2 DETERMINATION OF INDIVIDUAL ORGANISMS AND THEIR ACTIVITIES IN SITU

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1.0. INTRODUCTION AND BACKGROUND

1.1. Importance of the Individual Microorganism

Even more than for the plant and animal kingdoms, questions about the behavior and distribution of individual species of bacteria relate directly to the activity of the organisms and their function in the ecosystem. Autecological studies, therefore, are based on the individual species and its relationship to its environment irrespective of relationships with other organisms. Clearly, interspecific interactions are important, but they are of autecological importance only insofar as they effect the function of the microbe of interest. The environmental impact of microbial processes is invariably the sum of the contribution of each individual organism of the same or different *species*, located in close physical proximity. Microbiologists, rarely, if ever, deal with individual organisms (cells), except to quantify the total population density catalyzing a reaction of interest. Because of the difficulties in defining microbial species, microbiologists must depart slightly from the classical definition of autecology. Whereas autecology normally refers to studies of a population of a defined species and its environment, we will expand the usage to include functional groups defined as guilds (see Section 1.2).

This chapter deals with microbiological research methods and studies that are perhaps the closest to autecology in its classical usage. We will explore methods for identifying individual cells and for determining specific types of

activity. As will be seen, the sole purpose in this effort is to obtain a quantitative view of the numbers and distribution of individuals in situ. Often it is the distribution of an activity rather than a species that is of interest. Using the techniques described here, those activities can often be quantified using basic autecological concepts. The problems of defining species relationships in procaryotes are substantially greater than for the other kingdoms (see Section 2.0). This "problem" often benefits microbial ecology, however, for it tends to direct attention away from a species-centered approach, and instead allows us to focus on the important activities and functions carried out by the microbes.

1.2. Taxonomic Considerations

The fact that microbial autecology necessarily deals with individuals as units of a single group creates a serious problem in terms of identifying the group being examined. Numerical methods of grouping organisms are clearly superior when looked at from a genetic viewpoint. They allow examination of a large number of characteristics of isolates and determination of those with phenotypic similarities. However, such a classification scheme is not always relevant and is rarely useful in ecosystem ecology. Studies of microorganisms in the environment relate to what the cells do; specifically, what functions the organisms carry out. Innumerable studies revolve around the question, "How many cells are doing (or are capable of doing) the specific function that I am interested in without regard for the genetic label (species name) that a numerical analysis of a large number of isolates may provide?" In fact, it is only in certain studies that the species name has great relevance to any ecological question. For example, species identification can be important if the reactions of interest are unique to a single or few species. This would include environmental studies of pathogen behavior, of indicator organisms, or of functional types represented by a genetically limited collection of microbes such as the methanogens or autotrophic nitrifiers.

Ecosystems tend to be biologically redundant, that is, several mechanisms for accomplishing an essential function are generally available. Therefore, if environmental conditions change, a different set of organisms may become the dominant mediator of the reaction of interest. For example, in anaerobic habitats, many fermentors generate acetate from higher molecular weight substrates. To keep the process of organic matter decomposition operating, the acetate must be further oxidized. Two major groups of organisms carry out this function, the sulfate reducers and the methanogens. Although both groups of organisms can oxidize hydrogen, they also support the acetogenic proton reducers. If adequate sulfate is available, the sulfate reducers are the primary group responsible for acetate catabolism, whereas if sulfate is absent, methanogens convert the acetate to carbon dioxide and methane. Because several differ-

ent "species" capable of carrying out the same reactions (even at the same time) may coexist in a habitat, grouping of those organisms by major function is often desirable. Root (1967) used the term "guild" to describe groups of birds with similar feeding habits, although they were of different species. Wassel and Mills (1983) used the term to describe groups of bacteria clustered by numerical techniques, where the number of characters tested for the isolates was insufficient to claim relatedness near the species level. Guilds are often appropriate means of naming and considering functional groups in microbial ecology. Such terminology should be considered to replace the often misused term "population" when speaking of groups of organisms sharing the same function, for example, the guild of sulfate reducers, *not* the population of sulfate reducers. We shall use the term population to describe only those organisms that have such a high degree of phenotypic similarity as to be considered as species. We will use the term interchangeably with species, much as is done for the higher kingdoms. We suggest that others do likewise to avoid the confusion that microbial ecologists frequently encounter as a result of overuse and misuse of population.

The direct methods outlined in the later portions of this chapter often aid in the escape from the idea that we must give the organisms names. On the one hand, if we use some specific measure of activity, we can examine all the cells possessing that activity (all members of the guild) without regard for the "taxonomic label." This type of study must also be classified as autecological research. On the other hand, given an isolate that possesses a certain trait, use of specific identifying techniques, namely, immunofluorescence assays, can help us evaluate the behavior of that population (species) in complex ecosystems. Ultimately, it is necessary to combine identification techniques with activity measurements. It is essential to quantify the proportion of a population that is active. It is also important to know how many cells catalyzing the same reaction are of a specific guild.

Direct, sensitive assays are available for quantifying the activities of both guilds and populations. Several of the techniques are being widely used. A few research groups have taken the logical step and combined activity measures with species specific assays to begin to carry out the first true autecological studies that have accurate quantitative methodologies included as part of their protocols. Our purpose here is to provide a description of the techniques that are available at this time for use in direct examinations of individual cells in situ. Our treatment does not pretend to include all of the examples that could have been used. Many of the procedures are well documented and reviewed in other places, and we have sought to provide an entry into that literature, as opposed to inclusion of an exhaustive literature survey. We attempt here to make a case for the role of the direct, in situ procedures in the autecology of microorganisms, but we also include the appropriate warnings of the major limitations of methods currently being used. Further research on, and use of,

the approaches outlined here will help to establish these methods as the most acceptable means of examining specific groups of microbes in the environment.

1.3. Importance of Quantitative Considerations

Once a property of interest has been ascribed to an organism, whether the property is pathogenicity or some other function important within an ecosystem, the most frequently asked ecological questions are operational—is the organism of interest present and how abundant is it? Questions of the effect of various parameters, environmental and biological, are often answered by looking for changes in the abundance of the organism of interest. The first question, that of presence or absence, may be seen as a qualitative one, that is, the answer is either yes or no; however, it is obvious that the quantitative aspects of the method used to search for the microbe will determine whether presence of a very few cells or a great many cells will be required for detection. In the case of an organism that causes a disease, demonstration of presence is often adequate to answer the most pressing questions about it, while for organisms with more subtle activities, such as the oxidation of ammonium to nitrite, the abundance is of prime consideration.

If it is assumed that each cell contains the same amount of enzyme (a fact never conclusively demonstrated), then it follows that total activity should be directly proportional to the abundance of the organism. As we will see later, not all individuals of a population or guild contain the same levels of activity. The relative proportion of those containing the activity to the total number provides information as to the nutritional status of the group, be it population or guild, in that specific habitat. Accurate assessment of changes in either total numbers or in the percentage of active cells with time can lead to an understanding of the dynamics of the group, and how various conditions affect growth and survival of the population or guild in the habitat.

1.4. History of Enumeration

Plate counts have been the "mainstay" of quantitative microbiology since shortly after Mrs. Hesse suggested agar as a solidifying agent for Koch's cultural investigations. While a variety of methods are used to get the appropriate number of organisms on or in the medium, the basic principle of dilution to physically separate individual cells followed by counting of colonies that presumably arise from single cells can be attributed to Koch's early efforts in isolation of pathogens. Two general types of enumerations have been devised for the plate count (and its cousin, the most probable number technique— MPN), selective and nonselective. The selective counts assume that some material added to or deleted from the growth medium enhances development of a single type of organism while inhibiting the growth of all others. Included here for the purposes of this discussion are the differential media that allow growth of

many organisms, but which induce a pigment formation or cause some visible change in or about the colony so that individuals of one type are highlighted. Nonselective media, in theory, allow growth of all organisms present. The underlying objective for using the latter media is to enumerate total microbial (bacterial) communities. To this end, a variety of rich media have been used including yeast extract, animal and vegetable protein extracts, and mixtures of sugars and amino acids. Evaluations of effectiveness of various media has been in terms of those that produce the highest counts of total organisms, or of the organism in question.

Many individuals have decried the use of plate counts (and similar methods) even to the extent of saying that ". . . the plate count is the worst thing that ever happened to marine microbiology because it is so misused," (Hamilton, 1979). In many cases this observation is well founded, nevertheless, cultural counts remain the most frequently used means of gathering information on either total or specific microbial abundance. The use of plate counts in certain situations appears to be warranted by cost and time considerations, and, in many cases, provides a useful means of predicting some factor or determining some limit on use of microbiologically contaminated materials. This utility more likely relates to the good correlation of some symptom (such as increased occurrence of a disease or other perturbed state) with a reproducable colony count than to accurate quantification of the numbers of individuals.

Early microscopic observations indicated that there are more microbial cells in samples than are accounted for by plate counts or MPN enumerations (Starkey, 1939). Some feel that many of the cells observed with the microscope are nonviable or dormant (Jannash, 1965; Stevenson, 1978), but maybe not all of the organisms present are capable of growth on the medium used. Most recent research has shown that, while not all cells observed by direct counts are alive or active, the inability of many organisms (individual cells) to produce visible colonies on plates or turbidity in broth is the largest source of inaccuracy in cultural methods. It has been suggested, therefore, that all direct cell counts are overestimates of the true numbers of viable cells, while cultural counts are underestimates.

Differences between direct microscopic results and cultural counts may range, anywhere from 2 to 4 orders of magnitude. Even if only 10% of all the bacteria observed with a direct count method are viable, the differences between the two procedures can not be explained by viable versus nonviable cells; clearly the cultural methods fail to detect a tremendous number of bacteria in soil and water samples.

The search has thus begun for reasonable direct count methods. A variety of stains have been used, but direct visualization is often confounded by particles in the samples that react with stains and mimick bacteria. (This, of course, provides ammunition to those who contend that direct counts represented little or no advantage over cultural techniques.) The advent of the use of fluorescent dyes provides an advancement in our ability to directly observe microbial cells.

Particularly important are those chemicals that combine with biological materials, such as nucleic acids, with a high degree of specificity. Use of compounds such as acridine orange has permitted greatly improved differentiation of most bacteria (and other microbes) from particulate matter. But, quantitation is still a problem.

Initially, microscopes used routinely for fluorescence microscopy employed substage illumination. Thus, to be seen, samples had to be held on a transparent material, namely glass slides. While substage illumination is adequate for many stained preparations (e.g., tissue sections for histologic examination, or classical bacterial stains), resolution is reduced by the requirement for the light generated by the fluorochrome to pass through the cell body as it was directed toward the objective. Furthermore, estimating the number of bacteria in a volume of liquid smeared on a slide is neither accurate nor precise. A major breakthrough in enumeration of microbial populations came with the use of incident illumination, or epifluorescence. In this method the excitation beam is directed at the specimen from above, allowing the full fluorescence to be visualized. Extraneous light is excluded from the observer by a series of beam splitters and filters. This modification not only allowed for a brighter image, but also meant that opaque surfaces could be used to hold the cells (Mills and Maubrey, 1981). Using epifluorescence, a suspension can be quantitatively filtered, the surface of the filter examined, the particles present counted, and the numbers accurately extrapolated to the original sample.

Another modification in direct microbial counts came with the use of Nuclepore filters (Hobbie et al., 1977; Bowden, 1977). Tortuous pore filters often yield counts that are variable and usually lower than anticipated due to loss of cells into the depths of the filter. With Nuclepore membranes, the cells are retained on a flat surface. This simple fact increases both the yield and the precision of the direct count method.

While most investigators accept the fact that direct count methods are preferable in terms of accurately estimating the total abundance of microbes, it is clear that if the approaches and studies described in this chapter are truly representative of microbial ecology, the plate count will continue to be a very important tool. Any technique that examines a population requires, at some time or another, the isolation of organisms. Dilution-culture methods, such as are employed with plate counts, are the only means available for isolations. Even the most advanced methods, such as the fluorescent antibody (FA) techniques, require isolation of the organism presumed to be the one of interest. Difficulties with that presumption will be presented as part of the discussion of the FA technique.

As direct methods become more widely studied, modified and used, plate count and other cultural assays will likely be used primarily as rapid survey techniques, as a means of obtaining isolates for production of antibodies employed in direct assays, and as experimental analytical tools. Other uses would certainly include those such as water quality monitoring where large data sets on performance of the tests have been established.

2.0. INDIRECT METHODS

2.1. Use of Cultural Methods

While this chapter's purpose is to describe direct methods for the examination of the dynamics of guilds and populations in situ, it is useful to preface that discussion with one of the indirect assays provided by cultural methods. The reasons for doing so are twofold: (1) the direct and indirect techniques address the same questions, and (2) direct assays that utilize a single population or serotype rely on an initial survey by cultural methods followed by isolation of the organism of interest by classical plating or other dilution methods. A brief presentation of autecological approaches using plate counts or other cultural methods appropriately introduces the direct techniques for examining natural samples for the abundance dynamics of a specific population or guild.

Although frequent references are made to selective media, few media exist that truly inhibit growth of all organisms not of immediate interest. Most selective media have been developed in response to the need to isolate and enumerate pathogens from environmental samples. For example, Bordet-Gengou agar amended with penicillin inhibits growth of normal flora while allowing the growth of *Bordetella pertussis* (Krieg, 1981). Addition of sodium tellurite to agar suppresses growth of most Gram-positive and Gram-negative organisms with the exception of the *Corynebacterium* sp. Furthermore, the medium has the ability to differentiate among organisms that can grow successfully on the medium; *C. diptheriae* and similar organisms produce colonies that are black as a result of reduction of the tellurite (Krieg, 1981).

Some organisms are conveniently enumerated due to the extreme conditions in which they are active. For example, populations of the autotroph *Thiobacillus ferrooxidans* are most often quantified by an MPN technique using an organic-free medium containing abundant ferrous iron (as an energy source) at a pH of about 2.5 (Silverman and Lundgren, 1958). This is a highly selective medium for enumeration, although recent studies have demonstrated the ubiquitous presence of a heterotrophic contaminant that lives on the excretions of the autotroph (Harrison et al., 1980).

A simpler matter than looking for an individual population is the enrichment method for counting guild members. In fact almost all cultural techniques "select" for guild members rather than for a specific taxonomic group. For example, the ability to use a specific saccharide or amino acid as a sole source of carbon and energy can be used as a convenient selection criterion, simply by providing that substance as the only source of carbon and energy in the plates (this can be difficult in many marine situations where the agar itself may be metabolized by many of the organisms present in the sample). For those organisms that can utilize a compound in the presence of another carbon or energy source materials can be added to the medium to give direct evidence of the activity. Clear zones surrounding colonies catabolizing a lipid emulsion or a cellulosic substance in agar suspension are often used to enumerate individuals

TABLE 1. Examination of Various Guilds of Bacteria Existing in Beach Sands in the Straits of Magellan 2 yr After the Grounding of the VLCC Metula[a]

Guild	Sampling Date			
	May 19	June 3	June 19	August 13
Total	4.0×6	2.7×10^7	3.9×10^6	9.9×10^6
Starch	—	0	0	9.7×10^5
Chitin	5.2×10^5	7.7×10^5	2.7×10^5	2.0×10^5
Casein	—	6.0×10^5	4.0×10^5	7.1×10^5
Tween 20	—	7.3×10^6	4.0×10^5	1.6×10^6

[a]"Total" refers to counts made on a nonselective medium containing yeast extract and the guilds listed refer to the ability of colonies growing on the agar plates to produce a clearing zone in medium containing the indicated polymer. Unplublished data from the study reported in Colwell et al. (1978).

containing lipolytic or cellulolytic enzymes (Colwell et al., 1978). While these organisms may be from a variety of populations, the questions asked of the guild are identical to those asked of a species. For example — what is the effect of a pollutant on the numbers in the guild (and ultimately on the functions of the guild)? An example of the type of data collected in this manner is shown in Table 1 for a time course study of effects of crude oil on bacterial guilds in beach sands.

Direct plating techniques generally cannot be used to determine how population composition of the guild is altered, although some information is occasionally obtained by observing differences in colonial morphology. To answer questions such as this one, the numerical taxonomic approach is often employed, but the question is one of synecology rather than autecology, namely the relationship of the populations, one to another, and how the structure of the community may be altered.

Cultural methods are often used for autecological studies in laboratory simulations. If an organism is seeded into an environment or a sample of that environment in a laboratory situation, it is often possible to follow the dynamics of the population and correlate the results with measures of appropriately selected activities in the samples. If the sample is sterilized prior to inoculation, a nonselective medium may be used to provide information on the changes in numbers of the organism with time. If a selective or differential medium is used, a population can often be followed in the presence of other organisms. Some authors have utilized antibiotic resistant mutants of the organism of interest to mark the population inoculated into nonsterile samples (Danso et al., 1973). This method represents only a modification of the selective medium approach to following the dynamics of a population and is commonly used in attempts to trace the viability of genetically modified bacteria in the environment.

2.2. Problems with the Use of Cultural Methods

Despite the best efforts of scientists employing cultural techniques, the results, although often informative, are never accurate, and can easily lead to misconceptions about the organisms being considered. Many of the problems associated with the use of cultural counts in autecological studies are the ones leveled against the use of the plate count in general; that is, inaccuracy due to clumping of the cells, failure to spread the cells sufficiently on the plate, intercolony interactions, counting difficulties with spreading organisms, questions of counts of vegetative cells versus germinated spores, and so on. However, the major weakness of the cultural approach is that it is an indirect measure. While the question may be asked "How many members of the species or of the guild are present?" the actual question answered by any cultural technique is "How many organisms (or propagules) present are capable of growth on the medium I have chosen?" At best, the method provides information on the potential for organisms to carry out a specific function, whereas if properly used, the direct assays can tell which cells are actually carrying out a reaction in situ, or if the cells of a given population are viable.

Comparisons of results obtained with direct and indirect assay methods for individuals of a population or a guild suggest that the answers to the aforementioned questions are rarely, if ever, the same. Early work (Trolldenier, 1973) suggested that cultural counts could be related to direct counts by a scaling factor, that is, cultural counts recovered a constant fraction of the total number of cells present (see Fig. 1). This plot yielded a regression equation ($y =$
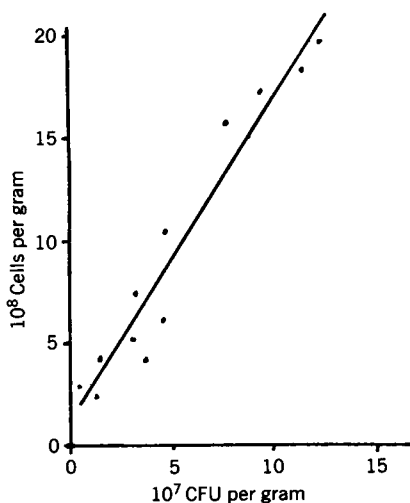

Figure 1. Recovery of microbes from soil by direct count (y axis) versus plate count (x axis). Figure redrawn from Trolldenier (1973).

900.2 + 15 [x − 49.5]) with $r = 0.970$. Several papers since that time have suggested that the relationship is not constant. Sample data in support of this concern are provided in Table 2. The good fit observed by Trolldenier (1973) is rarely seen. In the sample data, the regression actually has a substantially different form ($y = 4.37 \times 10^6 + 393.1x$) and $r = 0.07$, the r value indicates little relationship between the two counts.

While enumerating *T. ferrooxidans* in acidic to circumneutral waters, Baker and Mills (1982) found that the conventional MPN method employing the modified 9K medium (Silverman and Lundgren, 1958) underestimated the number of viable cells present at all sites tested. No cells were observed by the cultural method in the circumneutral waters even though the direct methods (described later) indicated the presence of viable organisms. It was further determined that if the appropriate preincubation procedures were conducted, namely, centrifugal separation of bacterial cells and sequential resuspension in liquid of progressively lower pH, *T. ferrooxidans* could be isolated from the higher pH environments. Thus, it became clear that *T. ferrooxidans* could survive periods of exposure to high pH waters and could be easily transported from one site to another in normal lake waters. This concept had not been previously realized, because MPN determinations in circumneutral waters always indicated that no organisms were present. Furthermore, it was the use of a direct assay for active cells of the population that disclosed this misconception.

Despite the lack of accuracy, cultural approaches in experimental situations usually produce results that successfully delineate overall trends in guild or population dynamics. As compared to the direct examination methods, the cultural techniques are less laborious and are often less expensive. Consequently, the classical methods, with appropriate modifications as new information becomes available, will continue to be utilized in many laboratory simulation exercises.

TABLE 2. Comparison of Direct (AODC) and Plate Counts at Stations in Lake Anna, Virginia in 1978[a]

Station	June	July	August	September
C1	8.1×10^5	9.4×10^5	5.1×10^6	4.8×10^6
	2.5×10^1	1.3×10^2	5.0×10^0	2.7×10^2
F1	1.5×10^6	1.0×10^6	6.3×10^6	6.7×10^6
	9.3×10^2	—	3.4×10^1	7.6×10^2
A2	1.8×10^6	6.6×10^5	6.1×10^6	1.1×10^7
	6.0×10^2	2.0×10^3	4.0×10^2	2.0×10^1

[a]Plate count enumerations employed one-half strength nutrient agar. The first of the two numbers given is the direct count in cells per milliliter, and the lower number is the number of colony forming units per millilter.

3.0. DIRECT METHODS

3.1. Direct Assays for Cellular Activity and Identity

Direct assays are of two types: those that are designed to determine the number and distribution of organisms capable of catalyzing a specific activity, for example, uptake of an amino acid, and those that are designed to determine the number and distribution of cells of a specific population, for example, *Legionella pneumophila*. There are some techniques that examine the numbers of total organisms, for example, the AODC (acridine orange direct counts) method; however, these methods have autecologic significance only when used in conjunction with another of the techniques, for example, to help show that silver granules formed during the microautoradiographic process are truly associated with cells. As stated previously, the purest autecological methods employ two types of assays, a population specific assay, namely, immunofluorescence (FA), to determine the total number of cells of a population present in the sample, and a direct activity-specific assay to determine how many of those organisms are active (viable). We will detail each of the assay types; activity measures being guild specific and FA techniques being population specific, and we will demonstrate the utility of each used alone or the two types together. We will describe several techniques that are considered to be general measures of microbial activity; that is, that ask how many cells in general are viable. These techniques may have great autecological significance if combined with the use of immunofluorescence (FA) techniques to examine a specific population. Those that have already been combined with FA methods will be detailed as such in Section 3.3. Because Jones (1979) published an excellent monograph covering the precise methodologic details of many of the techniques described herein, including appropriate filters, optics, vacuum pressures, and so on, we will not discuss such details.

3.1.1. Synthetically Active Bacteria.

Synthetically active bacteria (SAB) are those that continue to enlarge when in the presence of nalidixic acid (NA) and yeast extract. NA inhibits DNA synthesis within 15 min of exposure while only slightly inhibiting RNA and protein synthesis (Goss et al., 1965). The net effect is that cells continue to grow but are prevented from dividing. The cells appear enlarged as compared to the inactive cells present. Cells are visualized by acridine orange staining (other stains could be used) and epifluorescence microscopy.

Kogure et al. (1979) first used NA and yeast extract to examine bacteria near the Kuroshio current. He found that the numbers of SAB were 10–300-fold greater than colony forming units (CFU) obtained by the classical viable plate count method (CFU about 0.1% of SAB). Although this was an improvement over plate count recoveries, only 5–10% of the total AODC were active.

Kogure called the number of positive cells a "direct viable count" (DVC) as opposed to the "total direct count" (TDC) obtained with the AODC. The term

DVC implies that cells unaffected by either the NA or yeast extract are nonviable. This is probably not true as NA is generally more inhibitory to Gram-negative cells than to Gram-positive or eukaryotic cells, and not all bacteria are stimulated by yeast extract.

The effect of Kepone on the activity of the general community of Chesapeake Bay microbes was evaluated using SAB and ^{14}C amino acid uptake (Orndorff and Colwell, 1980). By this measure, the microbial community was apparently not affected by the in situ concentrations (< 0.01 ppm). However, when the concentration of Kepone was raised to 0.2 ppm, there was a 85 – 90% inhibition of amino acid metabolism measured by radiolabeled amino acid uptake and a 45 – 97% reduction in observed SAB.

Maki and Remsen (1981) compared SAB with INT (an indicator of cytochrome activity — see Section 3.1.4) in four different aquatic systems and found no significant difference in the abilities of the two methods to determine the percentage of active cells. A range of 4.3 – 14.7% active cells was obtained from both oligotrophic and eutrophic lentic freshwater systems in Wisconsin. Neither increasing the incubation time for the INT stain nor incubating INT with yeast extract plus NA significantly changed the percentage of active cells.

Tabor and Neihof (1984) recently compared SAB, INT, and microautoradiography (Section 3.1.5) using ^{3}H-acetic acid, ^{3}H-amino acids, and methyl-^{3}H-thymidine at the surface (1.0 m) and at depth (8.5 m) in Chesapeake Bay. They found from 3 to 77% SAB with the lowest values at both depths in March (3% at 1 m, 7% at 8.5 m). SAB in the surface water peaked at different times than those at depth, with 77% active at the surface in August and 50% active at 8.5 m in July and September. The correlation between the number of SAB and amino acid microautoradiographically active cells was higher than that between SAB and direct counts, and SAB and INT active cells. The proportion of SAB was found to be a significant function of temperature.

It is interesting to note that surface bacteria tended to give higher percentages of SAB than those at depth. The authors suggest that the anoxic hypolimnion is responsible for the decrease in activity. However, anoxic conditions encourage an increased number of fermentors and anaerobes, many of which may be killed or inhibited by the incubation conditions used for these tests.

While the SAB technique has been applied to general considerations of viable cells in the bacterial community, it could provide a valuable means of examining a population as well. If a fluorescent antibody method were coupled with the incubations, the number of synthetically active cells of that population could readily be determined. As with all immunofluorescence assays, this assumes that SAB cells are antigenically stable, that is, that the cells used to elicit the specific antibody response do not lose their reactive antigens (unlikely using polyclonal antibody) and that cells of other populations are unlikely to acquire cross reacting antigens. The SAB technique allows determination of those cells that are actively growing in the habitat. Thus questions about growth and reproduction of the population could be addressed directly by this means.

3.1.2. Frequency of Dividing Cells. Hagstrom, et al. (1979) introduced frequency of dividing cells (FDC) as an indirect method of measuring growth rate in aquatic environments. This would greatly expand the value of AODC in estimating productivity. The method assumes that growth rate (and therefore cell activity) is related to a cell cycle event (septum formation) (Woldringh, 1976). A positive relationship was demonstrated between FDC and growth rate with pure and mixed cultures isolated from the Baltic Sea, and seasonal changes in FDC covaried with ^{14}C-phytoplankton exudate uptake.

Newell and Christian (1981) used FDC to predict growth rates of coastal marine bacterial communities. Samples were filtered to exclude bacteriovores, although some bacteriovores may have passed the filters (Fuhrman and McManus, 1984), and the nutrient concentrations were manipulated. Regressing the natural logarithm of the growth rate (ln μ) on FDC gave a better fit (coefficient of variation $=$ 7%) than did a regression of the untransformed growth rate on FDC. Newell and Christian (1981) suggested that Hagstrom's (Hagstrom et al., 1979) straight–line fit with untransformed data was fortuitous and would not have been found if higher growth rates were examined.

There are problems with measuring the growth rate of a natural population. The large amount of point scatter around the regression line of ln μ on FDC suggested that it might be difficult to predict the growth rate of field samples using this method. Prediction of growth rates from FDC also assumes that the system is in a steady state, a tenuous assumption for a large pool of organisms with relatively short generation times. A further problem deals with starving cells that fragment and decrease their cell volume. Are these cells growing? FDC may be a valuable tool to estimate growth rates in nature, but many questions remain to be answered.

As with SAB, the FDC technique could also be applied to specific populations through the use of a fluorescent antibody to distinguish individual cells. The information gained would be largely the same as with the SAB method, except that there could be no possibility of inhibition of the cells by NA. This method holds promise, because it would require no manipulation of the samples. The FA could be applied to a sample in a manner similar to the acridine orange now used. The major drawback would be in finding adequate numbers of rare cells to provide a statistically valid sampling. That problem is, however, endemic to nearly all the FA methods.

3.1.3. Fluorescein Diacetate. Fluorescein diacetate (FDA) is a nonpolar, nonfluorescent derivative of fluorescein. Because of its nonpolar nature, the molecule is fat soluble and passively diffuses across the lipid phase of cell membranes (Rotman and Papermaster, 1966). Once inside the cell, nonspecific esterases deacetylate the compound. The deacetylated molecule (fluorescein) is fluorescent, polar, and will accumulate in intact cells. However, once cells are injured, (from ageing, freezing, puncture, surfactants, etc.) the dye leaks out and general background fluorescence is observed.

This technique was originally used for fluorometric assays of membrane-bound esterase (Rotman and Papermaster, 1966). It was considered to work only in intact membranes, and therefore fluorescence implied living cells. Schnurer and Rosswall (1982) observed that FDA is also hydrolyzed by proteases and lipases, as well as esterases. Brunius (1980) found that nonbiological hydrolysis occurs at high and low pH, in fresh uninoculated nutrient broth, and in inoculated nutrient broth. Furthermore, the presence of EDTA (ethylenediaminetetraacetic acid) increases cell permeability to FDA. Several workers have found that FDA may be useful for evaluating living fungal biomass (Schnurer and Rosswall, 1982; Swisher and Carrol, 1980; Soderstrom, 1977, 1979).

FDA does not seem to work as well with bacteria as with fungal hyphae, though Newell and Fallon (1982) observed a strong correlation between FDA positive bacterial cells and cells incorporating ^{3}H-thymidine ($r = 0.97$) in coastal water column and sediment samples. The FDA counts were 2–7 times higher than those obtained with the labeled nucleoside. Chrzanowski et al. (1984) used this method to detect active cells in fresh water. The FDA method gave estimates that 6–24% of the total cells were active. This was less than the estimate given by INT in the same study. The difference may have been due to a low permeability of FDA through the outer membrane of Gram-negative cells; the ester seems to penetrate Gram-positive bacteria, fungi, and other eukaryotic cells better than Gram-negative bacteria.

The FDA method of estimating active cells may be best for soils where fungi and Gram-positive cells are numerous. Bacterial cultures and mixed populations are quite variable in their ability to take up the stain (Jones and Simon, 1975).

This method has little hope of being combined with FA techniques, because the two fluorescences would compete. The only possibility for success would involve conjugation of the antibody with a fluorochrome that was excited by light from a different part of the spectrum than fluorescein. Such an adaptation would require switching illumination, but this is already done for combined methods such as FA and INT. In light of the several other methods that provide more information than FDA, combination of this method with FA is probably not worth pursuing.

3.1.4. Tetrazolium Salts. Tetrazolium salts serve as alternate electron acceptors, the reduced product being an insoluble pigmented formazan (Tsou et al., 1956; Nachlas, 1957). Originally, these compounds were used in histologic studies of eukaryotic cells (Kun and Abood, 1949; Cooperstein et al. 1950; Novikoff, 1959), where it was ascertained that the amount of formazan produced is a linear function of the amount of dehydrogenase present. Using plant cells and a variety of dyes [TNBT, NBT, MTT, INT, NT, BT, and TTC (see Table 3)], Gahn and Kalina (1968) found that all reductions were inhibited by the addition of malonate, a specific inhibitor of succinate dehydrogenase. Trevors (1984) compared INT and TTC (triphenyltetrazolium chloride) in soils

TABLE 3. Chemical Names for the More Common Tetrazolium Salts

Compound Abbreviation	Name
TTC	Triphenyl tetrazolium chloride
NT	2,2'(p-Diphenylene)-bis-(3,5-diphenyl)tetrazolium chloride
NT(BT)	Methoxy-derivative of NT
INT	2-(p-Iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride
NBT	2,2'-Di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-bi-phenylene)ditetrazolium chloride
MTT	3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyl tetrazolium chloride
TNBT	2,2'-5,5'-Tetra-p-nitrophenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene)ditetrazolium chloride

and found that the use of INT always resulted in higher activities than did TTC.

Packard (1971) estimated total combined phytoplankton and zooplankton respiration and found that maximum activity occurred when samples were incubated with NAD(P)H and succinate. He proposed that INT measured succinate dehydrogenase. Curl and Sandberg (1961) developed a quantitative method with INT to be used on shipboard. Optimum conditions were described (pH, temperature, reagents, concentrations, time, etc.) along with his finding that succinate enhanced the reaction. Although the method requires extraction of the reduced formazan, the paper is a valuable reference for experimental conditions.

Kenner and Ahmed (1975) found that diatoms and green algae did not reduce INT when tested in the presence of antimycin A, rotenone, or amytal. The authors suggested that the active site of formazan formation is the electron transfer from the cytochrome b–ubiquinone complex to cytochrome c_1.

Zimmerman et al. (1978) was probably the first to apply the technique to look directly at individual cells. This procedure allowed simultaneous determination of the percentage of respiring cells and the size distribution of respiring and nonrespiring cells. In the Baltic Sea coastal areas, most cells were 0.4 μm or less and 6–12% were actively respiring. In comparison, most cells in freshwater lakes and ponds were 1.6–2.4 μm with 5–36% respiring. Zimmerman raised the question of whether INT-negative cells are truly not actively respiring or are merely respiring at a rate below the limits of detection.

Other workers have found excellent correlation between INT reduction and oxygen consumption in a variety of systems including sediments (Jones et al., 1979), freshwater benthic and planktonic samples (Jones and Simon, 1979; Christensen and Packard, 1979), and sedimenting particulate material (51). Jones et al. (1979) found sediment results to be variable and not correlated with the trophic status of the lake. Iturriaga (1979) found that while the total numbers of bacteria exhibited no seasonal tendency, the percentage of INT positive cells was highest in the late summer and lowest in the spring. The study also

showed that the respiratory activity of sedimenting particles was lower than that of planktonic particles.

No information is available to determine if fermentors or anaerobes reduce INT. Since fermentors conserve energy by substrate phosphorylation and anaerobes tend to use electron carriers of lower potential than cytochrome b and cytochrome c_1, it is likely that these physiologic groups are INT negative. This may account for the apparent lower activities observed in sediments and on sedimenting particulate material. Alternatively, Baker and Mills (1982) observed that INT was rapidly reduced in the presence of ferrous iron. Formation of formazan prior to incorporation by the microbes would result in a lack of uptake and an artificially low number and size of crystals. Tetrazolium salts are known to be reduced to formazan in the presence of dithionite, diammonium sulfide, ascorbate, cysteine, glutathione, pyridine nucleotides, and several dehydrogenases (Nineham, 1955). The rate and extent of the reduction is a function of the specific Eh–pH conditions. Use of INT in any anaerobic environment or aerobic environment containing Fe^{2+} must be approached with caution.

Pamatmat et al. (1981) attempted to find a correlation between heat production in sediments and ATP or electron transport system (ETS) activity. There was no relationship between heat production and either ATP or ETS activity; however, there was a correlation between ATP and ETS activity in surface sediments. In 1980, two separate groups used INT to investigate microbial activity in marine systems (Harvey and Young, 1980; Christensen et al., 1980). Harvey and Young (1980) determined that the surface layer of a salt marsh estuary contained 16% respiring cells while subsurface water contained only 5.14%. Christensen et al. (1980) used pure cultures of marine pseudomonads to relate ETS activity to changes in physiologic state such as energy charge [(ATP + 1/2 ADP) / total adenylates]. As energy charge decreased, ETS activity measured by INT reduction concomittantly decreased, suggesting reduced cellular activity.

Tabor and Neihof (1983) recently suggested improvements to increase the sensitivity of the direct INT method. When formazan containing cells are examined, over 70% of the positive cells may be lost due to dissolution of the formazan crystal in the immersion oil, this problem was circumvented by isolating the organisms from the oil with a thin gelatin film. Clarity of the image may also be enhanced by transferring organisms collected on a filter to a gelatin film followed by an additional coating of gelatin. Using this method 61% of the total direct count of estuarine organisms examined by Tabor and Neihof were INT positive. The gelatin stripping method was used by Baker and Mills (1982) to demonstrate that in varying environmental conditions, from 1 to 80% of *T. ferrooxidans* cells were INT positive.

The reduction of INT has already been successfully coupled with the FA technique to produce a method for examining viable cells of a specific population (Baker and Mills, 1982; Fliermans et al., 1981). These techniques and their usage will be discussed in Section 3.3.

3.1.5. Microautoradiography. Microautoradiography (MARG) is potentially the most powerful method for the determination of single cell activity in situ. It is the most appropriate method for enumerating cells of guilds where members are delineated on their ability to incorporate a radiolabel. Major limitations involve the lack of suitable isotopes for some activities, for example, inorganic nitrogen incorporation and situations in which the important activity is determined as formation of a specific product. In such cases, MARG is unlikely to be of value.

There is extensive literature on the use of radioisotopes including a book by Rogers (1979) that provides a detailed discussion of theoretical considerations and technical problems of autoradiography. The most common activities evaluated include nucleic acid synthesis (Furhman and Azam, 1982; Bern, 1985; Karl, 1982; Moriarty and Pollard, 1982; Pollard and Moriarty, 1984), uptake of soluble organic matter (Ramsay, 1974; Ward et al., 1971; Stanley and Staley, 1977; Wright and Hobbie, 1965; Wright and Hobbie, 1966; Munro and Brock, 1968), and amino acid uptake (Tabor and Neihoff, 1984, Hoppe, 1976, Bright and Fletcher, 1983).

Brock and Brock (Brock and Brock, 1966; Brock, 1967; Brock and Brock, 1968) were the first to apply MARG to questions of microbial activity in situ. They found that the heaviest colonization of bacteria was in the axillary region of *Leukothrix mucor* where diffusion is limited and organic material may accumulate. Briefly, the method involves exposing a sample to a radiolabeled substrate, filtering the sample, and imbedding it in a gelatin matrix on a microscope slide. The slide is then coated with a photographic emulsion that is exposed by the radioactivity for approximately 1 week. After developing the emulsion, the silver grains associated with bacteria are counted. This is a fairly expensive and labor intensive procedure, but the activity defining the guild is probably more closely determined using this method than with any others.

Recently Tabor and Neihoff (1982) reported improvements in the MARG techniques and compared their results to those of Meyer-Reil (1978). The improvement is similar to the gelatin stripping procedure developed for the INT reduction assay. It leaves the cells imbedded in a gelatin matrix before exposure to the photographic emulsion. The resulting microautoradiogram is much clearer than that generated while the filter is in place. Tabor and Neihoff (1982) found 49, 50, and 73% of the direct count community active in the uptake of acetate, amino acids, and thymidine, respectively. When the specific activity of the amino acids was doubled, 94% of the cells become active in amino acid uptake. Substrate concentration is also an important variable when using this method and several concentrations should be evaluated. It may be difficult to compare results from one study to another in the absence of some standardized test concentration of both substrate and radioactivity.

Some common general problems with autoradiography include loss of activity due to chemical fixation of cells (Silver and Davoll, 1978), energy loss from beta particles from heat, pressure, or chemical reactions, chemography (Rogers, 1979), and inappropriate incubation times. Knoechel (1976) found that effects

of source geometry must be corrected for. A common simplifying assumption is that the grain count over a cell is proportional to the cell's radioactivity. This assumption is almost never realized with a mixed population, resulting in overestimation of the activity of large cells and underestimation of the activity of small cells. Relative grain densities around organisms are a function of cell geometry, specimen density, energy of tracer, and sensitivity of the photographic emulsion (Faust and Correll, 1977). Aggregating cells may contribute to underestimation of unlabeled cells.

There are numerous reports of methods for obtaining some kind of activity index. The simplest is the percentage of active cells (Faust and Correll, 1977; Fuhrman and Azam, 1982; Hoppe 1976). Ward (1984) devised relative activity as an index of CO_2 fixation on a per cell basis. He defined relative activity as the mean number of silver grains per cell ÷ the number of grains in a similar area of background. This measure is a relative one, and is not an absolute measure of CO_2 fixation.

3.1.5.1. Nucleoside Uptake. Incorporation of nucleosides, especially thymidine and adenine, has been used extensively as a measure of bacterial productivity. Concerns with measures of total uptake of labeled nucleosides have been voiced because of theoretical problems. It is generally assumed that only non-photosynthetic bacteria will incorporate exogenous thymidine since those cells contain the enzyme thymidine kinase (Fuhrman and Azam, 1982; Bern 1985; Moriarity and Pollard, 1982; Grivell and Jackson, 1968). Organisms lacking thymidine kinase incorporate radiolabeled thymidine with less specificity than those having preferred salvage pathways.

Some general processes that must be considered when examining uptake of nucleosides are (1) community potential for assimilating exogenous precursors, (2) intracellular and extracellular pools of structurally related compounds that may dilute the radioisotope, (3) balance between *de novo* and salvage pathways, (4) specificity and extent of macromolecular labeling (Wright, 1978; Karl, 1982). Qualitative and quantitative patterns of labeling may be influenced by the position of the label (Karl, 1981a). For example, a greater percentage of thymidine goes into DNA when the molecule is labeled with ^{14}C, than with methyl-^3H (Karl, 1982). Thymidine has been found to be incorporated into yeasts, slime molds, algae, and protozoa in nanomolar concentrations (Karl, 1982), and there are also reports of marine pseudomonads (Pollard and Moriarity, 1984) and anaerobes (Moriarity and Pollard, 1982) that do not incorporate exogenous thymidine. Lack of incorporation by some bacteria might be a severe problem in MARG assays; however, uptake by eucaryotes would present no problem since they are easily discernible microscopically from the bacteria.

Karl (Karl, 1981a; Karl et al., 1981b; Karl 1982) has examined the utility of $2-^3H$–adenine for detecting active cells in situ. Some of the major assumptions made when labeling populations with adenine are (1) most cells must transport and assimilate exogenous adenine, (2) addition of exogenous adenine must not expand the ATP pool, luxury uptake, or in situ stimulation of the rate of RNA

synthesis, (3) there should be no compartmentalization of ATP (an immediate precursor to RNA). Karl validated assumption (2) and found that 49 out of 50 random isolates were able to incorporate exogenous adenine. It was also found that the proportion of cells able to assimilate ^{3}H-adenine was greater than that of cells able to assimilate ^{3}H-amino acids.

3.1.5.2. Uptake of Organic Carbon Compounds. Using ^{3}H-glucose Meyer-Reil (1978) found no correlation between standing crop values (CFU, biomass, AODC) and the number of glucose-active cells in samples from Kiel Fjord beaches and Kiel Bight. While glucose is obviously not a suitable substrate for determining the relative number of active cells in the general community, it could be used effectively in a scheme combined with a fluorescent antibody to estimate viability in a population known to oxidize glucose. Several ^{14}C organics have been used to examine the activity of organisms in leaf litter (Ward et al., 1971) attached to sand grains (Munro and Brock, 1968), and in aquatic environments (Stanley and Staley, 1977). The results also lead to the conclusion that while a single compound might not be appropriate for general community work, guild examinations with or without a fluorescent antibody is certainly feasible.

Uptake kinetics of organic compounds need to be considered when estimating heterotrophic activity. Bacteria tend to have specific transport systems that become saturated at low nutrient concentrations while algae transport by Fickian diffusion with velocity of uptake increasing with increasing substrate concentration (Wright and Hobbie, 1965; Wright and Hobbie, 1966).

3.1.5.3. Other Labels. Labeled amino acids (Hoppe, 1976), and phosphorus (Faust and Correll, 1979) have also been used to assess microbial activity in situ. Hoppe (1976) used ^{3}H-amino acids to evaluate active heterotrophs with distance from shore. He found that the number of colony forming units from plate counts was from 0.01–6% of the total active heterotrophs determined using labeled amino acids (100% active), glucose (29%), aspartic acid (30%), and thymidine (81%). The amino acid mixture was taken up by the largest proportion of the total cells. The incubation time with the labeled compound must be long enough to label but short enough not to allow cells to multiply. Use of a labeled amino acid mixture tends to produce the highest percentage of active cells of all the labeling techniques (Tabor and Neihoff, 1984).

Using ^{33}PO$_4^{3-}$, Faust and Correll (1977) compared the relative importance of bacteria and algae in phosphorus cycling. They found that, overall, 95% of the phosphorus was assimilated by bacteria with algae being significant in phosphorus uptake only in the summer.

Obviously, activity or viability is actually defined by the conditions of the assay being used. Depending on which of the above techniques is used, the data vary widely (Table 4). This variation is understandable when the organismal capabilities needed to generate positive results in any given test are examined. Tests in which the cells expand in size (SAB) or divide (FDC) require the

TABLE 4. Various Estimates of the Percentages of Active Cells in Several Environments as Determined with Different Direct Assays[a]

Method	Saline Water		Freshwater		Soils/Sediments
FDA			6–24	(9)	2–83 (12)
INT	6–12	(1)	5.2–14.7	(10)	25–94 (13)
	5–16	(2)	5–36	(1)	20–90 (14)
	5–10	(3)			
	61	(6)			
SAB	5–10	(3)	4.3–9.7	(10)	
MARG + AODC	20–80[b]	(4)			
	28–50[b]	(5)			
	95[c]	(6)			
	2.3–56.2[c]	(7)	air/water 22–59 (10)		
MARG	63–85	(8) bacteria (^{33}P)			
+ SAFRANIN	28–42	(8) phytoplankton (^{33}P)			

[a]Values given are the percentages based on a direct count (usually AODC). Numbers in parentheses represent the literature citations:

[b] ^{3}H-thymidine.

[c] ^{3}H-amino acids.

[d] ^{3}H-glucose.

(1) Zimmerman et al. (1978).
(2) Harvey and Young (1980).
(3) Kogure et al. (1979).
(4) Riemann et al. (1984).
(5) Furhman and Azam (1982).
(6) Tabor and Neihoff (1982).

(7) Meyer-Reil (1978).
(8) Faust and Correll (1977).
(9) Chrzanowski et al. (1984)
(10) Maki and Remsen (1981).
(11) Hermansson and Dahlbak (1983).
(12) Lundgren (1981).
(13) Tabor and Neihoff (1984).
(14) Iturriaga (1979).

greatest integrated activity on the part of the cells. Measures of specific enzyme activity (FDA) or cytochrome activity (INT reduction) theoretically require that cells be intact and functional at some level and would be intermediate in terms of demands on the cells (Table 5). MARG techniques could be the least rigorous of all, since some labeled compounds may be passively transported across the membrane resulting in the enumeration of nonactive cells. The latter statement hinges, of course, on the compounds used in the studies, and on the general state of the samples. Nevertheless, it is clear that viability (or activity) may have different meanings, dependent on the test used. Individuals seeking to investigate cell specific activity in situ should select techniques based on the level of activity they wish to examine in the habitat being studied.

3.2. Population Specific Assays

3.2.1. Nonspecific Fluorescent Stains. The only direct assay for members of a population is by the immunofluorescence or FA technique. In order to place this method in its proper context, and also to provide a bit of information on the techniques used to examine guilds, especially with MARG assays, we will briefly discuss nonspecific stains as well.

3.2.1.1. Acridine Orange. Acridine orange (AO) is currently the most common nonspecific stain used to obtain total counts of bacteria. Acridines form random complexes with DNA and RNA by intercalating between stacked base pairs. This reaction separates the base pairs by twice their normal distance making acridines powerful mutagens, usually inducing frame-shift mutations (Mandelstam and McQuillen, 1976). Stained bacteria appear red or green when illuminated by epifluorescence microscopy. AO fluoresces green when it is complexed as the monomer with double stranded DNA and fluoresces red when it is complexed as a dimer to single-stranded RNA (ss RNA) or DNA (ss DNA), (Daley, 1979). Originally, it was thought that the color of fluorescence could be used to determine viability of the cells, namely, green cells should be the normal viable condition (not actively engaged in protein synthesis, that is, nonlog phase) and red cells should be dead and decomposing (ssDNA) or rapidly growing (ssRNA). Experience rapidly showed that this differentiation was so inconsistent as to be worthless in evaluating the physical status of cells.

Direct counting by any method is dependent on the quality of the microscope and the microscopist (Brock, 1984). Daley and Hobbie (1975) compared epifluorescent microscopic methods for determining numbers of marine and freshwater microorganisms. The size and nature of the filter used to trap bacteria (Fuhrman, 1981; Azam et al., 1983) are also very important, and prestaining filters with Irgalan Black helps to increase the visibility of the organisms by eliminating or reducing interfering fluorescence (Hobbie et al., 1977). Good agreement has been documented among AODC and scanning and transmission electron microscopic counts (Bowden, 1977; Larsson, 1978), suggesting that AO counts produce an accurate estimate of the true standing stock of bacteria. Bowden (1977) found that there was no significant difference in SEM and AO counts when 0.2-μm Nuclepore polycarbonate filters were used.

Daley (1979) has written as extensive review of the methodology and results

TABLE 5. Viability as Defined by the Several Direct Examination Techniques for Activities[a]

Techniques	Measures	Implications
MARG	Uptake	Indicates transport of a compound into the cells—active or passive
INT Reduction	Functioning cytochrome system	Indicates that the organism is currently respiring
SAB	Biomass accretion	Indicates that the cells are, in fact, growing larger—the biomass of the population is increasing
FDC	Growth rate	Indicates rate of cell division—increasing numbers

[a]The methods are ranked according to the level of activity measured, from general to specific.

obtained from direct epifluorescence microscopy. AO has been successfully used for enumeration of bacteria in water column (Moriarity, 1979), sediments (Rublee and Dornseif, 1978), and decomposing plant material and detritus (Robertson et al., 1982, Rublee et al. 1978).

3.2.1.2. Use of Other Fluorochromes. Two other fluorescent stains not extensively used for enumeration of bacteria are DAPI (4'6-diamidino-2-phenylindole), which is a DNA stain and may improve visualization of small ($< 1 \mu m$) cells (Porter and Feig, 1980), and FITC (fluorescein isothiocyanate). FITC stains proteins and is used extensively in immunology (see FA methods). Karl et al. (1981b) found that individual cells were easier to visualize in microautoradiographs when stained with DAPI than when stained with AO. It is likely that the use of DAPI will increase greatly, especially in marine studies where cells tend to be substantially smaller than in freshwater or soil habitats.

3.2.2. Immunofluorescence Assays. Antibody techniques take advantage of the antigenic diversity of organisms along with the immunologic specificity of antibodies. Antibodies were first used to identify the *Salmonella* serotypes responsible for human infection (Garvey et al., 1977). Once an organism is isolated, production of the antibody in a rabbit is fairly straightforward since bacteria tend to elicit a strong antibody response in rabbits. Briefly, whole cell suspensions are injected into the rabbit's marginal ear vein at 4-day intervals for a period of 16 days. The animal is rested for a week and the titer against the antigen is determined. If the titer is high, indicating a strong antibody response was achieved, the blood is harvested and the antibody is purified from the serum. The antiserum is termed polyclonal since antibody is made against more than one antigenic determinant site if whole cells are used. The antiserum must be tested against many strains of bacteria to determine its specificity and adsorbed with any cross-reactive strains. For example, there are over 200 serotypes of *Salmonella,* but only a few are generally important in human infection. At the same time *Pseudomonas* species tend to be very cross reactive. This is probably an extension of the general genetic plasticity of this genus. Testing antisera for cross-reactive populations isolated from the system of interest cannot be overemphasized. If cross-reactive species are found, then a decision must be made whether or not to adsorb the antisera. There are many good immunology texts that describe appropriate methods and controls necessary in all steps of antibody preparation (e.g., Garvey et al., 1977; Carpenter, 1975).

Bacteria to which the antibody is made are visualized by conjugating the antibody with a fluorescent dye (direct method), or by using a fluorescent tagged antibody against the rabbit antibody (indirect method). The indirect method is more sensitive and does not require much more time. An additional advantage to the indirect method is that one fluorescent tagged antibody may be purchased (usually goat anti-rabbit IgG) and used to visualize many specific rabbit antibodies made to different organisms.

Bohlool and Schmidt published an excellent review on the uses of the FA

method to enumerate bacteria in environmental samples (Bohlool and Schmidt, 1980). Counts of individuals using FA are generally one to three orders of magnitude greater than those generated using the most probable number method (Baker and Mills, 1982). Investigators have used FA to study the distribution of bacteria in a variety of aquatic and terrestrial environments (Gates and Pham, 1979; Apel et al., 1976; Ward and Frea, 1980; Stanley et al., 1979; Fliermans and Schmidt, 1975; Hermansson and Dahlbak, 1983).

The approach to quantifying cells with FA is similar to that used with other fluorescent dyes except that numbers of FA positive cells may be substantially less than the total count and more efforts must be made to concentrate and find the cells. This can become a problem when working in sediments or soils where cells tend to be attached to particles. There is also a problem with nonspecific fluorescence due to charge interactions of antibody and other particles, although this may be overcome by first staining the preparation with fluorochrome-conjugated gelatin (i.e., if FITC-conjugated antibody is used, counterstaining with Rhodamine-conjugated gelatin results in a brownish background with green fluorescing cells) (Bohlool and Schmidt, 1968).

Some disadvantages of FA are: (1) Because of the specificity of the stain, only one population (species, serotype) is detected. It is difficult to determine if the species is, in fact, environmentally important; and, (2) The stain also tags dead or inactive cells. To our knowledge, no one has determined how antigens disintegrate as cell function ceases.

The greatest weakness of the FA technique is the requirement for pure cultures of the organism of interest. For examination of the in situ distribution of an organism that is well known and characterized, this presents no problem. For example, studies of *Legionella pneumophila* proceed without difficulty, because the major biotype in the population has already been isolated, although new serotypes continue to appear in the literature. In cases where the organism of interest is one of the components of a natural assemblage, it is difficult to assess the importance or ecologic function of the isolate in the original sample. For studies that rely on obtaining an isolate from natural samples prior to making the antibody, the success of the entire study hinges on the ability of the investigator to get the "right" organism. For example, in studying the distribution of methanogens and sulfate reducers in an acidified lake, we have prepared antibodies to one isolate of each type. Unfortunately we do not know if the sulfate reducer is responsible for most or even a significant part of the sulfate reduction occurring in those sediments. We can make some statements about the likelihood of importance of the isolate after the study, when the sulfate reduction rates are correlated to the numbers of the isolate present in the sample. At that time (and not before) will we truly know if this organism is a good tracer of the sulfate reducing guild in these sediments. Of course, the same concerns can be raised for the methanogen. It is certainly possible to make antibodies against type strains available from various culture collections, but there is still a question of the relevency of that strain to the system under examination.

3.3. Combined Population and Activity Specific Assays

The FA technique is the key to population based autecological studies, but the greatest potential comes from combining two or more of the direct microscopic methods described previously. With the combinations we have essentially come full circle and are able to directly address the question of the abundance of active (viable) cells of a population in situ that was first addressed over 100 years ago with crude cultural methods. Unfortunately we have spent many pages building to this section, which will be one of the shortest. The use of combined methods for assaying the presence of viable cells of a specific population has barely been touched. The few available examples will be given here, along with an implicit demand for more research along these and similar lines.

Baker and Mills (1982) combined FA with INT (and called it the FAINT assay) to examine changes in the number of respiring *T. ferrooxidans* as distance from the source (pyrite mine tailings) increased. They found that the number of respiring organisms dropped more rapidly than the total number of *Thiobacillus* as the pH of the water increased (Table 6). Simultaneously the total number of cells and the number of actively respiring cells was found to increase. As pointed out earlier, the finding of viable *T. ferrooxidans* in circumneutral water, and the ability to isolate the organism whenever observed with the FAINT assay provides new information on how this bacterium moves from one source of energy to another.

Using the FAINT approach (Baker and Mills, 1982), Scala et al. (1982) examined the numbers of both *T. ferrooxidans* and *T. thiooxidans* in acid mine drainage from a variety of sources including metal sulfide ores and coal mines. Their results (Table 7) indicated that the two organisms occurred in roughly equal proportions at all of the 16 sites evaluated. The FA portion of the assay determined that the two organisms together accounted for only about 12 to 20% of the total numbers present by AODC determinations. The proportion of *Thiobacillus* sp. shown to be actively respiring by the FAINT procedure varied

TABLE 6. Distribution of Respiring Bacteria in Lake Anna, Virginia, as Determined by Combined Epifluorescence Staining and INT-reduction[a]

	Station[b] (10^3 cells mL)		
	C1	C3	A2
AODC	370	390	920
AODC + INT	74	230	580
FA	38	18	13
FAINT	30	9.2	2.9
MPN	0.0033	0	0
pH	3.2	4.2	6.9

[a]Comparisons are given for total cells by AODC and *Thiobacillus ferrooxidans* FA, FAINT, and MPN (Baker and Mills, 1982).
[b]Zero indicates below limit of detection, not true 0.

TABLE 7. Numbers of Total Cells, Respiring Cells, and Total and Respiring *Thiobacillus Ferrooxidans* and *Thiobacillus thiooxidans* in Several Samples of Acid Mine Drainage Waters from Different Mineral Sources[a]

Site	AODC 10^4 cells/ mL	FFA 10^3 cells/ mL	TFA 10^3 cells/ mL	FINT % of FFA	TINT % of TFA
Balaklala[b]	8.82	3.06	14.14	48.30	29.17
Weil[b]	82.20	57.77	50.13	51.25	46.90
Squaw Creek[b]	9.83	17.50	3.70	20.00	35.30
Mammoth[b]	10.24	2.45	4.96	35.57	46.65
Argo[c]	6.99	7.24	6.74	31.63	31.02
Contrary Creek[d]	47.80	9.02	6.19	31.00	15.00

[a]AODC and FA counts are expressed as cells per milliliter. INT reduction results are expressed as a percentage of the FA counts. Standard errors have been omitted for the sake of simplicity, but were always on the order of 5–30% of the listed value. A total of 16 sites were visited, and data similar to those shown here were seen at all locations. Values of pH at the various sites were all below 3.0 at the time of sampling.
[b]Iron mines in West Keswick mining district of California.
[c]Gold mine in Colorado.
[d]Abandoned pyrite mine in Virginia.

from site to site and ranged from 19 to 74%. In general, high or low percentages of active cells of each of the species examined covaried, that is, they could not be shown to be significantly different.

At the same time that Baker and Mills (1982) were using the FAINT assay for *T. ferrooxidans,* Fliermans et al. (1981) independently adapted a combined FA and INT reduction technique to examine the distribution of viable cells of *Legionella pneumophila* in situ. In later studies, (Fliermans and Harvey, 1984) the combined technique was used to show that an industrial biocide reduced neither the numbers nor percentage of INT-positive cells in cooling tower applications. INT reduction has also been combined with malachite green (MINT) to look at the number of active bacteria in sewage (Dutton et al., 1983), although this assay is not truly population specific. Actually, the MINT assay represents no significant difference from staining with AO or any other general stain.

MARG and epifluorescence microscopy (AO) are often combined to look at specific guilds of bacteria (Wright, 1978, Hermansson and Dahlbak, 1983; Harvey et al. 1984) in estuarine and coastal waters, air–water interface, and aquifers. The combination of FA and MARG has also been combined to examine population specific activities. MARG and FA techniques were combined to look at the activity of nitrogen cycle bacteria (Fliermans and Schmidt, 1975; Ward, 1984). Fliermans and Schmidt (1975) made a specific polyclonal antibody against *Nitrobacter agilis* and *Nitrobacter winogradskyi*. Using $NaH^{14}CO_3$ and $^{14}CO_2$ to follow carbon fixation, they determined that log phase cells were not uniformly active and that the highest percentage of activity

occurred in early log phase (84%). Fliermans and Schmidt correctly pointed out that theirs was a true study in autecology.

Ward (1984) used the antibody against *Nitrosococcus oceanus* and *Nitrosomonas marina* along with $^{14}CO_2$ to evaluate the autotrophic activity of ammonium oxidizers in seawater. Over 10^4 cells /L were found. Relative activity was positively correlated with the abundance of ammonium oxidizers, temperature, dark CO_2 assimilation, decreased oxygen concentration, and pheopigment concentrations in the upper photic zone. The relative activity peaked near the surface and again in the nitrite maximum region below the photic zone.

The frequency of dividing cells technique has also been combined with MARG (Riemann et al., 1984, Newell and Fallon, 1982) to produce quantitative growth data about the general community. Riemann et al. 1984 found that FDC activity approximated activity estimated from the uptake of (methyl-^{3}H) thymidine in Danish coastal environments with an increase in activity frequently occurring in the morning. Newell and Fallon, (1982) using similar methods in the water column and sediments of the Georgia coast, found a strong correlation between the two methods with FDC estimating two to seven times more activity than ^{3}H-thymidine. They estimated a secondary productivity of $2-4 \times 10^8$ cells $\cdot$ L^{-1} $\cdot$ hr^{-1} at 0.25-km offshore and $1-9 \times 10^7$ cells $\cdot$ L^{-1} $\cdot$ hr^{-1} at 15-km offshore. These studies, however, represent a comparison of methodologies rather than a combination for the purposes of autecology.

4.0. CONCLUDING COMMENTS AND SUGGESTIONS FOR FUTURE RESEARCH

It should be clear that a variety of techniques exist for approaching the original question of the distribution of active cells of a population or of a guild by examining individual cells in situ. What should also be clear is that use of these techniques is currently limited to a few research groups. The fact remains that fluorescent antibody work is tedious, and may border on impossible if large numbers of samples are involved. Furthermore, INT reduction and MARG are at least as time and labor consuming as FA procedures. Application of these methods, especially in combination, is not for the microbiologist looking for quick answers. The preparations for the experiments require a great deal of overhead in terms of operator time, and the analyses require time and effort devoted to, what must be candidly admitted, is very boring work. The results are, however, worth the time and effort. Direct examination of individual cells, especially when identified by immunofluorescence methods is the only way that truly accurate pictures of the behavior of populations and guilds in situ may be accomplished.

Data handling can often present problems. Most counts such as INT and MARG are made relative to AODC counts or FA counts made simultaneously on the sample. The AODC or FA counts are made on filtered samples allowing easy extrapolation of dilution factors, while the other counts are made on

smeared or gelatin-stripped subsamples stained with AO or a fluorescent anti-
body as a cell visualizer. The results are most often expressed as a percentage of
the AODC or FA. Any attempt to convert this percentage to the total number of
cells in a volume of sample presents difficulty. First there is a counting error
associated with the original count (AODC, e.g.). Counting two or more inde-
pendent samples allows a variance to be calculated for the counts. Similarly, if
multiple samples are run for the activity measure, a variance can be calculated
for the percentage of active cells. Combining those terms to produce a variance
term for the number of FAINT cells, (e.g.,) is less straightforward. Baker and
Mills (1982) approached the problem by randomly assigning each individual
INT determination to an individual FA determination to get a series of FAINT
values (in cells per milliliter) equal in number to the number of FA (and INT)
determinations. These values were then used to calculate a variance for FAINT
determinations. Scala et al. (1982) also used this procedure in their study of the
distribution of *Thiobacillus* spp. (Those calculations are not shown in Table 7.)
While the variance thus calculated was acknowledged to be contrived, its use in
parametric statistics was considered to be conservative, and the fact that few
significant differences were found in any comparisons except where the values
were so widely separated as to preclude the use of any statistical analysis lent
credence to the assertion that if an error arose it would have been a Type II error,
that of accepting a false null hypothesis, the most conservative type of error. It is
possible that an effect might be present but undetected, yet the authors would be
unlikely to have claimed a difference that was not present. Figure 2 indicates the
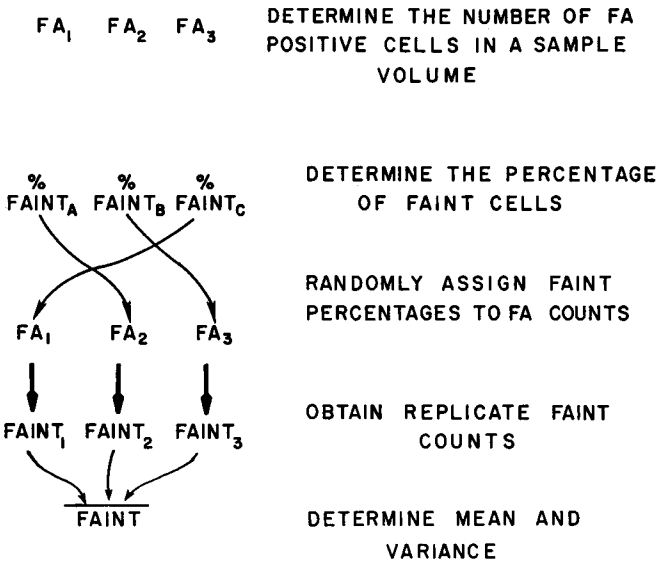process used to obtain a variance for FAINT counts.

FA_1 FA_2 FA_3 DETERMINE THE NUMBER OF FA
POSITIVE CELLS IN A SAMPLE
VOLUME

%   %   %   DETERMINE THE PERCENTAGE
$FAINT_A$ $FAINT_B$ $FAINT_C$ OF FAINT CELLS

RANDOMLY ASSIGN FAINT
PERCENTAGES TO FA COUNTS

FA_1 FA_2 FA_3

OBTAIN REPLICATE FAINT
COUNTS

$FAINT_1$ $FAINT_2$ $FAINT_3$

FAINT   DETERMINE MEAN AND
VARIANCE

Figure 2. Process for determining a mean and variance for FAINT counts. This scheme could be
used for any similar assay in which percentages are multiplied by separate counts to obtain a final
value. It is important that independent samples be used for the determinations.

While it may seem that several methods are available for examining populations and guilds, in situ, much work remains before the procedures will be commonplace. Methodological improvements are necessary. What, precisely, does INT reduction indicate about a cell in terms of viability versus activity? This question applies to any of the techniques. Does the SAB technique exclude slow growing organisms that may have an overwhelming effect on an important ecological transformation? What assurance is there that an organism enumerated by an FA technique is of quantitative significance in terms of the functions carried out by other members of the guild? There are many other questions appropriate to this area of microbial ecology, and more widespread examination of the techniques and their uses will not only reveal answers but additional questions as well. In the meantime, the data generated by users of these methods will continue to be recognized as the foundation of the future of microbial autecology.

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