Suitability of Flow Cytometry for Estimating Bacterial Biovolume in Natural Plankton Samples: Comparison with Microscopy Data[▽]

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The relationship between flow cytometry data and epifluorescence microscopy measurements was assessed in bacterioplankton samples from 80 lakes to estimate bacterial biovolume and cell size distribution. The total counts of 4',6'-diamidino-2-phenylindole-stained cells estimated by both methods were significantly related, and the slope of their linear regression was not significantly different from 1, indicating that both methods produce very similar estimates of bacterial abundance. The relationships between side scatter (SSC) and 4',6'-diamidino-2-phenylindole fluorescence and cell volume (microscopy values) were improved by binning of the data in three frequency classes for each, but further increases in the number of classes did not improve these relationships. Side scatter was the best cell volume predictor, and significant relationships were observed between the SSC classes and the smallest ($R^2 = 0.545$, $P < 0.001$, $n = 80$) and the largest ($R^2 = 0.544$, $P < 0.001$, $n = 80$) microscopy bacterial-size classes. Based on these relationships, a reliable bacterial biomass estimation was obtained from the SSC frequency classes. Our study indicates that flow cytometry can be used to properly estimate bacterioplankton biovolume, with an accuracy similar to those of more time-consuming microscopy methods.

Planktonic bacteria are important members of aquatic ecosystems, and the calculation of their biovolume is relevant to our understanding of their roles within the microbial food web and in the cycling of organic matter and nutrients. Direct epifluorescence microscopy (EFM) counts of samples stained with nucleic acid fluorochromes such as acridine orange or 4',6'-diamidino-2-phenylindole (DAPI) has for the last few decades been the standard method for determining bacterial abundance and biovolume in plankton samples (16, 26, 39). Based on these measurements, bacterial carbon biomass can be estimated by applying a general conversion factor (24, 28). However, despite improvements through the application of automated image analysis systems (29), microscopy counts are still time-consuming and require a considerable effort to obtain accurate measurements of bacterial cell volumes. In the early 1990s, flow cytometry (FC) was introduced as an alternative to EFM for estimating bacterial abundance in mixed natural assemblages (see references 13 and 30). FC has become a key tool in aquatic microbial ecology because it constitutes a rapid cell counting method and also makes it possible to process a high number of samples in a short time (33). Besides estimates of bacterial abundance, FC also provides information on single-cell parameters (e.g., light scatter values and specific channels of fluorescence) that can be useful for further discriminating distinct fractions of bacteria within mixed

assemblages and thus for analyzing the heterogeneity of bacterial communities (3, 11, 38). The increase in commercially available fluorochromes, the use of molecular techniques, the application of cell sorting, and technological progress have improved the methods for single-cell analysis and the discrimination of physiological and taxonomical diversity within bacterial assemblages (see references 5, 12, and 19). However, there are still many possibilities open for extracting data information on certain parameters of ecological interest (3), including biovolume and biomass.

It is still difficult to quantitatively translate FC signals to cellular properties, although positive relationships between cell size averages and averages of light scatter parameters (6, 13, 15, 17, 31, 32, 36) or DNA fluorescence (10, 11, 36) have been reported. Light scatter at different angles (forward angle side scatter or orthogonal angle side scatter [SSC]) is assumed to be proportional to bacterial size, although it is also affected by cell structure and chemical composition. The impossibility of obtaining FC and EFM measurements for the same individual cell, plus hardware differences among the instruments used, makes it difficult to estimate general relationships between light scatter and cell size. This partially explains why, until now, only average values have been compared and conflicting results have been reported (11).

In this study, we show the suitability of FC for estimating bacterial biovolume by comparing cytometry signals with EFM data from a large set of bacterioplankton samples from 80 lakes of contrasting characteristics. In contrast to other studies, we developed relationships between frequency classes of FC data (DAPI fluorescence and SSC signal) and of EFM data (cell volume). Based on these relationships, we show that an

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accurate prediction of bacterial biomass can be obtained from SSC data.

MATERIALS AND METHODS

Study sites and sampling. Samples were collected in 80 lakes distributed throughout the Pyrenees mountain range and covering a broad range of physicochemical and trophic conditions. For example, the lakes' surface areas ranged from 0.24 to 53.19 ha, maximum depths from 0.7 to 123 m, pHs from 4.53 to 8.96, conductivities from 5.2 to 190.9 $\mu\text{S cm}^{-1}$, levels of chlorophyll *a* from 0.03 to 19.07 $\mu\text{g liter}^{-1}$, and levels of dissolved organic carbon from nondetectable values to 9.42 mg C liter $^{-1}$. Lake samples (3 liter) were collected with a Ruttner sampler from 9 July to 10 August 2000 at a 1.5-fold Secchi disk value, corresponding to the summer chlorophyll maximum depth. In those lakes where the Secchi disk depth was larger than the lake's maximum depth (29 cases), samples were collected at 1 to 2 m above the bottom. The deep waters were not anoxic in any of the lakes. Immediately after sampling, samples (100 ml) were fixed with formalin (final concentration, 2% [vol/vol]) to preserve them for subsequent determination of bacterial abundance and size. In the laboratory, each sample was split into two subsamples, one for FC and one for microscopy analysis. The subsamples were stored at 4°C in the dark until analysis before 1 month.

FC. For FC analysis, 500 μl of lake water was stained with 20 μl DAPI to a final concentration of 1 $\mu\text{mol liter}^{-1}$. The pH was adjusted to ca. 7.4 by adding 20 μl of citrate buffer (1.25 $\mu\text{mol Na-citrate}$, 0.75 $\mu\text{mol K-citrate}$) before DAPI staining (18). Fluorescent microspheres of 1- μm diameter (40- μl TransFluo-Spheres 488/560; Molecular Probes, Leiden, The Netherlands) were added, serving as a counting standard and fluorescence reference. The absolute concentration of beads in the stock solution ($2.25 \times 10^7 \text{ ml}^{-1}$) was determined by flow cytometric analysis of gravimetrically measured volumes. The analysis was performed with a two-laser MoFlo (Dako Cytomation, Glostrup, Denmark) flow cytometer. The lasers were two water-cooled argon ion 4W Innova 90 C+ instruments (Coherent, Santa Clara, CA), tuned to 350 to 365 nm at the first and 488 nm at the second observation point. The output power levels were 50 mW and 100 mW at TEM00 for the UV and visible line, respectively. DAPI fluorescence from bacteria and yellow signals from the microspheres were measured from the UV laser at 445/40- and 580/30-nm band passes, respectively. SSC was measured at 488/10 nm using a 95/5 dichroic beam splitter, from 5% of the visible laser line signal. The detectors were R-1477 photomultiplier tubes (Hamamatsu, Hamamatsu City, Japan) at 450, 550, 550, and 650 W for DAPI, yellow, SSC and yellow, and red autofluorescence signals, respectively. Measurements were triggered on logarithmically amplified DAPI or yellow signals by using a custom multiple-trigger board (Dako Cytomation, Glostrup, Denmark). The log-amplified voltages were mapped onto 1,024 linearly spaced channels normalized to the microsphere's signal. The values for DAPI fluorescence and SSC correspond to these logarithmic channel numbers (range: 0 to 1,023). Between 5×10^3 and 8×10^4 cells were analyzed per sample.

EFM. Bacterial numbers were determined by EFM (Olympus BX-60, 100 $\times$ objective and UV excitation/long-pass emission filter set) using DAPI staining on black, 0.2- μm -pore-size Nuclepore filters (26). For each subsample, two replicate filters were processed. From each filter, a minimum of 200 bacterial cells were counted in at least 10 random fields. If the abundances calculated from two filters differed by more than 10%, an additional filter was processed.

The cell sizes and volumes of DAPI-stained bacteria were determined by image analysis. From each subsample, the images of a minimum of five fields containing ca. 400 bacterial cells were acquired by using a Cohu monochromatic video camera (Cohu, San Diego, CA). LUCIA 4.2 software (8 bit, 751 $\times$ 547 pixels; Laboratory Imaging, Prague, Czech Republic) was used to obtain bacterial length and width. Details on image processing (gray transformation, edge finding, and thresholding) are given elsewhere (27). The conversion to cell volume was calculated by applying the relationship $V = (w^2 \times \pi/4) \times (l - w) + (\pi \times w^3/6)$, where V is volume (in μm^3) and w and l are cell width and length (in μm), respectively. To transform biovolume into carbon biomass, the following allometric equation was used (24): $C = 120 \times V^{0.72}$, where C is carbon content (fg of C per cell).

Data analysis. The comparison between FC and EFM data was performed by dividing the data distributions into frequency classes. Distinct numbers of classes were tested for each parameter. For EFM parameters, the 25,113 cell observations were separated into two, three, four, five, or six classes, with an equal number of observations included in each partition of each class. For example, when cell volumes were separated into three classes, including 8,371 cell measurements each, the resulting intervals were $<0.08 \mu\text{m}^3$, 0.08 to $0.16 \mu\text{m}^3$, and $>0.16 \mu\text{m}^3$, whereas if data were separated into five classes, with 5,022 measurements each, the resulting class intervals were $<0.06 \mu\text{m}^3$, 0.06 to $0.09 \mu\text{m}^3$, 0.09

TABLE 1. Main features of lake bacterioplankton

Parameter	Value			
	Minimum	Maximum	Median	Mean ($\pm$ SD)
Samples ($n = 80$)				
Abundance ^a (10^6 cells ml^{-1})	0.029	3.87	0.64	
Abundance ^b (10^6 cells ml^{-1})	0.031	3.52	0.52	
Biomass ^b ($\mu\text{g C l}^{-1}$)	1.01	93.3	15.75	
Mean cell length ^b (μm)	0.74	2.60	0.99	
Mean cell width ^b (μm)	0.42	0.59	0.47	
Mean cell vol ^b (μm^3)	0.09	0.45	0.16	
Single cells				
SSC ^a (channel no.)		352.27		66.79 (85.89)
DAPI fluorescence ^a (channel no.)		604.29		125.83 (150.17)
Cell length ^b (μm)		61.39		1.06 (1.47)
Cell width ^b (μm)		1.43		0.46 (0.11)
Cell vol ^b (μm^3)		26.30		0.17 (0.43)

^a Parameter measured by FC.

^b Parameter measured by EFM.

to $0.14 \mu\text{m}^3$, 0.14 to $0.22 \mu\text{m}^3$, and $>0.22 \mu\text{m}^3$. For the FC parameters, classes were established after a careful analysis of the distribution shapes (see Discussion and Results); four and five classes were established for DAPI fluorescence and SSC signal data, respectively (DAPI, classes 1 to 4; and SSC, classes 1, 2, 3-1, 3-2, and 3-3).

The main trends of bacterioplankton data variation were explored with a principal component analysis (PCA) of the frequencies of the FC and EFM parameter classes. The PCA extracted the axes of main variability and provided a simultaneous ordination of data classes and sites (20). Furthermore, to evaluate the capacity to predict cell volume values from FC data, we used a stepwise multiple regression analysis with forward selection procedure (probability of F -to-enter, <0.05). This method established the set of independent variables, i.e., FC class frequencies, which explained a significant proportion of the variance of a dependent variable (i.e., each volume class) and provided a predictive model equation (20).

RESULTS AND DISCUSSION

Bacterial abundance. EFM analyses revealed that bacterial abundance in the 80 lakes varied by two orders of magnitude (from 0.03×10^6 to 3×10^6 cells ml^{-1}), and all cell size parameters, with the exception of width, showed a large range of values (Table 1). The broad range of single-cell results revealed a large heterogeneity of bacterial assemblages in these lakes, including quite large cells in many cases (Table 1). In 30 lakes, we found bacterial filaments within the 10- to 60- μm range, which represented between 0.25% and 7.34% (average, 1.17%) of the total abundance.

Similar data on abundance were retrieved for all lakes by FC analyses (Table 1). Linear regression of abundance data obtained by both methods demonstrated a highly significant correlation (Fig. 1), with a slope that was not significantly different from one ($P < 0.001$). Most discrepancies were smaller than 50%, and only six samples differed more than 100% between the two counting methods. These differences were not associated with the presence of filamentous bacteria (Fig. 1).

We obtained slightly higher abundances with FC, as indicated by the slope of the regression line (0.93) (Fig. 1) and the median of the ratio between both estimates (FC/EFM = 1.27). Several studies have reported the existence of a significant relationship between estimates of bacterial abundance with FC

FIG. 1. Relationship between bacterial abundance estimated by FC and EFM for the 80 lakes. Empty triangles indicate samples with short filamentous bacteria. The solid line shows the regression line ($y = 1.97 \times 10^{-3} + 0.931x$, $R^2 = 0.824$, $P < 0.001$), and the dashed line the 1:1 relation.

and EFM using different fixatives and DNA fluorochromes (7, 10, 21, 22, 30). However, in some data sets, there was a slight trend for FC to underestimate bacterial density, especially when small bacteria ($<0.06 \mu\text{m}^3$) were abundant (13, 23, 37).

FIG. 3. PCA of frequency classes from FC data (four DAPI fluorescence [○] classes, D1 to D4, and five SSC signal [□] classes, S1, S2, S3-1, S3-2, and S3-3) and from EFM data (three volume [*] classes, V1 to V3). Single points show lake scores. Scores for those lakes whose data are included as examples in Fig. 2 are labeled (A, B, C, and D).

In contrast, some authors suggest that EFM may underestimate bacterial counts because small bacterial cells can pass through some $0.2\text{-}\mu\text{m}$ -filter membranes (14).

Relationship between parameters measured by FC and EFM. There were no significant correlations between the sample average values of parameters measured by FC (DAPI flu-

FIG. 2. Some examples of data distributions for SSC (A and B) and DAPI fluorescence (C and D) measured by FC in lake bacterial assemblages. Represented samples are from Lake Asnos (A), Lake Gran de Mainera (B), Lake Bleu de Rabassoles (C), and Lake Ormiélas (D). The vertical lines mark the intervals of the classes (small numbers at top) considered for further analysis.

FIG. 4. Relationships between SSC- and EFM-measured volume frequencies (%) for the different classes established. Lines are regression fits. R^2 is indicated for those that are significant ($P < 0.001$). $n = 80$.

orescence and SSC signal) and those measured by EFM and image analysis (width, length, volume), because the distributions of values were far from normality and in some cases multimodal, as demonstrated by the histograms (Fig. 2). Therefore, a representation of the variability in the samples that was better than simple means was required to establish FC and EFM parameter relationships. For this purpose, we established a number of frequency classes. In the FC histograms, there were clear patterns that roughly corresponded to two basic types: one with a single, sharp peak at low channel numbers (Fig. 2A and C) and another type with several wider peaks

at large channel numbers (Fig. 2B and D). The former corresponded to histograms with exclusively small bacteria and was chosen as a starting reference class. To define its range, we selected all the histograms with that pattern (e.g., 31 from the 80 lakes for SSC), checked the range of the channel number mode (peak) (e.g., 0 to 25 for SSC), and selected the upper limit as the boundary of the class. To establish further classes, we considered the fact that, in the complex histogram patterns (e.g., Fig. 2B and D), the peaks were wider and smoother than in the histograms with only small bacteria. Thus, the higher the channel number segment considered, the wider the peaks in

TABLE 2. Classes established for SSC signal data and for cell volume measured by EFM, based on data set for all lakes^a

Class	Interval	Avg (± SD)	Mode	Frequency (%)		
				Avg	Minimum	Maximum
SSC (channel no.)						
SSC1	0–25	13.96 (1.55)	19.45	45.6	12.0	82.7
SSC2	25–50	35.62 (0.98)	33.97	24.3	11.2	38.3
SSC3	>50	63.87 (24.02)	108.74	30.1	5.6	73.4
Cell vol (μm³)						
V1	<0.08	0.058 (0.013)	0.036	32.65	3.68	60.23
V2	0.08–0.16	0.113 (0.021)	0.083	33.35	20.18	43.14
V3	>0.16	0.348 (0.719)	0.167	34	9.25	71.67

^a SSC data calculations for average and mode are from class sample means, whereas volume data for average and mode are calculated from all data of the class ($n = 8,371$). Class frequency averages and ranges for the 80 samples are also shown.

general. The best way to include in distinct classes the main peaks observed in the histograms (e.g., Fig. 2B and D) was to use class ranges that increased twofold between consecutive classes. Among the EFM parameters, cell widths showed little variability (Table 1); changes in cell length, in fact, determined cell size and shape (elongation) variability. Thus, the frequencies of cell length classes were highly correlated with those of cell volume classes.

To explore which combination of EFM classes better matched the SSC and DAPI classes, we performed a number of PCAs with the class frequency data for each lake. Consideration of three cell volume classes (V1, V2, and V3) provided the highest correspondence between EFM and FC parameter frequencies (Fig. 3). The first axis of the PCA analysis explained 68.7% of the data variability, whereas axis 2 explained 14.3% of the data variability. The main gradient was defined by the small versus the large class intervals of all the parameters considered and reflected not only a size rank but also a gradient of bacterial assemblage heterogeneity. It is notable that the SSC classes were close to the bacterial volume classes, whereas the DAPI classes followed a different pattern distribution in the PCA plot (Fig. 3). The larger classes of SSC (i.e., S3-1,

S3-2, and S3-3 in Fig. 3) plotted together around cell volume class V3, indicating that they did not provide substantially different information. As a consequence, the three classes were grouped into a new, unique class, S3 (bacteria with an SSC signal greater than 50), for further analysis.

Following the PCA exploration, stepwise multiple regression analysis was used to predict the cell volume class as a function of the FC parameter classes. The variable with the highest explanatory power for cell volume classes was S1 (54.5% of V1 variation, $P < 0.001$), followed by S3 (54.4% of V3 variation, $P < 0.001$). The other remaining variables were not significant, as expected from PCA analyses, and were not selected in the regression models. There were significant relationships between small SSC and small cell volume frequency classes as well as between the large classes (Fig. 4). Therefore, the percentage of each volume class can be estimated from SSC data as follows: $V1 = 6.79 + 0.568S1$, $V3 = 11.84 + 0.736S3$, and $V2 = 100 - (V1 + V3)$. The main characteristics of the SSC and volume frequency classes considered in the above equations are summarized in Table 2. From the mean cell volume (Table 2), we calculated the average cell C content of each volume class. Thus, we were able to estimate bacterial biomass

FIG. 5. Comparison between bacterial biomass measurements by EFM and predictions from SSC data, considering bacterial abundances estimated by EFM (A) and bacterial abundances estimated by FC (B). Solid lines are regression fits for the data points ($n = 80$) and dashed lines are the 1:1 relation.

for each sample by using volume frequencies and bacterial abundance. The ability of these equations to predict bacterial biomass was checked, considering only FC data to estimate cell size distribution (i.e., calculating the percentage of the volume classes from SSC data but considering abundance from EFM data) (Fig. 5A) or considering FC data to estimate both bacterial size and abundance (Fig. 5B). In both cases, the predicted values were significantly correlated with those estimated by EFM. In the case of EFM, the average cell carbon content per class could be estimated by following the same procedure as for FC, that is, averaging cell volume and then converting to C content. Alternatively, it could be estimated by applying cell C contents individually and then averaging. We found no significant differences between the results of these two approaches for any sample.

Allman et al. (2) hypothesized that, in natural bacterial assemblages, the existence of different specific cell structures that affect the refractive index could disrupt the expected relationship between cell size and light scattering. However, Bouvier et al. (6) found that, although the forward angle side scatter-biovolume relationship was species dependent, the variations of cell shapes and refractive indexes in natural marine assemblages were low. Our results show that the SSC can in fact be useful for rough size estimation and sufficient for an accurate estimation of bacterial assemblage biovolume. The bacterioplankton in the Pyrenean lakes studied can be characterized by the dominance of small cells and by the occasional occurrence of morphologically diverse and larger cells, mainly associated with the winter snow and ice cover (1, 8, 9). There were not very large filamentous bacteria ($>60\ \mu\text{m}$) in our samples, and only two cell size thresholds appeared important: $0.08\ \mu\text{m}^3$ and $0.16\ \mu\text{m}^3$. In some other alpine areas, very large filamentous bacteria appear to be more common during the ice-free season (25, 34, 35). Thus, whether our SSC calibration provides accurate results in those cases should be tested.

The units of measurement of SSC are arbitrary, and consequently, light scatter values change among instruments and stains used (see reference 4). However, there is no reason to believe that biovolume estimation by FC cannot be implemented in other type of instruments, because the procedure is based on the relationship between broad size classes and simple SSC classes. Further, no dependence on more sophisticated aspects of the instrument was found. Therefore, the method should be of broad application, but it necessarily includes a calibration for each instrument in order to establish a regression equation. However, once this calibration is established, it can be used in studies that require large numbers of biomass estimations without the parallel EFM work.

In developing calibrations for other systems, we suggest that a few key aspects be considered. The range of the first class of small cells is crucial for properly characterizing the heterogeneity of SSC distribution. Thus, it is convenient to have a relatively large number of samples with a high dominance of small cells to properly determine that range. Another important point to consider is the mean cell volumes of the respective classes from EFM. We believe that our values (Table 2) are representative of other bacterioplankton assemblages because of the large environmental diversity of our data set.

However, future assessments should confirm their applicability to other aquatic ecosystems.

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