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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]