Optimal Staining and Sample Storage Time for Direct Microscopic Enumeration of Total and Active Bacteria in Soil with Two Fluorescent Dyes

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Direct counting techniques, first developed for aquatic samples, can be used to enumerate bacteria in soil and groundwater sediments. Two fluorescent dyes, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) for actively respiring bacteria and 4′,6-diamidino-2-phenylindole (DAPI) for total bacteria, were tested for their usefulness in epifluorescent direct bacterial enumeration in soil. Both dyes can be used for the same soil sample without affecting enumeration results. Staining for 8 h with CTC and for 40 min with DAPI resulted in maximum numbers of stained cells. The optimal DAPI staining concentration is 10 mg liter^{-1}. After preparation, slides should be stored at 4°C and counted within 2 days for CTC and within 24 h for DAPI. Sodium PP$_i$ or sodium chloride solutions were used to desorb bacteria from soil prior to counting. Counts were significantly higher when sodium chloride was used.

Accurate methods of measuring biomass and microbial activity are essential to understanding microbial characteristics in soil subsurface environments. Many methods are available for this analysis. Three general approaches have been used to determine bacterial numbers: (i) the number of culturable bacteria has been determined by plate counts or most-probable-number techniques; (ii) direct counts have been made with stains specific to biological molecules to enumerate the total bacterial numbers; and (iii) direct counts have been made by using stains or microautoradiography to determine the number of active bacteria.

In the past, estimates of microbial number in the subsurface have relied upon most-probable-number or plate count methods. However, these methods cannot enumerate organisms that are unable to grow in the specific media chosen (1, 19). With these techniques, very small, variable numbers of CFU are typical of subsurface soil samples (2, 3, 8–10, 16, 17, 19). Also, plate count methods are very time-consuming (the incubation time is at least 1 week). Consequently, an alternative method, direct counting, was developed for bacterial enumeration.

Several dyes have been used to enumerate total bacterial numbers. The dye acridine orange (AO) has been widely used (2, 10, 18, 19). This method is based on the interaction of AO with nucleic acids (13). When the concentration of AO is kept relatively low, bacteria growing at high growth rates will fluoresce red-orange because of the predominance of RNA while inactive bacteria have mostly DNA and will fluoresce green (11). However, AO direct counting is not a good method to assess the activity of natural and undefined bacterial communities because the RNA/DNA ratio in a cell and thus the AO color reaction could be affected by growth media, AO staining concentration and procedure, and the method of cell fixation (if any), as well as cell taxonomy (12). Also, with the AO direct count method, it is hard to distinguish bacteria from nonliving, bacterium-sized particles such as clay, detritus, or colloids, which may also be stained or autofluorescent (9). Another

fluorescent dye, a DNA-specific dye, 4′,6-diamidino-2-phenylindole (DAPI), is superior to AO in total bacterial number enumeration, especially in instances of high background fluorescence (14). DAPI has also been recommended over another DNA-specific dye, mithramycin (4).

Several methods have been established for enumeration of active bacteria. The INT [2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride] staining technique is currently popular. INT can be reduced to insoluble INT-formazan crystals by the electron transport system of respiring organisms and observed microscopically by bright-field microscopy as an opaque red intracellular deposit (14). It may be difficult to see very small cells and their accumulated formazan deposits, because many small subsurface organisms are relatively poor INT reducers (18). This may result in an underestimation of the number of active cells. Another redox dye, CTC (5-cyano-2,3-ditolyl tetrazolium chloride), has recently been introduced for enumeration of active bacteria in water samples (15). CTC is readily reduced to insoluble, highly fluorescent, and intracellularly accumulated CTC-formazan through bacterial respiration. Fluorescence emission of CTC-formazan is primarily in the red region, with excitation by long-wavelength UV light. Thus, actively respiring bacteria can be distinguished from nonrespiring bacteria and abiotic material that typically emit in the blue or blue-green regions. The CTC technique is thus superior to INT staining.

Several techniques have been introduced to quantify both active and total bacteria in the same sample. For instance, INT can be used together with AO (2, 9, 18) or DAPI (6) to count active and total cells, respectively. However, optimum storage and staining conditions have not been conclusively identified.

It is not unusual for environmental microbiologists to collect large numbers of samples simultaneously. Because counting soil or sediment bacteria is time-consuming and not amenable to automation, some form of sample storage may be necessary. Fixatives may be used if the total number of bacteria is all that is desired. However, if the number of active bacteria is of interest, microorganisms must remain active until staining. Storage of unstained samples may lead to changes in microbial numbers through growth, death, predation, or other conditions

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that vary from the in situ environment. Understanding how to effectively store samples after they are stained may assist in sampling and experimental design. In this study, optimal staining conditions and sample storage time are evaluated for a combined DAPI and CTC technique of assessing total and active bacterial numbers in soil samples.

MATERIALS AND METHODS

Two sources of bacteria were used in this study, soil samples and pure cultures of *Pseudomonas putida*. Soil samples were collected from an agricultural field in Manhattan, Kans., immediately before each set of experiments. The *P. putida* cultures were obtained from Carolina Biological Supplies and grown in TSA medium. DAPI (total bacterial numbers) and CTC (actively respiring bacteria) dyes were used. DAPI was purchased from Sigma Chemicals Co., St. Louis, Mo., and CTC was purchased from Polyscience, Inc., Warrington, Pa.

To stain soil bacteria with CTC, 2.5 g of wet soil was placed in a sterile 125-ml Erlenmeyer flask and 22.5 ml of filter-sterilized 0.1% sodium PP_i solution (or 0.8% sodium chloride solution) was added after the pH had been adjusted to 7.0 with HCl. Then CTC was added to a final concentration of 2.0 mM, and the flasks were incubated at room temperature on a rotary shaker (160 rpm) for different times. After being shaken, the suspension was decanted into an amber vial and the larger particles were allowed to settle out for 2 min. An aliquot of the supernatant was filtered through a 0.2-μm-pore-size black Nuclepore membrane filter. Then, microscope slides were prepared and the numbers of active bacteria were counted. When simultaneous DAPI counts were to be made, cells were stained with DAPI as described below prior to filtration. Subsamples were pipetted from soil slurries for filtration, and at least 2 ml of liquid was filtered through a 0.2-μm black Nuclepore membrane filter with a Teflon filter tower (5) to get an even distribution of cells.

A similar technique was used to stain *P. putida* cultures with CTC. Log-phase cells (3 days after inoculation) were dislodged from culture plates with a sterile spatula after the plates were flooded with 100 ml of sterile deionized water. Five drops of Tween 80 was added to the solution, and the flooded plates were shaken for 2 h to break up aggregations of bacteria. The bacterial slurry (250 μl) was diluted with 750 μl of 0.1% sodium PP_i solution in 20-ml borosilicate glass scintillation vials, CTC was added to a final concentration of 2.0 mM, and the vials were shaken for 4 h.

For DAPI staining, an aliquot of supernatant was pipetted into an amber vial containing 10 ml of filtered water. Then DAPI was added to reach the final concentration of 10 mg liter^{-1}. After the suspension was stained, the filters were prepared as described previously. After the last drop of water had passed through the filter, the damp filter was air dried and mounted on a glass microscope slide with a drop of low-fluorescence Cargill immersion oil. Another drop of oil was added to the filter, followed by a coverslip. All of the above procedures were conducted in the dark, because both CTC and DAPI are light sensitive. Amber vials were used, and flasks, test tubes, and scintillation vials were wrapped with aluminum foil and electrical tape.

The mounted filters were microscopically examined, and all bacterial cells in the grid field were counted at a magnification of ×1,000. Generally, 8 to 20 fields per slide were counted. A Zeiss Axioskop 20 microscope or a Nikon Labophot 2A equipped with the appropriate filter blocks was used for epifluorescence counts.

To study CTC optimal staining times, the flasks with soil suspensions and CTC were shaken on a rotary shaker for 1, 2, 3, 4, 6.5, 11.5, 16, or 24 h at 160 rpm before microscope slides were prepared. For DAPI staining, cells were stained for 1, 3, 5, 10, 20, 40, 95, or 160 min before slide preparation. For slide storage experiments, cells from soil samples were stained with CTC and DAPI for 10 h and 40 min, respectively, while slides prepared with *P. putida* were stained with CTC and DAPI for 4 h and 10 min, respectively. Storage tests were done on slides with filters under immersion oil and coverslips. Slides were stored at room temperature (20°C), in an incubator (11°C), in a refrigerator (4°C), or in a freezer (−4 or −12°C). When the effect of light was tested, the slides were exposed to 100 μmol quanta m^{-2} s^{-1} (Li-COR; PAR sensor) from a fluorescent light bank. The slides were counted under the microscope immediately and after several days. To assess the effects of DAPI-CTC on CTC-DAPI, two sets of experiments were performed: (i) CTC and DAPI were used on a single slide, and (ii) CTC and DAPI were used on different slides. The staining times for CTC and DAPI were 10 h and 40 min, respectively. For comparison of the effects of sodium chloride and sodium PP_i as desorption and dilution solutions, a set of experiments were performed with 0.8% sodium chloride and 0.1% sodium PP_i solutions to prepare slides, respectively. All experiments were run in triplicate. Duncan's multiple-range test was used to compare differences at a significance level of 5%.

Emission and excitation spectra of DAPI- and CTC-stained *P. putida* cells were obtained with a Spex-Fluorolog 2, using a bacterial suspension stained as outlined above and diluted until a stable signal could be obtained. Unstained suspensions of cells were used to correct for background fluorescence.
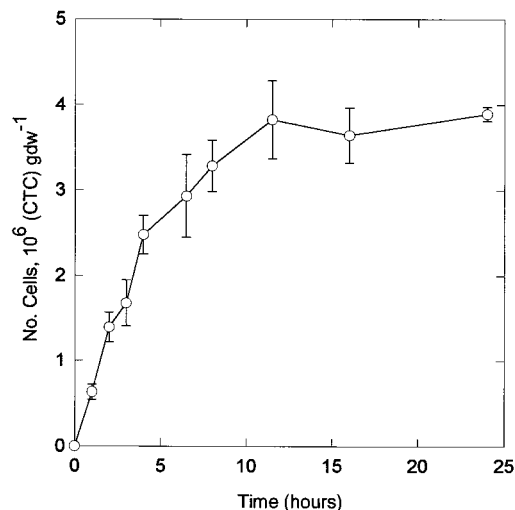

FIG. 1. Effect of staining time on CTC enumeration. Error bars indicate 1 standard deviation (SD); $n = 3$ for each time.

RESULTS AND DISCUSSION

The numbers of CTC-stained cells increased until 8 h of staining ($P \leq 0.05$) in soil samples (Fig. 1). The numbers of DAPI-stained cells were significantly different for each time step ($P \leq 0.05$) until 40 min of staining in soil samples (Fig. 2). Less staining time may be needed to enumerate bacteria in water samples (1 h) (15). Porter and Feig (14) showed that the staining time for DAPI in water samples was 10 min. Differences may be due to a decreased exposure of CTC and DAPI to bacteria in soil and/or to an increased respiration rate of active bacteria as a result of addition of nutrient media in other protocols. Numbers of active bacteria determined by CTC staining increased by 4 orders of magnitude in groundwater samples as a result of nutrient addition (15). In our study, to avoid preferential stimulation of inactive bacteria, no nutrients were added. Bacterial growth, however, could not be eliminated during staining, especially for CTC.

The final concentration of DAPI in this study was 10 mg
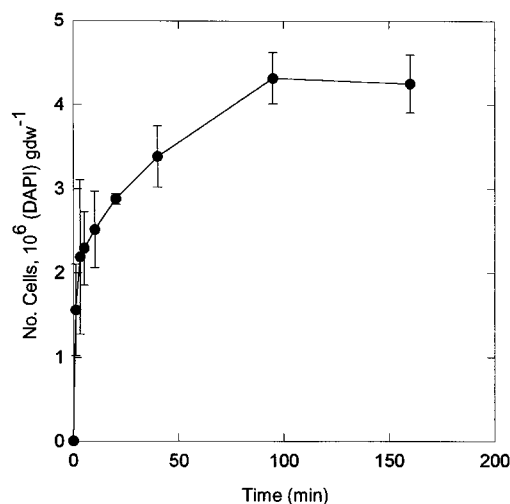

FIG. 2. Effect of staining time on DAPI enumeration. Error bars indicate 1 SD; $n = 3$ for each time.
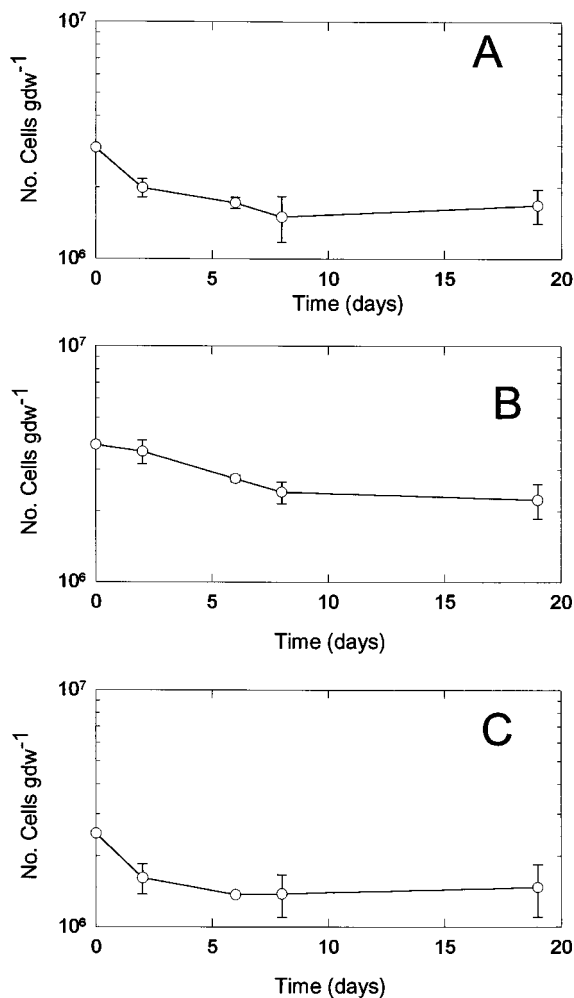
FIG. 3. Relationship between numbers of CTC-stained cells and slide storage temperature. (A) Stored at room temperature (20°C); (B) refrigerated (4°C); (C) stored in freezer (−12°C). Error bars indicate 1 SD; $n = 3$ for each time. Error bars are not visible when they are smaller than the symbols.

liter^{-1}. This concentration was also used by Dufour and Colon (6). Lower concentrations (0.01 to 3.5 mg liter^{-1}) have been used by others (4, 13–15). When we used several different concentrations (0.01, 1.0, 2.0, 6.6, 10, and 20 mg liter^{-1}), we counted very few bacteria when DAPI concentrations were 0.01 to 2.0 mg liter^{-1}. When DAPI concentrations of 6.6 and 10.0 mg liter^{-1} were used, more bacteria were seen and could be distinguished easily from the background. When 20 mg of DAPI liter^{-1} was used, excessive background fluorescence was observed. Other DNA-specific dyes, Hoechst 33258 and Hoechst 33342, have been reported to have distinct advantages over AO in enumeration of bacteria on surfaces which bind AO, such as polystyrene (13). However, we found that these dyes resulted in higher background fluorescence than DAPI (unpublished observations).

A significant decline in the numbers of active bacteria (CTC stained) in soil samples was observed after 2 days with all storage methods except storage at 4°C ($P \leq 0.05$) (Fig. 3). When slides were prepared from CTC-stained bacterial pure cultures, similar results were observed (Fig. 4). Storage in the freezer increased variance in the counts (Fig. 4E). Samples did not degrade more rapidly in the light than in the dark at room
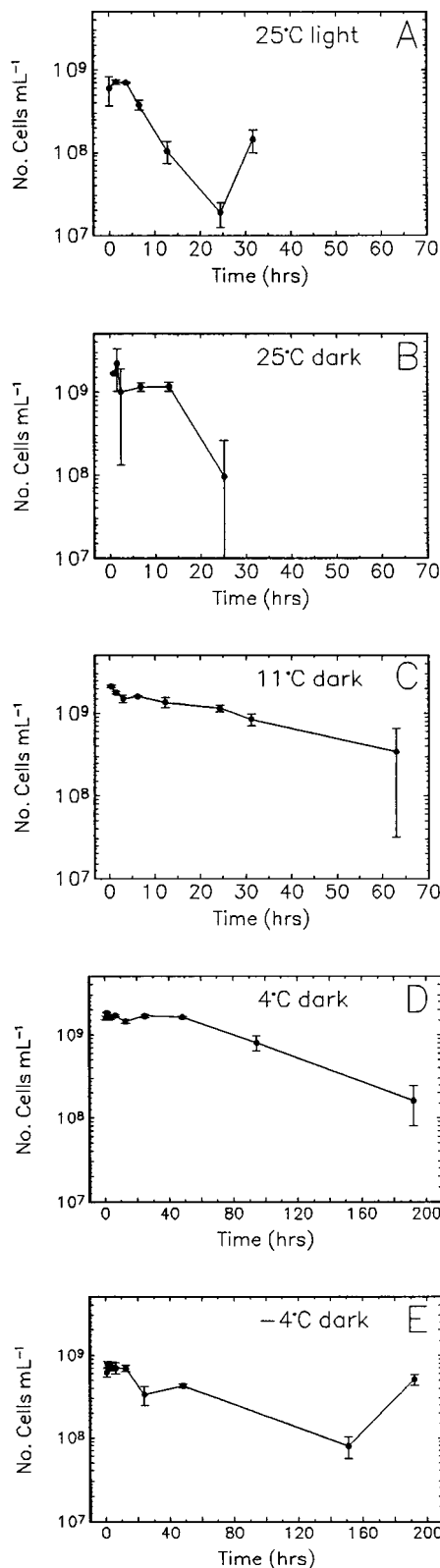

FIG. 4. Relationship between CTC counts of a *P. putida* culture and storage conditions. (A) 25°C in the light; (B) 25°C in the dark; (C) 11°C in the dark; (D) 4°C in the dark; (E) −4°C in the dark. Error bars indicate 1 SD; $n = 3$ for each time.
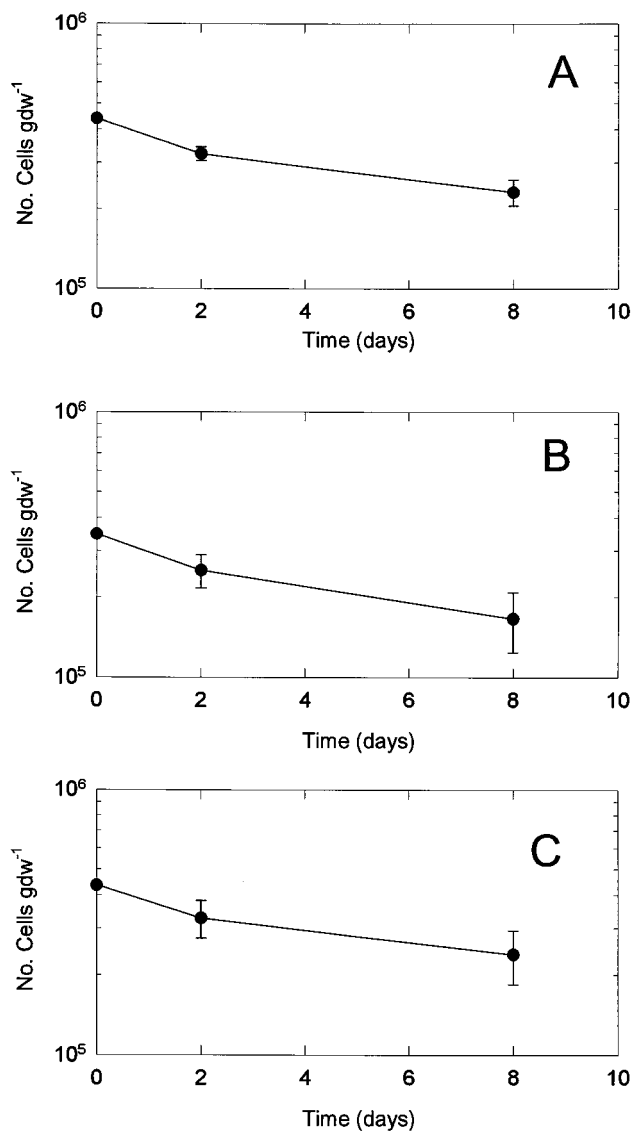
FIG. 5. Relationship between number of DAPI-stained cells and slide storage temperature. (A) Stored at room temperature (20°C); (B) refrigerated (4°C); (C) stored in freezer (−12°C). Error bars indicate 1 SD; $n = 3$ for each time. Error bars are not visible when they are smaller than the symbols.

temperature (Fig. 4A and B), suggesting that room light may not have a strong effect on storage of CTC-stained preparations. Therefore, we conclude that the most reliable storage method for CTC-stained slides is refrigeration and that the slides must be counted within 2 days of storage.

With DAPI-stained cells from soil samples, there were no significant differences ($P \leq 0.05$) among the three storage methods and total bacterial numbers decreased significantly within 2 days of storage (Fig. 5). Bacterial cultures stained with DAPI exhibited smaller numbers after several days of storage at room temperature; less sample degradation was evident for samples stored at 4°C (Fig. 6D). Storage in the freezer seemed to increase variance in the counts (Fig. 6E). Fluorescent lighting, commonly used in laboratories, did decrease bacterial numbers at room temperature. Therefore, DAPI slides should be counted immediately and stored in the dark to ensure accurate enumeration results.
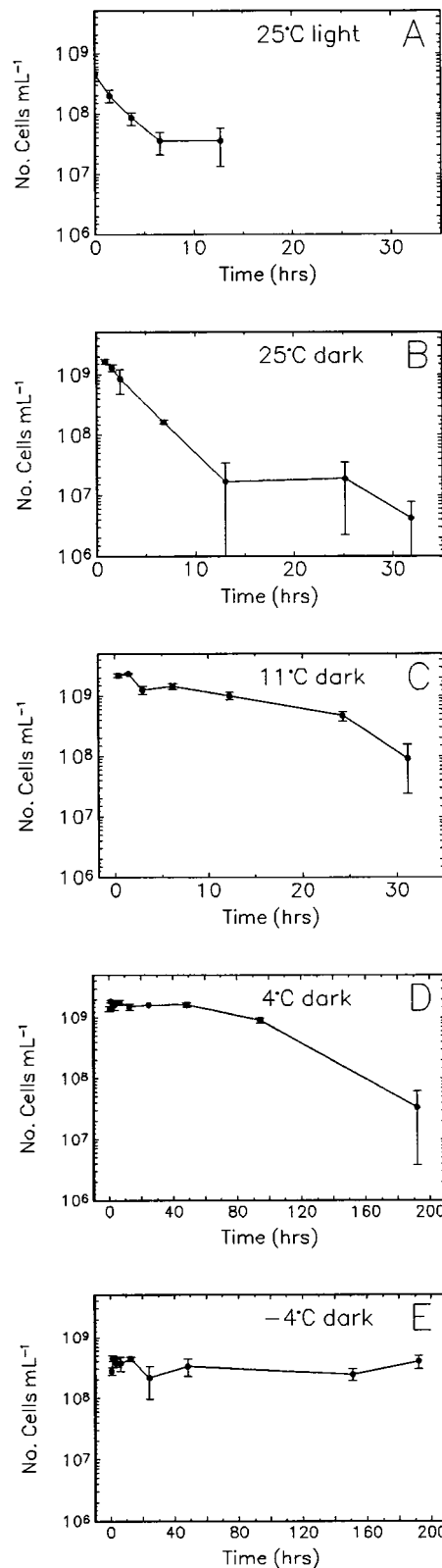

FIG. 6. Relationship between DAPI counts of a *P. putida* culture and storage conditions. (A) 25°C in the light; (B) 25°C in the dark; (C) 11°C in the dark; (D) 4°C in the dark; (E) −4°C in the dark. Error bars indicate 1 SD; $n = 3$ for each time.
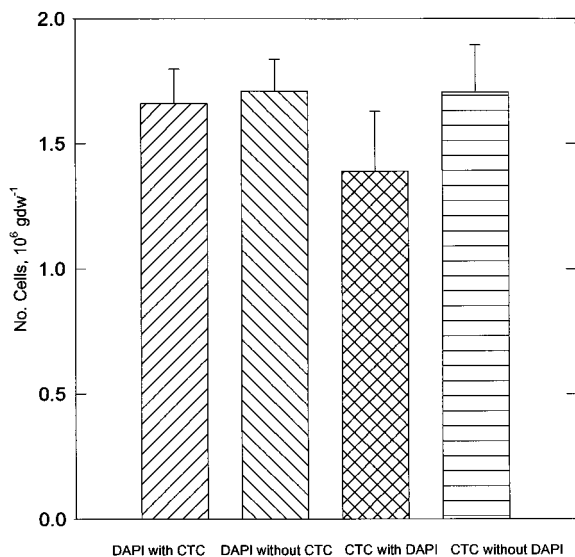
FIG. 7. Effects of DAPI or CTC staining on CTC or DAPI staining, respectively. Error bars indicate 1 SD; $n = 3$ for each time.
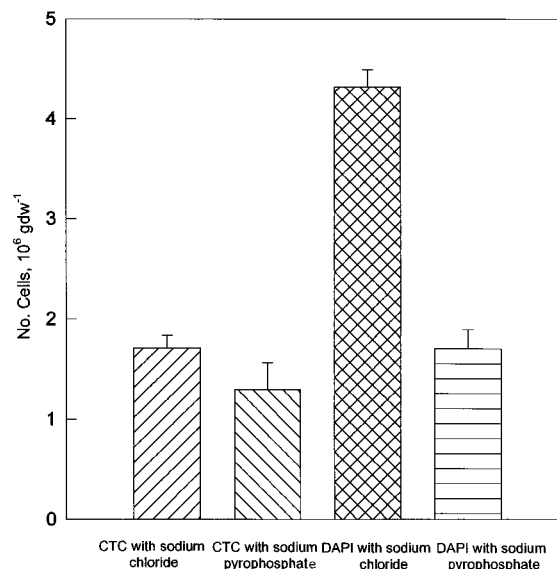

FIG. 9. Relationship between desorption effects of sodium chloride and sodium PP_i. Error bars indicate 1 SD; $n = 3$ for each time.

To compensate for sample degradation when many samples are prepared, it is tempting to fit the relationship between cell numbers and storage time and to correct for time of slide storage. The linear decrease on the log scale plots of bacterial numbers in many of our sample storage experiments suggests that it would be possible to predict sample deterioration with an exponential-decay model. With those simple models, the initial bacterial numbers in the samples could be calculated from numbers counted several days after slide preparation. However, this approach should be avoided if possible, because the decay constants in these models vary as a function of storage conditions and are different with pure bacterial cultures and soil samples (decay constants are not shown).

There was no significant effect of DAPI on CTC counts or of CTC on DAPI counts ($P \leq 0.05$) (Fig. 7). We found that the broad excitation peak of CTC-formazan is centered at 452 nm and that the emission peak of DAPI is at 470 to 480 nm (Fig. 8). This means that emission by DAPI-stained cells will be intercepted by CTC when both are in the same cell (either by direct absorption of photons, emitted by DAPI, or by Förster
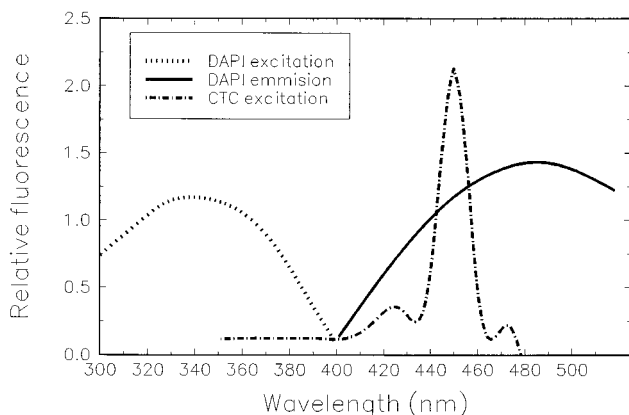
energy transfer). Thus, it is necessary to add CTC numbers to DAPI numbers when calculating total bacteria for samples in which both stains are used.

For soil samples, bacteria were desorbed from soil particles for slide preparation. Because of the importance of the desorption process, we compared desorption effects of sodium chloride with those of sodium PP_i. No significant differences were found ($P \leq 0.05$) for CTC-stained soil microorganisms (Fig. 9). However, DAPI-stained soil samples yielded significantly larger numbers ($P \leq 0.05$) when the sodium chloride solution was used. PP_i is often used to desorb bacteria from soil particles. In our study, however, it did not provide greater counts than saline. This merits further investigation. There is a higher sodium concentration in 0.8% NaCl solution (0.136 M) than in 0.1% $Na_4P_2O_7 \cdot 2H_2O$ solution (0.015 M). Even though increased cation concentrations in solution have been shown to increase bacterial adsorption to soil (7), numbers of suspended bacteria counted in solutions with a lower Na^+ concentration did not increase. Also, the microscopic field was observed to be much clearer with the sodium chloride solution than with the sodium PP_i solution. The higher background clarity may allow for more accurate enumeration.

The results of this study indicate that the dyes CTC and DAPI can be used on a single slide without significantly changing enumeration results. The minimum staining time is 8 h for CTC and 40 min for DAPI. The optimal DAPI concentration was found to be 10 mg liter^{-1}. Sodium chloride should be used rather than sodium PP_i. After the slides are prepared, they should be stored in a refrigerator (4°C) and counted within 2 days of storage for CTC and on the day of preparation for DAPI. Exposure to room light should be avoided, especially for DAPI-stained cells. When both stains are used, CTC numbers should be added to DAPI numbers for total bacterial counts. It is possible that as the direct counting procedures are applied to a wider variety of environmental samples, different (more restrictive) conditions of sample storage following staining will be necessary. For example, it is not known how active bacteria must be before they are stained by CTC. We hope that the research here serves as a reminder of some potential prob-

FIG. 8. Excitation and emission spectra for DAPI- and CTC-stained cell suspensions.

lems that may be encountered with direct enumeration techniques of total bacteria.

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