Microbial Ecology

Unravelling Microbial Communities with DNA-Microarrays: Challenges and Future Directions

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Abstract

High-throughput technologies are urgently needed for monitoring the formidable biodiversity and functional capabilities of microorganisms in the environment. Ten years ago, DNA microarrays, miniaturized platforms for highly parallel hybridization reactions, found their way into environmental microbiology and raised great expectations among researchers in the field. In this article, we briefly summarize the state-of-the-art of microarray approaches in microbial ecology research and discuss in more detail crucial problems and promising solutions. Finally, we outline scenarios for an innovative combination of microarrays with other molecular tools for structure-function analysis of complex microbial communities.

A Decade of DNA Microarray Research in Microbial Ecology

Microbial ecologists are confronted with challenges that plant or animal ecologists rarely experience. For example, when analyzing a few grams of bulk soil, a researcher interested in microbial communities deals with thousands of largely uncharacterized bacterial species and total numbers of individuals that exceed the number of humans on our planet [14, 23, 72]. Furthermore, the composition and activity patterns of these microbial communities vary on a microscale, with a multitude of highly complex interactions between different microorganisms. This enormous microbial biocomplexity can only be tackled using molecular methods that allow for high throughput and highly parallel analyses of the structure and function of such communities.

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In principle, DNA-microarrays fulfill these requirements—thousands of DNA probes targeting genes or gene products of interest can be immobilized on a solid support in a miniaturized manner and, after a single hybridization step with labeled target nucleic acids, the hybridization signal of each probe can be simultaneously recorded with a detector. Initially, such DNA microarrays were developed for transcriptome analyses of *Arabidopsis* cells [61] and have subsequently found widespread application for genome-wide monitoring of gene expression patterns in many eu- and prokaryotes. In 1997, David Stahl and his colleagues first introduced the microarray approach to environmental microbiology for microbial community composition analysis using a prototype array consisting of nine 16S rRNA-targeted probes for identification of selected nitrifying bacteria [25]. Since then, this field has grown rapidly (Fig. 1), and today many different microarray systems, which can consist of more than 30,000 probes [79], are available for detection of different target nucleic acids (for detailed reviews, please refer to [6, 27, 41, 42, 69, 85, 86]). Two main approaches are widely used. The so-called PhyloChips (or phylogenetic oligonucleotide arrays) target polymerase chain reaction (PCR)-amplified rRNA gene fragments or directly retrieved community rRNA (genes) and can, at least in principle, be designed to detect any microorganism. In contrast, functional gene arrays (FGAs) detect selected genes or gene families that encode key enzymes diagnostic for a certain metabolic pathway [60, 68, 80]. Therefore, these arrays are confined to diversity analysis of selected microbial guilds, for example, the methane-oxidizing bacteria [5, 66] or nitrogen-fixing prokaryotes [71].

Recently, several pioneering studies were published, which use PhyloChips or FGAs also for functional analysis of microbial communities [1, 17, 58]. In the so-called Isotope Array approach, a PhyloChip is used to

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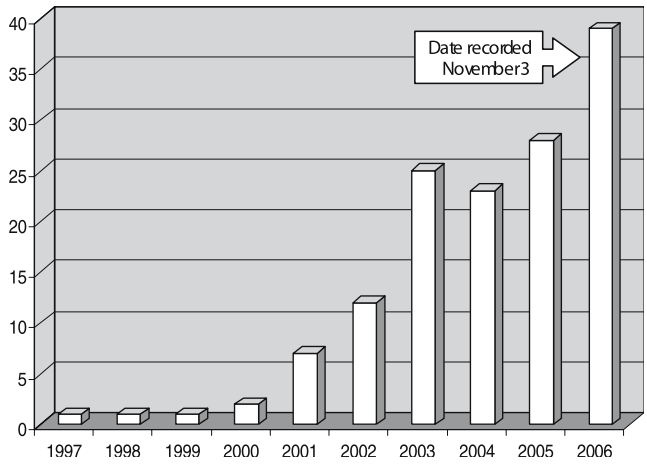

Figure 1. Number of microbial ecology-related DNA microarray publications per year. Literature search was performed on November 03, 2006 using the advanced search option of ISI Web of Science and yielded 139 records in total [Search string: (TS=microarray* OR TS=microchip*) AND (TS="microbial ecology" OR TS="environmental microbiol*" OR TS="microbial community" OR TS="microbial diagnostics" OR TS="microbial detection" OR TS="microbial identification" OR TS="determinative" OR TS="functional gene array" OR TS="community genome array" OR TS=PhyloChip OR TS="16S rRNA" OR TS="23S rRNA" OR TS="small subunit ribosomal" OR TS="large subunit ribosomal")]

monitor substrate utilization by microbial community members. This approach involves exposing a microbial community to a C-labeled substrate and, after a short incubation, measuring the incorporation of the label into the rRNA of probe-defined community members [1, 76, 78]. FGAs offer an alternative route where mRNA levels of defined catabolic genes in the environment are determined [7, 17, 58].

This position paper aims to summarize the current status and limitations of the application of microarray approaches in microbial ecology research and will discuss some possible solutions and future perspectives. Based on these considerations, we will provide our points of view on how to best integrate microarray technology into the increasingly interdisciplinary field of microbial ecology.

Microarrays—Key Issues, Major Limitations, (Partial) Solutions, and Consequences

During the first few years of application in microbial ecology research, several major issues and limitations of DNA-microarrays were recognized. These will be discussed in the following synopsis, which is complemented by Table 1.

Specificity of Microarrays. It has been proven very difficult to develop microarray protocols that allow all probes to hybridize only to their fully matched target sequence. The obvious difficulty of the microarray approach is that hundreds to thousands of different probes are hybridized simultaneously, but optimal (specific) hybridization conditions often vary between probes. Therefore, at a defined stringency, determined by the hybridization and washing buffer compositions and the hybridization/wash temperature, not all probes will produce specific signals—a fact that severely complicates interpretation of hybridization patterns.

There are several ways to deal with this problem. One conceptually appealing solution is to continuously read out the hybridized array while gradually increasing the stringency of the wash step [37]. The obtained nonequilibrium probe-target/nontarget dissociation curves allow the recording of signals for each probe at optimal stringency. This relatively tedious strategy has mainly been applied by using artificial target nucleic acids to better understand the dynamics of nucleic acid hybridization on microarrays [35, 73, 74] and still awaits full adaptation to its ultimate use for the analysis of complex microbial communities [20]. Alternatively, incomplete specificity of some probes can be tolerated and compensated for by applying the multiple-nested probe concept. In this article, each target organism/group is detected with a set of probes with parallel or hierarchical specificity and a positive signal of all, or a majority of, matching probes is required to confirm the presence of the target organism/group in the environmental sample [8, 18, 37, 38]. It should be noted that designing probes with nested specificity is straightforward if rRNA (genes) are to be targeted but almost impossible for FGAs as a result of degeneracy of the genetic code [41]. The successful application of such nested probe-carrying PhyloChips for microbial community structure analysis in environmental and medical systems has been demonstrated [34, 38, 39]. A special variant of an array system based on the multiple-probe concept exploits the use of the ligase detection reaction [3, 9, 10, 67]. Here, a target nucleic acid is only detected on the microarray if two specific probes bind adjacent to each other, thereby increasing the specificity of the assay.

Taken together, much effort and care must be invested to maximize the specificity of the microarrays before and during application, but past research has shown that the achievable specificity level is sufficient for meaningful analysis of environmental samples [40, 66]. Specificity testing for a newly developed microarray requires the use of extensive collections of reference nucleic acids representing target and suitable nontarget sequences for all probes on the array. This is also important because some probes do not function effectively on the microarray format and will therefore not produce the expected signal in response to the presence of the target organism [22]. Such false-negative probes must be removed from the microarray before its application [38].

Table 1. Some challenges and possible solutions in the development and application of DNA microarrays in microbial ecology

Challenges	*Problems*	*Solutions/Improvements*
In silico probe design	Identification of target group	•Creation/update of an aligned sequence database and subsequent phylogenetic analysis [43, 44] •If possible, select monophyletic target groups
	Assessing probe hybridization behavior (duplex-yield, cross-reactivity, etc.)	•Check probe specificity against up-to-date sequence databases [12, 19] •Evaluate theoretical hybridization behavior by calculating thermodynamic properties e.g. melting temperatures (T_m) and free energies (ΔG) for putative (i) perfectly matched and mismatched probe-target duplexes and (ii) inter- and intra-molecular interactions of probe and target molecules [40, 48]. Note that 100% accurate prediction of hybridization results is currently impossible [55].
Specificity	Some probes show false-negative or false-positive signals	•Evaluate microarray by individual hybridization with reference nucleic acids (set of test targets should contain at least one perfectly matched sequence per probe): Remove false-negative and highly cross-hybridizing probes
Uniform hybridization behavior	Different probes display different target-binding capacities	•Use probes with similar predicted T_m or ΔG [5] •Uniform oligonucleotide probe length plus addition of tertiary amine salts to hybridization/wash buffers [30, 47]
Detection limit	Low signal-to-noise ratio for a given probe-target hybrid	•High-quality probes (e.g., PAGE or HPLC-purified oligos, control of purified PCR-products by gel electrophoresis) [57] •3-dimensional slide formats and improved slide chemistry [87] •Improve labeling efficiency (e.g., avoid dye quenching effects) [13] •Use nanoparticles [88] •Enzymatic signal amplification [16]
	Low relative abundance of target organisms/genes	•Probe-based enrichment of target cells [65] •Target group-selective amplification by PCR [40] •Whole genome amplification strategies [24, 82]
	Steric hindrance during hybridization	•Optimize probe concentration for spotting •Introduce spacer elements between probe and slide surface [63] •Fragment target nucleic acids [64]
	Sensitivity thresholds differ among probes due to their different target binding capacities	•Determine range of sensitivity achievable with the microarray: Hybridize concentration series of target organisms/genes perfectly matching the probes with the lowest and highest duplex yield on the microarray
Quantification of target populations	DNA/RNA extraction, target amplification, labeling, and hybridization biases	•Pool nucleic acid extracts, obtained by employing different extraction strategies, prior to further analyses •Special PCR protocols alleviate biases [54, 59] •Spike known amounts of target DNA/RNA (labeled in a different color) to hybridization reaction as internal standard [5, 52]

Detection Limit of Microarrays. In microbial ecology research, most DNA microarrays are hybridized with nucleic acid mixtures derived from many different microorganisms. The ability to detect a certain nucleic acid target sequence by a DNA microarray depends on absolute copy number of the target and its relative abundance compared to other nucleic acid molecules in the hybridization mixture. Furthermore, one should keep in mind that microarray probes can dramatically differ in their duplex yield [38], which is directly linked to the detection threshold of the individual probes. Although for most microarray approaches a thorough evaluation of the detection limit is still missing, it has been estimated by several groups that DNA/RNA molecules, which are more abundant than approximately 0.05–5% in the labeled nucleic acid mixture, can be detected with current protocols and microarray formats [5, 11, 40, 46, 52, 53]. In other words, current microarrays can simultaneously detect the 20 to 2000 most abundant target populations in an environment. Low sensitivity

might be a severe limitation of a given microarray approach if, for example, a keystone species with relatively low abundance but large impact on the ecosystem is overlooked.

There are two main types of approaches for enhancing the sensitivity of DNA-microarrays. First, one can improve the signal-to-noise ratio of the hybridized probe spots. This might, for example, be achieved by using high-quality probes [57], by increasing the amount of probe immobilized per spot without increasing steric hindrance [25], by optimizing the labeling of the target [84], by increasing the hybridization efficiency [2, 50, 63], by enhancing signal emission [16, 31, 36], by more sensitive detection devices [4, 21, 49, 70, 83], or by exploiting special slide surface material for spotting [29, 33]. Another possibility to enhance sensitivity is to enrich the target nucleic acids of the entire microbial community or subgroups of microorganisms before microarray analysis. For example, the metagenome of an environmental sample can be randomly amplified using a whole-community genome amplification-based method [82]. Also, target gene fragments can be selectively amplified by using PCR primers specific for subgroups of interest [40]. Alternatively, one could enrich certain bacterial subgroups by fluorescence-activated cell sorting [62] or magnetic separation [65] after *in situ* hybridization with subgroup-specific probes before nucleic acid extraction and microarray analyses.

Quantification with Microarrays. The ecology of microbial communities can only be truly well understood if data on the abundance of the community members can be determined. Consequently, several research groups have investigated the potential of DNA microarrays for quantification. It has been demonstrated that within a certain range, an almost linear relationship exists between the signal of a defined probe and the abundance of its labeled target nucleic acid [18, 58, 71, 80, 81]. Deviations from linearity are caused by variations in spot size, probe density, and hybridization behavior between slides, problems that can at least partially be overcome by using reference DNA as an internal standard [11]. However, an additional problem that needs to be addressed is the differential binding capacity of different probes. For instance, probes targeting different regions of the 16S rRNA gene of a given organism can vary by a factor of 240 in signal intensity [40]. Bodrossy and colleagues showed that addition of known amounts of target nucleic acids labeled in a different color can be used to partially solve this problem [5]. However, to realize the quantitative potential of the microarrays fully, this approach requires the availability of all target nucleic acids (e.g., as cloned target gene fragments of uncultured organisms) present in the sample [52]. Furthermore, it should be noted that quantification of microbial populations in the environment is not only dependent on predictable microarray hybridization behavior, but would also require nonbiased nucleic acid extraction, target amplification (if applicable) and labeling. An additional complicating factor is the copy number of target genes or gene transcripts per cell, which often differs between bacterial populations (e.g., bacterial genomes contain 1–15 rRNA operons [32]). Thus, extrapolations of "quantitative" microarray data into absolute cell numbers are problematic. Whereas most of these limitations are also inherent to other established molecular tools for quantifying microorganisms in complex samples (e.g., quantitative PCR and quantitative dot blot hybridization), they do not apply to single-cell tools like fluorescent antibody staining or fluorescence *in situ* hybridization, which in turn have their own documented limitations [75].

Microarrays Identify Only the Known. The last few years have witnesses the rapid development of improved microarray systems and new strategies for increasing sensitivity and specificity of these high-throughput detection devices [28, 52]. For example, novel biomathematical approaches show great promise for filtering "noisy" hybridization data and thus for accurate identification and quantification of low-abundant target organisms in complex mixtures [46]. It is likely that this trend of innovation will continue, and we can thus expect that several of the above-mentioned limitations will be overcome in the future. However, even if we were to have DNA microarrays at hand that no longer suffered from sensitivity and specificity limitations, these tools would still only be suitable to identify those microorganisms in environmental samples for which specific probes can be designed. This rather trivial statement has two important consequences. First, the design of specific probes for microarray analyses is very much dependent on the quality and size of the probe target sequence database [41]. Submission of partial gene sequences, low sequence quality and insufficient coverage of the natural diversity of the respective genes in DNA sequence databases severely complicate probe design and accurate *in silico* judgment of theoretical probe specificities. Therefore, there is an urgent need to maintain and enlarge well-annotated, high-quality rRNA [12, 19, 44], and functional gene databases. Microbial ecologists should support these activities wherever possible, for example, by making specific gene databases publicly available and by submitting only high-quality sequences to public databases. Second, even the best-designed DNA microarray cannot identify microorganisms for which the target DNA sequence is still unknown. Most environments contain many prokaryotes not yet represented in current rRNA or functional gene libraries. Without this *a priori* knowledge of the breadth of microbial diversity of

the ecosystem under investigation, it remains impossible to design comprehensive DNA microarrays to monitor all microbial populations in a given environmental sample. If such data are available (e.g., by exhaustive rRNA sequencing approaches or metagenomic analyses), habitat-specific microarrays [51] can be designed and used to monitor spatial and temporal microbial community dynamics in a highly parallel manner. Another option is to apply microarrays targeting a known subgroup of microorganisms to monitor their presence in selected ecosystems and to ignore the yet unknown fraction of the communities. For example, this approach is extremely useful if one wants to understand the biogeography of a large and diverse group of known microorganisms [38, 39].

Replication and Confirmation of Microarray Analyses. As mentioned above, microarray technology does not always produce unambiguous results. For example, variations during spotting, nucleic acid labeling, hybridization, and recording of signals are difficult to avoid, although automation of some of these steps and data normalization is helping to reduce biases. A practical consequence of these combined variations is that fluorescence intensities near a predefined threshold value for positive probe signals must be interpreted with caution if only a single hybridization is performed. To partly account for variations inherently associated with microarray analyses, it is therefore recommended to perform at least three replicate hybridizations for each microarray experiment and to average the obtained data. However, replication cannot solve possible specificity or sensitivity problems associated with the microarray approach. Therefore, ambiguous microarray results regarding the presence (and abundance) of a target population in an environment should be confirmed by microarray-independent techniques. This strategy has the added benefit of allowing characterization of the microbial community in the sample of interest beyond the identification and community composition level. For example, deeper insights into the evolutionary history of the conspicuous microorganisms are obtained by employing subgroup-specific PCR primers for sequence retrieval and subsequent phylogenetic analysis [38, 39]. Furthermore, based on the microarray results and the newly obtained sequence data, specific probes or primers can then be selected/designed and applied in different (quantitative) formats to resolve the composition and to functionally characterize the microbial community at a higher resolution and accuracy (Fig. 2).

A Place for Microarray Analysis in Microbial Ecology

DNA microarrays offer microbial ecologists the possibility to characterize rapidly many samples for the presence and (with certain limitations) the abundance of many target organisms. Recent publications have demonstrated that, if carefully used, this cutting-edge technology is now ready for environmental application. However, one should realize that the development and evaluation of a new DNA microarray is very time-consuming and expensive and is thus particularly recommendable for long-term research projects involving many samples. This restriction obviously does not apply if a suitable and well-evaluated DNA microarray is already available.

Figure 2 shows how DNA microarrays nicely complement available molecular tools in microbial ecology. DNA microarrays and classical fingerprinting techniques (TGGE, DGGE, SSCP, T-RFLP), are optimally suited for rapid screening of microbial community composition in environmental samples and can thus provide important guidance for subsequent in-depth analyses. Compared to classical fingerprinting tech-

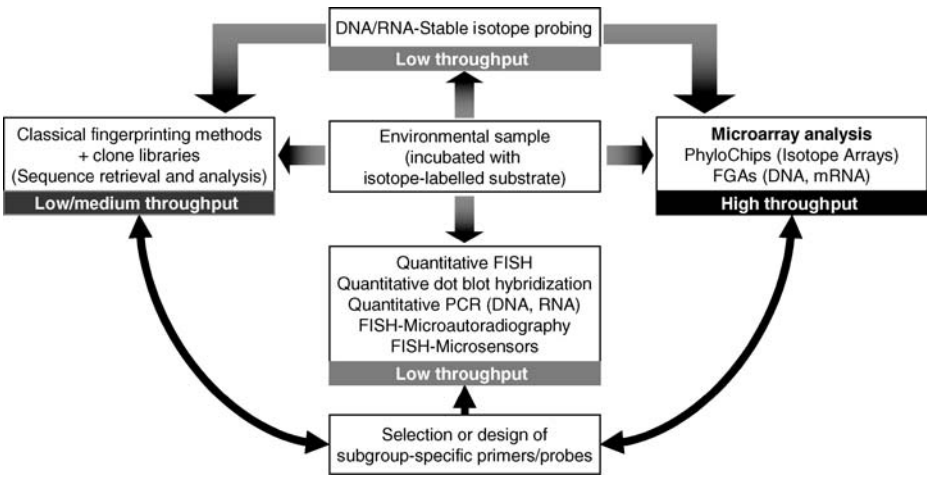

Figure 2. Integrating microarrays into the molecular toolbox for structural and functional analyses of complex microbial communities

niques, microarrays produce not only a "composition pattern" but also enable instant identification of the organisms detected. To achieve the same level of information, much more experimental effort is required if classical fingerprinting techniques are used—for example, for identification of the organisms "behind" a certain DGGE banding pattern, all bands must be separately excised, amplified, and sequenced. On the other hand, the traditional fingerprinting methods, in contrast to DNA microarrays, do not overlook unknown microorganisms and are thus often well suited for initial "quick-and-dirty" community screening. Taken together, DNA microarrays are, in our opinion, the preferred option for investigating the biogeography of defined groups of known microorganisms or for intensive monitoring of community dynamics in defined habitats for which the microbial diversity is largely known.

Today, DNA microarrays are mainly used in microbial ecology to infer the composition of microbial communities or of defined guilds. In addition to this application, we foresee that microarrays will become indispensable for the highly parallel analyses of the *in situ* function of microbes and their interactions with each other. We expect major breakthroughs in the near future by combining DNA microarrays with other recently developed techniques for structure–function analysis of microbial communities. For example, the composition of light and heavy DNA or RNA fractions obtained from stable isotope probing experiments [45, 56] can be compared by using microarray experiments to identify rapidly organisms with a defined ecophysiology. Furthermore, isotope arrays [1, 78] offer unique insights into the ecophysiology of microorganisms and, if several samples are taken at different time points during an incubation experiment, even allow microbial ecologists to begin deciphering microbial food webs. Given the rapid methodological development in the microarray field and the advent of the environmental genomics approach [15, 26], it might even become possible (at least in systems with relatively limited diversity) to monitor multiple gene expression patterns of uncultured microorganisms within their natural environment and to begin to understand how these patterns are influenced by changes in key abiotic and biotic parameters. Together with the suite of recently developed methods for monitoring the ecophysiology of microorganisms in the wilderness [76, 77] such insights should allow us to fully bring microbial ecology from the pure culture level "back to nature".

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