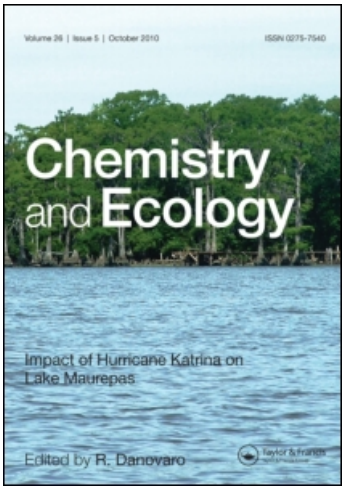

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DETERMINATION OF LIVING AND ACTIVE BACTERIOPLANKTON: A COMPARISON OF METHODS

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The purpose of this study was the quantification, through the comparison of different methods, of viable and metabolically active bacteria in marine environments. To quantify the living and active bacterioplankton fractions, we compared the total cell count (TC using DAPI staining), plate count on marine agar (CFU), and three viability-staining methods: nucleoid-containing cell count (NuCC), Live/Dead staining procedure (L/D) and direct viable count (DVC). With respect to TC (mean value $3.0 \pm 2.3 \times 10^5$ cell ml^{-1}) CFU represented less than 0.1% and DVC cells 1%, both showing significant differences. NuCC and L/D cells were 18.0% and 15.9% of TC, respectively, showing no significant differences and higher percentages in the Ionian Sea than the Adriatic Sea. Moreover, NuCC and L/D were two orders of magnitude greater than the culturable fraction, while active cells (DVC) exceeded CFU by one order of magnitude. The comparison of different staining methods allowed us to confirm the simultaneous presence of different physiological states within the bacterial population in natural marine environments. The NuCC and L/D methods gave comparable values to those of other authors, while the DVC procedure gave lower values than previously reported. This research provides information on the fraction of living and/or metabolically active bacteria in aquatic ecosystems. Since each method has its own detection limits, the study highlights the need to simultaneously compare the different methods to validate their results.

Keywords: Bacterioplankton; Living and active cells; Methods

1 INTRODUCTION

In oligotrophic environments, in which organisms <2 μm in size prevail, the role of heterotrophic bacteria and, in particular, the significance of the bacterial processes in organic matter cycles is recognized and known (Ducklow and Carlson, 1992; Azam *et al.*, 1993). Nevertheless, the actual percentage of prokaryotes which are actively involved in these processes remains unquantified. The utilization of procedures capable of quantifying all the viable cells within a bacterial natural community is crucial to determine the full impact of such cells within their ecosystem. Traditional total bacteria direct counting procedures give an overestimation as they include dead cells and bacterium-shaped particles (Zweifel and Hagström, 1995; Heissenberger *et al.*, 1996) and consequently do not determine the

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metabolically active and viable fraction (Zweifel and Hagström, 1995; Karner and Fuhrman, 1997; Muela *et al.*, 1999). Conversely, plate counts underestimate viable cells because living bacteria may undergo few cell divisions in unfavourable conditions, insufficient to produce bacterial colonies detectable by the observer (Button *et al.*, 1993; Bianchi and Giuliano, 1996). To obviate the restrictions of traditional total count and cultural methods, procedures including direct viable count (DVC) (Kogure *et al.*, 1979), microautoradiography (Tabor and Neihof, 1982), dilution culture (Button *et al.*, 1993), and microcolonies (Bianchi and Giuliano, 1996) have been developed to determine the true metabolically active population. In addition to the discrepancies between quantification techniques, studies are further complicated by the potential existence and significance of a viable but non-culturable population (Roszak and Colwell, 1987; Byrd *et al.*, 1991; Oliver *et al.*, 1995; Joux *et al.*, 1997; Whitesides and Oliver, 1997; McDougald *et al.*, 1998; Lebaron *et al.*, 1999; Muela *et al.*, 1999; Oliver, 1999; Weichart, 1999, 2000; Liu, 2000; Lleò *et al.*, 2001). However, the existence of such a population has been challenged (Bovill and Mackey, 1997; Bogosian *et al.*, 1998; Kell *et al.*, 1998; Barer and Harwood, 1999; Barer *et al.*, 1999; Bogosian and Bourneuf, 2001).

Among the methods developed to detect living, active and/or inactive, dormant and dead bacteria, we have chosen and simultaneously used three methods that are less expensive and less time-consuming than others and which involve easily utilizable equipment in field surveys. As these methods (direct viable count, nucleoid-containing cell count and Live/Dead staining count) differ in their physiological basis, our estimation of cellular viability in natural marine samples derives from the comparison of different results. We used the direct viable count (DVC) according to Joux and Lebaron (1997) for the detection of bacteria with active cellular synthesis; the nucleoid-containing cell count (NuCC) in agreement with Karner and Fuhrman (1997) to selectively distinguish bacteria with nucleoids from cells without nucleoids (ghosts) and bacterium-shaped particles (Zweifel and Hagström, 1995; Joux *et al.*, 1997; Muela *et al.*, 1999) and finally the Live/Dead (L/D) staining procedure to distinguish live and dead bacteria on the basis of membrane integrity (Alexander *et al.*, 1999; Boulos *et al.*, 1999; Defives *et al.*, 1999; Gasol *et al.*, 1999; Ericsson *et al.*, 2000; Berman *et al.*, 2001; Schumann *et al.*, 2003).

2 MATERIALS AND METHODS

2.1 Study Sites and Sampling

Samplings were performed during different oceanographic cruises by the Urania CNR vessel (E1 and AD offshore stations, July 2000 and April 2002, respectively) and by the R/V *Tethis* (A coastal station, October 2001) from three stations, two in the Ionian Sea (E1 and A) and one in the Adriatic Sea (AD) (Fig. 1). At stations E1, located at 37° 09′ 57″ N, 15° 22′ 59″ E, and AD, located at 41° 49′ 37″ N, 17° 44′ 34″ E, the samplings were carried out from surface to bottom (5–1540 m and 5–1191 m, respectively). At the coastal station (A), located at 37° 12′ 17″ N, 15° 14′ 54″ E, the sampling, from a depth of 25 to 55 m, was repeated after 4 h, to investigate short-term bacterioplankton variations. Samples were collected using Seabird CTD, equipped with a Rosette sampler, with twelve 8 l Niskin bottles.

2.2 Microbiological Determinations

Seawater samples for bacterioplankton direct counts were fixed on board with formaldehyde (final concentration 2%) and stored in the dark at 4°C. The total counts of bacterioplankton

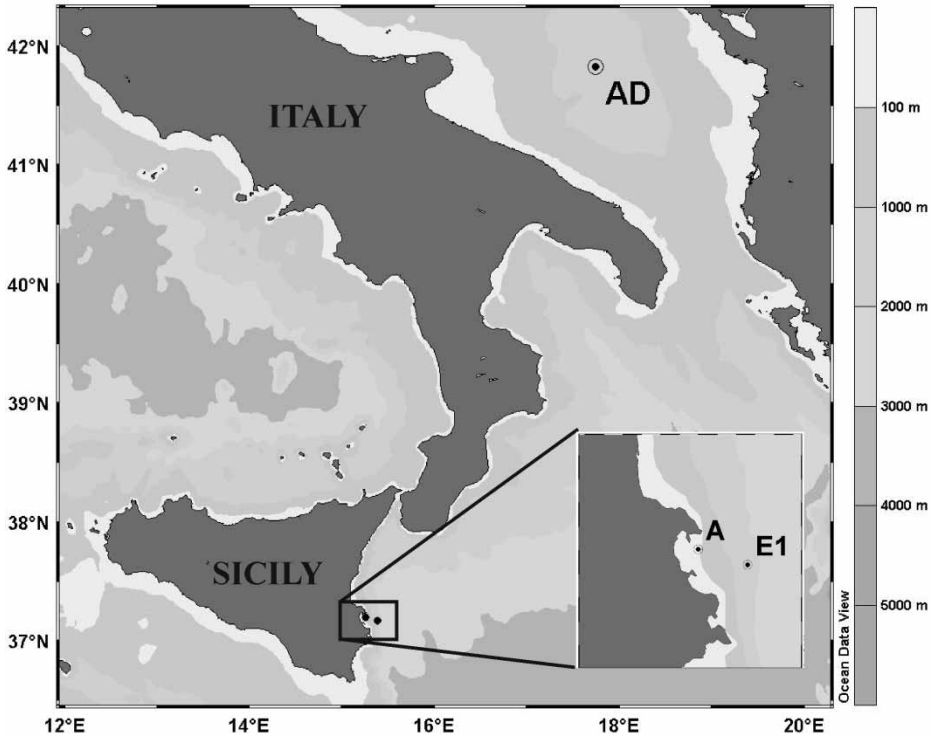

FIGURE 1 Sampling stations.

(TC) were performed using traditional DAPI stain (Porter and Feig, 1980). Samples, fixed with formaldehyde (final concentration 2% vol/vol), were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma) for 5 min (final concentration 10 $\mu g\ ml^{-1}$) and then filtered on black polycarbonate filters (0.2 μm pore size, Nuclepore).

NuCC counts were performed according to Karner and Fuhrman (1997) method. Samples were fixed with sodium azide (final concentration 0.5 M), stored at 4°C and analysed within 3 d. Samples were incubated with DAPI (final concentration 2 $\mu g\ ml^{-1}$) for 1 h, diluted four-fold with filtered deionized water, amended with Triton X-100 (final concentration 0.1% vol/vol) and incubated with DAPI (final concentration 2 $\mu g\ ml^{-1}$), in the dark, for 2 h. After filtration as in the TC procedure, 5 ml of isopropanol was added for 10 min to remove unspecific DAPI stain associated with non-bacterial particles and filtered again.

Samples for DVC, L/D and culturable bacteria (CFU) determinations were analysed within 2 h of sampling to avoid any modifications in viable/active bacterial counts due to storage.

DVC samples were incubated in the dark, at 20°C, for 24 h, with a cocktail of antibiotics (nalidixic acid, 20 $\mu g\ ml^{-1}$; piromidic acid, 10 $\mu g\ ml^{-1}$; pipemidic acid, 10 $\mu g\ ml^{-1}$; cephalexin, 10 $\mu g\ ml^{-1}$ (Sigma) and ciprofloxacin, 0.5 $\mu g\ ml^{-1}$ (ICN)) and nutrients (yeast extract (Difco), final concentration 50 $\mu g\ ml^{-1}$). After incubation, samples were fixed with formaldehyde (final concentration 2% vol/vol), stained with DAPI (final concentration 2.5 $\mu g\ ml^{-1}$) for 30 min and filtered as in the TC procedure (Joux and Lebaron, 1997). In our natural seawater samples, bacteria were found in a wide range of sizes, with a predominance of coccoid forms (mean size 0.4 μm) and the presence of some rod cells 0.8–1.0 μm long, as reported in the Ionian Sea by La Ferla *et al.* (2004). To avoid including

any false-positive cells in DVC counts, we chose to consider cells at least three times longer than the mean length of coccoid cells as elongated-fattened, taking into account that this decision may have led to an underestimation of DVC cells.

L/D bacteria were enumerated after staining with the Live/Dead BacLight Viability Kit (Molecular Probes, 1999). Samples (1 ml), stained with 1 ml of L/D stain mixture (final concentration 6 μmol l^{-1} SYTO 9 and 30 μmol l^{-1} propidium iodide), were incubated at room temperature, in the dark, for 15 min and then filtered as in the TC method.

All filters were mounted on clean microscope slides. The cells were enumerated at a magnification of 1000× under immersion oil with an epifluorescent microscopy (Axioplan Zeiss) equipped with a 100 W Hg lamp and DAPI filter set (G 365; FT 395; LP 420) for TC, DVC and NuCC procedures, while for the L/D stain procedure, the live (green fluorescent) and dead (red fluorescent) cells were viewed separately with fluorescein (BP 450–490; FT 510; LP 520) and rhodamine (BP 546/12; FT 580; LP 590) filter sets. To avoid the quick fading of the microscopic field under the rhodamine filter set, we lowered the lamp light intensity below 50%. Cell counts were performed on a minimum of 20 randomly selected fields, in two replicates, to avoid the analytical error and the heterogeneous distribution of bacterioplankton in the observed microscopic fields. The concentrations are reported as the mean value cell $\pm$S.D. ml^{-1}.

Culturable bacteria were determined by spread plate and incubation at 22°C for 7 d on Marine Agar 2216 (Zaccone *et al.*, 2002). Values were reported as colony-forming unit (CFU) counts. The environmental data (temperature, salinity, oxygen, particulated organic matter (POC), particulate organic nitrogen (PON), chlorophyll *a* (Chl-a tot), phosphate (PO_4) and nitrate (N tot) contents) were kindly provided by colleagues of the IAMC-CNR Institute of Messina. Statistical analyses were carried out by ANOVA and coefficient of correlation.

3 RESULTS

Table I lists the bacterial counts obtained with the various techniques. The mean value from all samples of total bacterioplankton (TC) was $3.0 \pm 2.3 \times 10^5$ cell ml^{-1}; CFU averaged $1.6 \pm 1.2 \times 10^2$ cell ml^{-1} and represented less than 0.1% of total counts; DVC represented 1% of TC; and living cells, *i.e.* NuCC and L/D, were 18.0% and 15.9% of TC, respectively.

The comparison between cell concentrations estimated by the different methods allowed us to quantify that NuCC and L/D were two orders of magnitude higher than CFU, while DVC exceeded CFU by one order of magnitude, showing significant differences by ANOVA ($P < 0.01$). The values of NuCC and L/D were one order of magnitude lower than TC, showing percentages ranging from 2.9% to 38.2% and from 3.1% to 32.6%, respectively. The maximum percentages of NuCC and DVC occurring at station E1 (1500 m depth) were due to the low corresponding value of TC.

By comparison, the minimum percentages of NuCC and L/D, observed at station AD (100 m depth sample), and of DVC, observed at station A1 (25 m depth sample), were due to the highest values of TC at both stations (Tab. I). TC, together with NuCC, L/D and DVC, did not show a clear decreasing trend with respect to depth at all stations (Fig. 2).

The physical, chemical and trophic parameters of the euphotic layers are reported in Table II. Temperature and salinity mean values showed differences between the Adriatic and the Ionian Seas, probably related to the different characteristics of these basins and to sampling seasons (April and October, respectively). The integrated values of trophic parameters (POC, PON and CHL-*a*) and inorganic nutrients were higher at the coastal stations (A1

TABLE I Bacterial counts detected by different methods.

Station	*m (depth)*	*TC (10^5)*	*CFU (10^2)*	*%*	*NuCC (10^4)*	*%*	*DVC (10^3)*	*%*	*L/D (10^4)*	*%*	*L/D tot (10^5)*	*%*
E1	5	3.1 ± 1.1	1.5	0.05	3.8 ± 1.9	12.5	1.3 ± 0.6	0.4	n.d.	n.d.	n.d.	n.d.
	25	1.7 ± 0.8	n.d.	n.d.	0.8 ± 0.4	4.4	1.0 ± 0.7	0.6	n.d.	n.d.	n.d.	n.d.
	50	2.5 ± 1.2	1.9	0.07	8.7 ± 3.3	34.1	1.6 ± 0.7	0.6	n.d.	n.d.	n.d.	n.d.
	75	1.8 ± 0.5	0.4	0.02	2.6 ± 0.9	14.0	1.8 ± 1.2	1.0	n.d.	n.d.	n.d.	n.d.
	100	1.5 ± 0.4	0.6	0.04	3.1 ± 0.9	21.3	1.2 ± 1.0	0.8	n.d.	n.d.	n.d.	n.d.
	200	1.8 ± 0.4	n.d.	n.d.	1.7 ± 0.8	9.3	1.9 ± 1.2	1.1	n.d.	n.d.	n.d.	n.d.
	400	1.0 ± 0.4	n.d.	n.d.	3.9 ± 1.3	34.3	1.5 ± 1.3	1.3	n.d.	n.d.	n.d.	n.d.
	700	1.8 ± 0.6	2.4	0.13	3.2 ± 1.5	17.9	1.8 ± 1.1	1.0	n.d.	n.d.	n.d.	n.d.
	1000	1.7 ± 0.5	1.7	0.10	2.7 ± 0.5	16.0	1.9 ± 1.0	1.1	n.d.	n.d.	n.d.	n.d.
	1500	0.8 ± 0.5	0.8	0.09	3.3 ± 1.3	38.2	1.8 ± 1.2	2.0	n.d.	n.d.	n.d.	n.d.
	1583	1.1 ± 0.4	0.9	0.08	3.0 ± 1.2	27.3	1.6 ± 1.1	1.5	n.d.	n.d.	n.d.	n.d.
Average ± S.D.		1.7 ± 0.6	1.3	0.07	3.3 ± 2.0	20.8	1.6 ± 0.3	1.0				
A1	25	8.5 ± 4.9	2.5	0.03	6.3 ± 1.8	7.4	2.5 ± 2.1	0.3	6.1 ± 2.3	7.1	1.7 ± 0.4	20.3
	35	2.4 ± 0.7	3.0	0.12	6.7 ± 1.7	27.9	2.6 ± 1.9	1.1	5.6 ± 2.6	23.4	1.6 ± 0.5	66.7
	45	2.3 ± 0.9	1.1	0.05	6.0 ± 1.4	26.5	2.4 ± 2.1	1.1	5.9 ± 2.1	26.4	1.5 ± 0.4	68.6
	55	4.7 ± 1.9	5.0	0.11	6.4 ± 1.3	13.5	2.6 ± 2.3	0.6	4.1 ± 1.9	8.8	1.3 ± 0.4	27.1
A2	25	2.3 ± 0.3	1.4	0.06	6.0 ± 2.3	26.2	3.5 ± 2.0	1.5	5.9 ± 1.2	25.6	1.6 ± 0.3	68.5
	35	4.6 ± 1.3	0.5	0.01	6.0 ± 1.7	13.0	2.2 ± 1.8	0.5	5.9 ± 1.4	12.9	1.7 ± 0.3	37.2
	45	1.7 ± 0.5	0.5	0.03	5.8 ± 2.3	34.5	1.9 ± 1.4	1.1	5.5 ± 1.0	32.6	1.6 ± 0.3	94.4
	55	2.0 ± 0.2	4.0	0.20	6.2 ± 2.0	31.4	2.6 ± 19	1.3	5.8 ± 1.4	29.4	1.5 ± 0.3	75.3
Average ± S.D.		3.6 ± 2.3	2.2	0.08	6.2 ± 0.3	22.5	2.3 ± 0.9	0.9	5.6 ± 0.6	20.8	1.6 ± 0.1	57.3
AD	5	7.4 ± 1.5	0.75	0.01	3.2 ± 0.7	4.4	n.d.	n.d.	3.4 ± 2.1	4.7	2.6 ± 1.0	35.7
	50	n.d.	1.6	n.d.	2.9 ± 0.6	n.d.	n.d.	n.d.	3.3 ± 1.2	n.d.	3.0 ± 1.0	n.d.
	100	9.6 ± 1.6	2.3	0.02	2.8 ± 0.7	2.9	n.d.	n.d.	2.9 ± 1.3	3.1	2.8 ± 1.0	28.9
	250	4.6 ± 1.5	0.19	0.00	1.9 ± 0.5	4.0	n.d.	n.d.	2.5 ± 1.1	5.5	2.1 ± 0.4	45.4
	500	1.5 ± 0.5	0.35	0.02	1.7 ± 0.7	11.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	800	3.0 ± 1.0	1.9	0.06	2.2 ± 0.6	7.4	n.d.	n.d.	1.9 ± 0.7	6.4	1.5 ± 0.5	49.5
	1100	1.6 ± 0.6	1.7	0.11	3.0 ± 0.7	19.0	n.d.	n.d.	2.1 ± 1.0	13.7	1.4 ± 0.4	92.5
	1191	3.1 ± 0.6	2.4	0.08	2.1 ± 0.6	6.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Average ± S.D.		4.4 ± 3.1	1.4	0.04	2.5 ± 0.6	8.0			2.7 ± 0.6	6.7	2.2 ± 0.7	50.4

Note: TC: total bacterial counts by DAPI stain; CFU: colony-forming units; NuCC: nucleoid-containing cell count; DVC: direct viable count; L/D: live bacterial count by Live/Dead stain; L/D tot: total bacterial count by Live/Dead stain; (%) CFU, DVC, NuCC, L/D and L/D tot percentages of TC, respectively. A1: Station A sampling at 14.00 h; A2: Station A sampling at 18.00 h; n.d.: not determined.

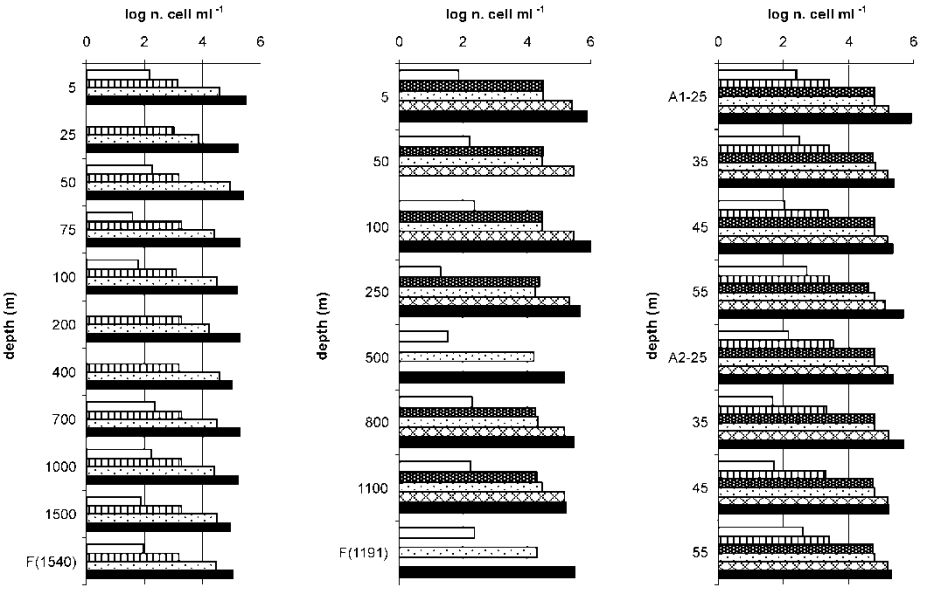

FIGURE 2 Comparison between bacterial densities determined by different methods: total bacterial counts by DAPI stain (black bars); colony-forming units (white bars); nucleoid-containing cell count (white bars with black dots); direct viable count (ruled bars); live (black bars with white dots) and total (meshed bars) bacterial counts by L/D stain. On the left: station E1; in the centre: station AD; on the right: station A. Station A sampling at 14.00 h (A1); station A sampling at 18.00 h (A2).

and A2) than at offshore stations (Tab. II), as well as all bacterial counts (Tab. I). This was also due to the sampling, performed in the photic layer, hence in a narrower range of depth (25–55 m) than the other stations.

In the Ionian Sea, at the offshore station E1, the mean values of all bacterial counts were about half those of the coastal stations, with significant differences (TC $F = 6.4$, $n = 19$, $P < 0.05$; NuCC $F = 15.5$ and DVC $F = 30.2$, $n = 19$ $P < 0.01$), while the percentages of NuCC, DVC and CFU were similar, suggesting that the viable and culturable fractions of the bacterial population were comparable at both stations.

Comparing the offshore stations of the Ionian and the Adriatic Seas, we observed higher TC values at station AD than at E1 ($F = 8.0$, $n = 18$, $P < 0.05$), while the differences in NUCC and CFU were not significant.

With respect to short-time investigation at station A, we did not find any significant variation between the two samplings at 14.00 h and 18.00 h. NuCC and L/D percentages were very similar (22.5% and 20.8%, respectively) and significantly higher than at Adriatic station AD (NuCC $F = 266.1$, $n = 16$, L/D $F = 73.6$, $n = 14$, $P > 0.01$), where, despite the highest mean value of TC, we found the lowest values of living cells (Tab. I).

TABLE II Environmental parameters of the euphotic layer.

	Temperature $^{\circ}C$	*Salinity psu*	*Oxygen mM*	*POC* $\mu g\ dm^{-3}$	*PON* $\mu g\ dm^{-3}$	PO_4 $\mu g\ dm^{-3}$	*N tot* $\mu g\ dm^{-3}$	*Chl-a* $\mu g\ dm^{-3}$
E1	18.36	38.58	246.3	47.9	6.8	0.02	0.72	0.11
A1	19.11	38.48	254.2	55.1	9.6	0.08	1.08	0.19
A2	18.22	38.47	257.0	81.4	12.5	0.23	1.16	0.32
AD	14.64	38.83	251.0	37.6	8.3	0.04		

Note: Data are depth-integrated and normalized. Values for temperature, salinity and oxygen are mean values.

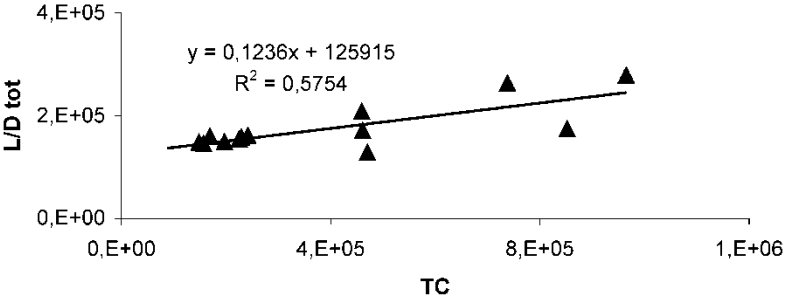

FIGURE 3 Relationship between bacterial density by DAPI stain (TC) and Live/Dead total count (▲, L/D tot).

Our observation of cells stained by the L/D staining procedure identified a population of mixed bacteria which was visible with both fluorescein (SYTO 9) and rhodamine (propidium iodide) filter sets. These mixed cells were counted separately and represented 5% (mean value) of total bacteria, ranging from 2.4 to 9.4%. To check that the cells counted using the single filter set did not include false-positive cells, we analysed superimposed images from fluorescein and rhodamine filter sets. The total bacterial count (L/D tot) performed using the L/D procedure (green + red + mixed cells) gave lower values than TC, but they did not show any significant differences by ANOVA ($F = 3.9$, $n = 40$, not significant for $P < 0.05$). We found a significant difference between TC and L/D tot fractions of microbial assemblage only at station A ($F = 5.9$, $n = 16$, $P < 0.05$).

Our DVC values, at both stations E1 and A, were low compared with the other methods. These low values can be explained by the difficulty in distinguishing between elongated/fattened cells and non-elongated/unfattened cells after the incubation period. To improve the accuracy of the method, we counted the DVC cells immediately after the slide preparation, thus avoiding the formation of precipitated masses (observed in laboratory experiments). However, it was not possible in this way to discern elongated cells. The period of incubation was increased to 48 h and the DAPI concentration to 10 $\mu g\ ml^{-1}$ (final concentration), but the number of DVC cells increased by only 10%.

The coefficient of correlation showed that TC was significantly related to L/D tot ($R^2 = 0.57$; $n = 13$; $P < 0.01$) but not to the other methods (Fig. 3). The values of NuCC and L/D cells were similar in the order of magnitude and in the mean value (Tab. I, Fig. 2) and did not show any significant differences (by ANOVA). Also, the direct comparison between living fractions of bacterioplankton (Fig. 4) showed highly significant

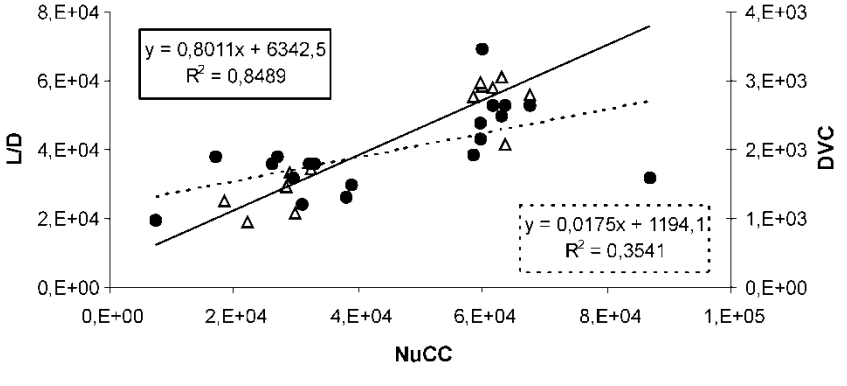

FIGURE 4 Nucleoid-containing cell count (NuCC) versus Live/Dead count (△, L/D) and direct viable count (●, DVC). The continuous regression line relates NuCC to L/D cells. The dotted regression line relates NuCC to DVC cells.

TABLE III Correlations between all microbial and environmental data from the three stations.

	n	*Temperature* 28	*Salinity* 28	*Oxygen* 28	*POC* 27	*PON* 27	PO_4 28	*N tot* 19	*Chl-a tot* 13
TC	26	0.20	0.08	0.34	0.10	0.16	−0.36	−0.43	0.24
CFU	24	0.18	−0.37	0.19	0.36	0.55 **	−0.09	−0.06	0.05
NuCC	27	0.45*	−0.70***	0.62***	0.67***	0.62***	0.10	−0.27	0.56*
DVC	19	0.20	−0.40	0.39	0.68***	0.57**	0.34	−0.14	0.69**
L/D	14	0.90***	−0.79***	0.68**	0.83***	0.68**	0.37	**− 0.56**	**− 0.41**
L/D tot	14	−0.37	0.82***	−0.01	−0.43	−0.37	−0.53	**− 0.70**	**0.35**

Note: $n = 7$ values are shown in bold; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

correlations between NuCC and L/D ($R^2 = 0.85$; $n = 14$; $P < 0.001$) and between NuCC and DVC ($R^2 = 0.35$; $n = 19$; $P < 0.01$), while the CFU fraction was correlated to NuCC at a low level of significance ($r = 0.40$; $n = 24$; $P < 0.05$). Moreover, many correlations were observed between the living fractions (NuCC, L/D and DVC) and organic matter (POC and PON), CHL-a and hydrological parameters (Tab. III).

4 DISCUSSION

This study represents a contribution to the evaluation of currently used bacterial enumeration and viability methods in seawater. The published data do not support the notion that most bacterioplankton cells are uniformly active, since not all bacterial populations grow at the same rates at a given time, and so there is a wide range of metabolic states in any given assemblage (Smith and del Giorgio, 2003). The methods we compared allow the detection of different physiological states in the natural microbial community. While DAPI staining gives the count of any particles containing DNA, with no distinction between living and non-living cells, the NuCC method removes unspecific DAPI retention by dead cells and non-bacterial particles. The L/D procedure assesses cell viability on the basis of membrane integrity, while the DVC method allows the detection of active cells through inhibition of duplication without affecting the other cellular metabolic activities.

The comparison of total bacteria by DAPI and L/D stains confirmed that TC and L/D tot represent the same total assemblage. Although the values of L/D tot were lower than DAPI TC, as already observed by Gasol *et al.* (1999), these differences were not significant. Other authors (Schumann *et al.*, 2003) found statistically significant differences between total bacteria stained by DAPI and L/D in 16 out of 24 investigated samples. We chose to calculate the percentages of culturable, metabolically active and living bacteria on DAPI TC, because it is one of the most widely adopted nucleic acid stains in bacteria counts.

The values of NuCC were lower than DAPI counts and their percentages were comparable with those of published data (Zweifel and Hagström, 1995; Heissenberger *et al.*, 1996; Karner and Fuhrman, 1997; Pinhassi and Hagström, 2000; Berman *et al.*, 2001) but lower than those reported by Gasol *et al.* (1999) in microcosm experiments in coastal water of the northwestern Mediterranean.

The L/D percentages were comparable with those of other environments (Boulos *et al.*, 1999; Defives *et al.*, 1999; Berman *et al.*, 2001) but lower than those reported by Gasol *et al.* (1999) and Schumann *et al.* (2003). This discrepancy can be explained by the different trophic characteristics between the coastal mesotrophic stations of South Baltic Sea, where Schumann *et al.* (2003) found higher percentages of intact membranes and total bacteria, and our stations. The coastal station A, even if located in a gulf, was characterized by the absence

of any terrestrial load; moreover, it showed typical trophic characteristics of an oligotrophic system (Tab. II), with low CHL-*a* and nutrient contents (Zaccone *et al.*, 2004).

L/D and NuCC did not show any significant differences, since a prerequisite for both staining methods is the presence of nucleic acids in the cells, and thus ghost cells cannot be detected. Nevertheless, some bacteria with intact membranes might not display a visible nucleoid region because their DNA can be dispersed in the cell in periods of low activity (Luna *et al.*, 2002). The similar values of NuCC and L/D and their comparison with previous studies validate the use of these two methods, confirming the current tendency to consider cells which have integer and polarized membranes and at least one genome as living cells (McDougald *et al.*, 1998; Lebaron *et al.*, 1999). Moreover, the good correlations between NuCC and the other living detection methods, as previously demonstrated by Gasol *et al.* (1999), indicate the presence of active or recently active cells. Ericsson *et al.* (2000) in their experiments on membrane integrity (detected by the L/D staining procedure), in a stationary-phase *Escherichia coli* population, showed that living bacteria (green) were capable of dividing, whereas dead bacteria (red) were not, confirming that reproductive ability is lost before the loss of membrane integrity.

The DVC counts were comparable with those of some authors (Kogure *et al.*, 1979, 1987; Roszak and Colwell, 1987) but lower than those reported for isolated strains, coastal seawater and lagoon water samples by Joux and Lebaron (1997). These results could depend on the predominance of small coccoid cells, whose elongation was not clearly distinguishable from larger cells, as previously described (Joux and Lebaron, 1997; Yokomaku *et al.*, 2000). To overcome the problem that the smaller than average elongated cells may be missed during the count, it would be useful to combine the DVC count with simultaneous studies of cell volumes. Recently, Yokomaku *et al.* (2000) developed an improved quantitative DVC procedure, which consists in the selective lysis of viable cells of water river samples by spheroplast formation following antibiotics and glycine incubation and a single freeze–thaw treatment, thus overcoming the difficulty in cell discrimination. Moreover, some marine bacteria have very long generation times (sometimes more than 1 week) and are probably not detectable during 1 or 2 d of incubation (Button *et al.*, 1993; Zaccone *et al.*, 2003). The increase in the incubation period could allow the detection of slow-growing cells. Finally, this low concentration (mean value 2.0×10^3), indicative of a small number of active/viable cells, could also be explained, at least partly, by the still debatable hypothesis that bacterial activity is correlated with size. This suggests that small bacteria are mainly inactive (dormant), whereas larger cells are active (Gasol *et al.*, 1995).

TC, together with NuCC, L/D and DVC, did not show a clear decreasing trend with respect to depth at all stations. With regard to the short-time investigation at station A, we did not find any significant variations on comparing the two samplings at 14.00 h and 18.00 h. This was probably due to the short temporal range considered and differs from other authors who have found clear diel cycles in NuCC abundance, together with bacterial activity, without significant differences in TC (Gasol *et al.*, 1998; Hagström *et al.*, 2001).

In the Ionian Sea, the differences observed in living counts between coastal and offshore stations were probably due to different contents in particulate matter and nutrients (both N and P). Thus, the bacterial abundance could have been affected by trophic availability, whereas living fractions did not change as a percentage of TC.

Differences between the offshore stations were observed only in the physical and chemical parameters, whereas the organic matter contents (POC and PON) were similar. The living fractions of all stations showed a direct relationship with temperature and an inverse relationship with salinity; moreover, they were strictly related to organic matter, indicating a possible effect of these parameters on bacterial vitality and activity. Thus, we can hypothesize that the low percentages of living bacteria in spring (station AD) were limited by

both temperature and substrate availability. According to Pomeroy and Wiebe (2001), who demonstrated a temperature–substrate interaction in experimental work in temperate waters, the seasonal changes together with organic matter were the most important factors affecting bacterial growth and viability.

The results seem promising for estimating the actual viable bacterioplankton fraction, giving a more accurate analysis of the effective abundance of bacterial biomasses and their activity in the environment.

Since each method presents its own detection limits and reveals different amounts of bacterial fractions, no suitable single protocol for the universal detection of cellular activity/viability has been found yet. In fact, different methods have different thresholds and metabolic "targets" for describing metabolic activity in bacterial communities (Smith and del Giorgio, 2003). As a consequence, our study highlights the need to compare the different methods simultaneously to validate their results. Only a combination of different methods, which takes into account the goal of the investigation, will be likely to produce an effective description of activity within bacterial assemblages. In our opinion, NuCC and L/D give good results because of their close relationships with the other methods and environmental parameters. We recommend their use, together with methods detecting community structure and activity, for a better understanding of the ecological valence of bacterial community in the ecosystem.

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