Microautoradiography: recent advances within the studies of the ecophysiology of bacteria in biofilms

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Abstract Microautoradiography (MAR) is a technique that allows the direct identification of active microorganisms and of their metabolic capabilities without prior enrichment or cultivation. Today, this technique is probably the only one that allows an in-depth investigation of the ecophysiology of uncultivated bacteria under their natural conditions, and it possesses, therefore, alone or in combination with other in situ techniques, a great potential to be used in comprehensive studies of biofilms and other ecosystems. Here, we present a brief description of the microautoradiographic technique, and recent examples of the type of information that can be obtained using MAR in combination with FISH or with other methods when investigating biofilm processes. Also, some limitations of the technique are discussed.

Keywords Microautoradiography; FISH; biofilm; uncultivated bacteria; ecophysiology; modeling

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Introduction

Microautoradiography (MAR) is a technique which enables a direct visualization of active microorganisms and their metabolic capabilities without prior enrichment or cultivation. With this technique, it is possible to investigate the ecological function of individual cells and enumerate the population of active cells. When combined with microbial identification via fluorescence in situ hybridization (FISH), information on the in situ metabolic capabilities of cultivated and uncultivated cells can be obtained. The combined MAR-FISH technique has been applied to the study of several complex microbial systems since it was first described by Lee *et al*. and Ouverney and Fuhrman in 1999. These microbial systems include marine samples (e.g. Ouverney and Fuhrman, 2000; Cottrell and Kirchman, 2000), activated sludge (e.g. Nielsen *et al.*, 1999, 2000; Kong *et al.*, 2004), and biofilms (e.g. Ito *et al.*, 2002), which reveal the broad potential of the technique. Presently, this technique is probably the only one that allows an in-depth investigation of the ecophysiology of cultivated and uncultivated bacteria under their natural conditions, and it possesses, therefore, alone or in combination with other in situ techniques, a great potential to be used in comprehensive studies of biofilms and other ecosystems. In this minireview we present a brief description of the microautoradiographic technique, and recent examples of the type of information that can be obtained using MAR in combination with FISH or with other methods when investigating complex microbial systems with focus on biofilms.

Microautoradiography

MAR was introduced in microbial ecology in the 1960s by Thomas Brock (Brock and Brock, 1966, 1968). The method is based on the capability of radiolabeled substrate taken up by individual prokaryotic cells to be visualized by means of a radiation-sensitive silver halide emulsion covering the radiolabeled organisms and subsequently processed by standard photographic procedures. The radiotracers used in microbial ecology are typically the soft beta emitters ^{3}H, ^{14}C, ^{33}P, and, in a few cases, the stronger beta emitter ^{32}P. Common to all radiotracers is their ability to form silver grains on top and near

the radiolabeled bacteria that can be clearly visualized by bright field or phase contrast microscopy. Low energy emitters give the highest resolution. Tritium, for example, yields a resolution of less than 0.5 μm, while ^{14}C and ^{33}P yield a resolution of 2–3 μm (e.g. Rogers, 1979). A detailed description of the procedures for MAR can be found in several studies (e.g. Rogers, 1979; Meyer-Reil, 1978; Tabor and Neihof, 1982; Carman, 1993; Andreasen and Nielsen, 1997, 2000; Nielsen and Nielsen, 2005).

MAR has been used in combination with various simple staining techniques (Meyer-Reil, 1978; Tabor and Neihof, 1982; Andreasen and Nielsen, 1997), and with microelectrodes (Pearl, 1984; Gieseke *et al*., 2005) to characterize microbial communities. When MAR is combined with FISH, bacterial identity can be linked to specific physiological traits (Figure 1). Slightly different approaches have been devised: MAR-FISH (MicroAutoRadiography-FISH, Lee *et al*., 1999), STAR-FISH (Substrate Tracking AutoRadiography-FISH, Ouverney and Fuhrman, 1999) and MICRO-FISH (MICROautoradiography-FISH, Cottrell and Kirchman, 2000).

The MAR-FISH procedure includes sampling of biofilms, and incubation under selected conditions, such as type and amount of radiotracer, incubation time, biomass concentration, temperature, and presence of inhibitors. After incubation, the samples are fixed, washed, and hybridized with relevant FISH probes or other stains. Subsequently, the liquid radiosensitive film is placed on top of the sample, dried, and exposed for typically 3–6 days before development and examination. The stained MAR-positive bacteria can be examined by a combination of bright field or phase-contrast and epifluorescence

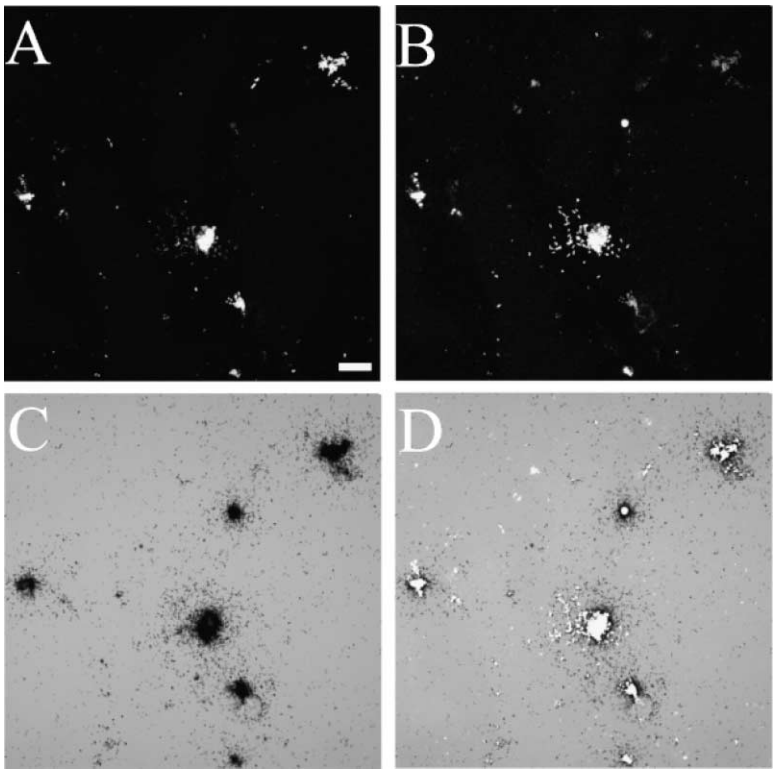

Figure 1 An example of MAR-FISH. The uptake of [^{14}C]-bicarbonate by microcolonies of the ammonium oxidizer *Nitrosomonas oligotropha* under aerobic conditions in a biofilm, detected by the combination of MAR and FISH. Images 1A and 1B show the FISH signal from *N. oligotropha* and a general probe for ammonia oxidizing *Betaproteobacteria*, respectively. 1C shows the corresponding MAR image of the uptake of [^{14}C]-bicarbonate. 1D shows the overlay of image 1A, B and C. Bar = 10 μm

microscopy or laser scanning microscopy. An up-to-date description of the protocol of the MAR-FISH technique can be found elsewhere (Nielsen and Nielsen, 2005).

Information obtained by MAR-FISH in biofilms

When MAR is applied to biofilms or other forms of biological aggregates, it is important to address the sort of information that is wanted. If the ecophysiology of the microorganisms is going to be determined under undisturbed in situ conditions, it is important to keep the 3-D structure intact during the incubation with radiotracer and other substrates, thus keeping intact microgradients of substrates, environmental parameters, and possible bacterial interactions (Table 1). Alternatively, the biofilm can be removed from the surface so the incubation can be performed on gently homogenized samples in suspension. Under these conditions, it is possible to control most variables such as the concentration of organic substrates and presence or absence of oxygen or other electron acceptors. In this case, information about the physiological potential of microorganisms is obtained, but not necessarily information about their actual activity under the defined conditions of a specific location within the biofilm.

As optimal MAR visualization requires samples with a thickness of a few micrometres, three options exist for the visualization of the radioactive microorganisms: for very thin biofilms, MAR (i.e. specifically the radiosensitive emulsion) can be applied directly on top of the biofilm surface after incubation; thicker biofilms must be removed, homogenized, and spread onto a glass slide before MAR is applied; or the biofilm can be embedded and cryosectioned in order to keep the structure intact and thus reveal the 3-D distribution of microorganisms and their activity down through the biofilm.

So far, only a few studies have been conducted with intact biofilms under in situ conditions. In a study related to biocorrosion, we were able to observe MAR-positive bacteria in thin biofilms directly on corroding metal surfaces and investigate the relative number of aerobic and anaerobic bacteria able to consume labeled acetate and bicarbonate. This approach makes it possible to locate bacteria around the corroding pits, thus revealing potential corroding bacteria and possible corrosion mechanisms (Kjellerup *et al.*, 2003; *submitted*). On corroding stainless steel surfaces many bacteria were observed in and around a pit. However, bacteria able to fix CO_2 in the absence of external organic substrates and thus possibly able to scavenge H_2 from the cathodic process were only present at a narrow location in the pit, indicating that corrosion took place in that area. In thick intact model biofilms containing different nitrifiers, the uptake of $^{14}CO_2$ was investigated under various substrate conditions in bulk water (Gieseke *et al.*, 2005). The substrate gradients (oxygen, ammonium, nitrite, nitrate) were measured by microsensors during the incubation. Subsequently, thin sections of the biofilms were analyzed by FISH and MAR, and the local uptake of $^{14}CO_2$ was quantified by

Table 1 Information obtained by MAR experiments with intact or homogenized biofilms

Biofilm	Incubation conditions	MAR	Information
Thin biofilms (a few micrometres)	Intact biofilms In situ conditions	On intact surfaces	Spatial distribution of active bacteria on surface
Thick biofilms	Intact biofilms Controlled substrate and environmental conditions	After cryosectioning	Spatial distribution of active bacteria within the biofilms
	Gently homogenized biofilm Controlled substrate and environmental conditions	After further homogenization or cryosectioning	Potential activity of probe-defined populations

beta-imaging. In this way, it was possible to identify the active nitrifiers throughout the biofilm, quantify the local and global uptake, and investigate at which local concentrations the different nitrifiers were active. These experiments with model biofilms show the great potential for detailed studies of thick natural biofilms using a suite of in situ methods, including MAR and FISH.

Gently homogenized biofilms have been used in several MAR studies. In an anaerobic biofilm, the dominating consumers of labeled formate, acetate, propionate, and bicarbonate were identified under sulfate-reducing conditions (Ito *et al.*, 2002). The substrate uptake characteristics of these compounds were compared between different phylogenetic subgroups of sulfate-reducing procaryotes (SRP) and compared with their characteristics described in pure culture studies. Furthermore, the capability of the SRP subgroups to utilize these compounds under nitrate-reducing conditions and aerobic conditions was investigated. Many bacteria belonging to the genus *Desulfobulbus* were able to take up organic substrate with nitrate as electron acceptor. Such results provide further insight into the complexity of the phylogenetic diversity and the physiological diversity of SRB populations inhabiting complex microbial biofilms. Kindaichi *et al.* (2004) used homogenized biofilms to study the interaction between nitrifying bacteria and heterotrophic bacteria in autotrophic nitrifying biofilms, where they identified several heterotrophs able to grow on compounds excreted by the heterotrophs. They observed that 50% of all bacteria of this "autotrophic" biofilm were in fact heterotrophic bacteria, revealing that the excretion of organic matter (based on bicarbonate fixation) was very significant to the development of the structure and function of the biofilm.

There are several studies with intact biological aggregates from wastewater treatment plants, where MAR studies have been applied to obtain information about the ecophysiology of specific non-cultured bacteria of great interest to biological N and P removal. Aspects of the ecophysiology of uncultured nitrite-oxidizers (*Nitrospira*) in biofilms and activated sludge have been investigated (Daims *et al.*, 2001). Besides fixation of labeled CO_2, *Nitrospira*-like bacteria were shown to assimilate pyruvate under aerobic conditions, thus showing potential mixotrophic activity. Recently, also potentially important denitrifiers were identified by molecular methods and further characterized by MAR (Ginige *et al.*, 2004; Thomsen *et al.*, 2004). Bacteria belonging to *Methylophilales* were found in enriched bioreactors supplied with methanol, while bacteria related to the genus *Aquaspirillum* were found in abundance in full-scale wastewater treatment plants with N-removal. The proposed denitrifying capabilities were shown by MAR observing uptake of labeled organic substrate with oxygen or nitrate (Ginige *et al.*, 2004) or oxygen, nitrate, or nitrite (Thomsen *et al.*, 2004) as electron acceptors. Based on these results it was suggested that these bacteria were able to denitrify. An additional indication of denitrifying capabilities was provided by Kong *et al.* (2004), who investigated the ecophysiology of uncultured polyphosphate-accumulating organisms (PAO) belonging to the genus *Rhodocyclus* in the *Betaproteobacteria*. Besides uptake of labeled organic substrate (acetate) with oxygen, nitrate, or nitrite as electron acceptor in short-term experiments (2–3 hours), these bacteria were also able to assimilate labeled acetate after a prolonged preincubation period (6–9 hours) with unlabeled acetate under the same electron acceptor conditions. This approach showed that these bacteria were most likely able to grow on both nitrate and nitrite and were thus able not only to accumulate polyphosphate but also to denitrify. The same type of MAR incubations also showed that these bacteria were able to store substrate under anaerobic conditions (e.g. acetate as poly-beta-hydroxy alkanoates, PHA). Uptake of labeled acetate took place in only 2–3 hours, indicating a saturation of the anaerobic storage capacity. Nile blue staining of the probe-defined bacteria confirmed that the storage product was formed (Kong *et al.*, 2004).

Simultaneous uptake of two different substrates in probe-defined bacteria can also be detected by MAR. *Rhodocyclus*-related PAO were able to take up both acetate and propionate or pyruvate and propionate simultaneously (Kong *et al.*, 2004). This can be shown by adding one labeled compound and one unlabeled compound in one experiment and then vice versa in another experiment. MAR-positive cells in both cases confirm the simultaneous uptake of the two substrates. Interestingly, some substrates could not be taken up as sole substrate (e.g. the amino acid leucine), but were taken up in the presence of acetate, maybe because leucine mainly acted as a nitrogen source.

So far, the information obtained by MAR has not been used directly for modeling purposes. However, the information obtained by MAR experiments can be extremely useful for the modeling of biofilm processes (Table 2). Enumeration of various functional groups by MAR can together with FISH enumeration give a quantitative description of the most dominant bacteria in terms of activity and abundance in a certain ecosystem. Together with an analysis of the degradation of various organic compounds, it is possible to identify the most important pathways of the degradation of organic matter in the biofilm and under which electron acceptor conditions they take place. Since it is also possible to count the number of silver grains on top of the bacteria produced as part of the MAR technique, substrate uptake information about individual cells can be obtained (Pearl and Stull, 1979; Nielsen *et al.*, 2000). A proper quantification requires procedures that ensure prevention of leakage from the cells and use of internal standards. With an improved fixation protocol and the use of an internal standard of bacteria with known specific radioactivity, the MAR-FISH method (Q-MAR, quantitative MAR) was further refined to quantitatively measure substrate uptake kinetics, such as average cell-specific substrate uptake rates and apparent K_s values (Nielsen *et al.*, 2003).

New developments of MAR

Besides the recent development of QMAR-FISH, a new variation of the MAR method has recently been developed, the HetCO_2-MAR (Hesselsoe *et al.*, 2005). This approach is based on the assimilation of CO_2 by most heterotrophic bacteria in various carboxylation reactions during biosynthesis, typically 1–10% of the biomass carbon by growing bacteria. Assimilation of $^{14}CO_2$ by heterotrophic bacteria was used for isotope labeling of

Table 2 Overview of information obtained by MAR-experiments useful for biofilm modeling

Type of information	Examples of data	References
Quantification of functional groups and identification of potential pathways in the degradation of organic matter	General enumeration of heterotrophs and autotrophs Enumeration of specific groups: nitrifiers, Fe(III)-reducers, sulfate reducers, methanogens, bacteria degrading specific organic compounds such as acetate or xenobiotic contaminants	Nielsen and Nielsen, 2002b Kindaichi *et al.*, 2004 Nielsen and Nielsen, 2002a Nielsen *et al.*, 2002 Kjellerup *et al.*, 2003 Yang *et al.*, 2003 Kindaichi *et al.*, 2004
Growth and substrate uptake kinetics for individual bacteria	Cell specific substrate uptake rate, substrate affinity (K_s), activity distribution within a population, uptake of dual substrates	Nielsen *et al.*, 2003 Kong *et al.*, 2004
Other physiological characterization of probe-defined population	Poly-P-accumulating activity, storage of organic substrate, denitrifying activity, mixotrophic activity	Nielsen *et al.*, 2000 Ginige *et al.*, 2004 Kong *et al.*, 2004, 2005

active microorganisms in environmental samples and visualized by MAR-FISH. The MAR signals were comparable with the traditional MAR-approach based on uptake of ^{14}C-labeled organic substrates. The HetCO_2-MAR approach was evaluated by investigating filamentous bacteria (*Microthrix parvicella*) forming foam in activated sludge. *M. parvicella* is able to take up oleic acid under anaerobic conditions as shown by the traditional MAR approach using ^{14}C-labeled oleic acid. By using the HetCO_2-MAR approach it was found that uptake of $^{14}CO_2$ in *M. parvicella* under anaerobic conditions did not take place, indicating no growth on oleic acid under these conditions. When O_2 or NO_3^- was added a significant $^{14}CO_2$ assimilation was observed indicating growth on the stored oleic acid. If NO_2^- was added no $^{14}CO_2$ assimilation took place, showing that the electron acceptors O_2 or NO_3^- but not NO_2^- could initiate growth. Such information could not have been derived using the traditional MAR procedure, so the new HetCO_2-MAR approach appears to differentiate better between substrate uptake and substrate metabolism that results in growth. Another obvious advantage is that isotope labeling of heterotrophic microorganisms is no longer restricted to the use of commercially available and often expensive labeled organic substrates, so any organic compound or mixture of organic substrates can in principle be investigated for potential uptake and metabolism just by looking at the uptake of $^{14}CO_2$. One potential disadvantage is that the MAR signal from the relatively strong beta emitter ^{14}C requires well homogenized samples or very thin cryosections if the bacteria are present in aggregates to get a clear single-cell signal. However, the novel HetCO_2-MAR approach expands significantly the possibility for studying the ecophysiology of uncultivated microorganisms.

Limitations of MAR-FISH

Although many of the recent studies show continuous progress in the development and application of MAR and MAR-FISH, MAR has some drawbacks and limitations. Extensive experience with both MAR and FISH is required, and experience and access to a LSM are preferred. MAR is time consuming (especially if Q-MAR is applied), and it is relatively expensive due to the cost of radiotracers. In some countries, the safety regulations require separate laboratories and equipment for isotope work. The availability of suitable radiolabeled substrates can be a problem. Often, the suppliers can only offer a limited selection of either [^{3}H] or [^{14}C]-labeled organic compounds, while more rarely sold compounds can only be produced upon request, and it can be very costly. The interpretation of a positive or negative MAR-signal must always be carried out carefully. If the cells investigated are MAR-negative, it is important to compare with positive controls to conclude if the cells are unable to take up the substrate, if the experimental design is poor, resulting in an insufficient amount of tracer incorporated in the cells, or if the incorporation is simply very low indicating a low yield on that particular substrate. Another problem with certain substrates is the risk of cross-feeding or partial substrate degradation. Labeled glucose, for instance, can be fermented under certain conditions and these labeled products can be taken up by other bacteria unable to consume glucose, and a false positive MAR-signal will appear (Kong *et al.*, 2004). The best way to test this is to add available inhibitors or add non-labeled fermentation products so the produced labeled products are diluted away. However, more in-depth studies of the ecophysiology require detailed evaluation of the possible biochemical pathways, the site of labeling in the organic substrate, and the incubation conditions.

Conclusions

MAR is probably the only technique today that allows an in-depth investigation of the ecophysiology of uncultivated bacteria under their natural conditions. Therefore, it

possesses, alone or in combination with other in situ techniques, a great potential to be applied in comprehensive studies of biofilms and other ecosystems. Quantitative information can be obtained about the dominant functional groups together with an identification of potential pathways in the degradation of organic matter. Also, growth and substrate uptake kinetics for individual bacteria as well as other physiological characteristics of the probe-defined population can be obtained. Investigations can be carried out on intact or gently homogenized biofilm samples. Much of this quantitative information can be used for modeling. Although the MAR technique has several limitations, recent studies show continuous progress in the development and application of the technique, thus demonstrating a great potential as a future tool to investigate complex microbial ecosystems.

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