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Direct Measurements of Natural Planktonic Bacterial Community Viability by Flow Cytometry

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A range of fluorescent viability dyes were used in conjunction with flow cytometry to rapidly enumerate viable bacteria from freshwater environments. Optimal labelling was achieved by using carboxyfluorescein diacetate or chemchrome B with a detergent-mediated permeabilization step. The viable bacterial count under optimal conditions was 7% in oligotrophic lake water and 75% in polluted river water.

It is widely recognized that conventional culture techniques may underestimate true viable bacterial numbers by several orders of magnitude. Culture on solid media is highly selective and decreases diversity, thus being unrepresentative of the natural community. Alternative approaches may improve viable counts (12, 13) and reduce selectivity (1, 25). The ability to rapidly assess the viability of bacterial populations would decrease bias due to sample storage and incubation (9), as well as assay time. Such methodologies have direct application in quality control of water supplies or foodstuffs. Flow cytometry (FCM), used in conjunction with fluorescent viability probes, has great potential for such a role (7, 8). In this study, we report concerning the use of viability dye conjugates, measured by FCM, for the rapid, automated assessment of viable planktonic bacteria from natural freshwater systems.

Fluorescent dyes exist as probes for different cellular functions. Membrane potential, examined by using rhodamine 123 (Rh123), has been successfully applied to viability assessment of a range of bacterial species in culture (5, 16, 19) and bacteria in a nonculturable state (20). However, the use of colored dyes may be limited in sites with a high level of particulate background material, as nonspecific staining may occur.

An alternative approach has been to use colorless fluorogenic esters, which are enzymatically cleaved within the cell to yield a fluorescent product, thus probing for enzyme activity. Retention of the product indicates membrane integrity, thus giving confidence in the assay. A commonly used dye has been fluorescein diacetate (2, 18), although the method has been criticized for its variability and irreproducibility with lake water populations (15). Hydrophobic fluorescein diacetate derivatives which are cleaved to form hydrophilic products, improving dye retention (21), have been developed. Examples include carboxyfluorescein diacetate (CFDA), calcein acetoxymethyl ester, and 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxy methyl ester (BCECF-AM). Chemchrome B (Chem B; Chemunex, Cambridge, United Kingdom) is a commercial preparation of unknown formulation which has the same mode of action. The potential of these dyes has been demonstrated only for pure bacterial cultures (4).

Water sampling. Windermere North basin (Windermere, Cumbria, United Kingdom) lake water and water from Church Beck, a stream running into Coniston Water (Coniston Water, Cumbria, United Kingdom), were collected during April 1992, solely for extensive studies using Rh123. Three sites along the stream were used, i.e., the point of discharge of a sewage outflow, a site approximately 50 m downstream from the outflow, and a site approximately 2 miles (ca. 3 km) upstream (Coppermine Valley, Coniston, Cumbria, United Kingdom). Sewage effluent was collected before it had made contact with the stream. These samples were processed in the laboratory within approximately 1 h of sampling.

Lake water samples for analysis using fluorogenic esters were collected from Windermere North basin in April 1993. Water was taken from approximately 10 cm below the surface, and incubations were initiated within 30 min. Water was also collected from the river Mersey (Fiddler's Ferry, Warrington, United Kingdom), downstream from a sewage plant (April 1993). These samples were processed within 1 h of sampling. No sample dispersal treatment beyond vigorous shaking for approximately 30 s was used for any sample.

FCM. Samples were analyzed with a Becton Dickinson FACStar Plus flow cytometer, maintained and operated as described previously (22). Fluorescence at 525 nm was detected through fluorescence detector 1 (FL1) set at a photomultiplier tube (PMT) voltage of 650, with a logarithmic gain. At these settings, the 0.5-μm-diameter fluorescent latex beads (Polysciences Inc., Warrington, Pa.) used to calibrate the machine appeared at mean channel numbers of 452 (forward scatter detector) and 828 (FL1).

All FCM analyses carried out on river Mersey water samples were performed by using an Argus 100 flow cytometer (Skatron Ltd., Newmarket, United Kingdom) maintained and operated as described previously (4, 5). Viability dye fluorescence was determined by using FL1 (520- to 560-nm emission filter) with a PMT voltage of 650 and a logarithmic gain. At the settings used to focus this machine, 1.0-μm-diameter latex beads appeared around median channel numbers of 87 (forward scatter detector; PMT voltage of 500; linear gain of 8.00) and 153 (FL1; PMT voltage of 650; logarithmic gain).

FCM output was analyzed by using the software provided with each machine. Cells were assumed to give a positive signal if their associated mean fluorescence intensity was greater than channel 200 (FL1) on the Becton Dickinson flow cytometer. This approximated to channel 50 on the Argus 100. In both cases, control samples had associated mean fluorescence intensities below this cutoff point. Events from control samples above this level were subtracted from sample counts.

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Cell viability measurements using membrane potential. Rh123 was tested for its ability to label cells according to membrane potential by the method of Diaper et al. (5). The ionophore gramicidin S was used as a control to dissipate membrane potential. Samples were analyzed by FCM, as described below.

Rh123 was found to be of limited value for environmental membrane potential studies, because of a high level of background fluorescence. To improve uptake of Rh123, sample pretreatment and labelling in Tris-EDTA (TE) (1× and 5× concentrations) were tested. Incubation times were extended up to 60 min. A shift in the histogram distributions from lake or stream water populations was observed with the addition of gramicidin S (indicative of dissipation of membrane potential), but the results did not allow a confident measure of viable numbers. More clear-cut results were found for samples taken from undiluted sewage effluent after pretreatment. However, the results were still not considered satisfactory. The use of Rh123 was thus discontinued for these studies.

Cell viability measurements using fluorogenic esters. A variety of fluorogenic esters were tested for their ability to label cells for FCM analyses. Stock CFDA (Sigma) was prepared at 1 mM in acetone (lake water studies) or dimethyl sulfoxide (river water studies). Calcein acetoxymethyl ester and BCECF-AM (Molecular Probes, Cambridge Biosciences, Cambridge, United Kingdom) stock solutions were prepared at 1 mM in dimethyl sulfoxide. Chem B was used as the commercial preparation. Cell samples were labelled by the addition of 10 μl of viability dye to 1 ml of sample. Controls were prepared by heat and formaldehyde fixation (6) before incubation. No washing steps were performed with these dyes.

Each dye labelling method was optimized by comparison of counts obtained after different treatments. The buffers investigated for labelling of cells with the fluorogenic esters were 10 mM Tris–1 mM EDTA (1× TE), phosphate-buffered saline (PBS), Chemunex labelling buffer (supplied with the viability kit), and lake water amended with filtered Tween 20 to a final concentration of 0.1%. Lake water samples (1 ml) were resuspended in buffer, when appropriate, after centrifugation (8,800 × *g* for 1 min) and careful removal of the top 900 μl to minimize disturbance of pelleted material. Incubations were then carried out with each dye at room temperature (approximately 20°C) and at 30 and 40°C for 10, 20, or 30 min. All samples were kept in the dark on ice for at least 10 but no longer than 30 min after labelling before analysis by FCM. Samples were incubated both with and without the Chem Red blocking reagent supplied with the Chemunex viability kit.

A variety of other counting methods were used to provide comparative data for the estimations obtained by using the fluorescent viability dyes. Direct viable counts (DVC) were performed by using the division-inhibiting antibiotic mixtures described by Kogure et al. (17). Acridine orange direct counts (AODC) were performed as described by Fry (10). Both DVC and AODC were performed with a final concentration of acridine orange of 5 μg/ml. Total respiring counts were carried out as described by Rodriguez et al. (24), with a final concentration of 5-cyano-2,3-ditolyltetrazolium chloride of 3.5 mM. All three counting procedures were performed using 0.22-μm-pore-size black membrane filters (Costar, High Wycombe, United Kingdom). Numbers of CFU were determined in triplicate by using R2A agar (23) and casein peptone starch agar (3). Dilution series were prepared both in PBS and in 0.22-μm-pore-size membrane-filtered lake water. Counts were made after 7 days of incubation at 18°C.

Data handling and statistical analysis. Triplicate counts were made for each procedure, with triplicate samples. Analysis of variance was performed by using MINITAB 8.21 (Minitab Inc., State College, Pa.) as described previously (11, 14), with the Minitab macros provided. In order to meet the analysis of variance assumptions of homogeneity of variance and normality of errors, $\log_{10}$ data transformation was required. In cases in which these assumptions were slightly violated, analyses were confirmed using the Kruskal-Wallis and Mood's median nonparametric tests (11). Minimum significant differences were calculated by the Tukey-Kramer method (11).

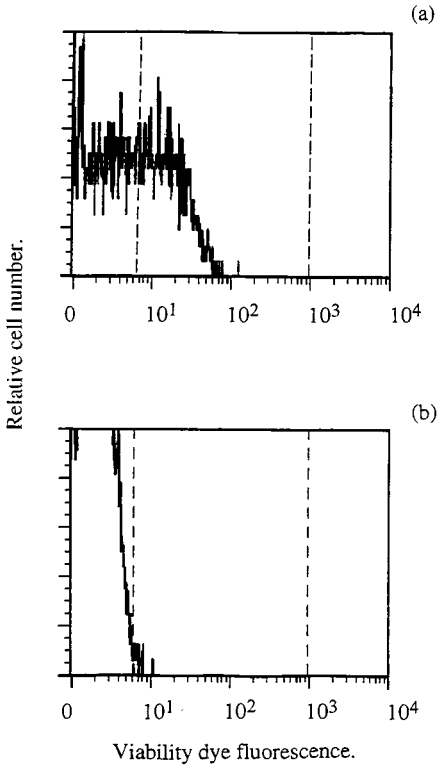

FIG. 1. FCM histograms demonstrating viability assessment of indigenous lake water bacteria using enzymatic viability assessment dyes. (a) Tween 20-amended sample labelled by using a cocktail of CFDA and Chem B incubated at 40°C for 20 min. (b) Control histogram for the sample used for panel a.

No signal indicative of viable cells was obtained with calcein acetoxymethyl ester in the environmental samples. A signal indicative of viable cells was obtained with BCECF-AM, but the count was not significantly different from that of the control ($P = 0.054$). The viable count was also one order of magnitude lower than those for the other enzymatic dyes. These two dyes were not used further.

For lake water populations, CFDA and Chem B gave clear signals indicative of viable cells (Fig. 1), and counts were significantly higher than control sample counts ($P = 0.025$ and $P < 0.000$, respectively). A higher-level background count was associated with CFDA, as Chem B control counts were 49% of CFDA control counts. However, the Chem B viable count was 71% of the CFDA viable count. More detailed analysis showed that of the treatments tested, only the choice of buffer had a significant effect (Fig. 2). Time and temperature effects were not significant (Fig. 2). Use of the Chem Red blocking reagent did not affect the viable count or produce a clearer result. The highest counts were obtained from water samples amended with Tween 20 (0.1% [vol/vol]), and similar trends were recorded for both dyes tested.

Broadly similar results were found with the river water pop-

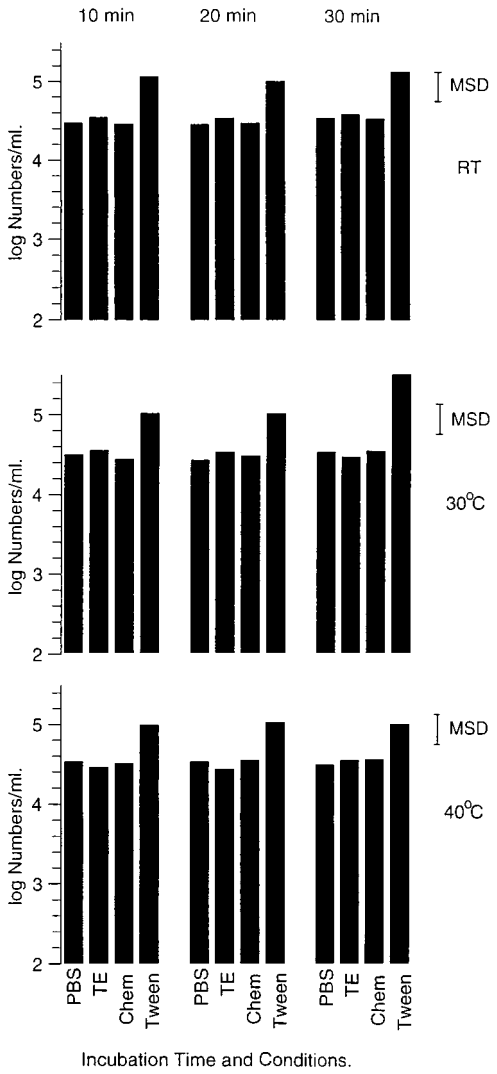

FIG. 2. Optimization of treatments for CFDA labelling of indigenous bacteria in Windermere lake water. Similar results were found with the alternative dye Chem B. Cell numbers were obtained from FCM enumeration of cells stained by the different treatments. MSD, minimum significant difference; RT, room temperature; TE, Tris-EDTA buffer, pH 8.0; Chem, chemchrome B labelling buffer supplied in manufacturer's kit; Tween, 0.1% Tween 20-amended lake water.

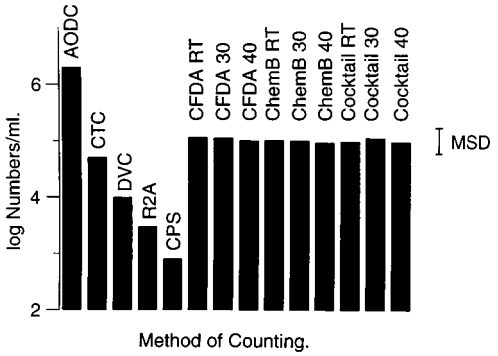

FIG. 3. Bar chart showing total and viable (FCM) counts of indigenous bacteria from Windermere lake water, obtained by different methods. MSD, minimum significant difference; CTC, respiring count obtained by using 5-cyano-2,3-ditolyltetrazolium chloride; R2A, CFU on R2A agar; CPS, CFU on casein peptone starch agar; RT, room temperature; 30, 30°C; 40, 40°C; Cocktail, sample labelled by using a mixture of CFDA and Chem B.

ulations. Again, BCECF-AM was found to be of limited use, while CFDA and Chem B gave clear signals, indicative of viable cells. Significant effects were again found only with the choice of buffer, with Tween 20 amendment producing maximum labelling.

Viable cell enumeration. FCM counts using CFDA, Chem B, and mixtures of the two under optimal conditions were compared with counts by other methods. Data from Windermere samples are presented in Fig. 3. CFU data were the lowest estimates of viable bacteria. Differences found between diluents used for the CFU counts (dilution series prepared in filter sterilized lake water or PBS) were not significant. R2A agar yielded significantly higher counts than casein peptone starch agar under these incubation conditions ($P < 0.000$; Fig. 3). The next lowest estimate of viable cells was that obtained by the DVC method, which was significantly higher than the CFU count but lower than the other viability estimates (Fig. 3). Total respiring counts were lower than, but not significantly different from, the enzymatic dye counts. No significant differences between any of the dye combinations were obtained (i.e., the use of dye cocktails did not increase viable numbers). The AODC was the largest count by one order of magnitude (Fig. 3), and it included both red and green fluorescent particles of bacterial cell size and shape. The differential red and green fluorescence exhibited by acridine orange was not used as an indicator of live and dead cells.

Results from viability assays under optimal conditions using river water samples showed differences from the lake water populations. CFDA yielded significantly lower counts than any other tested method, including CFU counting (Fig. 4). For this sample site, the CFU counts were not significantly different from counts obtained by using Chem B or from the DVC, but they were significantly different from the total (AODC) count (Fig. 4). The DVC and the Chem B counts were not significantly different from the total count (Fig. 4).

Concluding remarks. The results presented here were obtained from environmental samples processed as quickly as possible after sampling, thus minimizing population changes due to the bottle effect (9). Viable cell enumeration, using FCM and fluorescent dyes, was achieved within 2 h after removal of the sample from the site. Ferguson et al. (9) mea-

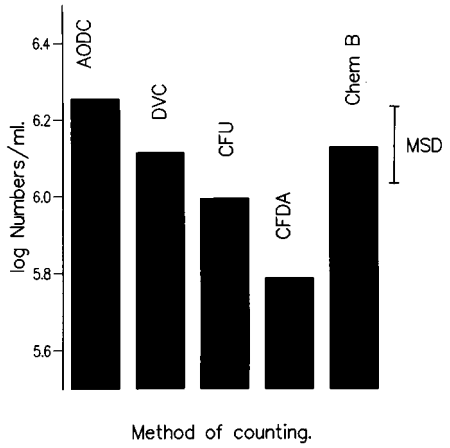

FIG. 4. Bar chart showing total and viable (FCM) counts of indigenous bacteria from river Mersey water, obtained by different methods. MSD, minimum significant difference.

sured changes in community structure only after 16 h of sampling, and thus it would be reasonable to assume that our results reflect the in situ community as closely as is practical.

The most effective method of cell labelling was to add detergent as a permeabilizing agent. A simple explanation for this fact is the lack of a damaging and inefficient centrifugation and/or resuspension step in the protocol. The requirement of a permeabilizing agent for these assays has been demonstrated previously (2, 4, 5, 19), and dye labelling in the absence of such an agent was not examined. Background fluorescence was not a problem when the enzymatic dyes were used (Fig. 1). However, overlap between background fluorescence and labelled cells cannot be disregarded, and it may lead to an underestimation of the true viable count.

The lack of a universal viability dye has been demonstrated previously (4, 7). It is likely that all labelling procedures will be selective to a greater or lesser extent. Of the methods tested here, the use of FCM with the viability dye conjugates was the most rapid and the least selective. FCM coupled with the enzymatic dyes CFDA and Chem B and with cocktails of the two provided an effective, rapid, and less selective alternative to plate counts. Both CFDA and Chem B were successful in labelling at least some lake water bacteria. Optimization of conditions for these dyes produced similar results for both dyes, possibly suggesting analysis of the same cell types in the samples. Although lake and river water samples were analyzed with two different flow cytometers, the results should be comparable. Our experience with the two machines has led us to believe that cells detected by one machine would also be detected by the other. The limiting factor in the analysis would, in our view, be cell labelling and not FCM differences. It is interesting that Diaper and Edwards (4) found that of the two dyes used here, Chem B labelled a greater range of laboratory-cultured bacterial species than did CFDA. This appeared to be reflected in samples from Fiddler's Ferry, which had a culturable fraction greater than that from lake water. Information about the remainder of the total population requires alternative approaches, such as the use of oxonol dyes (26), to enumerate dead cells. Counts could thus be made rapidly and easily by using FCM to enumerate both sections of the population and thus to attempt to further our understanding of the functioning of noncultivable or dormant bacteria in natural environments.

It thus seems likely that the methods presented here would require testing for each sample site of interest but that a satisfactory protocol will be available. The method could be used to supersede the plate count as an indicator of viable bacterial numbers. The multiparameter approach made possible by FCM may also allow simultaneous detection of immunofluorescently labelled cells, thus enabling detection and viability assessment of total and specific components of bacterial communities.

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