ELSEVIER

Linking microbial community structure with function: fluorescence *in situ* hybridization-microautoradiography and isotope arrays

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The ecophysiology of microorganisms has been at the heart of microbial ecology since its early days, but only during the past decade have methods become available for cultivation-independent, direct identification of microorganisms in complex communities and for the simultaneous investigation of their activity and substrate uptake patterns. The combination of fluorescence *in situ* hybridization (FISH) and microautoradiography (MAR) is currently the most widely applied tool for revealing physiological properties of microorganisms in their natural environment with single-cell resolution. For example, this technique has been used in wastewater treatment and marine systems to describe the functional properties of newly discovered species, and to identify microorganisms responsible for key physiological processes. Recently, the scope of FISH-MAR was extended by rendering it quantitative and by combining it with microelectrode measurements or stable isotope probing. Isotope arrays have also been developed that exploit the parallel detection offered by DNA microarrays to measure incorporation of labelled substrate into the rRNA of many community members in a single experiment.

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Introduction

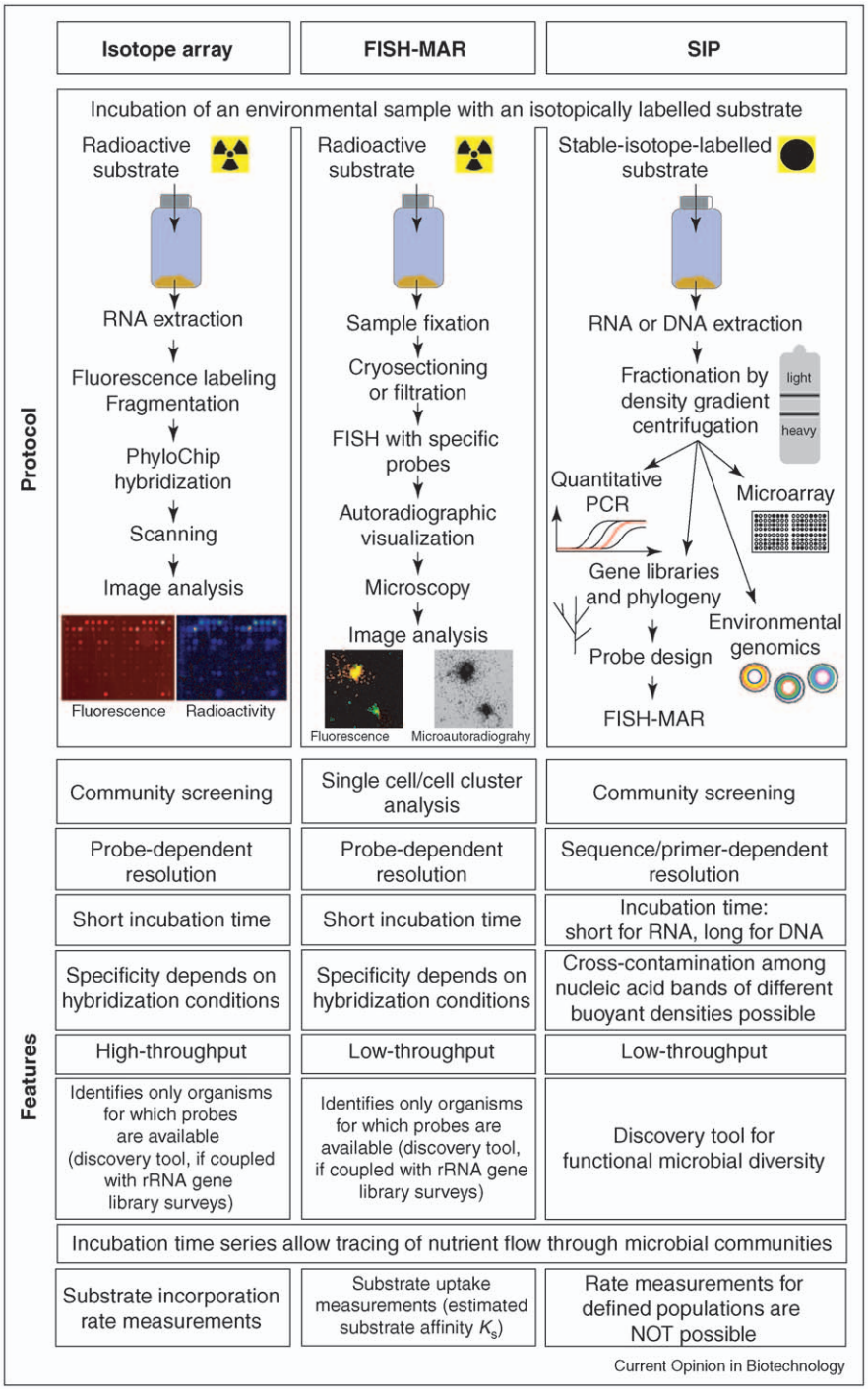
Since 1989, it has been possible to identify microorganisms *in situ* using fluorescently labelled ribosomal RNA-targeted oligonucleotide probes for fluorescence *in situ* hybridisation (FISH) [1]. Following this breakthrough, however, it was ten years before a method became available that also allowed specific functions to be assigned to the *in situ* detected microorganisms. In 1999, two research groups succeeded in combining FISH with microautoradiography (MAR) [2,3] and were thus able, after a short incubation of the environmental sample with radioactively labelled substrate, to observe under the microscope whether a probe-detected bacterium was capable of consuming the offered substrate under the incubation conditions applied (Figure 1). Such insights are of particular relevance for microbial ecology, as most microorganisms that thrive on our planet are not available as pure cultures and, even if the pure culture physiology of a particular bacterium is well known, it is still impossible to infer its ecophysiology as a member of a microbial community. Consequently, it is not surprising that FISH-MAR is now widely applied. However, this technique has two major limitations. Firstly, no more than seven bacterial populations can be specifically detected in a single FISH experiment, owing to the limited number of different fluorophores that can be applied simultaneously [4]. Keeping in mind that natural microbial communities can comprise thousands of species [5], then compiling a comprehensive list of those microorganisms that consume a specific substrate in the system of interest can quickly become very cumbersome, or even impossible. Secondly, not all environmental samples are well-suited for FISH analysis. For example, only a minor fraction of the resident bacteria will be detectable by FISH in bulk soil and thus most of these soil bacteria cannot be characterized by FISH-MAR. The so-called isotope array overcomes both problems by using rRNA-targeted DNA microarrays to measure incorporation of radioactively labelled substrate into the rRNA of the target organisms (Figure 1) [6••]. In principle, thousands of probes can be applied simultaneously in this approach, which should be applicable to any sample from which rRNA of sufficient quality and quantity can be purified.

In this review, we will describe new developments of the FISH-MAR approach, discuss the principle of the isotope array approach, and give examples of how these techniques have been used to reveal new and exciting insights into the ecophysiology of uncultured microorganisms.

FISH-MAR: features, new developments and applications

FISH-MAR has, with today's instruments and depending on the radiotracer used, a resolution of 0.5–2 μm and is thus a single-cell tool [7] (see also Update). However, in

Figure 1



Overview of the protocol and selected features of the isotope array (left), FISH-MAR (middle) and SIP approaches (right).

biofilms or other dense cell aggregates cryosectioning of the biomass, or efficient cell dispersal, is required before FISH and autoradiography to enable silver grains, which indicate the assimilation of the radioactive substrate, to be assigned to individual cells. For interpretation of FISH-MAR data, it is important to keep in mind that, in contrast to stand-alone MAR, this combined method does not measure total uptake of the radiolabelled substrates but only assimilation into macromolecules. Unincorporated labelled compounds are not retained inside the paraformaldehyde- or ethanol-fixed cells. Nevertheless, FISH-MAR is very sensitive compared with DNA- or

RNA-stable isotope probing (SIP) (Figure 1; see the articles by Friedrich and by Whiteley, Manefield and Lueders in this issue of *Current Opinion in Biotechnology*), as radiotracer incorporation into all macromolecules and not only into nucleic acids is detected. Therefore, FISH-MAR requires relatively short incubation times (generally a few hours) an thus minimizes cross-feeding of other bacteria that are not primary substrate consumers.

FISH-MAR can be applied to answer two categories of questions. Firstly, the ecophysiology of defined microorganisms of interest, for which a specific FISH probe is available or can be designed, can be investigated in great detail (e.g. [8]). Secondly, one can use this technique to hunt for and to quantify those microorganisms that are responsible for a certain physiological process in the environment (e.g. [9]). For this purpose, the incubation conditions (e.g. various electron acceptors) and radiolabelled substrate(s) are selected such that only microorganisms capable of catalyzing the process of interest will be active. To achieve such selectivity, it is often necessary to inactivate other physiological groups by the addition of specific inhibitors. Bacteria that are active under these conditions are identified using broad group-specific FISH probes and the results obtained then provide guidance for the selection of more specific FISH probes.

FISH-MAR and RNA- or DNA-SIP are complementary approaches that offer different insights and options and should thus ideally be used in parallel or in combination (see below) for the analyses of microbial communities. On the one hand, DNA-SIP has the unique advantage that it allows one to specifically harvest genomic DNA from those bacteria consuming a defined substrate. This DNA fraction is then available for all kinds of molecular analyses, including environmental genomics [10•]. On the other hand, SIP analyses blur all spatial information; for example, making it impossible to decide whether the active bacteria were located at the surface or the substratum of a biofilm. Furthermore, FISH-MAR allows one to investigate activity patterns within bacterial populations [11••,12•,13], which cannot be deciphered by DNA- or RNA-SIP.

Quantitative FISH-MAR

In most applications, FISH-MAR was used to qualitatively investigate the substrate uptake of bacteria (Table 1), although it is obvious that quantitative data would provide valuable additional insights into the ecophysiology of the studied organisms. First attempts to extract quantitative data were made by the Kirchman group (e.g. [14,15]) by measuring the percentage of the total silver grain area that can be assigned to probe-defined bacterial groups. However, this procedure does not take into account slide-to-slide variation in silver grain formation or possible saturation of silver grain formation on some highly active populations after prolonged exposure times; thus, it would not be suitable to infer, for example, cell-specific uptake rates. Therefore, a multistep protocol for more precise quantification of substrate uptake by probe-defined bacteria via FISH-MAR was recently developed [11••] (see also Update) (Figure 2). For this purpose, it is necessary to first construct a standard curve using a pure culture of a microorganism with similar morphological properties to the target organism (e.g. a filamentous bacterium). To this end, the pure culture is incubated in separate experiments with different amounts of radioactively labelled substrate. Following MAR and microscopy, the counted number of silver grains per cell (or per μm^2 if a filament is analyzed) is plotted against the counts per minute (CPM) per cell (or μm^2) calculated from total cell counts obtained by scintillation counting of an aliquot of the incubated biomass. For these, and all subsequent experiments, it is important to optimize the length of exposure to ensure that a linear relationship between exposure time and silver grain

Table 1

Selected applications of FISH-MAR that have extended our knowledge on the physiology of the target organisms.

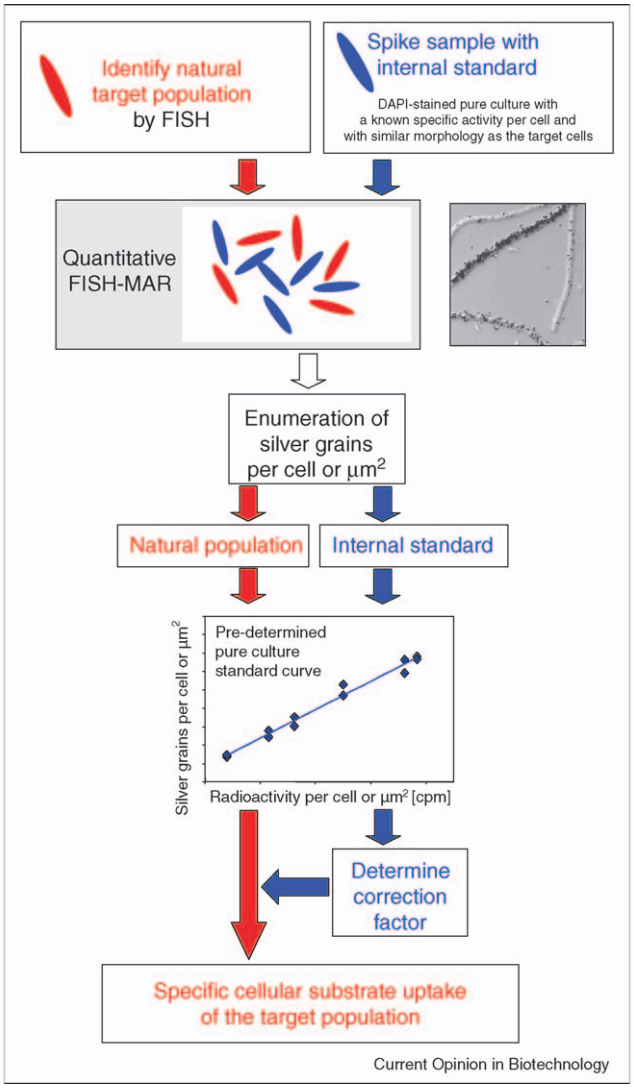
Investigated organism(s)	Habitat	Key biological findings obtained using FISH-MAR
Marine prokaryotic plankton [19•]	North Atlantic water column	A large fraction of mesopelagic and bathypelagic prokaryotes is metabolically active; uptake of bicarbonate is largely restricted to *Archaea*
Marine planktonic *Bacteria* [3]	Surface water (Pacific)	Uptake of dissolved amino acids
Marine planktonic *Archaea* [40]	Mid-depth ocean waters (Mediterranean, Pacific)	Uptake of dissolved amino acids from nanomolar concentrations
Marine prokaryotic plankton [18]	North Atlantic water column	Uptake of L-aspartic acid by a higher proportion of *Archaea* than *Bacteria* in deep waters
Marine *Roseobacter*-related bacteria and some γ-*proteobacteria* [48•]	Surface water (Gulf of Mexico and Mediterranean)	The fraction of cells incorporating DMSP was higher in these two populations than in any other identified group
Marine planktonic *Bacteria* [15]	Surface water (Gulf of Maine, Gulf of Mexico, and Sargasso Sea)	*Roseobacter*-related bacteria assimilated more DMSP than any other identified group, but did not account for most of the total DMSP assimilation; a large and diverse group of bacteria showed uptake of DMSP
Marine bacteria related to *Pelagibacter ubique* (SAR11) [12•]	Surface water (Gulf of Maine, Sargasso Sea, and North Carolina coast)	Responsible for a large fraction of DMSP and amino acid assimilation

Table 1 (*Continued*)

Investigated organism(s)	Habitat	Key biological findings obtained using FISH-MAR
Marine bacteria related to *Pelagibacter ubique* (SAR11) [42]	North Atlantic seawater	Major contribution to bacterial biomass production; differential utilization of low- and high-molecular-weight dissolved organic matter (glucose, free amino acids, protein)
Marine bacterioplankton [49]	Coastal North Sea	Anoxic uptake of glucose, indicating facultatively anaerobic metabolism of phylogenetically different pelagic bacteria
Marine *Proteobacteria* and members of the *Bacteroidetes* [50]	North Atlantic seawater	Differential use of dissolved organic matter components by different phylogenetic groups of plankton bacteria
Estuarine plankton *Bacteria* [14]	Delaware estuary water	Differential use of thymidine and leucine by different phylogenetic groups of plankton bacteria along a salinity gradient
Freshwater bacterioplankton [51]	Eutrophic freshwater lake	Uptake of cyclic AMP by a significant fraction of freshwater bacterioplankton; addition of cAMP to cultivation media facilitates the enrichment of uncultured plankton bacteria
Rhodocyclus-related polyphosphate-accumulating organisms (RPAO) [30]	Activated sludge	No direct assimilation of glucose, but use of fermentation products derived from glucose; ability of at least some RPAO to denitrify with NO_2^- and NO_3^-
Uncultured *Rhodocyclus*-related bacteria, *Actinobacteria* [31]	Activated sludge systems	Contribution of *Actinobacteria* to enhanced biological phosphorus removal in wastewater treatment
Tetrasphaera-related *actinobacterial* polyphosphate-accumulating organisms (APAO) [32••]	Activated sludge	Contribution of APAO to enhanced biological phosphorus removal in full-scale wastewater treatment plants; different substrate usage of APAO and RPAO
Unidentified β-*proteobacteria* other than *Rhodocyclus*-like bacteria [33]	Activated sludge	Contribution to enhanced biological phosphorus removal in wastewater treatment
Uncultured nitrite-oxidizing *Nitrospira*-like bacteria [23]	Activated sludge and nitrifying biofilm	Autotrophic CO_2 fixation and uptake of pyruvate (mixotrophy) under nitrifying conditions
Ammonia- and nitrite-oxidizing bacteria [21]	Artificial model biofilm	Vertical zonation of nitrifying activity in the model biofilm
Uncultured *Chloroflexi* and members of the *Bacteroidetes* [24]	Nitrifying biofilm obtained from a wastewater treatment plant	Uptake of *N*-acetyl-D-[1-^{14}C]glucosamine, indicating a possible role in the degradation of carbon compounds produced by (nitrifying) bacteria
Uncultured *Chloroflexi* and members of the *Bacteroidetes* [25••]	Nitrifying biofilm obtained from a wastewater treatment plant	Uptake of carbon fixed primarily by nitrifying bacteria: under nitrifying conditions mainly by *Bacteroidetes*, after switch to non-nitrifying conditions mainly by *Chloroflexi*
Anaerobic ammonium oxidizers (*Candidatus* Brocadia anammoxidans, *Candidatus* Kuenenia stuttgartiensis) [52]	Bioreactor enrichments	Confirmation that these organisms autotrophically fix CO_2 *in situ*
Uncultured bacteria related to the *Methylophilales* [22•]	Activated sludge	Uptake of methanol; contribution to denitrification in wastewater treatment
Uncultured *Curvibacter*-related and some other bacteria [53]	Activated sludge	Contribution to denitrification in wastewater treatment
Uncultured mycolic-acid-containing actinomycetes (mycolata) [36]	Activated sludge	Selective uptake of oleic acid, but no other tested substrate, by mycolata responsible for activated sludge foaming
Candidatus Microthrix parvicella [16•]	Activated sludge	Anaerobic storage of oleic acid; metabolic activity with O_2 or NO_3^- as electron acceptor
'*Candidatus* Microthrix parvicella' [37]	Activated sludge	Reduction of organic substrate uptake after addition of polyaluminium chloride to activated sludge
Meganema perideroedes [8]	Activated sludge	Uptake of various organic substrates under aerobic conditions; reduced substrate spectrum and uptake rate under denitrifying conditions; no indication for fermentative metabolism
Meganema perideroedes, *Thiothrix* sp. [11••]	Activated sludge	*In situ* substrate affinity (K_S) for acetate of both populations
Iron-reducing bacteria [9]	Activated sludge	Detection of novel iron-reducing γ-*proteobacteria* that oxidize acetate
Various prokaryotes including sulfate reducers and methanogens [54]	Corroding heating systems	Unexpected metabolic activity, under aerobic conditions, of prokaryotes from (presumably anaerobic) heating systems
Uncultured *Achromatium* species [55]	Freshwater sediment	Utilization of inorganic carbon and acetate by different, coexisting *Achromatium* species
Actinobacteria [56]	Freshwater streams and aquacultures	Frequent occurrence of metabolically active *Actinobacteria* in aquatic environments
Halophilic square *Archaea* [57]	Solar saltern crystallizer pond	Assimilation of amino acids and acetate, but not of glycerol and bicarbonate
Chloroflexaceae-related bacteria (*Chloroflexus* spp. and type C) [20]	Photosynthetic microbial mats from an alkaline hot spring	Uptake of acetate, but no assimilation of CO_2

DMSP, dimethylsulfoniopropionate; RPAO, *Rhodocyclus*-related polyphosphate-accumulating organisms; APAO, *Tetrasphaera*-related *actinobacterial* polyphosphate-accumulating organisms.

Figure 2



Schematic flow diagram of the multistep quantitative FISH-MAR approach.

number exists. In the next step, the environmental sample is incubated with the radioactively labelled substrate and then spiked with pure culture cells with a defined specific radioactivity and pre-stained with the fluorescent DNA-binding dye 4′-6-diamidino-2-phenylindole (DAPI). After FISH-MAR, the number of silver grains on top of the probe-labelled target cells and the DAPI-stained internal standard cells are counted and the CPM of the target cells inferred from the standard curve. For this purpose, it is important to correct for experimental variation by using a factor calculated from the standard curve and the number of silver grains on the internal control cells. From these data, the specific activity per target cell can finally be calculated. If specific activities are inferred after incubations with different substrate concentrations, it is even possible to measure the substrate affinity K_s of the uncultured target organism [11••].

Heterotrophic MAR

A major problem of traditional FISH-MAR is that homogenously isotope-labelled complex organic substrates are often not commercially available. Microbial consumers of such substrates can nevertheless be identified by FISH-MAR by simultaneously incubating the sample with unlabelled complex substrate and labelled $^{14}CO_2$ [16•]. This technique, coined HetCO_2-MAR, makes use of the phenomenon that most heterotrophic bacteria assimilate CO_2 during biosynthesis in various carboxylation reactions. HetCO_2-MAR is therefore an inexpensive option for encompassing ecophysiological studies of heterotrophic bacteria, as only a single labelled compound is required for all experiments. Another major advantage of HetCO_2-MAR over traditional FISH-MAR is that it allows one to better differentiate between substrate incorporation without growth (e.g. into storage compounds) and actual growth of the substrate-consuming microorganism, because growth is generally required for active carboxylation reactions. It is important to note that HetCO_2-MAR experiments often require the addition of inhibitors of autotrophic prokaryotes to avoid false-positive results.

Combining FISH-MAR with other techniques

As expected, MAR can also be combined with other fluorescent staining techniques like live/dead stains (e.g. the redox dye 5-cyano-2,3-tolyl-tetrazolium chloride; CTC) [17] or variations of the standard FISH protocol, such as catalyzed reporter deposition-FISH [18,19•] (see also Update). The scope of FISH-MAR is extended more significantly by combining this technique with microelectrodes, which measure concentrations of dissolved compounds in the microenvironment of the target organisms. Application of this approach to hot spring microbial mats revealed that uncultured *Chloroflexaceae* relatives, which occurred close to the mat surface in the direct vicinity of cyanobacteria and tolerated high O_2 concentrations, may grow photoheterotrophically on organic carbon provided by the autotrophic cyanobacteria [20]. In another study, nitrogen turnover and oxygen consumption in a nitrifying biofilm were measured with microelectrodes and the activity of nitrifying bacteria was monitored by FISH-MAR on the level of individual microcolonies. Furthermore, inorganic carbon assimilation patterns in the biofilm were elegantly detected by β microimaging, although this does not provide single-cell resolution [21]. Despite the use of an artificial biofilm, which was obtained by embedding separately grown microbial biomass in agarose, this example illustrates that combinations of FISH-MAR and microelectrodes have a high potential for correlating the activity of distinct microbial populations with substrate turnover rates in a local microenvironment.

As FISH-MAR requires the use of oligonucleotide probes with defined specificity, it depends on *a priori* knowledge about the phylogenetic affiliation of the studied organisms. If such knowledge is not available, then how does one decide which probes should be used in FISH-MAR experiments? This obstacle can be overcome by combining FISH-MAR with SIP. In the first step, SIP is used to obtain the rRNA gene sequences of those organisms that synthesized nucleic acids (and thus were metabolically active) during incubation with a stable isotope tracer. Subsequently, new specific oligonucleotide probes can be designed based on these rRNA gene sequences. The new probes are then used for FISH-MAR after incubation of the environmental sample with the same substrate, which must now be radioactively labelled [22•]. The FISH-MAR experiments, which require only short incubation times, are performed to confirm the results of SIP. They are less biased by cross-feeding than SIP experiments and can be used to exclude false-positive SIP results owing to contamination of the heavy nucleic acid fraction with nucleic acids from the light fraction.

Applications of FISH-MAR

As a detailed description of all FISH-MAR applications would be beyond the scope of this article, only two examples are described here: the use of FISH-MAR for analyzing microbial communities in wastewater treatment plants and in marine ecosystems (the compilation in Table 1 also includes other FISH-MAR applications).

Wastewater treatment plants are interesting model systems for microbial ecology, because they offer unique and easily accessible opportunities to study nutrient fluxes, interactions, and niche differentiation in complex microbial communities under well-defined environmental conditions. The removal of nitrogen in nitrification-denitrification cascades is one of the most important processes in biological wastewater treatment. As one of the first reported applications of FISH-MAR, the uptake of different carbon sources by *Nitrospira*-like bacteria, which are important nitrite oxidizers, was studied in nitrifying activated sludge and biofilm samples [23]. These experiments confirmed the autotrophic carbon fixation by these yet uncultured microbes, and also showed that the same bacteria are able to use pyruvate as an additional carbon source, and possibly also as an energy source. In two recent studies, FISH-MAR was used to monitor the carbon flow from nitrifiers to heterotrophic bacteria after autotrophic CO_2 fixation [24,25••]. The reported discovery of niche differentiation among heterotrophic bacteria, which used carbon sources provided by the autotrophic nitrifiers, by this approach demonstrates the high potential of FISH-MAR for studying the sequestration of organic compounds in complex microbial food webs.

Although the ability to nitrify is apparently restricted to a few prokaryotic lineages [26–28], the use of nitrate or nitrite as terminal electron acceptors under anoxic conditions (denitrification) is a widespread metabolic activity among prokaryotes [29]. The large diversity of the denitrifiers makes it extremely difficult to identify the key players of this guild in wastewater treatment plants or natural habitats. This problem was addressed by the combination of FISH-MAR with SIP, which allowed the discovery of a methylotrophic denitrifier enriched from activated sludge [22•]. Besides nitrogen elimination, enhanced biological phosphorus removal is a key step of wastewater treatment needed to prevent the accumulation of high nutrient loads in waters into which treated wastewater is discharged. This process is catalyzed by polyphosphate-accumulating organisms (PAOs), which have thus far resisted cultivation. However, application of FISH-MAR has yielded interesting insights into their ecophysiology [30–31]. For example, *Rhodocyclus*-related PAOs do not directly take up glucose, but instead use fermentation products derived from glucose by other, coexisting bacteria [30]. Furthermore, FISH-MAR provided evidence that novel PAOs related to the *Actinobacteria* occur and are active in activated sludge [31,32••]. One of the most serious problems in biological wastewater treatment is the foaming and bulking of activated sludge caused by excess proliferation of filamentous bacteria. The classification of these mostly uncultured filaments has a long history [34,35], but efficient strategies to fight sludge bulking require insight into their physiology and interactions with other organisms. Such knowledge has recently been collected, using FISH-MAR, for several important filamentous bacteria [8,11••,16•,36,37].

Although wastewater treatment is quantitatively the most important biotechnological application worldwide, the oceans are by far the largest ecosystems on the planet. The majority of marine microorganisms has not been cultured yet, and thus cultivation-independent methods like FISH-MAR are the only means to explore the physiological potential of most *Bacteria* and *Archaea* in the sea. Not long ago, it was first shown that non-thermophilic *Archaea* constitute a large fraction of the marine prokaryotic plankton [38,39]. FISH-MAR revealed that these organisms can incorporate amino acids present in only nanomolar concentrations in their environment [3,40], and showed that these organisms take up inorganic carbon [3] and may thus be key primary producers in the oceans. Consistent with the latter finding, a marine crenarchaeote that grows chemolithoautotrophically by oxidizing ammonia to nitrite has been described recently [27]. Another large fraction of marine prokaryotic plankton is made up by organisms related to the γ-proteobacterium *Pelagibacter ubique* ('SAR11'), which is considered important for global carbon cycling and has recently been cultured in the laboratory [41]. As shown by FISH-MAR, members of the SAR11 clade do not utilize different components of dissolved organic matter equally *in situ*, but may have a preference for low-molecular-weight

substances which would render them especially important for the turnover of monomers rather than polymers [42]. Moreover, the same organisms may also play significant roles in the nitrogen and sulfur cycles, as demonstrated by FISH-MAR experiments with radiolabeled amino acids and dimethylsulfoniopropionate [12•].

The isotope array

The principle of identifying radioactively labelled microorganisms using rRNA-targeted oligonucleotide probes was recently also adapted to the microarray format. In this so-called isotope array (Figure 1), community rRNA is first extracted from an environmental sample that was incubated with a radioactively labelled substrate, then covalently linked with a fluorescent dye, fragmented, and hybridized with an rRNA-targeted microarray. Subsequently, fluorescence and radioactivity probe signals are quantified with a fluorescence scanner and a β imager, respectively. Two main advantages render the isotope array methodologically appealing for the analysis of complex microbial communities. The multiple probe hybridization format offers the opportunity to identify many microorganisms with a defined metabolic ability in a single microarray experiment. Furthermore, the ratio between radioactivity and fluorescence of a probe spot provides a unique, quantitative measure of how efficiently a probe-defined population has incorporated the labelled substrate into its rRNA.

Proof of the isotope array principle was recently accomplished; rRNA of active β-proteobacterial ammonia-oxidizing bacteria became labelled upon a short incubation of activated sludge samples with radioactive bicarbonate under nitrifying conditions and this rRNA could be subsequently detected based on its fluorescence and radioactivity after hybridization with a prototype microarray [6••]. In a follow-up study, a fully evaluated microarray for recognized as well as yet uncultivated members of the β-proteobacterial order *Rhodocyclales*, including the above-mentioned *Rhodocyclus*-related PAOs [43], was used to determine substrate specificity of these microorganisms in a full-scale wastewater treatment plant (M Hesselsoe *et al.*, unpublished data). In separate experiments, different short-chain fatty acids were added to the activated sludge as electron donors and incubated under oxic or denitrifying conditions. Analogous to $HetCO_2$-MAR, ^{14}C-bicarbonate was exploited as a general activity marker in the presence of an inhibitor of autotrophic CO_2 assimilation. Diverse members of the *Rhodocyclales*, including *Zoogloea* species and the *Rhodocyclus*-related PAOs, were simultaneously identified and shown to be actively involved in denitrification in this wastewater treatment plant.

Although conceptually simple, the effectiveness of the isotope array approach strongly depends on the availability of suitable microarrays and their analytical performance (i.e. their specificity and sensitivity). Thus, it is anticipated that widespread application of the isotope array approach will be strongly facilitated by continued efforts in developing and optimizing rRNA-targeted oligonucleotide microarrays [43–46].

Conclusions

FISH-MAR is rapidly becoming an indispensable tool in microbial ecology for investigating the function of microorganisms within their natural communities. Armed with this and other related techniques, like the isotope array [47], directly linking the huge amount of available biodiversity data with biogeochemical processes and ecosystem functioning is no longer a dream. Furthermore, we anticipate major future applications of FISH-MAR and isotope arrays for testing hypotheses generated by analyses of environmental genomics data. The major beauty of these approaches is that they unambiguously demonstrate consumption of a substrate under the incubation conditions applied. Such a definite conclusion cannot be reached from environmental transcriptomics or proteomics data because, for example during physiological inhibition, mRNA or even a key physiological protein might be detectable although the target organism did not perform a certain physiological activity at the time of sampling. Ultimately, FISH-MAR and isotope arrays will not only be used to decipher the function of individual populations in a microbial community, but will also reveal key physiological interactions between the different members of these communities. Thus, in the long run, they will help to provide more solid grounds for theoretical modelling of such communities.

Update

In wastewater treatment systems, glycogen-accumulating organisms (GAOs) often outcompete PAOs for the same substrates and thus hamper efficient phosphorus removal. The ecophysiology of these yet uncultured γ-proteobacterial GAOs, the activity of which is detrimental to the performance of enhanced biological phosphorus removal, has recently been investigated by FISH-MAR [58].

Furthermore, a comparison of the number of active prokaryotes in a drinking water reservoir, as analyzed by FISH, catalyzed reporter deposition-FISH, and MAR, has demonstrated that MAR provides the highest numbers and FISH the lowest [59]. Detailed protocols for FISH-MAR, including latest methodological advances, have also recently been published [60].

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- • of special interest
- •• of outstanding interest

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