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Minireview

# Tips and tricks for high quality MAR-FISH preparations: Focus on bacterioplankton analysis

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ABSTRACT

The combination of microautoradiography and fluorescence *in situ* hybridization (MAR-FISH) is a powerful technique for tracking the incorporation of radiolabelled compounds by specific bacterial populations at a single cell resolution. It has been widely applied in aquatic microbial ecology as a tool to unveil key ecophysiological features, shedding light on relevant ecological issues such as bacterial biomass production, the role of different bacterioplankton groups in the global carbon and sulphur cycle, and, at the same time, providing insights into the life styles and niche differentiation of cosmopolitan members of the aquatic microbial communities. Despite its great potential, its application has remained restricted to a few laboratories around the world, in part due to its reputation as a "difficult technique". Therefore, the objective of this minireview is to highlight the impact of MAR-FISH application on aquatic microbial ecology, and also to provide basic concepts, as well as practical tips, for processing MAR-FISH preparations, thus aiming to contribute to a more widespread application of this powerful method.



Autoradiography has been known for more than a century. It has, in fact, contributed to the discovery of radioactivity, since Niepce in 1867, and later Becquerel in 1896, observed that uranium caused blackening of photographic emulsions [44]. The application of autoradiography to microbial ecology was introduced in the 1960s with the work of T.D. Brock, who estimated the *in situ* growth rate of morphologically conspicuous marine bacteria by measuring the incorporation of radiolabelled thymidine [12].

Microautoradiography in combination with fluorescence *in situ* hybridization – known as MAR-FISH [26], STAR-FISH [39] or Micro-FISH [15] – is a powerful technique to track the incorporation of radiolabelled compounds by specific (*i.e.* probe targeted) bacterial populations, in either cultures or environmental samples. Together with fluorescence *in situ* hybridization combined with Raman microscopy (Raman-FISH) [22], and halogen *in situ* hybridization coupled with nano-scale secondary-ion mass spectrometry (HISH-NanoSIMS) [34], MAR-FISH represents one of the few available techniques that allow the analysis of the *in situ* physiology of single cells of uncultured microorganisms, which represent the great majority of environmental microbes. Single cell resolution is highly appreciated for studies linking microbial identity and activity. Besides allowing the phylogenetic identification of the cells, it also provides valuable information concerning their size, shape and spatial localization, which are all relevant traits for a deeper understanding of bacterial-mediated processes.

Since its first appearance in the late 1990s, MAR-FISH has been applied successfully, mostly in the fields of aquatic microbial ecology and waste water treatment. Some of the key findings in the field of aquatic microbial ecology are summarized in Table 1. For examples on waste water treatment applications please refer to the reviews by Okabe et al. [38] and Wagner et al. [57].

One of the most relevant ecophysiological features that was established early on by the application of this technique, was the corroboration that planktonic *Bacteria* and *Archaea* incorporated amino acids at the nanomolar concentrations characteristic of their environment [39,40], highlighting their importance in the cycling of dissolved organic matter (DOM) and concluding that at least some of the marine *Archaea* were heterotrophic. Soon after these discoveries, the first *in situ* evidence was obtained that the most abundant wide-ranging phylogenetic groups of marine bacterioplankton differed in their uptake of distinct classes of DOM: *Alphaproteobacteria* would be mainly specialists in using low molecular weight compounds, while *Bacteroidetes* would mainly incorporate high molecular weight substrates [15]. Additional studies have shown that different bacterial groups exhibit further preferences related not only to substrate quality but also to substrate concentration, and that these preferences often go beyond the main phylogenetic divisions (*i.e.* phyla, classes) [5,7].

These findings have been crucial for establishing that it would be necessary to consider more than a single compartment for modelling the role of heterotrophic bacteria in the carbon cycle.

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**Table 1**
Summary of key findings in aquatic microbial ecology, derived from the application of MAR-FISH.

| Environment | Target organisms | Main results | Reference |
|---|---|---|---|
| Pacific and Mediterranean marine waters | *Bacteria*, *Archaea* | Planktonic *Bacteria* and *Archaea* incorporate amino acids at environmental concentrations. | [39,40] |
| Delaware Bay estuary and Atlantic Ocean at the Indian River Inlet | Main clades of estuarine bacteria (*Alpha-*, *Beta-*, *Gammaproteobacteria*, *Bacteroidetes*) | The main clades of marine bacteria differ in their preferences for high and low molecular weight substrates. | [15] |
| Coastal North Sea | Heterotrophic picoplankton populations (SAR11, *Roseobacter*, DE2, SAR86, *Euryarchaea*) | Different prokaryotic populations – including members within the same class – differ in their consumption of low molecular weight substrates according to their concentration. | [5,7] |
| Mediterranean Sea | Main clades of marine bacteria (*Alpha-* and *Gammaproteobacteria*, *Bacteroidetes*), and subpopulations of *Alphaproteobacteria* (SAR11, *Roseobacter*) | There are substantial changes in the activity of specific groups throughout the year. A relatively high proportion of the main clades took up ATP in this phosphorous-limited environment. | [2] |
| Coastal North Sea | Main clades of marine bacteria (*Alpha-* and *Gammaproteobacteria*, *Bacteroidetes*) and subpopulations of *Gammaproteobacteria* (*Alteromonas*, *Pseudoalteromonas*, *Vibrio*) | Widespread ability of heterotrophic bacterioplankton to thrive under anoxic conditions. The activity of some members was even favoured by these conditions. | [6] |
| North Atlantic surface and deep waters | *Bacteria*, *Crenarchaea*, *Euryarchaea* | Widespread ability of D-amino acids incorporation by the prokaryotic plankton, especially by *Crenarchaea* in deep water layers. | [51] |
| Dilution cultures from Arctic seawater | Marine bacteria (*Beta-* and *Gammaproteobacteria*, *Bacteroidetes*) and subpopulations of *Gammaproteobacteria* (*Alteromonas-Colwellia*, *Oleispira*, Arctic96B-16) | *Beta-* and *Gammaproteobacteria* incorporate moderate amounts of bicarbonate in the dark. The different gammaproteobacterial groups differ in this capacity. | [1] |
| Delaware Bay Estuary and South China Sea | Main clades of estuarine bacteria (*Alpha-*, *Beta-*, and *Gammaproteobacteria*, *Bacteroidetes*) | The contribution of the main clades to biomass production varies along salinity gradients. Bacterial abundance and activity are only partially correlated. | [14,61] |
| Gossenköllesee and Schwarzsee ob Sölden (Austrian alpine lakes) | Main clades of freshwater bacteria (*Alpha*-and *Betaproteobacteria*, *Actinobacteria*, *Bacteroidetes*) and the betaproteobacterial subcluster *Limnohabitans* | Main clades of freshwater bacteria incorporate leucine rather than thymidine, *Actinobacteria* being a remarkable exception. | [41] |
| North Atlantic, Gulf of Mexico, Mediterranean Sea | Main clades of marine bacteria (*Alpha*-and *Gammaproteobacteria*, *Bacteroidetes*) | Despite DMSP uptake appearing as a widespread ability, *Alphaproteobacteria*, especially *Roseobacter*, are the main organisms involved. | [30,56] |
| Mediterranean Sea | Main clades of marine bacteria (*Alpha-*, *Beta-*, and *Gammaproteobacteria*, *Bacteroidetes*) | The proportion of the bacterial community assimilating DMSP varies seasonally, it sharply increases in summer. | [54] |
| Gulf of Mexico, Mediterranean Sea, Gran Canaria Island and Sargasso Sea | *Prochlorococcus*, *Synechococcus* and diatoms | The main components of marine phytoplankton (unicellular cyanobacteria and diatoms) incorporate DMSP sulphur, a mechanism diverting its emission to the atmosphere. | [55] |
| Mediterranean Sea | Main clades of marine bacteria (*Alpha-*, *Beta-*, and *Gammaproteobacteria*, *Bacteroidetes*) and subpopulations of *Alphaproteobacteria* (SAR11, *Roseobacter*) | The main clades of marine bacteria have different sensitivity to natural levels of incident solar radiation. This difference is also marked between members of the *Alphaproteobacteria* class. | [3] |
| North Pine Dam (Australia) Římov Reservoir (Czech Republic) | *Actinobacteria* | Despite exhibiting a low content of nucleic acids, *Actinobacteria* constitute a very highly active fraction of freshwater microbial communities. | [19,36] |
| Římov Reservoir (Czech Republic) | Main clades of freshwater bacteria (*Beta-* and *Gammaproteobacteria*, *Bacteroidetes*), and the betaproteobacterial subcluster *Limnohabitans* | The *Limnohabitans* bacteria are very active members of the freshwater bacterial community. Furthermore, their activity is stimulated in treatments with enhanced bacterivory and phosphorous addition. | [18] |
| Castillos Lagoon (Uruguay) | *Betaproteobacteria* and its subpopulations *Polynucleobacter* B, *Polynucleobacter* C and *Limnohabitans* | Sharp environmental transitions are reflected in abrupt changes of bacterial physiology. The main freshwater betaproteobacterial clades differ in their response. | [8] |
| Grosse Fuchskuhle Lake (Germany) | *Betaproteobacterial clades* Polynucleobacter C and *Limnohabitans* and members of the actinobacterial clade ACI | Niche differentiation between Polynucleobacter C subcluster and *Limnohabitans* clade assessed through differential incorporation of low molecular weight substrates. | [13] |
| Coastal and mid-North Atlantic, Sargasso Sea, Coastal North Sea | SAR11 | Members of the SAR11 clade are highly active in the uptake of LMW substrates, particularly at low concentrations. | [7,28,29] |
| Lake Zürich (Switzerland) | LD12 | LD12 bacteria are able to utilize several monomeric substrates, exhibiting a marked preference for glutamine and glutamate. | [46] |

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At the same time, they point to a high metabolic diversification among microbial communities, thus raising the question of how many categories/functional groups should be considered for biogeochemical modelling. This is a matter subject to intense debate among microbial ecologists, since, for example, the question "*What level of phylogenetic diversity is an appropriate target if we are to better understand the functional role of microbes in marine biogeochemical cycles?*" was one of the main focuses of the workshop entitled "*The microbial view of marine biogeochemical cycles*" held in Banyuls in 2010 [21].

Other key ecophysiological features of the marine bacterioplankton, shown through the application of MAR-FISH, include the corroboration that there is a widespread ability for anaerobic metabolism among the common bacterioplankton groups [6]. This was revealed through the incorporation of radiolabelled glucose in anoxic conditions by members of *Alpha-* and *Gammaproteobacteria* and *Flavobacteria* in a shallow coastal system, and pointed to a high metabolic flexibility of the bacterioplankton assemblages, suggesting also a potential for habitat exchange between the water column, organic particles and sediment surfaces. Another kind of special metabolic property discovered by using MAR-FISH was the preferential prokaryotic incorporation of the D-enantiomers of amino acids with increasing water depth in marine systems, suggesting that mesopelagic bacterioplankton members (particularly the *Crenarchaea*) may rely on the consumption of bacterial DOM [51]. More recently, it was demonstrated that certain members of the bacterioplankton (mainly affiliated to *Beta-* and *Gammaproteobacteria*) were able to incorporate moderate amounts of bicarbonate in the dark, concluding that this metabolic feature could be relevant for their survival under resource-depleted conditions, thus supporting bacterial metabolism in particular environments [1].

MAR-FISH has also been applied to infer the contribution of the main bacterioplankton groups to the bacterial biomass production in aquatic systems [14,61]. From these studies, it was shown that, to a certain extent, the assimilation of biomass production tracers (leucine/thymidine) by bacterial groups is explained by their abundance, suggesting that abundance and activity are partially correlated, but also that other features could certainly play a role in defining the contribution of different bacterial groups to total production. Moreover, MAR-FISH has been applied to evaluate differences in the microbial incorporation of thymidine and leucine at a single cell level, which showed that, at least for freshwater communities, the incorporation of leucine was not only far more widespread than the incorporation of thymidine, but also that the incorporation pattern was related to the bacterial community composition [41].

A particularly rich example of the application of MAR-FISH, for identifying those members within a complex microbial community that are responsible for a given biogeochemical process, has been the characterization of the planktonic processing of dimethylsulphoniopropionate (DMSP), an algal osmolyte involved in climate change [29,30,54–56]. From these studies, it has been clearly established that certain members of the heterotrophic bacterioplankton (particularly SAR11, *Roseobacter* and some *Gammaproteobacteria*), as well as the autotrophic plankton (*Prochlorococcus*, *Synechococcus*, and even diatoms) were the main groups involved in the utilization of DMSP, and that their relative contribution to this process depended on environmental factors, such as the trophic state and seasonality.

In addition, this technique has been used as a tool to infer the life style and niche differentiation of bacterioplankton clades at fine phylogenetic resolution (*i.e.* genus, species). Remarkably, this kind of work has been carried out mainly on freshwater systems, probably in part due to the existence of a suite of probes targeting small coherent populations, particularly among *Betaproteobacteria*. Examples of such studies include analysing the effects of bottom up *versus* top down control on the abundance and activity of members of the *Limnohabitans* (formerly R-BT) cluster [18], the demonstration that members of the main betaproteobacterial clades (*Polynucleobacter* B and C, and *Limnohabitans*) differed in the metabolic adjustments exhibited across sharp environmental transitions [8], and also in the pattern of incorporation of different carbon sources [13]. Recently, MAR-FISH has been used to assess the incorporation of 14 different radiolabelled molecules, which has provided detailed fingerprinting of substrate preferences for the alphaproteobacterial cluster LD12 [46]. The results obtained prompted the authors to conclude that members of this freshwater cluster would have a lifestyle similar to SAR11, their sister marine clade, which, in turn, has been shown to specialize on low molecular weight substrates [7,28,29].

Despite the proven power of the MAR-FISH technique, its application has remained restricted to a relatively small number of laboratories, presumably due to three main factors: (i) the concerns of working with radiolabelled compounds, (ii) the tedious microscopic evaluation which it implies, and (iii) the prejudice that it is a very difficult technique. Regarding the first issue, clearly, safety measurements should be employed when handling radioactive materials, as applies to any dangerous chemicals (*e.g.* 4′,6-diamidino-2-phenylindole; DAPI). However, the amount of radioactivity handled in common applications is very low, usually far below levels regarded as risky to human health [52,58]. As an example, by drinking a whole flask of the standard size (1 mCi) of a tritiated compound, one would be exposed to less radioactivity than in a full-body computed tomography scan. The second issue of time-consuming evaluation was indeed more critical for a long time. However, with the advent of automated microscopy routines [60], the evaluation of MAR-FISH preparations has become a smooth, high-throughput process. The present work, therefore, addresses the third issue and is aimed at the MAR-FISH methodology, in order to attempt to demystify the process of sample preparation. It will be shown that the method is no more difficult than any standard technique with a common application in microbial ecology. Firstly, some basic concepts of the autoradiographic process and its combination with fluorescence *in situ* hybridization are provided, then, a complete procedure for analysis of bacterioplankton samples is introduced and analysed step by step, considering potential pitfalls.

## The autoradiographic process

The nuclear emulsions used for microautoradiography are suspensions of silver bromide crystals in gelatine [25]. When these crystals are hit by electrons from the radioactive source, a so called "latent image" is formed due to the presence of a metallic silver nucleus inside the exposed crystals. Through photographic development, this latent image is converted into a true image and, in the presence of the developing agent, the metallic silver nucleus catalyses the conversion of the entire crystal into metallic silver. The crystals in which the silver cations are not reduced to metallic silver are subsequently dissolved out by the fixative, leaving a pattern of silver grains on the emulsion that follows the location of the radioactivity sources [44].

## Combination of autoradiography with fluorescence *in situ* hybridization

The rationale and protocols for fluorescence *in situ* hybridization (FISH), and its variant fluorescence *in situ* hybridization with catalysed reporter deposition (CARD-FISH) have been presented in depth in a series of methodological reviews [9,42,43].

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**Box 1: Summary of the protocol for MAR-FISH performance after incubation with radiolabelled substrates**

1. Stop incubation by adding paraformaldehyde (PFA) at a 1% final concentration. Fix for 1 h at room temperature.
2. Filter subsamples onto polycarbonate filters to obtain an optimized cell density (diameter: 25 mm, pore size 0.2 μm).
3. Rinse the filters twice with 5 mL 1× phosphate buffered saline (PBS).
4. Cut the filters into eight (or more) triangular pieces.
5. (Perform the *in situ* hybridization by FISH or CARD-FISH.)
6. Immobilize the filter pieces onto the slides using molten 2% agarose as an adhesive.
7. In the dark room, melt photographic emulsion for 30 min at 43 °C in a water bath.
8. Add an equal volume of agarose solution (0.2%, w/v) to the emulsion to obtain a 1:1dilution, and mix gently.
9. Coat the glass slides containing the filters with the diluted emulsion and put them on a cold metal bar, allow them to dry for a few minutes (*ca.* 5 min).
10. Place the slides in a lightproof box wrapped in aluminum foil and expose them at 4 °C (exposure times need to be optimized for each experiment).
11. Develop the slides by using Kodak specifications (2 min in developer, 10 s in deionized particle-free water [$dH_2O$], 5 min in fixer, and final washing for 5 min in $dH_2O$).
12. Allow to air dry, preferably overnight in a desiccator.
13. (Perform the *in situ* hybridization by FISH or CARD-FISH.)
14. Stain with DAPI. For difficult samples use the following settings: stain with a final concentration of 1 μg $mL^{-1}$, at 4 °C for 10 min, rinse for 1 min with deionized water and then for 30 s with ethanol (80%).
15. Mount in a mixture of Citifluor/Vectashield.

Basically, the FISH protocol consists of a hybridization step where the whole cells are in contact with a buffer containing the probe, and a subsequent washing step which removes the unbound probe. In the CARD-FISH protocol, due to the use of horseradish peroxidase labelled probes, further steps linked to cell permeabilization and signal amplification are needed. Thus, compared to the FISH protocol, the CARD-FISH technique is longer and includes several incubation and washing steps that represent further "aggression" to the sample. This needs to be taken into account when it is combined with autoradiography (details provided below).

The standard MAR-FISH procedure is depicted in Fig. 1, and the detailed protocol is presented in Box 1 . The FISH or CARD-FISH procedure can be applied either before or after the autoradiogram preparations. In the first case, no extra care is needed, and the samples are hybridized according to Amann and Fuchs [9]. This is the most common and simple MAR-FISH procedure. However, on certain occasions it might be preferable to produce the autoradiograms first, and only then perform the hybridizations. This is typically the case when working with short-lived isotopes (such as $^{35}$Sulphur), or when one needs to return to a sample already prepared, in order to perform a second hybridization with an additional probe (Fig. 2). This is a very important feature of the technique because it is not destructive and all radioactive signals are collected at once, it is possible to go back to an autoradiogram to explore further issues concerning the identity of the microorganisms involved in any substrate uptake.

## Tips and tricks

### *Choice of isotopes*

The isotopes suitable for microautoradiography (MAR) are mainly β emitters, their disintegration is due to the loss of nuclear electrons. The β particles emitted by a given isotope have a range of initial energy and the shape of the initial energy spectrum, as well as its maximum energy value, varies between isotopes. For example, the energy spectrum of tritium is skewed towards the lowest energy values, with most particles containing around 2 keV and a maximum of 18 keV, whereas the spectrum of $^{32}$P has a maximum of approximately 600 keV, although its particles can reach up to 1600 keV [44].

The initial energy of each particle determines how far it can travel, and thus impact on the nuclear emulsion where it generates a pattern of grains. Thus, incubations with tritiated substrates are preferable because of their low radiation energy, which causes silver grain deposition sufficiently close to the cells incorporating the substrate [11,48]. Although $^{14}$C and $^{35}$S can also be suitable, in contrast, autoradiograms with $^{32}$P may be difficult to interpret since the high-energy electrons can expose the emulsion a long way from the radioactive source [11,44]. Other types of isotopes suitable for MAR are those which lose energy as low energy γ photons, examples of such isotopes include $^{125}$I, $^{55}$Fe, and $^{51}$Cr [44].

Enough radioactivity must be incorporated by the cells in order for the emulsion to be exposed, preferably after exposure times of a few hours or days [11]. It is better to use substrates with higher activities than to perform longer incubations [11]. Too long incubation times may result in undesired transformations of the substrate (*e.g.* metabolization and additional incorporation of a derivative), and changes in the original microbial community in terms of abundance or composition.

Specific radioactivity should not be less than 1 Ci/mmol [48], it frequently ranges between 5 and 60 Ci/mmol [11]. The tracers should be added as the highest specific radioactivity available, in order to avoid increasing the organic concentration, thus altering the system [11]. A typical concentration used for incubation with monomers (amino acids, sugars) in aquatic systems is 10 nM [8,39,46,50]. However, the optimum radiotracer concentration depends on the purpose of the study, and on the combination of the specific substrate and environment (*e.g.* oligotrophic/eutrophic). Concentrations mimicking the environmental concentration range of the substrates (*e.g.* 0.1–10 nM for amino acids in the sea [17]) are advisable for analysing their *in situ* metabolization by the microbial community, although higher concentrations should be used when aiming for potential activities (*e.g.* the degradation of xenobiotics [59]). Moreover, it has been observed that even common tracers (*e.g.* sugars, amino acids) are unequally incorporated by different prokaryotic groups based on their concentration [5,7]. Thus, to determine the optimum tracer concentration, according to the purpose of the study, it is advisable to evaluate a series of conditions first. A useful and less time consuming approach is to infer the concentration from bulk measurements of total incorporation.

### *Fixation*

Aldehydes used for fixation may react with autoradiographic emulsions by producing background grains and desensitizing the emulsion. Therefore, the common fixative used in microbial ecology, paraformaldehyde, needs to be removed before the sample comes into contact with the emulsion [11]. This is achieved by carefully washing the sample with phosphate buffered saline (PBS) after filtering.

It is assumed that assimilation into macromolecules, rather than total substrate uptake, is monitored by MAR because cells

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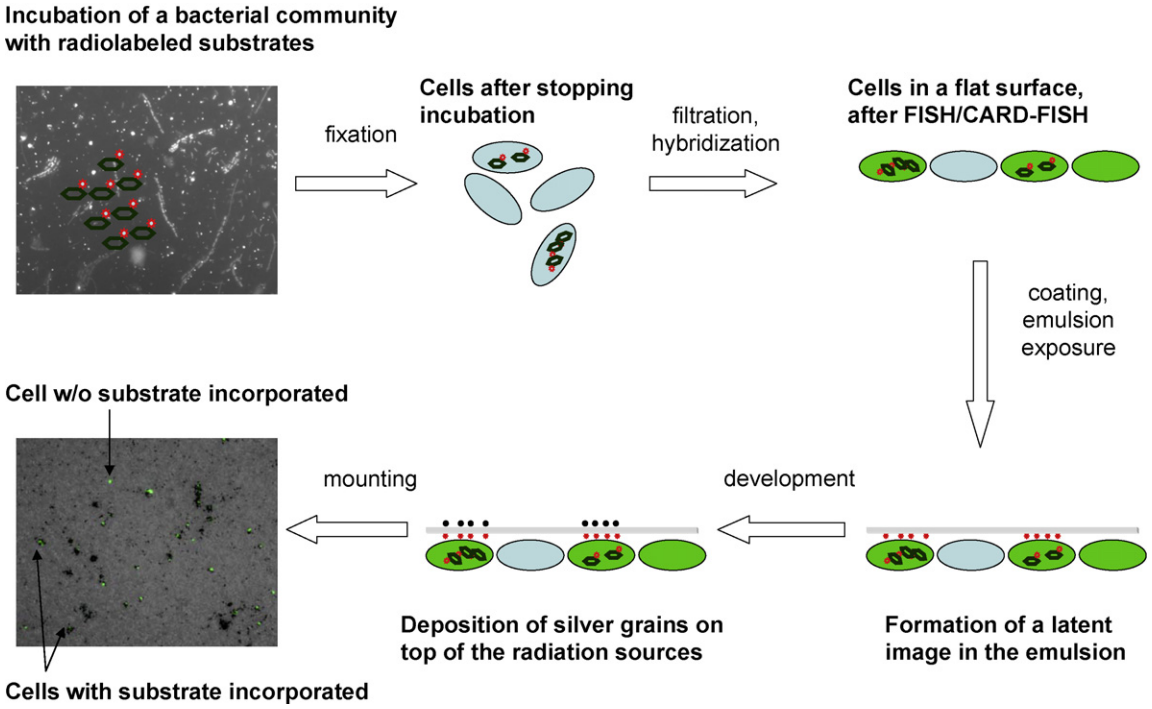


**Fig. 1.** Schematic representation of the standard procedure for obtaining MAR-FISH preparations from bacterioplankton.

fixed with paraformaldehyde lose unincorporated radioactive compounds [24].

*Filtration*

As both FISH and MAR need to be carried on a solid surface, the cells have to be retained on filters (or on glass slides for concentrated samples, such as sediments). The suitable filters for this purpose are polycarbonate track-etched (Nucleopore) filters, as they represent a flat surface of homogeneous pore size, which does not produce background for either of the two techniques. Other filters which also fulfil these requirements are the Anodisc filters. However, their use is not recommended due to their brittle nature, which makes them difficult to handle.

The amount of sample to filter is the one required to obtain a density of cells which allows between 100 and 300 bacterial cells to be identified in a field of view of approximately 100 $\mu m \times 100\ \mu m$. A lower density results in a more time consuming evaluation, especially if the target organisms are rare, whereas a higher density may cause difficulties in assigning the MAR signals to individual cells. This may be particularly relevant for large multicellular bacterial aggregates, normally for a couple of cells standing closely together it is still quite straight forward (Fig. 3).

*Preparation of slides for autoradiograms*

For performing the hybridization step, the filters are cut into triangular pieces (sectors of the circular filter) normally, eight correct-sized pieces can be obtained from a 25 mm filter. The filter pieces need to be attached to a slide to provide a support for coating with emulsion. They can be glued with a variety of means, but the best and most inert form is to previously coat the clean empty slides with a very thin layer of molten high strength agarose (2%, w/v). This has to be carried out immediately before attaching the filters, while the agarose is still fluid, in order to avoid the filters detaching afterwards during development.

As many as six filter pieces can be easily attached on a slide, it is advisable to put the replicates of the same treatment on the same

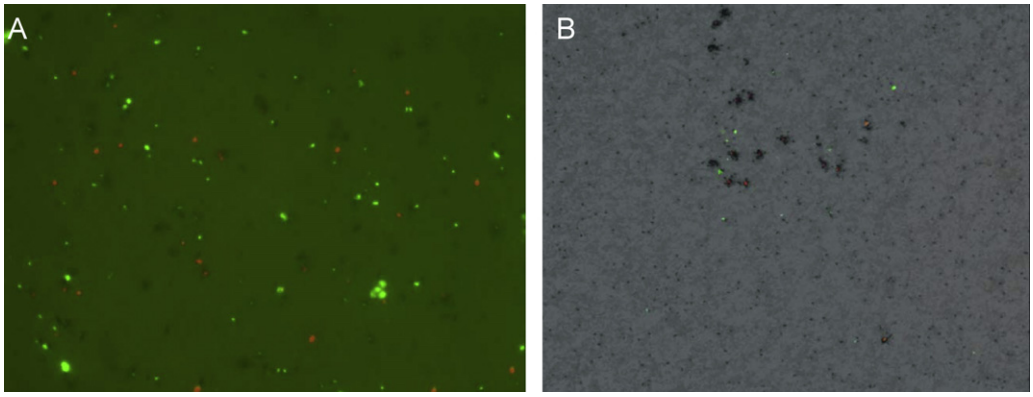


**Fig. 2.** Micrographs of MAR-FISH preparations showing double hybridizations performed on the same sample. Panel A: Field of view from epifluorescence. The green cells are *Alphaproteobacteria* and the red cells are *Polaribacter* spp. Panel B: Field of view combining epifluorescence and transmitted light. The green cells are SAR11 and the red cells are *Roseobacter* spp. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

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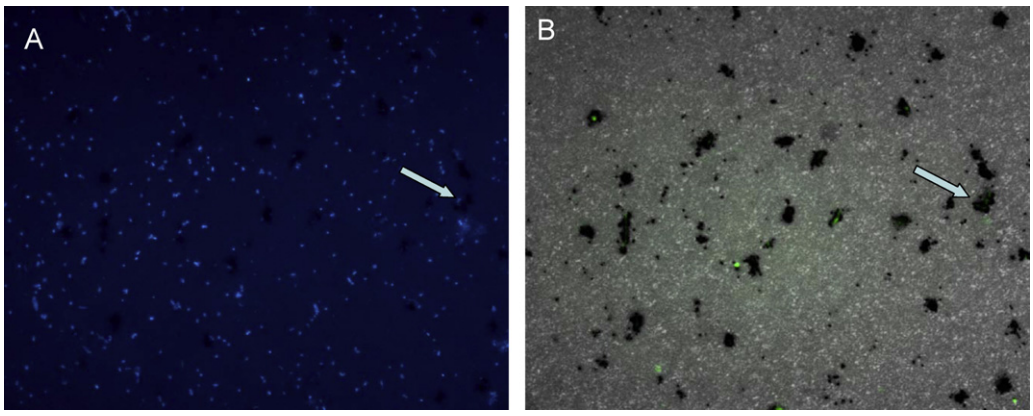


**Fig. 3.** Micrographs from the same field of view of an autoradiogram. Panel A: All cells stained with DAPI. Panel B: Hybridized cells. The arrows point to an aggregate and a very close single cell, showing that the MAR signal derived from the MAR positive cells in the aggregate is easily assigned to the corresponding cells, and does not interfere with the MAR negative cell.

slide, in order to ensure that the autoradiogram process is the same for all of them. Also, as the emulsion coating is best done in complete darkness, it is useful to cut one edge of the slide using a glass cutter, so its orientation can be identified by feeling its contour.

*The dark room*

The emulsion (*e.g.* Eastman Kodak NTB-2 liquid nuclear track) must be handled only in the dark room, preferably in complete darkness, or at most, with the room illuminated by a safelight filter appropriate for the emulsion type (*e.g.* Wratten #2 for the Eastman Kodak emulsions) [11,26]. The darkroom must be absolutely light tight and the temperature should be kept preferably under 23 °C, as higher temperatures may interfere with the exposing and developing procedures [11,44]. Care is also needed to block all light that may leak from luminous surfaces, such as indicator bulbs. It is important to keep the dark room clean, especially from emulsion spills, which can be removed easily with a solution of 1% potassium hydroxide [44].

*Emulsion handling*

The emulsion should be kept refrigerated and protected from any form of radiation, and it should not be frozen at any stage [25]. Before use, it has to be molten at 43 °C, also in the dark room.

The molten emulsion forms a too thick layer when applied to a surface; for the visualization of microbial cells it is better to dilute it to half strength with an inert solution [11]. The best diluent is high strength agarose 0.2% (w/v) [6], which, in turn, will be critical if hybridization needs to be performed after the autoradiogram is developed. It is highly recommended to aliquot the fresh emulsion in order to avoid re-melting that can lead to spoiling. Normally, the emulsion is aliquoted in 10 mL portions inside plastic slide containers, and this quantity is enough to coat approximately 20 slides in a row.

While coating the slides, the emulsion must not be shaken but rather mixed through gentle inversion, since shaking introduces air bubbles that distort the autoradiograms. It is useful to have a standard protocol for performing coating, in order to ensure a uniform thickness of the emulsion layers in all preparations, since this is a factor that determines the quantity of grains produced [44]. A working protocol is to dip a slide in the molten emulsion for 5 s, gently rolling the container, and then letting it drip for 3 s. Then, the back of the slide is cleaned with a paper tissue, before the slide, with the cells facing up, is carefully placed on top of a cold metal surface for a few minutes to allow it to cool down, harden, and dry.

As the nuclear emulsion dries, it becomes more sensitive to exposure by light, radiation and physical damage. Therefore, touching the slides on the top should be avoided, in order to prevent the generation of fingerprints that will obscure the signals resulting from the radioactive decay of the sample. In this step, it is strongly recommended not to use any light at all. However, if it is absolutely necessary, one should not work closer than 1 m from the safelight [11,44].

*Exposure conditions*

The formation of the latent image is a reversible process, since the silver nucleus may ionize back to silver bromide, causing the fading of the image before chemical development. Latent image fading is enhanced by high temperatures, oxidizing agents, and water [44]. Thus, in order to prevent fading of the image, it is important to reduce as much as possible the humidity of the environment where the slides will be exposed [31,48]. Exposure at low temperatures (typically 4 °C) is preferable because it also reduces the rate of chemical processes that may increase the background, although it decreases the sensitivity of the emulsion as well [11].

If the exposure time is too long, there may be a high density of silver grains obscuring the underlying cell, rendering it unidentifiable. Also, since the latent image may fade, prolonging the exposure time will not necessarily intensify a weak image when the sample contains insufficient radioactivity. It is preferable to use short exposure times (a maximum of up to 1 week) [11], although for certain compounds, incorporated in very small amounts, exposure times are typically in the order of 20 days [54].

For each experiment, it is necessary to test a series of exposure times, in order to find the correct one (*i.e.* the time at which the maximum number of MAR positive cells is achieved). If bulk measurements of isotope incorporation are concomitantly carried out, the values obtained will be indicative of the optimal exposure time to achieve good MAR-FISH signals. An example of this empirical rule is presented in Table 2 for different incubations of

**Table 2**
Empirical relationship between bulk tracer molecule incorporation and optimal exposure time for MAR-FISH. The recommended times are usually a good guide, but may vary according to the substrate.

| Bulk incorporation (nanomoles) | Recommended exposure time |
|---|---|
| $<1 \times 10^{-05}$ | At least 6 days |
| $3 \times 10^{-05}$ to $9 \times 10^{-05}$ | 72 h |
| $1 \times 10^{-04}$ to $3 \times 10^{-04}$ | 48 h |
| $3 \times 10^{-04}$ to $7 \times 10^{-04}$ | 24 h |
| $>4 \times 10^{-03}$ | 12–18 h |

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coastal bacterioplankton with a commonly used amino acid (tritiated leucine, $n = 23$) and sugar (tritiated glucose, $n = 8$), for a range of radiotracer concentrations between 0.1 and 100 nM, with specific activities between 1.29 and 5.14 TBq $mmol^{-1}$.

*Chemical developing of the autoradiograms*

During developing, an oxido-reduction reaction occurs between the reducing agent (*e.g.* hydroquinone) and the silver bromide crystals, which is catalysed by the metallic silver nuclei produced during exposure. During this reaction, the cationic silver in the exposed crystals is reduced to metallic silver.

Developing works as an amplification process. It increases the size of the deposit of metallic silver in a crystal until it attains a threshold at which it can be recognized, at the specific conditions employed for viewing [44]. Increasing the contact time with the chemical developer adds more silver to the silver deposit. The process ends when all the silver available in the crystal has been converted to metallic silver. At this point, the developed grain occupies a volume of up to three times that of the original crystal [44].

Since the emulsion is extremely light sensitive when it is in contact with the developer, developing must be carried out in total darkness [11,25]. Some of the factors affecting photographic development are: (i) the nature of the developing agent, because some developers are more powerful reducing agents than others, (ii) the developing time, as ultimately every crystal in the emulsion could be developed, and (iii) indirectly the temperature, because it controls the rate of the process. Therefore, it is critically important to work under 23 °C. Developing conditions might need to be optimized for all these factors in order to achieve the best signal-to-noise ratio.

After chemical development, fixation removes the undeveloped silver bromide crystals that remain in the emulsion, this is usually undertaken in a solution of thiosulphate [25]. The thiosulphate does not affect the developed grains but forms soluble complexes with ionic silver, dissolving away the silver bromide. The end point of fixation is considered to be twice the time required for the emulsion to become transparent [44].

As nuclear emulsions contain a higher percentage of silver bromide than photographic emulsions, and the fixation rate decreases rapidly as the products of fixation accumulate in the solution, it is important not to exceed the capacity of the fixer solution by over use [44]. Usually, the amount of developer and fixer needed for each experiment should be prepared and kept for a maximum of 1 month. From this stock, 200 mL of developer and 200 mL of fixer are used to process a maximum of 20 slides in a single day, and this aliquot is discarded after use. The Milli-Q water should be changed after each round of developing (approximately every 7–8 slides).

*Background signals*

In every autoradiogram, silver grains appear that are not derived from the radiation of the experimental source, originating background signals. One of the causes of this phenomenon is over-development. Even in the absence of metallic silver, the probability of development is finite, and some crystals that were not exposed will nevertheless become developed grains. Therefore, the development process is merely adjusted to produce the optimal ratio between grains at the source of radioactivity and non-specifically formed background grains. Other causes for background include: (i) exposure to light (safelight filters only ensure that the wavelength of the light falling on the emulsion is the least harmful), (ii) background due to pressure by accidentally touching the emulsion, or due to too fast or prolonged drying (because the shrinking of gelatine in the emulsion exerts pressure on the silver bromide crystals), (iii) chemography, such as certain reactive groups, mainly from reducing agents, can produce a latent image, (iv) contamination of the emulsion due to improper cleaning, (v) environmental radiation, and (vi) spontaneous background formation due to the high sensitivity of nuclear emulsions, which is a factor that becomes more critical with emulsion ageing [44].

To overcome the problem of false positive signals due to non-specific background, it is mandatory to always include a negative control (*i.e.* an incubation where the tracer was added after the cells were killed with paraformaldehyde). This incubation is evaluated by the same procedure, and the number of positive MAR cells obtained is then subtracted from the numbers obtained in the other incubations. Usually the percentage of MAR positive cells in the killed controls is approximately 1%.

*Factors affecting the efficiency of autoradiograms*

A practical definition of the efficiency of an autoradiogram refers to how close the relationship is between the number of β particles which impacted the emulsion and the number of grains produced as a consequence [44]. A variety of factors concerning the sample, the emulsion, and the conditions of exposure and development affect this relationship. Some of the factors include the specific isotope (due to the differences in the initial energy of the β particles), the distance between the sample and emulsion, the thickness of the sample, the backscattering of β particles due to attachment of the sample to a support (*e.g.* on a glass slide), the thickness of the emulsion, the sensitivity of the emulsion, the fading of the latent image, the temperature and duration of exposure, and the specific developer and development conditions used [44].

*Performing CARD-FISH after the autoradiography*

As previously mentioned, the CARD-FISH protocol implies a series of incubations and washings which may destroy the autoradiogram by disrupting the thin layer of nuclear emulsion. To avoid this, it is necessary to first of all use the nuclear emulsion diluted 1:1 with high strength (>1200 g $cm^2$ at 1%) agarose 0.2% (w/v) (*e.g.* SeaKem® LE agarose). Other agaroses and concentrations are not suitable because they do not maintain the correct balance between melting at 43 °C, and remaining hard throughout the CARD-FISH procedure (see Alonso [4] for a detailed comparison of the performance of different agarose types and brands). Then, the hybridizations are performed inside humid chambers (*i.e.* Falcon tubes) embedded with a simple version of the hybridization buffer, as used in monolabelled FISH [43]. As the function of the buffer is merely to maintain the correct formamide concentration in the atmosphere of the chamber, and it does not enter in contact with the sample, there is no need to include all the components of the more complex (and expensive) buffer formulation used for CARD-FISH. The washings are carried out by carefully placing the slides into boxes containing the buffers, without shaking. The same types of trays used for developing are a good option to use for the washings in this case.

*Counterstaining and evaluation*

After the preparations are completely dry, they need to be stained with DAPI. Normally, simply applying a mixture of Citifluor/Vectashield where DAPI has been included is sufficient. However, sometimes, it is difficult to obtain a good quality DAPI signal. In these cases, it is useful to perform a separate DAPI staining at 4 °C, and extend the staining time.

The hybridized cells and the silver grains are visualized by combining transmitted and fluorescence light in any normal epi-fluorescence microscope.

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**Table 3**
Example of coefficients of variation (CV) of triplicate MAR-FISH evaluations, according to the number of hybridized cells counted, and the percentage of MAR positive cells within each hybridized population.

| Set of triplicates | Average FISH (*n*) | Average MAR+ (%) | CV |
|---|---|---|---|
| 1 | 14 | 14 | 0.31 |
| 2 | 14 | 20 | 0.51 |
| 3 | 14 | 30 | 0.26 |
| 4 | 33 | 14 | 0.20 |
| 5 | 41 | 11 | 0.18 |
| 6 | 27 | 20 | 0.38 |
| 7 | 18 | 33 | 0.64 |
| 8 | 62 | 13 | 0.25 |
| 9 | 61 | 13 | 0.31 |
| 10 | 32 | 26 | 0.43 |
| 11 | 281 | 3 | 0.25 |
| 12 | 12 | 86 | 0.07 |
| 13 | 54 | 31 | 0.16 |
| 14 | 24 | 94 | 0.06 |
| 15 | 330 | 8 | 0.33 |
| 16 | 209 | 21 | 0.11 |
| 17 | 335 | 16 | 0.21 |
| 18 | 528 | 11 | 0.08 |
| 19 | 497 | 12 | 0.12 |
| 20 | 519 | 12 | 0.05 |
| 21 | 222 | 32 | 0.07 |
| 22 | 104 | 84 | 0.05 |
| 23 | 157 | 59 | 0.15 |
| 24 | 864 | 11 | 0.12 |
| 25 | 162 | 71 | 0.03 |
| 26 | 714 | 19 | 0.12 |
| 27 | 367 | 37 | 0.16 |
| 28 | 1204 | 12 | 0.41 |
| 29 | 538 | 27 | 0.10 |
| 30 | 582 | 30 | 0.11 |
| 31 | 653 | 29 | 0.12 |
| 32 | 1554 | 17 | 0.11 |
| 33 | 1319 | 30 | 0.11 |

*How many cells to count?*

The evaluation of MAR-FISH experiments is preferably carried out in triplicates. To attain good reproducibility between replicates, it is important to achieve a certain threshold of counted cells. With the aim of defining this threshold, samples have been evaluated with a hierarchical set of probes, targeted to bacterial taxa that formed between <1% and 68% of all DAPI counts [6]. These bacterial groups exhibited the whole range of possible MAR fractions of MAR positive cells (from 1% to 100%). The coefficients of variation (CV) between replicates were calculated for each set of triplicates (Table 3). When the CVs were plotted as a function of the number of hybridized cells and the percentages of MAR positive cells, the higher CVs (>0.3) were found at combinations of low numbers of hybridized cells (<60) and low proportions of MAR positive cells (<33%). Altogether, the number of examined cells was the most decisive factor for precision. Based on these results, it was concluded that in order to obtain CVs below 0.2 for triplicate evaluations, at least 50 MAR-positive hybridized cells of each target bacterial group should be counted for each replicate [6].

The evaluation of MAR-FISH preparations can be carried out manually. However, due to the obligatory switch between epifluorescence and transmitted light for each field, it is a time consuming process, which is certainly not recommended when many samples need to be evaluated. Soon after the appearance of MAR-FISH in the field of aquatic ecology, researchers started using custom-developed semi-automated approaches for image acquisition and evaluation [6,14]. More recently, a fully automated routine for image acquisition, along with a highly flexible and powerful programme for image analysis have been developed by Zeder and co-workers [60]. The employment of these combined routines for acquisition and image analysis permits a fast, accurate, and high throughput evaluation of samples, thus greatly expanding the scale at which single studies can be performed, allowing for example the simultaneous evaluation of multiple substrates [8,46].

## Protocol variants

The MAR-FISH protocol proposed here is derived from the combination of CARD-FISH with the autoradiographic protocol introduced by Meyer-Reil in 1978 [32]. A variant of MAR-FISH, based on the autoradiographic protocol of Tabor and Neihof [49], which is also commonly used for bacterioplankton analysis, was the procedure initially proposed for marine bacteria by Ouverney and Fuhrman [39].

However, this latter MAR-FISH protocol possesses several drawbacks. It is based on FISH rather than CARD-FISH, and the application of FISH often yields lower detection rates in environmental samples (see Bouvier and del Giorgio [10] for a review). This limitation has been overcome by the substitution of FISH by CARD-FISH [50]. However, this protocol still implies the critical step of transferring the cells from the filter to a thin layer of solidified emulsion, which inevitably causes cell loss. Cottrell and Kirchman [15] observed that after transferring the cells to the emulsion, approximately 50% of all cells still remained in the filters. By experience, this percentage seems to be highly variable and depends on several handling factors. Moreover, the risk of background generation is increased due to the obligatory use of light at distances shorter than the manufacturer's indications, and common accidental pressure while placing the filters on top of the emulsion. In addition, a high fraction of preparations (up to 30%) can be lost due to the detachment of filters during transfer and developing. Also, difficulties are encountered during evaluation, as very heterogeneous numbers of cells per microscopic field are found in a single preparation. As a consequence, this protocol is rather cumbersome and time-consuming, thus providing limited capacity for an accurate evaluation of numerous samples. Nevertheless, it does have some advantages over the protocol presented here, since the cells lie on top of the emulsion, the visualization of hybridized cells is less dependent on hybridization quality and on the number of grains in the vicinity of cells, and, in addition, the quality of DAPI staining is often better.

When working with an abundant and phylogenetically narrow population, the drawbacks of the protocol which relies on the transfer of bacterial cells may not be too severe. However, when whole communities have to be analysed, experience shows that it is preferable (and completely under the control of the operator) to adjust the hybridization and exposure conditions in order to ensure good quality signals, rather than cope with the hazardous consequences of transferring cells from hybridized filters. A comparison of the two protocols with their advantages, disadvantages and possible suggestions for improvement was presented by Alonso [4].

Protocol variants are also found for the processing of other types of samples, such as sediments [27], soils [45], biofilms [38] and waste water treatment sludge [37].

## Autoradiographic signal quantification

After obtaining the information concerning which population is incorporating a given compound, the next immediate question is if this process can be quantified in order to evaluate relative or absolute substrate uptake rates within mixed communities. Attempts have been made to achieve such quantification for MAR-FISH preparations, and these have been based mainly on the area of the silver grains surrounding the target cells. The simplest approach for assessing the relative contribution of

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different groups to the community incorporation of tracer consists of comparing the area of silver grains associated with the target groups and, based on this, inferring which particular group may be mainly responsible for the uptake. Examples of this approach are given in the work of Cottrell and collaborators [14,16,28–30]. This analysis may provide similar results as calculating the contribution of each group based on the number of MAR positive cells [14], but it can also yield very different results [14,30], especially when long exposure times are used [14]. This is possibly due to the fact that the silver grain area actually depends on a variety of factors, as detailed above in the sections devoted to the autoradiographic process. To allow for comparisons between groups, an advisable approach would be to use double hybridizations in a single autoradiogram [7], in order to guarantee that the same conditions are applied to the analysis of each target population. Moreover, care is needed to ensure that the autoradiogram efficiency is constant during the evaluation [44]. It is noteworthy that this method is only suitable for comparing preparations from the same batch of samples, and it cannot be used to compare samples originating from different sampling events (*e.g.* to compare environments, seasons, *etc.*).

An approach aimed at circumventing this problem was presented by Sintes and Herndl [47], who showed that the average halo around the MAR positive cells co-varied linearly with the bulk uptake measurements along the incubation time. They proposed that, once the adequate incubation time to ensure a linear response is established for a given set of samples, the actual uptake rates could be calculated for the individual cells based on the area of the silver grain halo and the bulk uptake value. It is important to bear in mind that high differences in uptake rates can be found between sites, seasons, experiments, *etc.*, therefore, the adequate incubation time needs to be established for each new sample [47]. Furthermore, all the other steps in the procedure (*e.g.* fixation, exposure time, emulsion, *etc.*) should be kept constant to ensure comparability between different samples, since, as detailed earlier, changes in any of these factors may result in differences in the grain area obtained for a given bulk uptake value.

The most adequate method for calculating absolute amounts of incorporated radioactive tracer requires the utilization of appropriate radioactive standards, such as cells with similar shape and size to those present in the sample, labelled with a known amount of radioactivity [11,44]. An example of this approach, applied to waste water treatment samples, has been shown by Nielsen et al. [35]. In that study, pure bacterial cultures with similar morphology to the target cells, and containing a known quantity of radioactive label, were used as internal standards either spiked into the activated sludge sample or immobilized apart from the sample, but on the same slide [35]. Although the introduction of the adequate internal standards reduces the effect of some sources of variability (*e.g.* emulsion thickness, exposure time length), carefully controlled experiments need to be carried out in order to minimize the effect of the ample array of factors influencing the efficiency of autoradiograms [35,44].

## Final considerations

Microautoradiography is a robust long-standing technique, which, particularly in combination with *in situ* fluorescence hybridization, has allowed the recovery of direct evidence of several key ecophysiological traits of environmental bacteria. The latest advances in automated microscopic image acquisition and analysis [60] have boosted its potential for permitting high-throughput evaluations, which greatly expanded the scale at which experiments can be performed. Furthermore, as a non-destructive technique, it has the potential to be combined with a suite of methodologies, such as the fluorescence *in situ* hybridization applied to functional genes in its different versions, which include GeneFISH [33], *in situ* rolling circle amplification-fluorescence *in situ* hybridization (RCA-FISH) [20], and two-pass tyramide signal amplification-fluorescence *in situ* hybridization (two pass TSA-FISH) [23], with the aim of linking bacterial identity, functional gene identification, and substrate processing. It could also be potentially combined with combinatorial labelling and spectral imaging-fluorescence *in situ* hybridization (CLASI-FISH) [53], that would allow for an extensive survey of substrate tracking by looking at different populations at the same time with confocal laser microscopy. A particularly interesting combination is that with HISH-NanoSIMS [34], as both techniques would benefit from the advantages of each, thus compensating for their opposite drawbacks: An initial application of MAR-FISH would be the most suitable approach for identifying and quantifying the proportion of cells within groups engaged in substrate incorporation at a high processing scale and, afterwards, selected cells of the target groups could be used for calculating actual incorporation rates by measuring their isotopic content with the NanoSIMS instrument.

In summary, this “ancient” method is in fact rather easy and highly versatile, even to allow its combination with the current “cutting-edge” techniques for bacterial identification and activity assessments, thus providing substantial information at a single cell resolution level.

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