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Tetrazolium Reduction-Malachite Green Method for Assessing the Viability of Filamentous Bacteria in Activated Sludge

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A method was developed to assess the activity of filamentous bacteria in activated sludge. It involves the incubation of activated sludge with 2(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride followed by staining with malachite green. Both cells and 2(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride-formazan crystals can be observed in prepared specimens by using bright-field microscopy. This procedure allowed us to distinguish between inactive and actively metabolizing filaments after chlorine application to control the bulking of activated sludge.

Filamentous organisms are normally present in activated-sludge flocs, providing a structural matrix to which zoogleal organisms attach (15). However, their outgrowth from the floc in quantities exceeding 10 km per g of suspended solids causes sludge-settling properties to significantly deteriorate (14). Because there are over 20 different types of filamentous organisms which can occur in activated sludge (4; P. F. Strom and D. Jenkins, 51st Annu. Conf. California Water Pollut. Assoc., San Diego, Calif., 1979), it is often difficult to determine what caused the outgrowth of a specific filamentous organism. Moreover, in many cases in which a specific cause can be identified, plant flow schemes and operational capabilities are too inflexible to allow implementation of the appropriate remedies in a timely manner. To avoid these difficulties, nonspecific techniques such as chlorine application are commonly used to control poorly settling (bulking) sludge. Chlorination of activated sludge is effective when the dosage is high enough to kill off the extended (and exposed) filaments, but not so high as to significantly inhibit the floc-forming microorganisms. Physiological activity assays such as dissolved oxygen uptake or dehydrogenase activity (2, 5, 9) do not differentiate between the activities of the zoogleal and the filamentous components of the flocs. Less sensitive indicators (e.g., morphological observation by using phase microscopy and sludge volume index) are available to assess whether a given chlorine dosage is having the desired effect. A rapid and sensitive technique for determining the specific activity of filamentous organisms in activated sludge would enable more precise use of chlorine (or other toxicants such as hydrogen peroxide) in controlling bulking and could possibly be applied in a bioassay technique to predict effective dosage ranges.

Bacterial metabolism can be assessed by measuring the activity of the electron transport system, which is based on the reduction of tetrazolium salts (13). Electron transport system activity was shown to be related to respiration (3). Zimmermann et al. (16) developed a technique based on the reduction of 2(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to INT-formazan crystals by bacterial electron transport system activity. INT acts as a hydrogen acceptor, and respiring (active) bacteria will accumulate water-insoluble red INT-formazan crystals. This procedure was used to examine the levels of respiring bacteria in the surface layer of a salt marsh (6) and in freshwater environments (11). However, in all of the above procedures it was necessary to combine the INT technique with epifluorescence. Thus, it was necessary to first identify the bacterial cell with epifluorescence and then switch to bright-field microscopy to observe the formazan crystals. This study deals with the use of bright-field microscopy and a new staining technique for the examination of the activity of filamentous bacteria in activated sludge after chlorine addition.

A continuous-flow activated-sludge unit consisting of a 20-liter aeration basin with an integral clarifier was seeded with activated-sludge culture from the University of Florida (Gainesville) wastewater treatment plant. The operating conditions were as follows: feed, domestic settled sewage; hydraulic detention time, 8.5 h; mean cell residence time, 6 days; dissolved oxygen concentration, 1.0 mg/liter; and mixed liquor volatile suspended solids, 1.7 g/liter. The sludge volume index (1) increased from 70 to 250 ml/g in the period of 1 week, coinciding with the proliferation of the filamentous bacterium *Sphaerotilus natans*.

One ml of a 0.2% (wt/vol) solution of INT

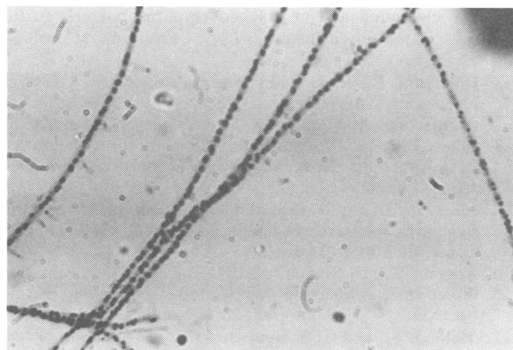

FIG. 1. Filamentous bacteria stained by the INT-malachite green method (×1,600).

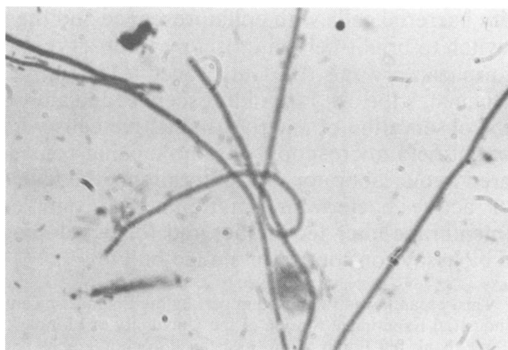

FIG. 3. Filamentous bacteria, in the presence of 25 μg of chlorine per liter, stained by the INT-malachite green method (×1,600).

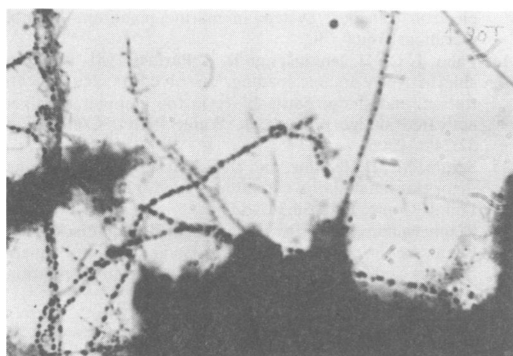

FIG. 2. Sludge floc, with associated filamentous bacteria, stained by the INT-malachite green method (×1,600).

(obtained from Eastman-Kodak, Rochester, N.Y.) was added to 10 ml of mixed liquor, and the mixture was incubated at room temperature, in the dark, for 20 to 30 min, as reported by Zimmermann et al. (16). The INT solution was sterilized via membrane filtration and can be stored in a refrigerator at 6°C for up to 1 month. After fixation with 0.1 ml of 37% formaldehyde, two drops of the mixture were spread on a clean glass slide, covering a surface area of approximately 6 cm^2. The smears were air dried and gently fixed with heat. The dry smears were covered with a 0.05% (wt/vol) aqueous solution of malachite green (MC/B, Norwood, Ohio) for a period of 1 min. The malachite green was then drained completely (do not wash) off the slide (the residual stain creates no problems), and the smear was allowed to dry before examination under oil immersion (×1,600), using bright-field microscopy.

Our goal was to develop a staining technique which would allow us to observe, in the same field, active (with red formazan crystals) as well as inactive filamentous bacteria. A counterstain, which would make epifluorescence unnecessary, was thus needed. Various counterstains (methylene blue, alcian blue, and malachite green) were tried, and malachite green was the most suitable (data not shown). Figures 1 to 3 show the results of the INT-malachite green technique. Figure 1 shows *S. natans* from a bulking sludge. The filaments are stained in green, within which the formazan crystals appear as dark red spots. Figure 2 shows active bacterial filaments extending from a sludge floc. After treatment of activated-sludge mixed liquor with 25 μg of chlorine per liter, almost no formazan crystals could be detected within bacterial filaments (Fig. 3).

Other methods have been proposed for distinguishing between inactive and actively metabolizing bacteria. Autoradiography (12) is a complicated procedure. Epifluorescence (7) is controversial with regard to that type of distinction (8). Kogure et al. (10) reported a nalidixic acid-yeast extract procedure for distinguishing between active and nonactive bacteria. Nalidixic acid prevents DNA synthesis and causes the elongation of actively respiring bacteria in the presence of yeast extract. The red fluorescent elongated cells may be viewed via epifluorescence (11). This method is not suitable however for the observation of certain cultured cells (11) and sewage pond bacteria (Bitton, unpublished data). By combining this method with the INT procedure, fluorescence may be blocked by the dark formazan crystals (11). Another drawback is the growth of nalidixic acid-resistant bacteria (11). The combination of the INT method with epifluorescence makes it necessary to first iden-

tify bacterial cells with epifluorescence and then switch to bright-field microscopy to observe the formazan crystals (6). Our procedure is simple, eliminates the use of epifluorescence, and allows the observation of inactive and active cells with bright-field microscopy. It is now being considered in our laboratory for determining the levels of active bacteria in environmental samples (membrane-filter technique) and for developing a bioassay for control of sludge bulking.

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