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The Immunofluorescence Approach in Microbial Ecology

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1. Introduction

Fluorescent markers appropriately conjugated to antibody proteins provide the basis for a method to visualize those antibodies as they participate in antigenantibody reactions. The method is referred to as the fluorescent antibody (FA) or immunofluorescence (IF) technique; it has been in widespread and successful use in medical microbiology and in pathology as a highly sensitive and specific cytochemical staining procedure for many years. Most current applications of the technique are for the localization of cellular and viral antigens in tissues and for the rapid detection and identification of infectious agents.

The same features of the FA technique that make possible the detection and identification of microbial pathogens in animal tissues provide its potential for autecological application in microbial ecology. Autecology, as an approach whereby the individual microorganism is studied directly in its natural environment, has been virtually unavailable to microbial ecology because of technical difficulties imposed by the small size and nondescript morphology of microorganisms and by the physical complexities of the natural environment. Direct microscopic examination of the natural environment could become a powerful tool for autecological study if a method were available to recognize one specific microorganism among all others present in the field of view. Such a capability is inherent in the FA technique. Because extremely small amounts of fluorochrome-marked antibody may be detected against a dark background by fluo

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rescence microscopy (Goldman, 1968), microorganisms which bind the antibody may be visualized with great sensitivity. The companion property of specificity results from the great precision of the antigen-antibody reaction. Developments to exploit the unique potential of FA for microbial ecology have come about mostly in the past decade with greater availability of the necessary equipment and facilities and with the resolution of certain technical difficulties.

1.1. Theory and General Considerations

The theory for FA as applied to microbial ecology is simple (see Fig. 1). An isolate of some particular interest is used as an antigen for the preparation of active antiserum. Antiserum to the isolate is labeled with a fluorescent dye, usually fluorescein isothiocyanate (FITC), and the labeled antiserum (FA) is then applied as a stain to a sample of a natural environment. If the microorganism of interest is present in the natural material, the FA will combine specifically with it in an antigen-antibody reaction. When the stained preparation is examined by fluorescence microscopy, the outline of the microorganism of interest is seen by virtue of light emitted from FA bound to its surface. Other organisms present in the same field are devoid of antibody and hence invisible. Figure 2 illustrates how IF can recognize the desired bacteria among all the other microorganisms present in a natural sample. The preparation is a smear from human feces which

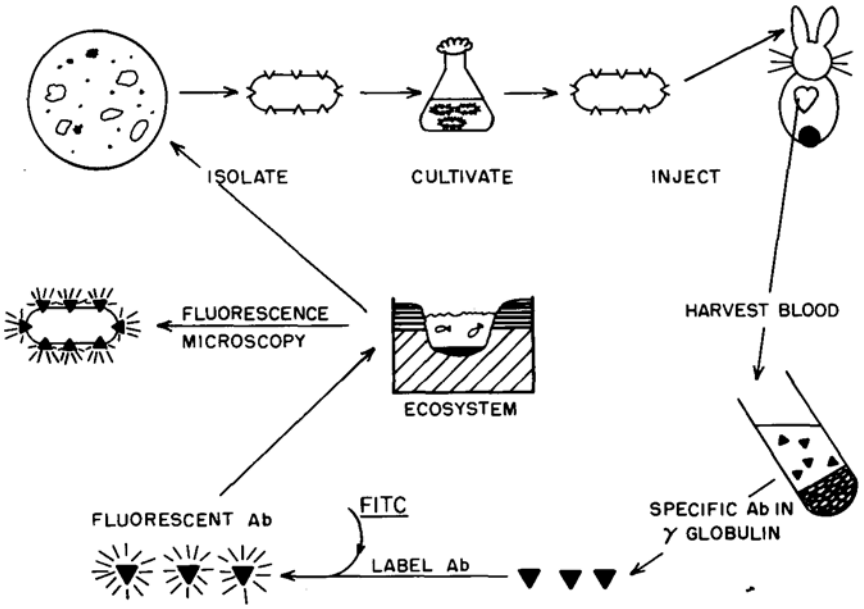

Figure 1. Theory and general considerations in the application of immunofluorescence to autecological studies of microorganisms in natural samples.

was artifically inoculated with *Salmonella typhimurium.* When the smear is stained with a common fluorescent stain such as acridine orange (IA), a wide variety of unidentifiable microorganisms could be seen, but when the specific FA is used, only the organism of interest stains.

Two methods of FA staining are in common usage, the "direct" and the "indirect." For the direct FA, the antibody against the microorganism of interest is itself labeled. The major advantage of the direct method resides in the ability to work with a single antibody reagent. In the indirect or "sandwich" method, the specific antibody is applied unlabeled and is detected on the surface of the cell by a second antibody which is fluorescent and which is directed against the specific antibody. The specific antibody normally is prepared in rabbits injected with the antigen of interest, and the indicator antibody is prepared in goats in response to rabbit globulin. The goat anti-rabbit serum is labeled with a fluorochrome. An obvious advantage of the indirect method is that a single fluorescent antibody can be used to detect the localization of various specific antibodies.

There are a number of background references devoted to the theory, principles, and practical considerations of FA techniques in general. All are oriented to the many applications of FA for use in medical microbiology and pathology. None includes recognition of the much more recent development of the use of FA in microbial ecology. The treatises of Goldman (1968), Kawamura (1977), and Nairn (1975) are the most extensive and should be accessible to the microbial ecologist concerned with the application of FA or the interpretation of FA data. Goldman in particular gives a fine account of the history wherein the central contributions of Albert Coons and co-workers at Harvard University are appropriately highlighted. A small, highly practical, and extremely useful manual published by Cherry *et al.* (1960) unfortunately is out of print, but worth a search on the part of the initiate to FA techniques. Basic immunological procedures starting with the handling of animals are covered in a well-illustrated text by Garvey *et al.* (1977).

The fluorescence microscope is a conventional light microscope equipped with a light source of suitable intensity and wavelength and with necessary filters. Information on the various components of suitable fluorescence microscopy systems is readily available from technical representatives of the major microscope manufacturers.

Appropriate controls are important in all FA procedures, but particularly so when applied to ecological questions. Microbial ecology may call for antibodies to microorganisms whose serology is unknown, and for staining of natural materials of great physical, chemical, and biological diversity. Controls relating to the specificity of the FA reagents and other types of controls as they pertain to applications in microbial ecology were outlined by Schmidt (1973).

1.2. Development of FA for Microbial Ecology ,

The first paper clearly indicating the potential of FA for microbial ecology was that of Hobson and Mann (1957). They demonstrated that several bacteria

isolated from rumen could be detected in *situ* by staining rumen contents with appropriate fluorescent antibodies. Surprisingly, this interesting work was not extended by the authors or other rumen microbiologists for many years. Development of FA for the most complex of natural ecosystems, those of terrestrial environments, began with the paper by Schmidt and Bankole (1962). This and subsequent papers (Schmidt and Bankole, 1963, 1965) were concerned with detection of the fungus *Aspergillus flavus* in soil. Isolates of the fungus were cultured in the vicinity of buried glass microscope slides and subsequently observed on those contact slides by immunofluorescence. The specificity of the FA was found to be adequate for autecological study of *A. flavus,* and background fluorescence could be controlled by the use of certain filters.

Whereas the autecological study of certain fungi by FA appeared feasible for terrestrial habitats, such was not the case for bacteria. The major problem was that of nonspecific adsorption of the FA to soil materials (nonspecific staining). Due to the small size of bacteria, the amount of FA deposited on the cell is very low relative to that localized on a fungal hypha; consequently, the microscopy demands the most efficient lighting and filter systems possible. Required also is a desirable dark, nonfluorescing background. This is necessary not only because the sites of fluorescence are so small that they must be seen in contrast, but also because nonspecifically fluorescing background materials may obscure or even resemble bacteria. Levels of nonspecific staining that could be controlled for fungi with inefficient filter combinations or tolerated because of their larger size and distinctive morphology could not be tolerated for bacteria.

The problem was avoided to some extent in work reported by Hill and Gray (1967). FA techniques were used for differentiation of *Bacillus subtilis* and *B. circulans* in a very sandy forest soil which did not give excessive background fluorescence. Attempts on their part to improve the background with a variety of reagents were largely unsuccessful. Limitations imposed by nonspecific staining were illustrated in the photomicrographs of Schmidt *et al.* (1968). They characterized an FA for a strain of the symbiotic nitrogen fixer *Rhizobium japonicum.* The FA was shown to be highly specific at the strain level and capable of detecting that strain in unknown, wholly natural field soils. Despite the obvious utility of the approach, it was shown that specifically stained cells were obscured and artifacts were troublesome in certain microscopic fields where the conjugate attached nonspecifically to soil colloids and soil films.

Difficulties with respect to nonspecific adsorption of FA to soil background were satisfactorily resolved by Bohlool and Schmidt (1968). A dilute gelatin solution was partially hydrolyzed at a high pH and applied to a soil preparation before staining with the labeled antibody. Such pretreatment with the gelatin apparently saturated the sites of nonspecific adsorption. Subsequent application of the FA resulted in specific staining of the antigens with no interference from the gelatin and with no adsorption of FA to nonspecific sites. By conjugating the gelatin with a fluorochrome of contrasting color to that used on the antibody, the gelatin not only prevented nonspecific staining but also served as a counter-

stain which made it possible to see the specifically stained bacteria in relation to the gelatin-labeled portions of the microenvironment on a contact slide. Rhodamine isothiocyanate was the fluorochrome conjugated to the hydrolized gelatin.

Early applications made of FA techniques in microbial ecology were summarized in a review by Schmidt (1973). The number was relatively small, and the nature of the reports reflected a concern primarily for the detection and identification of different microorganisms in their natural habitats. It was noted that FA in microbial ecology was still largely in a developmental phase and that few serious autecological problems had been addressed.

One inherent limitation that hampered more effective use of FA for problems dealing with microbes in terrestrial environments especially was the qualitative nature of the technique. Many problems require information gained from enumeration of microorganisms in relation to a process or to a change in the environment. Enumeration data provide a basis for the estimation of biomass, growth rate, and growth response to environmental variables. Such information is obtainable by other techniques only for extreme environments where species diversity is sharply limited (Brock, 1971); hence, it was highly desirable that the unique specificity of the FA be directed to quantitative examination of the normal habitat with characteristic complexity and diversity.

The basic problem in enumerating FA-stained bacteria is again size related. Because of the magnification needed, the area of the field of view is very small, and the amount of soil that can be examined per field without significant interference is correspondingly small. As a consequence, populations must be high in order to encounter a reasonable number of bacteria. Total direct counts by conventional microscopy, as an example, use a maximum of about 1 mg of soil/ cm^2. To count one bacterium per field under such circumstances, at approximately 1000 X magnification, the cell density must be about 10^6 to 10^7/g soil. The problem is less severe for aquatic systems since microorganisms in a given volume may be concentrated onto a membrane filter and observed with incident light fluorescence microscopy. The maximum of dispersed soil that may be examined in a similar fashion is about that deposited during filtration of 0.1 ml of a 1/10 soil dilution. In order to count 1 bacterium per field under such circumstances, the cell density must be about 2 X 10^6 /g. Quite obviously if quantitative autecology were to be carried out at realistically low population levels for the terrestrial system, it would be necessary to separate the bacteria from soil and concentrate them on a surface for FA staining.

A method devised for quantitative FA examination of soil bacteria was first outlined by Bohlool and Schmidt (1973a) and later presented in more detail by Schmidt (1974). Essential steps in the method involve dispersion of a diluted soil sample to release bacteria into suspension, flocculation to remove soil particulates from the supernatant fluid, filtration of a portion of the supernatant fluid through an appropriately pretreated membrane filter, and finally then microscopic enumeration of the specific bacterium on the FA-stained membrane filter surface. Methodological details may be modified somewhat to accommodate to

the special properties of the particular soils under examination. The availability of suitable quantitative FA procedures may be expected to provide a new and workable approach to problems in microbial ecology that are currently refractory.

2. Nitrogen-Fixing Bacteria

Papers on nitrogen fixation and the bacteria associated with the process probably comprise a substantial majority of the literature of microbial ecology. Despite their obvious biogeochemical importance and the research attention devoted to them, the nitrogen-fixing bacteria are little known with respect to their biology and activity in natural environments. Only the symbiotic nitrogen fixers, with emphasis on the root-nodule bacteria of legumes, have been studied intensively in their nitrogen-fixing milieu. The root nodule presents a discrete and manipulatable niche where the bacteria occur in pure culture. Niches for the free-living nitrogen fixers and for free-living stages of symbiotic fixers are in the mixed-culture communities of natural habitats, which are inaccessible to direct study. It is not surprising that aspects of the nitrogen-fixing bacteria were among the first of the ecological applications of FA techniques.

2.1. Rhizobia

Various methods are available to study rhizobia once they are concentrated in their main niche in nature, the legume root nodule. FA is a useful adjunct to these methods for the rapid identification and characterization of strains in the nodule isolates, but the main promise of FA in *Rhizobium* ecology lies in the study of events prior to nodulation. Prenodulation events are those that involve the ecology of the *Rhizobium* strain as it adapts to the soil, responds to the rhizosphere of the developing legume host, somehow recognizes the appropriate nodulation site on precisely the right legume root, and begins to interact with the plant to form a functional nodule. Such events are of great practical importance to the effective management of *Rhizobium* and its host legume, but little detailed information is available because the complexity of the plant-soil-bacterium interactions has permitted only indirect experimental approaches. The FA technique is of special pertinence to the ecology of free-living rhizobia because it is the only method to provide the potential for direct investigation of the *Rhizobium* in the soil. Its attractiveness is further enhanced for such studies by the existence of a substantial background literature on the serology of the genus (Graham, 1963; Holland, 1966; Vest *et al.,* 1973; Dudman, 1977).

The first paper to report an autecological study of rhizobia was that of Schmidt *et al.* (1968), a paper that was concerned with *R. japonicum.* A number

of important aspects emerged from this study. It was found that the FA-staining reaction was highly strain specific: FA prepared against 1 strain did not crossreact with other strains of *R. japonicum* belonging to at least 6 other serogroups, nor with other rhizobia or 65 unidentified soil bacteria isolated from 12 soils. Specificity at the strain level is highly desirable for study of the rhizobia because the ecological questions are usually asked in terms of a particular strain of interest because of its superior ability to fix nitrogen. Also demonstrated was the ability to detect the antigen strain on contact slides during its growth in an autoclaved soil. The technique moreover detected FA-reacting bacteria on contact slides in a field soil whose rhizobial content was unknown. This presumptive evidence for the natural occurrence of the antigen strain was strengthened when it was found that the same soil could be used as an inoculant of soybeans to produce nodules whose bacteroids cross-reacted with the specific FA. A final aspect of this study was the evidence that FA was useful to detect and identify a bacteroid strain directly in nodule crushes.

Although FA detection in soil of a specific *R. japonicum* strain was clearly feasible, Schmidt *et al.* (1968) noted the nonspecific adsorption of FA to soil particulates and soil films. Further modification of the technique to overcome nonspecific staining was successful (Bohlool and Schmidt, 1968). In subsequent experiments, the FA technique as modified to control nonspecific staining was used (Bohlool and Schmidt, 1970) for the detection of two distinct serotypes of *R. japonicum* in a range of nonsterile field soils.

R. leguminosarum was studied by Zvyagintzev and Kozhevin (1974) following inoculation at 10^7 to 10^8 cells/g in a soddy-podzolic and chernozem soil. A variation of the direct smear technique was used for quantification by indirect IF. No specificity control data were presented, which is unfortunate, for the authors made the interesting observation that the addition of glucose favored the development of *R. leguminosarum* in the soils. The ability of rhizobia to compete with other bacteria in soil for available substate is a highly important ecological feature, and it is imperative that FA data reporting a free-living growth response of rhizobia include appropriate controls to insure that only the specific *Rhizobium* was detected.

All of the work cited, together with the development of suitable procedures to permit the enumeration of bacteria in natural habitats by FA (Bohlool and Schmidt, 1973a; Schmidt, 1974), was either preliminary in the sense of assessing the FA technique for ability to detect rhizobia in the complex soil environment, or developmental as was necessary to overcome the limitations that were encountered. None of the ecological questions that have been difficult or impossible to approach by indirect methods had yet been addressed by FA.

Considerable attention has been focused on the inability of desirable inoculant strains of rhizobia to compete with indigenous strains in field soils (Vest *et al.,* 1973). The soil-adapted strains commonly account for the majority-of nodules even though high populations of a potentially better nitrogen-fixing strain

are added as inoculant. Factors affecting the competitive success of given rhizobia have not been resolved, and studies of competition between strains have had to rely on the end result of competitive interactions-the nodule. Competition studies have not yet taken advantage of FA to examine the obviously important population dynamics of competing strains in the soil and rhizosphere before the race has been won. FA can be used in the terminal analysis of nodule rhizobia for strain identification purposes (Schmidt *et al.,* 1968; Trinick, 1969) and hence constitutes a convenient means to identify the strain or strains that have been successful under various competition conditions (Jones and Russell, 1972; Bohlool and Schmidt, 1973b; Lindemann *et al.,* 1974).

Jones and Russell (1972) carried out a highly artificial study involving two serologically distinctive strains of *Rhizobium* trifold, separately or in a pureculture mixture, and aseptically grown clover seedlings. FA was used to serotype the nodules. A more extensive but still preliminary study of competition was reported by Bohlool and Schmidt (1973b). They measured the ability of *R. japonicum* strain USDA 110 to nodulate soybeans in competition with the resident rhizobia of a silt loam soil. Varying numbers of strain 110 were added to soil containing a low but constant population of indigenous *R. japonicum,* and the soil was used to inoculate soybeans. Relatively high inoculant/resident ratios were needed to overwhelm the resident populations. When the log of the numbers of introduced strain 110 was plotted against the percentages of strain 110 nodules, a sigmoidal curve resulted. The authors suggested that curves similarly derived for any given soil might be descriptive of the resident competition barrier to be overcome by an inoculant strain. Strain 110, added to the test soil and then allowed to incubate, apparently failed to persist since it formed progressively fewer nodules in competition with indigenous strains with increasing time.

Competition experiments of another sort were carried out by Lindemann *et al.* (1974) with detailed IF examination of nodules to provide evidence that two serologically different strains of *R. japonicum* may occur in a single soybean nodule. Strains USDA 117 and 138 were used in 50:50 mixtures at different population levels to inoculate soybeans grown in Leonard jars. At an inoculation density of 10^8 rhizobia/plant, 32% of the nodules contained both strains, and this double infection declined as inoculum density decreased. Strain 138 generally was more competitive than strain 117. The widely accepted assumption that only a single strain of *Rhizobium is* to be found in a given nodule was found not necessarily to be the case.

Double infection was also demonstrated in nodules of lentils grown in the field (May, 1979). Seeds pelleted with mixtures of serologically distinct strains *of Rhizobium leguminosarum* were planted in a Hawaiian inceptisol. IF typing *of* the resultant nodules revealed that over 30% *of* nodules could contain two strains, if two highly competitive strains (e.g., Hawaii 5-0 and NZP5400) were used as inoculum. On the other hand, when the pair in the inoculurrtincluded a poor competitor (e.g., NZP5400 and 128A12), then only 6% *of* the nodules were

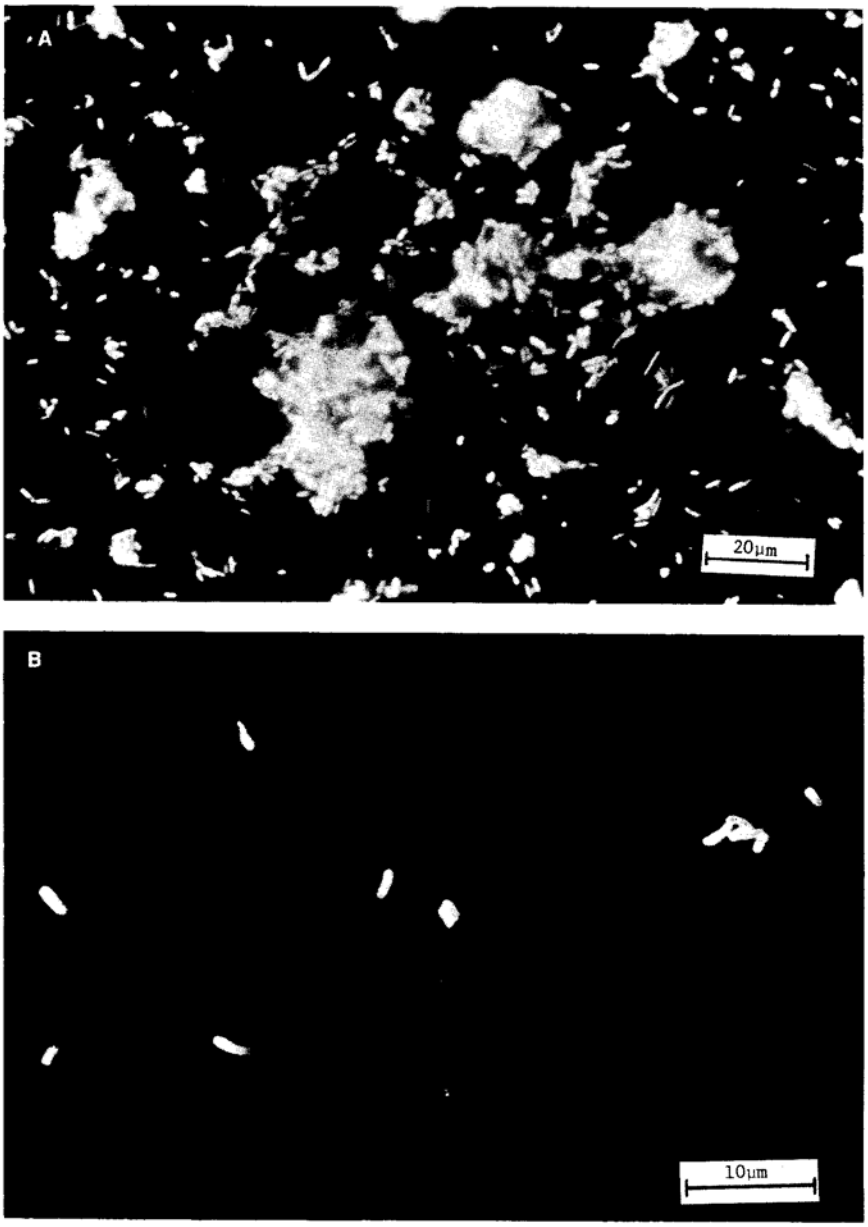

Figure 2. Smear from human feces which was artificially inoculated with *S. typhimurium* Succ-L. A: Stained with acridine orange. B: Stained with FA against *S. typhimurium* Succ-L.

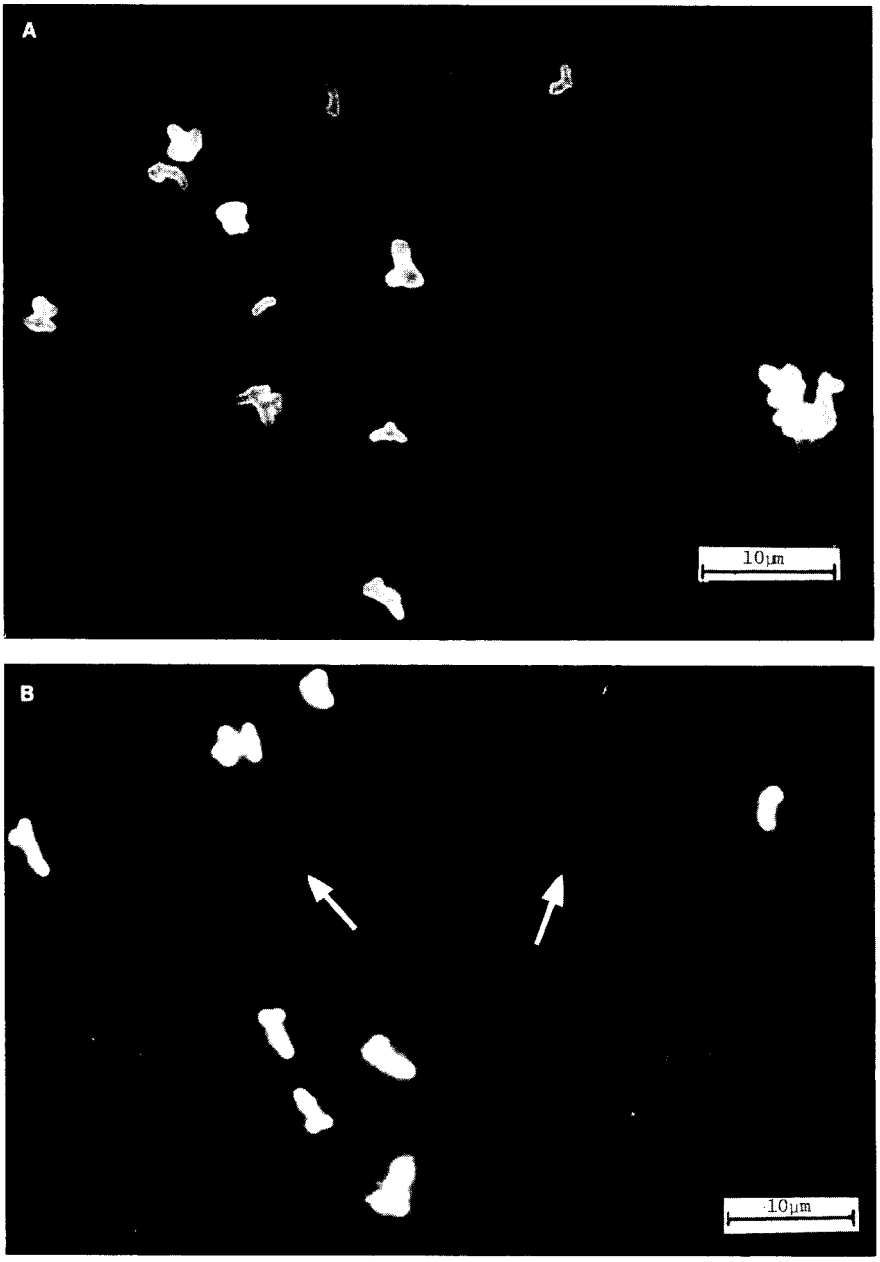

Figure 3. Mixed infection in nodules of lentils grown in the field. Smears from two nodules from the same plant were stained with FA against strain Hawaii 5-0. A: All the bacteroids stain (single strain in the nodule). B: Only a proportion of bacteroids stain (two strains in the same nodule). The unstained bacteroids (arrows) in B are visualized by the use of a double-light system using a dark-field condenser. From May (1979) and May and Bohlool (1979).

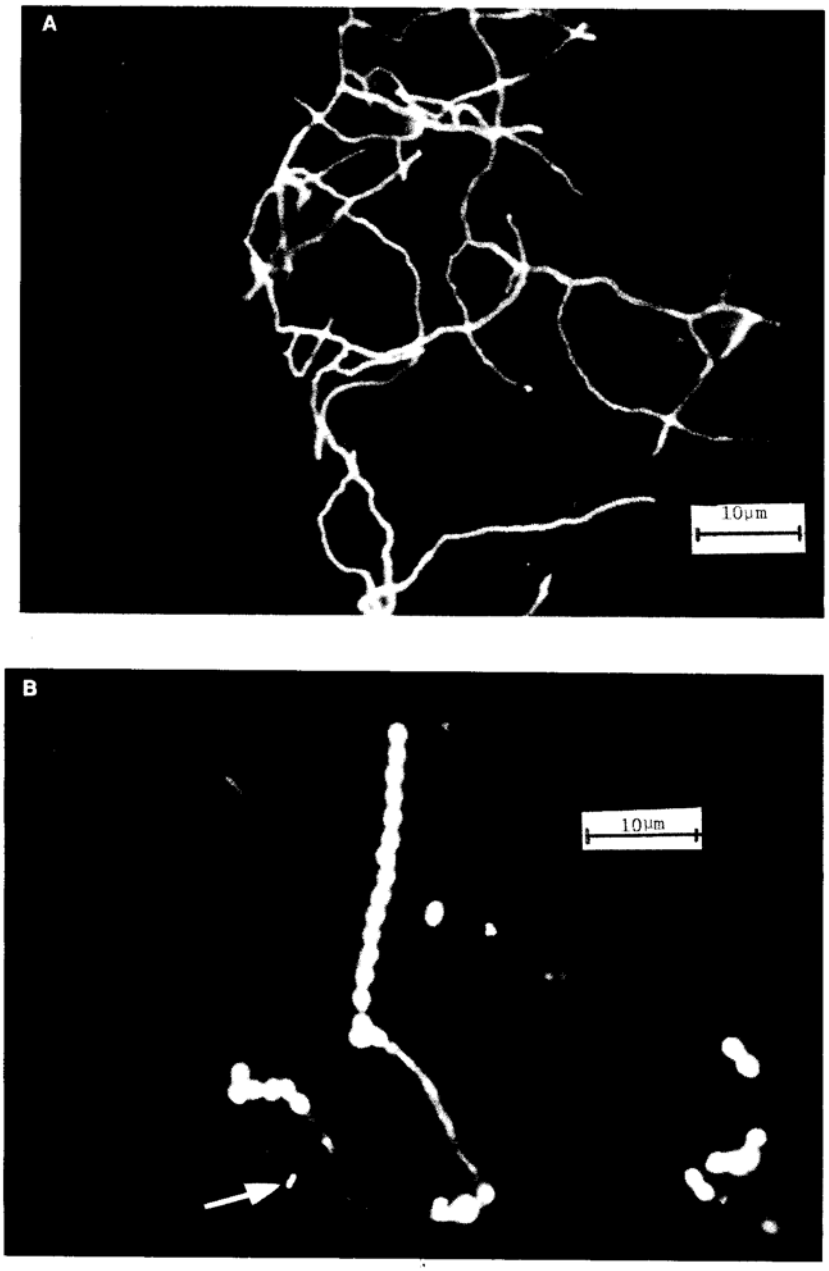

Figure 4. Problems associated with (A) heterologous cross-reaction and (B) nonspecific staining. A: FA against *R. japonicum* strain Nitragin 61A72 cross-reacts with an unidentified soil actinomycete growing in autoclaved soil. From Bohlool and Schmidt (1970). B: FA against *R. japonicum* strain USDA31 nonspecifically stains conidia of a soil *Fusidium* growing, together with USDA31, in autoclaved soil. Arrow depicts a cell of strain USDA31 attached to the fungal filament. From Bohlool and Schmidt (unpublished).

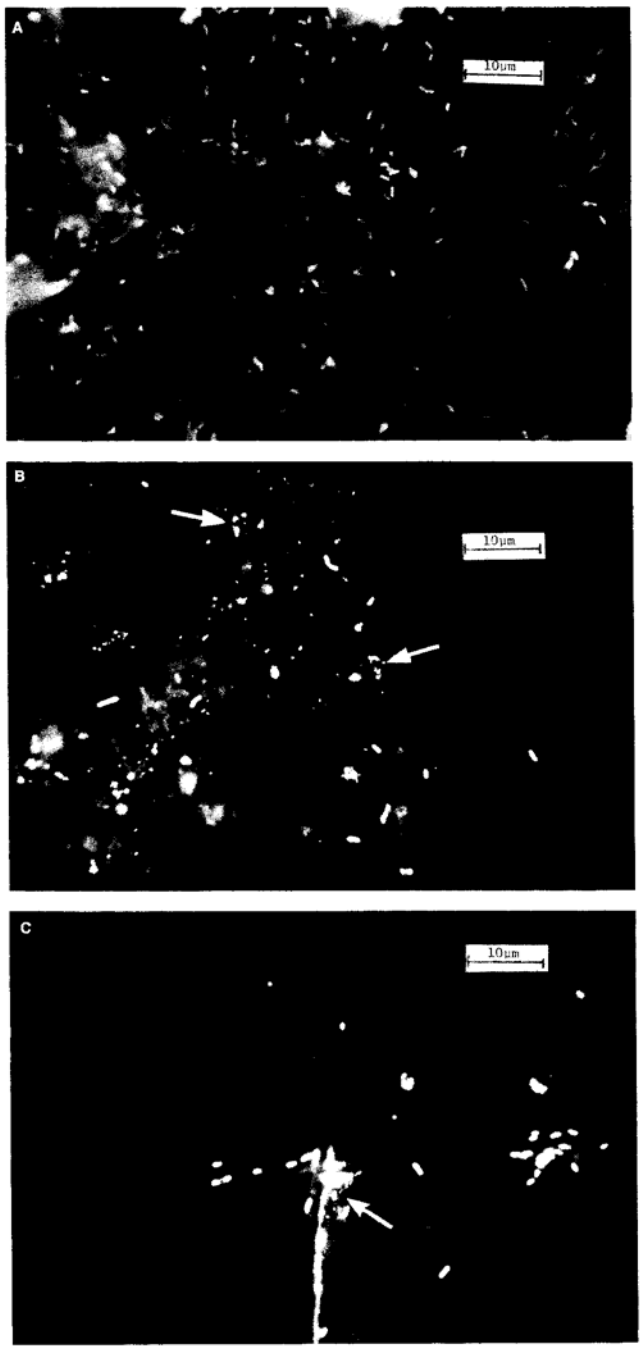

Figure 5. Disintegration of *R. japonicum* USDA110 cells in normal soils. A: Intact cells at 1 day. B: Intact cells and antigen debris (arrows) at 8 days. C: Intact cells and solubilized antigen (arrow) along filament of unidentified soil fungus.

doubly infected. Figure 3 shows IF typing of two nodules from the same lentil plant: A, nodule formed by a single strain and B, nodule containing two strains.

The procedures to use FA to quantify soil bacteria "in situ" were developed with R. japonicum as a model system and were applied to study the growth rate of that species in soil. Bohlool and Schmidt (1973a) made FA counts of strain 110 during growth of the bacterium in a sterilized silt loam soil. The growth curve was conventional and closely similar to that obtained by plate count, with a mean generation time of 14 hr by either procedure. Growth features of the same strain inoculated into nonsterile soil and followed by FA were strikingly different (Schmidt, 1974). Growth rates were extremely slow, with a mean doubling time of 240 hr in one of the test soils and 365 hr in another. These limited studies merely showed that FA could be used to study growth rates in soil and that growth in natural mixed-culture habitats probably bears little relation to pure-culture growth. The population features of both indigenous and introduced rhizobia in soil make up a highly important segment of rhizobial ecology which should be pursued.

The ecological question of *Rhizobium* response to the rhizosphere of developing legumes is one that should profit from direct FA examination. Indirect evidence by plant-dilution assay has indicated that rhizobia in the rhizosphere of host legumes increase greatly in numbers as compared to nonrhizosphere soil (Rovira 1961; Tuzimura and Watanabe, 1961; Robinson, 1967). Such data have been interpreted as "specific stimulation" by a number of authors (Nutman, 1965; Brown et al., 1968; Vincent, 1974), as indicating that a host legume somehow selectively enhances growth of its symbiont over other bacteria and other rhizobia. Such a rhizosphere effect may merely mean that rhizobia are good rhizosphere bacteria. The nature of the rhizosphere response and its significance to nodulation are unresolved problems.

The first direct study of the population dynamics of a *Rhizobium* in rhizospheres was carried out with FA specific for the serotype *R.* japonicum USDA 123 (Reyes and Schmidt, 1979). Indigenous populations of strain 123 were only a few hundred to a few thousand per gram of field soil. Rhizosphere effects from field-grown soybean plants were modest, reaching a maximum of about 10^4 to 10^5 cells of strain 123/g inner rhizosphere soil. Comparably slight rhizosphere effects were observed with field corn. Pot experiments with the same soil gave data comparable to that derived from field soils. No evidence suggestive of the specific stimulation theory was found in any of these experiments.

Vidor and Miller (1980a) used IF techniques to study four strains of *R.* japonicum heavily inoculated (10^7/g) into two normal Ohio soils. All strains introduced into a *R.* japonicum-free soil dropped to about 10^6/g level, which was maintained for 180 days. Strains inoculated into a soil with an indigenous *R. japonicum* population died off rapidly as compared to the other soil, and evidence suggestive of a lytic bacteriophage was presented. This work is of particular interest in that it illustrates the feasibility of a direct approach in the evalu-

ate of saprophytic competence in soil of specific strains and some of the factors involved in rhizobia-soil population interactions. In a companion study, Vidor and Miller (1980b) followed population changes by FA in the same *R. japoni*cum strains in soils supporting soybean plants. Populations of strain 110 increased substantially from 10^3 to 10^6/g soil, whereas cell densities of the other strains increased only slightly. Release of rhizobia from nodules to augment soil populations as observed by Reyes and Schmidt (1979) was also noted in this study.

2.2. Asymbiotic Nitrogen-Fixing Bacteria

The capacity for nitrogen fixation by free-living procaryotes is scattered among numerous genera. Recent listings (Knowles, 1977; LaRue, 1977) attest to the considerable biological and ecological diversity of forms with this potential. But the extent to which the nitrogen-fixing capacity of these organisms is actually expressed in nature is an unresolved problem that is crucial to an understanding of the nitrogen economy of the biosphere. Despite advances that have been made in the detection and assay of the nitrogen-fixation process, process chemistry will provide data of little predictive value unless related to the microbiology responsible for the process. Asymbiotic nitrogen fixation is tied to growth, and the nitrogen fixed may be suspected to vary in magnitude and rate with the growth response of the bacteria responsible. The problem thus involves the identification of the causative organisms in a nitrogen-fixing habitat and the quantification of their growth responses in relation to the atmospheric nitrogen they assimilate. The contribution of FA to asymbiotic nitrogen fixation will be in direct proportion to its capacity to identify the nitrogen fixers in a natural environment and to measure their growth. Very little has been done to date, and all of that has been highly preliminary.

2.2.1. Azotobacter

A great many media with highly selective properties have been devised for the isolation of specific microorganisms; however, those that may be used to quantify a specific microorganism in its natural environment with adequate efficiency are exceptional. Culture techniques fail to mimic the natural environment well enough or lack precision, or both. The genus *Azotobacter* should be well suited to selective plating based on simple, nitrogen-free medium, and such media have served to follow the fate of *Azotobacter* inoculated at high densities relative to the soil population, as with *Azotobacter paspali* (Brown, 1976). Selective plating is less effective at sizes of *Azotobacter* populations normally encountered in soil, since plates at low dilutions develop colonies of oligonitrophiles which obscure the *Azotobacter* (Duncan and Rosswall, 1974). As ideally suited for selective plate enumeration as this genus may appear to be, there remains an urgent need for a reliable method of enumerating natural populations of *Azotobacter* and other nitrogen-fixing bacteria (Postgate, 1972).

The only published reports dealing with FA study of *Azotobacter* are those of Tchan and DeVille (1970) and DeVille and Tchan (1970). They prepared an anti *Azotobacter vinelandii* conjugate with FITC and an anti *Azotobacter chroococcum* conjugate with rhodamine and mixed the two in equal proportions to detect the two *Azotobacter* species in inoculated soil smeared on slides. Distinction between the two species was excellent with the bivalent antibody stains. Control of both autofluorescence and nonspecific adsorption was achieved with methylene blue treatments. In the second of these papers (DeVille and Tchan, 1970), the authors report a procedure to quantify the occurrence of *Azotobacter* in soil by FA. They prepared serial dilutions of soils, smeared a known volume of soil dilution on a slide, and stained with FA. After noting the presence or absence of Azotobacter-stained cells in each smear, they used MPN (most-probable-number) tables to obtain estimates of the population. The validity of this approach is questionable on both practical and theoretical grounds. In practice, cell densities must be unusually high in order to detect a specific microorganism in a soil dilution (Schmidt, 1974). MPN statistical estimates are based on dilution to extinction so that some of the inocula for replicate tubes have one bacterium per unit volume. In the MPN procedure, the bacterium develops to a detectable level in positive tubes; if the inoculum itself is examined directly, as proposed by DeVille and Tchan, the likelihood of finding one bacterium in the 5000 or more microscope fields constituting a 1-cm^2 smear is extremely small. Failure to detect fluorescent bacteria in such endpoint smears could not, in theory, be considered as negative unless all 5000 fields were examined.

Specificity data were not included in the above reports, but the implication of the data was that the *Azotobacter* FAs were species specific. This was found not to be the case for an A. *chroococcum* FA which cross-reacted with certain strains of A. *vinelandii* (Schmidt, unpublished data). The serology of the genus has received little attention, and data are conflicting. Petersen (1959) found that A. *chroococcum* and A. *vinelandii* had agglutinogens in common and had serological affinities to *Beijerinckia* species. Norris and Kingham (1968) reported that antisera to strains of A. *chroococcum* and *A. vinelandii* did not cross-react with 31 strains representative of 5 heterologous species of *Azotobacter.* Both agglutination and precipitin reactions were restricted to homologous species, but considerable strain specificity was reflected in the data for A. *chroococcum,* whose serum agglutinated only the homologous strain and none of six others of the same species. Review of the Russian work by Rubenchik (1963) indicated both serological cross-reactivity between A. *chroococcum* and A. *vinelandii* and strain specificity for A. *chroococcum* serum. Serological relationships are equally unclear among other azotobacters.

Nonspecific staining is a factor that should receive particular attention in the ecological study of *Azotobacter* by FA. It has been observed (Schmidt, 1973) that certain soil organisms, notably some fungus spores, have been termed "universal acceptors," because they cross-react with any conjugate. Cysts of *A. chroococcum* were shown by Good (1972) to be included among the universal

acceptors. Vegetative cells of *A. chroococcum* only stained specifically, but once out of log-phase growth, cyst occurrence followed in culture, and cysts stained indiscriminately with all conjugates used. Nonspecific staining of *A. chroococcum* cysts was readily controlled by application of the rhodamine-gelatine conjugate of Bohlool and Schmidt (1968) without interference with specific staining. Cyst formation is also a feature of *A. vinelandii, A. beijerinckia,* and *A. paspali,* so that nonspecific adsorption of antibody must also be considered in any immunodetection system involving these species.

Extremely interesting and significant problems remain concerning the kind, distribution, and growth features of *Azotobacter* as related to the nitrogenfixing process in soil and rhizosphere. The consensus has been that *Azotobacter* probably adds little to the supply of fixed nitrogen in the biosphere, but the disclaimer, in the absence of adequate technology, has been the possibility of the specialized niche as illustrated by the *A. paspali Paspalum* association (Dobereiner *et al.,* 1972). FA appears to have excellent potential for the exploration of the specific *Azotobacter* in the special niche, but clearly its application must be preceded by more preliminary work in which careful attention is paid to controls.

2.2.2. Beijerinckia

Ecological questions as to the significance of *Beijerinckia* to nitrogen fixation are very similar to those of *Azotobacter.* The genus has been isolated occasionally from temperate soils, but it occurs commonly in tropical soils, where its special habitat for nitrogen fixation could be the rhizosphere (Knowles, 1977). Leaf surfaces of tropical plants may also provide a selective environment conducive to nitrogen fixation since Ruinen (1961) reported high populations of *Beijerinckia* on rain-forest vegetation in Indonesia.

The two reports of FA study of *Beijerinckia* were both concerned with a temperate-soil isolate obtained from the rhizosphere of rice (Diem *et al.,* 1977, 1978). The first of these was introductory, documenting the specificity of the FA as appropriate for detection of the antigen strain. None of 4 species of *Beijerinckia* cross-reacted, nor did any of 6 species of *Azotobacter* or any of 44 unidentified rhizosphere bacteria. Occurrence of the isolate in sterilized and nonsterile soil after inoculation was demonstrated indirectly by plating and subsequent FA examination of characteristic colonies. Colonization of the rice rhizosphere by the *Beijerinckia* isolate was followed by FA examination of plants grown in soil underlaid with agar in the laboratory. Roots imbedded in agar at the soil-agar interface were observed by direct IF, and the isolate could be seen distributed in scattered sites on root surfaces and in adjacent rhizospheres. Little ecological information was obtained in this study beyond the demonstration of at least brief persistence of *Beijerinckia* in the vicinity of rice roots. The feasibility of the approach was not fully resolved in these experiments in view of the artificial nature of the root environment used and the interfering autofluorescence noted for the roots.

2.2.3. Azospirillum

A report by Dobereiner and Day (1976) noted high acetylene-reduction activities in the rhizospheres of tropical grasses as a function of the occurrence of a spiral-shaped nitrogen-fixing bacterium. The bacterium was found to be concentrated in loosely organized subepidermal patches of root tissue identified as the site of nitrogen fixation. The plant-bacteria relationship was described as associative nitrogen fixation. The bacteria now have been designated as making up the new genus Azospirillum, with two species A. lipoferum and A. brasilense (Tarrand et al., 1978).

The considerable interest that has focused on associative symbioses involving Azospirillum has brought attention to the relevant ecological questions: the nature of the niche in the root, the events involved in establishing the niche, the behavior of Azospirillum in the soil and rhizosphere apart from the niche, and the fate of Azospirillum inoculants. All these are autecological questions immediately suggestive of FA approaches.

Schank et al. (1979) have issued the first report of FA detection of Azospirillum. Antibodies prepared against four strains of A. brasilense were applied by indirect IF. Specificity of the one FA examined most critically appeared to be excellent with respect to absence of cross-reaction with known cultures, including A. lipoferum, Azotobacter paspali, and numerous random soil isolates. Azospirillum isolates obtained from Brazil, Florida, Venezuela, and Ecuador generally cross-reacted with one or more of the four antisera, but four such-cultures failed to stain. The data indicate the specificity, as in most systems, is at the strain level, although many strains appear to share antigens of more than one serotype. The authors call attention to one antiserum, UM125A2, as highly strain specific, but their data do not support such a conclusion. Cross-adsorption studies are obviously needed to arrive at serogroups, and the use of adsorbed serum would probably be advisable in future studies if other workers encounter similar cross-reactivity at the strain level. Preliminary attempts to detect Azospirillum in root and rhizosphere preparations were generally encouraging. Crossreactive bacteria were found unevenly distributed in the root mucigel, but no localizations or rudimentary root structures indicative of a niche were observed. Problems with respect to root autofluorescence and nonspecific binding of the fluorescent goat anti-rabbit reagent were noted.

3. Nitrifying Bacteria

FA techniques are particularly appealing for ecological study of the nitrifying bacteria because such studies have been almost entirely blocked by the complexities of natural habitats and the specialized biology of the nitrifiers. The bacteria are slow-growing chemoautotrophs refractory to the usual microbiological approaches for examination, enumeration, and isolation. As a result, essentially nothing is known with respect to two basic aspects of nitrifying popu-

lations: the diversity of nitrifiers within a given population and the *in situ* nitrifying activities of the individual components of a population. It has only been possible to arrive at rough statistical estimates of the overall nitrifying population by means of the MPN method; the method has high statistical uncertainty, requires many weeks' incubation, and is selective only for certain components of the population (Belser and Schmidt, 1978b). The peculiar problems of the microbiology of the nitrifiers was discussed by Schmidt (1978).

More thorough understanding of the microbiology underlying nitrification is needed to control the process in its many applications such as nitrogen management in field crops, nitrogenous waste disposal in sewage systems, and prevention of groundwater and lake contamination with nitrate. With adequate information, it will be possible to construct models with useful predictive capabilities. Prospects for this are still somewhat remote, but there are indications that FA will make unique and significant contributions toward that goal.

3.1. Nitrite Oxidizers

Bacteria responsible for the second stage of nitrification, the oxidation of nitrite to nitrate, were the first of the nitrifiers to be studied by FA. Fliermans *et aL* (1974) prepared antisera against *Nitrobacter agilis* and N. *winogradskyi* and evaluated them as to specificity. Low-level cross-reactions between the sera were removed by cross-adsorption. All 15 *Nitrobacter* isolates that had been obtained from a variety of natural environments were found to react with one or the other of the 2 adsorbed FAs, but no cross-reaction was found in extensive tests that included 5 isolates of ammonia-oxidizing autotrophs and 668 heterotrophic bacteria from soil, water, and sewage. Staining of the *Nitrobacter* isolates with the highly specific FAs indicated that nitrite oxidizers were composed of only two serotypes, with the implication that *Nitrobacter* FAs were possibly species specific and that diversity among nitrite oxidizers was surprisingly limited. Both implications have proved not to hold, since *Nitrobacter* isolates obtained more recently by the nonenrichment procedures of Belser and Schmidt (1978b) were of several different serotypes than those cited above (Stanley *et al.,* 1979). Aside from the application of FA to the problem of diversity, this study included ecological data in the form of the detection of *Nitrobacter* in various ecosystems. A subsequent refinement of the FA technique, using autoradiography combined with FA to distinguish between metabolically active and inactive cells, was developed with *Nitrobacter* as a model (Fliermans and Schmidt, 1975). A soil known to contain *Nitrobacter agilis* by FA detection in the Fliermans *et al.* (1974) study was treated with nitrite and incubated to enrich for *Nitrobacter.* The soil was then exposed to $^{14}C0_2$ briefly and processed for combined FAautoradiography analysis. *Nitrobacter* cells were detectable by their specific fluorescence, and about 40% of these cells had incorporated the $^{14}C0_2$ as evidenced by silver-grain development in the autoradiographic emulsion directly above the cells.

Development of procedures to enumerate specific bacteria in soil (Bohlool and Schmidt, 1973a; Schmidt, 1974) was particularly pertinent to the autecology of the nitrifiers as a possible alternative to the MPN method. Schmidt (1974) inoculated a partially sterilized soil with a strain of N. *winogradskyi* originally isolated from that same soil, added nitrite as substrate, and followed *Nitrobacter* growth by FA along with nitrate formation. The data showed that the log plot of the population paralleled the log plot of nitrate formation and pointed to the success in this modified natural system of relating the dynamics of a bacterial population to the dynamics of the process which it carries out. This kind of approach offers for the first time the possibility of linking the nitrification process to its microbiological base in terms of growth constants and substrate conversion rates-an ecological problem of first-order significance to control of the nitrification process and the development of useful predictive models. Subsequent extension of this application of FA to wholly natural systems has not been fully successful because of technical problems in recovering nitrifiers from soil and the existence of multiple serotypes in many nitrifying populations (Belser and Schmidt, 1978a).

Growth of *Nitrobacter* in soil following nitrite addition was observed by Fliermans and Schmidt (1975) with a bivalent FA. Numbers increased from 4.8 X 10^3 to 3.6 X 10^6/g. Other direct FA enumerations of *Nitrobacter* in natural habitats were reported by Rennie *et al.* (1977) in a study relating to the controversy of whether roots are or are not nitrifier inhibitors. The rhizospheres of soybeans and wheat examined in this study manifested no inhibitory effects on *Nitrobacter.* *Nitrobacter* counts in various soils with a combined *N. winogradskyi-N. agilis* FA preparation were all in the 10^4-10^5/g range (Rennie and Schmidt, 1977a). Stanley *et al.* (1979) applied standard quantitative FA protocols to enumerate *Nitrobacter* in lake-water columns. The ability to quantify a microorganism in nature greatly expands the variety of autecological questions that may be posed and the extent to which they may be probed. Use of FA to enumerate *Nitrobacter* in soils, water, sediments, and sewage is clearly possible if the *Nitrobacter* serotypes therein are ones for which FA reagents are available; what is needed now is more information on the serotypec diversity of the nitrite oxidizers and the distribution of the sero-types in nitrifying environments. Little such data are now available.

3.2. Ammonia Oxidizers

Detailed study of the ammonia oxidizers by FA was begun somewhat later than that of the nitrite oxidizers. Belser and Schmidt (1978b) looked at three of the four genera of terrestrial ammonia oxidizers. Most of the strains examined were isolates of *Nitrosomonas;* these fell into four serotypes. The 11 isolates of *Nitrosolobus* were included in 3 serotypes, and 6 of 7 isolates of *Nitrosospira* fit into 2 serotypes. No intergeneric cross-reactions were observed. The obvious conclusion was that the ammonia oxidizers are a much more diverse

group than has been generally recognized. With additional isolates and additional FAs, the number of serotypes increased (Belser and Schmidt, 1978c).

In subsequent work, Belser and Schmidt (1978b) examined the question of diversity in ammonia oxidizers with emphasis on the nitrifying population of a single soil. MPN media of several kinds were used for microscopic examination, isolation, and enumeration. Depending on the MPN medium used, either *Nitrosomonas* or *Nitrosospira* was the most abundant genus in that soil. *Nitrosolobus* was seen occasionally in MPN tubes at the lower dilutions. This was the first report of the coexistence of multiple genera of ammonia oxidizers in the same environment, and the finding points out clearly that the widely accepted textbook convention of equating the process of ammonia oxidation with the term *Nitrosomonas is* a myth. The point is significant because the various genera differ in growth rate, biomass, yield, and substrate-oxidizing activity (Belser and Schmidt, manuscript in preparation).

Isolates obtained from the single soil cited above were reacted with 16 different FAs for ammonia oxidizers (Belser and Schmidt, 1978c). There were 7 *Nitrosomonas, 12 Nitrosospira,* and 1 *Nitrosolobus* isolate. Of these, five of the seven *Nitrosomonas* isolates were stained effectively by the use of four FAs; six of the *Nitrosospira* were identifiable with four FAs. The one *Nitrosolobus* was stained well by only one of the three FAs used. A greater degree of crossreactivity would certainly be more convenient to allow for a genus-specific, strain-comprehensive FA reagent. From the above results, it would appear that at least five FAs would be needed to cover the *Nitrosomonas* present in this soil and five or more FAs for the *Nitrosospira* component. Thus, composites involving perhaps a dozen FAs would be required for this particular soil assuming all serotypes had been isolated. The existence of a large number of serotypes poses obvious practical problems for achieving the ideal of detection and enumeration of an entire nitrifying population. Further experiments are needed to evaluate the perhaps more reasonable objective of determining the genera and strains that predominate in numbers and activities and to develop the appropriate FA reagents. The specter of infinite serological variability appears not to be real since FAs prepared against both ammonia oxidizers and nitrite oxidizers isolated from Minnesota soils were shown to stain isolates from diverse parts of the world (Fliermans *et al.,* 1974; Schmidt, unpublished).

4. Sulfur- and Iron-Oxidizing Bacteria

The chemolithotrophic bacteria that use reduced forms of sulfur and iron as sources of energy and reducing power are widespread in nature. They play an important role not only in biogeochemical cycling of these elements but also in acid and iron pollution of lakes and rivers.

4.1. Thiobacillus in Coal-Refuse Material

One of the most abundant substances associated with coal deposits is pyrite, which occurs in a variety of forms. One of these, iron pyrite, consists of both iron and sulfur in the reduced form. It therefore provides an ideal substrate for sulfur- and iron-oxidizing bacteria of the genus *Thiobacillus.* These bacteria can oxidize either sulfur (T. *thiooxidans)* or both sulfur and iron (T. *ferrooxidans)* and produce large quantities of sulfuric acid and ferric hydroxide (Lundgren *et al.,* 1971). The consequences of these microbial processes in coal-refuse disposal are highly acid mine drainage waters and metal precipitates ("yellow-boy"), which constitute a major water-pollution problem in coal-producing regions.

Iron pyrite oxidizes in air spontaneously, but only at pH values above 5.0. Abiotic acid production therefore becomes self-limiting once the pH of the refuse reaches this value. Below this pH, biological oxidation, mainly carried out by thiobacilli, becomes the major contributing factor to acid production in coal-refuse piles. For a description of a pH-dependent succession of iron bacteria in pyrite material, see Walsh and Mitchell (1972).

Both the iron- and sulfur-oxidizing bacteria can be isolated from coal-refuse piles and acid mine drainage waters (Colmer *et al.,* 1950; Tuttle *et al.,* 1969). However, few quantitative studies have examined these bacteria *in situ.*

Belly and Brock (1974) used $^{14}CO_2$ uptake by coal-refuse material as a measure of total autotrophic activity and showed that it was related to the mostprobable-number of iron-oxidizing bacteria. These results, however, only represent the overall $^{14}C0_2$-fixing potential of the sample, and the data do not relate directly to the autecology of the organisms involved. Apel *et al.* (1976) are the only authors who have used immunofluorescence (indirect) for detection and enumeration of T. *ferrooxidans* in acid mine environments. The specificity of the FA used in these studies was reported to be adequate in that their reagent did not react with a variety of other microorganisms, including other members of the genus *Thiobacillus.* Apparently, however, it was necessary to adsorb the FA with a number of unrelated bacteria in order to achieve the level of specificity reported.

The authors were not able to observe stained bacteria directly colonizing the coal-refuse particles. This they attributed to low numbers of bacteria and intense fluorescence of the particles. They did, nevertheless, show that when the bacteria were washed from the particles and concentrated by centrifugation, T. *ferrooxidans* could be seen and enumerated. Bohlool and Brock (1974a), on the other hand, had very little difficulty distinguishing *Thermoplasma acidophilum* (a thermophilic acidophilic mycoplasma) colonies in similar samples. They used the direct immunofluorescence technique, with the rhodomine-gelatin conjugate to suppress background fluorescence. Brightly fluorescing cells of T. *acidophilum* could be seen colonizing particles and films on microscope slides buried in the

field in contact with coal-refuse material or immersed in the stream draining the refuse pile.

4.2. Sulfolobus in Hot *Springs*

Sulfolobus acidocaldarius is a sulfur-oxidizing facultative autotroph that inhibits sulfur-rich environments that are extremely hot, 55-92°C, and acid, pH 0.9-5.0 (Brock *et al.* 1972; Brock, 1978). These bacteria have been shown to have an optimum temperature for growth of 70-75°C with a lower temperature limit of 55°C. They are also obligately acidophilic, with a growth optimum of pH 2-3. In nature, the major mode of growth for this organism is probably by chemolithotrophic oxidation of elemental sulfur, although its ability to grow heterotrophically has been demonstrated (Brock,1978).

Questions about the evolution, biogeography, and ecology of *Sulfolobus* in such extremes of temperature and pH are both intriguing and significant from an evolutionary point of view. Immunofluorescence has been useful in gaining some understanding of the origin, distribution, and activity of this organism in its natural environment.

4.2.1. Distribution and Biogeography

Sulfolobus has a wide geographical distribution. It has been found in acidic geothermal areas in North America, El Salvador, Dominica, Italy, Japan, and New Zealand (see Brock, 1978).

Bohlool and Brock (1974b) used immunofluorescence and immunodiffusion to classify into serogroups *Sulfolobus* isolates, "serostrains," cultured from different hot-spring environments. Distribution of serologically distinguishable populations was then studied quantitatively using the membrane-filter immunofluorescence technique. It was shown that several specific "serostrains" could exist in the same spring. This was compatible with the results of Mosser *et al.* (1974b), who had reported isolating different "temperature-strains" from the same source. One of the "serostrains" was found in all the springs sampled, but their relative numbers varied greatly. Numbers as high as 10^{7}-10^{8} cells/ml of a specific strain could be found in several springs. In flowing springs, the majority of the cells were located in the "sediment" of the flowing channel, with only a few in the water while in nonflowing pools, large numbers were suspended in the water.

In a later study, Bohlool (1975) found that several *Sulfolobus* isolates from New Zealand hot springs cross-reacted with FAs against Yellowstone organisms. The antigenic relatedness of New Zealand and Yellowstone populations raises interesting questions of dispersal and evolution of *Sulfolobus* (for further discussion, see Brock, 1978).

4.2.2. Growth Rate in Nature

The stable and steady-state nature of hot springs makes it relatively easy to measure *in situ* growth rates of the populations. Mosser *et al.* (1974a) applied a chemostat approach to measure the steady-state growth rate of *Sulfolobus* in several Yellowstone hot springs. Several springs were enriched with sodium chloride, and the rate of dilution of the chloride ion was determined over a period of time. Since the springs maintained a constant volume, temperature, pH, and *Sulfolobus* numbers, the authors suggested that chloride dilution rates in the spring could be used as an estimate of the rate the bacterial populations have to grow to maintain their numbers. To ascertain that the bacteria were being diluted at the same rate as the chloride ion, formaldehyde-fixed cells of a serologically distinct isolate of *Sulfolobus* were also added to several of the springs at the time of NaCl addition, and the decrease in their numbers was measured using immunofluorescence. The loss rates for formaldehyde-fixed cells were shown to agree reasonably well with those for chloride dilution. Half-times for cell and chloride dilution, presumably equivalent to bacterial doubling time, were estimated at about 10-20 hr for springs ranging in volume from 20 to 2000 liters, but 30 days or more for 2 larger springs of about 1 million liters' capacity.

5. Bacteria Involved in the Methane Cycle in Lakes: Methanogens and Methylotrophs

Decomposition of organic matter in most anaerobic environments leads to the formation of methane gas. The bacteria (methanogens) that are responsible convert the fermentation products ($C0_2$, H_2, formate, and ethanol) of other anaerobes in these environments to methane. They belong to a highly specialized obligately anaerobic group of bacteria that occupies the terminus of the food chain in the sediment.

In aquatic systems such as lakes and lagoons, the methane gas, being poorly soluble in water, escapes into the overlaying water column, where it serves as a source of carbon and energy for the aerobic and microaerophilic methaneoxidizing bacteria (methylotrophs).

5.1. Methanogens in Lake Sediments

Strayer and Tiedje (1978) used immunofluorescence to study the distribution of *Methanobacterium fonnicicum* in several lake sediments, anaerobic sewage sludge, and bovine rumen fluid. FA-reactive bacterial cells could be detected directly in some of the samples. FA enumeration of the specific methanogen in one lake sediment revealed numbers as high as 3.14×10^6/g dry sedi-

ment, which was at least one order of magnitude greater than MPN counts of the total methanogenic population. The specificity of the FA used in the above studies was satisfactory, since the FA did not react with 9 other methanogens, including M. *formicicum* strains, and 24 heterotrophic bacteria, many of which had been isolated from the study site.

5.2. Methylotrophs in Lake Sediments and Waters

Using direct immunofluorescence, Reed and Dugan (1978) studied the distribution and abundance of two different methylotrophs in the water column and sediments of Cleveland Harbor. *Methylomonas methanica* was detected at all the sites examined. Its numbers were found to increase with depth, with the largest population found in the sediment. *Methylosinus trichosporium,* on the other hand, could only be detected in two of the stations and only in the sediments and 1 m above. These findings are consistent with other published observations that methane oxidation takes place only directly above the sediments where the O_2 concentration is less than 1.0 mg/liter. The antisera prepared for these studies proved quite specific in that they reacted only with their homologous bacteria and not with any of 25 other bacteria, some of which were methylotrophs isolated from Cleveland Harbor.

6. Anaerobic Bacteria

Methods for enumeration of specific anaerobic bacteria from natural samples often involve tedious anaerobic culture techniques and prolonged incubation. In most instances, proper media have not been formulated for selective enrichment and isolation of the desired anaerobe. Immunofluorescence provides a possible rapid shortcut in detection and enumeration of a specific anaerobe directly in the sample of interest. An excellent example has already been noted (Section 5.1) in the recent study of Strayer and Tiedje (1978) on the distribution of the obligate anaerobe M. *formicicum* in lake sediments.

One of the first applications of immunofluorescence in microbial ecology concerned the detection and identification of anaerobic bacteria in the rumen fluid (Hobson and Mann, 1957). In a later study, Hobson *et al.* (1962) reported successful detection of *Selenomonas ruminantium* directly in stained smears of sheep-rumen contents. Their FA prepared against the S. *ruminantium* isolate cross-reacted with another rumen organism, *Veillonella alcalescens;* however, the FA could be made specific by adsorbing with the cross-reactive organisms.

Garcia and McKay (1969) and Garcia *et al.* (1971) have applied immunofluorescence for detection and identification of two anaerobic bacteria, *Clostridium septicum* and *Sphaerophorus necrophorus,* in natural and clinical specimens.

6.1. Clostridium in Soil

Clostridia are spore-forming anaerobic bacteria which are widely distributed in soils and are known to be involved in anaerobic decomposition of plant and animal constituents. Many clostridia are also causative agents of a variety of diseases of man and animals. In normal, unamended soil, clostridia are thought to occur mostly in the spore stage. However, Garcia and McKay (1969) combined qualitative immunofluorescence with viable counts to show that vegetative cells added to soils did not sporulate immediately and were capable of growth and multiplication in normal soils. The results of these studies were limited to qualitative FA assessment, for no attempts were made at quantification.

6.2. Sphaerophorus in Soil and Animal Tissues

S. necrophorus, a gram-negative nonsporing obligate anaerobe, is known to cause hepatic abscesses in cattle and necrotic gangrenous lesions in man and animals, and it is often associated with enzootic foot-rot infections in livestock. It was shown to survive well in soils incubated anaerobically or moistened to 80% of water-holding capacity. The species-specific fluorescent antibody against an isolate of S. *necrophorus* facilitated the detection of the organisms in liver abscesses, viscera, ruminant contents, and soils (Garcia *et al.,* 1971).

7. Public Health and Water Pollution Microbiology

7.1. Bacteriological Indicators of Fecal Pollution

Most human diseases that are transmitted by water originate from human feces. Common among these are diseases caused by strains of enteropathogenic *Escherichia coli, Salmonella, Shigella, Vibrio, Leptospira,* enteric viruses, and a host of parasitic protozoal cysts and worm larvae.

Assessment of the sanitary quality of water has traditionally relied on quantitative detection of indicator organisms rather than on specific pathogens. Part of the reason is that, even in badly polluted waters, pathogens are ordinarily present in very low numbers. Furthermore, monitoring of every pathogenic agent in every water supply becomes an inefficient and costly endeavor.

7.1.1. Enterobacteriaceae

The "coliform" group of bacteria-those aerobic and facultatively anaerobic, gram-negative, non-spore-forming rods capable of lactose fermentation with gas formation within 48 hr at 35°C (American Public Health Association, 1975)-are the most commonly used indicators of fecal pollution. However, the "fecal

coliforms-the subgroup of coliforms that can ferment lactose with gas production at 44.5°C (mainly *E.* coli)-are more characteristic of feces of man and other warm-blooded animals and thus, are, more reliable indicators of fecal contamination.

Aside from the reliability considerations in the use of the indicator concept, another factor of prime importance in a water-quality monitoring system is the rapidity and ease with which large numbers of samples can be processed. Application of immunofluorescence for presumptive mass screening and monitoring of water supplies offers a practical, and perhaps economic, alternative to conventional techniques. It has been field-tested for use in rapid detection of enteropathogenic *E.* coli (Cherry *et al.,* 1960; Pugsley and Evison,1974; Abshire, 1976), *Shigella flexneri* and S. *sonnei* (Thomason *et al.,* 1965), and *Salmonella typhi* (Thomason and Wells, 1971) in fecal smears and for identification of typhoid carriers (Thomason and McWhorter, 1965). Using polyvalent FAs for salmonellae, Cherry *et al.* (1972) screened enrichments of several surface waters and found 60% more positive samples than they could detect by conventional culture methods. These applications were later extended to the detection of *Salmonella* in foodstuff and water (Cherry *et al.,* 1975).

The results of all of these applications have suggested that IF could be a valuable tool in early diagnosis and control of some diarrheal diseases and epidemic conditions. However, as had been emphasized by Cherry and Moody (1965), FA detection of certain members of Enterobacteriaceae must only be considered presumptive and can in no way replace isolation and definitive identification of the etiologic agent. The major limitation to exploitation of FA is the well-known antigenic relatedness and cross-reactivity among the various genera of this family.

The introduction of nonfluorescent membrane filters has made the quantitative detection of low numbers of specific bacteria in water a reality. Cells from a large volume of water can be concentrated on black membrane filters and the desired bacteria identified by staining with appropriate FAs. The approach was first used to quantify low numbers of E. coli and *Shigella guarrabara* artificially inoculated into water (Danielsson, 1965; Danielsson and Laurell, 1965; Guthrie and Reeder, 1969), but this potential has never been field-tested directly in water-quality assessment.

7.1.2. Fecal Streptococci

Another normal inhabitant of the intestine of warm-blooded animals which has often been suggested as an indicator of fecal contamination is the group of gram-positive bacteria collectively termed fecal (or Lancefield's Group D) streptococci.

Abshire and Guthrie (1971) and Pavlova *et al.* (1972, 1973) have shown that IF may be used to identify Group D streptococci isolated from contaminated

waters. Pugsley and Evison (1975) used Group D-specific FAs to count colonies of fecal streptococci on nonfluorescent membrane filters preincubated on enrichment media for 10 hr. However, reports of direct enumeration of these organisms from natural waters are difficult to find.

Whereas the fecal coliform test does not differentiate between human and animal fecal contamination, it has been suggested that the ratio fecal coliform fecal streptococci (FC : FS) could indicate this distinction (Geldreich, 1970). The explanation advanced is that in human feces and domestic sewage, the FC : FS ratio is often greater than 4, while in farm-animal feces and effluents from packing houses, feedlots, and dairy farms, the same ratio is generally less than 1. The main objection to the use of the FC : FS concept as an indicator of human vs. animal source of fecal pollution arises from the fact that the two organisms survive differently once they are discharged into water. Therefore, viable counts downstream from the effluent discharge do not reveal the true bacteriology at the source. IF, on the other hand, can overcome this limitation because it can identify viable and nonviable cells alike. More effort should be directed toward developing improved FA reagents and techniques so that the indicator organisms can be quantified directly in the suspected sample.

7.2. Legionnaires' Disease Bacteria *(Legionella)* in Nonepidemic-Related Habitats

The etiologic agent of Legionnaires' disease, *Legionella pneumophila,* has been isolated from a number of man-made and natural environments. However, the organisms have been studied primarily in clinical specimens or pure cultures obtained from infected hosts. Isolates from air-conditioning cooling towers have been shown to be associated with four different outbreaks of Legionnaires' disease (Center for Disease Control, 1978).

Cherry *et al.* (1978) have developed a direct IF procedure for detection of Legionnaires bacteria in lung scrapings, histological sections, and fresh lung tissue obtained at biopsy or autopsy. Their preliminary data indicated that serologically identical or related organisms were common in soils and soil animals from several locations, but none of the cross-reacting organisms was isolated or identified.

Recently, Fliermans *et al.* (1979) have been successful in applying IF for quantitative detection of *Legionella* in samples of lake water concentrated by continuous centrifugation. Confirmatory evidence was obtained by injecting the same water samples into guinea pigs and isolating organisms identified as *L. pneumophila* by morphological, cultural, physiological, and serological characteristics.

Quantitative IF has also provided the tool to study the growth of this organism in association with blue-green algae in the laboratory (Tison *et al.,* 1979). It was shown that a *Legionella* isolate (serogroup 1) from an algal mat community in a

man-made thermal effluent (45°C) was capable of rapid multiplication when grown in association with the blue-green alga *Fischerella sp.* in mineral salts' medium.

8. Plant Pathogenic Bacteria

Although the potential of immunofluorescence for applications in plant pathology was recognized quite early (Paton, 1964), progress in this field has been rather slow. Published information on such applications are limited to a few scattered and preliminary reports.

Paton (1964) demonstrated that the technique was effective in locating *Pseudomonas syringae* growing intra- and extracellularly in leaves and tubers of various plants. Auto fluorescence of plant tissue was a problem but could be controlled by either acetone extraction followed by treatment with dimethylformamide or by treating with a saturated mercuric chloride solution followed by several rinses in EDTA. Nonspecific staining of plant tissue was reported to be minimized if the serum was preadsorbed with rat-liver homogenate and the slides counterstained with lissamine rhodamine-conjugated preimmune serum prior to FA staining. Some evidence was presented indicating that the bacteria grown in culture were not antigenically identical to those *in vivo.* Acid treatment of *in* vivo bacteria slightly improved the staining reaction of FA reagent produced against culture-grown cells, suggesting that an antigenic layer had been removed from the bacterial surface. Likewise, antibodies produced against *in vivo* cells reacted better with cells *in vivo* than *in vitro.*

Auger and Shalla (1975) were able to identify the causative bacteria of Pierce Disease (PD) of grapevines in host plant tissue and insect vector. The conventional means of diagnosing PD in vines involves transmission, via grafting or insect vector, to another susceptible plant followed by prolonged incubation (24-55 days) for the development of symptoms. Immunofluorescence proved to be a specific and rapid alternative for the diagnosis of PD in infected plants and contaminated insect vectors.

Allan and Kelman (1977) found that FAs prepared against a strain of *Erwinia carotovora* var. *atroseptica* (Eca) was highly specific against Eca in that it reacted only with isolates characterized as *E. carotovora* var. *atroseptica* and not with any of a large number of strains belonging to *E. carotovora* var. *carotovora, E. chrysanthemi, E. ariodeae, E. carnegiana,* and *E. amylovora.* They were able to use the reagent to detect *E. carotovora* var. *atroseptica* in artificially inoculated soils, infected potato tubers and leaves, and fruit flies and maggot flies which had been feeding on pure cultures of bacteria in the laboratory or infected potatoes in the field.

Other applications of immunofluorescence in plant sciences have involved

the identification of a nitrogen-fixing strain of *Chromobacterium lividum* in various tissues of leaf-nodulated plants (Bettelheim *et al., 1968)* and detection of a bacterial contaminant in nodule squashes of some leguminous plants (Van der Merwe *et al., 1972).*

9. Filamentous Fungi

A large variety of techniques have been developed for the study of fungi from soil and other habitats (for a review, see Parkinson, *1973).* Among these, the plate count has been the most frequently used method for quantitative estimation of fungal biomass. This technique has been shown, however, to have serious limitations in that it is, for the most part, selective for those fungi that occur as spores. Direct methods also have intrinsic limitations but nevertheless often afford a realistic view of microorganism *in situ.* The usefulness of direct observation is greatly enhanced when the identity of the microorganism can be established. IF provides such an opportunity.

Schmidt and Bankole explored the potential of IF in recognizing strains of *Aspergillus flavus* grown in sterile soil in the presence of five other soil fungi (Schmidt and Bankole, *1962, 1963, 1965).* The FA prepared against one strain of *A. flavus* reacted strongly with all other *14* strains of *A. flavus* but not with *17* isolates of species of *Aspergillus* other than *A. flavus.* Four other aspergilli, particularly *A. sydowi,* cross-reacted intensely. Most other fungi tested reacted weakly or not at all.

Schmidt *et al. (1974)* found IF to be especially promising in the study of certain ectomycorrhizae. FAs prepared against cultures of two ectotrophic mycorrhizal fungi, *Thelephora terrestris* and *Pisolithus tinctorius,* were effective and specific microscopic stains for identification of the homologous fungus on contact slides and in "Hartig Nets" in the mycorrhizal structure. T. *terrestris* FA gave 0 and P. *tinctorius* FA gave only *5* cross-reactions of moderate intensity with *31* diverse nonmycorrhizal fungi. Most of the heterologous cross-reactions could be eliminated by adsorption with the cross-reacting fungus, without signifcantly affecting the activity of the homologous system.

Eren and Pramer *(1966)* have used IF to visualize the nematode-trapping fungus, *Arthrobotrys conoides,* in artifically inoculated soils.

Many of the other applications of IF in mycology are either for diagnosis of mycotic infections (for a review, see Goldman, *1968)* or for detection of specific fungi in samples of infested grains (Warnock, *1971, 1973).*

Questions about the ecology of important members of the fungal flora-i.e., plant pathogens and decomposers-have not been approached using direct techniques. IF provides an attractive opportunity, but its potentials and limitations have not been fully explored.

10. Other Applications

There are only a few other noteworthy examples of the use of IF in ecologically oriented studies.

Gray and his co-workers have used IF for studies on the distribution, growth, and spore formation and germination of *Bacillus subtilis* in soil (Hill and Gray, 1967; Siala *et al.,* 1974; Siala and Gray, 1975). They were able to differentiate between the spores and the vegetative cells of this organism *in situ,* using FAs prepared against the respective antigens. It was shown that *B. subtilis is* present mainly in the vegetative form in an acid horizon of a forest soil but as spores in the alkaline horizon of the same soil profile. Vegetative cells on glass slides in contact with an acid forest soil initially declined in numbers but later grew after fungal hyphae were established on the glass slides. Growth did not occur in sterile soil, nor in alkaline soil, until fungal growth was stimulated. Likewise, spore germination did not occur in the same acid soil unless fungi were also growing. Roots of *Finus sylvester* growing in the soil was shown to inhibit both vegetative growth and spore germination.

The distribution of *Leptospira* in field soils and waters was studied by Henry *et al.* (1971) using direct IF. Microscope slides in contact with soil or water in the field were colonized within 4 to 14 days with leptospiral- like cells that reacted with fluorescent antibodies against a saprophytic leptospira, serotype *patoc* Patoc I. The presence of leptospires at various sites, as detected by IF, correlated well with data which had been obtained earlier by culture methods.

The thermophilic acidophilic mycoplasma, *Thermoplasma acidophilum,* was studied in spontaneously heated coal-refuse piles in strip-mining regions by Bohlool and Brock (1974a) using IF. FA-reactive cells could be found colonizing refuse particles on glass slides buried in the field. Although some degree of crossreaction was found for most of the stains tested, IF/cross-adsorption revealed several antigenically distinct serogroups. Survey of their geographical distribution showed that different serogroups could inhabit the same site, whereas the same serogroup could be found in piles from different geographical areas.

IF was used by Farrah and Unz (1975) to establish the role of a strain of *Zoogloea ramigera* in the formation of flocs and finger-like zoogloeae in microbial films that develop on the surface of stored samples of activated sludge. It was shown that some of the fingerlike projections consisted almost entirely of cells of one IF-reactive type, whereas others were devoid of reactive cells.

Fliermans and Schmidt (1977) have applied IF for an autecological study of the unicellular cyanobacterium, *Synechococcus cedrorum.* Their FA reacted brightly and specifically and was thus effective in specifically enumerating S. *cedrorum* cells in water samples from the water column of a stratified lake.

IF has also been applied to study the distribution of *Aeromonas hydrophila* in thermal effluents (Hazen and Fliermans, 1979) and its association with the

ciliate Epistylis in the epizootic red-sore lesions in large-mouth bass (Hazen *et* al., 1978).

11. Problems and Limitations

In general, the effectiveness of any technique cannot surpass the competence of the technician. There are, however, intrinsic limitations and possible sources of error for all techniques that the user should be thoroughly familar with in making sound interpretations and conclusions. This is especially true of IF because the results are often based on subjective evaluation of microscopic observations, and, particularly so, when dealing with highly complex and heterogeneous materials, such as soils and sediments. For these reasons, controls of various kinds are essential in all applications of IF. These were outlined in a previous review by Schmidt (1973).

The most important considerations in the application of IF to natural samples relate to (1) the specificity of the antibody to be used and problems with nonspecific staining (NSS), (2) the interference from auto fluorescence of, or nonspecific adsorption (NSA) of FA to, the background, (3) the stability of the antigen under different growth conditions and environments, (4) the inability to distinguish between live and dead cells, and (5) the efficency of recovery of the desired cells from the sample for quantification.

11.1. Specificity

The FA-specificity spectrum is one of the first criteria that must be established after the preparation of an FA reagent. The question of specificity must be raised for every new system to be used. In some instances, i.e., Enterobacteriaceae and *Rhizobium,* extensive serological data are already available from published records and can be valuable in selecting the right strain for study. Even for such systems, however, the specificity features of the new reagent must be confirmed for the application planned.

The degree of specificity desired varies with the type of ecological question under study; there could be too *much* or *not enough* specificity in a particular system. For instance, if attention is focused on a specific strain for any particular reason (i.e., a highly effective *Rhizobium* strain or an enteropathogenic strain of E. coli), then an FA that will react only with that strain is highly desirable. If, on the other hand, the study involves organisms responsible for a specific process, e.g., methanogens or chemosynthetic ammonium or nitrite oxidizers, then a species-specific reagent is needed. In general, FAs can be made more specific by adsorption with cross-reacting organisms; an example of removal of cross-reaction by this means is found in the study of Fliermans *et* al. (1974). Likewise, strain-

specific FAs may be pooled to obtain a more species-specific FA reagent, but the effectiveness of this approach is limited by the number of serotypes that may be involved in the natural population under study, since only about 6-8 active FAs may be pooled because of dilution effects.

Although examples of cross-reaction between totally unrelated organisms are rare, it deserves some attention, nevertheless, since a few examples have been reported. The cross-reaction of OX-19, OX-2, and OX-K strains of *Proteus vul*garis with typhus and spotted-fever-group rickettsiae is a well-documented case and forms the basis for the Weil-Felix serologic test for diagnosis of these diseases (see Joklik and Willett, 1976). Heidelberger and Elliot (1966) reported crossreaction of pneumococcal antisera with exopolysaccharides of *Rhizobium* and *Xanthomonas.* Dudman and Heidelberger (1969) later found the *Rhizobium* cross-reaction to be due to a pyruvate subgroup on the polysaccharide moiety.

More pertinent to ecological applications of IF is the observation by Bohlool and Schmidt (1970) that an FA prepared against a strain of *R. japonicum* stained a soil actinomycete isolate specifically (Fig. 4a). Cross-reactions of this type, involving antigens shared by unrelated forms, have not been reported in any other ecological study. Continued attention should be given to this possibility though, for actinomycetes may fragment in pieces that resemble single-cell bacteria.

Another potential problem related to specificity is that some microorganisms (usually fungal spores) are "universal acceptors" (Schmidt, 1974) in that they will stain with any FA, including the preimmune control. Figure 4b illustrates this phenomenon. The soil fungus *Fusidium* and a strain of *R. japonicum* (USDA 31) were grown together in sterilized soil and contact slides stained with USDA 31-FA. Notice that only the *R. japonicum* cells (one attached to a fungal mycelium) and the fungal conidia stained, while the mycelia are negative. It is likely that the surfaces of many fungus spores are highly absorptive and trap any sort of protein material. The fact that A. *chroococcum* cysts also act as universal acceptors of FA and that this appears to be a trapping reaction which may be blocked by gelatin-rhodamine has been mentioned (Section 2).

11.2. Autofluorescence and Nonspecific Adsorption

Autofluorescence of material in the sample and nonspecific attachment of FA to the background can mask the specifically stained cells and seriously limit IF observations. Autofluorescence is specially troublesome with plant material, but it varies greatly with the type of plant used. Hughes et *al.* (1979) had difficulty in using fluorescein-labeled material to stain soybean root hairs because of autofluorescence of root hairs in the fluorescein region of the spectrum. In our experience, most of the interfering autofluorescence of soybean roots is associated with the main root and not root hairs; furthermore, it can be controlled by the use of various extractors and the gelatin-RhITC counterstain (Bohlool and Schmidt, 1968). When autofluorescence presents a serious problem to IF investi-

gation, a variety of modifications can be tried, such as the use of other fluorochromes and filter systems, treatment with extractors, and the application of counterstains.

Problems of NSS encountered in the application of IF to soil systems has been discussed in an earlier section (1.2). A counterstain, consisting of hydrolyzed gelatin conjugated to rhodamine, was highly effective in overcoming NSS in a variety of soils and also in some animal and human tissue (Bohlool and Schmidt, 1968). This was also found to be effective in reducing NSS with mycorrhizal sections (Schmidt et *al.,* 1974), other plant material (unpublished), and coalrefuse material (Bohlool and Brock, 1974a).

11.3. Antigen Stability

A major concern in the application of any marker technique in ecological studies is the stability of the marker under different conditions and environments. Does the organism growing in nature maintain its antigenic integrity sufficiently so that it could be recognized by antibodies prepared against cells from artificial media? Unapproachable as this question seems, there exists enough indirect evidence to suggest that the environment has little if any effect in modifying structural antigens. FAs against cultured rhizobia have been shown to react equally well with cells grown in four different media (Bohlool, 1968), bacteria growing in various sterilized soils (Schmidt et *al.,* 1968; Bohlool and Schmidt, 1968, 1973a), and bacteriods in nodules (Schmidt et *al.,* 1968; Trinick, 1969; Bohlool and Schmidt, 1973b; Lindemann et *al.,* 1974; May, 1979). Antigen stability is the premise underlying the widely used practice of serotyping nodules by quick agglutination tests (Means et *al.,* 1964).

Further indirect evidence of antigen stability comes from the finding that certain specific strains of *Rhizobium* could be detected in natural soils using FA prepared from rhizobia grown in culture medium (Bohlool and Schmidt, 1970). Presumptive evidence for the presence of two strains of *R. japonicum* in a range of natural soils was further strengthened by the observation that nearly every soil which contained FA-reactive cells, when used to inoculate soybeans, caused nodule formation, and some proportion of the nodules contained bacteroids which also reacted with the same FA. Conversely, when a particular FA failed to detect reactive bacteria in a soil, that soil either did not nodulate soybeans or, if it did, none of the nodules contained bacteriods that reacted with the FA.

More definitive evidence of the stability of rhizobial antigens in soils over a 12-year period was obtained by Diatloff (1977) in Australia. Two serologically distinct strains of *Rhizobium* for Lotononis *bainesh* were introduced into an isolated field station where Lotononis-pangola grass pastures were established but no indigenous Lotononis rhizobia were previously present. Stabilities of four rhizobial characters-colony color, effectiveness, antibiotic sensitivity, and antigens-were assessed from nodule isolates. It was shown that antigens and colony

characteristics were not changed over a period of 5-12 years of residence in the soil, whereas effectiveness and antibiotic sensitivity had undergone slight modifications.

Yet another line of evidence attesting to the remarkable stability of the antigens of bacteria stems from the world wide distribution of many cross-reacting strains. A few examples will suffice: Fliermans *et al.* (1974) found that FA prepared against a *Nitrobacter agilis* strain from Minnesota stained nitrite-oxidizing chemosynthetic bacteria from soils of Kentucky, Morocco, and Iceland; strains of *Nitrosomonas* and *Nitrosospira* isolated from a Minnesota soil yielded FAs which stained cells in ammonia-oxidizing samples of a tidal bay in New Zealand as well as isolates of ammonia oxidizers isolated from those samples (Belser and Schmidt, unpublished); Bohlool (1975) found that *Sulfolobus* FAs prepared against hot-spring isolates from Yellowstone National Park, U.S.A., reacted with *Sulfolobus* isolates from hot springs in New Zealand; and the same isolate of *Nitrobacter* was found to be entirely constant with respect to FA-staining properties whether grown in strictly autotrophic conditions or in strictly heterotrophic medium in the absence of nitrite (Stanley and Schmidt, unpublished). Despite great differences in geographical distribution, environmental pressures, and growth conditions, it appears that major antigenic components of bacteria remain essentially unchanged.

11.4. Viability

IF cannot differentiate living from dead cells because both will stain brightly and specifically. Dead microorganisms, however, are not expected to persist in nature very long, for they would soon be decomposed and consumed by other organisms. Some evidence for this was obtained by Bohlool and Schmidt (1973b), who observed that heat-killed (65°C for 3 h) R. *japonicum* cells were decomposed in soil and completely cleared within 1-2 weeks. Live cells (from culture) were also degraded in the soil, but it took much longer for them to decline in numbers. Figure 5 illustrates how intact cells are degraded into pieces of fluorescent debris, which are cleared completely after a period of time. The duration of persistence will perhaps vary considerably with the type of cell, their numbers, and the environment. Figure 5c illustrates degradation of cells by an unidentified soil fungus.

Escherichia coli is one of the few bacteria that can be enumerated in normal soil by selective plating. Although *E. coli* does not grow in soil, the die-off rate of a population added to soil may be observed. Data reported by Schmidt (1974) in a comparison of selective plate counts with FA counts of E. coli as a function of time in contact with a soil population, reflect the relatively rapid clearance of dead bacteria from soil. Between 3 and 10 days there was a large decline in the number of viable *E. coli* cells (plate count), but only a small percentage of the dead cells was sufficiently intact to be recognizable by IF.

Even more rapid disappearance of *Nitrobacter* cells occurred in several soils

once added nitrite was exhausted by an actively nitrifying population (Rennie and Schmidt, 1977b). Cell numbers peaked at about 8 days after addition of substrate to the soils, at which time the nitrite had been completely oxidized; between 8 and 14 days, the numbers of FA-detectable cells dropped almost to initial levels. These data together with the common sense realization that dead cells do not pile up in most natural environments suggest that inclusion of nonviable cells in counts is unlikely to be a major source of error in IF enumeration.

11.5. Quantification

Problems associated with the enumeration of FA-stained bacteria in natural materials are most pronounced in the case of soil populations. The microscopic field is extremely small in area, and only a limited quantity of soil may be tolerated in the field lest bacteria be buried or masked. As a consequence, populations must be large in order to encounter a reasonable number of cells from a tolerable deposition of soil. Problems related to microscopic field size and cell densities may be handled by passing a volume of sample through a suitable membrane filter, one that is adequate to trap a convenient number of cells per field of filter. Practical limitations are dictated by inverse relationships between the size of the specific population and the amount of suspended particulates in the water. To quantify realistically low population levels in soils or sediments, it is necessary to separate cells from soil and concentrate them on a surface for FA staining. Development and evaluation of a procedure for the enumeration of a specific population in natural habitats were reported by Bohlool and Schmidt (1973a) and Schmidt (1974).

Limitations associated with IF quantification of soil organisms derive largely from the efficiency with which cells can be released from soil particulates and retained in a supernatant fluid following removal of those particulates. Trapping of cells below the surface in the fibrous matrix of cellulose acetate membranes and masking by residual soil particulates are additional, but likely less important, sources of error. Estimates of the efficiency with which bacteria can be recovered from soil are not easily obtained, for there are no means to obtain fully reliable standards. Enumeration by plate count is the best standard for evaluation of recovery by quantitative FA, but adequate selective plating procedures are available for very few soil organisms. A reasonable compromise is to introduce a known inoculum of the organism of interest into sterilized soil and to estimate recovery by both plating and IF as the pure culture grows. In all such studies, the many limitations of the plate count itself, especially with respect to clumping and statistical variability, must be kept in mind. Recovery estimates obviously are still difficult for organisms, such as the nitrifiers, which are poorly amenable to plating.

The few recovery data thus far available are from procedures based on the protocol of Bohlool and Schmidt (1973a). It is clear that recovery varies with

the microorganism and with the soil so that modifications in procedural details may be necessary. Bohlool and Schmidt (1973a) reported recoveries for *R. japonicum* USDA 110 of 25-130% at various growth stages in a sterilized soil. Recovery of viable E. coli cells from nonsterile soil after 1 and 3 days was 89 and 64%, respectively, relative to a plate count reported by Schmidt (1974), who also observed that attempts to recover A. *chroococcum* from normal soil (selective plating) were less successful than for E. coli. *R. japonicum* strain USDA 123 was recovered from a Waukegan soil with an estimated 30% efficiency (Reyes and Schmidt, 1979). Vidor and Miller (1980a), using the same procedure but 1% $CaCl_2$ as flocculant, recovered *R. japonicum* strain USDA 110 from a Rossmoyne soil with 80% efficiency, but with only 20% efficiency from a Miami silt loam. Recoveries such as those reported do not pose serious limitations so long as they are consistent for a given system and for populations in the 10^5 or 10^6/g range. Low rates of recovery, even if consistent, become a technical limitation when the cell of interest is present in the soil at densities below 10^5 /g.

Some tropical soils which fix added rhizobia rapidly and irreversibly with respect to the usual soil-release procedures of IF enumeration were encountered by Kingsley and Bohlool (unpublished). A strain of chick-pea *Rhizobium* was studied in a sand perlite mixture from which it could be released and counted by FA with more than 90% efficiency. Addition of only small amounts of a

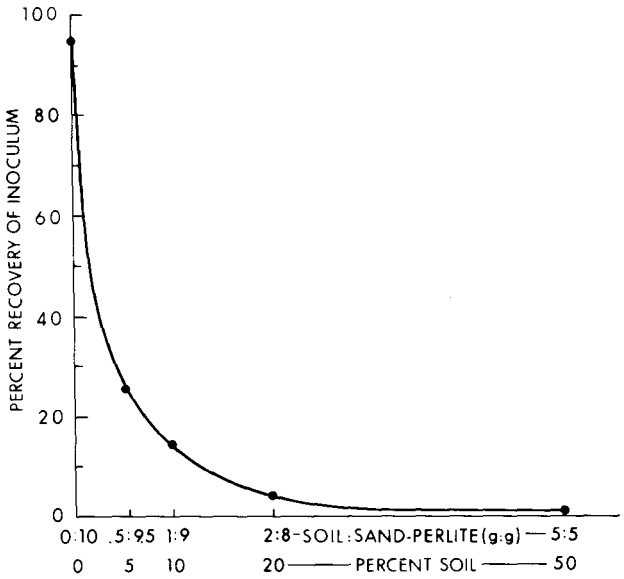

Figure 6. Effect of increasing concentration of a tropical soil (a Hawaiian Oxisol) on recovery of chick-pea *Rhizobium* strain ICRISAT 3889 from a sand-perlite mixture. From Kingsley and Bohlool (unpublished).

Hawaiian Oxisol to the mixture decreased recoveries dramatically to less than 1% (Fig. 6).

New techniques as well as modifications to enhance recovery from refractory soils or of refractory organisms are in order for situations where recovery is found to be a limitation. Work underway with rhizobia-soil systems (Donaldson, Bezdicek, and Sharma, unpublished; Wollum and Miller, unpublished) has focused on density gradient centrifugation procedures as a means to achieve consistently high recoveries. Preliminary results from both groups indicate recovery rates near 100% in tests with heavily inoculated soil.

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