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MICROSCOPY

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7.1 INTRODUCTION

One widely applied method for studying the microbiology of activated sludge is microscopy. It provides an insight into the hidden world of microbes, which cannot be seen otherwise by the naked eye. In this chapter we describe protocols for the microscopic examination of activated sludge samples. These include staining techniques to give an understanding the huge taxonomic and functional diversity found among the microbes in activated sludge. Direct observations and staining can enable the differences between bacterial, fungal and protozoan populations to be distinguished. Studying these microorganisms effectively requires the correct use of the microscope to reveal differences in their shape and size and to diagnose cellular structures. Standard light microscopes with appropriate performance for routine purposes are available from several manufacturers, but using more sophisticated microscopic techniques can enhance the level of information generated.

In this chapter the basic principles of the light and fluorescence microscope are explained and methodologies for relevant staining techniques and data interpretation are provided. The aim of the experimental protocols outlined here is to serve as a user-friendly guide for cell morphological examination characterization, staining techniques (e.g. DAPI: 4',6-diamidino-2-phenylindole dihydrochloride, Neisser, Gram, Nile Blue) for detecting cell viability and intracellular accumulation of storage compounds including poly-hydroxy-alkanoates (PHA) and poly-phosphate granules (poly-P) and *in situ* identification of targeted microbial populations using Fluorescence *in situ* Hybridization (FISH). Combinations of these techniques provide powerful tools for elucidating metabolic features of cells at a single cell level. Combining FISH with staining techniques is often problematic, and so careful planning is required to ensure that the interpretation of this information is unequivocal. This chapter provides the basis for carrying out standard protocols.

7.2 THE LIGHT MICROSCOPE

The purpose of the microscope is to provide sufficient magnification to distinguish between the objects examined. The most commonly used microscope is a bright field microscope that projects a focussed beam of light onto the image on a glass slide. Nowadays, almost all these microscopes are (*i*) binocular (Figure 7.1), meaning that both eyes are used to view the object, making persistent use less tiring, and (*ii*) compound, where more than one lens system is used to achieve the required sample resolution.
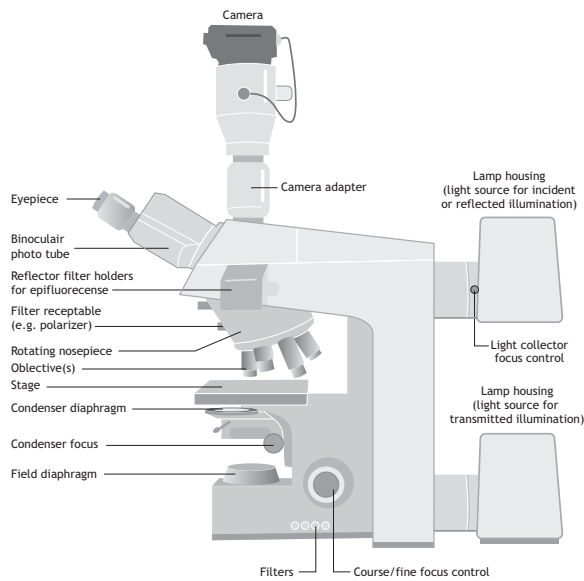
Figure 7.1 The compound microscope.

Figure 7.2 The light path in microscopy illustrating the concept of the numerical aperture and condensers.

The eyepiece (ocular) lenses and the objective lenses provide the resolving and magnifying power of the microscope, while the condenser lens system, by focussing the light source onto the specimen (Figure 7.2), maximises the resolution of the systems by increasing the numerical aperture or light-capturing ability of the objective lenses. Its position is critical for optimization of the performance, and therefore it should supply a cone of light capable of filling the objective lens aperture. The resolution reflects the ability of the microscope to discriminate between two closely positioned entities. If they are closer than the resolution distance then they appear blurred in the microscope image. Actual size, image size and magnification are related as follows:

Image size = Actual size · Magnification   Eq. 7.1

The distance between two distinct objects is referred to as the resolution d and its relationship to numerical aperture (NA) and wavelength (λ):

$$d = \frac{1.22 \cdot \lambda}{N \cdot \sin \alpha} \quad \text{or} \quad d = \frac{1.22 \cdot \lambda}{NA_{objective} + NA_{condenser}} \qquad \text{Eq. 7.2}$$

Where,
λ is the light wavelength;
α equals one-half of the objective's opening angle and;
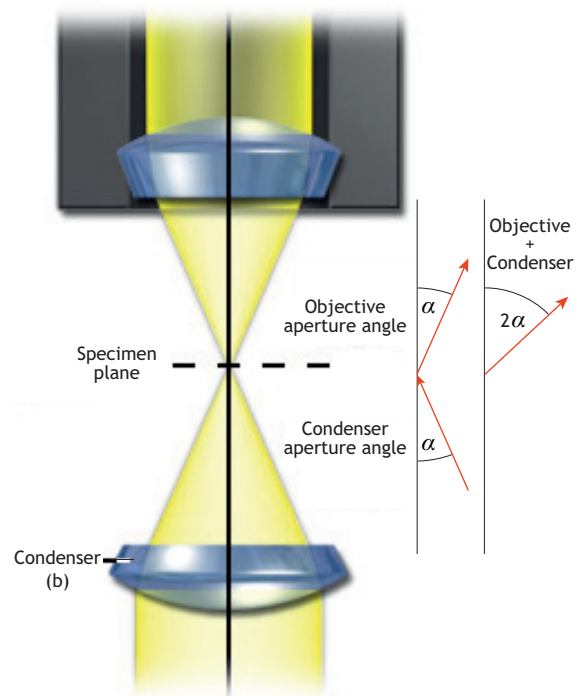N is the refractive index of the immersion medium used below the objective lens.

At the highest magnifications used (100×), because it has a low light-capturing ability (high NA), it is necessary to increase the refractive index (RI) of the medium between the specimen and the objective lens. For light microscopes, the highest resolution is obtained when the condenser aperture angle matches the objective. Lenses in air as a medium cannot have NA that exceed 0.65, whereas in water and certain immersions such as oils the objective lens NA can be increased theoretically to ca. 1.515, and the resolution of the microscope is then approx. 0.2 µm, suitable for viewing/studying most bacteria, but not viruses.

Reflections and loss of illumination intensity can be eliminated by using immersion oil with a RI that matches the RI of the lens glass. The NA of an objective is also partially dependent upon the amount of correction for any optical aberration. Highly corrected objectives have much larger NA for the respective magnification (Table 7.1).

Table 7.1 Examples of objectives and their numerical aperture (NA) and optical correction.

Magnification	Plan Achromat (NA)	Plan Fluorite (NA)	Plan Apochromat (NA)
2×	0.06	0.08	0.10
4×	0.10	0.13	0.20
10×	0.25	0.30	0.45
20×	0.40	0.50	0.75
40×	0.65	0.75	0.95
40× (oil)	n/a	1.30	1.40
63×	0.75	0.85	0.95
63× (oil)	n/a	1.30	1.40
100× (oil)	1.25	1.30	1.40

7.2.1 Standard applications of light microscopy

The microscope must be set up and used properly to generate high quality images. The routine for setting up the microscope as described below is recommended. More experienced users may omit some of the steps in the procedure as they may have gained familiarity with the microscope and the samples under examination already. Microscopes are precision instruments, sensitive and expensive to repair or replace. Thus, they require careful handling and maintenance. The following advice should be observed:

- Avoid large temperature variations, store the equipment in a cool and dark place, and protect it from dust. Always use a dust cover when it is not in use.
- Objective lenses must be cleaned using a lens tissue. Do not dry wipe any lens as this may lead to scratching. Begin by blowing off dust or any loose material with a pressurized optical duster.
- Be careful not to spread residual immersion oil onto other oculars or clean objectives. Use a commercial cleaner or solvent (e.g. 70 % ethyl alcohol - EtOH) to remove any oil or grease. Flooding or placing solvents directly onto the lens is discouraged, and a lens tissue must always be used.
- Keep the microscope clean at all times.
- Objectives immersed in oil must be cleaned immediately after use by wiping with a lens tissue. If not, the oil may dry and thus need removal with a suitable lens cleaning solution. Do not use any solvents (e.g. alcohol) as this might lead to lens damage.

7.2.2 Low power objective

At the first setup of the microscope for optimal performance, one should always start using a low magnification, e.g. 10× objective or lower. The eyepieces should be adjusted to a position suitable for viewing with both eyes. Then the next steps should be followed:

- Lower the stage and insert the prepared slide so that the specimen is centred in the cone of light after passing through the condenser.
- Lower the lens until it is located a few millimetres from the slide surface. Look down the ocular while carefully turning the coarse focus knob until the image becomes visible, and then by adjustment with the fine focus knob bring the specimen image into sharp focus.
- Adjust the iris diaphragm to optimise the contrast to where the image is sharp. With the eyepiece taken out, check that approx. $^2/_3$ to $^3/_4$ of the back lens is light-filled. If not, adjust the condenser position until the iris diaphragm is sharply focussed and, if necessary, readjust the iris diaphragm until it superimposes on the circle of light. The best resolution is when the condenser is raised to near its maximum height.
- Reinsert the eyepiece and examine the preparation using the fine focus adjustment and adjustable mechanical stage.

7.2.3 High power objective

In a good quality microscope the objectives should be parfocal, meaning that all the objectives have very similar focus settings.

- Rotate the nosepiece to the high power objective and then the fine adjustment to sharpen the image.
- Adjust the iris diaphragm to increase the illumination and optimise contrast and then re-examine the specimen.

7.2.4 Immersion objective

Check the required medium designated for the high magnification objective (usually written on the objective or indicated by a black (oil) or blue (water) ring). Add a small drop of immersion oil or water (< 2 cm in diameter) on the slide while rotating the objective lens into place. The space between the slide and the lens should be filled with the immersion medium.

Some microscopes are only equipped with oil or water objectives. These should be used by adding a small drop

of the required medium onto the slide and slowly lowering the lens until it touches the medium, shown by a clear change in the light cone. Then perform fine adjustment while viewing through the ocular lenses until the image becomes sharp. If this fails, repeat the whole procedure. Avoid using any media other than those designated. In other words, do not shift from an oil immersion objective to a water or air objective or any other combinations. Clean and dry the slide before changing objectives.

7.2.5 Important considerations

When carrying out microscopic investigations one should consider:

- Empty magnification occurs when the image is enlarged beyond the physical resolving power of the microscope. Therefore optimal magnifications are usually achieved using 500-1,000 times the NA of the objective. For this reason avoid using eyepieces of high additional magnification, if the objective does not supply sufficient resolution. For example:
  63× (objective) · 12.5× (eyepiece) = 787.5.
- Once the condenser has been focused properly, it should not be necessary to further adjust it.
- The iris diaphragm needs to be changed, so the cone of the light matches the NA of the objective and should therefore be readjusted each time the objective is changed.
- Less experienced users have a tendency to only use one eye while looking through the oculars. Keep using both eyes open. This might require some practice to get used to it.
- Air bubbles can markedly impair the image quality when using immersion lenses. These can be detected by removing the eyepiece and examining the objective rear focal plane through the microscope observation tubes. If observed, gently squeeze the cover slip with a nail. Alternatively, clean the objective lenses and specimen slide and then carefully reapply the oil.
- Dirt or dried oil on the front lens of the objective (or on the slide from previous inspections) might cause blurred images from unwanted scattered light. Clean the lens with a dedicated lens tissue.
- Uneven focus in the field of view or difficulties in obtaining sharp images might be because of how the slide is arranged on the stage. Check that the slide is positioned correctly.
- Problems with focusing might be because the cover slip is adhering to the objective lens. Affix the cover slip with clamps, glue or tape.

- Use the correct thickness for the cover slip. The objectives used on most biological microscopes are designed for use with No. 1.5 cover slips (0.17 mm thickness). Check this with the supplier of the cover slips.
- Use the correct immersion oil and avoid mixing oils with different RI. Usually immersion oil with a RI of 1.515 can be used for all objectives having NA greater than 0.95. Check with the supplier if in doubt.
- Air bubbles can produce shadows or unclear zones in the field of view, but can usually be overcome by elevating the objective and re-focussing, or by checking to see whether sufficient immersion medium is present between the objective and the cover glass. Remove and clean the slide and the objective if the nuisance persists.
- Insufficient illumination can be due to incorrect adjustment of the condenser or an inadequately opening the sub-stage diaphragm.

7.2.6 Bright-field and dark-field illumination

The principle of the light microscope is based on a bending and scattering of the light passing from the light source through the sample into the eyepiece lenses. Variations in the RI of the components in the specimen allow the generation of images based on contrasts, transmissions and reflections.

An efficient imaging with high resolution and proper control of contrast and depth is obtained by aligning the microscope to optimize the brightness and illumination of the image (see the section on Köhler illumination).

The most commonly used light microscope technique is with Bright-field (BF) illumination. This technique is suited for objects with high natural absorption such as plant cells or pigmented cells. However, most bacterial cells are small and appear as transparent objects in BF thus lacking sufficient contrasts. Staining can compensate for this low contrast, and most of the common microbiological staining protocols (Gram, Neisser etc.) are used together with BF.

Phase contrast (Ph) microscopy is an optical-microscopy technique that converts phase shifts in light passing through a transparent specimen to brightness changes in the image. It allows samples with low contrasts to be studied and therefore is suited for examining unstained fixed specimens and live cells. These samples types have very low absorbance

differences compared to their surrounding medium, and such differences in refractive index cannot be detected by BF microscopy. In Ph microscopy an annulus in the condenser aperture generates a hollow cone of zero-order illumination that is projected onto the back focal plane of the objective. A matching phase ring in the objective lens absorbs non-diffracted light and shifts the wavelength slightly, thereby enhancing the contrast. This approach works best with relatively thin specimens (< 5 µm). Ph requires the use of designated objectives in conjunction with aligned phase rings.

7.2.7 Fluorescence microscopy

Some molecules contain fluorochromes that can absorb photons from light with specific energies and thereby become 'excited'. This state lasts for approximately 10^{-7} seconds and it ends by emitting a photon with a slightly lower energy, and hence longer wavelength. These fluorochromes exist as components of naturally occurring compounds frequently found in nature (e.g. pigments such as chlorophylls, vitamins etc.). However, new chemically synthesised compounds with high extinction coefficients are nowadays widely applied to label specific biomolecules and to stain tissues and cells for their identification. The diversity of these fluorescent markers, stains and labels targeting biomolecules, and dynamic dyes for measuring chemical conditions (pH, Ca^{2+}, oxygen etc.) is growing rapidly (Table 7.2).

Table 7.2 Dyes commonly used in microscopy of complex microbial systems.

Stain	Synonym or chemical name	Target	Chemical structure	Excitation/Emission
DAPI	4',6-diamidino-2-phenylindole dihydrochloride	A cell-permeable DNA-binding dye preferential to adenine and thymine-rich DNA	$C_{16}H_{15}N_5 \cdot 2HCl$	λex ~359 nm, λem ~461 nm; λex 340 nm, λem 488 nm (only DAPI), λex 364 nm, λem 454 nm (DAPI-DNA-Komplex:100 mM NaCl, 10 mM EDTA, 10 mM Tris, pH 7)
Propidium Iodide	2,7-diamino-9-phenyl-10 (diethylaminopropyl)-phenanthridium iodide methiodide	A membrane-impermeable fluorescent nucleic acid stain	$C_{27}H_{34}N_4 \cdot 2I$	Excitable at 536 nm and emits at 617 nm (red)
Calcofluor	Calcofluor White	Ca^{2+} fluorescent probe; nonspecific fluorochrome binding to alginate, cellulose and chitin in cell walls	$C_{40}H_{44}N_{12}O_{10}S_2$	λex 355 nm, λem 433 nm
Nile Red	9-(diethylamino)-5H-benzo[a]phenoxazin-5-one	Lipophilic stain	$C_{20}H_{18}N_2O_2$	λex 552 nm, λem 636 nm
Nile Blue	Basic Blue 12, Nile Blue sulphate	Lipophilic stain targeting neutral fats	$C_{20}H_{20}N_3O \cdot 4SO_4$	λex 630 nm, λem 665 nm
DCFDA	2',7'-dichlorofluorescein diacetate	A cell-permeable fluorogenic stress probe targets reactive oxygen species (ROS) and nitric oxide (NO)	$C_{24}H_{14}Cl_2O_7$	λex 504 nm, λem 524 nm
Hoechst 33342	Bisbenzimide	A lipophilic fluorescent stain for DNA labeling; an A/T-specific DNA minor groove ligand	$C_{27}H_{28}N_6O \cdot 3HCl$	λex < 380 nm, λem 450-495 nm
Congo Red	C.I. Direct Red; Cosmos Red; Cotton Red; Direct Red	An amyloidophylic dye that specifically stains stacked β sheet aggregates	$C_{32}H_{22}N_6O_6S_2 \cdot 2Na$	N/A

Crystal Violet	Gentian Violet; Hexamethylpararosaniline chloride	Component of Gram staining that allows one to recognize the difference between gram-positive and gram-negative bacteria	$C_{25}H_{30}N_3 \cdot Cl$	N/A
Sudan Black B	Solvent Black 3; Fat Black HB	A fat-soluble diazo dye used for staining of neutral triglycerides and lipids	$C_{29}H_{24}N_6$	λex ~596-605 nm, stains blue-black
Neutral Red	Basic Red 5; Toluylene Red	Vital dye used as an indicator and biological stain	$C_{15}H_{17}N_4 \cdot Cl$	N/A
BCECF-AM	Spiro(isobenzofuran-1(3H), 9'-(9H)xanthene)-2',7'-dipropanoic acid	A membrane-permeable fluorescent indicator for measurement of cytoplasmic pH	Mixture of $C_{30}H_{20}O_{11}$, $C_{35}H_{28}O_{15}$ and $C_{40}H_{36}O_{19}$	λex 505 nm, λem 520 nm
Acridine Orange	3,6-Bis(dimethylamino)acridine	A fluorescent nucleic acid binding dye which interacts with both DNA and RNA	$C_{17}H_{19}N_3$	When bound to DNA, it emits green fluorescence (Em = 525 nm) and when bound to RNA, it emits red fluorescence (Em = ~650 nm).
Phalloidin-TRITC	Phalloidin-tetramethylrhodamine B isothiocyanate	A fluorescent stain used to identify filamentous actin	$C_{62}H_{72}N_{12}O_{12}S_4$	λex 540-545 nm, λem 570-573 nm
Bisbenzimide	Hoechst 33342 trihydrochloride	A/T-specific DNA minor groove ligand widely used fluorochrome for visualizing cellular DNA	$C_{27}H_{28}N_6O \cdot 3HCl$	λex 350 nm, λem 461 nm
Fluorescein isothiocyanate	FITC; Fluorescein 5-isothiocyanate	Amine-reactive reagent for the FITC labelling of nucleotides and proteins	$C_{21}H_{11}NO_5S$	λex 492 nm, λem 518 nm
Rhodamine B isothiocyanate	RBITC	A fluorescent probe for labelling of nucleotides and proteins	$C_{29}H_{30}ClN_3O_3S$	λex 543 nm, λem 580 nm
Sudan IV	Scarlet Red, Fat Ponceau R	Staining triglycerides, lipids and lipoproteins	$C_{24}H_{20}N_4O$	N/A
Safranin	Basic Red 2	Stain Gram negative bacteria	$C_{20}H_{19}ClN_4$	N/A
1,3,6,8-Pyrenetetrasulfonic acid tetrasodium salt	Tetrasodium 1,3,6,8-pyrenetetrasulfonate; HPTS	A fluorescent probe and pH indicator	$C_{16}H_6O_{12}S_4 \cdot 4Na$	λex 454 nm, λem 511 nm
5-Cyano-2,3-di-(p-tolyl) tetrazolium chloride	CTC	Vital stain	$C_{16}H_{14}ClN_5$	λex 490 nm, λem 630 nm

Fluorescent molecules have very specific absorption and emission wavelengths (high specificity) and follow the Lambert-Beers law, making them suitable for quantitative determination under a large range of physiological and physical conditions. Application of appropriate light source and filters for discriminating shorter-wavelength excitation and longer-wavelength emission light provides highly selective conditions for visualizing an individual fluorescent molecule with low interferences. Most fluorescence microscopes are equipped with epi-illumination/excitation and incorporate specific dichroic mirrors or chromatic beam splitters to allow a separation of the desired fluorescence emission light from any unabsorbed reflected excitation light and transmission of light not deriving from the applied fluorochrome. Fluorescence microscopy can be readily combined with other microscopic techniques including Ph microscopy.

Alignment of the microscope (Köhler illumination) is the most critical step for obtaining optimal resolution for all light microscopes. The following steps are advised:

- Centre and focus the light source. This can be carried out by removing the light diffuser (if applicable) and then, without the condenser, project the light onto a piece of paper placed in the condenser carrier. Fill the circular area with the image of the light source.
- Adjust the iris diaphragm located nearest to the light source (field iris) until you see sharp edges.
- Focus by adjusting the condenser-focusing knob. Both the specimen and the iris diaphragm should be in focus.
- Centre the image of the field iris using the condenser-centring knobs (usually located on the condenser).
- Open the field iris so that the edges lie just beyond the field of view.
- Optimize the image contrast, depending on the specimen, by adjusting the condenser iris diaphragm. Do not use this aperture to control light intensity.
- Adjust light intensity with the illuminator rheostat or by inserting neutral density filters (for colour photography).

Specimen contrast with a correct Köhler illuminated microscope is obtained by adjusting the condenser diaphragm. Illumination intensity is varied by adjusting the voltage to the light source or by neutral density filters in front of the illuminator.

7.2.8 Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) has become widely used among microbial ecologists due to its superior image quality. This increase in image quality comes from exchanging the light source used in epifluorescent microscopy with lasers that have much high intensities per area. This allows so-called pinholes, which prevent light that derives from out-of-focus information from reaching the photomultipliers (Figure 7.3), to be applied. This provides the basis for less blurry images and furthermore allows optical sectioning. The equipment used for CLSM is considered specialized equipment that requires skilled operators, and is outside the scope of this chapter.
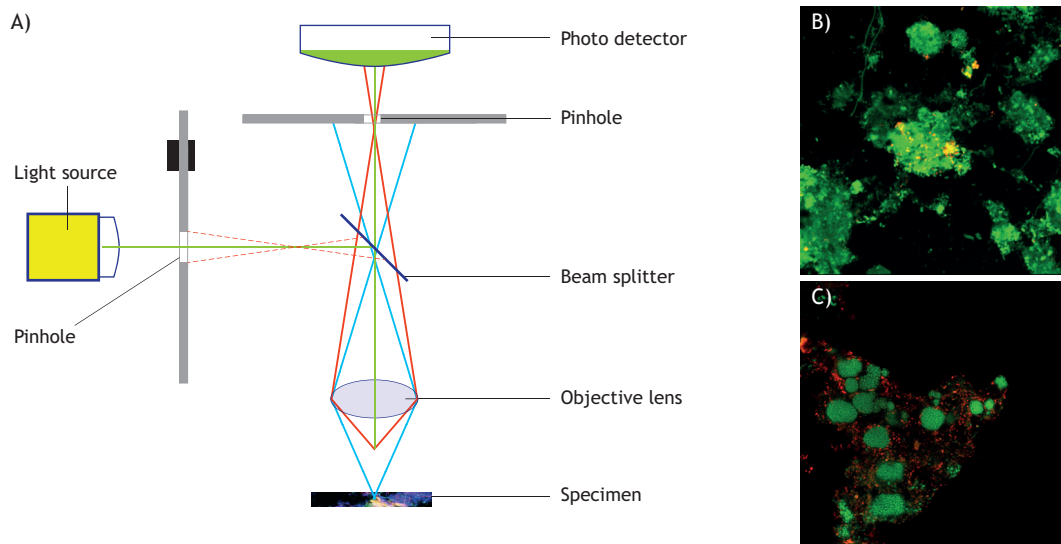

Figure 7.3 (A) Principle of the confocal laser scanning microscope. Images (B) and (C) show the same sample after fluorescence *in situ* hybridization (FISH) visualized by epifluorescence microscopy, and by a confocal laser scanning microscope, respectively (images: J.L. Nielsen).

7.3 MORPHOLOGICAL INVESTIGATIONS

In activated sludge systems most bacteria responsible for the removal of pollutants (e.g. C, N and P) grow in flocs and facilitate the settling and separation of the treated effluent. This floc-formation process is a result of the aggregation of wastewater components and an active excretion of extracellular polymeric substances (EPS) by 'floc-forming bacteria'. The flocs consist of bacteria often clustered into micro-colonies, organic and

inorganic particles, and of the filamentous bacteria that provide the scaffolding or matrix backbone of the floc around which microorganisms aggregate.

Settling and the compaction properties of the activated sludge are directly related to the floc size and structure, and reflect its chemical, physical and biological components. The presence and abundance of filamentous bacteria are especially important for good sludge and settling properties. Imbalance between floc-forming bacteria and filamentous bacteria can lead to sludge bulking, where filaments extend from the floc surface and form inter-floc bridges, or disperse growth (pin point flocs, which results from too few floc-forming bacteria). Filamentous bulking can also result from the generation of large, irregular and open flocs. Excessive amounts of (certain) floc-forming bacteria occasionally lead to an overproduction of hydrophobic EPS, which may result in the formation of weak and buoyant flocs (so-called zoogloeal bulking) (Eikelboom, 2000; Jenkins *et al.,* 2004).

Morphological examinations alone or in combination with chemical analyses (EPS, ions etc.) and physical measurements (settling properties, compactness etc.) of the sludge can therefore reveal important plant operational and maintenance information. It is recommended to combine the information obtained from microscopic examination with direct plant observation, e.g. colour of the mixed liquor (activated sludge), percentage of the aeration basin surface covered by foam, depth of the sludge blanket in the secondary clarifier, the type of floating material on the surface of the clarifier (effervescent with bubbles *versus* greasy and sticky, etc.).

The general microscopical characterisation of flocs includes descriptions of their size, form and overall structure, the presence of organic fibres and inorganic particles, or single cells, and the recognition of different morphotypes of filamentous bacteria and their quantities. More details about the microscopical characterisation of activated sludge can be found in the manuals by Eikelboom (2000), Jenkins *et al.* (2004) and Seviour and Nielsen (2010).

7.3.1 Microscopic identification of filamentous microorganisms

For an unequivocal and precise identification of these filamentous bacteria it is considered important to find suitable control measures for bulking (Nielsen *et al.,* 2009; Seviour and Nielsen, 2010). This precise

identification requires the application of molecular tools including FISH and 16S rRNA amplicon sequencing (see Chapter 8). A few filamentous morphotypes in activated sludge can be characterized and to some extent tentatively identified using the manuals by Eikelboom (2000) and Jenkins *et al.* (2004). However, nowadays it is clear that most of filamentous microorganisms cannot be characterised adequately in this way. Therefore, the recommendation here is to use microscopy in association with molecular methods to allow for the identification *in situ* of these and other bacteria, and to restrict the morphological studies to preliminary characterizations and, where appropriate, to confirm the molecular data. Since there has been a long tradition of microscopic morphological characterisation and 'identification' of filamentous bacteria in activated sludge, especially by the industry, and since this approach is still widely used, these methods will be outlined here.

Characterization of filamentous bacteria is based on the analysis of their morphological features, reactions to the Gram and Neisser staining, and physiological traits, such as the accumulation of intracellular elemental sulphur granules. Eleven features were assessed for distinguishing the filamentous species in the manual by Eikelboom (2000):

- Shape and length of the filaments.
- Shape of the cells.
- Filament diameter.
- Motility-gliding.
- Attached growth of other bacteria.
- Branching.
- Septa between adjoining cells (visible or not visible).
- Presence of a sheath.
- Granules of stored internal compounds (especially sulphur granules).
- Gram staining.
- Neisser staining.

Microscopy has been used to estimate the abundance of filamentous bacteria in activated sludge and their immediate influence on settling properties. For indexing into levels, the so-called Filament Index (FI) is an efficient semi-quantitative measure. The scale ranges from 0 (filaments absent) to 5 (excessive numbers of filaments) (Figure 7.4). This evaluation is simple to perform at low magnification (10×) phase contrast, and is considered reliable. It is recommended by Eikelboom (2000) to carry out such quantification regularly (every 1-2 weeks) or when changes in sludge settling properties are observed.
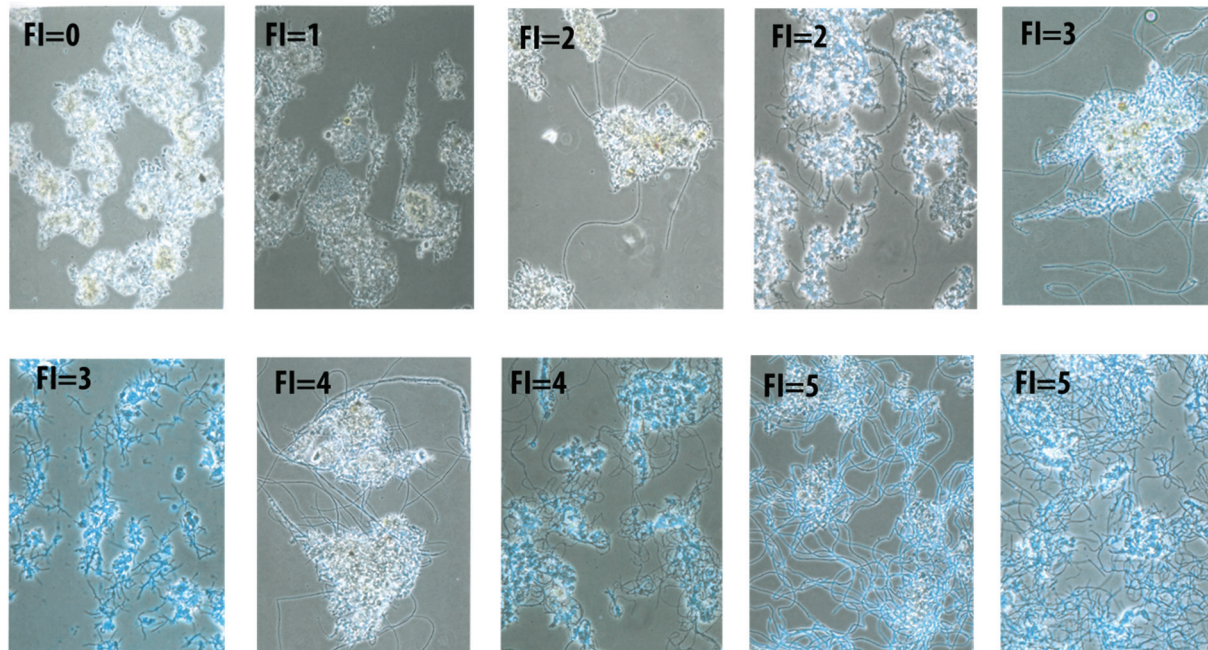
Figure 7.4 Filament indices (photos: Eikelboom, 2000 by courtesy of IWA Publishing).

7.3.2 Identification of protozoa and metazoa

The microbial communities in activated sludge consist, besides prokaryotes, of bacteriovorous and carnivorous protozoa and metazoa. These are important for shaping the composition of the sludge community and improving the quality of the final effluent, and are widely used to monitor plant performance. Their ecological importance comes from feeding on floc-associated and freely suspended bacteria, and they therefore reduce the turbidity of the final discharged liquid phase. The protozoa can be divided into flagellates, amoebae, free-swimming and crawling ciliates, attached ciliates, while the metazoa include rotifers, nematodes and oligochaete worms. The description of the protozoal and metazoan fauna may provide clues for reasons for the poor plant operation and often correlates with its physicochemical parameters. Table 7.3 shows some generalizations commonly used to predict plant performance and effluent quality. These generalizations should be used with caution (Seviour and Nielsen, 2010). Frequent examinations of the presence of protozoa can be used to obtain information about under- or overloading of the plant, whether the plant is sufficiently aerated, has suitable sludge retention time, etc.

More specific bio-indicators for plant performance might be found when applying higher taxonomical resolution. However, it is a time-consuming task that requires a high degree of expertise and experience. Furthermore it is still unclear how important some of these protozoa are as indicators of the wastewater treatment plant performance. Recent studies have applied FISH to identify protozoa (Xia *et al.,* 2014), but because of practical difficulties, only a small number of FISH probes for these have been published so far. Protozoan species such as *Vorticella picta* have been used as indicators for low organic sludge loading, while *Vorticella microstoma* and *Opercularia coarctata* are indicators for high organic loading (Madoni, 1994). Other studies have proposed that *Acineta tuberosa*, *Zoothamnium* and *Euplotes sp.* are good indicators for high-quality effluent and thus well-functioning plants (Salvado *et al.,* 1995).

Table 7.3 Generalizations of the presence and abundance of protozoa and metazoa for the evaluation of the performance of an activated sludge plant.

Microorganism	Characteristics	Examples	Overabundance indications	Manifestation in wastewater treatment plant
Flagellates	High numbers found during recovery from toxic discharge or low dissolved oxygen (DO) concentration	-	High organic loading	Poor performance
Amoebae	High during start-up and recovery	-	High organic loading	Unstable operation
Free-swimming ciliates	Occur under good floc formation	Euplotes, Aspidisca	High organic loading	Well-operating plant
Attached ciliates	Under low DO or toxicity, stalked ciliates will leave their stalks	Vorticella	Low organic loading	Well-operating plant
Crawling ciliates	-	-	-	Good performance
Rotifers	Require high DO	Euchlanis	Low organic loading	Well-functioning and stable plant
Higher invertebrates	-	Nematodes, Targrades, Annelids	Low organic loading	High ammonia loads as Tardigrades and Annelids are susceptible to ammonia toxicity

7.4 EXAMINING ACTIVATED SLUDGE SAMPLES MICROSCOPICALLY

7.4.1 Mounting the activated sludge sample

Two approaches are used for light microscopic examination: (*i*) wet mounts suitable for examining sludge characteristics such as floc structure, filament morphology, protozoa and metazoa identification, and (*ii*) fixed smears for stained preparations.

A wet mount is prepared by adding a drop (ca. 1 cm diameter) of fresh activated sludge onto a glass slide and then gently placing a cover slip on top of the drop. If the drop is too large, then it might be more difficult to focus on the sample or the cover slip will float and the specimen will then leak around it and risk contaminating the microscope. If too small, the specimen will contain interfering air bubbles and will prematurely dry out. Sealing the edges along the cover slip with nail polish or vaseline will reduce these problems. The slide is then ready for examination.

A fixed smear immobilises the biomass onto the slide, but may reduce specimen contrast. The sample is thinly smeared onto the slide and then can be easily spread using a pipette. The smear is allowed to air dry at room temperature. Heat fixing by passing the slide through a flame is usually not necessary for activated sludge samples and runs the risk of harming the sample.

Hydrophobic slide surfaces from remnant grease used in the cutting process frequently create problems as they can cause the smear to dry out irregularly. Thus, slides should be degreased before use. Acid washing of slides and coating the glass slide with gelatine or poly-L-lysine are recommended. Acid-washed slides are prepared by leaving them in a preheated (60 °C) 1 M HCl solution overnight and then rinsing them first in distilled water (dH₂O) followed by 95 % ethanol solution before drying. For improved sample adhesion, dip the slides in 0.5 % (*w/v*: weight/volume percent) gelatine solution for 5 min at 70 °C. Alternatively, acid-washed slides can be dipped in 0.01 % poly L-lysine solution for 5 min at room temperature. Slides are then air-dried in a vertical position in a dust-free environment. Fixing the sample at a higher temperature (40-60 °C) to the gelatine or poly-L-lysine coated slides improves its adherence to the coated slides.

Immediate deposition of the activated sludge sample onto the slide will usually maintain the spatial structure of the flocs. However, a gentle homogenization of flocs prior to immobilization of the sample often helps staining protocols to visualize cell structures of interest, and this can be achieved by gently rubbing two glass slides with a 20 µL sample against each other, or more efficiently with a tissue grinder.

The cryosectioning procedure can reveal high specimen resolution while keeping its spatial organisation intact. This procedure involves embedding the sample in paraffin or a polymerizing resin (e.g. Tissue-Tek O.C.T.). Paraffin-embedding usually ensures undamaged tissue slices but requires sample heating and de-waxing with xylene, while cold polymerizing resin application is performed at ambient temperature and requires no further chemical treatment. A simple cryosectioning procedure involves mixing the activated sludge (or biofilm) sample and embedding material (Tissue-Tek O.C.T.) in the lid of an Eppendorf tube. After allowing the embedding material to migrate into the sample overnight at 4 °C, it is transferred to liquid nitrogen. Sectioning into 5-20 μm thin slices is carried out on a cryotome at -20 °C. The slices should be immediately placed on a slide (at room temperature), where they melt and are allowed to dry on the bench for 3 hours. Afterwards, further staining of the sample can now be done.

7.4.2 Gram staining

The Gram stain is a differential stain to distinguish between two major bacterial groups, Gram-positive (stained purple) and Gram-negative (stained red) cells (Figure 7.5).
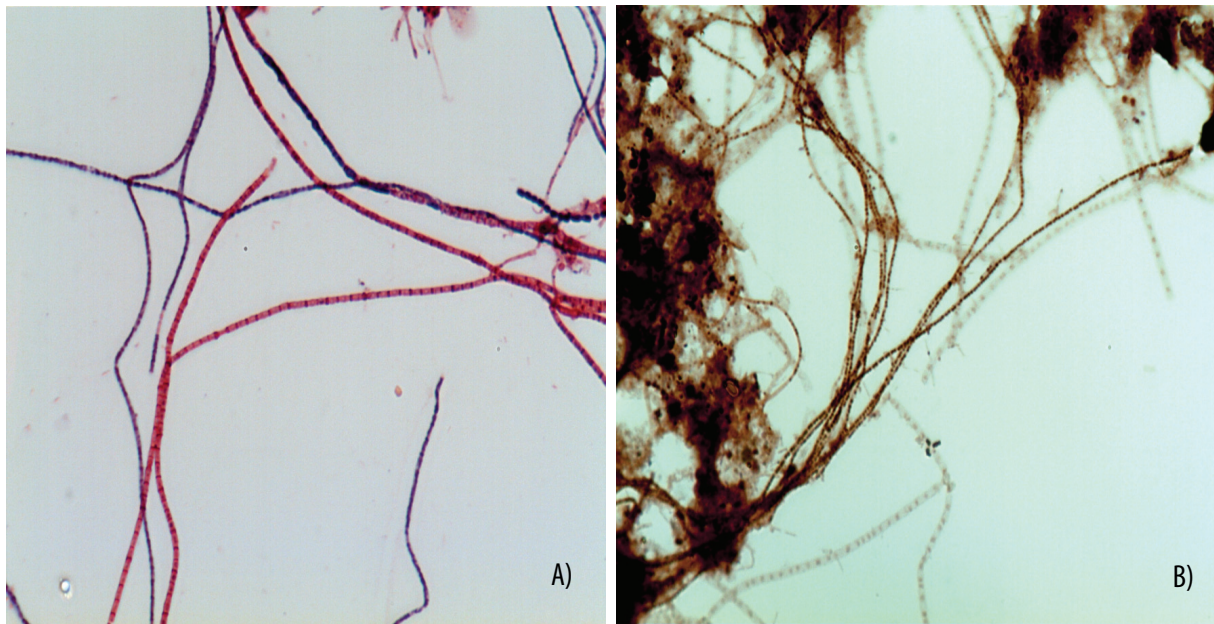

Figure 7.5 Examples of typical Gram (A) and Neisser (B) staining of an activated sludge sample (photo: B. McIlroy).

This staining technique reflects basic differences in their cell wall composition and organisation. Gram-positive cell walls contain high levels of peptidoglycan, which contracts in the presence of the decolouriser (70 % ethanol), hence retaining the crystal violet/iodine complex. In Gram-negative bacteria, layers of peptidoglycan are present, but in much lower quantities. Furthermore Gram-negative cells have a lipopolysaccharide membrane lying outside the peptidoglycan. In the presence of the decolouriser, this membrane is disrupted, and the cells therefore become leaky, allowing the crystal violet/iodine complex to be washed out. A suitable counterstain (safranin) is then applied to the cells, which are therefore stained red.

7.4.2.1 Reagents and solutions for Gram staining

- **Crystal violet solution**
  Crystal violet stock (solution A): Dissolve 20 g Crystal violet (85 % dye) in 100 mL of 95 % ethanol. Filter the sample.

Oxalate stock (solution B): Dissolve 1 g ammonium oxalate in 100 mL water.
Working solution: Dilute solution A (1:10) in solution B. The working solution can be stored at room temperature up to one year in the dark.

• **Gram's iodine solution**
Dissolve 1 g iodine crystals and 2 g potassium iodide in 5 mL water. Then add 240 mL distilled water and 60 mL of 5 % (*w/v*) sodium bicarbonate solution. Mix thoroughly. The solution can be stored at room temperature for up to one year in the dark.

• **Counterstain**
Mix 2.5 g safranin O with 100 mL of 95 % ethanol. Can be stored for up to 1 year at room temperature.

• **Decolourizing solution**
Mix equal volumes of 95 % ethanol and acetone. Can be stored for up to 1 year at room temperature.

Commercial and ready-to-use Gram staining kits are available from assorted suppliers.

7.4.2.2 Procedure

a. Prepare a fixed smear, hopefully achieving a monolayer of cells as described earlier.
b. Flood the sample with crystal violet solution and let it stand for 30 seconds.
c. Gently wash the stain off with running tap water for ~5 seconds. Shake off excess water.
d. Flood the smear with Gram's iodine solution for 1 min.
e. Gently wash the stain off with running tap water for ~5 seconds. Shake off excess water.
f. Decolourize with decolourizing solution by slowly dropping the solution above the smear on the slightly tilted slide. This should allow the decolourizing solution to slowly flow down across the smear. Continue until no more purple colour is eluted from the smear (excessive decolourization will decolourize Gram-positive cells, and too moderate decolourization may give false Gram-positive cells; false Gram stain reactions signals can also be seen with stored and starved samples, where Gram-positive cells stain Gram negatively).
g. Gently rinse off the stain with running water for ~5 seconds. Shake off excess water.
h. Cover the slide with counterstain (safranin) for 30 seconds.
i. Gently rinse off the stain with running water for ~5 seconds. Shake off excess water.

j. Allow the slide to air-dry, or carefully blot with filter paper.

A well-prepared smear should be barely visible to the naked eye. Examine the slide by adding a drop of immersion oil in the centre, and examine using a 100× Bright field objective. Do not use a cover slip. A Gram-stained slide can be stored indefinitely at room temperature in the dark. Gram-positive cells appear purple and Gram-negative cells appear red. The colour can vary from blue to almost black. A blue filter will enhance the contrast. Some cells can appear as intermediates or unevenly stained, and are referred to as Gram-variable.

Problems with large flocs and too dense smears can be overcome by sample dilution or gentle sample homogenisation. Hexidium iodide (HI) is a fluorescent binding dye that allows the Gram status of a culture to be determined by its differential absorption through the bacterial cell walls (Haugland, 1999).

7.4.3 Neisser staining

Neisser staining is useful for detecting the presence of metachromatic granules, so-called Babes-Ernst polar bodies, in bacterial cells (Figure 7.5). Under defined pH, both methylene blue and crystal violet will bind to these polar bodies (volutin bodies including poly-P), but not to the rest of the bacterial cell. The polar bodies become visible as dark dots. The Neisser staining is based on the dye binding at high pH to negatively charged materials in the cell walls or granules. It is useful for detecting filaments deeply located within floc and for staining of phosphate-accumulating bacteria (PAOs) responsible for enhanced biological phosphate removal (EBPR).

7.4.3.1 Reagents and solutions for Neisser staining

• **Methylene blue solution**
Dissolve 0.1 g methylene blue in 100 mL distilled water. Add 5 mL 96 % ethanol and 5 mL glacial acetic acid. Filter the sample.

• **Crystal violet solution**
Dissolve 0.33 g crystal violet in 100 mL distilled water and 3.3 mL 96 % ethanol. Filter the sample.

• **Counter-staining solution**
Mix 33.3 mL 1 % chrysoidine solution with 100 mL distilled water. Alternatively use 0.2 % Bismark brown.

- **Working solution**

   Prepare a fresh mixture containing two parts methylene blue solution and one part crystal violet solution.

Commercial and ready-to-use Neisser staining kits are available from several suppliers.

7.4.3.2 Procedure

a. Prepare a fixed smear as described earlier.
b. Flood the sample with the freshly prepared working solution and leave to stand for 15 s.
c. Gently rinse off the stain with running water and ensure both sides of the slide are rinsed.
d. Flood the smear with the counter-staining solution and leave for 1 minute.
e. Gently rinse off the stain with running water and ensure both sides of the slide are rinsed.
f. Let the slide air dry or use filter paper.
g. Examine under the microscope with oil immersion using a high magnification Bright field objective, direct illumination and no cover slip.

A well-prepared smear should be barely visible to the naked eye. A Neisser stained slide can be stored at room temperature in the dark indefinitely. Neisser-positive cells appear blue/grey or purple while Neisser-negative cells appear yellow/brownish. Frequently, exocellular sheaths and polysaccharide capsular material also stain. Neisser-negative cells have little contrast and may be difficult to see.

7.4.4 DAPI staining

The DAPI nucleic acid stain preferentially stains double-stranded (ds) DNA which then emits a blue-fluorescent light. Its affinity appears to be associated with clusters rich in adenine and thymine (AT), and DAPI binds especially to the minor groove of the DNA double helix, where the natural fluorescence is enhanced because of the displacement of water molecules from both DAPI and the minor DNA groove (Trotta and Paci, 1998). DAPI also binds to RNA, although in a different binding mode that might involve AU-selective intercalation (Tanious *et al.,* 1992). Other molecules have been reported to adsorb DAPI, including poly-P, where the complex emits light of a wavelength in the yellow range. The specificity of the DAPI stain depends on the protocol and on the concentration used. Cells containing poly-P in concentrations higher than 400 µmol g^{-1} dry weight can be visualized when DAPI is applied at a concentration of at least 5 to 50 µg mL^{-1}. At higher concentrations DAPI also binds to, and fluoresces with, other cellular constituents such as lipids. At lower concentrations, the resulting blue fluorescence is related primarily to its binding to DNA. Two forms of the dye are frequently used, DAPI dihydrochloride and the more water soluble DAPI dilactate.

7.4.4.1 Reagents and solutions for DAPI staining

- **DAPI stock solution**

   Prepare a 5 mg mL^{-1} DAPI stock solution by dissolving it in distilled water (dH$_2$O) or dimethylformamide (DMF). The DAPI dihydrochloride may take some time to completely dissolve and may require mild sonication. Other solvents (e.g. PBS: Phosphate-buffered saline) are problematic because of solubility difficulties. For long-term storage, the DAPI stock solution can be aliquoted and stored at -20 °C. For short-term storage it can be kept at 4 °C, if protected from direct light exposure.

7.4.4.2 Procedure

Counter-staining by DAPI can be performed directly with liquid samples or fixed smear samples as described earlier.

a. For samples in suspension: mix DAPI stock solution to a final concentration of 1 µg mL^{-1} directly into a small volume sub-sample. Allow the dye to migrate into the sample and bind at room temperature for 5 to 30 min (depending on the nature of the sample). Protect it from direct exposure to light. Remove excess dye by centrifugation and rinse the sample with sterile water until any background fluorescence is minimized.

b. For immobilized samples: Embed the smeared sample with a 1 µg mL^{-1} DAPI solution. Allow it to migrate into the sample and react at room temperature for 5 to 30 min, depending on the nature of the sample. Keep it protected from direct exposure to light. Remove any excess DAPI by centrifugation and rinse with plenty of sterile water until any background fluorescence is minimal. Immobilize the suspended sample as described for the smeared sample. Let it air dry. A DAPI-stained slide can be stored at -20 °C indefinitely. Apply a drop of antifading agent (Citifluor, Vectashield or a mixture hereof) directly onto the slide with the sample. Cover it with a cover slip and examine it under epifluorescence microscopy. The excitation

maximum for DAPI bound to dsDNA is 358 nm, and the emission maximum is 461 nm.

7.4.5 CTC staining

The 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) is a redox dye that produces fluorescent formazan (CTF) crystals in reduced environments such as in active respiring cells with an active electron transport. The formazan is deposited intracellularly as large crystals and can therefore be used as a cellular redox indicator. Although it has been described as an non-specific indicator it is still valuable as a fast indicator of filamentous activity in activated sludge samples. An advantage of the CTC activity stain is that it does not usually require any washing step, as the non-reduced CTC does not absorb light above 400 nm.

7.4.5.1 Reagents and solutions for CTC staining

- 1 % (*v/v*) aqueous solution of CTC (5-cyano-2,3-ditolyl tetrazolium chloride).

7.4.5.2 Procedure

a. 200 μL 50 mM are added to a test tube containing 2 mL of the sludge sample. Let the sample incubate for 1-4 h at room temperature with moderate agitation.
b. The stained cells can be visualized by filtering the sample onto a 0.2 μm black polycarbonate filter or simply add a small drop on a microscope slide and let it air-dry.
c. Filters are air-dried and mounted with immersion oil on glass slides. Examine through the microscope using an excitation of 450 nm and an emission of around 630 nm.
d. Formazan-containing cells are relatively photo-stable and can be stored at 4 °C for several days if required.

7.5 FLUORESCENCE *in situ* HYBRIDIZATION

Hybridization with fluorescently-labelled DNA oligonucleotides has allowed visualization of individual microbial cells in complex environments and their *in situ* identification. This technique can provide important information for quantitative enumeration of targeted microbial groups of interest and their spatial organization within the sample. It does not discriminate between cells that can or cannot yet be cultured.

The FISH technique, described in details elsewhere (Nielsen, 2009), is based on the principle of hybridizing fluorescently-labelled DNA probes to target sites of ribosomal rRNA in permeabilized whole cells of interest (Figure 7.6).

The probes are small DNA fragments designed to hybridize specifically to their complementary target sequences in the rRNA structures in metabolically active target cells. One powerful aspect of FISH is that it is possible to design a probe to target a narrow phylogenetic group (down to the species level) or one which can target members of a whole bacterial phylum or any other higher phylogenetic hierarchal group. Only cells with the proper target sequence in their 16S rRNA molecule will hybridize with the DNA probe used, and because an individual cell will contain multiple ribosomes, a sufficient fluorescence signal is generated to allow its detection microscopically.

Despite the fact that the method can be applied widely in microbiology, some limitations of FISH have been recognized. Although activated sludge is usually characterized by a high level of metabolic activity resulting in high cellular ribosomal counts and high fluorescence signals, some cells might emit detectable levels of fluorescence, for several reasons. Such low emission signals might be compensated for partially by using fluorochromes with higher extinction coefficients, enzymes that amplify signal intensities (Card-FISH, see detailed protocols in Pernthaler and Pernthaler, 2007) or by using multiple labelled probes (DOPE-FISH, Stoecker *et al.*, 2010). The FISH technique is sometimes limited by penetration of the probe through the wall/membrane and into the targeted cell, and so a false negative is the outcome, as often seen with the foam-stabilising Mycolata (Seviour and Nielsen, 2010). Thus, some cell pre-permeabilisation is essential. Nor will they fluoresce if the probe is designed to target an inaccessible region of the rRNA, and helper probes will need to be designed and applied to overcome this problem (Fuchs *et al.,* 2000). Sample autofluorescence is also often a serious problem with activated sludge samples. Methodological problems and pitfalls of the FISH technique have been reviewed elsewhere (Moter and Göbel, 2000). Important factors that influence the sensitivity and quality of the FISH technique are described by Bouvier and Del Giorgio (2003). These can be summarized as follows:
- Auto-fluorescence from naturally fluorescing compounds in the sample or cells.
- Probe sequence (binding energy, self-complementarity, etc.).

- Probe concentration.
- Type of probe.
- Microorganism (the nature of the membrane might influence permeability and undergo cell lysis).
- Target site on rRNA (accessibility might be hindered).
- Fixation procedure (might chemically modify the sample/cells).
- Presence of deoxyribonucleases (DNases), or peroxidases (for Card-FISH).
- Hybridization time.
- Temperature.
- Salt concentration.
- Formamide concentration.
- Washing time.
- Adsorption phenomena.

Information on probe sequence and stringency can be found in literature and in probeBase (Loy *et al.,* 2003) and can be coupled to multiple fluorochromes. In general, fluorochromes such as Cy3, Cy5, FLUOS, TAMRA, and various Alexa variants, which provide low fading and high intensity signals without compromising permeabilization, work well. Custom synthesis of oligonucleotide probes with or without fluorochromes can be purchased from plenty of internet-based suppliers.

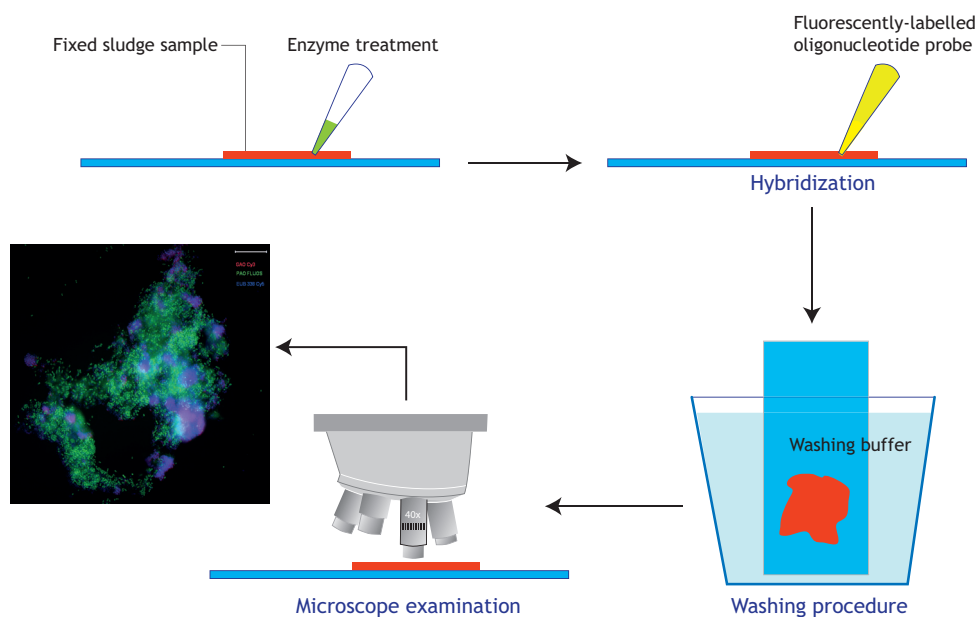The FISH procedure for each probe/sample should be optimized based on an empirical approach.

Figure 7.6 Schematic presentation of the FISH procedure.

7.5.1 Reagents and solutions for FISH

- **Fixative (8 % Paraformaldehyde, PFA) for Gram-negative cells**
  Mix 4 g PFA in 30 mL dH$_2$O at 60 °C, add a drop of 2 N NaOH to facilitate it dissolving (work in the fume hood); add 16.6 mL 3 × PBS (see below). Adjust to 50 mL with dH$_2$O. Filter the solution through a 0.22 μm polycarbonate filter to remove any particulate material. The solution should be used fresh, but it can be stored for a few days in the fridge or in aliquots at -20 °C.

- **Lysozyme for cell permeabilisation**
  Dissolve lysozyme to a final concentration of 10 mg mL^{-1} (~360,000 U mL^{-1}) in 0.05 M EDTA 0.1 M^{-1} Tris-HCl (pH 8.0). Prepare fresh when needed, and keep it on ice; it can be stored in aliquots at -20 °C.

- **Proteinase K for cell permeabilisation**
  Dissolve proteinase K (from *Tritirachium album*) to 20,000 U mL^{-1} in Tris-EDTA buffer (0.01 M EDTA, 0.1 M^{-1} Tris-HCl, pH 8.0). Prepare fresh when needed, and keep it on ice; it can be stored in aliquots at -20 °C.

- **3 × phosphate-buffered saline (3 x PBS)**
  Mix 0.1 M NaH$_2$PO$_4$ with 0.1 M Na$_2$HPO$_4$ until pH 7.4 is reached. Mix 22.8 g NaCl into 300 mL of this phosphate solution and add dH$_2$O to 1,000 mL. Autoclave, and store at room temperature.

- **Tris-EDTA buffer (TE buffer)**
  0.01 M EDTA, 0.1 M Tris-HCl, pH 8.0. Sterilize by filtration and store at 4 °C.

- **1 M Tris-HCl, pH 8.0**
  Dissolve 121.1 g Tris in 800 mL dH$_2$O, add 42 mL concentrated HCl, allow it to cool, adjust pH and fill to 1 L with dH$_2$O. Autoclave, and store at room temperature.

- **5 M NaCl**
  Dissolve 292.2 g NaCl in 800 mL dH$_2$O, and fill to 1 L with dH$_2$O. Sterile filtration, and store at room temperature.

- **Sterile distilled H$_2$O (dH$_2$O)**
  Autoclave sterile filtered dH$_2$O.

- **10 % Sodium dodecylsulfate (10 % SDS)**
  Heat 50 g SDS (electrophoresis-quality) in 400 mL dH$_2$O to 70 °C, and adjust pH with concentrated HCl to 7.2, fill to 500 mL; no sterilization required. Store at room temperature.

- **0.5 M EDTA**
  Dissolve 18.6 g EDTA in 80 mL dH$_2$O by adjusting the pH to 8.0 (ca. 2 g NaOH pellets required), and fill to 100 mL with dH$_2$O. Sterilize by filtration and store at 4 °C.

7.5.2   Procedure

a. Sample collection. Fresh samples are collected and fixed immediately as follows (can be kept at 4 °C for 2-3 days without impact on the cells).

b. Fixation. The sample is usually fixed for both Gram-negative and Gram-positive cells, unless the target population is known.

(i) Gram-positive cells: Mix equal volumes of activated sludge (mixed liquor) and 96 % ethanol. Keep in the freezer (-20 °C).

(ii) Gram-negative fixation: Centrifuge 5 mL sample for 8 min at approximately 3,400 × g. Remove the supernatant and replace it with cold 4 % PFA/PBS. The cell suspension is fixed for 3 h at ~4 °C. Centrifuge for 8 min at approximately 3,400 × g. Remove the supernatant (PFA) and discard appropriately. Add 5 mL of cold tap water and mix before centrifugation for 8 min at approximately 3,400×g. Remove the supernatant. Repeat this step once more. Add 5 mL of cold sterile filtered tap water and mix. The sample is now ready for the FISH procedure.

The fixed sample can be kept at -20 °C for several months. Prior to usage it must be treated as follows: Centrifuge for 8 min at approximately 3,400×g. Remove the supernatant and replace by a cold solution of 1:1 PBS/EtOH. If the sample has been kept in 1:1 PBS/EtOH, it must be washed once and re-suspended in tap water prior to immobilization.

c. Immobilisation on slides. Spread 15 μL of the sample on a cover slip or in each well of a Teflon-coated slide. Use the pipette tip to evenly distribute the sample. Allow the samples to dry in a fume hood until completely dry (15-30 min). Drying at 46 °C appears to improve the binding of more dilute samples to the surface of the slide compared with drying at room temperature.

d. Dehydration. Dehydrate the slides in EtOH by stepwise increasing concentrations as follows: 3 min in 50 % ethanol followed by 3 min in 80 % ethanol and by 3 min in 96 % ethanol. The ethanol can be used several times, so can be stored in a fume cupboard. Allow the slides to air-dry completely before FISH probe hybridisation. DAPI-stained samples must not be dehydrated, as they fade rapidly in the presence of ethanol.

e. Permeabilization. Some cells (e.g. Mycolata) require pretreatment to bring about permeabilization of their cell wall/envelope. This can best be achieved enzymatically with exposure to Lysozyme and proteinase K, or chemically (mild acid treatment). Which enzymes are used, their concentrations and incubation conditions etc. depend on the nature of the sample and cells, and should be optimized for each sample. However, many activated sludge samples can be FISH-probed without any enzymatic or chemical permeabilization or by applying mild treatment. Where necessary the methods detailed in the following protocols should be used:

(i) Permeabilization with lysozyme
- Apply 10-15 μL cold lysozyme (36,000-360,000 U mL^{-1}) to the sample on a slide or slide well. Place the slide in a horizontal position, in a 50 mL polyethylene tube, containing a tissue paper soaked with 2 mL of dH$_2$O.
- Incubate the slide for 10-60 min, depending on the nature of the cells, at 37 °C.
- Wash the slide 3 times in dH$_2$O, and once in absolute ethanol, and allow it to air-dry.

The slide can now be stored at -20 °C for several months.

(ii) Permeabilization with proteinase K
- Apply 10-15 μL cold proteinase K (2,000-20,000 U mL^{-1}) per slide or slide well and transfer to a 50 mL polyethylene tube lined with a moisturized tissue paper. Incubate for 20-60 min at 37 °C.
- Wash the slide 3 times in dH$_2$O, then once in absolute ethanol, and let the slide air-dry.

At this point the slide can be stored at -20 °C for several months.

(iii) Permeabilization with mild acid hydrolysis
- Submerge the slides with dehydrated cells in hydrochloric acid (1 M HCl) at 37 °C for 30 min.
- Wash the slide with dH$_2$O, then once in absolute ethanol, and let the slide air-dry.

f. Hybridisation. For optimal hybridization of fluorescent oligonucleotide probes all the factors affecting the success and outcome of the approach need to be taken into account every time a new system is examined.

(i) Prepare hybridisation buffers (Table 7.4) containing appropriate hybridization stringency conditions for each oligonucleotide probe that can be found in probeBase (Loy *et al.,* 2003). If the probe has not been properly tested, these conditions will need to be determined for each new probe in an empirical fashion.

(ii) Transfer 8 μL of hybridisation buffer onto the slide within an area of 1-2 cm^2 on a glass slide or into each well of a Teflon-coated slide with one or more wells.

(iii) Add 1 μL of each gene probe (probe concentration 50 ng μL^{-1}) and mix carefully (avoid contact with the sample) with the hybridization buffer (sterile pipettes must be used for all work with gene probes). If more gene probes are added to the same well the order is unimportant. Equimolar concentrations of each probe should be used. Place the slide horizontally into a sterile Greiner tube (50 mL) with a piece of cotton paper wetted with 1-2 mL of the same hybridisation buffer. Place the tube in the hybridisation oven (46 °C) for 1½ hour.

Table 7.4 Hybridization buffer (46 °C) for the FISH procedure.

FA %	FA μL	dH$_2$O μL	5 M NaCl μL	1 M Tris/HCl μL	10 % SDS μL
0	0	1,600	360	40	2
5	100	1,500	360	40	2
10	200	1,400	360	40	2
15	300	1,300	360	40	2
20	400	1,200	360	40	2
25	500	1,100	360	40	2
30	600	1,000	360	40	2
35	700	900	360	40	2
40	800	800	360	40	2
45	900	700	360	40	2
50	1,000	600	360	40	2
60	1,200	400	360	40	2
65	1,300	300	360	40	2
70	1,400	200	360	40	2

g. Washing.
(i) Prepare 50 mL washing buffer (Table 7.5) and preheat in a water bath to 48 °C before washing.
(ii) After hybridisation, carefully remove the slide from the Greiner tube using a pair of tweezers.
(iii) Pour a few millilitres of the preheated washing buffer on the top of the slide (Caution! Not directly on the sample) to remove excess probe solution from the wells.
(iv) Put the slide into the preheated (48 °C) 50 mL Greiner tube containing the washing buffer for 15 min in a 48 °C water bath to wash out the unbound probes.
(v) Again remove the slide carefully with a pair of tweezers. Rinse with cold dH$_2$O by dipping the slide in a glass beaker to remove any crystallised NaCl.
(vi) Allow the slides to air-dry.

Table 7.5 Washing buffer (48 °C) for the FISH procedure.

FA %	1 M Tris/HCl (pH 8,0) µL	10 % SDS µL	5 M NaCl µL	0.5 M EDTA µL
0	1,000	50	9000	0
5	1,000	50	6,300	0
10	1,000	50	4,500	0
15	1,000	50	3,180	0
20	1,000	50	2,150	500
25	1,000	50	1,490	500
30	1,000	50	1,020	500
35	1,000	50	700	500
40	1,000	50	460	500
45	1,000	50	300	500
50	1,000	50	180	500
55	1,000	50	100	500

h. Microscopy (EPI-fluorescence and CLSM). The dried slides can now be examined microscopically.

 (i) Place a small drop of Citifluor (or Vectashield, or a mixture hereof) mounting fluid on the slides and put a cover slip on top. Make sure that the Citifluor is distributed to all the wells before microscopy.

 (ii) If the slides are not evaluated on the same day of hybridisation they can be stored at -20 °C for some weeks, where the intensity of the fluorescence signal remains virtually unchanged.

7.6 COMBINED STAINING TECHNIQUES

Many different staining techniques can be combined to provide a better understanding of the relationships between bacterial identification and their function. This approach is very powerful and can provide ecophysiological information at the single cell level in complex communities. Thus they allow detection of intracellular storage compounds such as poly-P and PHA in populations of interest, and help explain which organisms are responsible for the chemical transformations in processes such as enhanced biological phosphate removal. They can also identify cells that are able to synthesise specific extracellular enzymes or cells whose surfaces are hydrophobic, which is an important cell feature linked to foaming (Table 7.6).

Most of the bacteria in complex mixed natural communities such as those in activated sludge cannot be isolated and grown in pure culture, and even establishing enrichment cultures in the laboratory is likely to modify the selective natural biotic and abiotic parameters. So it

is necessary to study their functions directly in the natural habitats. Many such studies have been carried out through use of combined methodologies as those described here, with the hope that other investigators will adopt them in their studies (e.g. Nielsen *et al.,* 2010a,b).

Table 7.6 Staining techniques that can be combined to link identification with function.

Identification	FISH, Gram	References
Substrate uptake	Microautoradiography	Nierychlo *et al.,* 2015
Surface properties	Microsphere adhesion to cell	Nielsen *et al.,* 2001
Surface components	Lectins, antibodies	Böckelmann *et al.,* 2002
Exoenzymatic activity	Enzyme-linked fluorescence	Nielsen *et al.,* 2002, 2010a Kragelund *et al.,* 2007 van Ommen Kloecke and Geesey, 1999
Internal storage products	PHA, poly-P, Sulphur	Nielsen *et al.,* 2010b

An example is given below on how to apply the FISH technique in combination with (*i*) DAPI staining and (*ii*) PHA staining to detect and identify the bacteria capable of taking up poly-P (PAOs) and bacteria accumulating large quantities of bioplastics in the form of PHA in activated sludge systems designed to remove phosphorus microbiologically. Such data will contribute toward a better understanding of the complex microbiology of these processes (e.g. how PAOs may outcompete other organisms) to pave the way for an eventual knowledge-based optimization of the wastewater treatment plants with EBPR. It is remarkable that most PAOs have never been isolated in pure culture, but are studied either as enrichment cultures or in full-scale systems.

DAPI staining can detect all bacteria from its non-specific binding to DNA. However, DAPI has also been known to stain intracellular poly-phosphate granules in bacterial cells when applied at elevated concentrations. The fluorescence spectra of DAPI-DNA complexes exhibit a maximum fluorescence emission at around 450 nm (blue), whereas DAPI-poly-P complexes produce a so-called bathochromic shift with emission around 525-550 nm (green-yellow). While DNA is usually stained by working solutions containing 1 µg mL^{-1} DAPI, poly-P detection is carried out by incubating cells with 50 µg mL^{-1} DAPI.

7.6.1 FISH – DAPI staining

7.6.1.1 Reagents and solutions for DAPI staining

- **DAPI stock solution and storage** (see Section 7.4.4.1)

- **Reagents used for FISH** (see Section 7.5.1)

7.6.1.2 Procedure

a. Perform FISH as described above using FISH probes designed to target the PAO, and the appropriate conditions for fixation, immobilisation and probe hybridisation.

b. After incubation in the dark for 30 min at room temperature (longer staining might be required for some samples), remove any excess DAPI by centrifugation and rinse with plenty of sterile water until any background fluorescence is minimal.

c. Allow to air-dry. A FISH and DAPI stained slide can be stored at -20 °C indefinitely.

d. Apply a drop of anti-fading agent (Citifluor, Vectashield or a mixture) directly onto the sample. Cover it with a cover slip and examine under the epifluorescence microscope. The excitation maximum for DAPI bound to dsDNA is 358 nm and 525-550 nm for poly-P. The emission maximum is 461 nm.

A filter set with a bandpass excitation maximum at 350 nm and a 500 nm long pass emission filter, is combined with the appropriate dichroic mirror (see the manufacturers' instructions).

The best results for the combined use of FISH with DAPI-poly-P staining are obtained with probes tagged with red-shifted fluorescence dyes such as Cy3 for FISH.
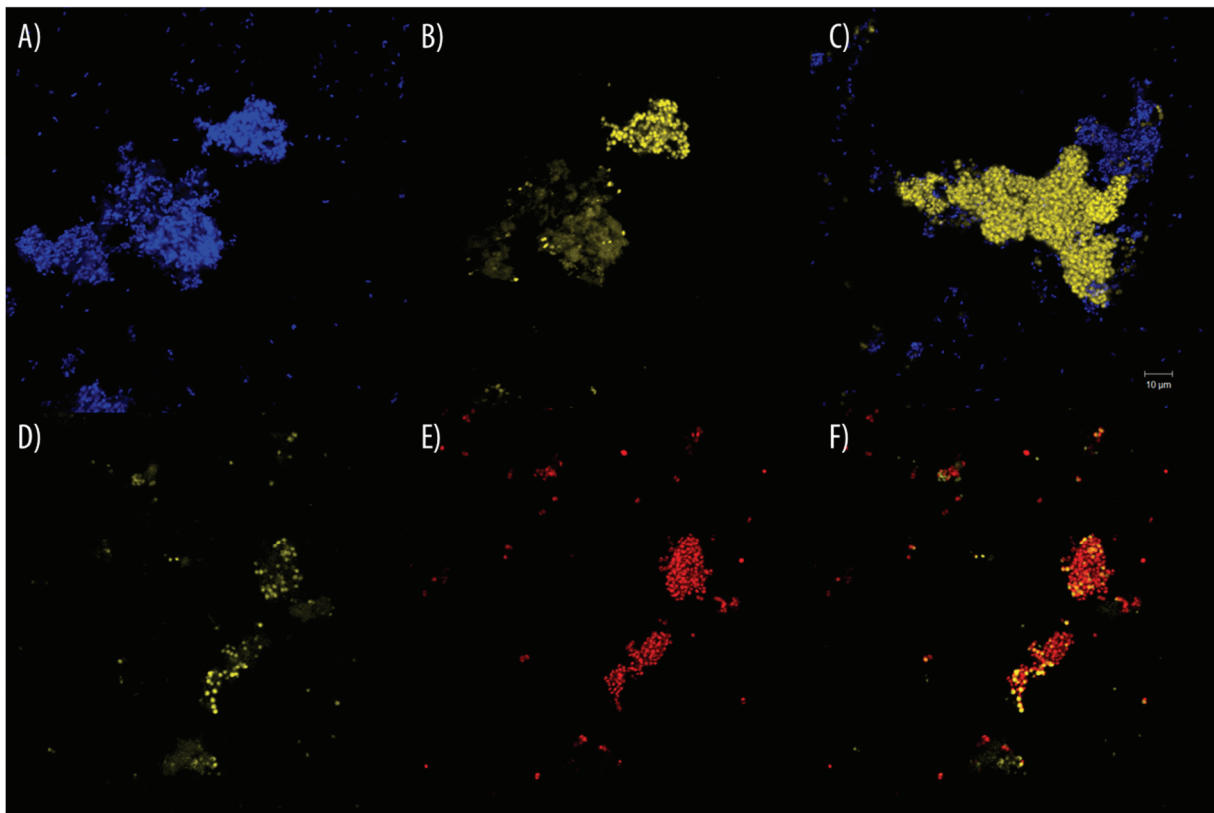

Figure 7.7 Combined use of DAPI staining (blue) for poly-P staining (yellow) and FISH (red) for identification of the cells. Image (A) and image (B), and (D), (E) and (F) show the same microscopic field, respectively.

7.6.2 FISH – PHA staining

7.6.2.1 Reagents and solutions for PHA staining

- **Reagents used for PHA staining**
    (i) 1 % (*v/v*) aqueous solution of Nile Blue
    (ii) 8 % (*v/v*) acetic acid

- **Reagents used for FISH** (see Section 7.5.1)

7.6.2.2 Procedure

a. Dilute freshly collected activated sludge with water to a final concentration of approximately 1 g MLSS L^{-1}, and transfer 20 μL to a gelatine-coated microscope slide. Allow to dry. Homogenization can be helpful to increase resolution.

b. Stain the sample by dipping the slide into a suspension of 1 % aqueous solution of Nile Blue (heated to 55 °C) for 10 min.

c. Remove the excess stain by carefully rinsing with dH_2O at room temperature.

d. Wash the stained cells for 1 min in 8 % acetic acid.

e. Remove the excess acetic acid by carefully rinsing with dH_2O.

f. Allow the slide to air-dry.

g. Add a drop of dH_2O to the slide, and examine with an epifluorescence microscope (excitation wavelength 630 nm). A positive PHA staining response will be shown by the presence of fluorescent PHA granules inside the cell, and be readily distinguishable from negatively-stained cells.

h. Record any field of interest with a charge coupled device (CCD) camera or a laser-scanning microscope and store the coordinates for that field on the microscopic micrometre stage. Alternatively mark the position of the light cone with a pencil on the side of the slide.

i. Remove the slide from the stage, and let the slide air-dry.

j. Fix the sample directly on the slide with PFA or EtOH followed by a permeabilization step (if required) as described under the FISH procedure. Allow the slide to air-dry.

k. FISH is carried out as described in the Section 7.5. For clear differentiation of the fluorescence signals from PHA staining and FISH hybridization, ensure oligonucleotide probes labelled with a strong fluorochrome clearly distinguishable from the PHA emission spectra (such as FLUOS, Cy5) are used.

l. Relocate the co-ordinates where the PHA images were acquired on the microscope stage, and evaluate the FISH signal. Obtain the images before and after applying the FISH and evaluate the identity of those cells with positive PHA staining.

PHA staining can be carried out on ethanol or PFA fixed samples, but fixation directly on the slide between the PHA staining and the FISH procedure should be avoided.

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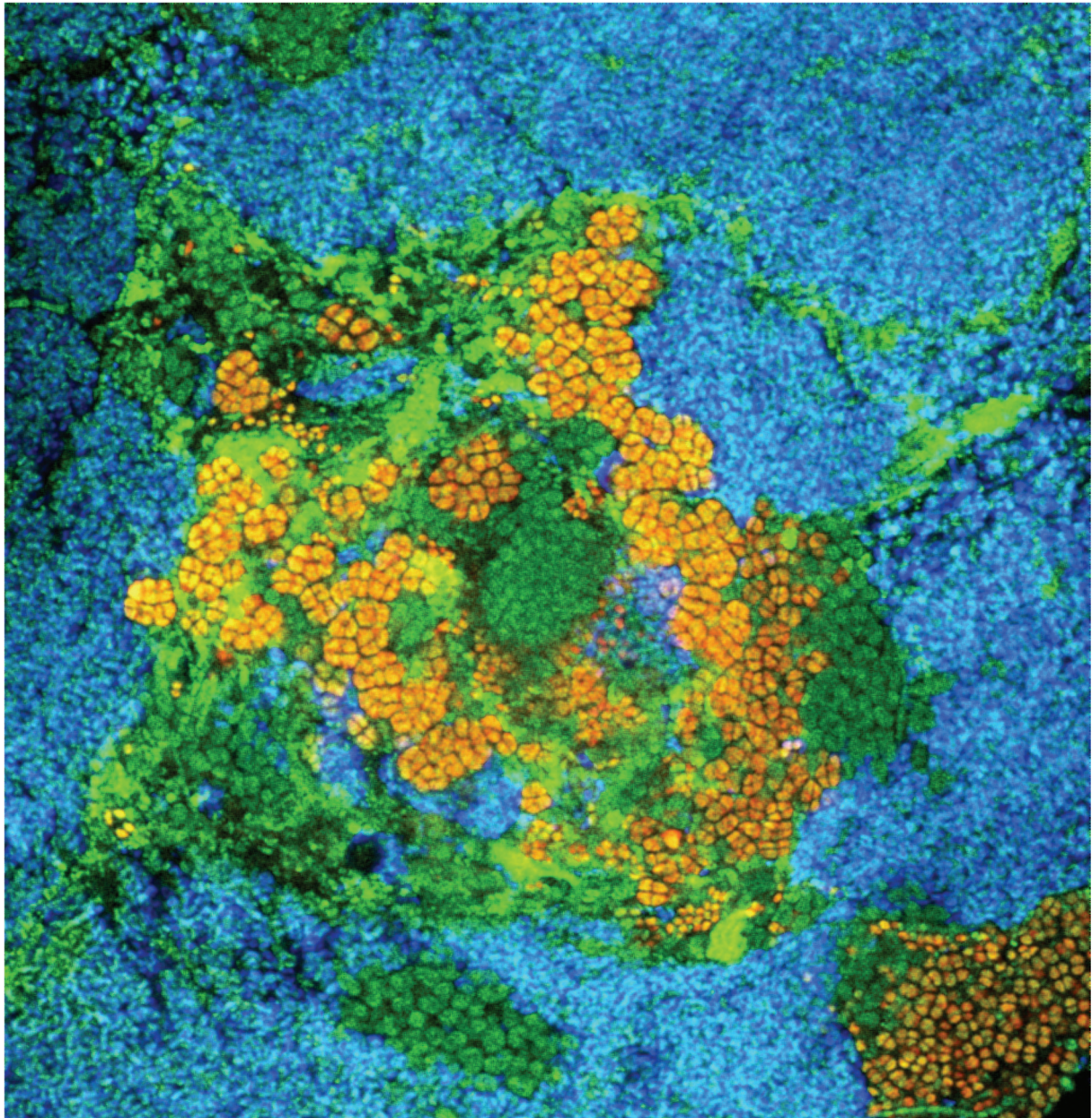
Figure 7.8 Result of the application of FISH procedure on the sludge sample from an EBPR removal reactor. Blue: Accumulibacter (PAOs targeted by probe PAOmix – Cy5); red: Defluviicoccus (GAOs targeted probe DF2mix – Cy3); green: Eubacteria (targeted by probe EUB338 mix), (image: McIlroy, 2016).