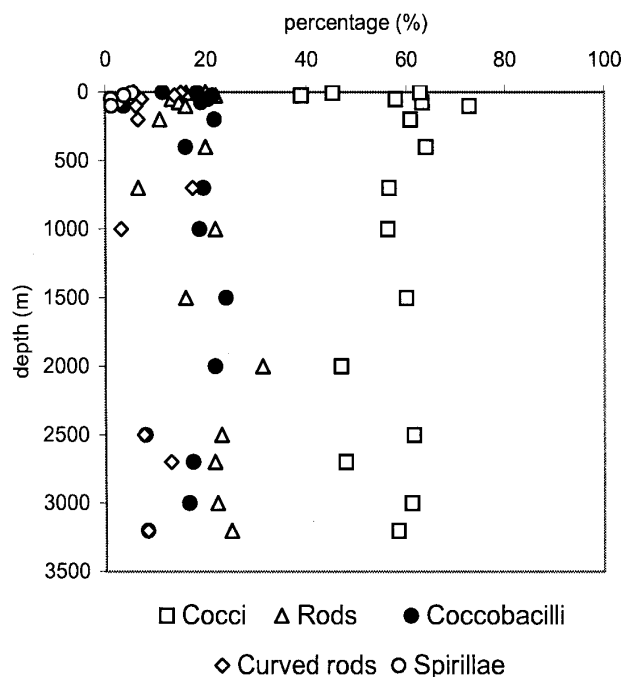


FIG. 4. – Distribution with depth of the different morphotypes expressed as percentages.

### Morphometric analysis

Five morphotypes of bacteria were distinguished: cocci, rods, coccobacilli, vibrios and spirillae. Cocci were the most common morphotype as they contributed 54% to total bacteria; coccobacilli and rods showed similar proportions (17% and 18%, respectively); vibrios amounted to 9% of the total bacteria; and spirillae were fairly negligible (2%).

The morphometric data set per bacterial types is reported in Table 1. Cocci, including *Synechococcus* and *Prochlorococcus*, averaged 0.44  $\mu\text{m}$  in diameter; most coccobacilli were about 0.40 to 0.62  $\mu\text{m}$  in length and 0.24 to 0.40  $\mu\text{m}$  in width; rods were about 0.24 to 0.38  $\times$  0.62 to 0.72  $\mu\text{m}$  in size; vibrios were generally about 0.19 to 0.29  $\times$  1.58 to 1.70  $\mu\text{m}$  in size. The distribution patterns of the different morphotypes along the water column are shown in Figure 4. The proportions of cocci were higher than those of other morphotypes at every depth, ranging from 39% at 25 m to 73% at 100 m. On the whole, lower percentages occurred in the upper 25 m. The rod-shaped bacteria had homogeneous proportions from the surface to the bottom, with a slight decrease at 700 m (6%) and a peak at 2000 m (31%). Coccobacilli were also homogeneously distributed along the water column (about 20%), while the proportions of vibrios were rather variable but <20%.

Spirillae occurred only in surface samples (<10%) and disappeared below 100 m.

### DISCUSSION

To our knowledge, no studies have so far dealt with bacterial abundances or morphometry in the deep Ionian Sea, with the exception of a study in superficial, intermediate and deep waters of the Ionian Sea by Zaccone *et al.* (in press), who determined cell densities ranging between 0.4 and 1.7  $\times 10^5$  cells  $\text{ml}^{-1}$ .

Our data show that in the upper 200 m bacterial abundance ranged from 0.45 to 11.4  $\times 10^5$  cell  $\text{ml}^{-1}$ , whereas below 200 m depth it ranged from 0.18 to 4.49  $\times 10^5$  cell  $\text{ml}^{-1}$ . Bacterial density was similar to that found by Tanaka and Rassoulzadegan (2002) in the northwestern Mediterranean (from 0.22 to 21  $\times 10^5$  cell  $\text{ml}^{-1}$  throughout the depth interval 0-2000 m) and by Van Wambeke *et al.* (2002) in the Gulf of Lions (from 3 to 12  $\times 10^5$  cell  $\text{ml}^{-1}$  in the upper 165 m). During summer stratification in the Catalano-Balearic Basin, Pedrós-Alió *et al.* (1999) found bacterial abundances increasing from 2  $\times 10^5$  cell  $\text{ml}^{-1}$  at surface to 5  $\times 10^5$  cell  $\text{ml}^{-1}$  at depths close to the DCM and decreasing below 200 m down to 0.4  $\times 10^5$  cell  $\text{ml}^{-1}$ . In the Levantine Basin of the southeastern Mediterranean Robarts *et al.* (1996) reported lower bacterial numbers ranging from 0.40 to 3.90  $\times 10^5$  cell  $\text{ml}^{-1}$  in the upper 200 m.

As noted above, the abundances detected at Station C08 were the highest in the study area and could presumably depend on water mass hydrological characteristics.

Cell volumes of the bacterial populations investigated widely ranged from 0.003 to 0.927  $\mu\text{m}^3$  with a mean value of 0.0593  $\mu\text{m}^3$ . The high standard deviations were due to the different patterns in biovolume distribution shown in Figure 3. Robarts *et al.* (1996) measured mean volume of 0.049  $\mu\text{m}^3$  for cocci and 0.107  $\mu\text{m}^3$  for rods and vibrios in the southeastern Mediterranean. In the northern Gulf of Mexico, Jochem (2001) estimated 0.04 and 0.01  $\mu\text{m}^3$  cell volumes for coccoid and rod-shaped bacteria respectively.

The data obtained from each station of our study, with the exception of stations KM3 and C08 (this latest sampled only down to 544 m), showed regional patterns in size-distribution with depth. Surprisingly, the highest volumes were detected at depth at the stations away from the coast and east of the

shelfbreak in the Ionian Sea (L1 and KM4), while some pelagic stations (G99, T08 and KM3 bis) were characterised by smaller peaks along the entire water column.

These findings suggest that suitable conditions sustaining the cell growth could occur at depths also; hence, the variability observed in the size of bacterial cells along the water column is probably due to the different availability of organic matter and inorganic nutrients in the seawater masses which characterise the Mediterranean Sea.

Psenner (1993) has determined that cell size derives from the balance between the growth and the frequency of division and then depends on environmental conditions, such as temperature and the quantity and quality of substrates (Thingstad and Rasmuzadegan, 1995). According to Gasol *et al.* (1995) there is a linear relationship between bacterial size and activity. Differences in bacterial strain morphologies may depend on growth-limiting factors (Vrede *et al.*, 2002). Albright and McCrae (1987) have reported the predominance of large cells in the surface and deeper waters of Howe Sound (Canada). In the deep Mediterranean Sea, Zaccone *et al.* (in press) has observed relatively high specific activity in bacterial production rates owing to the organic load derived from young water mass intrusion.

The simultaneous occurrence of different bacterial morphotypes in marine environments has been well documented (Jochem, 2001; Acinas *et al.*, 1999; Robarts *et al.*, 1996; Viles and Sieraki, 1992). In the Ionian Sea, the bacterial community was mainly composed of cocci representing 54% of total bacteria. Their contribution together with coccobacilli (71%) was comparable to those reported by Robarts *et al.* (1996), Wiebe and Pomeroy (1972), Ferguson and Rublee (1976) in studies carried out in pelagic and coastal ecosystems. The predominance of cocci, including cyanobacteria, in oligotrophic and/or deep environments might be explained by the adaptation to a low organic substrate concentration due to their better surface-to-volume ratio than rod-shaped and curved bacteria (Jochem, 2001). In contrast, the proportion of rods and curved bacteria (vibrios and spirillae) in the Ionian Sea was higher than the 13% reported by Robarts *et al.* (1996) in the southeastern Mediterranean and the 10% detected by Jiao and Ni (1997) in the western and central Pacific.

The distribution of morphotypes with depth indicates the high contribution of coccal forms in oligotrophic environments like the Ionian sea. Neverthe-

less, greater forms, like rods and curved rods, occurred not only at the surface but also at depth, suggesting the growth factor availability within deep and intermediate water masses.

LPS determination in seawater samples is a method that is not currently adopted and no studies in the Mediterranean Sea or in deep environments exist to our knowledge. LPS measurements varied from 0.02 to 0.30 ng ml<sup>-1</sup> with a mean value of 0.08 ± 0.07 ng ml<sup>-1</sup>. On the whole, such concentrations were similar to or lower than those reported for Antarctica by La Ferla *et al.* (1995; 0.03-0.70 ng ml<sup>-1</sup>), Crisafi *et al.* (2000; 0.02-0.20 ng ml<sup>-1</sup>) and La Ferla *et al.* (2001; 0.08-0.19 ng ml<sup>-1</sup>). LPS distribution down the water column showed a net decrease below 250 m depth, as already noted by Maeda *et al.* (1983) in subtropical areas.

On the basis of our results the LPS-test is to be considered a valid support to other methodologies owing to its easy and quick applicability for studying bacterial biomass in marine environments. Maeda *et al.* (1983) and Watson *et al.* (1977) already considered the distribution of LPS concentration as a good indicator of bacterial biomass, resulting from the relation between bacterial number and LPS content. The linear correlation (P<0.01) derived from the regression analysis of our results confirms this finding. Anyhow, what seems even more interesting is the close correlation of the biomass derived from biovolumes vs the biomass derived from LPS. It is presumable that the comprehensive biomass, depending on both abundance and cell volume, is mainly modulated by cell densities. We cannot explain the significance of these observations, but the amount of LPS does not seem to depend on cell size. Moreover, a different pattern was shown for euphotic and aphotic zones from the ratio computed between LPS concentrations and biovolumes, and the amounts of LPS per cell in the euphotic zone was about three times higher than in the deep waters. The significance of this ratio is not easy to explain. Further laboratory and field experiments could in future clarify these relationships. We suggest that LPS is an important cellular component that should be further considered in the context of the marine microbial ecology.

During the last two decades several allometric formulae and linear conversion factors have been used to determine carbon content by converting cell volume to biomass (Posch *et al.*, 2001). We compared the value of 23.24 fg C cell<sup>-1</sup> derived from the LPS quantification to those obtained from the appli-

cation of several formulae (Loferer-Kröbächer *et al.*, 1998; Theil-Nielsen and Søndergaard, 1998; Norland, 1993; Fry, 1990; Lee and Fuhrman, 1987). If the allometric relation proposed by Loferer-Kröbächer *et al.* (1998) is applied to our morphometric data, the biomass estimates, i.e. 19.19 fg C cell<sup>-1</sup>, fit well with the result obtained from LPS analysis. For this reason, we recommend the use of this allometric relation specifically created for DAPI-stained bacteria, as recently suggested by Posch *et al.* (2001).

The mean values of cell carbon content derived from the bacterial sizing and the LPS analysis are not very different from the classical and widely-used factor of 20 fg C cell<sup>-1</sup> (Lee and Fuhrman, 1987). Nevertheless, the more recent detailed treatments on this topic by Gundersen *et al.* (2002), Vrede *et al.* (2002) and Zubkov *et al.* (2000) have enhanced the debate on the physiologically unreasonable application of a constant conversion factor and demonstrated that biomass assessment is highly dependent on the choice of cell-specific conversion factors. In this study we have also highlighted the high variability in each cell carbon content, hence the extrapolation of the most correct conversion factor must consider the locally derived biomass estimates.

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## REFERENCES

- Acinas, S.G., J. Antòn and F. Rodríguez-Valera. – 1999. Diversity of free-living and attached bacteria in offshore western Mediterranean waters as depicted by analysis of genes encoding 16S rRNA. *Appl. Environ. Microbiol.*, 65: 514-522.
- Albright, L.J. and S.K. McCrae. – 1987. Annual cycle of bacterial specific biovolumes in Howe Sound, a Canadian West Coast Fjord Sound. *Appl. Environ. Microbiol.*, 53: 2739-2744.
- Bratbak, G. – 1985. Bacterial biovolume and biomass estimation. *Appl. Environ. Microbiol.*, 49: 1488-1493.
- Bratbak, G. – 1993. Microscope methods for measuring bacterial biovolume: epifluorescence microscopy, scanning electron microscopy, and transmission electron microscopy. In: P.F. Kemp, B.F. Sherr, E.B. Sherr and J.J. Cole (eds.), *Handbook of methods in aquatic microbial ecology*, pp. 309-317. Lewis Publisher, USA.
- Bruni, V., R. La Ferla, M.L.C. Acosta Pomar and L. Salomone. – 1995. Structural differences of the microbial community in two sites of the Terra Nova Bay (Ross Sea, Antarctica): a statistical analysis. *Microbiologica*, 18: 409-422.
- Caron, D.A., H.G. Dam, P. Kremer, E.J. Lessard, L.P. Madink, T.C. Malone, J.M. Napp, E.R. Peele, M.R. Roman and M.J. Youngbluth. – 1995. The contribution of microorganisms to particulate carbon and nitrogen in surface waters of the Sargasso Sea near Bermuda. *Deep-Sea Res.*, 42: 943-972.
- Christian, J.R. and D.M. Karl. – 1994. Microbial community structure at the US-JGOFS Station ALOHA: inverse methods for estimating biochemical indicator ratios. *J. Geophys. Res.*, 99: 14269-14276.
- Crisafi, E., F. Azzaro, R. La Ferla and L.S. Monticelli. – 2000. Microbial biomass and respiratory activity related to the ice-melting upper layers in the Ross Sea (Antarctica). In: F.M. Faranda, Guglielmo L., Ianora A. (eds.), *Ross Sea Ecology*, chapter 14, pp. 171-180. Springer-Verlag, Berlin Heidelberg.
- Ducklow, H.W. and C.A. Carlson. – 1992. Oceanic bacterial production. In: K.C. Marshall (ed.), *Advances in Microbial Ecology*, Vol. 12, pp. 113-181. Plenum Press, New York.
- Ducklow, H. – 2000. Bacterioplankton production and biomass in the oceans. In: D. Kirchman (ed.), *Microbial ecology of the oceans*, Chapter 4, pp. 85-120. New York: Wiley.
- Ferguson, R.L. and P. Rublee. – 1976. Contribution of bacteria to standing crop of coastal plankton. *Limnol. Oceanogr.*, 21: 141-145.
- Fry, J.C. – 1990. Direct methods and biomass estimation. *Meth. Microbiol.*, 22: 41-85.
- Fukuda, R., H. Ogawa, T. Nagata and I. Koike. – 1998. Direct determination of carbon and nitrogen contents of natural bacterial assemblages in marine environments. *Appl. Environ. Microbiol.*, 64(9):3352-3358.
- Gasol, J.M. and P.A. Del Giorgio. – 2000. Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Sci. Mar.*, 64: 197-224.
- Gasol, J.M., P.A. Del Giorgio, R. Massana and C.M. Duarte. – 1995. Active versus inactive bacteria: size-dependence in a coastal marine plankton community. *Mar. Ecol. Progr. Ser.*, 128: 91-97.
- Gundersen, K., M. Heldal, S. Norland, D.A. Purdie and A.H. Knap. – 2002. Elemental C, N, and P cell content of individual bacteria collected at the Bermuda Atlantic Time-series Study (BATS) site. *Limnol. Oceanogr.*, 47(5): 1525-1530.
- Herbert, R.A. – 1990. Methods for enumerating microorganisms and determining biomass in natural environments. *Methods Microbiol.*, 22: 1-39.
- Jiao, N.Z. and I.H. Ni. – 1997. Spatial variation of size-fractionated chlorophyll, cyanobacteria and heterotrophic bacteria in the central and western Pacific. *Hydrobiologia*, 352: 219-230.
- Jochem, F.J. – 2001. Morphology and DNA content of bacterioplankton in the northern Gulf of Mexico: analysis by epifluorescence microscopy and flow cytometry. *Aquat. Microb. Ecol.*, 25: 179-194.
- Klein, B., W. Roether, B.B. Manca, D. Bregant, V. Beitzel, V. Kovacevic and A. Lucchetta. – 1999. The large deep water transient in the Eastern Mediterranean. *Deep-Sea Res. I*, 46: 371-414.
- La Ferla, R., A. Allegra, F. Azzaro, S. Greco and E. Crisafi. – 1995. Observation on the microbial biomass in two stations of Terra Nova Bay (Antarctica) by ATP and LPS measurements. *Mar. Ecol.*, 16(4): 307-315.
- La Ferla, R., F. Azzaro, M. Azzaro and G. Maimone. – 2001. Microbial biomass and respiratory activity in two sites in the Ross Sea. 2<sup>nd</sup> International Conference on the Oceanography of the Ross Sea (Antarctica), 8-13 October 2001, Terme Ischia (Naples).
- La Ferla, R., M. Azzaro, G. Civitarese and M. Ribera d'Alcalà. – (in press). Distribution patterns of carbon oxidation in the Eastern Mediterranean Sea: evidence of changes in the remineralization processes. *J. Geophys. Res.*, special issue.
- Lee, S. and A. Fuhrman. – 1987. Relationship between biovolume and biomass of naturally derived bacterioplankton. *Appl. Environ. Microbiol.*, 53: 1298-1303.
- Loferer-Kröbächer, M., J. Klima and R. Psenner. – 1998. Determination of bacterial cell dry mass by transmission electron microscopy and densitometric image analysis. *Appl. Environ. Microbiol.*, 64: 688-694.
- Maeda, M. and N. Taga. – 1979. Chromogenic assay method of lipopolysaccharide (LPS) for evaluating bacterial standing crop in seawater. *J. Appl. Bacteriol.*, 47: 175-182.
- Maeda, M., W.J. Lee and N. Taga. – 1983. Distribution of



- lipopolysaccharide, an indicator of bacterial biomass, in subtropical areas of the sea. *Mar. Biol.*, 76: 257-262.
- Malanotte-Rizzoli P., B. B. Manca, M. Ribera d'Alcalà, A. Theocharis, A. Bergamasco, D. Bregant, G. Budillon, G. Civitarese, D. Georgopoulos, G. Korres, A. Lascaratos, A. Michelato, E. Sansone, P. Scarazzato and E. Souvermezoglou. – 1997. A synthesis of the Ionian Sea hydrography. Circulation and water mass pathways during POEM phase I. *Progr. Oceanogr.*, 39 (3): 153-204.
- Massana, R., J.P. Gasol, P.K. Bjørnsen, N. Blackburn, A. Hanström, S. Hietanen, B.H. Hygum, J. Kuparinen and C. Pedrós-Alió. – 1997. Measurement of bacterial size via image analysis of epifluorescence preparations: description of an inexpensive system and solutions to some of the most common problems. *Sci. Mar.*, 61(3): 397-407.
- Naganuma, T., E. Ikemoto, S. Sukizaki, Y. Tsuji and H. Hotta. – 1990. Vertical distribution of bacterial abundance in the Northwest Pacific Ocean. *J. Oceanogr. Soc. Japan*, 46: 107-110.
- Nagata, T. – 1986. Carbon and nitrogen content of natural planktonic bacteria. *Appl. Environ. Microbiol.*, 52(1): 28-32.
- Norland S. – 1993. The relationship between biomass and volume of bacteria. In: P.F. Kemp, B.F. Sherr, E.B. Sherr and J.J. Cole (eds.), *Handbook of methods in aquatic microbial ecology*, pp. 303-307. Lewis Publisher, USA.
- Pedrós-Alió, C., J-I. Calderón-Paz, N. Guixa-Boixereu, M. Estrada and J.M. Gasol. – 1999. Bacterioplankton and phytoplankton biomass and production during summer stratification in the northwestern Mediterranean Sea. *Deep Sea Res. I*, 46: 985-1019.
- Porter, K.G. and Y.S. Feig. – 1980. The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr.*, 25: 943-948.
- Posch, T., M. Loferer-Krößbacher, G. Gao, A. Alfreider, J. Pernthaler and R. Psenner. – 2001. Precision of bacterioplankton biomass determination: a comparison of two fluorescent dyes, and of allometric and linear volume-to-carbon conversion factors. *Aquat. Microb. Ecol.*, 25: 55-63.
- Psenner, R. – 1993. Determination of size and morphology of aquatic bacteria by automated image analysis. In: P.F. Kemp, B.F. Sherr, E.B. Sherr and J.J. Cole (eds.), *Handbook of methods in aquatic microbial ecology*, pp. 339-345. Lewis Publisher, USA.
- Robarts, R.D., T. Zohary, M.J. Waiser and Y.Z. Yacobi. – 1996. Bacterial abundance, biomass, and production in relation to phytoplankton biomass in the Levantine Basin of the southeastern Mediterranean Sea. *Mar. Ecol. Prog. Ser.*, 137: 273-281.
- Simon, M. and F. Azam. – 1989. Protein content and protein synthesis rates of planktonic marine bacteria. *Mar. Ecol. Prog. Ser.*, 51:201-213.
- Tanaka, T. and F. Rassoulzadegan. – 2002. Full-depth profile (0-2000 m) of bacteria, heterotrophic nanoflagellates and ciliates in the NW Mediterranean Sea: vertical partitioning of microbial trophic structures. *Deep Sea Res. II*, 49: 2093-2107.
- Theil-Nielsen, J. and M. Søndergaard. – 1998. Bacterial carbon biomass calculated from biovolumes. *Arch. Hydrobiol.*, 141: 195-207.
- Thingstad, T.F. and F. Rassoulzadegan. – 1995. Nutrient limitations, microbial food webs, and “biological C-pumps”: suggested interactions in a P-limited Mediterranean. *Mar. Ecol. Prog. Ser.*, 117: 299-306.
- Van Wambeke, F., S. Heussner, F. Diaz, P. Raimbault and P. Conan. – 2002. Small-scale variability in the coupling/uncoupling of bacteria, phytoplankton and organic carbon fluxes along the continental margin of the Gulf of Lions, Northwestern Mediterranean Sea. *J. Mar. Syst.* 33-34: 411-429.
- Vrede, K., M. Heldal, S. Norland and G. Bratbak. – 2002. Elemental composition (C, N, P) and cell volume of exponentially growing and nutrient-limited bacterioplankton. *Appl. Environ. Microbiol.*, 68(6): 2965-2971.
- Viles, C.L. and M.E. Sieraki. – 1992. Measurement of marine picoplankton cell size by using a cooled, charge coupled device camera with image-analyzed fluorescence microscopy. *Appl. Environ. Microbiol.*, 58: 584-592.
- Watson, S.W., T.J. Novitsky, H.L. Quinby and F.W. Valois. – 1977. Determination of bacterial number and biomass in the marine environment. *Appl. Environ. Microbiol.*, 33: 940-946.
- Watson, S.W. and J.E. Hobbie. – 1979. Measurement of bacterial biomass as lipopolysaccharides. In: J.W. Conseron and R.R. Colwell (eds.), *Native aquatic bacteria: enumeration, activity and ecology*, pp. 82-88. Philadelphia, ASTM.
- Wiebe, W.J. and L.R. Pomery. – 1972. Microorganisms and their association with aggregates and detritus in the sea: a microscopic study. *Mem. Ist. Ital. Idrobiol.*, 29 (Suppl.): 325-342.
- Zaccone R., L.S. Monticelli, A. Seritti, C. Santinelli, M. Azzaro, A. Boldrin, R. La Ferla and M. Ribera d'Alcalà. – (in press). Bacterial processes in the intermediate and deep layers of the Ionian Sea in winter 1999. *J. Geoph. Res.*, special issue.
- Zubkov, M.V., M.A. Sleight, P.H. Burkil and R.J.G. Leakey. – 2000. Bacterial growth and grazing in contrasting areas of north and south Atlantic. *J. Plankton Res.*, 22: 685-711.

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