96-021

Biofouling, 1996, Vol. 9(4), pp. 269–277
Reprints available directly from the publisher
Photocopying permitted by license only

EVALUATION OF PHYSIOLOGICAL STAINING, CRYOEMBEDDING AND AUTOFLUORESCENCE QUENCHING TECHNIQUES ON FOULING BIOFILMS

CHING-TSAN HUANG, GORDON A MCFETERS and PHILIP S STEWART†

Center for Biofilm Engineering, Montana State University
Bozeman, MT 59717, USA

(Received 30 June 1995; in final form 15 January 1996)

Physiological staining, cryoembedding, cryosectioning and autofluorescence quenching techniques were evaluated for their applicability to undefined mixed population biofilms collected from environmental or engineered systems. Four different biofilms from two cooling towers, a paper mill machine and the effluent ditch of a wastewater treatment plant were tested. The redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) was used in combination with the DNA stain 4′,6-diamino-2-phenylindole (DAPI) to distinguish respiring and nonrespiring cells. Positive CTC staining, as evidenced by the development of pink or red color, was successful in all samples examined except for paper mill biofilm. The structural integrity of frozen sections deteriorated when biofilms contained rigid or fibrous material. Autofluorescence generally impaired the ability to distinguish specific staining from natural background fluorescence. Two physical and three chemical methods were tested to quench autofluorescence. Quenching with crystal violet reduced most of the autofluorescent interference and still maintained physiological staining intensity, but contrast between CTC staining and residual autofluorescence was poor. Autofluorescence and the difficulty of sectioning thick biofilms containing abiotic materials limit the applicability of cryoembedding/staining techniques to fouling biofilms.

KEYWORDS: biofilms, physiological staining, cryoembedding, cryosectioning, autofluorescence quenching, crystal violet

INTRODUCTION

Biofilm formation and persistence creates problems in engineered systems by increasing the resistance to mass, momentum, and energy transfer and by mediating chemical or biological reactions at the substratum. Detrimental effects of biofilm formation on system performance range from operational problems to economic losses. The main strategy of biofilm control is the use of antimicrobial agents, such as chemical biocides or antibiotics, to kill biofilm microorganisms and/or remove them from the surface. Research on biocide action requires a reliable and convenient approach for the measurement of biocide efficacy, *i.e.* the distinction of physiologically active and inactive microorganisms. The traditional technique of assessing biocide efficacy has relied on enumerating surviving bacteria by colony formation on agar plates. This approach is time-consuming, may overestimate biocidal efficacy (Yu & McFeters, 1994), and can not provide spatial information within biofilms. In order to more completely evaluate the

† Corresponding author

effect of a biocide against biofilms, techniques that reveal changes in physiological activity within biofilms during disinfection while preserving spatial information are needed.

One such approach is the use of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and 4′,6-diamino-2-phenylindole (DAPI) to distinguish respiring and nonrespiring cells in conjunction with cryoembedding (Rodriguez *et al.*, 1992; Ramsing *et al.*, 1993; Schaule *et al.*, 1993; Yu & McFeters, 1994, Yu *et al.*, 1994). This new approach allows for the visualization of spatial patterns of respiratory activity within biofilms during disinfection (Huang *et al.*, 1995). The technique has succeeded with laboratory-grown biofilms. Industrial/natural biofilms differ, however, from laboratory biofilms in important ways that could influence the ability to apply physiological staining/cryoembedding methods. Compared with laboratory-grown biofilms, most industrial and natural biofilms are thicker, microbially undefined, and contain abiotic material. Inherent fluorescence in industrial/natural biofilms can significantly impair the detection of fluorescent probes within bacterial cells. There are several papers which have proposed methods for quenching autofluorescence. A single or dual-laser method was used to reduced autofluorescence in flow cytometry (Steinkamp & Stewart, 1986; Alberti *et al.*, 1987). This approach is not applicable to mixed culture samples. Ramsing *et al.* (1993) found autofluorescence of sulfate-reducing bacteria was substantially reduced following ethanol, $NaBH_4$ and HCl treatment. Another group reported that Evans Blue quenched glutaraldehyde-derived autofluorescence of collagen-based biomaterials (Werkmeister *et al.*, 1990). Halldén *et al.* (1991) reported the autofluorescence of alveolar macrophages from smokers was quenched by crystal violet. The research to date related to autofluorescence quenching only deals with pure cultures of bacteria and animal cells or with defined biomaterials. In this paper, the application of CTC staining, cryoembedding, cryosectioning and autofluorescence quenching methods are evaluated in various fouling biofilms.

MATERIALS AND METHODS

Biofilm Systems

1. Biofilm A from the fill material of a simulated cooling tower. Biofilms were cultivated in laboratory scale recirculating water systems inoculated with a mixed culture of bacteria and algae and 5 g of fresh soil. Dechlorinated municipal tap water was continuously recirculated over high efficiency PVC fill material with a residence time of 24 h. Water temperature averaged approximately 30°C and the pH was 8.0 ± 0.5. A slurry of a bentonite/kaolinite (3:7) clay mixture was also added to effect 50 mg l^{-1} final concentration for 1–2 months after inoculation. The systems were sampled after approximately 13 months of operation. Biofilm thickness, as estimated visually, ranged from 2–5 mm.

2. Biofilm B from the effluent tubing of a cooling tower. Artificial seawater (pH 8.0 ± 0.2) was continuously fed into an outdoor open recirculating cooling tower. Biofilms were grown on silicone tubing placed in the tower effluent for two months. The temperature was controlled at 25–30°C. Biofilm thickness, as estimated visually, was less than 1 mm.

3. Biofilm C from an acid paper mill. Biofilms were grown on the clear leg wall of a floatation saveall from an acid fine paper machine. The pH of the mill fluid was 4.9 and the temperature ranged between 43 and 49°C. Wood fibers, alum, rosin size, starch and a polymer retention aid were present in the bulk fluids. Isothiazolone was fed slowly into

the system, but the residual biocide at the saveall was thought to be too low to kill biofilm cells. Biofilms were harvested for analysis after 3–4 weeks of continuous operation. Biofilm thickness, as estimated visually, was 3–5 mm.

4. Biofilm D from an effluent ditch of a wastewater treatment plant. Polycarbonate coupons were set in an effluent ditch at the city of Bozeman, wastewater treatment plant for 6 months. Biofilms consisted of a variety of aquatic bacteria, algae and protozoa. The water temperature varied between 5 and 25°C during the period and the pH was approximately 7 at the time of sampling. Biofilm thickness, as estimated visually, was 1 to 3 mm. One sample was treated with 2 mg ml^{-1} chlorine for 1 h.

Physiological Staining, Cryoembedding and Cryosectioning

CTC staining, cryoembedding and cryosectioning were performed as described elsewhere (Yu *et al.*, 1994) with minor modification. Briefly, biofilm samples were stained with 0.04% CTC for 2 h, then fixed with 5% formaldehyde for 5 min. Samples without CTC staining were used as controls. After staining and fixation, separate biofilm samples were embedded with Tissue-Tek® OCT compound (Miles Incorporated, Elkhart, IN) for 0, 0.5, 1, 2, 4, and 6 h, respectively, before being placed on dry ice. After the embedding agent was completely frozen, biofilms were separated from the substratum, turned over and put back on the dry ice. Another thick layer of embedding medium was dispensed on the partially embedded biofilms. Embedded biofilms were cut into 5 μm thick sections using a Reichert-Jung cryocut 1800 (Leica Incorporated, Deerfield, IL). Sections were mounted directly on glass slides for observation and further treatment.

Autofluorescence Quenching

(1) Light microscopy method. Autofluorescence is not a problem with visible light microscopy. Attempts were therefore made to visualize the red color of CTC-formazan in sections by ordinary light microscopy. 10-μm sections were used to increase the visibility of red CTC-formazan crystals.

(2) Narrow spectrum method. Autofluorescence can sometimes be eliminated by use of appropriate filters, especially if the autofluorescence occurs within a narrow wavelength band. Three different excitation regions, ultra-violet, blue and green were tested to eliminate autofluorescence. The filter combinations used are listed in Table 1.

(1) Ethanol-sodium borohydride-HCl (ESH) treatment. ESH treatment was performed according to the method of Ramsing *et al.* (1993). Biofilm sections were dehydrated with 50, 80, and 100% ethanol for 5 min each, then submerged in 50 mM $NaBH_4$ (in 100 mM Tris-Cl) in the dark for 30 min. After rinsing with distilled water, sections were treated with 0.2 N HCl (in 0.1% Triton X-100) for 5 min, followed by counter staining with 1 μg ml^{-1} DAPI for 2 min.

Table 1 Parameters of Olympus BH-2 microscope filters used to filter autofluorescence

Excitation region	Excitation filter	Dichroic mirror	Barrier filter
U (Ultraviolet)	U (UG-1)	DM-400	L-420
B (Blue)	B (BP-490)	DM-500	AFC+O515
G (Green)	G (BP-545)	DM-570	O590

(2) Evans Blue treatment. Biofilm sections were treated with 0.5% Evans Blue (in phosphate buffered saline and 0.1 M sodium azide) for 2 min, then gently washed with distilled water and air dried. Sections were counterstained with DAPI as described above. *(3) Crystal Violet treatment.* Biofilm sections were stained with 2 mg ml^{-1} crystal violet for 2 min, then gently washed with distilled water, air dried and counter stained with DAPI.

RESULTS AND DISCUSSION

Cryoembedding and Cryosectioning

Cryoembedding and cryosectioning were generally successful on all but the thickest biofilm samples. For the four samples and six time intervals tested, the infiltration time in the embedding medium prior to freezing had little effect on the quality of the sections. Separation of the frozen biofilm from the substratum was achieved for all specimens. Biofilm A was separated from the PVC substratum by lifting one end of the substratum with a razor blade. Biofilm B, which grew attached to a curved thick silicone tube, took longer to completely freeze. The embedding medium tended to spill over the biofilms and only a thin layer of embedding agent covered the samples. This was solved by engraving a channel in the dry ice block and burying the silicone tubing in it. Biofilm C was obtained without substratum and samples were embedded on a stainless steel slide or in a Cryomold® (Miles Incorporated, Elkhart, IN). Biofilm D grew on polycarbonate slides and samples were easily separated by gently bending the slide. In general, the embedding time and biofilm type did not affect the embedding results but the thickness, rigidity and curvature of the substrata did affect the specific embedding protocol.

Most samples tested cut well but some of the thickest ones did not give satisfactory sections. For example, it was difficult to cut paper mill samples (Biofilm C) thicker than 4 mm and recover a usable section. Thicker specimens yielded sections of uneven thicknesses with consequent difficulty in focusing when observing the section using a high magnification objective. Splitting also occurred during cryosectioning of other thick samples such as Biofilms A and D. These problems may be due to the presence of fibers and to insufficient strength of the embedding agent to support thick samples through cutting.

Physiological Staining

Even without magnification or illumination, most biofilm samples developed visible red color during CTC staining. Biofilms A, B and D showed obvious red color. Biofilm C exhibited non-uniform faint pink color. The occurrence of red color indicated that respiratory activity existed within these biofilms. The development of less apparent color change in Biofilm C might be a result of the low pH (the sample pH was around 5), which could inhibit CTC reduction (Smith, 1995), prior biocide treatment or shipment time after separation from papermaking fluids.

Autofluorescence Quenching

Examples of broad spectrum autofluorescence from industrial/natural biofilms are shown in Figures 1a and 2a. Red, green and blue autofluorescence from cells and/or abiotic materials (*e.g.* clay, soil, *etc.*) was evident in the biofilm section. It was impossible to identify respiring cells without quenching this inherent fluorescence.

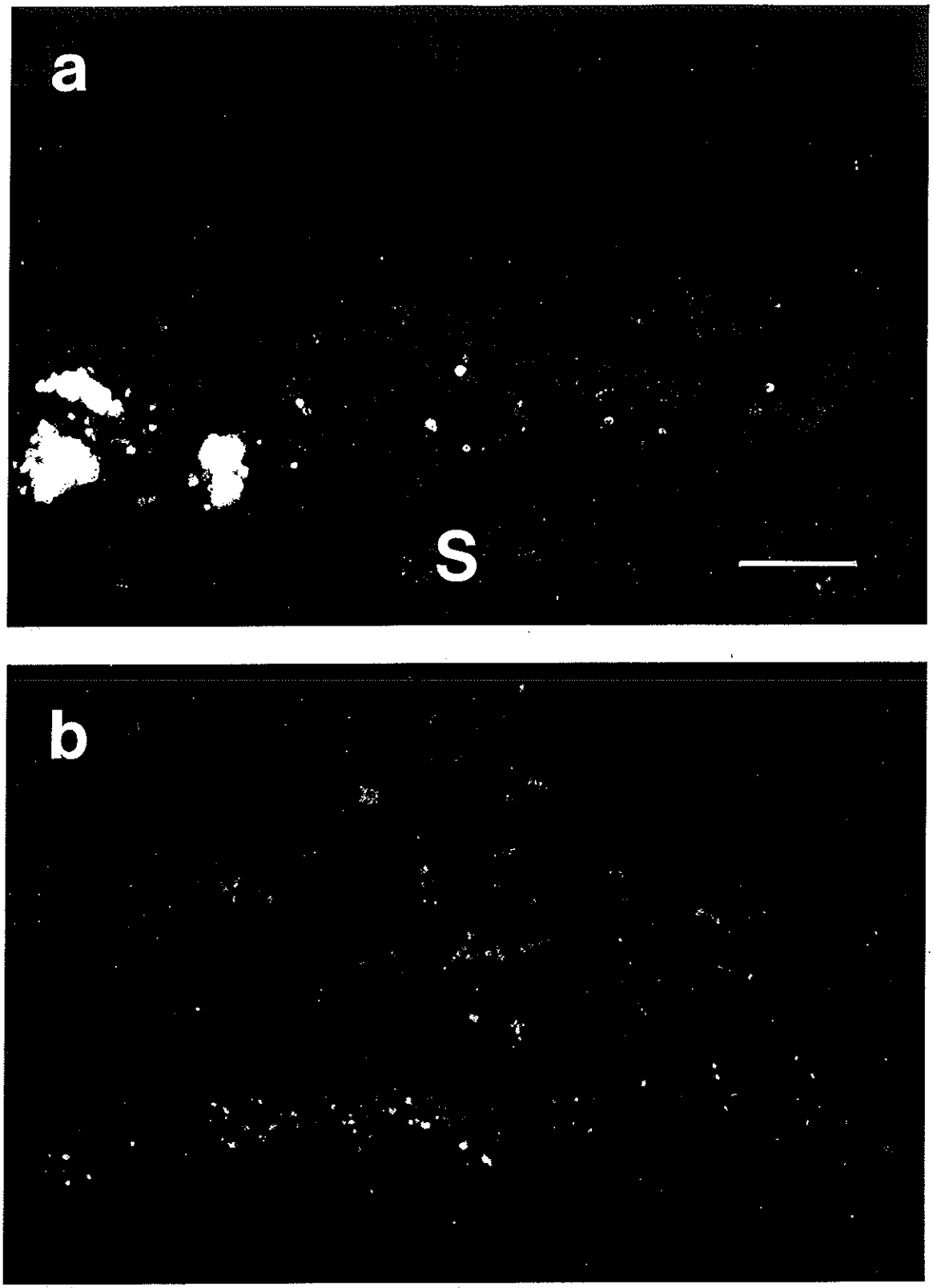


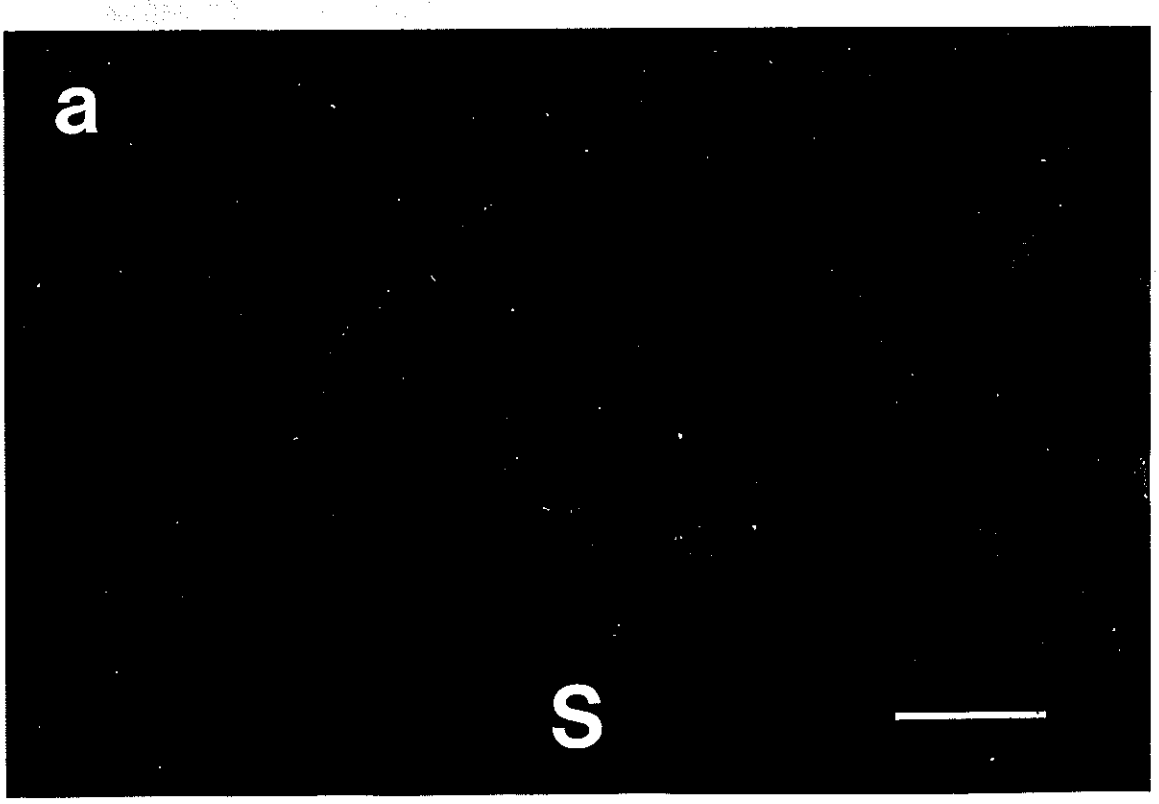
Fig. 1 Micrographs of Biofilm A in cross-section without CTC staining. a = before ESH treatment; b = after ESH treatment. S = substratum. Bar = 50 μm. (See Color Section.)

Light microscopy revealed faint red color corresponding to CTC-formazan, which could also be observed with fluorescent microscopy at the same spot. However, light microscopy methods appear to have little use because CTC-formazan color under light microscopy is not intense enough to distinguish between respiring and nonrespiring cells. Using the B, U and G filters of an Olympus BH-2 microscope also failed to eliminate the inherent fluorescence (micrographs not shown). Another possible approach is through the use of an additional narrow-bandpass filter that allows only red light in the region of 615–625 nm to pass through. While this may cut off most of the intrinsic fluorescence, it may also reduce the total light intensity to less than 1% (Ramsing *et al.*, 1993).

Figures 1a and b illustrate Biofilm A from the fill material of a cooling tower without CTC staining before and after ESH treatment, respectively. Autofluorescence completely impaired the use of fluorescence probes before quenching. The inherent fluorescence was shifted to green after ESH treatment. However, this protocol did not work for the CTC probe because ethanol or other organic solvents such as acetone or ether used for dehydration dissolved the CTC-formazan crystals.

Evans blue treatment led to a shift in the autofluorescent spectrum to longer wavelengths. For the samples tested, Evans blue treatment turned all the autofluorescence into bright red (micrographs not shown). This was incompatible with the use of CTC because CTC-formazan also fluoresces red.

Crystal violet has an absorption spectrum that overlaps with the emission spectrum of the autofluorescence. Crystal violet treatment can shift autofluorescence to a dark red color and permit FITC-labeled immunostaining of surface antigens of yeast cells (Hed, 1977). There is also a potential for crystal violet treatment in combination with CTC staining. Figure 2a shows Biofilm A with CTC staining before quenching; respiring cells could not be distinguished due to the autofluorescence. After crystal violet treatment, autofluorescence was shifted to dark red (micrograph not shown). Respiring cells with small red CTC-formazan crystals, illustrated in Figure 2b, were seen clearly after counter



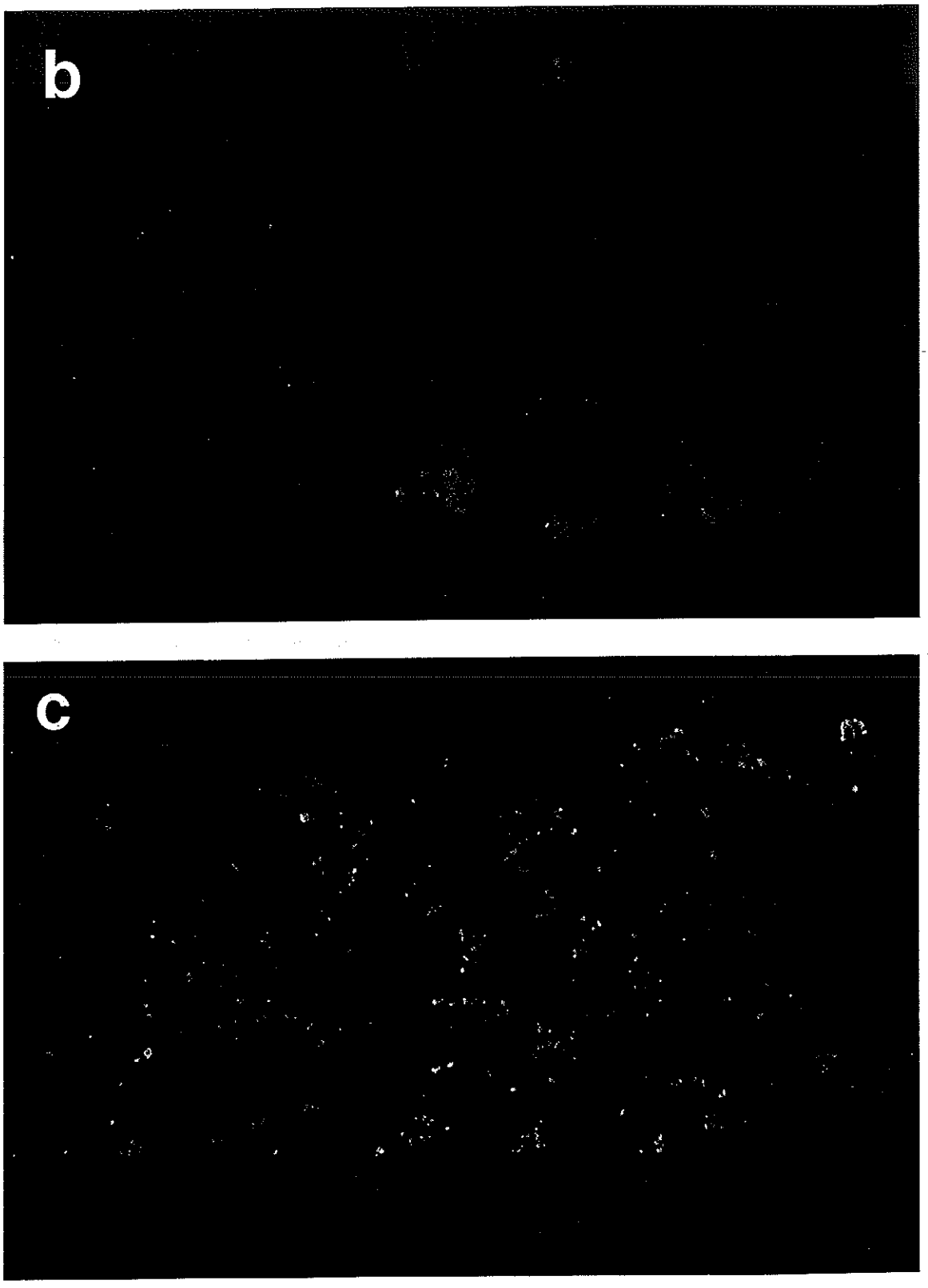


Fig. 2 Micrographs of Biofilm A in cross-section treated with crystal violet and counterstained with DAPI. a = with CTC staining, before quenching; b = with CTC staining, after quenching; c = without CTC staining, after quenching. Figure 2a and b are images from the same cross-section; Figure 2c represents a different sample from the same biofilm system. S = substratum. Bar = 50 μm. (See Color Section.)

staining with DAPI. In contrast, Biofilm A without CTC staining did not show similar red CTC-formazan crystals after crystal violet treatment and DAPI counterstaining (Fig. 2c).

Of the protocols tested, quenching autofluorescence with crystal violet provided a potential method for the use of physiological probes in fouling biofilms from industrial systems. This approach was applied to a sample of Biofilm D (wastewater treatment plant) that had been treated with 2 mg ml^{-1} chlorine for 1 h. The result, shown in Figure 3, reveals nonrespiring cells (green) and some respiring cells (red) in the upper layer of the biofilm.

Summary

The applicability of fluorescent staining and cryoembedding techniques to industrial and environmental fouling biofilms was evaluated. These methods are attractive in that they have the potential to provide information about the microscale spatial distribution of activity within biofilm. All of the biofilm samples examined, which came from four different systems, exhibited broad spectrum autofluorescence that impaired the ability to detect staining with the respiratory indicator CTC. Of several physical and chemical methods that were tested for their ability to quench autofluorescence while retaining CTC staining, post-treatment with crystal violet was the most successful. Even with this method, however, the contrast between CTC staining (bright red) and residual autofluorescence (dark red) was poor in some cases. Because of this, the technique is qualitative for the types of biofilm considered in this study. A second barrier to general

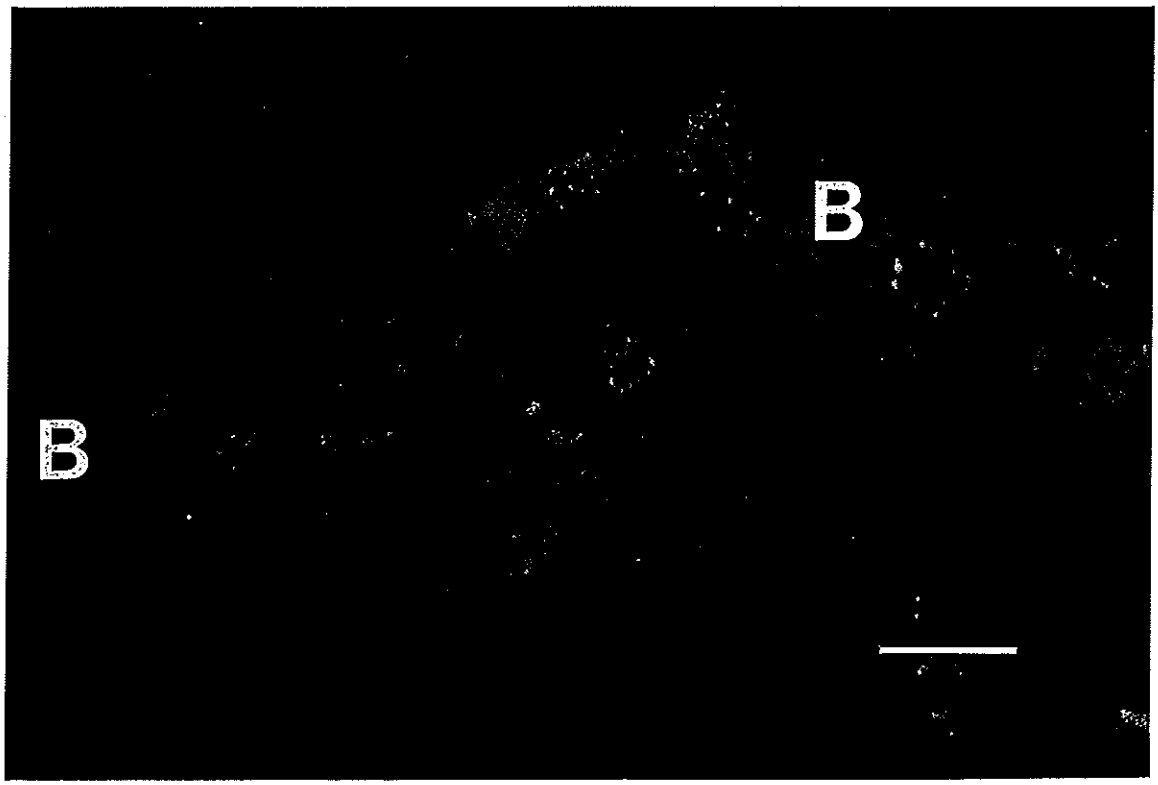


Fig. 3 Micrograph of Biofilm D in cross-section with CTC staining. The biofilm was exposed to 2 mg l^{-1} chlorine for 1 h prior to CTC staining. Green cells represent nonrespiring cells and red bacteria indicate respiring cells. Shown is the upper layer of the biofilm near the biofilm-bulk fluid interface (B). Bar = 10 μm. (See Color Section.)

application of this technique was the inability to obtain sections of thick (> 4 mm) biofilms or films containing significant amounts of abiotic material. Unlike most laboratory-grown biofilms, fouling biofilms from industrial systems contain undefined mixed microbial populations and may include such diverse abiotic material as corrosion products, scale inhibitors, slit, and fibers. These features of industrial biofilms are probably responsible for their inherent fluorescence and difficulty of sectioning. Despite these impediments, the combination of CTC staining with cryosectioning has the potential to provide some qualitative information about spatial patterns of microbiological activity within fouling biofilms.

Acknowledgements

This project was supported by the Centre for Biofilm Engineering at Montana State University, a US National Science Foundation-sponsored Engineering Research Center (cooperative agreement EEC-8907039), by a grant from the US National Science Foundation EPSCoR program, and by Calgon Corporation and Nalco Chemical Company. The authors are grateful to Drs R M Donlan, L R Robertson and F P Yu for providing samples and helpful discussion. The technical assistance of Gayle Callis and Eric Wentland is also appreciated.

References

Alberti S, Park D R, Herzenberg L A (1987) A single laser method for subtraction of cell autofluorescence in flow cytometry. *Cytometry* **8:** 114–119

Halldén G, Sköld C M, Eklund A, Forslid J, Hed J (1991) Quenching of intracellular autofluorescence in alveolar macrophages permits analysis of fluorochrome labeled surface antigens by flow cytofluorometry. *J Immunol Methods* **142:** 207–214

Hed J (1977) The extinction of fluorescence by crystal violet and its use to differentiate between attached and ingested microorganisms in phagocytosis. *FEMS Microbiol Lett* **1:** 357

Huang C-T, Yu F P, McFeters G A, Stewart P S (1995) Nonuniform spatial patterns of respiratory activity within biofilms during disinfection. *Appl Environ Microbiol* **61:** 2252–2256

Ramsing N B, Kühl M, 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

Rodriguez G G, Phipps D, Ishiguro K, Ridgway H F (1992) Use of fluorescent redox probe for direct visualization of actively respiring bacteria. *Appl Environ Microbiol* **58:** 1801–1808

Schaule G, Flemming H-C, Ridgway H F (1993) Use of 5-cyano-2,3-ditolyl tetrazolium chloride for quantifying planktonic and sessile respiring bacteria in drinking water. *Appl Environ Microbiol* **59:** 3850–3857

Steinkamp J A, Stewart C C (1986) Dual-laser, differential fluorescence correction method for reducing cellular background autofluorescence. *Cytometry* **7:** 566–574

Smith J J (1995) Survival, physiological response and recovery of enteric bacteria exposed to a polar marine environment. PhD dissertation, Montana State University

Werkmeister J A, Tebb T A, Peters D E, Ramshaw J A M (1990) The use of quenching agents to enable immunofluorescent examination of collagen-based biomaterials showing glutaraldehyde-derived autofluorescence. *Clin Mater* **6:** 13–20

Yu F P, McFeters G A (1994) Rapid in situ assessment of physiological activities in bacterial biofilms using fluorescent probes. *J Microbiol Methods* **20:** 1–10

Yu F P, Callis G, Stewart P S, Griebe T, McFeters G A (1994) Cryosectioning of biofilms for microscopic examination. *Biofouling* **8:** 85–91