Variations of bacterial-specific activity with cell size and nucleic acid content assessed by flow cytometry

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ABSTRACT: In most aquatic bacterial communities, it is possible to discriminate bacterial cells with a high nucleic acid content (HNA) from those with a low nucleic acid content (LNA) by flow cytometry. The distribution of leucine incorporation rate per cell (specific activity) within the fraction of bacteria with a high apparent nucleic acid content was investigated in coastal seawater using flow cytometry and cell sorting techniques. Bacterial cells from a natural seawater sample were first labeled with tritiated leucine, stained with a fluorescent nucleic acid stain and then sorted based on their fluorescence and scatter signals, assuming that fluorescence was proportional to the cellular nucleic acid content and scatter signals to biovolume. Our results clearly demonstrated that specific activity was heterogeneously distributed within the group of HNA cells and increased with both scatter and fluorescence signals. This shows that important differences in terms of cell-specific rates of activity exist within the HNA group and that specific activity is positively correlated with both the nucleic acid content of cells and their biovolume. We hypothesized that this heterogeneity may be partly due to the structure of bacterial communities. Therefore, further investigations were made by analyzing the nucleic acid content and scatter properties of a set of 10 species isolated from the same coastal area. Our results confirm that both fluorescence and scatter values vary greatly between species at a given growth stage and between growth stages for a given species. Variations reported at different growth stages suggest that both parameters are sensitive to the metabolic activity of individual cells and confirm the positive relationship reported in this study between scatter and fluorescence parameters and specific activity within the HNA cellular fraction of natural communities. Interestingly, it was shown that HNA and LNA cells were present in late stationary phase cultures. This suggests that LNA cells found in natural communities may be dead or dying cells.

KEY WORDS: Nucleic acid content · Scatter · Cellular activity · Flow cytometry · Cell sorting

INTRODUCTION

Recently, there has been an increasing interest in discriminating metabolically active cells from inactive cells in aquatic ecosystems (Sherr et al. 1999, Gasol & del Giorgio 2000, Berman et al. 2001). The detection and enumeration of active cells participating in the production of bacterial biomass would be of great help in aquatic microbial ecology. Furthermore, this parameter would be ecologically more relevant than total cell counts including inactive and dead cells (Joux & Lebaron 2000).

Several authors recently suggested that in aquatic bacterial communities, the fraction of cells with a high nucleic acid content (HNA) are more representative of the fraction of growing cells than total (Gasol & del Giorgio 2000, Lebaron et al. 2001) and respiring (Servais et al. 2001) cell counts. The nucleic acid content of individual cells is generally determined by flow cyto-

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metry after staining the cells with SYBR Green dyes (Marie et al. 1997, Lebaron et al. 1998b). In a recent study, it was shown that HNA cells were responsible for most of the total bacterial production in various aquatic environments and that the average specific activity (SA) of these HNA cells varied greatly depending on the trophic status of the ecosystems (Lebaron et al. 2001). Nevertheless, bacterial production did not correlate very well with HNA cell counts, suggesting that cell SAs may vary within this cellular fraction ranging from slow- to fast-growing cells (Lebaron et al. 2001).

The positive relationship between bacterial biovolume and scatter signals has been recently reported in a few studies (Troussellier et al. 1999, Bouvier et al. 2001). In a previous study investigating the relationships between side scatter (SSC) and SA, cell-specific leucine incorporation rate was shown to increase with cell size, and small cells were less active than medium-sized cells (Bernard et al. 2000). Therefore, at least this relationship may contribute to the heterogeneity of activity within the fraction of HNA cells since SSC signals can vary widely within a natural community (Lebaron et al. 2001). Although the nucleic acid content determined by fluorescence intensity and the cell size estimated by light scatter values vary within the fraction of HNA cells, the contribution of both parameters to the heterogeneity of cell SAs is not well understood. To our knowledge, the relationships between fluorescence signals and cell SA within a size class have never been investigated at the cellular level, probably due to the lack of appropriate techniques. Recently, it was shown that the SSC signal of virus-infected phytoplanktonic cells was lower than that of non-infected cells (Brussaard et al. 1999). Thus, if we assume that active cells are preferentially infected by viruses, viral infection may also contribute to the heterogeneity of activity within HNA cells by affecting both fluorescence (nucleic acid content) and scatter (relative cell size) signals of HNA cells.

Information on the heterogeneity of cell SAs within the fraction of HNA cells is of great importance in the field of aquatic microbial ecology to improve our interpretation of data obtained by flow cytometry as well as for our general understanding of the ecological significance of HNA cells. This information is also relevant for the determination of the proportion of HNA cells represented by fast-growing cells.

In this study, we used flow cytometry and cell sorting techniques to investigate the relationships between both fluorescence and SSC signals and cell SA within 2 natural bacterial communities. The variations of both fluorescence and scatter parameters were then investigated further by studying individual strains isolated from the same sample that had been brought into culture.

MATERIALS AND METHODS

Water samples and study design. Samples were collected in June (Expt 1) and October (Expt 2) 2000 from the Banyuls-sur-Mer harbor (42° 28′ N, 3° 08′ E) on the Mediterranean coast of France. Five liter samples were taken just below the surface, and the samples were processed in the laboratory within 1 h of collection. The study design is reported in Fig. 1.

Enumeration and isolation of culturable cells. Colony-forming units (CFU) were only counted on the October sample using the R2A (Difco Laboratories) medium prepared with 75% (vol/vol) filtered (0.45 µm pore size) aged seawater (R2Am). The seawater sample was serially diluted in autoclaved seawater, and five 100 µl replicates of each dilution were plated. Counts were made after 7 and 15 d of incubation at room temperature. The percentage of CFU was determined as the ratio of CFU to total cell counts. After incubation and counting, the colonies were carefully examined, and the colony morphotype was used as an indicator of the strain type (Lebaron et al. 1998a). One colony of each morphotype was picked and purified by at least 2 subcultures on the same medium. Each isolate was assigned the prefix BH, corresponding to Banyuls harbor.
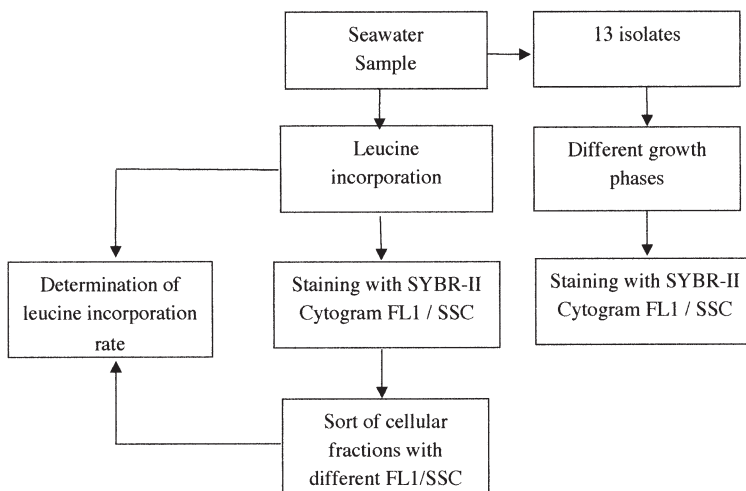

Fig. 1. Flow chart of the strategy used to compare (1) the cell-specific leucine incorporation rate of sorted cells with different fluorescence and scatter signals, and (2) cytograms obtained from a natural sample with those of isolates at different growth stages. FL1: green fluorescence; SSC: side light scatter

Enumeration of total bacterial cells, HNA and low nucleic acid content cells by flow cytometry. For flow cytometric analyses, a 1 ml sample was incubated with 0.5 µl of SYBR II (nucleic acid stain; Molecular Probes) for 15 min at room temperature in the dark (Lebaron et al. 1998b). Cells were counted with the FacsCalibur flow cytometer (Becton Dickinson) equipped with an air-cooled argon laser (488 nm, 15 mW). For cultures, bacterial cells were also stained with SYBR I and SYTO 13, 2 other nucleic acid dyes commonly used in the literature, to compare the fluorescence signals with those obtained with SYBR II and to determine which stain provided the best signal:noise ratio at the different stages of the growth curve. The staining conditions for SYBR I were similar to those of SYBR II, whereas SYTO 13 was used at 5 µM (final concentration) and incubation was performed in the dark for 30 min (Lebaron et al. 1998b). Stained bacterial cells, excited at 488 nm, were discriminated and enumerated according to their right angle light scatter (SSC, related to cell size) and green fluorescence (FL1) emission measured at 530 ± 30 nm. The precise volume analyzed and subsequent cell concentration estimations were calculated by weighing the sample before and after a 5 min run of the cytometer. Fluorescent beads (1.002 µm; Polysciences Europe) were systematically added to each analyzed sample, and cell fluorescence emission and light scatter values were normalized by dividing cellular by bead signals. All cytometric analyses were performed on a logarithmic scale. In a plot of FL1 versus red fluorescence (FL3) we were able to distinguish photosynthetic prokaryotes from non-photosynthetic prokaryotes. The group of HNA cells was discriminated from the group of cells with a low nucleic acid content (LNA) (Lebaron et al. 2001). Each subgroup was delimited on the SSC versus FL1 plot by drawing a gate (region), and cell abundances were determined in each subgroup. The cytometric noise corresponded to particles that could not be assigned to any population and was sometimes close to the LNA subgroup.

Bacterial production. The ^{3}H-leucine incorporation method (Kirchman et al. 1985, Servais 1992) was used in this study to estimate bacterial production. Incorporation of ^{3}H-leucine (151 Ci mmol^{-1}, Amersham) was measured at 80 nM (5 nM of tritiated leucine and 75 nM non-radioactive leucine), and this concentration was shown to saturate leucine incorporation in the different aquatic systems tested (data not shown). Samples were incubated in the presence of tritiated leucine for 1 to 2 h in the dark at the temperature of seawater. After incubation, cold trichloroacetic acid (TCA) was added (final concentration 5%), and the samples were filtered through 0.2 µm pore size cellulose acetate membranes before rinsing filters 4 times with 5 ml of cold 5% TCA. Radioactivity associated with the filters was estimated by liquid scintillation and incorporation rates into proteins, expressed in pmol l^{-1} h^{-1}, were calculated.

Leucine incorporation followed by cell sorting using flow cytometry. In order to estimate the contribution of HNA cells and LNA cells to total bacterial activity, we used a procedure similar to that developed by Servais et al. (1999) and applied by Bernard et al. (2000). This procedure consisted of labeling bacteria with ^{3}H-leucine and then sorting different bacterial subpopulations by flow cytometry in order to estimate the contribution of each sorted subpopulation to the activity of the whole bacterial community. In this study, subpopulations were sorted after ^{3}H-leucine labeling on the basis of their FL1 associated with their nucleic acid content and their SSC in order to determine the specific leucine incorporation rate of the bacteria present in these subpopulations. In parallel, total bacteria were sorted after similar ^{3}H-leucine labeling to estimate the average specific leucine incorporation rate of the total bacterial community and the bacterial leucine incorporation. From a practical point of view, leucine was incorporated at 80 nM final concentration (saturating concentration in the studied environment). Only radioactive leucine (151 Ci mmol^{-1}, Amersham) was added to maximize the detection limit of labeled bacteria. A 5 ml (Expt 1) or a 15 ml sample (Expt 2) was incubated at *in situ* temperature during 2 h in the dark and in the presence of ^{3}H-leucine. Incubation was stopped by the addition of formaldehyde (2% final concentration). Labeled bacteria were then stained with SYBR II following the procedure used to determine total counts. Labeled and stained bacterial cells were then sorted with the FacsCalibur flow cytometer in different gates defined on the cytogram, where FL1 was plotted against SSC (Fig. 2 for Expt 1 and Fig. 3 for Expt 2). A first gate (total bacteria) was defined around the total bacterial population, and this gate was divided into several gates surrounding different subpopulations. We selected on the flow cytometer the 'single sort mode', where a sort occurred only if a single target cell was identified. This result gave a high purity with less emphasis on recovery and the most precise counts of sorted cells. Sorted cells were thus enumerated and collected at the outlet of the flow cytometer directly onto a 0.2 µm pore size membrane (acetate cellulose Sartorius filter). The sort was ended when the number of sorted cells ranged between 200 000 and 400 000 cells to obtain a measurable radioactivity on the filter. Ten milliliters of cold 5% TCA was added to the filter in order to precipitate macromolecules and to rinse the membrane. After 10 min, TCA was eliminated by filtration, and the radioactivity associated with the sorted bacteria was estimated by liquid scintillation.
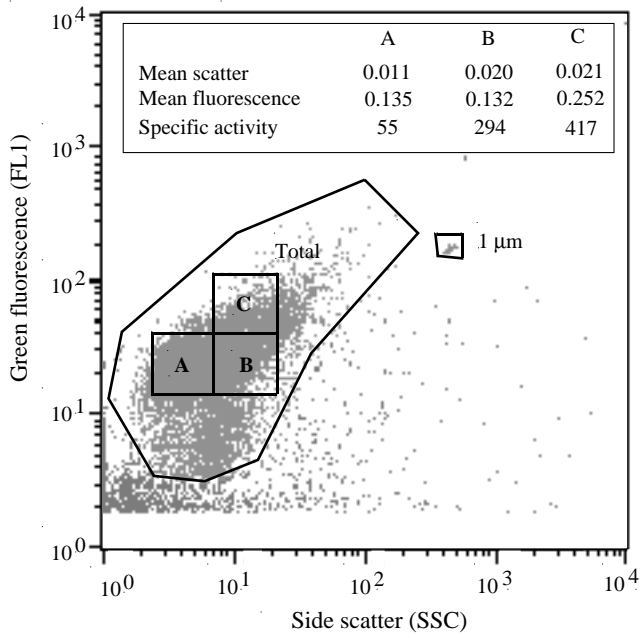
Fig. 2. Flow cytometric analysis of natural seawater collected in Banyuls harbor (Expt 1). The total bacterial community is delimited by a wide gate (total), and 3 other cellular groups differing by their scatter or fluorescence values are delimited by Gates A, B and C. For Gates A, B and C, mean scatter and fluorescence relative values in reference to fluorescent beads (1 μm) and specific activity determined after cell sorting (10^{-21} mol leucine l^{-1} h^{-1}) are given in Table 1

The average SA of the total bacterial community (moles of leucine incorporated $cell^{-1}$ h^{-1}) was determined by dividing the radioactivity incorporated by the bacteria sorted in the total bacteria gate by the number of sorted cells in this gate (total sorted population). Similarly, the average SA of the other subpopulations was calculated on the basis of the radioactivity associated with the cells sorted in different sub-populations. Labeling experiments were replicated for some subpopulations during Expt 2 to estimate the reproducibility of the whole procedure. As a control of the sorting procedure, three 100 μl aliquots were also collected before SYBR II staining, filtered onto a 0.2 μm pore size membrane (acetate cellulose Sartorius filter), rinsed several times with cold 5% TCA and radioassayed in order to estimate SA of the total bacterial community (total unsorted population) independently from the sorting procedure.

Identification of isolates. Based on their colony morphotype, 13 isolates were selected for characterization by the small-subunit rDNA gene (SSU rDNA) analysis. To check for species redundancies between isolates, the *Hha*I restriction fragment length polymorphism (RFLP) pattern analyses of their SSU rDNAs were compared (Lebaron et al. 1998a). The almost complete

SSU rDNA sequences were determined for the 10 strains (BH1 to BH10) having different RFLP patterns (Genome Express). Sequences were then compared to those available in public databases by using the BLAST (Basic Local Alignment Search Tool) server (Altschul et al. 1990) in order to ascertain their closest relatives.

Flow cytometry analysis of isolates. The 10 isolates corresponding to different ribotypes (as determined after RFLP analysis) were grown at 20°C in R2Am broth. For growth experiments, 100 ml of fresh R2Am medium was inoculated with an overnight culture to a final concentration of 1% (v/v). Incubation was performed at 20°C under continuous shaking. Growth was monitored spectrophotometrically at 600 nm, and 30 ml subsamples were taken and fixed (2% formaldehyde, final concentration) at different stages of the growth curve for flow cytometric analysis.

RESULTS

Cell-sorting experiments

These experiments were performed on 2 seawater samples collected in the Banyuls harbor. For each experiment and after leucine incorporation, 1 subsample was used to determine the activity of bacteria at the community level and another subsample was stained with SYBR II. Then, total cells and different cellular groups were identified and sorted within the fraction of HNA cells using flow cytometry. Radioactivity within each sorted fraction was determined and the specific bacterial activity calculated.

For the first experiment, 3 cellular fractions were identified (A, B and C; Fig. 2). The groups of cells were sorted according to their mean light scatter signals (A and B) and fluorescence intensities (B and C). The SA of total unsorted cells was slightly higher (12%) than that of total cells after cell sorting. The slight difference may be due to the presence of some cells outside the drawn gate or to the loss of some active cells during the sorting procedure, which may reject aggregated cells. Cells in Gate B had a similar mean fluorescence intensity to those in Gate A but a 1.9 times higher mean scatter signal and a 5 times higher SA. Cells in Gate C had a similar mean scatter signal to those in Gate B but a 1.9 times higher mean fluorescence intensity and a 1.4 times higher SA. Therefore, for a similar increasing factor (1.9-fold) in fluorescence or scatter signals, the resulting increase of SA was much more important in the case of an increase of scatter signals. The heterogeneity of SA within the fraction of HNA cells was confirmed in this first experiment and further investigated in a second experiment.
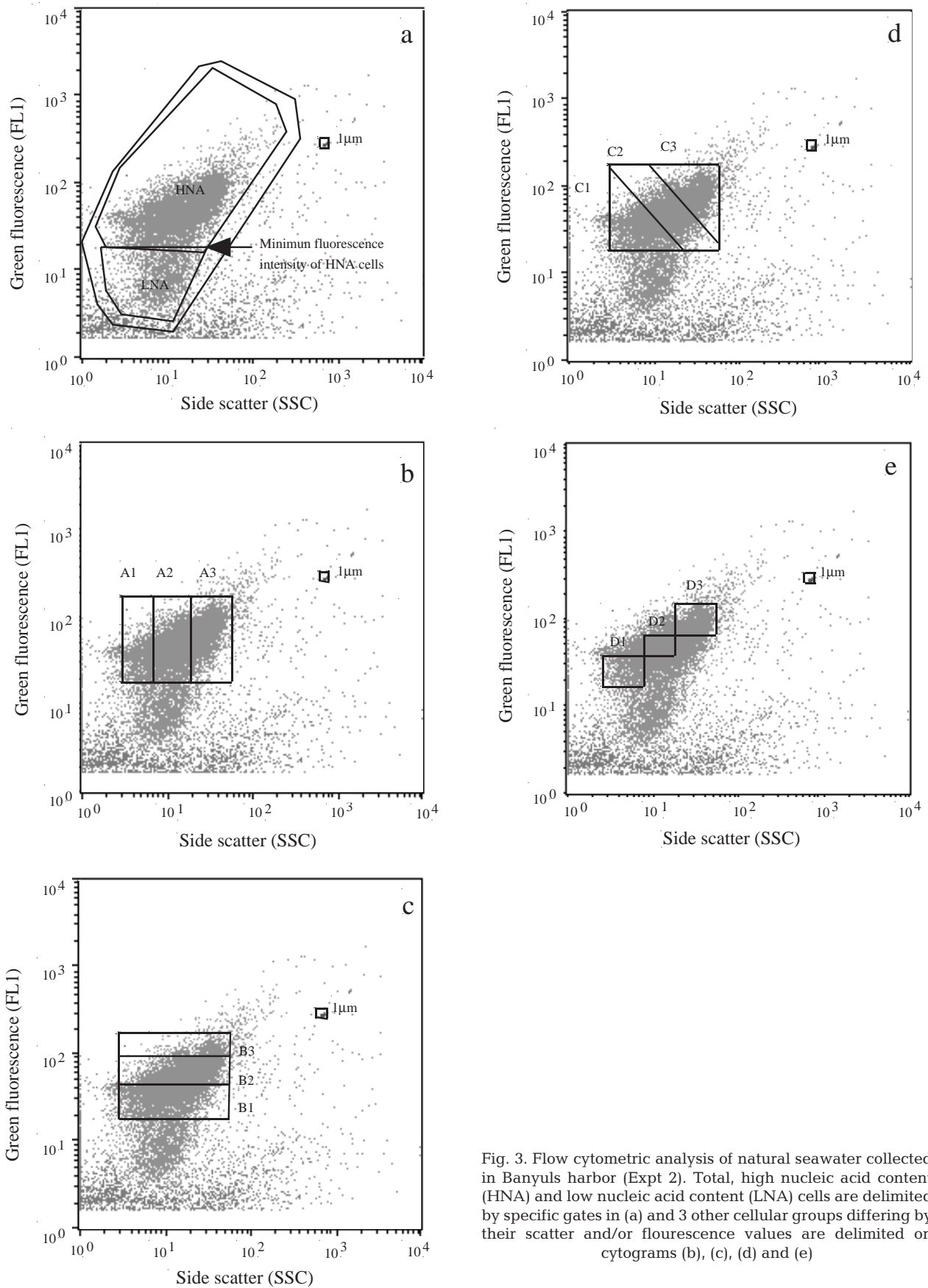
Fig. 3. Flow cytometric analysis of natural seawater collected in Banyuls harbor (Expt 2). Total, high nucleic acid content (HNA) and low nucleic acid content (LNA) cells are delimited by specific gates in (a) and 3 other cellular groups differing by their scatter and/or flourescence values are delimited on cytograms (b), (c), (d) and (e)

Table 1. Bacterial abundance, relative fluorescence, relative scatter (SSC) and the specific activity of leucine incorporation (SA) in the total bacterial community (unsorted and sorted) and in the subsamples analyzed in October 2000 (sample from Banyuls-sur-Mer harbor). Scatter and fluorescence values are normalized in reference to fluorescent beads. HNA: high nucleic acid content; LNA: low nucleic acid content; nd: not determined

	Cell abundances (10^9 l^{-1})	Fluorescence	SSC	SA (10^{-21} mol leu l^{-1} h^{-1})
Total unsorted	nd	nd	nd	226
Total sorted	2.30	0.128	0.025	228 ± 15
LNA	0.30	0.038	0.015	25 ± 3
HNA	1.90	0.153	0.027	259 ± 12
A1	0.25	0.128	0.008	53
A2	0.89	0.139	0.018	189
A3	0.75	0.0247	0.040	502
B1	0.72	0.106	0.017	208
B2	0.81	0.200	0.028	307
B3	0.20	0.366	0.042	471
C1	0.53	0.109	0.011	93
C2	0.74	0.129	0.025	281
C3	0.45	0.210	0.045	457
D1	0.19	0.119	0.007	61
D2	0.50	0.191	0.019	207
D3	0.26	0.350	0.047	460

For the second experiment, total cells, and LNA and HNA cellular fractions were drawn on the cytogram, and the fraction of HNA cells was divided into 12 gates (Fig. 3). All fractions were sorted after leucine incorporation and SAs were determined (Table 1). The activity of total sorted cells was similar to that of unsorted cells, confirming that the cell-sorting procedure had no effect on the activity of cells. HNA cell counts were significantly higher than LNA counts, and their average SA was 10 times higher than that of LNA cells. Within the HNA fraction, the activity of cells increased strongly from Gate A1 to A3, corresponding to an important increase of the mean SSC signals. Similarly, the activity of cells increased from Gates B1 to B3, C1 to C3 and D1 to D3. The highest increases of activity were reported for Gates A and D (factors of 9.47 and 7.5, respectively), corresponding to the highest SSC variations (factors of 5.0 and 6.7, respectively). Inversely, the highest increase of fluorescence intensities found in Gate B (factor 3.4) corresponded to the lowest increase in activity (factor 2.26). After computing the mean SSC, fluorescence and activity values for the 12 cellular groups, the correlation between mean SSC values and activity was highly significant (r^2 = 0.982, n = 12, p < 0.01), whereas that between mean fluorescence intensities and activity was poorer (r^2 = 0.680, n = 12) (Fig. 4). This suggests that SA is more variable at a given fluorescence intensity than at a given scatter value.

Flow cytometric analysis of isolates at different growth stages

A set of 10 species was isolated from the natural seawater sample used for the second experiment. The fraction of culturable cells was 1.4% of total cell counts. These isolates were identified or related to *Bacillus halodurans* (BH1), *B. fumarioli* (BH9), *Vibrio orientalis* (BH2), *V. alginolyticus* (BH7), *V. splendidus* (BH4), *Pseudoalteromonas atlantica* (BH3 and BH5), *P. Riboflavi* (BH10), *Idiomarina loihiensis* (BH6) and *Tenacibaculum mesophilum* (BH8). The mean light scatter and fluorescence signals of all species were determined at different stages of the growth curve from lag phase to late stationary phase. Three dyes commonly used in aquatic bacterial ecology were compared because these dyes have different quantum yields depending on whether they bind to RNA or to DNA (Lebaron et al. 1998b). Strong correlations were found between the fluorescence signals obtained with the 3 dyes and, in all cases, the highest fluorescence values were obtained with SYBR dyes. These good correlations were observed when data were grouped together as 1 data set or separated by isolate or by growth phase (data not
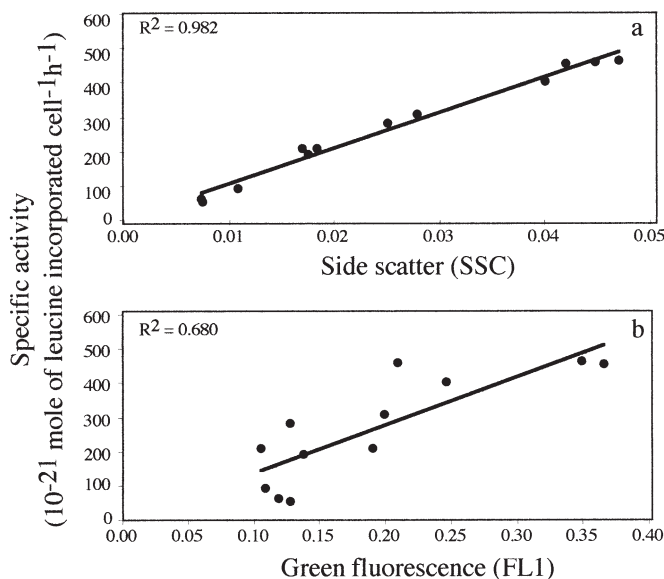

Fig. 4. Correlations between the specific activity of cells determined from 12 sorted cellular groups (A1 to A3, B1 to B3, C1 to C3, D1 to D3; see Fig. 2), and SSC (a) and FL1 (b) values determined for each group
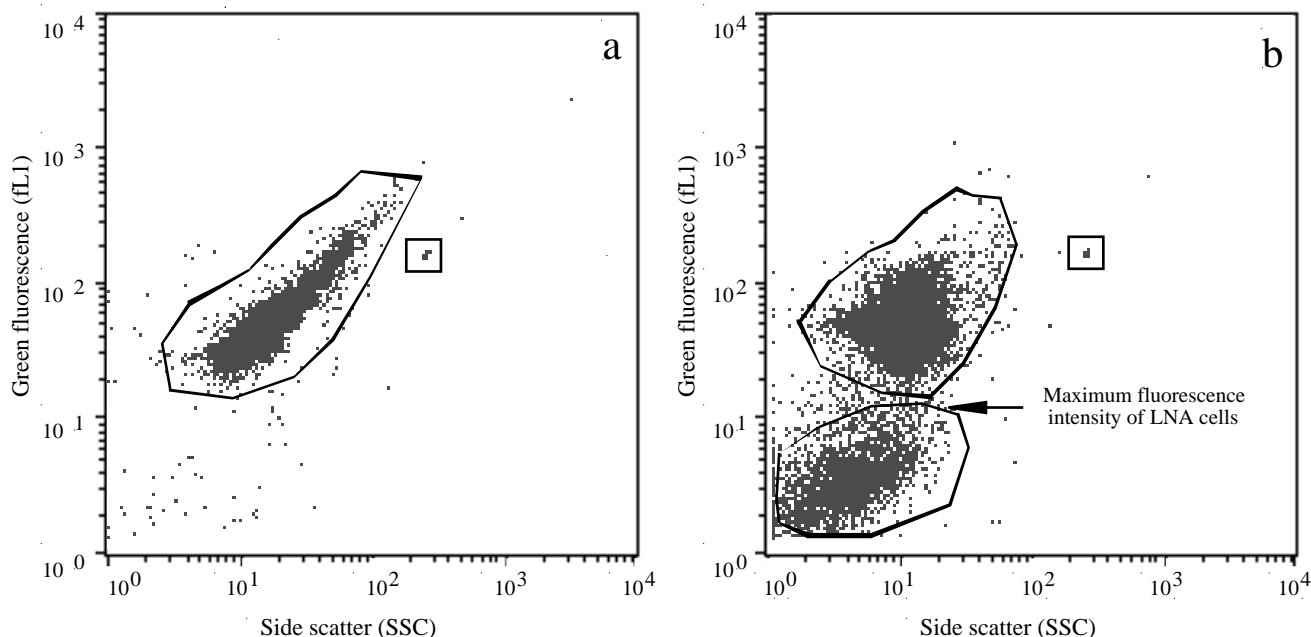
Fig. 5. Cytograms representing the distribution of fluorescence and scatter values of strain BH10 at 2 growth stages: (a) log phase and (b) late stationary phase

shown). For all strains and growth phases, light scatter signals were independent of the staining procedure (data not shown). The variations of mean fluorescence and scatter values obtained with SYBR II for the 10 species and at different growth stages were analyzed (data not shown). For each growth stage, great variations of fluorescence intensities and light scatter signals were observed between species and, as expected, between growth stages for a given species. Even so, for both parameters, the inter-species variations were the highest (sometimes more than 10 times). During the growth phase, the lowest scatter and fluorescence signals were higher than the mean values reported for HNA cells in the natural community, suggesting, as expected, that cells in culture have no common cellular properties with those in natural communities. However, it was interesting that for most species the fluorescence distribution changed during the stationary phase, and 2 cellular groups were discriminated (see Fig. 5b). These groups appeared in late stationary phase differing by their fluorescence intensity and should be compared to HNA and LNA groups discriminated in natural communities. The mean scatter and fluorescence signals determined for each group in late stationary phase and for the different isolates are reported in Table 2. The mean relative fluorescence value of LNA cells determined for isolates (0.031) was quite similar to the mean fluorescence value (0.042) reported for LNA cells in a set of 4 marine natural communities (data from Expt 2; Lebaron et al. 2001) and

was significantly lower than the mean relative fluorescence value (0.19) of HNA cells in natural communities. The mean value of maximum fluorescence intensities of LNA cells determined for isolates (0.068) was also quite similar to that of LNA cells (0.067) in natural communities and was much lower than the minimum fluorescence intensity of HNA cells (0.086) in natural systems.

DISCUSSION

Heterogeneity of SA within HNA cells

Two subgroups of bacteria that have roughly similar SSCs but differ significantly in FL1 are frequently identified in aquatic ecosystems (Li et al. 1995, Jellett et al. 1996). These 2 groups of bacteria are generally associated with bacteria with LNA (LNA group) and with HNA (HNA group). However, the ecological significance of these groups remains unclear. The relationships between SA of individual bacterial cells and their fluorescence or light scatter properties as determined by flow cytometry have been addressed in a few recent studies (Gasol et al. 1999, Servais et al. 1999, Bernard et al. 2000, Lebaron et al. 2001). It was shown that for different aquatic ecosystems, the SA of bacteria increases from small to medium-sized cells and also with their nucleic acid content. Gasol et al. (1999) suggested that HNA cells are the active and dynamic

Table 2. Mean SSC and fluorescence intensities of HNA and LNA cellular populations as determined for (1) different bacterial strains in late stationary phase and (2) natural seawater samples. In parentheses: minimum and maximum values (min./max.) determined for each cellular population

	Fluorescence		Light scatter	
	HNA	LNA	HNA	LNA
Strains				
BH1	0.808	0.068	0.110	0.030
	(0.387/–)	(–/0.131)	(0.027/–)	(–/0.115)
BH2	0.178	0.023	0.048	0.020
	(0.062/–)	(–/0.050)	(0.011/–)	(–/0.056)
BH3	0.220	0.023	0.061	0.012
	(0.062/–)	(–/0.045)	(0.021/–)	(–/0.032)
BH4	0.373	0.020	0.058	0.010
	(0.061/–)	(–/0.043)	(0.008/–)	(–/0.035)
BH5	0.234	0.024	0.025	0.027
	(0.148/–)	(–/0.080)	(0.008/–)	(–/0.074)
BH6	0.308	0.019	0.035	0.016
	(0.099/–)	(–/0.068)	(0.008/–)	(–/0.061)
BH7	0.308	0.037	0.096	0.039
	(0.101/–)	(–/0.063)	(0.031/–)	(–/0.104)
BH8	0.791	0.035	0.084	0.015
	(0.138/–)	(–/0.069)	(0.004/–)	(–/0.047)
BH9	1.210	0.025	0.558	0.062
	(0.170/–)	(–/0.073)	(0.077/–)	(–/0.218)
BH10	0.276	0.030	0.037	0.016
	(0.168/–)	(–/0.062)	(0.011/–)	(–/0.053)
Natural samples				
Expt 2	0.15	0.038	0.027	0.015
	(0.065/0.510)	(0.016/ 0.065)	(0.004/0.064)	(0.004/0.024)
Harbor[a]	0.215	0.044	0.038	0.017
	(0.117/0.533)	(0.034/0.084)	(0.006/0.085)	(0.005/0.023)
SOLA 1[a]	0.170	0.047	0.021	0.014
	(0.090/0.478)	(0.022/0.065)	(0.005/ 0.065)	(0.005/0.026)
SOLA 2[a]	0.204	0.038	0.024	0.015
	(0.074/0.392)	(0.017/0.053)	(0.005/0.062)	(0.005/0.025)

[a]For details on the origin of these samples, see Lebaron et al. (2001)

members of the bacterioplankton community. Lebaron et al. (2001) have confirmed that HNA cells are responsible for most of the community activity, but the correlation between HNA counts and bacterial production was low, and these authors suggested that this was due to an heterogeneity of SAs within this group.

In this study, we determined the distribution of SA within HNA cells depends on their cell size and nucleic acid content. Our results clearly demonstrated that SA is heterogeneously distributed within the group of HNA cells and increases with both scatter and fluorescence signals. Cells with the highest scatter signals have 10 times more SA than those with the lowest signals, and the same tendency was found, to a lesser extent, for fluorescence intensity (Fig. 3, Table 1). It shows that important differences in terms of cell-specific rates of activity exist within the HNA group and that activity is positively correlated with both the nucleic acid content of cells and their biovolume (assuming that scatter signals are related to cellular

volumes). A similar relationship between DNA-related fluorescence and growth rate was shown by Button & Robertson (2001). Therefore, cell SA of HNA cells increases along both fluorescence and scatter axes. Bacterial groups that were initially called bacteria group II by some authors (Jellett et al. 1996, Marie et al. 1997) correspond to HNA cells, whereas bacteria group III or II-b determined by the same authors as cells with high DNA and high scatter values correspond, as expected by the authors, to the most active bacterial cells. This is congruent with the finding that these cells were more abundant in phytoplankton-rich waters (Li et al. 1995). Although the relative abundance of HNA cells provides an assessment of bacterial metabolic activity that complements bacterial production estimates, a clear discrimination between subgroups with different mean scatter and fluorescence values is much more difficult and probably impossible. This is because activity is gradually distributed along fluorescence and scatter axes, as suggested by the mean SA of each sorted fraction, but also because fluorescence and, to a lesser extent, scatter properties vary between species for a given SA.

The heterogeneity in fluorescence and scatter properties may be related to differences in morphology, physiology or taxonomy. Variations in fluorescence signals may be due to heterogeneity in the nucleic acid content of cells or to a difference in the effectiveness of staining the DNA of different species. For a given species, the apparent DNA content may be affected by DNA conformation, topology or stain accessibility. These variations were further investigated by comparing the fluorescence and scatter properties of some isolates at different growth rates.

Variations of fluorescence and light scatter between species

The ecological significance of both LNA and HNA cell fractions as determined from natural bacterial communities remains unclear. If HNA and LNA cells differ by their physiological state and if we assume that HNA and LNA cells are, respectively, active and

inactive cells, one way to improve our understanding of this fluorescence distribution would be to analyze this distribution at different growth stages of isolates including late stationary phase.

Although nutrient conditions in culture are far from reproducing those in natural systems, changes in activity may be analyzed under such conditions. Some of the 10 isolates analyzed in this study are representative of marine bacterial species that are considered to be the major species found in most marine ecosystems including offshore and inshore waters (Pukall et al. 1999, Hagström et al. 2000). Our results confirm that both fluorescence and scatter values vary greatly between species at a given growth stage and between growth stages for a given species. Variations reported at different growth stages suggest that both parameters are sensitive to the metabolic activity of individual cells. This is congruent with the positive relationship reported in this study between scatter and fluorescence parameters and SA within the HNA cellular fraction of natural communities. However, scatter and, to a lesser extent, fluorescence values are also species-dependent at a given growth stage including stationary phase, and these variations may also contribute to the heterogeneity of the SA of individual cells within the HNA cellular fraction.

Interestingly, LNA and HNA subpopulations were present in all late stationary phase cultures of isolated species (Fig. 5b). The LNA subpopulation was not detectable in log phase cultures and at the first parts of the stationary phase. This suggests, if we assume that LNA cells found in natural communities are related to those found in pure culture under nutrient limitation (late stationary phase), that these cells correspond to inactive and dying cells as previously hypothesized for natural communities (Lebaron et al. 2001). The comparison of maximum and minimum fluorescence values reported in both pure cultures and natural communities is congruent with this hypothesis.

However, there is a very narrow limit between the maximum fluorescence value of LNA cells in pure cultures (Fig. 5) and the minimum fluorescence intensity of HNA cells reported in natural communities (Fig. 3). This is why the discrimination between LNA and HNA fractions is sometimes difficult and probably dependent on the structure and physiological state of these communites.

A fundamental question underlying the utility of flow cytometry and nucleic acid staining assays is whether all the HNA cells participate in bacterial production. We showed that the discrimination of HNA cells by flow cytometry allows the discrimination of cells participating in the whole community activity, but cell-specific incorporation rates remain highly heterogeneous but positively correlated with both fluores-

cence and scatter signals. We are not aware of other studies showing that the fluorescence and scatter signals of LNA cells in natural communities are similar to those found in late stationary phase cultures of marine isolates. If we assume that these are dying or dead cells, then the fraction of LNA cells observed in natural aquatic communities should be only considered as organic particles that do not participate in community functions.

LITERATURE CITED

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403

Berman T, Kaplan B, Chava S, Viner Y, Sherr BF, Sherr EB (2001) Metabolically active bacteria in Lake Kinneret. Aquat Microb Ecol 23:213–224

Bernard L, Courties C, Servais P, Troussellier M, Petit M, Lebaron P (2000) Relationships between bacterial cell-size, productivity and genetic diversity in aquatic environments using cell sorting and flow cytometry. Microb Ecol 40:148–158

Bouvier T, Troussellier M, Anzil A, Courties C, Servais P (2001) Using light scatter signal to estimate bacterial biovolume by flow cytometry. Cytometry 44:188–194

Brussaard CPD, Thyrhaug R, Marie D, Bratbak G (1999) Flow cytometric analysis of viral infection in 2 marine phytoplankton species, *Micromonas pusila* (prasinophyceae) and *Phaeocystis pouchetii* (prymnesiophyceae). J Phycol 35:941–948

Button DK, Robertson BR (2001) Determination of DNA content of aquatic bacteria by flow cytometry. Appl Environ Microbiol 67:1636–1645

Gasol JM, del Giorgio PA (2000) Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. Sci Mar 64:197–224

Gasol JM, Zweifel UL, Peters F, Furhman JA, Hagström Å (1999) Significance of size and nucleic acid content heterogeneity as measured by flow cytometry in natural planktonic bacteria. Appl Environ Microbiol 65: 4475–4483

Hagström Å, Pinhassi J, Zweifel UL (2000) Biogeographical diversity among marine bacterioplankton. Aquat Microb Ecol 21:231–244

Jellett JF, Li WKW, Dickie PM, Boraie A, Keplay PE (1996) Metabolic activity of bacterioplankton communities

assessed by flow cytometry and single carbon substrate utilization. Mar Ecol Prog Ser 136:213–225

Joux F, Lebaron P (2000) Use of fluorescent probes to assess physiological functions of bacteria at a single-cell level. Microb Infect 2:1523–1535

Kirchman D, K'Nees F, Hodson R (1985) Leucine incorporation and its potential as a measure of protein synthesis by bacteria in natural aquatic systems. Appl Environ Microbiol 49:599–607

Lebaron P, Ghiglione JF, Fajon C, Batailler N, Normand P (1998a) Phenotypic and genetic diversity within a colony morphotype. FEMS Microbiol Lett 160:137–143

Lebaron P, Parthuisot N, Catala P (1998b) Comparison of blue nucleic acid dyes for the flow cytometric enumeration of bacteria in aquatic systems. Appl Environ Microbiol 64:1724–1730

Lebaron P, Servais P, Agogue H, Courties C, Joux F (2001) Does the high nucleic acid content of individual bacterial cells allow us to discriminate between active cells and inactive cells in aquatic systems? Appl Environ Microbiol 67:1775–1782

Li WKW, Jellett JF, Dickie PM (1995) DNA distributions in planktonic bacteria stained with TOTO or TO-PRO. Limnol Oceanogr 40:1485–1495

Marie D, Partensky F, Jacquet S, Vaulot D (1997) Enumeration and cell cycle analysis of natural populations of marine picoplankton by flow cytometry using the nucleic acid stain SYBR Green I. Appl Environ Microbiol 63:186–193

Pukall R, Paüker O, Buntefub D, Ulrichs G and 5 others (1999) High sequence diversity of *Alteromonas macleodii*-related cloned and cellular 16S rDNAs from a Mediterranean seawater mesocosm experiment. FEMS Microb Ecol 28:335–344

Servais P (1992) Bacterial production measured by ^{3}H-thymidine and ^{3}H-leucine in various aquatic ecosystems. Arch Hydrobiol Beih Ergeb Limnol 37:73–81

Servais P, Courties C, Lebaron P, Troussellier M (1999) Coupling bacterial activity measurements with cell sorting by flow cytometry. Microb Ecol 38:180–189

Servais P, Agogué H, Courties C, Joux F, Lebaron P (2001) Are the actively respiring cells (CTC+) those responsible for bacterial production in aquatic environments? FEMS Microb Ecol 35:171–179

Sherr BF, del Giorgio PA, Sherr EB (1999) Estimating the abundance and single-cell characteristics of respiring bacteria via the redox dye CTC. Aquat Microb Ecol 18:117–131

Troussellier M, Courties C, Lebaron P, Servais P (1999) Flow cytometric discrimination of bacterial populations in seawater based on SYTO 13 staining of nucleic acids. FEMS Microbiol Ecol 29:319–330