Cytometry
PART A
Journal of the International Society for Advancement of Cytometry

Detection of Extracellular Phosphatase Activity at the Single-Cell Level by Enzyme-Labeled Fluorescence and Flow Cytometry: The Importance of Time Kinetics in ELFA Labeling

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Received 29 July 2008; Revision Received 29 October 2008; Accepted 3 November 2008

Grant sponsors: INSU-CNRS (The French program PROOF-PECHE), The PAI Barrande project 2005-06-009-01.

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Published online 2 December 2008 in Wiley InterScience (www.interscience.wiley.com)

DOI: 10.1002/cyto.a.20686

Abstract

ELF97 phosphate (ELF-P) is a useful compound for assessing the phosphorus-related status of planktonic aquatic populations. The technique has been successfully applied to phytoplankton and more recently to heterotrophic prokaryotes in both freshwater and marine samples. We have used a recently developed protocol that enables the detection by flow cytometry of ELF alcohol (ELFA), the product of ELF-P hydrolysis. This protocol allows for identification of the fraction of cells able to express phosphatase activity (i.e., ELFA-labeled). This protocol is also very valuable in the study of time kinetics in this ELFA-labeling. The percentage of ELFA-labeled cells, the relative median ELFA fluorescence per cell, and the absolute ELFA fluorescence were determined in both freshwater (lake) and marine samples. The incubation time necessary to reach a stable percentage of active cells with maximal fluorescence intensity varied widely among samples. We highlight very subtle but important problems of discrimination between active and nonactive cells and of estimation of per-cell activity and we underline the importance of studying time kinetics of ELFA-labeling to determine the appropriate incubation time and thus making sample comparisons more relevant. Working on time kinetics of ELFA-labeling is promising for phosphomonoester hydrolysis rate determination at single cell level. © 2008 International Society for Advancement of Cytometry

Key terms

alkaline phosphatase; ELF phosphate; heterotrophic bacteria; kinetics

As presented at the 4th International Conference on analysis of microbial cells at the single-cell level (May 22nd–24th 2008, Bad Schandau, Dresden, Germany, http://qbab.aber.ac.uk/sc2008/) new methods developed in the recent years, such as the ones presented by Harz et al. (1) or Zordan et al. (2), have improved analysis of what occurs at the single-cell level, allowing us to begin to understand why microbes behave in the way that they do either both in culture [see (3–5)] and in the natural environment (this study). Monitoring cell activity at the single-cell level, using substrates which turn fluorescent after biological activity, allows observing the presence and dynamics of subpopulations. These approaches at the single-cell level are already adding to our understanding of microbial physiology.

Phosphorus (P) is essential to life (6,7). Although dissolved inorganic phosphorus (DIP) is directly available for microorganisms, its concentration in the surface waters of the open ocean is significantly lower than that of dissolved organic P (DOP) (8), which often makes it a limiting factor for growth. Thus, DOP is considered as an alternative P source. But DOP compounds have to be hydrolyzed outside the cells before being utilizable as a P source and for this purpose, organisms synthesize phosphohydrolytic ectoenzymes like alkaline or acid phosphatases (9). These

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enzymes are specific to phosphomonoester, which represent about 75% of the high-molecular-weight DOP pool (10).

Although bulk measurements with soluble substrates have been widely used to quantify the kinetic parameters of phosphatase activity (PA) (9,11,12), they provide global information at the community level but do not provide information about the origin of the activity. In contrast to bulk assay, ELF-97 [2-(59-chloro-29-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone]-phosphate (ELF-P) is a substrate, which upon enzymatic hydrolysis, rapidly provides an insoluble fluorescent precipitate of ELF alcohol (ELFA) marking the site of PA (11,13,14). Flow cytometry which performs fast analysis at the single cell level has been applied to detect ELFA-labeled cells in field samples. The result is the observation of several subpopulations that could not be distinguished using bulk measurements (15,16).

In this study, we show that the analysis of ELFA-labeled samples by flow cytometry allows quantitative assessment in both fresh and marine waters of the extracellular PA for heterotrophic prokaryotes (referred to as bacteria in the text). We underline the importance of studying time kinetics in ELFA labeling to determine the appropriate incubation time and make sample comparisons more relevant. We also highlight very subtle but important problems of discrimination between active and nonactive cells and of the estimation of per-cell activity.

Materials and Methods

Samples

Surface freshwater samples from two mountain lakes located in the Bohemian Forest (South Bohemia, Czech Republic) were taken on November 16, 2006. Lake Plešné (48°47′N, 13°52′E) is an acidified mesotrophic lake (17) and Lake Prášilské (49°29′N, 13°14′E) is an acidified oligotrophic to mesotrophic lake (18). Samples were filtered onto 200-μm membranes immediately after sampling to remove large particles. Samples from Lake Plešné were also filtered onto 10-μm porosity membranes before ELF-P labeling treatment to remove large cells and filaments that could otherwise clog the flow cytometer.

Marine samples from the SOFCOM site (43° 14′ 30 N, 05° 17′ 30 E) in the Mediterranean Sea (Marseilles Bay) were taken from 10-m depth on July 25th 2007.

DIP concentrations in both lake and seawater samples were below the detection limit (30 nM) of the standard Strickland and Parsons'(19) molybdate blue method.

Sample Processing

The protocol used in this study to prepare samples for detection of ELFA-labeling of heterotrophic prokaryotes by flow cytometry has been previously described in Duhamel et al. (15). These samples can be preserved for up to 4 months in liquid nitrogen before analysis (15).

Marine samples from oligotrophic areas such as the SOFCOM site must be concentrated before incubation with the ELF-P substrate (15). Fourteen milliliters of the sample were added to 15-ml Beckman (Fullerton, California) polyallomer centrifuge tubes and centrifuged for 30 min at 20,000 g. The supernatant was discarded with care by aspiration using a sterile pipette. The remaining bacterial cells in the pellet were incubated with 100 μl of the 1/20 A_{kit}/B_{kit} solution of ELF-97 Endogenous Phosphatase Detection Kit (E6601) from Invitrogen (Carlsbad, California), for different time intervals (up to 18 h), at in situ temperature (i.e., 19.4°C). The reaction was stopped with 400 μl of a solution of phosphate buffered saline at 10 mM (pH 7.5) containing a 5% formaldehyde solution (4% final concentration). After 15 min the sample was re-suspended in 1.5 ml of 0.2 μm-filtered water of the same sample before analysis by flow cytometry.

In lake samples, bacterial abundance was sufficiently high ($>10^6$ cell ml^{-1}) for ELFA-labeling without a pre-concentration step. Samples were incubated for different time intervals (between 0 and 120 minutes) with pure ELF-P substrate (E6588, Invitrogen; 20 μM final concentration) at room temperature as described by Nedoma and Vrba (20). Following incubation, 2 ml aliquots were dispensed into cryotubes. The reaction was then stopped by the addition of a phosphate buffer (KH_2PO_4/K_2HPO_4, pH 6, 10 mM final concentration) and the cells were fixed with formaldehyde (2% v/v final concentration, 15 min). The samples were preserved in liquid nitrogen until analysis by flow cytometry.

A 4′,6-diamidino-2-phenylindole (DAPI) solution (2.5 μg ml^{-1} final concentration) was used to discriminate between ELFA-labeled bacteria (labeled with both ELF and DAPI) and non ELFA-labeled bacteria (thus labeled with DAPI only). Samples were incubated 15 min with DAPI in the dark and at room temperature immediately preceding analysis by flow cytometry.

Flow Cytometry

Flow cytometry analyses were carried out on a MoFlo cell sorter (Dako, Carpinteria, California) equipped with a water-cooled Enterprise II Argon laser (Coherent, Santa Clara, California) providing 352 nm (UV) and 488 nm (blue) laser beams set at a regulated 50 mW output power for the UV line. Each particle (cell) was characterized by five optical parameters: two scatter parameters based on the 488-nm laser beam (forward and right-angle light scatters), and three fluorescences related to ELFA (530 ± 40 nm) and DAPI (405 ± 30 nm) fluorescence emissions after UV excitation, and to chlorophyll (>640 nm) after blue excitation. DAPI (Ex/Em: 358/461 nm) and ELFA (Ex/Em: 360/530 nm) were both excited by UV light and showed an overlap of their emission spectra (15). DAPI emission was collected after a 405 ± 30-nm bandpass filter set up just in front of the detector. The power of the UV laser was sufficiently high to collect a sufficient signal using this band-pass filter, whereas the DAPI maximal emission was centered ~461 nm. ELFA emission was collected after a 530 ± 40-nm bandpass filter set up in front of the corresponding photomultiplier tube.

All the parameters were acquired in logarithmic scale. Acquisition was triggered on the right-angle light scatter signal. ELFA-positive and -negative bacteria were discriminated and counted on the cytogram ELFA fluorescence intensity versus

DAPI. Compensation was necessary to completely remove the spillover between ELFA and DAPI signals on the cytogram.

The autotrophic picoplankton was gated out from the heterotrophic bacteria based on the red fluorescence of chlorophyll collected after a 640-nm long pass filter.

The sheath tank was filled with 0.2 μm filtered distilled water. Sheath pressure was maintained constant at 60.0 PSI, and sample pressure at 60.1 PSI. The sample flow rate was maintained lower than 8,000 events per second to avoid doublets and hard coincidences. A minimum of 50,000 bacteria were analyzed per sample. Analyses were performed during 1 or 2 min. The exact volume analyzed was derived from a preliminary calibration of the cytometer flow rate. A sample was weighed before and after a 3-min analysis to determine the flow rate (in μl s^{-1}). The procedure was repeated three times and the average flow rate calculated.

Flow cytometry data were acquired and analyzed with the Summit V4.3 software (Dako). All parameters are in arbitrary units. To compare the data between samples and to determine the ELFA relative fluorescence of each bacteria a solution of 2 μm microspheres (Fluoresbrite, Polysciences, Warrington, Pennsylvania) was added to each sample. These beads are both blue- and UV-excitable and emit both in the DAPI and ELFA emission wavelength range. The relative median fluorescence per cell of cells labeled with ELFA was determined by dividing the median fluorescence intensity of ELFA-labeled cells in the ELF channel (in arbitrary units per cell, A.U. $cell^{-1}$) by the median fluorescence intensity of beads in the same channel. Absolute ELFA fluorescence (in arbitrary units per ml, A.U. ml^{-1}) corresponds to median cell associated ELFA fluorescence multiplied by the number of ELFA-labeled cells.

Results

Freshwater Samples

Time course studies for ELFA-labeling were first carried out using freshwater samples. Surface water samples were taken on both Lake Prášilské and Plešné (Fig. 1). Two types of control sample were initially made: blank and time-0 samples. The blank samples correspond to 2 ml lake samples fixed with the stopping solution of phosphate buffer (10 mM final concentration) containing formaldehyde (2% v/v final concentration). The time-0 samples correspond to ELFA-labeled samples in which incubation was stopped immediately after the ELF-P substrate addition (using the same stopping solution as for the blank samples). As expected, no significant abundance of ELFA-labeled cells nor of ELFA induced-median fluorescence per cell was measured in the blank and time-0 sample controls. The proportion of ELFA-labeled cells increased with the incubation time, from ~0% to up to 30.3 $\pm$ 0.8% and up to 53 $\pm$ 5% after 120 min, for Lake Plešné and Prášilské, respectively. In Lake Plešné, the proportion of ELFA-labeled cells did not reach a plateau within the 2 h of the experiment, while in Lake Prášsilské a plateau was reached after 1 h only.

In Lake Plešné samples, the relative ELFA median fluorescence per cell showed no statistical difference between samples incubated during different times ($P < 0.05$), with the exception of 60 min, at which time an increase of 0.21 $\pm$ 0.01 A.U. $cell^{-1}$ was recorded ($P < 0.05$) (one-way ANOVA, all pairwise multiple comparison procedures (Holm-Sidak method)). In Lake Prášilské samples, the relative ELFA median fluorescence per cell showed a lag-time of 30 min (0.18 $\pm$ 0.01 A.U. $cell^{-1}$) followed by an increase of up to 0.26 $\pm$ 0.01 A.U. $cell^{-1}$.

In Plešné Lake samples, the value of the absolute ELFA fluorescence increased consistently from (16 $\pm$ 7.1) $\times$ 10^2 A.U. ml^{-1} after a 10-min incubation to (5.5 $\pm$ 0.4) $\times$ 10^4 A.U. ml^{-1} after a 2-h incubation. As in this study, Nedoma et al. (21) did not find any plateau phase in absolute ELFA fluorescence for Lake Plešné within the duration of the experiment. In Lake Prášilské, the value of the absolute ELFA fluorescence increased from (3.8 $\pm$ 0.4) $\times$ 10^4 after a 10-min incubation to (29 $\pm$ 2) $\times$ 10^4 A.U. ml^{-1} after a 120-minute incubation. As no statistical difference was found for samples incubated for between 60 and 120 min ($P < 0.05$), we consider that a plateau phase was reached in terms of absolute ELFA fluorescence after 60 min of incubation in Lake Prášilské.

Marine Samples

To determine the cell activity (i.e., the hydrolysis rate, or the amount of substrate hydrolyzed or product formed per unit of time and per cell), it is necessary to discriminate between active and nonactive cells. Figure 2 presents a series of cytograms acquired from a time kinetic experiment conducted on the marine samples from the SOFCOM site. Nonactive cells are labeled with DAPI only (region ELF− on Fig. 2), whereas active cells are labeled both by DAPI and ELFA (regions ELF+/− and ELF+ on Figure 2, see below for explanations on these zones). The term "active cells" rather than "cell activity" was preferred here because of this continuum of activity intensities between inactive and active cells. This continuum makes the very limit between activity and nonactivity only vaguely defined. Figure 2 illustrates this when looking at ELFA-labeling intensity by flow cytometry.

The cluster of ELFA-labeled cells (region ELF+) increased with incubation time both in abundance and in fluorescence intensity. Since the number of ELFA-labeled cells and the median ELFA fluorescence did not vary after 12 h of incubation, it was possible to determine a region corresponding to cells with very low activity, named ELF+/− on Figure 2, and located between the ELF+ and ELF− regions. We hypothesize that this region corresponds to a transient phase between non ELFA-labeled cells and fully ELFA-labeled cells as the active enzymatic sites of the cells process the ELF-P hydrolysis.

The number of cells exhibiting a low amounts of ELFA fluorescence (i.e., in the ELF+/− region, Fig. 2) dramatically decreased with incubation time (86% of the cells after 1 h of incubation to <33% after at least 3 h of incubation, Fig. 2). The same decrease was observed for the fluorescence intensity per cell, ranging from 0.06 to 0.04 A.U. $cell^{-1}$ after 1 h and 6 h of incubation, respectively. After 6 h of incubation, both the fluorescence intensity per cell and the number of active cells remained stable in both ELF+ and ELF+/− regions. The absolute ELFA fluorescence corresponding to the cells in the

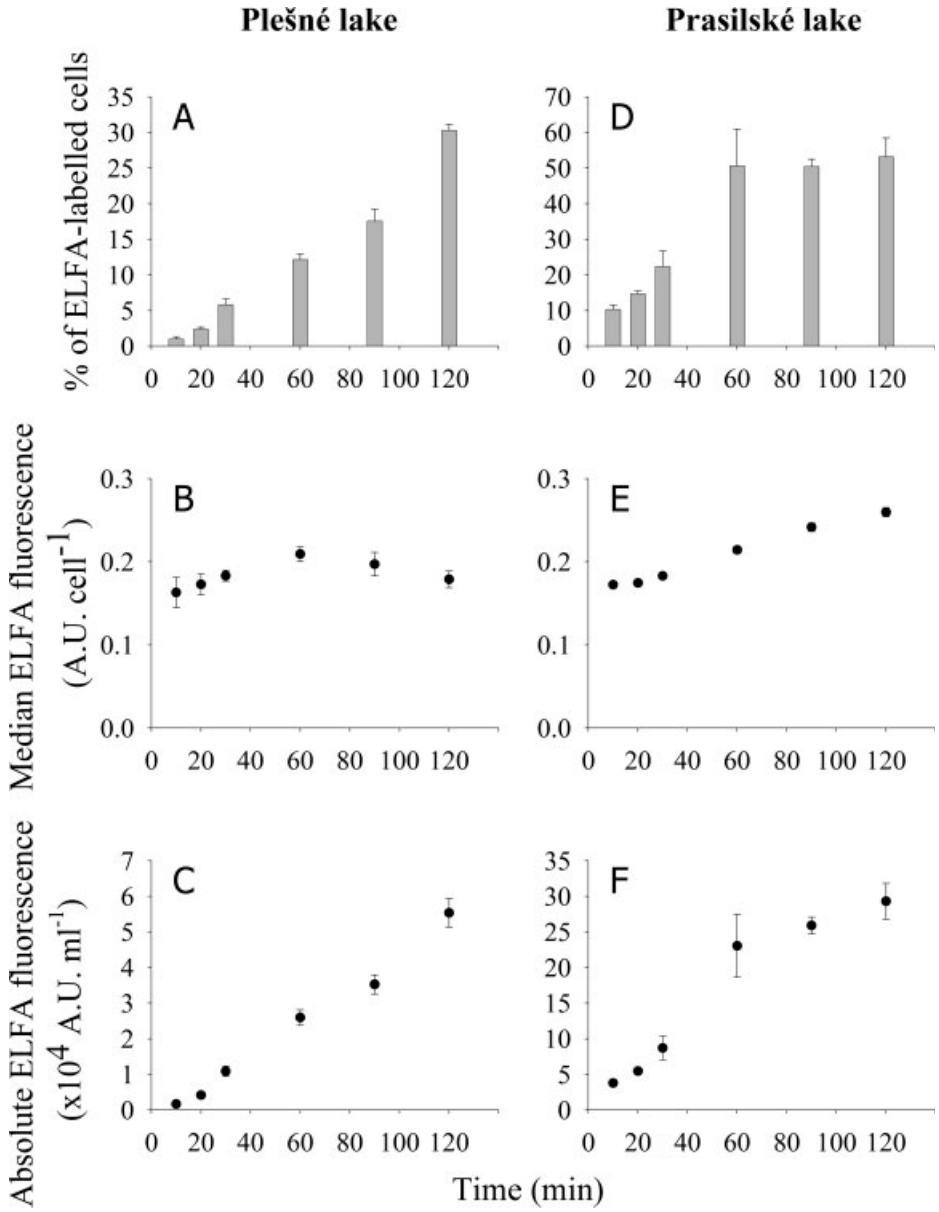

Figure 1. Time course following the proportion of ELFA-labeled cells (%, **A, D**), and relative median fluorescence per cell of cells labeled with ELFA (Arbitrary Unit per cell; A.U./cell^{-1}, **B, E**) and the absolute ELFA fluorescence ($\times 10^4$ A.U. ml^{-1}, **C, F**) in Plešné and Prášilské Lakes water samples incubated with ELF-P for between 0 and 120 min. Error bars represent the standard deviation between triplicates.

ELF+ regions increased from 6.1×10^3 A.U. ml^{-1} after 1-h incubation to 1.5×10^4 A.U. ml^{-1} after 6 h of incubation.

Discussion

As far as the ecosystem functioning is concerned, it is fundamental to better understand interactions between microbes and the P cycle. The detection of extracellular PA at the single cell level by flow cytometry should bring new insights into that field of research. Ten years of ELF-P substrate utilization have brought new understanding about phosphomonoester utilization by microorganisms. Nevertheless, many questions remain unresolved and the information provided by using this tool are principally qualitative (i.e., results expressed as percentage of ELFA-labeled cells). One of the limits in using ELF-P as a quantitative tool to assess PA is the lack of information dealing with time kinetics of the precipitate formation. A major difficulty comes from the diversity of activity levels in a natural sample. Indeed, there is a continuum of activity intensities among cells rather than clear difference between active and inactive cells.

Previous studies using epifluorescence microscopy to detect ELFA labeling underlined the difficulty in determining a threshold between ELFA-labeled and non-ELFA-labeled cells (15,22,23). Indeed, because all cells do not present the same level of PA, a natural sample incubated with ELF-P will show a wide range of ELFA fluorescence intensities per cell. Since after 6 h of incubation, both the fluorescence intensity per-cell and the percentage of active cells remain stable in both ELF+ and ELF+/− regions, hydrolysis of ELF-P is probably quenched by the precipitates which either destroy the enzymes or make

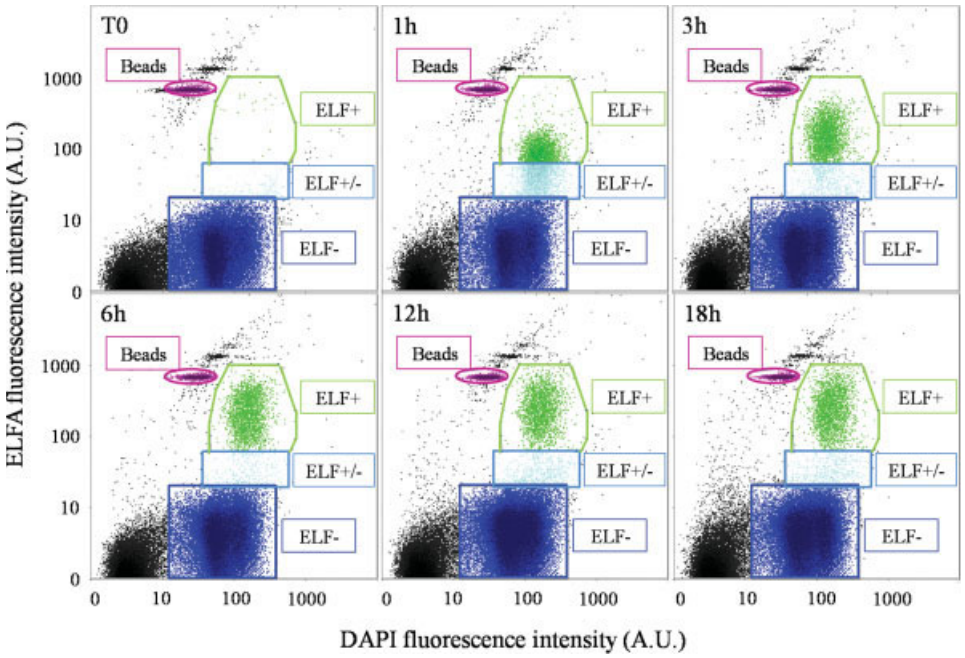

Figure 2. Cytograms showing a time course of ELFA-labeling for marine samples taken from SOFCOM site. Sample incubation times were 0 (T0), 1, 3, 6, 12, and 18 h. Areas of interest are circled for calibration beads (Beads), ELFA-labeled cells (ELF+), cells with small amount of ELFA precipitate (ELF+/−) and non ELFA-labeled cells (ELF−). The Y-axis represents the ELFA fluorescence intensity (A.U.) and the *x*-axis represents the DAPI fluorescence intensity (A.U.). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

them inaccessible to the substrate. At this point, the region ELF+/− contains cells presenting a lower ELFA-induced fluorescence (i.e., amounts of ELFA precipitates) which could be the result of very weak enzyme activity due to a lower number of enzymatic sites per cell.

Because ELFA is insoluble, it precipitates at the site of enzymatic activity (24). It thus seems reasonable to suppose that before comparing cell levels of activities in a natural sample, it is necessary to make sure that all enzymatic sites are completely labeled by the substrate. For this, time kinetics have to be done as a prerequisite for the estimation of PA at the single cell level, as previously suggested in Nedoma et al. (21). Consequently, it may be that the accumulation of ELFA precipitates destroys the enzymes or makes the enzymatic sites inaccessible for further substrate. This phenomenon should not be confused with a saturation of enzyme sites, even though both phenomena may occur together. This phenomenon is illustrated by the plateau phase observed both in the proportion of ELFA-labeled cells and in the ELFA median fluorescence per cell after certain incubation time when all the active sites of PA become inaccessible for further ELF-P hydrolysis.

In Lake Prášilské samples and in marine samples the proportion of ELFA-labeled cells reached a plateau phase after 1 and 6 h, respectively. However, in Lake Plešné samples, the proportion of ELFA-labeled cells did not reach the plateau phase within the time of the experiment (2 h). This suggests that a longer incubation time was needed and that all active cells may not have been labeled, which makes comparisons with other samples inappropriate.

Although no plateau was reached for relative ELFA median fluorescence per cell in Lake Prášilské samples, a plateau phase was reached after 1-h and 6-h incubation periods in Lake Plešné samples and in marine samples, respectively. In other fresh water samples, Dignum et al. (16) (shallow eutrophic lake) and Nedoma and Vrba (20) (acidified oligotrophic mountain lake), did not find any maximum in relative ELFA median fluorescence within the experimental period (1 h and 25 min, respectively). Our results suggest that the incubation time chosen in these studies was too short to reach the ELFA median fluorescence maximum, as well as underlining the importance of studying such kinetics as a prerequisite for the estimation of PA at the single cell level.

These different incubation periods necessary to reach a plateau phase, in both the proportion of ELFA-labeled cells and the relative ELFA median fluorescence, appear to be related to the activity level of the sample. Indeed, in a sample showing high PA level the enzymatic sites are likely to quickly be fully labeled.

As previously developed by Nedoma et al. (21) for microscopy applications, a calibration between flow cytometry and spectrofluorometry is conceivable for the quantification of increase in absolute ELFA fluorescence over time. The addition of beads in samples as an internal standard for flow cytometry enables both a direct comparison between samples as well as quantitative measurements in terms of relative ELFA fluorescence. However, Nedoma et al. (21) used the initial phases of incubation for comparison between the linear increases in fluorescence measured both by spectrophotometry and by cyto-

metry, which are not suitable for determination of the percentage of active cells since it requires longer incubation periods. Thus, activity can only be reliably measured per population (i.e., active + nonactive), and not per single "active" cell, even if the activity is determined at the single cell level.

In this article, we show that the analysis of ELFA-labeled samples by flow cytometry allows quantitative assessment of the extracellular PA for heterotrophic prokaryotes in both fresh and marine waters. We also highlight very subtle but important problems of discrimination between active and nonactive cells and of estimation of per-cell activity. We believe they can be extended to other enzymatic activities. Future experiments to assess the bacterial diversity in each of these subpopulations would be very informative in the determination of which cells are the real performers of the PA. The combination of ELF and Catalyzed reporter deposition Fluorescence In Situ Hybridization (CARD-FISH) (25) labeling appears to be a promising development in this field. Finally, we highlight the importance of studying time kinetics in ELFA-labeling to determine the appropriate incubation time and to increase the relevance of sample comparisons. Working on time kinetics of ELFA-labeling is also promising for phosphomonoester hydrolysis rate determination at single cell level.

Acknowledgments

The authors like to acknowledge Michel Denis and the Plateforme Régionale de Cytométrie pour la Microbiologie PRECYM (COM, UMS 2196). They are also grateful to the Gordon and Betty Moore Foundation (P.I., David M. Karl).

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