In situ methods for assessment of microorganisms and their activities

Rudolf Amann* and Michael Kühl†

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.

Addresses
*Junior Group for Molecular Ecology, Max-Planck-Institut für marine Mikrobiologie, Celsiusstrasse 1, D-28359 Bremen, Germany; e-mail: ramann@mpi-bremen.de
†Microsensor Research Group, Max-Planck-Institut für marine Mikrobiologie, Celsiusstrasse 1 D-28359 Bremen, Germany; e-mail: mkuehl@mpi-bremen.de

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Abbreviations

FISH	fluorescence *in situ* hybridization
GFP	green fluorescent protein
PCR	polymerase chain reaction

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-

cleotides [9•] resulted in an increase of fluorescence of at least one order of magnitude. Similar results were obtained with an indirect approach in which biotin-labeled probes mediated binding of streptavidin–horseradish peroxidase conjugates that subsequently catalyzed immobilization of fluorescein-labeled tyramide [10•]. Even though TSA® yields very bright fluorescent signals, it should be noted that FISH of whole fixed cells relies on penetration of the probe molecules across the cell periphery to intracellular target molecules. This is more easily achieved with the much smaller fluorescently labeled oligonucleotides than with biotinylated probes, and, consequently, in aqueous samples more cells could be detected with Cy3 (carbocyanine dye)-labeled oligonucleotides [11] than with TSA®-based methods [9•]. Treatment of aqueous samples with chloramphenicol, an inhibitor of protein synthesis and RNA degradation, was reported to further increase the percentage of cells detectable with a general 16S rRNA-targeted, fluorescent oligonucleotide probe [12•].

Lanoil and Giovannoni [13•] have demonstrated that identification of bacterial cells can also be achieved by chromosomal painting, a method originally developed to identify genetic material on eukaryotic chromosomes. They prepared probes from purified genomic DNA by nick-translation, which resulted in a statistical mixture of fragments with an average length of 50–200 nucleotides. After removal of cross-hybridizing fragments even two closely related serotypes of *Salmonella choleraesuis* could be distinguished. The authors [13•] point out that by preparing the probes from operons encoding metabolically important functions they could be used to analyze for defined activities as well as identification purposes. *In situ* reverse transcription was shown by Chen *et al.* [14•] to bear potential for characterization of genetic diversity and activity of bacteria. This technique relies on the initiation of reverse transcription of rRNA or mRNA at specific primers and the incorporation of labeled nucleotides in the resulting cDNA. Potential disadvantages of this technique are similar to those encountered for *in situ* PCR, a method recently described by the same group [15]. Cells need to be permeabilized for the quite large polymerase molecules to gain access and transcription initiation from nonspecifically bound primers or internal priming sites may result in background signals. The future will show whether these methods are robust enough to be applied to the *in situ* detection of specific genes and gene products in complex environmental or medical samples.

Of the numerous applications of FISH for the identification and localization of individual bacterial cells we have selected a few from the environmental field to highlight recent trends. Several studies have achieved *in situ* identification of so far uncultured bacteria based on 16S rRNA sequences directly retrieved from samples as different as acanthamoebae [16•], the epibiotic microflora of the hydrothermal vent annelid *Alvinella pompejana* [17••], activated sludge [18•], and soil [19•]. Taking into account 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-conferred 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

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 O_2, CO_2, H_2, H_2S, NO_3^-, NO_2^-, NH_4^+, CH_4 [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 O_2, H_2, 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.

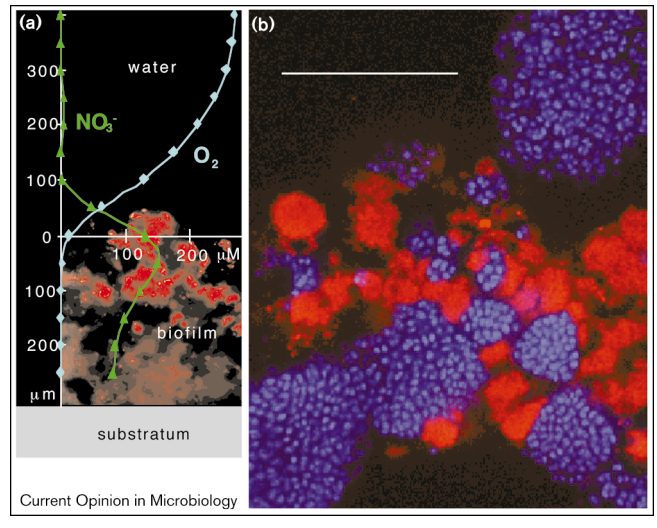
It thus becomes important to combine traditional microbiology with knowledge about the gradients present in the natural habitat of microbes. To understand the function and regulation of isolated microbes it is necessary to study them while they are exposed to such gradients (i.e. in gradient growth systems). 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 O_2, H_2S, 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 O_2 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

Figure 1



Examples of fluorescence *in situ* hybridization teichniques. **(a)** *In situ* hybridization combined with microsensor measurements. *In situ* hybridization of a vertical biofilm slice with a carboxytetramethylrhodamine-labeled probe (NIT3) specific for the genus *Nitrobacter* (red stain cluster) correlated to oxygen and nitrate gradients measured by microelectrodes. Magnification, ×400. Adapted from Schramm *et al.* [63]. **(b)** Confocal microscopic image of a bacterial aggregate thin section after simultaneous hybridization with a Cy3-labeled probe specific for nitrite-oxidizing *Nitrospira* sp. (red) and a Cy5-labeled probe specific for ammonia-oxidizing *Nitrosospira* sp. (blue). Scale bar indicates 20 µm (A Schramm, M Wagner, unpublished data).

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, Sticher *et 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* strain had been genetically engineered to carry a transcriptional fusion of the *alk*B promoter of *Pseudomonas oleovorans* and the promoterless *lux*AB 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 *lac*Z 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 *Nitrosomas* 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 Kühl, 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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