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Applications of Laser Scanning Microscopy for Analysis of Aquatic Microhabitats

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ABSTRACT Laser scanning microscopy was used 1) to analyze Mn oxide–encrusted biofilms and particles in marine Mn-oxidizing enrichment cultures, 2) to optimize fluorescence in situ hybridization protocols of 16S ribosomal RNA-targeted oligonucleotide signature probes for single bacterial cell identification in particles from a wetland, and 3) to develop a combined immunofluorescence-microautoradiography procedure for analysis of the distribution of ^{14}C-labeled organic compounds and ^{14}C-mineralizing bacteria in groundwater seep sediments. The results demonstrated the wide applicability and benefits of using laser scanning microscopy for analysis of complex microbial assemblages.

INTRODUCTION

Laser scanning microscopy (LSM) has had a large impact on cell biology over the last decade, but its impact on microbial ecology is only now being felt. Given the advantages of confocal optical sectioning and 3D imaging for visualization of internal structure of semitransparent specimens (Caldwell, 1993; Caldwell et al., 1992a), it is surprising that LSM has not been more widely applied for analysis of microbial habitats (microhabitats). Indeed, only a few account have been published on LSM applications for analysis of microorganisms in environmental samples (Distel and Cavanaugh, 1994; Ghiorse, 1993; Korber et al., 1993, 1994; Lawrence et al., 1991; Lens et al., 1994; Wolfaardt et al., 1994). All but two of these reports (Distel and Cavanaugh, 1994; Ghiorse, 1993) are analysed of laboratory-generated biofilms.

Conventional epifluorescence microscopy (CEFM) is still the method of choice among microbial ecologists for directly analyzing microbial assemblages in natural material (Kemp et al., 1993); CEFM imaging can be improved significantly by using high-sensitivity CCD cameras for detection and deconvolution image analysis software to produce confocal images electronically (Caldwell et al., 1992a; Slayter and Slayter, 1992). Thus, deconvolution confocal CEFM is being considered by some researchers as a solution for high resolution imaging of complex microhabitats (see, for example, Ramsing et al., 1993).

However, deconvolution confocal software has not yet been widely applied to environmental samples. According to Caldwell et al. (1992a), deconvolution confocal imaging using CEFM will be possible only under ideal conditions where objects in the specimen are not too closely packed. Furthermore, as Caldwell et al. (1992a) pointed out, deconvolution confocal images produced by CEFM are not likely to be of the same quality as those produced by LSM. Thus, it seems unlikely that deconvolution confocal CEFM will ever rival confocal LSM for high resolution analysis of natural biofilms or semitransparent particles in natural waters, soil, and sediment particles.

High resolution imaging aside, many useful microscopic techniques based on CEFM have been developed in recent years for detection, abundance, and biomass estimates of microorganisms in aquatic microhabitats (Kemp et al., 1993). Single-cell identification techniques are now available using immunofluorescence (IF) staining and fluorescence in situ hybridization (FISH) of 16S ribosomal RNA (rRNA)-targeted oligonucleotide signature probes (16S riboprobes); activities of single cells can be measured by microautoradiography (MARG). These methods have been adapted from methods originally developed as research tools in cellular and molecular biology. Like the cellular and molecular methods, the microscopic methods adapted for microbial ecology can be improved by using the LSM, especially when samples containing many types of microorganisms associated with various organic and mineral phases are examined.

The purpose of this article is to describe LSM-based procedures being developed in our laboratory for high resolution analysis of microhabitats in natural material. Methods are described that 1) improve visualization of acridine orange (AO)–stained bacteria within semitransparent matrices of Mn oxide–containing biofilms and particles, 2) facilitate optimization of 16S

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riboprobe–FISH protocols for identification of methanotrophic bacteria and Mn-oxidizing sheathed bacteria in particulate material from a local wetland area, and 3) enhance microscale analysis of fluorescent antibody-labeled bacteria vis-à-vis the distribution of the ^{14}C label from ^{14}C-glucose in organic sediment particles using a combined IF-MARG procedure. Because of the varied nature of the research problems being addressed, we include a brief overview of pertinent background literature as a prelude to a discussion of the results obtained for each problem.

MATERIALS AND METHODS

AO-Staining of Mn Oxide–Containing Biofilms

Enrichment cultures for Mn-oxidizing oligotrophs were set up in 3 L erlenmeyer flasks following procedures described by Ghiorse and Hirsch (1982). The medium consisted of 0.25 μm filter-sterilized natural seawater (500 mL) supplemented with 0.0025% each of peptone and yeast extract (Difco) and 0.01% $MnSO_4$ or natural seawater with 0.01% $MnSO_4$ only. The cultures were inoculated with portions of 0.8 μm pore size nylon membrane filters (142 mm diameter) (MSI, Westboro, MA) onto which suspended particles from 20 L of prefiltered (7 μm) seawater from Vineyard Sound, MA, had been concentrated (J.W. Moffett, Woods Hole Oceanographic Inst., Woods Hole, MA). After several weeks of incubation at room temperature (22–25°C), discontinuous Mn oxide–containing biofilms developed on the upper surfaces of the filter. Initially, small brown colonies appeared and, during several weeks of incubation, the colonies grew larger, in some cases spreading over the entire surface of the filter. After 1–2 months of incubation, Mn oxide–containing biofilms formed at the air-water interface, and suspended particles containing brown Mn oxide were visible in the flasks. These observations are similar to those described previously for isolation of Mn-oxidizing budding bacteria from enrichment cultures inoculated with fragments of Baltic Sea ferromanganese concretions (Ghiorse and Hirsch, 1982).

For examination of the biofilm on the filter surface, small segments of the filters were stained in a large drop (~0.5 ml) of 0.01% AO solution for several minutes. During staining the filter became increasingly colored yellow-orange due to its uptake of AO. Stained filter segments were washed in a 1 M NaCl solution, rinsed in distilled water, mounted in distilled water, biofilm side up, and sealed with vaspar (a 1:1 mixture of petroleum jelly and paraffin). Mn oxide–containing particles were removed from the flask with a sterile loop or pipet and similarly mounted and sealed. Specimens for optical sectioning were sealed with 18 mm^2 coverslips to minimize vertical displacement of the coverslip by oil immersion lenses during optical sectioning. Mounted specimens were examined immediately in the LSM or stored in the dark at 4°C for several hours before examination without noticeable deterioration of the sample.

Bacterial Cultures and Wetland Detrital Samples

Cultures of *Methylomonas albus* BG-8 (a Type I methylotroph) and *Methylosinus trichosporium* OB3b (a Type II methylotroph) (both obtained from Lorie Buchholz, Center for Great Lakes Studies, Milwaukee, WI) were grown in liquid medium under an atmosphere containing 25% methane as described by Tsien et al. (1990). *Leptothrix discophora* SS-1 (ATCC 43182) (Adams and Ghiorse, 1986) and SP-6 (ATCC 51168) (Emerson and Ghiorse, 1992) were grown in liquid (PTYP) medium. PTYP contained (per liter of distilled water) 0.25 g each of peptone and trypticase (Difco), 0.5 g yeast extract (Difco, Detroit, MI), 0.6 g $MgSO_4 \cdot 7\ H_2O$, 0.07 g $CaCl_2 \cdot H_2O$, 2.38 g HEPES (N-2-hydroxyethylpiperazine-H′-2-ethanesulfonic acid), pH 7.2, 1.0 ml 10 mM $FeSO_4 \cdot 7\ H_2O$ solution, and 5.0 ml of 20% Na pyruvate. *Escherichia coli* B/r (M. Shuler, Department of Chemical Engineering, Cornell University) was grown in nutrient broth (Difco, Detroit, MI) at 37°C on a gyratory shaker operated at 150 RPM. *Sphingomonas paucimobilis* RSP-1 was isolated from an enrichment culture in which organic sediment was supplemented with phenanthrene (Sandoli, 1994). The sediment was obtained from a coal tar–contaminated groundwater drainage seep area at a coal-tar disposal site (Madsen et al., 1991). For IF-MARG experiments, RSP1 cells were grown in a mineral salts base (MSB) medium (Stanier et al., 1966) with 20 mM glucose added as the carbon and energy source. All liquid cultures except *E. coli* were incubated at 25°C on a gyratory shaker operated at 150 RPM until they were in the logarithmic stage of growth.

Detrital particles were obtained from the root zone of duckweed plants (*Lemna* spp.) in August 1993 at our Sapsucker Woods wetland field site (Ghiorse, 1984). The samples were fixed by adding 37% formaldehyde solution to a final concentration of 3.7% formaldehyde.

Optimization of 16S Riboprobe FISH Protocol

Type I (10 γ, 5′-GGTCCGAAGATCCCCCGCTT-3′) and II (9 α, 5′-CCCTGAGTTATTCCGAAC-3′) 16S riboprobes for methylotrophs (Tsien et al., 1990) and universal (U, 5′-GWATTACCGCGGCKGCTG-3′, W = A or T, K = G or T) and negative control (C, 5′-GTGCCAGCMGCCGCGG-3′, M = A or C) 16S riboprobes (Giovannoni et al., 1988) were synthesized and labeled at their 5′ ends with Texas Red (TR) or fluorescein isothiocyanate (FITC) according to the general procedures outlined by DeLong et al. (1989). A *Leptothrix-Sphaerotilus* group 16S riboprobe (PS-1, 5′-GATTGCTCCTCTACCGT-3′) was synthesized and labeled at the 5′ end with FITC. The PS-1 probe was designed by comparing 16S rRNA sequence information obtained for the *Leptothrix-Sphaerotilus* group (Siering and Ghiorse, 1994) with sequences extracted from Genbank/EMBL and The Ribosomal Database Project (Deveraux et al., 1984; Larsen et al., 1993). PS-1 was shown to identify several strains of Mn-oxidizing sheathed bacteria in the genus *Leptothrix* and one strain of *Sphaerotilus natans* (Siering and Ghiorse, 1994). All oligonucleotide probes were synthesized and labeled commercially (Oligos etc., Inc., Wilsonville, OR; Cornell Center for Advanced Technology in Biotechnology).

For in situ hybridization, bacterial cells in the middle or late exponential phase of growth were fixed in 3.7% formaldehyde on ice, washed twice in phosphate

buffered saline solution (0.145 M NaCl, 0.1 M NaH_2PO_4, pH 7.4), smeared on chromated gelatin-coated glass slides (DeLong, 1993), and air-dried. The air-dried smears were further dehydrated and the cells permeabilized in a solution containing 3.7% formaldehyde and 90% ethanol (Braun-Howland et al., 1992). The smears were then rinsed in distilled water, air-dried, and subjected to three different hybridization and washing protocols based on those described by Tsien et al. (1990) and Poulsen et al. (1993). These protocols were varied to optimize hybridization and stringency of washing conditions for the target cells. After hybridization and washing, the specimens were mounted in an antifading reagent containing 0.75 M NaCl, 0.005 M EDTA, 0.1 M Tris-HCl, pH 7.8 (5× SET), 50% glycerol, and 0.1% p-phenylenediamine. Slides were sealed with vaspar or clear nail polish and examined immediately in the LSM, or they were stored in the dark at 4°C for 36–48 h before examination with little loss of fluorescence intensity.

Combined IF-MARG Procedure

To develop a combined IF-MARG procedure, we followed the general procedures and recommendations of Fliermans and Schmidt (1975) and Ward (1984). First, an indirect IF staining procedure was developed for recognition of *Sphingomonas paucimobilis* RSP-1 cells in sediment samples (for a general description of IF staining methods see Campbell, 1993). RSP-1 cells were injected into rabbits to raise polyclonal antibodies. The RSP-1 rabbit antiserum was tested and shown to be specific for recognition of RSP-1 cells by assessing cross-reactivity of related Gram-negative bacteria using an indirect IF staining procedure (Sandoli, 1994). The same staining procedure was shown to stain RSP-1 cells added to the sediment using 0.1% agar to bind cells and sediment particles to glass slides in air-dried smears.

A MARG procedure was developed following the general procedures and recommendations of Brock and Brock (1968), Meyer-Reil (1978), and Tabor and Neihof (1982). Accordingly, a glucose mineralization experiment was set up in a sealed biomineralization vial containing 0.5 g sediment plus 1.5 μC of $U^{14}C$-glucose and approximately 10^8 mid-log phase, glucose-grown RPS-1 cells in a 5 mL slurry made with the MSB-glucose medium (Sandoli, 1994). After approximately 5 h, during which mineralization of glucose (measured by $^{14}CO_2$ evolution) reached 10% of the ^{14}C added to the vial, 37% formaldehyde solution was added to the slurry to a final concentration of 2% formaldehyde to stop metabolism. The slurry was diluted tenfold with MSB, and molten agar was added to a final concentration of 0.1% to aid binding of slurry contents to glass microscope slides. Subsamples (5 μL) were smeared on the slides and air-dried. Working in darkness, the slides were coated with diluted Kodak NTB-2 Nuclear Track™ emulsion (1 part emulsion:2 parts sterile distilled water) at 45°C. The liquid emulsion was drained from the slides, and they were placed on a cool surface to solidify and air-dry the emulsion. Slides were exposed in a light-tight, dry container kept at 4°C for 10 days. Exposed slides were removed from the box, developed in Kodak D-19 developer, fixed, rinsed, and dried according to manufacturer's instructions.

The developed MARG slides were subjected to an indirect IF-staining procedure to stain cells through the emulsion (R. Sandoli and K. Stuart, unpublished results). In this procedure, the RSP-1 antiserum and a phycoerythrin (PE)-conjugated anti-rabbit IgG secondary antibody (Sigma) were diluted 1:50 in NaCl-free PB solution containing 2.8 mM $NaH_2PO_4 \cdot H_2O$ and 7.2 mM $Na_2HPO_4 \cdot H_2O$, pH 7.2. After treatment for 1 h at 37°C with a blocking reagent containing 10 mg · mL^{-1} bovine serum albumin (BSA), each antibody solution was applied for 24 h at 37°C. After each antibody application, slides were washed by flooding them ten times with 1 ml of PBS (PB plus 0.15 M NaCl) followed by rinsing in distilled water. Stained slides were mounted in PBS containing 0.1% propyl gallate as an antifading agent, sealed with vaspar, and examined in the LSM immediately. Storage of sealed slides for up to 2 weeks at 4°C in darkness did not adversely affect the fluorescence intensity of labeled cells.

Microscopy

All samples were examined in a Zeiss LSM-210 laser scanning microscope equipped with an external argon-ion laser (488 and 514 nm lines) and an internal helium-neon laser (633 nm) as well as conventional light sources for transmittance and epifluorescence. In this instrument, the focused laser beam is scanned through the objective lens across a defined area of the specimen by galvanometer scanning mirrors driven by a digitally controlled scan generator. Our instrument is equipped with oil immersion objective and condenser lenses (NA 1.3 or 1.4). Laser scanned confocal imaging is done only in the reflectance or epifluorescence modes. Laser scanned confocal and nonconfocal epifluorescence (EF), differential interference contrast (DIC), brightfield (BF), and phase contrast (PC) imaging are used routinely. Both epifluorescence and transmitted images of the same field can be viewed simultaneously using a photomultiplier detector for the epifluorescence images and a Si diode detector for the transmitted images. Conventional EF, DIC, BF, and PC images can be viewed through ×10 eyepieces or through an attached color CCD video camera (model VI-470; Optronics, Goleta, CA) on a color video monitor (Trinitron model PVM 1343MD; Sony, San Jose, CA).

Both the laser scanning and conventional optical systems are controlled through the LSM-210 control panel software (LSM version 2.08; Carl Zeiss, Inc., Thornwood, NY). Control panel commands allow for selection of imaging mode. laser wavelength, laser scan rate, ×20–160 electronic zoom, laser attenuator filters, and photomultiplier band width filters. The software also contains programs for z and phi-z sectioning, stereo imaging, split-screen and overlay functions, measuring and positioning functions, line and frame averaging, image filtration, and other image processing functions. Specimen position is controlled by high resolution, x-y stage and z focusing motors. Digital images and imaging parameters produced in the laser scan modes are stored temporarily on a 2 MB frame grabber or permanently on a 32 MB disk drive in the host computer. The

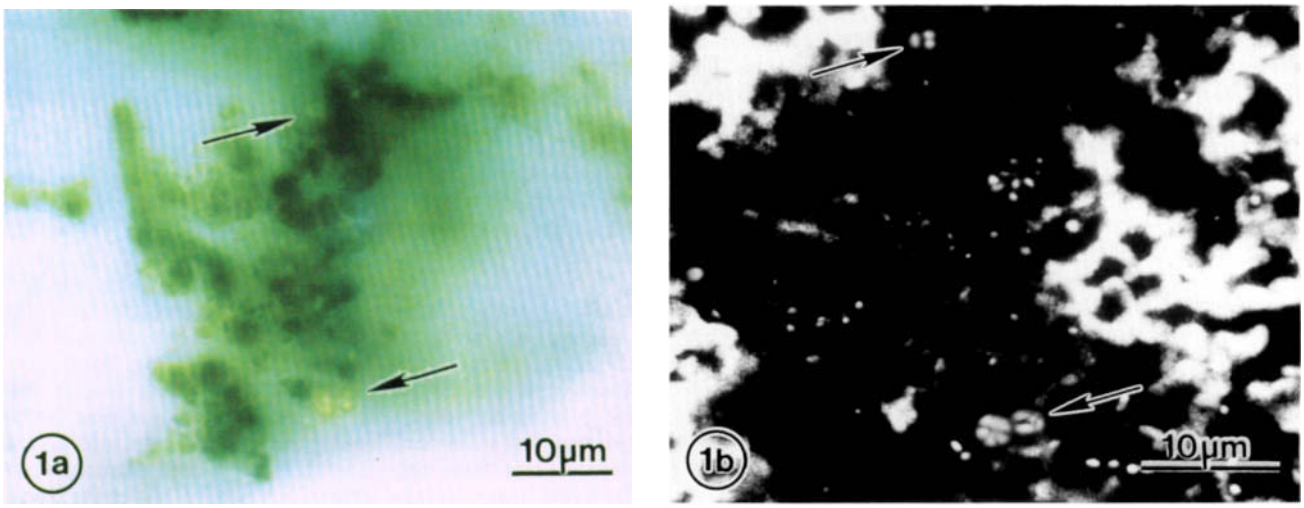

Fig. 1. Bacteria in a Mn oxide–containing biofilm on the surface of a nylon filter in a low-nutrient, Mn^{2+}-containing, seawater enrichment culture. **a:** Color photomicrograph of conventional epifluorescence image made with the CCD camera (arrows show clumps of bacterial cells). **b:** Image composed of six overlaid confocal optical sections taken in the central area of the field shown in panel a (arrows show same clumps of cells). Cells are clearly imaged within the semitransparent biofilm microcolony by confocal optical sectioning in panel b. Note also the structure of the 0.8 μm nylon filter shown in the right-central area of panel b. LSM imaging parameters and processing: Objective lens = phase contrast × 100 (NA 1.4); argon laser line = 488 nm, laser current = 4.00 amps, attenuation filter = 0.1; zoom factor = 40; scan rate = 8 sec per 512 × 512 × 8 bit frame, 16 lines averaged; contrast (C = 641); brightness (B = 465). Constant imaging parameters were used during z-sectioning. A series of confocal images were stored on the hard drive and then overlaid directly using the z-sectioning software.

digital images can be transferred via an IEEE linkage to a 20 MB mass storage cartridge (Bernoulli, Box II; Iomega, So. Roy, UT) through an image analysis computer (Kontron, ELEKTRONK GmbH, Eching, Germany, VIDAS 2.1). Laser scanned images are displayed on a RGB video monitor (Trinitron model CPD-1402E character display monitor; Sony, San Jose, CA) and recorded on a Sony UP-850 black-and-white videographic thermal printer or on 35 mm photographic film through a color video image recorder (MATRIX Instruments Inc., Orangeburg, NY). Conventional images are recorded through the LSM-210 camera portfitted with a Nikon dual-channel optivar unit. One channel is connected to a Zeiss MC100 35 mm camera system, the other to the CCD camera. The CCD images are recorded on the black-and-white printer or on a color printer (model UP-3000; Sony, San Jose, CA).

Kodak Plus X (black-and-white) and Kodak 100 HC or EK160T (color) slide film were used for recording primary images. In some instances color slides or black-and-white video prints were copied on Polaroid (Type 55) negative film.

RESULTS AND DISCUSSION

Visualization of AO-Stained Bacterial Assemblages in Mn Oxide–Containing Biofilms

High resolution imaging and accurate microscale analysis of microbial assemblages inside semitransparent or opaque specimens is a difficult task. Imaging problems in such specimens can be overcome to some degree by using chemical treatments to clear the mineralized portions of the sample and ultrathin sectioning followed by transmission electron microscopy (TEM) to visualize fine structural details inside the particles (Ghiorse, 1980, 1984; Ghiorse and Hirsch, 1979). However, such images do not necessarily lead to a better understanding of intermediate-scale (1–100 μm) microhabitat structure because of the high magnifications used in the TEM. There are also problems of incomplete preservation or extraction artifacts that may adversely affect interpretation of TEM results. Staining with nucleic acid–reactive fluorochromes and subsequent analysis by CEFM is very useful for distinguishing cells from noncellular material inside particles at the intermediate scale (Ghiorse and Ehrlich, 1993); but CEFM analyses are often hampered by blurry images resulting from nonspecific fluorochrome staining and high background fluorescence.

The LSM has superior ability to overcome these problems by virtue of confocal imaging and optical sectioning which allow high resolution light microscopic images to be produced at intermediate levels of magnification (up to × 16,000). We have found that confocal imaging and optical sectioning greatly improved our ability to visualize the distribution of fluorescent bacterial cells in Mn oxide–containing biofilms growing on the surface of nylon filters (Fig. 1). Most of the bacterial cells in these samples were invisible when viewed by CEFM due to the high background fluorescence produced by AO bound in the nylon filter (Fig. 1a). Confocal optical sectioning was used to visualize the distribution of bacteria in the sample. A series of confocal optical sections was obtained and then overlaid to create a clarified EF image (Fig. 1b) of the distribution of AO-stained bacterial cells within the matrix of the Mn oxide–encrusted biofilm. Similar results were obtained

when suspended Mn oxide–containing particles were examined (Fig. 2). When viewed by PC (Fig. 2a) and EF (Fig. 2b), rod-shaped, coccoid, and filamentous bacteria were found to be intimately associated with the particle composed of highly fluorescent matrix material. However, the spatial relationships between the cells and other components of their microhabitat were poorly resolved. Confocal optical sectioning and stereo imaging were used to elucidate the spatial relationships of clusters of intertwined bacterial filaments (Fig. 2c) and microcolonies containing oval and coccoid cells (Fig. 2d).

As expected from previous work (Ghiorse, 1980; Ghiorse and Hirsch, 1982), only a small fraction of the bacteria observed in these biofilms could be isolated in axenic cultures. A few Mn-oxidizing coccoid, vibroid, and *Hyphomicrobium*-like budding bacterial isolates were obtained. Bacteria with similar morphologies were seen in the enrichment culture samples, but, with the exception of one coccoid isolate that resembled the coccoid bacteria seen in Figures 1b and 2d, most of the isolated bacteria were not major components of the biofilm or particle communities (W.C. Ghiorse, unpublished results).

Optimization of 16S Riboprobe–FISH Protocols for Identification of Single Bacterial Cells in Natural Assemblages

As part of an effort to develop microscale analytical methods for general use in environmental microbiology and microbial ecology, we have optimized published 16S riboprobe–FISH protocols for identification of single cells in natural assemblages. Like other workers in this rapidly developing area of research (DeLong, 1993; Manz et al., 1992; Ramsing et al., 1993; Raskin et al., 1994a,b; Wagner et al., 1994), we have found that there are many components of the published protocols which may not always be needed for successful results (see discussion below). However, there is still much work to be done before standardized 16S riboprobe–FISH procedures and protocols can be prescribed. Therefore, in anticipation of optimizing these procedures and protocols, we found it useful to identify the common steps included in most 16S riboprobe–FISH protocols used for environmental applications (Table 1). Probe design, synthesis, labeling, and testing (steps 1 and 2 in Table 1) are very important preliminary steps (for reviews, see Amann et al., 1990, 1992a, 1992b; DeLong, 1993; Stahl and Amann, 1991); steps 3 and 4 (Table 1) are crucial intermediate steps aimed at determining optimal imaging conditions and establishing probe specificity and are needed for interpretation of results when a given protocol finally is applied to environmental samples (steps 5 and 6 in Table 1). In our optimization work, we found that the digital fluorescence intensity signal of confocal and nonconfocal EF images produced in the LSM was especially useful for the comparisons of fluorescence intensities required in steps 3–6 (Table 1).

Because 16S riboprobe–FISH techniques depend on a complex set of molecular interactions that may vary from probe to probe, cell to cell, and sample to sample, we also found it useful to analyze and identify the key procedures governing the success of any FISH protocols. Preservation of cellular morphology, the stability of the ribosome structure, and the permeability of the cell envelope to the probe are of major importance for cell recognition and access of the probe to ribosome target sites in cells. To optimize fixation and permeabilization treatments (step 3, Table 1) some knowledge of the effects of fixative chemicals in cellular constituents is helpful. Formaldehyde solution has been the fixative of choice in most FISH protocols. Usually this fixative is prepared by diluting a commercially prepared 37% formaldehyde solution (containing 10–15% methanol) in buffer or by dissolving powdered paraformaldehyde in an appropriate buffer by heating it above 60°C and elevating the pH above 12. Formaldehyde rapidly penetrates the cell and cross-links cellular proteins, and it induces less nonspecific autofluorescence than glutaraldehyde (the preferred protein cross-linking agent of electron microscopy). Air-drying and mild heat fixation also have been used to fix gram-positive bacteria (Jurtshuk et al., 1992), but this procedure is not recommended for microhabitat analysis because of the morphological alterations that can be caused by drying and heating cells and other delicate microhabitat structures. Specific permeabilization procedures may be required to allow for probe penetration into the cells. Gram-negative cells are more easily permeabilized than gram-positive cells (Beveridge and Davies, 1983). Usually this is done by exposing cells to alcohol during or after fixation. Commercially available 37% formaldehyde solutions containing methanol may aid in permeabilization, but usually a separate dehydration treatment (e.g., treatment with a graded series of ethanol solutions) is included in most protocols (Braun-Howland et al., 1992). Some gram-positive cells, especially those with waxy cell walls, may present severe permeability problems (Manz et al., 1992; Wagner et al., 1994). Cell wall–damaging solvents (e.g., acetone or ether) or enzyme treatments (e.g., lysozyme, protease), chelating agents (e.g., citrate, EDTA) and detergents (e.g., SDS, Nonidet, Triton) may be needed to aid permeabilization of problematic bacterial cell walls.

Optimal stringency of hybridization and washing conditions (step 4, Table 1) can be predicted roughly on the basis of the conditions predicted for base pair hydrogen bonding between the nucleotide sequence of the probe and its complementary target sequence. The key elements here are probe length (number of bases) and conditions that regulate base pair hydrogen bonding such as temperature, salt concentration, and formamide concentration. Probe concentration and hybridization buffer composition (i.e., sodium phosphate or Tris-HCl buffer, pH 7.0–7.8, EDTA or sodium citrate) are also important considerations. Temperature, salt, and formamide concentrations usually are varied to control stringency (specificity); buffer and probe concentrations usually are held constant. Stringency can be controlled by adding formamide to the hybridization mixture, altering the salt concentration, or changing the temperature of hybridization and washing. Low salt concentrations, high temperatures, or high formamide concentrations are used to increase stringency. Ideally, the highest stringency conditions should remove all nonspecifically bound probe molecules except

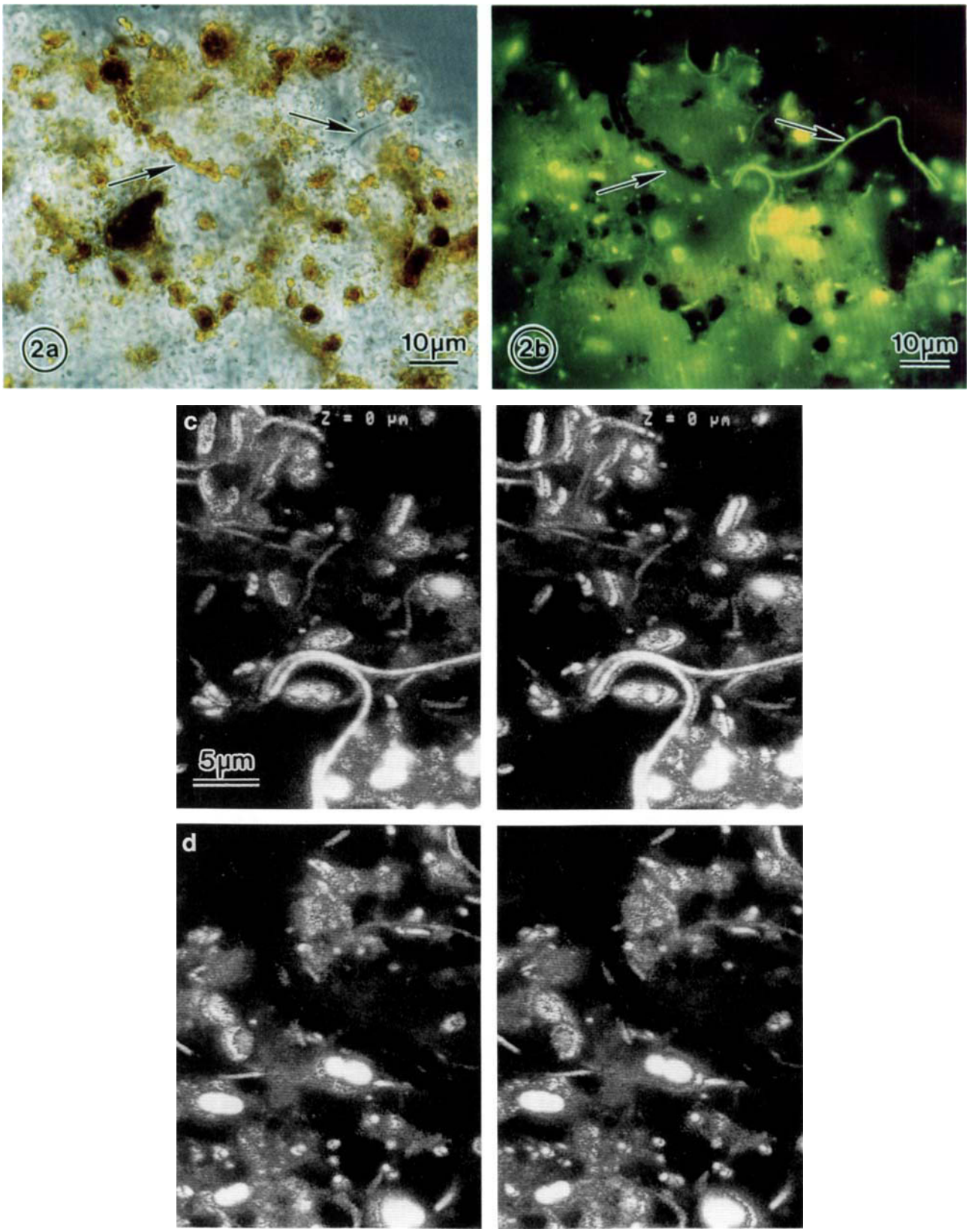

Fig. 2. Color photomicrographs showing conventional phase contrast (**a**) and epifluorescence (**b**) images of a Mn oxide–encrusted particle in another enrichment culture (arrows indicate obvious filamentous structures in each micrograph). Note the brown Mn oxide in panel a and the green and yellow fluorescence of matrix material in panel b. Also note the bright AO fluorescence of bacterial cells and the generally high background fluorescence of the matrix in panel b. **c,d:** Stereo images of central portions of same field shown in panels a b. Panel c shows the area near the curved filament seen in the center of panel b. Panel d shows an area in the vicinity of the arrow to the left of the curved filament. The stereo pairs were made by overlaying four confocal optical sections taken at 1 μm depth intervals. Note the improved resolution and depth relationships in the stereo images. LSM imaging parameters and processing (c,d): Objective lens = phase contrast ×100 (NA 1.4); argon laser line = 488 nm, laser current = 4.00 amps, attenuation filter = 0.1; zoom factor = 40; scan rate = 8 sec per 512 × 512 × 8 bit frame, 16 lines averaged; contrast (C = 641); brightness (B = 465). Imaging parameters were kept constant during z-sectioning. A series of confocal images was stored on the hard drive and then overlaid as right/left stereo pairs using the z-sectioning software.

TABLE 1. Procedural steps used in development of 16S riboprobe–FISH protocols for environmental applications[1]

1.	Design, synthesize, and label specific oligodeoxynucleotide probe
2.	Test specificity and fidelity against target and nontarget nucleic acids
3.	Optimize fixation and permeability conditions with cultured cells of target organisms
4.	Optimize stringency of hybridization and washing conditions with cultured cells of target organisms.
5.	Decide if a single compromise protocol can be used or if multiple optimized protocols are needed for detection of target populations in environmental samples
6.	Test final protocol(s) on environmental samples with target cells and negative control cells added

[1]References: Braun-Howland et al. (1992); DeLong (1993); Manz et al. (1992); Ramsing et al. (1993); Wagner et al. (1994).

those bound to the targeted complementary sequence. Other components frequently used in hybridization mixtures, including Denhardt's solution (an equal part mixture of Ficol, polyvinylpyrrolidone, and bovine serum albumin), dextran sulfate solution, polyadenylic acid or low molecular weight DNA solution, dithiothreitol, and detergents, are not universally applied in hybridization protocols. They may improve results, however, by improving probe accessibility (detergents, dithiothreitol) and blocking nonspecific binding sites (Denhardt's solution, dextran sulfate, nucleic acids). Hybridization times vary from 30 min to overnight depending on the type of sample and the hybridization temperature.

FISH results (steps 3 and 4, Table 1) are best judged by using a microscopic assay to estimate fluorescence intensity of target cells. The cells should contain enough ribosomes (cells in the logarithmic phase of growth are generally used) to allow for relatively high fluorescence intensity signals. The fluorescence intensities of target cells labeled with specific signature probes are compared with those of cells from the same cultured probed with positive (universal) and negative control probes under the same hybridization and washing conditions.

Following these guidelines, we developed a useful LSM procedure to estimate fluorescence intensity during optimization of FISH protocols for Types I and II methylotrophic bacteria (Table 2; Fig. 3). In this procedure, predetermined brightness and contrast settings were used to produce all images; all other imaging parameters were held constant. The appropriate brightness and contrast settings were determined by previewing all of the samples to define the range of fluorescence intensities of all cells being compared. Representative images of cells in each sample were then recorded and stored on the LSM hard drive. Later, the relative fluorescence intensities of cells in the stored images (e.g., Fig. 3) were estimated and rated on a scale from 0 to 10. The objective was to determine which protocol had produced the highest fluorescence intensity with the highest specificity. In the example shown (Table 2; Fig. 3), a sample containing a mixture of Type I and Type II methylotrophic bacteria and one containing *E. coli* were hybridized with 16S riboprobes that identified Type I and Type II methylotrophic bacteria. When the resulting images (Fig 3) were examined systematically and rated for relative fluorescence intensity (Table 2), we found good discrimination between the two types of methylotroph cells using the appropriate probes in the original hybridization protocol of Tsien et al. (1990) (Table 2, protocol 1). However, we also found that the Type I probe did not discriminate well between *M. albus* BG-8 and *E. coli* B/r under these hybridization and washing conditions (Table 2). We found a similar lack of discrimination when the protocol of Poulson et al. (1993) (protocol 3) was used. Protocol 2, a hybrid protocol using the hybridization conditions of protocol 3 and washing conditions of protocol 1, distinguished between all three organisms (see middle row, Table 2 and Fig. 3). It should be noted that the universal probe, which was designed to hybridize with all ribosomes (Giovannoni et al., 1988), uniformly gave the highest relative fluorescence intensity values (10) for all organisms and all protocols (results not shown); the negative control probe, which was designed not to hybridize with any ribosome (Giovannoni et al., 1988), uniformly gave the lowest values (0–2) (results not shown).

These experiments allowed us to identify protocol 2 as the optimum FISH protocol for discrimination of cultured strains of methylotrophic bacteria, but the results also suggested that further modifications of protocol 2 may be warranted for future applications to wetland samples. For example, lower salt and higher temperature washing conditions may allow us to distinguish better between Type I and II methylotrophs in samples from the wetland.

The relative fluorescence intensity estimation procedure we used (Table 2; Fig. 3) can be made more quantitative using image analysis densitometry software (e.g., Image 1.49, Research Services Branch, National Institutes of Mental Health, Image Processing and Analysis, NIH Bethesda, MD). With this image analysis program, it is possible to compare mean pixel intensity values in defined areas of images to quantify fluorescence intensity measurements (Wu and Errington, 1994). Also, it is possible to calibrate such analyses using fluorescence standards (e.g., Multispeck^{Tm}; Molecular Probes, Inc., Eugene, OR). Such quantitative analysis and calibration will be useful for comparison of results obtained from different experiments in the future.

The procedures outlined above were also used to optimize stringency conditions of a FISH protocol for the PS-1 16S riboprobe, which we are developing to identify Mn-oxidizing sheathed bacteria of the genus *Leptothrix* in wetland samples (Siering and Ghiorse, 1994). First, the fluorescence intensity method described above was used to determine optimal hybridization and stringency of washing conditions (Fig. 4). As expected, the greatest fluorescence intensities of the target cells, *L. discophora* SS-1, were observed in samples washed under the lowest stringency conditions, and the lowest intensity was associated with high stringency washing (compare Fig. 4a with 4d); moderate washing produced intermediate fluorescence intensities (Fig. 4b,c).

When wetland particle samples were hybridized with PS-1 and washed under low and moderate stringency conditions, at least two different unknown types of fil-

TABLE 2. Relative fluorescence intensity values of cells in LSM images of methylotrophic bacteria and Escherichia coli *(see Fig. 3) using three different 16S riboprobe–FISH protocols*

	Relative fluorescent intensity[1]							
	Type I probe[2]				Type II probe			
	Methylotrophs[3]		Control[3]		Control		Methylotrophs	
Protocol 1[4]	OB3b	0	**B/r**	**5**	B/r	2	**OB3b**	**3**
	BG-8	**7**					BG-8	0
Protocol 2[5]	OB3b	2	B/r	0	B/r	2	**OB3b**	**5**
	BG-8	**3**					BG-8	1
Protocol 3[6]	OB3b	0	**B/r**	**3**	B/r	2	**OB3b**	**8**
	BG-8	**4**					**BG-8**	**4**

[1]After hybridization and washing the smears were viewed in the LSM-210 using constant imaging parameters as described in Fig. 3. The universal and negative control probes (Giovannoni et al., 1988) gave relative intensity values of 8–10 and 0–2, respectively, for all three target organisms in all three protocols. Values higher than negative control are in bold type.
[2]Fluorochrome-labeled oligonucleotide probes (Type I: 5′ FITC; Type II: 5′ Texas Red; universal: 5′ FITC; control: 5′ FITC) were added at a concentration of 5.0 ng/μL. Design, synthesis, and labeling of signature oligonucleotide probes for Types I (10 λ) and II (9 α) methylotrophic bacteria are described by Tsien et al. (1990).
[3]*Methylomonas albus* BG-8 (Type I) and *Methylosinus trichosporium* OB3b (Type II) were used as mixed methylotrophs. *Escherichia coli* B/r was a negative control.
[4]Protocol 1: Exponential cells were fixed, dehydrated, permeabilized, and smeared on gelatin-chromate-coated slides. Probing at 37°C was carried out per Tsien et al. (1990) for 16 h.
[5]Protocol 2: Permeabilized, dehydrated smears (as in protocol 1) were hybridized per Poulsen et al. (1993) but washed according to Tsien et al. (1990).
[6]Protocol 3: Smears (as in protocol 1) were hybridized per Poulsen et al. (1993) for 4 h but including 30% formamide in the wash solution.

amentous bacteria were labeled (Fig. 5). One of the unknown bacteria was similar in size and morphology to cultivated strains of *L. discophora* (arrows, Fig. 5a,b); the other filaments contained much larger cells than the cultivated strains. Surprisingly, the larger diameter cells hybridized more strongly with the PS-1 probe, as indicated by the persistence of fluorescence of these cells after washing under moderate to high stringency conditions (Fig. 5c,d).

These preliminary results not only demonstrate the applicability of LSM-assisted 16S riboprobe–FISH methods for use on environmental samples but also showed that the Type I, Type II, and PS-1 probes will be useful in studies of the methylotrophic and Mn-oxidizing communities in the wetland. Past work has established the dominance of *Leptothrix* spp. at certain times of the year (Ghiorse, 1984) as well as the involvement of methylotrophs in regulating seasonal methane fluxes from the wetland (Miller et al., 1994). However, the unexpected labeling of unknown filamentous cells shown in Figure 5 illustrates the uncertainty that can arise in 16S riboprobe–FISH studies of natural communities. In this case, the unknown organisms were labeled by the PS-1 probe which was originally designed to recognize laboratory-grown strains. Obviously, there is a need to further test the specificity of this probe and to compare the FISH results to those obtained by other morphological, genetic, and biochemical characterization techniques applied to the same samples. The LSM procedures described above will greatly facilitate this kind of work in the future.

Development and Application of an IF-MARG Procedure for Determination of Single Cell ^{14}C-Mineralization Activity and the Microscale Distribution of ^{14}C-Labeled Chemicals in Groundwater Seep Sediments

Since its first applications in soil microbiology and microbial ecology in the 1960s and 1970s (Bohlool and Schmidt, 1968, 1980; Fliermans et al., 1974), the immunofluorescence (IF) staining technique has been applied extensively for autecological studies of aquatic microorganisms (Campbell, 1993). Microautoradiography (MARG) was also developed for use in microbial ecology in the 1960s (Brock and Brock, 1966, 1968); it too has been applied extensively to aquatic microorganisms (Carman, 1993; Meyer-Reil, 1978; Tabor and Neihof, 1982). In the MARG technique, active cells are recognized by their association with autoradiographic silver grains in the emulsion. Staining with a fluorescent dye such as AO is used commonly to locate bacterial cells. Identification of specific bacteria can be achieved by combining IF staining with MARG (Fliermans and Schmidt, 1975; Ward, 1984).

We were interested in developing a combined IF-MARG procedure for use in microscale studies of pollutant bioavailability and biodegradation in coal tar–polluted groundwater seep sediments (Sandoli, 1994). In theory, such a combined IF-MARG method could be used to determine the microscale distribution of a ^{14}C-labeled pollutant chemical located by MARG in sediment particles, relative to the active ^{14}C-mineralizing bacterial cells in the sample identified by IF staining. The high resolution and superior imaging capabilities of the LSM-210 made it the instrument of choice in the development of a combined IF-MARG procedure.

We first developed an indirect IF staining procedure using the RSP-1 antiserum and a phycoerythrin (PE)-labeled anti-rabbit secondary antibody. We then demonstrated that the indirect IF staining procedure was capable of staining the *S. paucimobilis* RSP-1 cells in sediment samples, and we showed that the RSP-1 cells mineralized ^{14}C-glucose in a sediment slurry. Next, a MARG procedure was developed to detect RSP-1 cells that had taken up or metabolized the ^{14}C-glucose in the sediment slurry. Finally, we combined the methods to create a functional IF-MARG procedure (Sandoli, 1994). A critical step was development of a procedure for staining RSP-1 cells through the MARG emulsion.

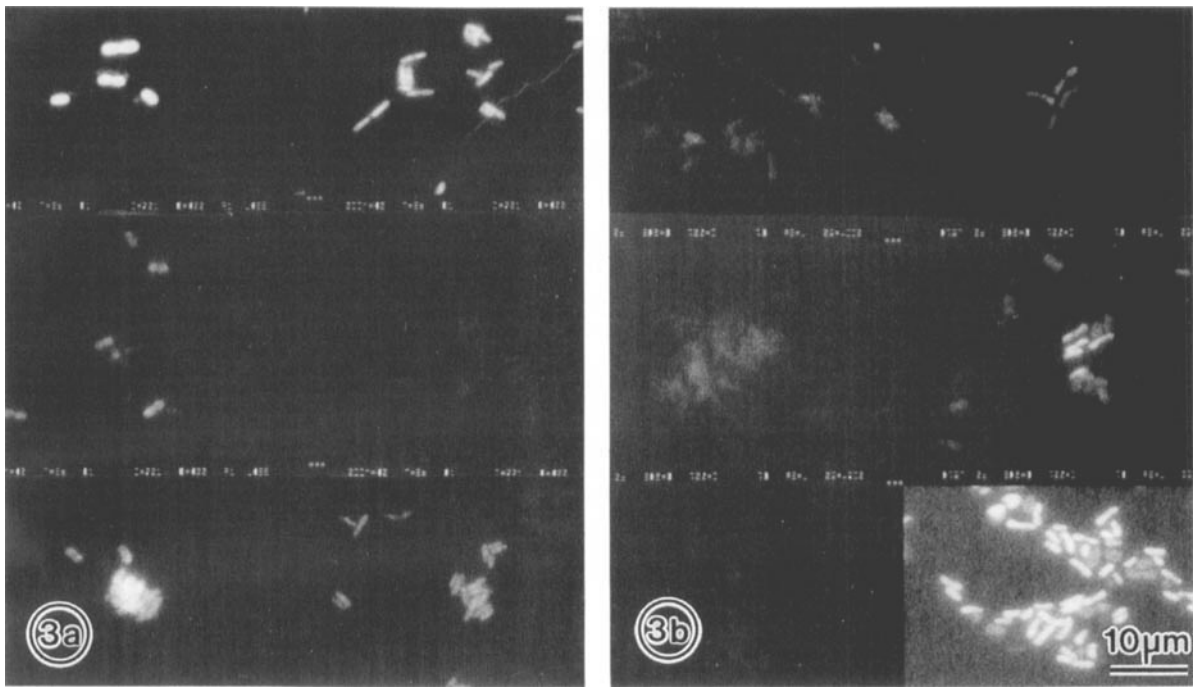

Fig. 3. Laser scanned fluorescence images used to estimate relative fluorescence intensity values obtained from the three FISH protocols listed in Table 2. The results from each protocol are represented by four micrographs arranged in the three horizontal rows in panels a and b. Protocol 1 is in the top row, protocol 2 in the middle, and protocol 3 in the bottom row. **a:** Results from hybridization with the Type I probe. **b:** Results with the Type II probe. The column on the left in panel a and on the right in panel b contain images of a mixture of the two types of methylotroph cells. Coccoid cells are *M. albus* BG-8, a Type I methylotroph; rod-shaped cells are *M. trichosporium* OB3b, a Type II methylotroph. The right-hand column in panel a and the left-hand column of panel b contain images of the *E. coli* B/r cells. Note that protocol 2 (middle row of images in panels a and b) gave the best discrimination among the three types of cells. Imaging parameters: Objective lens = phase contrast ×100 (NA 1.4), argon laser excitation = 488 nm (Type I probe labeled with FITC) or 514 nm (Type II probe labeled with TR), laser current = 4.00 amps; attenuation filter = 0.1, electronic band filter (B1); zoom factor = 40; scan rate = 8 sec per 512 × 512 × 8 bit frame, 16 lines averaged; contrast (C = 701), brightness (B = 200). Each image was obtained using identical imaging parameters. Stored images were displayed with the four-way split-screen function, printed on the Sony videograph printer without further processing, and then compared for relative fluorescence intensity. For illustration, the videograph prints were copied on Polaroid BW negative film and printed at uniform exposure and contrast.

This step was achieved by applying the primary and secondary antibody staining solutions for extended periods (24 h at 37°C for each antibody). During development of this procedure, problems with wrinkling and peeling of the emulsion were overcome by omitting NaCl from the staining solutions (R.L. Sandoli and K. Stuart, unpublished results).

Once the final IF-MARG protocol was developed (Sandoli, 1994), LSM was applied to study the distribution of ^{14}C from ^{14}C-glucose relative to the distribution of ^{14}C-mineralizing bacterial cells in the sample (Fig. 6). We found conventional DIC imaging to be most useful for screening for active cells (indicated by their association with silver grains) (Fig. 6a). Cells located in this way were identified by PE-labeled antibody fluorescence using CEFM (not illustrated). High resolution LSM analysis involving precise location of cells and silver grains associated with organic particles was best achieved with laser scanned BF images (Fig. 6b) combined with laser scanned nonconfocal (Fig. 6c) and confocal EF (Fig. 6d) images. This combination of LSM imaging techniques proved most useful for determining the location of cells relative to silver grains and analyzing their spatial relationships in sediment particles. It should be noted that confocal optical sectioning (Fig. 6d) greatly improved the clarity of the fluorescence images of labeled cells attached to particles by virtually eliminating out of focus blurriness and increasing the depth of focus (compare Fig. 6c with 6d). Using the combined imaging techniques it was observed that the ^{14}C label from ^{14}C-glucose associated strongly with organic particles and that only a small fraction (approximately 10%) of cells took up the label (Sandoli, 1994). These results clearly demonstrated the advantages of using the LSM imaging for analysis of IF-MARG results in samples containing particulate material.

CONCLUSIONS

The results presented in this paper show the wide applicability and advantages of using LSM for analysis of complex microbial assemblages in aquatic microhabitats. The results also set the stage for further development of LSM-based procedures for studying bacterial distribution and community structure by single-cell identification, and they emphasize the utility of LSM

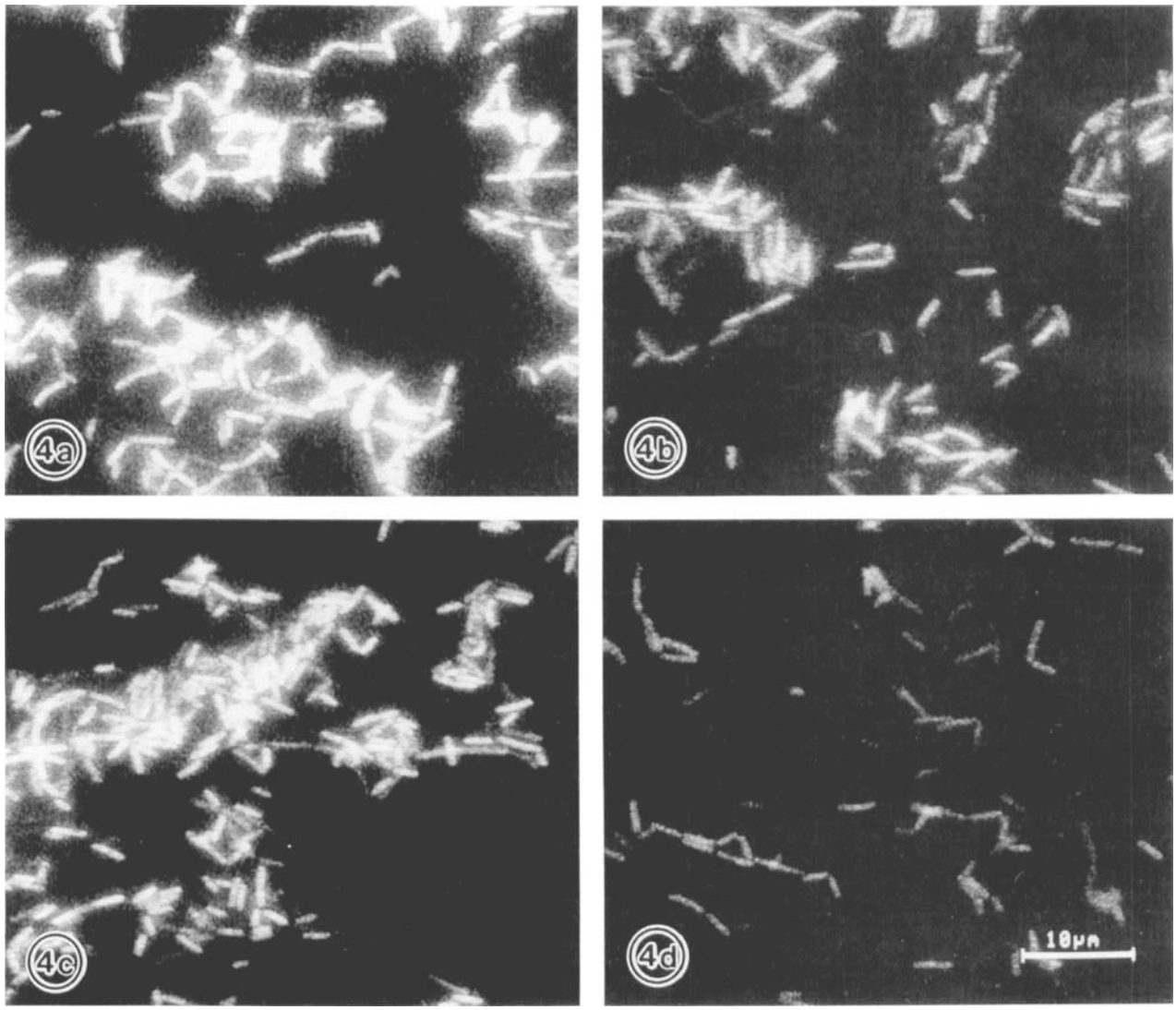

Fig. 4. Laser scanned fluorescence images (nonconfocal) showing the effect of four different stringency washing conditions on relative fluorescence of *Leptothrix discophora* SS-1 cells hybridized under identical conditions with the FITC-labeled PS-1 16S roboprobe. This probe identifies Mn-oxidizing sheathed bacteria in the genus *Leptothrix* as well as one strain of *Sphaerotilus natans* (Siering and Ghiorse, 1994). Exponential phase cells were prepared for hybridization as described in Materials and Methods. Hybridization was carried out for 4–8 h at 37°C in a solution containing 5.0 ng/µL of the PS-1 probe following protocol 2 (Table 2) (Poulson et al., 1993). After hybridization, excess probe was removed by flooding the slide on ice-cold 5× SET (Tsien et al., 1990). Stringency conditions were varied by washing slides three times for 10 min each at 37°C and at 50°C with various dilutions of 5× SET. Slides were examined in the LSM under constant imaging conditions as described in Fig. 3. **a:** Low stringency (37°C, 1× SET). **b:** Moderate stringency (37°C, 0.2× SET). **c:** Moderate stringency (50°C, 1× SET). **d:** high stringency (50°C, 0.2× SET). Note the general decline in relative fluorescence intensity with increasing stringency. Imaging parameters: The parameters were the same as described in Fig. 3 except that a ×63 (NA 1.4) phase contrast lens was used, contrast (C = 670) and brightness (B = 371) values were predetermined as described in the text and the scan rate was 2 sec. All micrographs were printed under identical photographic conditions.

for high resolution analysis of complex particulate material in natural samples. The development of LSM-based techniques reported in this work encourages us to believe that development of even better LSM-based procedures will be possible in the future. For example, we envision the possibility of a combined FISH-MARG method that would allow more flexible identification of active bacterial cells than the combined IF-MARG method. A combined FISH-MARG method would not require that target cells be isolated. Also, it may be possible to improve the current sensitivity limits of FISH techniques, which are now limited by the number of ribosomes in target cells, by using gene amplification procedures to amplify the FISH signal. Amplification techniques such as in situ PCR or antibody amplification of antigen (e.g., biotin or dixoygenin)-labeled oligonucleotide probes are currently used in mammalian cytogenetics for gene mapping and subcellular lo-

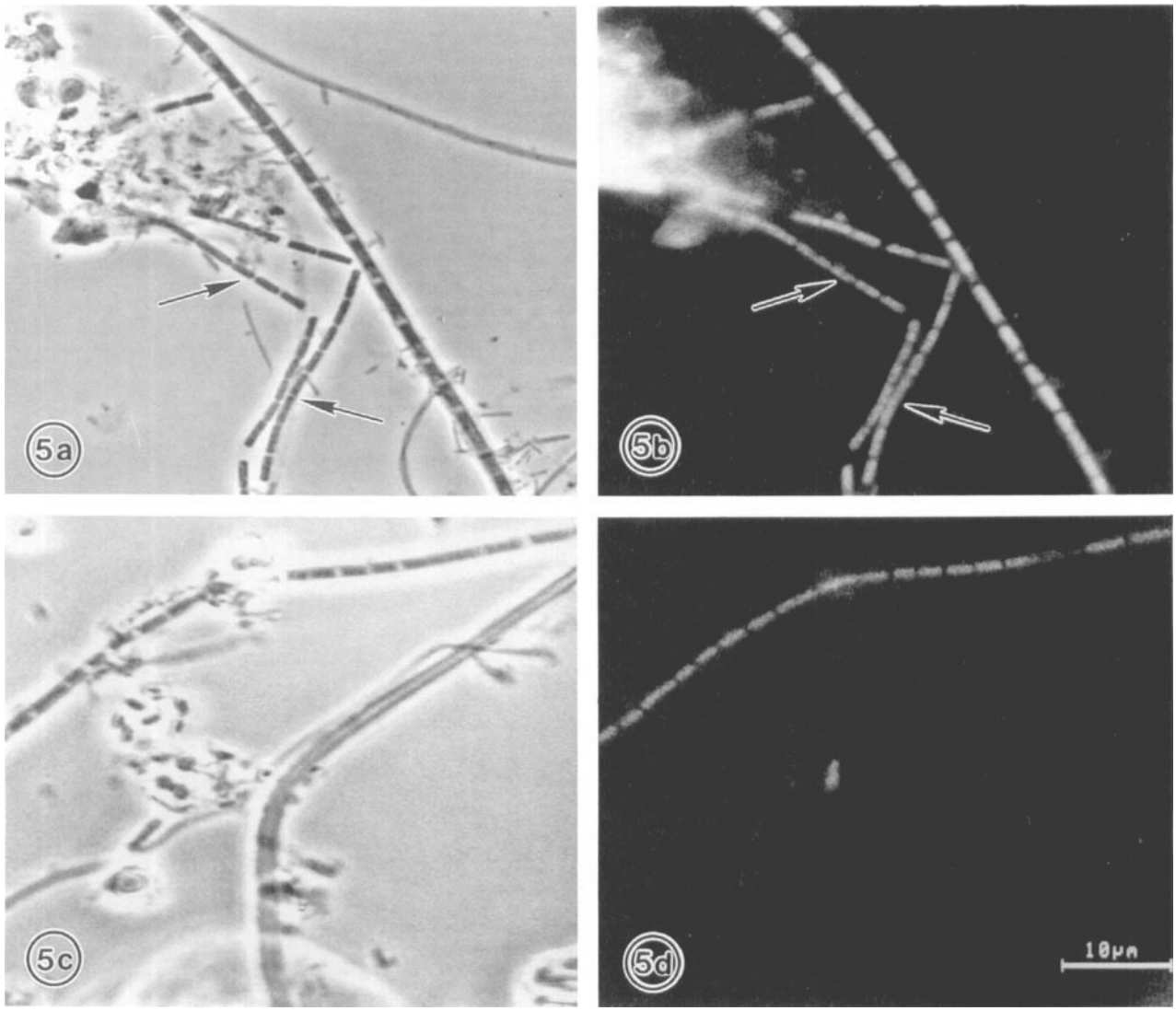

Fig. 5. Laser scanned epifluorescence (nonconfocal) and matching laser scanned phase contrast images of previously unidentified filamentous bacteria labeled by the PS-1 16S FISH riboprobe in a sample of particulate material from Sapsucker Woods wetland. Samples were hybridized as described in Fig. 4 and washed at low stringency (37°C, 1 ×SET) **(a,b)** and moderate stringency (50°C, 1 ×SET) **(c,d)**. Note that under low stringency conditions a greater number of morphological types of bacteria were labeled (arrows in a,b) and cells generally fluoresced more intensely than under moderate stringency conditions (compare a,b with c,d). Imaging parameters. The same parameters described in Fig. 4 were used except for different contrast and brightness values: (a) C = 670; B = 370; (b) C = 850; B = 172; (c) C = 698; B = 372; (d) C = 865; B = 172. The scan rate in panels a and b was 8 sec.

calization of DNA and RNA sequences (McNeil et al., 1991; Ward et al., 1991). Such techniques can be adapted for use on environmental samples with the assistance of LSM analysis in the future.

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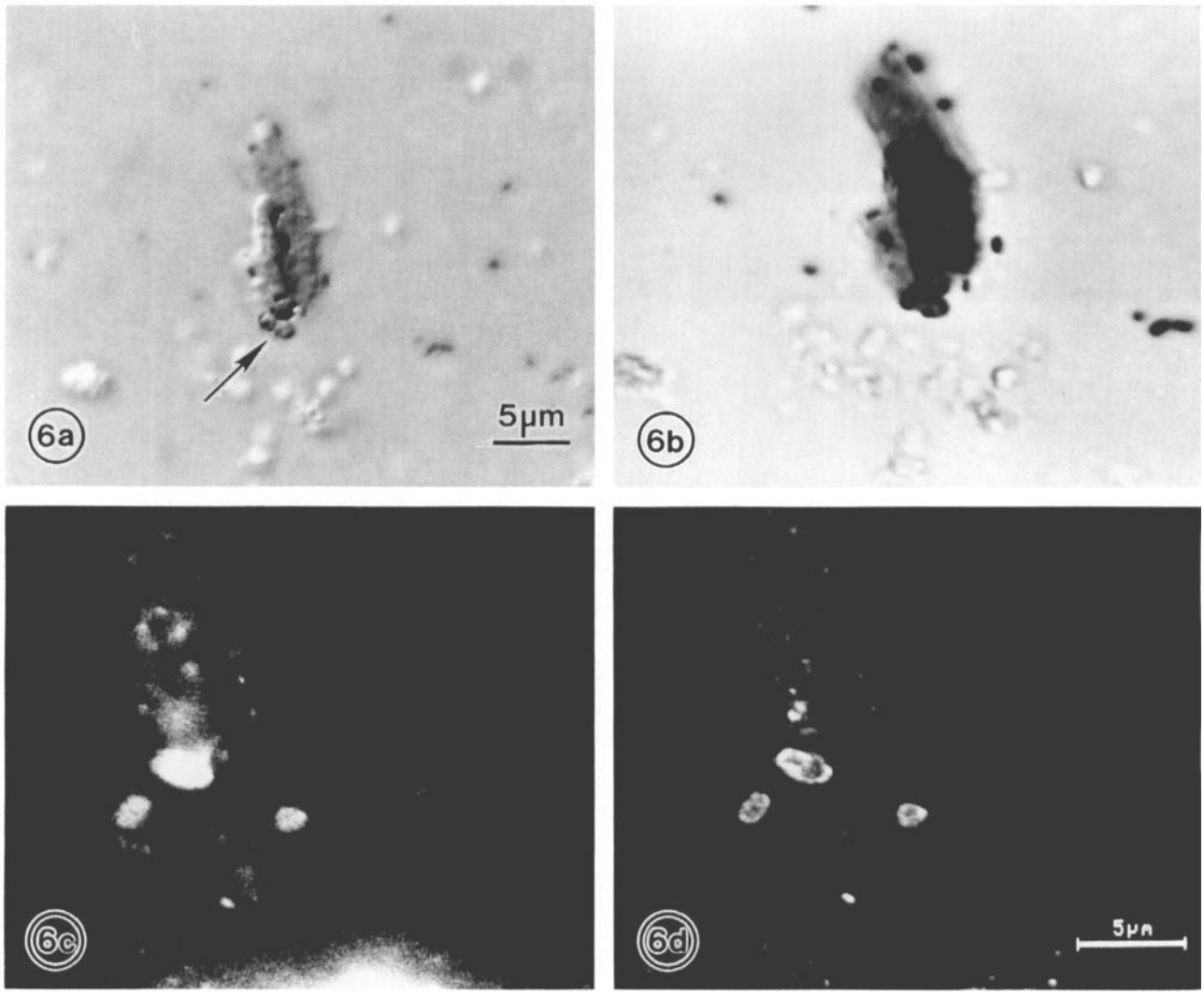

Fig. 6. Conventional DIC (image made with the CCD camera) (a) and three laser scanned images (b,c,d) of the same organic sediment particle in an IF-MARG preparation of sediment slurry spiked with ^{14}C-glucose and *Sphingomonas paucimobilis* RSP-1 cells. a: DIC image showing one active cell (arrow) attached to the particle and surrounded by autoradiographic silver grains (dark particles). b: BF image showing the peripheral distribution of silver grains associated with the organic particle and the edges of the cell. c: EF (nonconfocal) image made through a long pass ($\lambda > 590$ nm) emission filter for detection in this field. Note the bright fluorescence of the cell depicted in panel a as well as two additional cells (not clearly seen in panel a). d: Confocal EF image composed of four optical sections taken at 1 μm depth intervals and then overlaid to produce the final composite image. Note the improvement in resolution and clarity of this image compared to the nonconfocal fluorescence image in panel c. LSM imaging parameters and image processing (b,c,d): Objective lens = ×100 (DIC) (NA 1.4); argon laser line = 488 nm, laser current = 4.00 amp; attenuation filter = 0.1; zoom factor = 50; scan rate = 8 sec per 512 × 512 × 8 bit frame, 16 lines averaged; contrast and brightness of the final images were enhanced using the LSM-210 image analysis software. The composite optical section image (panel d) was produced by overlaying four stored confocal images using the z-sectioning software.

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REFERENCES

Adams, L.F., and Ghiorse, W.C. (1986) Physiology and ultrastructure of *Loptothrix discophora* SS-1. Arch. Microbiol., 145:126–135.

Amann, R.I., Krumholz, L., and Stahl, D.A. (1990) Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol., 172:762–770.

Amann, R.I., Stromley, J., Devereux, R., Key, R., and Stahl, D.A. (1992a) Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. Appl. Environ. Microbiol., 58: 614–623.

Amann, R.I., Zarda, B., Stahl, D.A., and Schleifer, K.-H. (1992b) Identification of individual prokaryotic cells using enzyme-labeled, 16S-rRNA-targeted oligonucleotide probes. Appl. Environ. Microbiol., 58:3007–3011.

Beveridge, T.J., and Davies, J.A. (1983) Cellular response of *Bacillus subtilis* and *Escherichia coli* to the Gram stain. J. Bacteriol., 156: 846–858.

Bohlool, B.B., and Schmidt, E.L. (1968) Nonspecific staining: Its con-

trol in immunofluorescence examination of soil. Science, 162:1012–1014.

Bohlool, B.B., and Schmidt, E.L. (1980) The immunofluorescence approach in microbial ecology. Adv. Micorbial Ecol., 4:203–241.

Braun-Howland, E.B., Danielsen, S.A., and Nierzwicki-Bauer, S.A. (1992) Development of a rapid method for detecting bacterial cells in situ using 16S rRNA-targeted probes. Biotechniques, 13:928–932.

Brock, M.L., and Brock, T.D. (1968) The application of micro-autoradiographic techniques to ecological studies. Fur Theoretische und Angewandte Limnologie, 15:1–29.

Brock, T.D., and Brock, M.L. (1966) Autoradiography as a tool in microbial ecology. Nature, 209:734–736.

Caldwell, D.E. (1993) The potential of scanning confocal microscopy in studies of groundwater communities. International Symposium on Subsurface Microbiology, Bath, England. E-01.

Caldwell, D.E., Korber, D.R., and Lawrence, J.R. (1992a) Confocal laser microscopy and digital image analysis in microbial ecology. Adv. Microbial. Ecol., 12:1–67.

Campbell, L. (1993) Immunofluorescence method for the detection and characterization of marine microbes. In: Handbook of Methods in Aquatic Microbial Ecology. P.F. Kemp, B.F. Sherr, E.B. Sherr, and J.J. Cole, eds. Lewis Publ., Boca Raton, FL, pp. 295–300.

Carman, K.R. (1993) Microautoradiographic detection of microbial activity. In: Handbook of Methods in Aquatic Microbial Ecology. P.F. Kemp, B.F. Sherr, E.B. Sherr, and J.J. Cole, eds. Lewis Publ., Boca Raton, FL, pp. 397–404.

DeLong, E.F. (1993) Single cell identification using fluorescently labeled ribosomal RNA-specific probes. In: Handbook of Methods in Aquatic Microbial Ecology. P.F. Kemp, B.F. Sherr, E.B. Sherr, and J.J. Cole, eds. Lewis Publ., Boca Raton, FL, pp. 285–294.

DeLong, E.F., Wickham, G.S., and Pace, N.R. (1989) Phylogenetic stains: Ribosomal RNA-based probes for identification of single microbial cells. Science, 243:1360–1363.

Deveraux, J.P., Hoeberli, P., Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res., 12:387–395.

Distel, D.L., and Cavanaugh, C.M. (1994) Independent phylogenetic origins of methanotrophic and chemoautotrophic bacterial endosymbioses in marine bivalves. J. Bacteriol., 176:1932–1938.

Emerson, D., and Ghiorse, W.C. (1992) Isolation, cultural maintenance, and taxonomy of a sheath-forming strain of *Leptothrix discophora* and characterization of manganese-oxidizing activity associated with the sheath. Appl. Environ. Microbiol., 58:4001–4010.

Fliermans, C.B., and Schmidt, E.L. (1975) Autoradiography and immunofluorescence combined for autecological study of single cell activity with *Nitrobacter* as a model system. Appl. Microbiol., 30: 676–684.

Fliermans, C.B., Bohlool, B.B., and Schmidt, E.L. (1974) Autecological study of the chemotroph *Nitrobacter* by immunofluorescence. Appl. Environ. Microbiol., 27:124–129.

Ghiorse, W.C. (1980) Electron microscopic analysis of metal-depositing microorganisms in surface layers of Baltic Sea ferromanganese concretions. In: Biogeochemistry of Ancient and Modern Environments. P.A. Trudinger, M.R. Walter, and B.J. Ralph, eds. Australian Academy of Science and Springer Verlag, New York, pp. 345–354.

Ghiorse, W.C. (1984) Biology of iron- and manganese-depositing bacteria. Annu. Rev. Microbiol., 38:515–550.

Ghiorse, W.C. (1993) Use of the confocal laser scanning microscope for detecting viable, but unculturable subsurface bacteria. International Symposium on Subsurface Microbiology, Bath, England. p. B 33.

Ghiorse, W.C., and Ehrlich, H.L. (1993) Microbial biomineralization of iron and manganese. In: Iron and Manganese Biomineralization Processes in Modern and Ancient Environments. R.W. Fitzpatrick and H.C.W. Skinner, eds. Catena, Cremlingen-Destedt, Germany, pp. 75–99.

Ghiorse, W.C., and Hirsch, P. (1979) An ultrastructural study of iron and manganese deposition associated with extra-cellular polymers of *Pedomicrobium*-like budding bacteria. Arch. Microbiol., 123: 213–226.

Ghiorse, W.C., and Hirsch, P. (1982) Isolation and properties of Mn-Fe-depositing budding bacteria from Baltic Sea ferromanganese concretions. Appl. Environ. Microbiol., 43:1464–1472.

Giovannoni, S.J., DeLong, E.F., Olson, G.J., and Pace, N.R. (1988) Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J. Bacteriol., 170:720–726.

Jurtshuk, R.J., Blick, M., Bresser, J., Fox, G.E., and Jurtshuk, P. (1992) Rapid in situ hybridization technique using 16S rRNA segments for detecting and differentiation, the closely related Gram-positive organisms, *Bacillus polymyxa* and *Bacillus macerans*. Appl. Environ. Microbiol., 58:2571–2578.

Kemp, P.F., Sherr, B.F., Sherr, E.B., and Cole, J.J., eds. (1993) Handbook of Methods in Aquatic Microbial Ecology. Lewis Publ., Boca Raton, FL.

Korber, D.R., Lawrence, J.R., Hendry, M.J., and Caldwell, D.E. (1993) Analysis of spatial variability within MOT^+ and MOT^- *Pseudomonas fluorescens* biofilms using representative elements. Biofouling, 7:339–358.

Korber, D.R., James, G.A., and Costerton, J.W. (1994) Evaluation of fleroxacin activity against established *Pseudomonas fluorescens* biofilms. Appl. Environ. Microbiol., 60:1663–1669.

Larsen, N., Olsen, G.J., Maidak, B.L., McCaughey, M.J., Overbeek, R., Macke, T.J., Marsh, T.L., and Woese, C.R. (1993) The ribosomal data base project. Nucleic Acids Res., 21:3021–3023.

Lawrence, J.R., Korber, D.R., Hoyle, B.D,. Costerton, J.W., and Caldwell, D.E. (1991) Optical sectioning of microbial biofilms, J. Bacteriol., 173:6558–6567.

Lens, P., Massone, A., Rozzi, A., and Verstraete, W. (1994) The effect of sulfate reducing bacteria on the treatment performance of aerobic biofilm receptors. In: Second International Symposium on Environmental Biotechnology. Chameleon Press Ltd., London, pp. 58–60.

Madsen, E.L., Sinclair, J.L., and Ghiorse, W.C. (1991) In situ biodegradation: Microbiological patterns in a contaminated aquifer. Science, 252:830–833.

Manz, W., Amann, R., Ludwig, W., Wagner, M., and Schleifer, K.-H. (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: Problems and solutions. System. Appl. Microbiol., 15:593–600.

McNeil, J.A., Johnson, C.V., Carter, K.C., Singer, F.H., and Lawrence, J.B. (1991) Localizing DNA and RNA within nuclei and chromosomes by fluorescence in situ hybridization. GATA, 8:41–58.

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.

Miller, D.N., Yavitt, J.B., Madsen, E.L., and Ghiorse, W.C. (1994) Methane fluxes and population dynamics of methanotrophic bacteria in a wooded wetland. Abst. Annu. Meet. Amer. Soc. Microbiol. N-61, p. 326.

Poulsen, L.K., Ballard, G., and Stahl, D.A. (1993) Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. Appl. Environ. Microbiol., 59:1354–1360.

Ramsing, N.B., Kühl, M., and Jørgensen, B.B. (1993) Distribution of sulfate-reducing bacteria, O_2, and H_2S in photosynthetic biofilms determined by oligonucleotide probes and microelectrodes. Appl. Environ. Microbiol., 59:3840–3849.

Raskin, L., Poulsen, L.K., Noguera, D.R., Rittmann, B.E., and Stahl, D.A. (1994a) Quantification of methanogenic groups in anaerobic biological reactors by oligonucleotide probe hybridization. Appl. Environ. Microbiol., 60:1241–1248.

Raskin, L., Stromley, J.M., Rittmann, B.E., and Stahl, D.A. (1994b) Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. Appl. Environ. Microbiol., 60:1232–1240.

Sandoli, R.L. (1994) Isolation and Characterization of a Phenanthrene-Degrading *Sphingomonas paucimobilis* Strain and Its Use in Macro- and Micro-Scale Approaches for Studying Bioavailability of Compounds in Sediments. MS thesis, Cornell University, Ithaca, NY.

Siering, P.L., and Ghiorse, W.C. (1994) Development and application of 16S ribosomal RNA-targeted probes for detection of iron- and manganese-oxidizing sheathed bacteria in environmental samples. Abst. Annu. Meet. Amer. Soc. Microbiol. N-154, p. 342.

Slayter, E.M., and Slayter, H.S. (1992) Light and Electron Microscopy. Cambridge University Press, New York.

Stahl, D.A., and Amann, R. (1991) Development and application of nucleic acid probes in bacterial systematics. In: Sequencing and Hybridization Techniques in Bacterial Systematics. E. Stackebrandt and M. Goodfellow, eds. John Wiley and Sons, Chichester, England, pp. 205–248.

Stanier, R.Y., Palleroni, N.J., and Doudoroff, M. (1966) The aerobic

pseudomonads: A taxonomic study. J. Gen. Microbiol., 53:1010–1019.

Tabor, P.S., and Neihof, R.A. (1982) Improved microautoradiographic method to determine individual microorganisms active in substrate uptake in natural waters. Appl. Environ. Microbiol., 44:945–953.

Tsien, H.C., Bratina, B.J., Tsuji, K., and Hanson, R.S. (1990) Use of oligodeoxynucleotide signature probes for identification of physiological groups of methylotrophic bacteria. Appl. Environ. Microbiol., 56:2858–2865.

Wagner, M., Erhart, R., Manz, W., Amann, R., Lemmer, H., Wedi, D., and Schleifer, K.-H. (1994) Development of an rRNA targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for in situ monitoring in activated sludge. Appl. Environ. Microbiol., 60:792–800.

Ward, B.B. (1984) Combined autoradiography and immunofluorescence for estimation of single cell activity by ammonium-oxidizing bacteria. Limnol. Oceanogr., 29:402–410.

Ward, D.C., Lichter, P., Boyle, A., Baldini, A., Menninger, J., and Ballard, S.G. (1991) Gene mapping by fluorescent in situ hybridization and digital imaging microscopy. In: Etiology of Human Disease at the DNA Level. J. Lindsten and U. Pettersson, eds. Raven Press, Ltd. New York, pp. 291–303.

Wolfaardt, G.M., Lawrence, J.R., Headley, J.V., Robarts, R.D., and Caldwell, D.E. (1994) Microbial exopolymers provide a mechanism for bioaccumulation of contaminants. Microb. Ecol., 27:279–291.

Wu, L.J., and Errington, J. (1994) *Bacillus subtilis* SpoIIIE protein required for DNA segregation during asymmetric cell division. Science, 264:572–575.