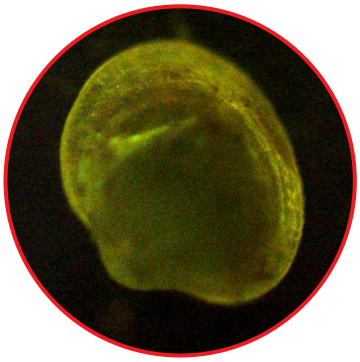
BIOFOULING METHODS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Introduction

Biofouling is the accumulation of unwanted biological material at an interface and we normally associate it with the growth of organisms on surfaces in aquatic environments, be they hard or soft, living or non-living, surfaces. The organisms making up the unwanted biological assemblage may range in size from nanoscale viruses to large macroscopic algae several meters long, and the methods required to study these assemblages are accordingly diverse.

Although the study of biofouling has taken off in recent decades, with the term first appearing in the literature in the mid-1970s, the issue has been noted for millennia, and the term antifouling has a much more antiquated usage associated with the use of tars, paints, and copper sheathing to control the growth of biofouling on ships in days gone by. This reflects the huge impact that biofouling has on vessels, causing both drag and corrosion. Indeed, much of the current driving force behind research into biofouling is the need of the global merchant marine fleet and also navies to reduce the cost of propulsion. This economic driver has the benefit of also reducing the global fleet's carbon footprint, that is, the same performance but with less fuel. More recently, with the advent of large off-shore engineering projects, such as oil and gas installations, and coastal projects, such as power stations and desalination plants, the awareness of the impact of biofouling on both hydraulics and corrosion has increased considerably outside of the sphere of shipping. This concern is further driving the need for more research into both fundamental processes and novel antifouling technologies.

Biofouling and antifouling research is now a substantial academic field with its own journal and a biennial conference. It was also the focus of a recent Wiley-Blackwell textbook, Durr and Thomason's (2010) **Biofouling**, which brought the literature in the field up to date. That book was a key review of the current boundaries but contained only a summary of research methods. Conversely, the aim of this book, **Biofouling Methods**, is to be an essential companion to the former work by providing a "cook book" of practical recipes for those who are currently working in, or just entering, the biofouling field. We have strived to ensure that the book includes methods are that tried and tested as well as those at the cutting edge, thus encompassing the full diversity of the field. We expect this book to become the essential methodological reference for all those working on biofouling and antifouling in academia, namely aquatic biologists, ecologists, environmental scientists, and also for research and development technologists in the antifouling industry. It will also be relevant to anyone who has to monitor biofouling, such as aquaculture producers, managers of off-shore and coastal installation in the oil, gas and desalination sectors, amongst others. This book will also be useful for some specialized practical courses and for graduate and postgraduate students undertaking their own research.

The book is organized in two parts:

1. Methods for Microfouling *(Part Editor: Sergey Dobretsov)*
2. Methods for Macrofouling, Coatings and Biocides *(Part Editors: Jeremy C. Thomason, David N. Williams)*

Each chapter aims to cover a brief history of the method(s) to ensure suitable acknowledgement of the original inventors, includes some examples of the successful use of the method, and examples of the questions that can be answered with the method. Each chapter may cover several methods in a clearly defined subarea. The materials and equipment and methods are described in sufficient detail that the method can be readily implemented and troubleshooting hints and tips are given to permit rapid problem solving along with suggestions with examples for data analysis and presentation. Some chapters vary from this theme, particularly where there is little experimental methodology to describe and we were not overly prescriptive to the authors.

We hope that this book serves its purpose and that you find the methods described here to be useful for your research.

Sergey Dobretsov
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David N. Williams
(Felling, UK)

Guide to methods


What do you want to do?
Study micro-fouling
Visualise microbes in biofilms
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Grow biofilm microbes
Chapter 4, Chapter 7, Chapter 10 & 13
Study biofilm community dynamics
Chapter 4, Chapter 5 & 6
Measure biofilm properties
Chapter 2, Chapter 5
Study biofilm communities
Chapter 4, Chapter 5 & 6
Sample and measure biofilms in the field
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Test biocides
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Part I
Methods for Microfouling

Part Editor: Sergey Dobretsov

1 Microscopy of biofilms

Abstract

Identification, visualization and investigation of biofouling microbes are not possible without light, epifluorescence and electron microscopy. The first section of this chapter presents methods of quantification of microbes in biofilms and Catalyzed Reporter Deposition Fluorescent *in situ* hybridization (CARD-FISH). The second section provides an overview of Laser Scanning Confocal Microscopy (LSCM) imaging, which focuses mainly on the Fluorescent *in situ* Hybridization Technique (FISH) technique. This technique is very useful for visualization and quantification of different groups of microorganisms. The third section describes the principles of transmission (TEM) and scanning (SEM) electron microscopy.

Biofouling Methods, First Edition. Edited by Sergey Dobretsov, Jeremy C. Thomason and David N. Williams.

Section 1 Traditional light and epifluorescent microscopy

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1.1 Introduction

Light microscopy is among the oldest methods used to investigate microorganisms [1, 2]. Early microscopic observations are usually associated with the name of Antony van Leeuwenhoek, who was able to magnify microorganisms 200 times using his designed microscope [1]. A modern light microscope has a magnification of about 1000× and is able to resolve objects separated by 0.275 μm. This resolving power is limited by the wavelength of the used light for the illumination of the specimens. Several light microscopy techniques, such as bright field, dark field and phase contrast, enhance contrast between microorganisms and background [1]. Fluorescent microscopy takes advantage of the ability of some materials or organisms to emit visible light when irradiated with ultraviolet radiation at a specific wavelength. Phototrophic organisms have a natural fluorescence due to the presence of chlorophyll in their cells [3]. Other organisms require additional dyes in order to become fluorescent.

Light microscopy is a simple and cheap method [2]. It is commonly used for observation of relatively large (>0.5 μm) cells of microorganisms (Figure 1.1). In comparison, epifluorescent microscopy provides higher resolution and is generally used for observation of bacteria or cell organelles. The pros and cons of these methods are presented in Table 1.1.

Epifluorescent stains allow quick and automatic counting of bacteria using flow cytometry (discussed later in this chapter). Epifluorescent microscopy is preferable over scanning electron microscopy (SEM) (Chapter 1, section 3) for bacterial size and abundance studies [4]. While direct light microscopy measurements can be highly sensitive to low cell numbers, electron microscopy methods are not. Light and epifluorescent microscopy has the advantage over electron microscopy that a larger surface area can be assessed for a given amount of time [5]. Two fluorescent stains are widely used to stain microbial cells, namely 4',6-diamidino-2-phenylindole (DAPI), which binds to DNA [6] (Figure 1.2), and acrydine orange, which binds to DNA and RNA as well as to detritus particles [7]. Therefore, the estimated number of bacteria stained with DAPI is on average 70% of bacterial counts made with acrydine orange [8]. The use of DAPI stain allows a longer period between slide

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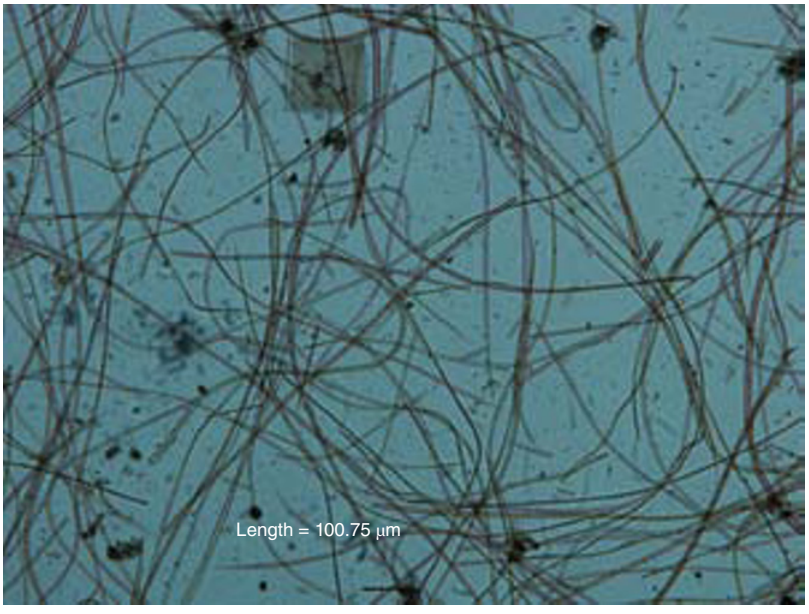

Figure 1.1 Microfouling community dominated by different cyanobacteria, diatoms and bacteria under a light microscope. Magnification 100×. Picture by Julie Piraino. For color detail, please see color plate section.

Table 1.1 Pros and cons of light and epifluorescent microscopy.

Method	Pros	Cons
Light microscopy	• Relatively inexpensive method (<$500) and does not require specialized equipment • Simple sample preparation. In order to increase contrast, object can be stained	• Visualization of small microorganisms (>0.5 mm) is difficult • Only large cell organelles (such as nucleus) can be visualized • Counting of bacteria is difficult
Epifluorescent microscopy	• Small microorganisms, such as bacteria, can be visualized and easily counted • Photosynthetic organisms, such as diatoms and cyanobacteria, do not require staining • Specialized selective probes allow staining of different cell organelles or different groups of microorganisms	• Require specialized equipment, relatively expensive (>$10 000) equipment (epifluorescent microscope with UV lamp) • Usually requires staining with fluorescent probes

preparation and counting, since DAPI fluorescence fades less rapidly than acrydine orange. DAPI staining does not allow accurate measurement of the size of the bacterial cells, since it could only stain the specific part of the cell containing DNA [8]. Visualization of bacteria in dense biofilms is highly difficult. This problem can be overcome to a certain extent by using confocal scanning laser microscopy (CSLM) (Chapter 1, part 2). DAPI staining has been intensively used for determination of bacterial abundance in water samples [9] as well as in biofilms [10]. This can be useful for the determination of the efficiency of biocides (Chapter 2).

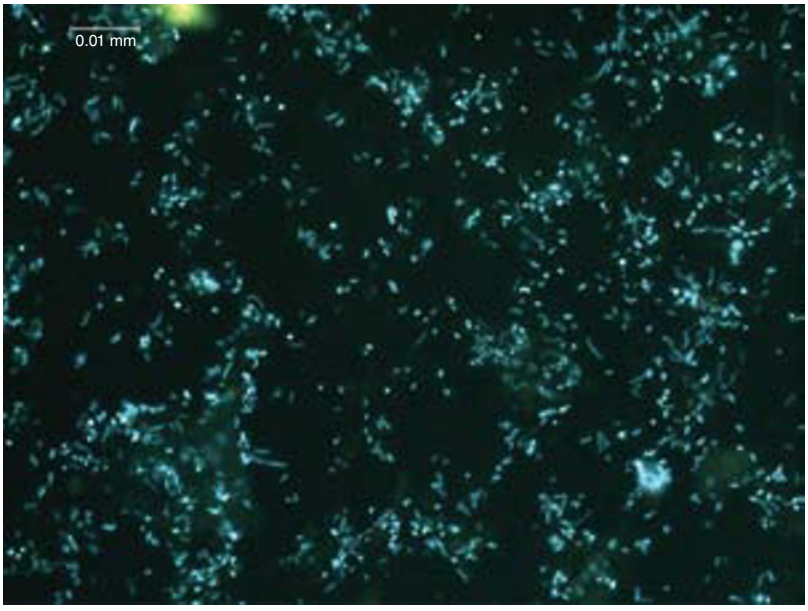

Figure 1.2 Bacterial cells stained with DAPI visualized under an epifluorescent microscope. Magnification 1000×. For color detail, please see color plate section.

Fluorescent *in situ* hybridization (FISH) allows quick phylogenetic identification (phylogenic staining) of microorganisms in environmental samples without the need to cultivate them or to amplify their genes using the polymerase chain reaction (PCR) [11] (Table 1.2, Figure 1.3). This method is based on the identification of microorganisms using short (15–20 nucleotides) rRNA-complementary fluorescently labeled oligonucleotide probes (species, genes or group specific) that penetrate microbial cells, bind to RNA and emit visible light when illuminated with UV light [12]. Common fluorescent dyes include Cy3, Cy5 and Alexa®. In comparison with other molecular methods (Chapter 3), FISH provides quantitative data about abundance of bacterial groups without PCR bias [13]. The FISH-based protocol is presented later in this chapter (Chapter 1, section 2); here the modified protocol of catalyzed reporter deposition fluorescent *in situ* hybridization (CARD-FISH) is described. CARD-FISH is based on the deposition of a large number of labeled tyramine molecules by peroxidase activity (Figure 1.3), which enhances visualization of a small, slow growing or starving bacteria that have a small amount of rRNA and, thus, give a weak FISH signal [14]. Additionally, CARD-FISH can be used for the visualization and assessment of the densities of microorganisms in the samples that have high background fluorescence, such as algal surfaces, fluorescent paints, phototrophic biofilms and sediments [14–16]. In this procedure, FISH probes are conjugated with the enzyme (horseradish peroxidase) and after hybridization the subsequent deposition of fluorescently labeled tyramides results in substantially higher signal intensities on target cells [16]. The critical step of CARD-FISH is to ensure probe microbial cell permeability with cellular integrity, especially in diverse, multispecies microbial communities [17]. Recent improvements in CARD-FISH samples preparation, permeabilization and staining techniques have resulted in a significant improvement in detection rates of benthic and planktonic marine bacteria [14, 15].

Table 1.2 Common probes used in FISH and CARD-FISH and their specific conditions. Detailed information about rRNA-targeted oligonucleotide probes can be found in the public database ProbeBase (http://www.microbial-ecology.net/default.asp) [19, 20].

Probe	Sequence (5′-3′) of the probe	Target group	Formamide (%)	Reference
Universal				
EUB338	GCT GCC TCC CGT AGG AGT	Most of bacteria	20–35	[21]
Eury806	CAC AGC GTT TAC ACC TAG	Euryarchaea	20	[22]
NONEUB	ACT CCT ACG GGA GGC AGC	Non-specific to bacteria (control for EUB338)	20	[23]
Group specific				
ALF968	GGT AAG GTT CTG CGC GTT	Alphaproteobacteria except Rickettsiales	20	[24]
GAM42a*	GCC TTC CCA CAT CGT TT	Most Gammaproteobacteria	35	[25]
CF319a	TGG TCC GTG TCT CAG TAC	*Bacteroidetes* (most *Flavobacteria*, some *Bacteroidetes*, some *Sphingobacteria*)	35	[26]
BET42a**	GCC TTC CCA CTT CGT TT	Betaproteobacteria	35	[25]
LGC354C	CCG AAG ATT CCC TAC TGC	*Firmicutes* (Gram-positive bacteria with low G+C content)	35	[27]
HGC69A	TAT AGT TAC CAC CGC CGT	*Actinobacteria* (high G+C Gram-positive bacteria)	25	[28]
Genes specific				
GV	AGG CCA CAA CCT CCA AGT AG	*Vibrio* spp.	30	[29]
Species specific				
PseaerA	TCT CGG CCT TGA AAC CCC	*Pseudomonas aeruginosa*	30	[30]

*GAM42a requires competitor GCC TTC CCA CTT CGT TT that increases chances of specific binding.
**BET42a requires competitor GCC TTC CCA CAT CGT TT that increases chances of specific binding.

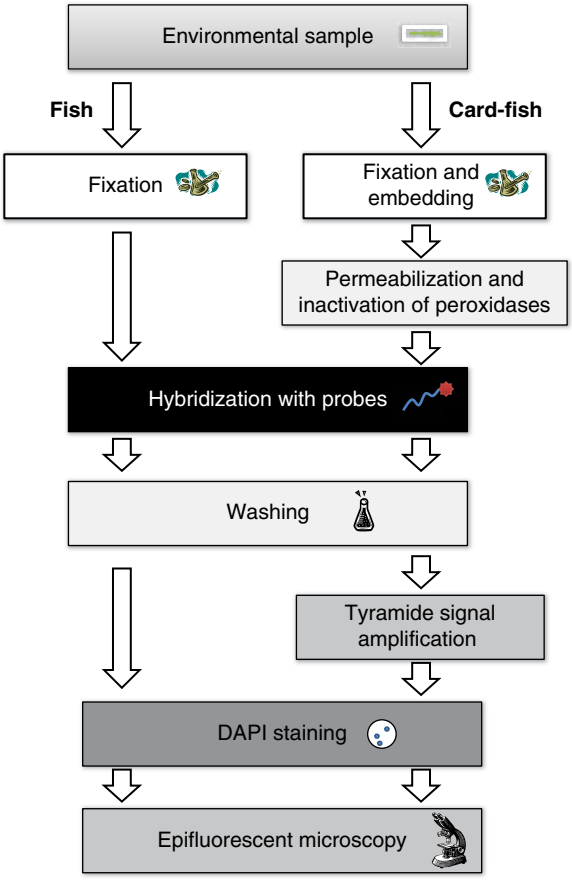

Figure 1.3 Outline of fluorescent *in situ* hybridization (FISH) and catalyzed reporter deposition fluorescent *in situ* hybridization (CARD-FISH).

1.2 Determination of bacterial abundance

1.2.1 Material and equipment

The materials and equipment necessary for counting bacteria in biofilms using DAPI staining are listed in Table 1.3.

1.2.2 Method

1. Add a few drops of DAPI solution in order to fully cover the biofilm.
2. Stain for 15 minutes in the dark. Stained samples should be processed within 2–3 days in order to avoid loss of bacterial numbers [18].
3. Place a cover slip.
4. Remove excess water using filter paper.
5. Place immersion oil on the top of the cover slip.
6. Using 100× objective count bacteria in 20 fields of view selected randomly. In the case of digital camera coupled with an epifluorescent microscope, an automatic counting of