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Determination of the Number of Respiring *Thiobacillus ferrooxidans* Cells in Water Samples by Using Combined Fluorescent Antibody–2-(*p*-Iodophenyl)-3-(*p*-Nitrophenyl)-5-Phenyltetrazolium Chloride Staining

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Fluorescent antibody staining was combined with 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride reduction in a procedure termed FAINT to allow for the direct microscopic determination of specific actively respiring populations of bacteria in a variety of aquatic habitats. The FAINT procedure is simple, precise, and appropriate for use in a wide variety of autecological studies. The distribution of *Thiobacillus ferrooxidans* was examined by FAINT enumerations in both acidic and nonacidic sites. Comparisons among the FAINT technique and fluorescent antibody staining alone or most-probable-number determinations in 9K broth showed that the use of most-probable-number determinations resulted in an underestimation of the number of viable cells by one to three orders of magnitude, whereas fluorescent antibody counts resulted in an overestimation of the number of viable *T. ferrooxidans*. The amount of difference was not consistent but varied, depending on the sample site.

Direct counting of microbial cells stained with fluorescent dyes such as acridine orange or fluorescein isothiocyanate is a well-established method for determining the total standing crop of microorganisms in natural habitats (13, 17, 18, 22, 25). However, it is not possible to differentiate specific types of bacteria by using these stains, nor is it possible to distinguish between live and dead cells (6, 17, 33). To differentiate specific types of bacteria, several workers have used immunofluorescent staining techniques to determine the population size of a variety of bacteria in natural samples, including species of *Rhizobium* (5, 34, 35), *Nitrobacter* (14, 16, 32, 37), *Thiobacillus* (1, 20), *Legionella* (9, 15), *Thermoplasma* (3), and several methanogens (38, 39).

Several methods have been employed to discern live cells from dead cells. Microautoradiography has been combined with direct staining methods to distinguish active from inactive cells in detrital, marine, and estuarine communities (7, 23, 28, 31). Kogure et al. (27) recently proposed the use of incubations in the presence of nalidixic acid to distinguish synthetically active cells from inactive cells, and Orndorff and Colwell (29) applied the technique to a study of heterotrophic microbial communities in a Kepone-polluted section of the James River.

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Alternative electron acceptors in the form of tetrazolium salts have been used to measure electron transport activity in samples of zooplankton, phytoplankton, and bacteria (8, 10, 11, 12, 26). The reduction of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) to extractable formazan has been shown to be correlated with oxygen uptake and respiratory activity (10). In a recent modification of the INT technique, Iturriaga and Rheinheimer (24) and Zimmerman et al. (40) proposed direct microscopic examination of samples incubated in the presence of INT to allow for the determination of respiratory activity on a cell-by-cell basis, and Harvey and Young (21) applied the direct INT technique to the enumeration of respiring bacteria in salt marsh samples.

The combination of fluorescent-antibody (FA) staining with a method such as microautoradiography or INT reduction for the determination of cellular activity would make it possible to determine how many viable cells of the type of interest are in a sample. Fliermans and Schmidt (16) combined FA staining and microautoradiography for the direct microscopic enumeration of viable *Nitrobacter* cells in soil systems. We report here a simple method for combining FA staining and INT reduction (FAINT) to determine the numbers of viable *Thiobacillus ferrooxidans* cells in natural samples. The results indicate that the FAINT method provides a rapid, accurate measure of species-specific numbers of

active cells and is more sensitive than conventional cultural techniques by at least one order of magnitude.

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MATERIALS AND METHODS

Preparation of antiserum. *T. ferrooxidans* cells (ATCC 3598) to be used for antibody preparation were grown in 9K medium, harvested, and freed of inorganic precipitates by the procedures of Silverman and Lundgren (36). After harvesting, the cells were washed with phosphate-buffered saline (PBS [0.1 M PO_4^{-3}–0.85% NaCl, pH 7.6]) and resuspended in PBS at a concentration of approximately 10^9 cells ml^{-1}.

A New Zealand white rabbit was injected intravenously with increasing doses (1×10^8, 5×10^8, 1×10^9, and 5×10^9) of *T. ferrooxidans* cells over a 2-week period. The rabbit was then rested for 1 week and bled through an ear vein. After the initial induction period, antibody titers were maintained in the rabbit by periodic injections of 10^6 cells.

Antiserum was prepared from the collected blood samples by the procedures of Garvey et al. (19) as described by Gates and Pham (20). By using the slide agglutination procedure (2), the antiserum was tested for specificity against known strains of gram-positive and gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhimurium*, and *Staphylococcus aureus*) and 86 strains of heterotrophs isolated from the environment where this procedure was to be implemented as an analytical tool. In addition, the preparation was tested against nine strains of *Thiobacillus* species, including *Thiobacillus thiooxidans* (ATCC 8085), and eight environmental isolates of sulfur-oxidizing autotrophs unable to oxidize iron. If any of the slide agglutination tests yielded questionable results, the strain was further tested by a complete FA stain. In the few cases where cross-reactivity with a strain was observed, the cross-reacting antibodies were removed by placing washed cells of the interfering strain into the preparation and allowing adsorption to the antigen for a 16-h period at 4°C. The cells and the adsorbed antibody were then removed from the serum. Retesting of the treated antiserum always demonstrated complete removal of the interfering antibodies.

Sample collection and preparation. Water samples were collected from Lake Anna, Louisa County, and Peacock Hill Lake, Albemarle County, Va., in April 1981. Sampling sites were chosen to reflect a variety of aquatic habitats, including areas with a high probability and those with a low probability of the presence of *T. ferrooxidans* cells. Specific conductance, pH, dissolved O_2, and temperature were measured at each site as the samples were taken. Culturable *T. ferrooxidans* cells were determined at each site, using a five-tube most-probable-number (MPN) procedure in 9K broth (36).

Samples for FA counts were fixed immediately upon collection by the addition of formaldehyde (2% final concentration). Samples for activity determinations, using FAINT staining, were filtered through a 0.2-μm filter (Nuclepore Corp.) to collect the cells. The filters were then placed in a 30-ml sterile centrifuge tube containing 10 ml of a 0.2% solution of INT (J. T. Baker Chemical Co.) in PBS and incubated in the dark for 10 min. After incubation, formaldehyde was added to a final concentration of 2% to fix the cells. Fixed samples were stored in the dark and returned to the laboratory for further processing.

FA staining. Samples were stained by using the indirect staining technique of Gates and Pham (20). Bovine serum albumin (20 mg/ml) or hydrolyzed gelatin was used to reduce nonspecific background fluorescence (4, 20), and 200 ml of PBS was used at each washing step. For each cell observed, the intensity of fluorescence was rated on a scale of 0 (no fluorescence) to +4 (brilliant fluorescence). Cells showing +3 to +4 fluorescence were counted as *T. ferrooxidans*.

FAINT staining. In the laboratory, the filters which had been incubated in the presence of INT were transferred to a staining tower, and the INT solution in which the filter had resided was passed through the filter to collect any cells which may have been dislodged from the membrane during transport. The cells were then stained, using the indirect FA procedure (20). After staining, the cells were transferred from the filter surface to a microscope slide, using the gelatin-stripping technique of Tabor and Neihof (P. S. Tabor and R. A. Neihof, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, N80, p. 186). Briefly, this procedure involves placing the filter face down on a microscope slide which has been coated with a thin layer of a gelatin-chrome alum mixture (40) by dipping the slide in a beaker of the molten preparation and allowing the excess to run off. The solution is allowed to dry overnight, and the filter is carefully stripped off, leaving the cells embedded in the gelatin. The surface is then coated with a second layer of the gelatin solution to prevent dissolution of the formazan crystals by the immersion oil.

Individual cells visible with the FA stain were examined under alternating epifluorescent-transmitted illumination to determine whether they contained formazan crystals. For each sample processed, a minimum of 20 FA-positive cells were rated. Concentrations of actively respiring microorganisms were determined by multiplying the number of *T. ferrooxidans* determined by the FA counts by the percentage of cells that were FA positive and contained formazan crystals, i.e., the percentage of cells that were FAINT positive.

Optimization of INT reduction assay. The effect of the incubation pH on INT reduction was determined by suspending pure cultures of *T. ferrooxidans* cells in either distilled water acidified to pH 2.6 or PBS at pH 7.6. INT (0.02% final concentration) was added to the preparations, and the suspensions were incubated in the dark for 18 h. Samples were periodically withdrawn, and the percentage of cells containing formazan crystals was determined with phase-contrast microscopy (24).

The optimal time for the incubation of cells in the presence of INT was determined by incubating *T. ferrooxidans* suspended in 0.02% INT in PBS. Two incubation series were used. In the first series, the cells were exposed to INT for 10 min and then fixed by the addition of formaldehyde (2% final concentration). The second series was incubated in the presence of

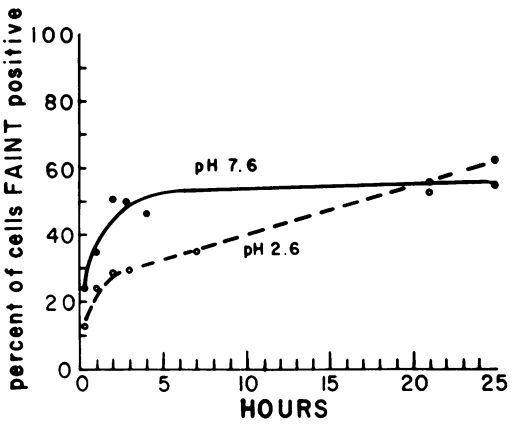

FIG. 1. Relationship of pH to the time of development of visible formazan crystals in bacterial cells.

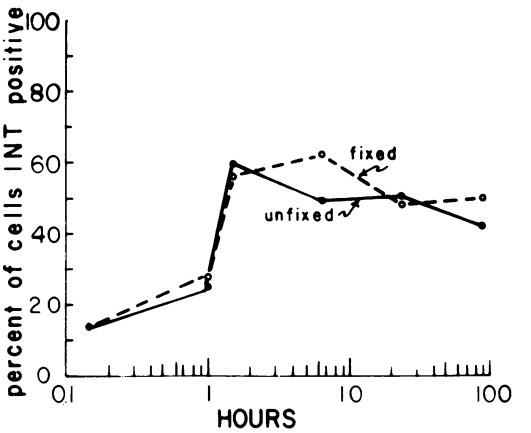

FIG. 2. Time for development of visible formazan crystals in cells fixed or not fixed with formaldehyde after 10 min of incubation with INT. Note the apparent deformation of the early portion of the curve due to the logarithmic scale of time.

INT without any added formaldehyde. Samples were periodically withdrawn from each treatment, and the percentage of INT-positive cells was determined.

The possibility that non-biological, extracellular reduction of the INT might occur in the presence of reducing agents such as Fe^{2+} was tested by adding INT to cultures of *T. ferrooxidans* in 9K broth, to sterile 9K broth, and to the Fe^{2+} solution used to make 9K broth. Samples were removed and examined microscopically for the presence of extracellular formazan crystals.

Recovery of T. ferrooxidans cells from neutral solutions. *T. ferrooxidans* cells originally grown on 9K media were transferred to PBS. After 4 days of incubation in PBS, INT counts (with acridine orange as the fluorescent stain) were made to determine the percentage of the cells which were still active. Subsamples of the culture were transferred to 9K medium to test for the presence of culturable *T. ferrooxidans* cells. Separate subsamples were sequentially centrifuged and suspended for 2 h in acidified (H_2SO_4) distilled water (pH 6.0 and 4.0) before being inoculated into 9K media.

RESULTS

FA staining. Antiserum for *T. ferrooxidans* showed no cross-reactivity with any culture of heterotrophic bacteria, including those isolated from the sampling sites under examination. There was a very slight cross-reactivity (slide agglutination, +/−; fluorescence, 0/1) between *T. ferrooxidans* antiserum and several *Thiobacillus* spp. demonstrated not to be *T. ferrooxidans* by their inability to oxidize Fe^{2+}. The cross-reacting antibodies, however, were easily removed by adsorbing them overnight with the non-Fe^{2+}-oxidizing *Thiobacillus* isolates.

A nonspecific background fluorescence which could not be completely blocked by the use of either bovine serum albumin or hydrolyzed gelatin was present in the samples; however, washing of the filters with 200 ml of PBS between each of the reaction steps reduced the background to a level (0/1) that did not interfere with counting of the cells. Comparisons of acridine orange and FA counts on suspensions of *T. ferrooxidans* cells or on water samples to which *T. ferrooxidans* cells were added indicated that all of the added *T. ferrooxidans* cells were counted with the FA stain.

FAINT staining. Formazan crystals in *T. ferrooxidans* appeared as dark-red deposits when viewed with transmitted light. Usually, but not always, the formazan crystals were deposited in polar regions of the cells. Since extracellular deposition of formazan crystals was occasionally noted, only those crystals which could be seen within a fluorescent cell were counted as indicating actively respiring cells. No intracellular formazan deposits were evident in samples of *T. ferrooxidans* cells which had been killed by the addition of formaldehyde before the addition of INT.

The formation of visible formazan crystals in the cells took considerably longer at pH 2.0 than at pH 7.6, although similar results were obtained at the two pH values after 15 h of incubation (Fig. 1). The color of the formazan crystals under acid conditions was never as intense as at the higher pH, which made distinction of INT-positive cells at the low pH values extremely difficult.

Samples which were fixed after a 10-min exposure to INT and examined after sufficient time for growth of the formazan crystal (2 h) showed no difference in the percentage of cells which were INT positive when compared with samples incubated for 2 h with no fixation (Fig. 2).

The addition of INT to cultures of *T. ferrooxidans* cells in 9K media resulted in the virtually

TABLE 1. Environmental parameters of the sites sampled for the presence of *T. ferrooxidans*[a]

Station	Parameter			
	Specific conductance (siemens cm^{-1})	pH	Temp (°C)	Dissolved O_2 uptake (mg $liter^{-1}$)
C1	300	3.2	11.6	6.6
C4	171	4.4	6.4	—[b]
A2	95	6.9	15.6	7.0
F1	160	7.2	15.0	8.8
L1	85	6.8	6.9	—
PH	75	6.7	9.3	11.5

[a] The sites were not necessarily sampled simultaneously.
[b] —, Measurement not made.

instantaneous reduction of INT with concomitant formation of extensive extracellular formazan deposits. Extensive abiological INT reduction was also found to occur when INT was added to either 9K solution or the Fe^{2+} solution used in 9K media.

Enumeration of *T. ferrooxidans* cells in field samples. Site C1 in Lake Anna is highly contaminated with acid mine-drainage (AMD), as can be seen by the low pH and high conductivity of the water (Table 1). Site C4 is 1.1 km downstream and represents an intermediate zone of AMD contamination. Site A2 is 2 km downstream from site C1 and, as a result, has a higher pH and a lower conductivity than the upstream station. Sites F1, L1, and PH were chosen as control sites where viable *T. ferrooxidans* cells were not expected to occur because none of these sites receives AMD input.

The MPN technique consistently gave the lowest estimates of the number of *T. ferrooxidans* cells present (Table 2). By this technique, *T. ferrooxidans* cells could be detected only at site C1, and the number there was low. FA staining indicated that *T. ferrooxidans* cells were present at sites C1, A2, and F1. No *T. ferrooxidans* cells were detected at site PH with any method.

Activity measurements, using the FAINT technique, indicated that use of direct microscopic counting of FA-stained cells resulted in an overestimation of the number of viable cells at all sites where *T. ferrooxidans* cells were present. Active cells accounted for approximately 80% of the *T. ferrooxidans* cells at site C1 but only about 50% of the *T. ferrooxidans* cells observed at site A2. FAINT-positive cells were detected in only one of the three replicate samples taken at station F1, and for that sample, the actively respiring cells were only 14% of the total FA count.

Recovery of *T. ferrooxidans* cells from neutral solutions. No culturable *T. ferrooxidans* cells could be recovered when cells which had been incubated in PBS for 4 days were inoculated directly into 9K broth, even though 7% of the cells observed in the inoculum were INT positive. When the cells were gradually acclimated to the acidic medium by successive passage through intermediate pH solutions, however, it was possible to recover culturable Fe^{2+}-oxidizing *T. ferrooxidans* cells when the cells were finally inoculated into the 9K broth.

DISCUSSION

The FAINT technique is based upon two assumptions: (i) the antibody preparation being used is specific and (ii) INT reduction reflects metabolic activity within the cell. The problems involved in preparing specific antibodies and the techniques necessary to achieve this goal have been reviewed by Schmidt (33) and, more recently, by Bohlool and Schmidt (6). Extensive screening of the agglutinated antiserum preparation against non-*T. ferrooxidans* isolates from the sampling sites indicated that the antiserum used in the present study was species specific. Schmidt (33) has reported on the preparation of specific antiserum to *T. ferrooxidans*, and the antiserum preparations used by Gates and Pham (20) and Apel et al. (1) showed little cross-

TABLE 2. Numbers of cells of *T. ferrooxidans* (FA), culturable *T. ferrooxidans* (MPN), and respiring *T. ferrooxidans* per milliliter in samples of water from AMD-contaminated and uncontaminated sites

Station	AMD	No. (mean) of *T. ferrooxidans* cells determined by indicated technique[a]:		
		FA	FAINT	MPN
C1	+[b]	7.7×10^4 (0.08)	5.6×10^4 (0.15)	33
C4	+/−	4.1×10^4 (0.19)	1.8×10^4 (0.24)	0
A2	+/−	2.7×10^3 (0.04)	4.1×10^2 (0.29)	0
F1	−	5.4×10^2 (0.50)	1.4×10^1 (1.0)	0
L1	−	4.6×10^3 (0.15)	5.4×10^1 (1.0)	0
PH	−	0	0	0

[a] Values for direct counts are the means and coefficient of error obtained from three replicate water samples.
[b] +, Presence of AMD; −, absence of AMD.

reactivity. Although there is insufficient information available concerning the serology of the thiobacilli to completely eliminate the possibility of cross-reactivity between antiserum preparations and non-*T. ferrooxidans*, the previous reports and our own findings indicate that it is possible to obtain species-specific antibody preparations for the FA study of *T. ferrooxidans*.

Nonspecific background fluorescence could not be completely eliminated in the indirect FA procedure employed, although the background could be reduced to a noninterfering level. The use of either bovine serum albumin (1, 20) or hydrolyzed gelatin (4) has been shown to reduce or eliminate some types of background fluorescence by adsorbing to nonspecific binding sites. In our experience, bovine serum albumin and hydrolyzed gelatin reduced but did not eliminate background fluorescence. Further reductions in background fluorescence could be achieved only with extensive washing of the filter with PBS, indicating that low-level background fluorescence may be a limitation inherent in the use of an indirect FA procedure. It is likely that there would have been no significant background fluorescence had a direct FA procedure been employed.

Sensitivity of the INT reduction process to acidity similar to that reported here was previously observed in studies in which the quantification of extractable formazan was used as an estimate of total activity (12). In the present work, this sensitivity did not impose a significant limitation on the use of the FAINT technique in acidic environments, since it was possible to satisfactorily measure respiratory activity by removing the cells from their natural environment and suspending them briefly in a neutral buffer. Although in some systems the sudden shift in pH involved in the procedure might result in a lethal stress to some of the cells present, the results presented here indicate that such a stress is not a problem in FAINT determinations of *T. ferrooxidans*.

The abiological extracellular reduction of INT by agents such as Fe^{2+} necessitates that extreme care be taken in the determination of the number of INT-positive cells in a sample. Intracellularly, INT is an alternate acceptor for the electrons generated by the oxidation of the ubiquinone-cytochrome *b* complex (30). As such, it is subject to reduction not only intracellularly by viable microorganisms but also by compounds which are moderately strong reducing agents (such as Fe^{2+}) that might be found in reducing or anoxic natural environments. Thus, the INT portion of the FAINT technique may not be directly applicable to environments such as reducing sediments or hypolimnetic waters. Because of the possibility of abiological extracellular INT reduction, the determination of the number of INT-positive cells present in a sample must be made only when the presence of a fluorescing cell with an intracellular formazan crystal can be directly demonstrated (40).

As noted previously, the second assumption of the FAINT technique is that the reduction of INT to formazan is an accurate reflection of metabolically active cells. That intracellular INT reduction does not occur in killed bacterial cultures has been amply demonstrated in both our work and the studies of Zimmerman et al. (40) and Iturriaga and Rheinheimer (24). Furthermore, the present research has shown that, in situations where INT-positive cells could be detected but no *T. ferrooxidans* cells could be enumerated with conventional MPN techniques in 9K media, it was possible to recover iron-oxidizing *T. ferrooxidans* cells when they were slowly acclimated to the lower pH of the 9K medium. Although these data do not prove unequivocally that a one-to-one correspondence exists between INT-positive cells and viable, physiologically active cells, such an inference is supported by the data.

Using the FAINT technique, we have demonstrated the occurrence of active populations of *T. ferrooxidans* in both acidic and neutral aquatic habitats. Viable *T. ferrooxidans* cells were rarely detected at either of the sites which had no recorded input of AMD. It is important to note, however, that *T. ferrooxidans* was detectable at a concentration of 5.0×10^2 cells ml^{-1} at site F1, using FA staining alone. It was only by combining the use of FA with cell-specific activity measurements that it could be demonstrated that the number of viable *T. ferrooxidans* cells was insignificant at site F1.

Bohlool and Schmidt (6), in their recent review of the application of FA staining to microbial ecology, contended that the difference between total population counts obtained with FA staining and the actual number of viable cells in the environment is negligible since dead cells do not persist in nature. Our data indicate that the number of inactive *T. ferrooxidans* cells in nonoptimal habitats can be considerable (20% at station C1; over 80% at station F1). Therefore, in stressed environments, direct counts obtained with FA alone may lead to overestimates of the number of viable cells and thus an overestimate of the potential impact of the organisms on their environment. Zimmerman et al. (40) and Harvey and Young (21) have presented evidence obtained by using combined acridine orange staining-INT reduction which indicates that inactive cells can be numerous in natural samples. Our work extends these observations to the case of a single type of bacterium, *T. ferrooxidans*.

The results of this research indicate that the combination of immunofluorescent staining with the use of INT as an indicator of electron transport activity provides a simple and sensitive method for the enumeration of metabolically active specific populations of bacteria. Fliermans (C. B. Fliermans, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, N26, p. 177) has applied a similar technique in a study of *Legionella pneumophila*. We feel that the FAINT technique is applicable to autecological studies of a wide variety of natural populations of respiring bacteria because of the distinct advantages of comparative ease of preparation and lack of substrate specificity limitations that are found in other direct measures of microbial activity in natural habitats.

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LITERATURE CITED

1. **Apel, W. A., P. R. Dugan, J. A. Filppi, and M. S. Rheins.** 1976. Detection of *Thiobacillus ferrooxidans* in acid mine environments by indirect fluorescent antibody staining. Appl. Environ. Microbiol. **32**:159–165.
2. **Bailey, W. R., and E. G. Scott.** 1974. Diagnostic microbiology. C. V. Mosby Co., St. Louis, Mo.
3. **Bohlool, B. B., and T. D. Brock.** 1974. Immunofluorescence approach to the study of the ecology of *Thermoplasma acidophilum* in coal refuse material. Appl. Microbiol. **28**:11–16.
4. **Bohlool, B. B., and E. L. Schmidt.** 1968. Nonspecific staining: its control in immunofluorescence examination of soil. Science **162**:1012–1014.
5. **Bohlool, B. B., and E. L. Schmidt.** 1973. Persistence and competition aspects of *Rhizobium japonicum* observed in soil by immunofluorescence microscopy. Soil Sci. Soc. Am. Proc. **37**:561–564.
6. **Bohlool, B. B., and E. L. Schmidt.** 1980. The immunofluorescent approach in microbial ecology, p. 203–242. *In* M. Alexander (ed.), Advances in microbial ecology, vol. 4. Plenum Publishing Corp., New York.
7. **Brock M. L., and T. D. Brock.** 1968. The application of microautoradiographic technique to ecological studies. Mitt. Int. Ver. Limnol. **15**:1–29.
8. **Casida, L. E.** 1977. Microbial metabolic activity in soil as measured by dehydrogenase determinations. Appl. Environ. Microbiol. **34**:630–636.
9. **Cherry, W. B., B. Pittman, P. P. Harris, G. A. Hebert, B. M. Thomason, L. Thacker, and R. E. Weaver.** 1978. Detection of Legionnaires disease bacteria by direct immunofluorescent staining. J. Clin. Microbiol. **8**:329–338.
10. **Christensen, J. P., T. G. Owens, A. H. Devol, and T. T. Packard.** 1980. Respiration and physiological state in marine bacteria. Mar. Biol. **55**:267–276.
11. **Christensen, J. P., and T. T. Packard.** 1979. Respiratory electron transport activities in phytoplankton and bacteria: comparison of methods. Limnol. Oceanogr. **24**:576–583.
12. **Curl, H., Jr., and J. Sandberg.** 1961. The measurement of dehydrogenase activity in marine organisms. J. Mar. Res. **19**:123–128.
13. **Daley, R. J., and J. E. Hobbie.** 1975. Direct counts of aquatic bacteria by a modified epifluorescent technique. Limnol. Oceanogr. **20**:875–882.
14. **Fliermans, C. B., B. B. Bohlool, and E. L. Schmidt.** 1974. Autecological study of the chemoautotroph *Nitrobacter* by immunofluorescence. Appl. Microbiol. **27**:124–129.
15. **Fliermans, C. B., W. B. Cherry, L. H. Orrison, and L. Thacker.** 1979. Isolation of *Legionella pneumophila* from nonepidemic-related aquatic habitats. Appl. Environ. Microbiol. **37**:1239–1242.
16. **Fliermans, C. B., and E. L. Schmidt.** 1975. Autoradiography and immunofluorescence combined for autecological study of single cell activity with *Nitrobacter* as a model system. Appl. Microbiol. **30**:676–684.
17. **Floodgate, G. D.** 1979. The assessment of marine microbial biomass and activity, p. 217–253. *In* R. R. Colwell and J. Foster (ed.), Aquatic microbial ecology. Maryland Sea Grant Publications, College Park, Md.
18. **Francisco, D. E., R. A. Mah, and A. C. Rabin.** 1973. Acridine orange epifluorescent technique for counting bacteria in natural waters. Trans. Am. Microsc. Soc. **92**:416–421.
19. **Garvey, J. S., N. E. Cremer, and D. H. Susdorf.** 1977. Methods in immunology, 3rd ed. W. A. Benjamin, Reading, Mass.
20. **Gates, J. E., and K. D. Pham.** 1979. An indirect fluorescent antibody staining technique for determining population levels of *Thiobacillus ferrooxidans* in acid mine drainage waters. Microb. Ecol. **5**:121–127.
21. **Harvey, R. W., and L. Y. Young.** 1980. Enumeration of particle-bound and unattached respiring bacteria in the salt marsh environment. Appl. Environ. Microbiol. **40**:156–160.
22. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33**:1225–1228.
23. **Hoppe, H. G.** 1976. Determination and properties of actively metabolizing heterotrophic bacteria in the sea investigated by means of micro-autoradiography. Mar. Biol. **36**:291–302.
24. **Iturriaga, R., and G. Rheinheimer.** 1975. Eine einfach Methode zue Auszahlung von Bakterien mit aktivem Elecktronentransport system in Wasser- und Sedimentproben. Kiel. Meeresforsch. **31**:83–86.
25. **Jones, J. G., and B. M. Simon.** 1975. An investigation of errors in direct counts of aquatic bacteria by epifluorescent microscopy with reference to a new method for dyeing membrane filters. J. Appl. Bacteriol. **39**:317–329.
26. **Kenner, R. A., and S. I. Ahmed.** 1975. Measurements of electron transport in marine phytoplankton. Mar. Biol. **33**:119–127.
27. **Kogure, K., U. Simidu, and N. Taga.** 1978. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. **25**:415–420.
28. **Meyer-Reil, L.-A.** 1978. Autoradiography and epifluorescence microscopy combined for the determination of number and spectrum of actively metabolizing bacteria in natural waters. Appl. Environ. Microbiol. **36**:506–512.
29. **Orndorff, S. A., and R. R. Colwell.** 1980. Effect of kepone on estuarine microbial activity. Microb. Ecol. **6**:357–368.
30. **Packard, T. T.** 1971. The measurement of respiratory electron-transport activity in marine phytoplankton. J. Mar. Res. **29**:235–244.
31. **Ramsay, A. J.** 1974. The use of autoradiography to determine the proportion of bacteria metabolizing in an aquatic habitat. J. Gen. Microbiol. **80**:363–373.
32. **Rennie, R. J., V. V. Reyes, and E. L. Schmidt.** 1977. Immunofluorescence detection of the effects of wheat and soybean roots on *Nitrobacter* in soil. Soil Sci. **124**:10–15.
33. **Schmidt, E. L.** 1973. Fluorescent antibody technique for the study of microbial ecology. Bull. Ecol. Res. Commun. (Stockholm) **17**:67–76.
34. **Schmidt, E. L.** 1974. Quantitative autecological study of microorganisms in soil by immunofluorescence. Soil Sci. **118**:141–149.

35. **Schmidt, E. L., R. O. Bankole, and B. B. Bohlool.** 1968. Fluorescent-antibody approach to study of rhizobia in soil. J. Bacteriol. **95**:1987–1992.

36. **Silverman, M. P., and D. G. Lundgren.** 1959. Studies on the chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans*. I. An improved medium and a harvesting procedure for securing high cell yields. J. Bacteriol. **77**:642–647.

37. **Stanley, P. M., M. A. Gage, and E. L. Schmidt.** 1979. Enumeration of specific populations by immunofluorescence, p. 46–55. *In* J. W. Costerton and R. R. Colwell (ed.), Native aquatic bacteria: enumeration, activity and ecology, ASTM/STP 695. American Society for Testing and Materials, Philadelphia, Pa.

38. **Strayer, R. F., and J. M. Tiedje.** 1978. Application of the fluorescent-antibody technique to the study of a methanogenic bacterium in lake sediments. Appl. Environ. Microbiol. **35**:192–198.

39. **Ward, T. E., and J. I. Frea.** 1980. Sediment distribution of methanogenic bacteria in Lake Erie and Cleveland Harbor. Appl. Environ. Microbiol. **39**:597–603.

40. **Zimmerman, R., R. Iturriaga, and J. Becker-Birck.** 1978. Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. Appl. Environ. Microbiol. **36**:926–935.