**In situ methods for assessment of microorganisms and their activities**

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Recent technical developments in the field of molecular biology and microsensors are beginning to enable microbiologists to study the abundance, localization and activity of microorganisms *in situ*. The various new methods on their own bear high potential but it is the combination of studies on structure and function of microbial communities that will yield the most detailed insights in the way microorganisms operate in nature.

**Introduction**

Many bacteria and other microorganisms usually do not have enough morphological detail for easy identification. Microbiology has, consequently, relied on cultivation for identification, which has proven difficult for many environmentally or medically important microorganisms [1,2]. Even though new microorganisms continue to be isolated, it is estimated that so far only a small fraction, possibly below 10%, of the extant microorganisms have been grown in pure culture and characterized [1]. Consequently, we are still unable to identify many microorganisms, including the causative agents of certain diseases, or to understand the role of microbes in the regulation of globally important mineralization processes. The lack of knowledge is most severe for complex, multispecies microbial communities. Here, populations are frequently arranged in a very specific way (e.g. in biofilms) and such communities have activities that can not be achieved by individual microorganisms [3]. Even when all bacteria can ultimately be cultured (which is quite unlikely), progress in the understanding of the ecology of complex microbial communities will therefore still require studies on the activity and distribution of microbes directly in minimally disturbed samples.

Information that is important for studying microbial ecology may be subdivided into the following categories:

- **diversity, structure and function.** We can ask questions such as: what organisms are present in a given ecosystem? How many cells of a certain species are in a defined spatial element at a given time? What is the *in situ* activity of an individual microbial cell in an environment defined by physicochemical parameters that may be modulated by other biological entities? This review focuses on recent developments that have significantly enhanced our ability to address structure and function of microbial communities *in situ*. A thorough review on new developments and findings in the third category, the field of microbial diversity, is beyond its scope.

**In situ identification and localization**

Studies in this area are still mainly based on the rRNA approach to microbial ecology and evolution [1,4]. The main reasons are that comparative analysis of 16S (and 23S) rRNA sequences is today the most commonly used method for studying the phylogeny of microorganisms, and that rRNA sequences can be obtained from environmental or medical samples without cultivation. This direct retrieval is facilitated by the polymerase chain reaction (PCR) exploiting highly conserved primer binding sites on the 16S and 23S rRNA genes (e.g. near the 5′ and 3′ end of the 16S rRNA gene). Consequently, the number of publicly accessible 16S rRNA sequences has been increasing rapidly in the last decade and is now exceeding 10,000 [5••,6••]. Based on these sequence collections rRNA-targeted oligonucleotide probes (chemically synthesized, single stranded, short [usually 15–25 nucleotides in length] DNA molecules) can be designed in a directed way. These probes may be targeted to signature sites of the rRNA molecules characteristic for defined taxonomic entities such as species, genera, families, orders, or even domains, since the rRNA molecules also have conserved signatures that separate the three lines of descent, the Archaea, Bacteria and Eucarya [7]. Sets of probes, therefore, allow for a rapid assignment of cells or rRNA of interest to major groups [1]. *In situ* identification of individual microbial cells with fluorescently labeled, rRNA-targeted oligonucleotide probes, the so-called phylogenetic stains [8], is based on the high cellular content of usually more than 1000 ribosomes, and consequently as many 16S and 23S rRNA molecules.

There were several interesting technical developments in the area of fluorescence *in situ* hybridization (FISH) in the past year, all aimed to increase sensitivity of *in situ* identification of small environmental bacterial cells. Tyramide System Amplification (TSA®, NEN Research Products) combined with horseradish peroxidase labeled oligonu-
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...the vast undiscovered microbial diversity it is interesting that whole fixed cells, sorted in a flow cytometer on the basis of parameters such as light scatter, DNA content or probe-conferring fluorescence, could be used for subsequent retrieval of almost full length 16S rRNA sequences [20**]. Using this technique defined fractions of the total cells in a sample can be selected for molecular identification. As soon as 16S or 23S rRNA sequences are available, specific probes can be designed in minutes. With public software packages like ARB [21**] probe target sites can be selected and tested against all other available rRNA sequences [6**,7]. Successful in situ identification, however, also requires permeabilization of the target cells prior to hybridization. In the case of the filamentous bacterium Microthrix parvicella, a frequent cause of activated sludge bulking and foaming, permeabilization has proven difficult and ultimately required enzymatic treatment of the Gram-positive cell wall [22].

New group-specific probes have been developed that enlarge the set of group-specific, rRNA-targeted oligonucleotide probes available for a rapid classification of single cells by FISH (e.g. for Gram-positive bacteria linked with activated-sludge foaming [23] and for thermophilic bacteria present at deep-sea hydrothermal vent sites [24,25]). It is also notable that traditional isolation of bacteria has been the basis for two nice studies applying FISH. Hess et al. [26**] demonstrated that hydrocarbon-degrading Azoarcus sp. strains isolated from a diesel fuel contaminated laboratory aquifer made up only 1–2% of all bacteria present in this system. Kalmbach et al. [27**], in contrast, used FISH to identify those strains that are the major constituents in drinking water biofilms from 234 strains originally isolated from these communities of considerable interest for public health. These two studies clearly show that FISH and cultivation are not competing but complementary techniques.

The recombinant green fluorescent protein (GFP) technology [28] has emerged as a technique for the in situ monitoring of live bacteria. Eberl et al. [29*] examined its potential for ecological investigations in activated sludge by combining the detection of GFP with FISH. It has to be realized, however, that the introduction of the gfp gene converts a native bacterium into a genetically modified one with potentially altered behavior.

Microenvironment and microbial activity

The microbial world and its inhabitants are subject to physicochemical constraints that differ from those met by larger organisms. The relevant spatial scale within this world is micrometers, viscous forces predominate, and diffusion rather than advection is the relevant transport mechanism for the solute exchange between bacteria and their biotic and abiotic surroundings [30–32,33**]. Many microbes stick to each other or to surfaces rather than being freely suspended single cells, and microbial biofilms, microcolonies and aggregates are hot spots of activity

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in which microbes can influence their environment. Typically, steep gradients of physicochemical parameters are present in such microbial communities over distances ranging from a few micrometers up to some tenths of a millimeter. Microbial life is thus a life in constantly changing gradients, which are affected by changes in environmental variables, their effect on microbial activity and vice versa. Tools and techniques to directly monitor the microenvironment and activity of microbes in their natural habitats have become available and are largely based on the use of microsensors. In the following, we summarize the most recent developments and give examples that show their potential for applications in microbiology. A more detailed discussion of microsensors and their use in microbial ecology is, however, impossible in this context and the reader is referred to recent reviews [34,35,36,37].

### Measuring the microenvironment

Measuring techniques to probe the microenvironment must be minimally invasive in terms of disturbance of firstly, the delicate three-dimensional organization of microbial communities, secondly, the steep physicochemical gradients present over micrometer distances, and, thirdly, other microenvironmental conditions such as boundary layers, diffusion and flow patterns on the microscale. Special measuring devices, microsensors, with tip diameters of <1 μm have been developed and increasingly applied in microbial ecology since their introduction in the late seventies by Revsbech (reviewed in [34,36]). As a result of their small dimensions, microsensors can be used directly in undisturbed samples of microbial communities, in the lab or in the field, where they can resolve, with a high spatial and temporal resolution, the gradients of light [38], pH, temperature, and important metabolites such as O₂, CO₂, H₂, H₂S, NO₃⁻, NO₂⁻, NH₄⁺, CH₄ [37,39,40,41,42,43,44]. The measured gradients contain information on the distribution and dynamics of important variables, and from these gradients the zonation of processes and even their rates can be estimated [34,39,41].

Microsensors mostly rely on pure electrochemical [34] or, more recently, optical sensing principles [35,38] for detecting the variable of interest. Several important environmental variables, however, cannot be measured in this way. A solution is to combine biological catalyzers (i.e. enzymes or whole cells) with more traditional measuring principles. The function of such biosensors is often hampered by the fact that the biological material needs well-defined conditions to function properly and most biosensors, therefore, find only limited application in environmental studies. Recently, two new types of microbiosensors were described that are applicable directly in natural microbial communities: the first methane microsensor [42] and the first nitrate microsensor that works in sea water and other complex media [43]. A detailed account of the measuring principles is beyond the scope of this review but the sensor principles rely strongly on the use of bacteria, which function well in microgradients that are established in the microsensors tip compartment. The bacteria thus grow in a near optimal setting and this leads to very robust and stable sensors.

Microsensors only allow for relative few point measurements in natural samples. Thus, sample heterogeneity cannot be fully addressed despite the fact that most microbial communities exhibit a pronounced spatial heterogeneity. Furthermore, even microsensors can affect the local microenvironment in some cases and are therefore not totally noninvasive; extrapolation of microsensor data to larger entities of the investigated community is problematic. Recently, a new approach for high spatial resolution studies was developed based on the use of planar optical sensor foils for oxygen in combination with imaging techniques [45,46]. Here, the sensor foil can be mounted on the inside of a transparent sample container, and by monitoring the sensor foil from outside with a charge-coupled device (CCD) camera, changes in the oxygen-dependent luminescence of the sensor foil can be monitored and used for measuring the two-dimensional oxygen distribution in the sample. With this approach, the two-dimensional oxygen distribution within an area of several square centimeters can be monitored noninvasively with a spatial resolution of 25–100 μm (i.e. a resolution similar to that obtained with microsensors) [45]. Consequently, this new technique enables studies on larger heterogeneous areas of natural microbial habitats.

### Application of microsensors in microbiology

The use of microsensors enables microbiologists to test some of their assumptions about the microbial habitat and to learn more about the organization and regulation of important metabolic processes. As an example, the hindguts of termites were for a long time regarded as homogeneous anoxic fermenters, miniature analogues to the mammalian rumen. Recent microsensor studies in termite guts have demonstrated, however, that this analogy does not hold [44]. The termite gut microenvironment thus appears to be highly structured into compartments with steep lateral and radial gradients of O₂, H₂, and pH. Termite gut microbiologists must thus take into account the fact that bacteria and other microorganisms in the gut have to adapt to these gradients.

It thus becomes important to combine traditional microbiology with knowledge about the gradients present in the natural habitat of microbes. To understand the function and regulation of isolated microbes it is necessary to study them while they are exposed to such gradients (i.e. in gradient growth systems). While several studies have demonstrated the potential of this approach in even very simple gradient systems (e.g. [47–49]), the concept seems to be only slowly acknowledged in traditional microbiology, which is largely based on batch
and chemostat culture techniques. One problem with setting up gradients in the laboratory is the fact that microbes live in a multitude of gradients in their natural habitat. Recently, more advanced gradient growth systems were described that allow such multidimensional gradients to be set up and used in microbiological studies. A system for growing phototrophic bacteria in experimental O2, H2S, pH, and light gradients was described and used for the first pure culture studies of green and purple bacteria growing in gradients [50,51]. An even more flexible two-dimensional gradient growth system, that allows multiple opposing gradients to be set up, was used to study and isolate bacterial populations from oil contaminated soil [52]. Isolates were characterized by their growth in the gradient chambers along with microsensor measurements and genotypic fingerprinting.

The presence of anaerobes in aerobic environments has been shown by comparing the distribution and activity of sulfate-reducing bacteria to the oxygen distribution in microbial mats as measured with microsensors. This has led microbiologists to reconsider the role of sulfate reducers in such communities, and oxygen tolerant species have now been isolated [53,54]. Microsensor analysis can also help answer questions regarding the absence of certain microorganisms from an apparently suitable environment. Planktonic aggregates of fecal material, plankton cells and other debris have long been considered to potentially harbor anoxic microniches, where anaerobic bacteria (e.g. sulfate-reducing bacteria and methanogens) could prevail in an otherwise aerobic environment. With oxygen microsensors, such anoxic niches were indeed found in some marine aggregates but anaerobic respiratory activity could not be measured with microsensors nor could anaerobic bacteria be detected by molecular techniques [55]. The absence of anaerobes from such anoxic compartments seems counterintuitive, but a closer analysis of the obtained microsensor data showed that anoxia is probably only an ephemeral phenomenon in fresh aggregates with a high O2 consumption, and carbon limitation will be reached within a few hours to days leading to oxygenated aggregates [55]. Anaerobes would thus have very little time to grow and colonize such aggregates.

Besides microsensors there are several other ways to measure metabolic activities in situ. A straightforward one is the application of radiolabeled substrates followed by microautoradiography. Andreasen and Nielsen [56] used a panel of six substrates to study their specific uptake by various filamentous bacteria in activated sludge. Karner and Fuhrman [57] used incorporation of tritium-labeled amino acids to estimate the fraction of actively growing cells in marine bacterioplankton and found a good correlation with the number of cells hybridizing with a universal 16S-rRNA-targeted probe. It has been argued before that detection of rRNA by FISH is a good indication for in situ activity, or at least for potential for in situ activity [1]. Presence of high amounts of ribosome in a cell, of course, only indicates the potential to synthesize proteins and gives no information on a particular type of metabolic activity. Localization of specific activities requires either in situ detection and quantification of particular mRNAs (e.g. [14,15,58]) or the direct immunological detection of specific proteins [59]. Alternatively, cells can act as sensors on their own. For example, Stichet al. [60] reported the development and characterization of a whole-cell bioluminescent sensor for bioavailable middle-chain alkanes in contaminated groundwater samples. Applying standard methodology, an Escherichia coli Z reporter strain had been genetically engineered to carry a transcriptional fusion of the alkB promoter of Pseudomonas oleovorans and the promoterless luxAB genes of Vibrio harveyi. The luciferase activity of the resulting whole-cell biosensor is induced by middle-chain alkanes such as octane. One important prerequisite for reliable alkane measurements is the saturation of the cellular luciferase with decanal. Poulsen et al. [61] combined the application of a chromogenic luxZ reporter strain with its identification in mixed culture by a fluorescently labeled, rRNA-targeted oligonucleotide probe and thereby achieved monitoring of gene expression and quantitation of beta-galactosidase activity in single cells in situ.

Conclusions

While both molecular and microsensor techniques alone find numerous applications in microbiology, we see the
largest potential in the combined use of these techniques, where the data gained from FISH on fine scale distribution of specific microbial populations are correlated to activity measurements at a similar resolution with microsensors. The first of such studies focused on distribution and activity of sulfate-reducing bacteria in biofilms [62]. Schramm et al. [63] used FISH to visualize the spatial organization of ammonia- and nitrite-oxidizing bacteria in biofilms, and, by use of microsensors, could correlate their distribution to the nitrification activity within the biofilm. For the particular biofilm investigated they proved that members of the genera Nitrosomonas and Nitrobacter were the key catalysts of this environmentally important process. With the current state of the art of FISH and microsensor techniques, it is no longer a problem to perform such combined studies in various environments. The next step is to use the techniques in an ecological context to address important open questions about microbial diversity, community structure and activity in nature. Various such studies are underway; for example, using molecular techniques, a high level of bacterial diversity was shown in a hot spring mat despite the fact that only a limited number of morphologically distinct strains could be isolated with traditional techniques [64**]. This led to the hypothesis that similar morphotypes would consist of various genetically different populations with different adaptations of bacterial photosynthesis to temperature [65*,66**]. The presence of various niches was recently confirmed by microsensor measurements (M Küh, unpublished data).

Combined in situ studies of microbial activity and population dynamics encompassing controlled perturbation experiments will allow us to investigate such systems even further. Microbiology in general could, in our opinion, largely benefit from such a multidisciplinary analysis of the structure and function of complex microbial communities.

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References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:

• of special interest
** of outstanding interest


The second large database for 16S and 23S RNA sequences.


15. Amann R, Springer N, Schonhuber W, Ludwig W, Schmidt EN: Another example of the transfer of a technique common to eukaryotic cell biology to microbiology research has proven important and successful.


18. Beautiful pictures on the red-green discrimination of two closely related bacterial populations.

A large study combining 16S rRNA sequence retrieval and fluorescence in situ hybridization with surprising results; for example, the detection of potentially pathogenic members of the genus Arcobacter in high numbers.


A whole new bacterial phylum that might be widespread in the environment is revealed by the RNA approach.


Cells can be considerably enriched by flow sorting prior to the retrieval of 16S rRNA. This can make the search for new bacterial diversity more directed.

Erhart R, Bradford D, Seviour EM, Seviour RJ, Amann RI, the best tools for the directed design of rRNA-targeted oligonucleotide probes.


A freely available, comprehensive software package with, perhaps, currently the best tools for the directed design of rRNA-targeted oligonucleotide probes.


A very detailed data set on oxygen consumption, nitrogen and sulfur cycling in a biofilm, where first data on the role of nitrite in these processes are presented.


The most recent review of microsensor techniques available for environmenal analysis.


A new biosensor based on a special mutation of a denitrifying bacterium. The sensor tip contains a microscale chemostat, wherein a nitrate microsensor for use in both marine and freshwater environments was realised.


The first microprofiles of methane in biofilms and sediments were measured with a new microbiosensor, which works with methane oxidizers kept in a gradient microchamber at the sensor tip. Clever use of microbiology in designing of a new class of biosensors.


A new biosensor based on a special mutation of a denitrifying bacterium. The sensor tip contains a microscale chemostat, wherein a nitrate microsensor for use in both marine and freshwater environments was realised.


Microsensor measurements demonstrate the presence of steep opposing gradients of oxygen and hydrogen in the termitic gut. This paper gives a totally new picture of the termitic gut microenvironment along with some microbiological considerations about the importance of various metabolic processes.


In this study a new technique for imaging the two-dimensional oxygen distribution at high spatial resolution is outlined and demonstrated for the first time in heterogeneous biofilms. It represents an elegant approach for measuring distribution in various heterogeneous natural samples and consequently bears high potential for many applications in microbiology.


A new gradient growth system is used in combination with microsensors and microbiological and molecular techniques. One of very few examples in which all these techniques are used in concert.


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64. Ward DM, Santegoeds CM, Nold SC, Ramsing NB, Ferris MJ, Bateson MM: Biodiversity within hot spring microbial mat communities: molecular monitoring of enrichment cultures. *Antonie van Leeuwenhoek* 1997, 71:143-150. Excellent review of how the use of molecular techniques can reveal more details on the population structure of a natural microbial community than the information obtained from traditional microbiological techniques, and how enrichment procedures can bias the obtained view of the community structure.
