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MAGDALENA DOMAŃSKA[1], RAMONA KUHN[2],
JANUSZ ŁOMOTOWSKI[3], EWA STAŃCZYK[3]

# FISH METHOD FOR IDENTIFICATION OF MICROBES IN WASTEWATER DISTRIBUTION SYSTEMS

The fluorescence in situ hybridization (FISH) method is widely used to identify various types of cells. In comparison to cultivation dependent methods, identification of microbes by FISH is easier and generally takes several hours. A review of improvements to the FISH method has been presented, its advantages and disadvantages, as well as examples of application. Particular consideration was given to the efficiency of identification of microbes in samples taken from sewerage. The effectiveness of the method was confirmed by the results obtained in samples from the membrane bioreactor.

## 1. INTRODUCTION

In the last two decades there has been a rapid progress in culture-independent, qualitative methods for exploring microbial communities [1]. The methods most widely used in molecular biology for the study of microorganisms include polymerase chain reaction (PCR), gel electrophoresis and its modifications, cloning of DNA sequences, sequencing of DNA, DNA micromatrices, green fluorescent proteins (GFP), southern hybridization, northern hybridization and fluorescence *in situ* hybridization [2]. Fluorescence *in situ* hybridization (FISH) technique using oligonucleotide probes labeled with fluorochromes was described in 1989 by Edward F. DeLong [3]. A year later it was presented by Amann who significantly contributed to the development of this method [4, 5]. This revolutionary approach enabled scientists rapid identification of microorganisms in environmental samples.

[1]Wroclaw University of Environmental and Life Sciences, Faculty of Environmental Engineering and Geodesy, Environmental Engineering Institute, pl. Grunwaldzki 24, 50-323 Wrocław, Poland, corresponding author, M. Domańska, e-mail: magdalena.domanska@up.wroc.pl

[2]Brandenburg University of Technology, Siemens-Halske-Ring 8, 03046 Cottbus, Germany.

[3]Kielce University of Technology, Faculty of Civil and Environmental Engineering, Chair of Environmental Engineering and Protection, al. Tysiąclecia Państwa Polskiego 7, 25-314 Kielce, Poland.

FISH is an advanced tool for identifying morphological features of microorganisms, specifically if their morphology is incompatible with the standard description. Identification of microorganisms with the FISH method can be performed owing to the availability of special molecular probes containing a sequence of the genetic material complementary to the known sequence of RNA or DNA. During the analysis, the RNA sequences attach to the corresponding sequences of the genetic material of the bacteria to be detected. Owing to the fluorescent molecule attached to the acid, the microorganisms become well visible under the microscope [6].

The technique is based on targeting 16S or 23S ribosomal RNA (rRNA) with a designed complementary RNA probe. In more detail, such standard probes are rRNA-targeted oligonucleotides with an attachment at the 5′ end. The attachment is commonly a single fluorescent dye molecule such as fluorescein, Texas Red, indocarbocyanine dyes and others. Nowadays, the user only needs to submit the complementary sequence and wanted dyes to a company of his or her choice and receives the probe within few days. The labeled probe is transferred into the cell to the ribosomes, where it binds to the complementary RNA sequence if available. Laser light of a fluorescence microscope promotes then the electron of the dye to a higher energy level for a short time, and this results in a photon emission, the so-called specific fluorescence signal of the probe. The intensity of the fluorescence signal increases with the increase in the number of ribosomes. The increase originates from the increased protein synthesis or, in other words, from increased cell activity. Thus, the FISH signal can also provide information on the active state of the target microorganism.

The paper presents the potential of FISH method to determine the microbial composition of the activated sludge.

## 2. MODIFICATIONS AND IMPROVEMENTS TO THE FISH METHOD

FISH is a reliable and rapid method as its performance takes up to several hours. Visualization is possible with using standard fluorescence microscope or confocal laser scanning microscope (CLSM) and also in combination with other microscopes, such as electron microscope, RAMAN spectroscope in combination with a microscope or nanoSIMS. Most standard epifluorescence microscopes are equipped with sets of filters, which allow one to separate recording at least two or three differently labeled probes and one nucleic acid such as DAPI (4′,6-diamidino-2-phenylindole). Then this image recording can be combined into a single image with a software package for the image analysis. Progress based on advanced confocal laser scanning microscopy with multiimage analysis, as well as special tools such as spectral imaging or FISH protocols using quantum dots for labeling probes, allow simultaneous combination of a much larger number of differently labeled probes [7]. However, a low ribosome content of the sample is a frequent cause for an insufficient signal

intensity [8]. This is why the FISH technique has been subject to continuing improvements (Table 1) [9].

Table 1

Improvements to the FISH method reported in the time span of 1998–2003 [9]

| Task | Problem | Solution |
|---|---|---|
| Probe design and evaluation | limited accessibility of probe target site | check in probeBase |
| | | helper probes |
| | | PNA probes |
| | no pure culture available | clone-FISH |
| | determination of optimal hybridization conditions | digital image analysis |
| Detection in complex samples | low abundance | enrichment by filtration, flow cytometry, substrate addition |
| | target cells not detectable (low ribosome content) | CARD-FISH |
| | | polyribonucleotide probes |
| | target cells not detectable (insufficient permeabilization) | PNA probes |
| | | enzymatic pretreatment modification of fixation |
| | bad signal/noise ratio | self-ligation probes |
| | | polyribonucleotide probes |
| | | CARD-FISH |
| Quantification in complex samples | biofilms/cell aggregates hamper manual counting | Spike-FISH |
| | | digital image analysis |
| Analysis of general metabolic activity | ribosome content does not always correlate with activity | FISH/MAR |
| | | FISH/BrdU |
| | | rRNA open spacer probes |
| Analysis of specific function | phylogeny rarely reflects physiology | FISH/MAR |
| | | FISH/BrdU + inhibitors of substrates |
| Phylogenetic assignment/sorting of rDNA clones | | Clone-FISH + flow cytometry |

In the past years FISH has been improved by developing and implementing new labeling and detection methods, as well as by combining FISH with other techniques, which significantly enhanced its sensitivity and expanded its potential for detection [8]. Below is a review of these developments:

- MAR-FISH consists in combining microautoradiography (MAR) with fluorescence *in situ* hybridization (FISH), i.e. in radioisotopic, using organic and inorganic substrates radiolabeled with $^{3}H$ or $^{14}C$. This technique does not only enable one to examine labeled components in complex microbial systems but also to identify metabolic activity of bacteria at a single cell level [10, 11]. The limitation of this method is

the lack of taxonomic resolution of FISH probes and inability to use many probes simultaneously [12].

- FISH is also combined with microsensors. Microsensors are an effective means for evaluation of biological activity in the biofilm. With their microelectrodes, it is possible to measure the concentrations of substrates and products in the biofilm interior. The combination of the two techniques is well suited, e.g., for the determination of sulfate reduction or changes in the biofilm's substrate, and provides reliable direct information on the relationship between microbial activity and the occurrence of specific microorganisms in biofilm communities [11].
- CARD (catalysed reporter deposition)-FISH involves horseradish peroxidase (HRP) and tyramide signal amplification, which makes it possible to significantly enhance the signal intensity of hybridized cells and identify cells at low ribosome content of the sample [2, 8]. Unfortunately, the protocol is more complicated and multicolor imaging options are very laborious and limited [13].
- Clone-FISH. In this method, identification performed with FISH is preceded by generating the expression of the 16S or 18S rRNA targeted gene in *Escherichia coli*. Probes can also be designed by making use of the Cat-FISH method [2, 14].
- CLASI-FISH (FISH combined with combinatorial labeling and spectral imaging) and DOPE-FISH (FISH combined with double labeling of oligonucleotide probes) solve the problem of how to simultaneously identify many microbial populations. The methods also offer an approximately twofold enhancement of signal intensity [8, 13]. In CLASI-FISH there is a possibility of detecting 28 target organisms simultaneously. However, this approach exploits two probes for each target organism, hybridizing to the same binding site, which affects reducing signal intensity [13].
- RING-FISH (with RING standing for recognition of individual genes) is a method used for the identification of individual genes in a single bacterial cell by using high concentrations of polynucleotide probes for the increase in sensitivity and visualization [15].
- FISH-DVC (direct viable count) is applied in the analysis of water samples with low bacterial counts [11, 16].
- RCA-FISH (with RCA denoting rolling circle amplification) is a technique designed to strength a signal amplification for low or single copy number targets like genes on microbial genomes, e.g., to identify the genes responsible for denitrification processes [8].
- FISH-BrdU. In this method, microorganisms are identified using 5-bromo 2-deoxy-uridine (BrdU). There is no need to use paraformaldehyde for cell fixation or formamide for DNA denaturation. The method is believed to be safer than classical FISH techniques [17];
- Spike-FISH. In this approach, *Escherichia coli* cells are used as the internal standard to determine absolute numbers of ammonia oxidizing bacteria in activated

sludge. Quantification is based on an internal standard, which is introduced by spiking the samples with known amounts of *E. coli* cells [18].

- RAMAN-FISH extends and complements current technologies such as FISH--microautoradiography and stable isotope probing, and can be applied (with Raman microspectroscopy) for investigations of complex microbial communities at a single--cell resolution [19];
- nanoSIMS is based on the visualization of oligonucleotide probe-conferred hybridization signal in single microbial cells and isotopic measurement using high resolution ion microprobe (NanoSIMS) [20].

The solutions mentioned above represent only a few combinations of the FISH method. More examples have been described by Volpi and Bridger [15].

## 3. EXAMPLES OF APPLICATION

As a versatile method enabling identification of microbial species and their visualization on the surface examined [21], FISH has found wide acceptance in many different fields. A common use of FISH is in research on the morphology of biofilms growing on surfaces of contact with natural water, tap water and wastewater. Another major use where FISH is applicable includes observations of membrane biofouling in microfiltration, nanofiltration and reverse osmosis installations. Serious problems arise when the biofilm develops on the internal walls of plastic water pipes that are widely used in distribution systems [22]. Rapid identification of the microbes colonizing the biofilm offers the possibility for an effective control of their growth. FISH has successfully been applied for the detection of *Cryptosporidium parvum*, as well as for the identification of viable *Cryptosporidium parvum* oocysts and oocytes in water [23]. Combined with flow cytometry, FISH enables the detection of *Cryptosporidium parvum* oocytes in samples with low bacterial counts.

FISH-based methods have revolutionized investigations into the morphology and microbial composition of activated sludge. They enable the following bacteria to be mapped into the structures of activated sludge flocs and granules: AOB (ammonium-oxidizing bacteria) and NOB (nitrite-oxidizing bacteria) [24, 25], denitrifying bacteria [8] and phosphorus-accumulating bacteria [26]. All of these bacteria have their characteristic colours, but extracellular polymeric substances (EPS) fail to display a specific pigmentation.

## 4. APPLICATION POSSIBILITIES OF FISH METHOD FOR ACTIVATED SLUDGE

FISH performance is carried out easily within few steps. First the water sample has to be fixed. According to Amann [5], for most gram-negative bacteria paraformaldehyde is good to be used whereas ethanol is better for gram-positive bacteria. The

fixation step is very important, otherwise degradation of ribosomes is risked. This could lead to underestimation of the fluorescence signal. For some water samples, it is necessary to perform enzymatic digestion after fixation. Especially this is required for samples with very dense biofilm formations that impede direct diffusion of the probe into the target cell. Enzymes like lysozyme or trypsin break down most polysaccharides and proteins, respectively, and therefore can enhance the transport of the probe through the biofilm into the cell. The hybridization step is then directly carried out on the sample itself. Therefore the sample is spotted on teflon coated microscope slides and air-dried. In a next step, the sample is dewatered through stepwise incubation in ethanol from low to high percentage, i.e. from 50% to 98%, respectively. After dewatering, the hybridization step is carried out through adding a mixture of salts, formamide and detergents, and including the probe directly into the sample, which will be then incubated in the dark for at least two hours. Subsequently, the sample has to be washed of residual probes and can be analyzed under the fluorescence microscope.

### 4.1. EXPERIMENTAL

*Sample preparation*. MBR11 was operated to perform biodegradation of carbon and nitrogen compounds. The MBR had the volume of 0.12 $m^3$ and was equipped with a Zenon Zee-Weed hollow fiber membrane module (0.93 $m^2$). The reactor was fed with municipal wastewater providing an average of 400 mg COD/$dm^3$.

For the FISH experiment, the sludge samples were fixed in 4% paraformaldehyde. 0.01 $cm^3$ well suspended sludge was placed on teflon coated wells of microscope slides fixed with 1% gelatine. The slides were air dried. The FISH procedure was carried out according to Amann et al. [4] but a detailed procedure may also be found elsewhere [1, 27].The following probes for hybridization were applied: EUB338 mix (universal oligo probe which covers 90% bacteria from bacteria domain) [4, 28], ALFA968 (alpha-proteobacteria) [29], NSO1225 (ammonia-oxidizing bacteria) [30], THAU646 (*Thauera* spp.) [31]. Probes ALFA968, NSO1225, and THAU646 were labeled with the sulfoindocyanide dye CY3. EUB338 mix was labeled with fluorescein isothiocyanate (FITC). Hybridization was performed with different stringency at 20% for EUB338 mix and ALF42a probes, and 35% for NSO1225 and THAU464 probes. The hybridization solution contained 20% or 35% formamide, 0.9 mM NaCl, 20 mM *tris*-HCl (pH 7.2), and 0.01% sodium dodecyl sulfate. 2500 ng of each fluorescence labeled probe were mixed with the hybridization solution. Approximately 0.01 $cm^3$ of that mix was added to each sample well and incubated in a hybridization chamber at 46 °C for 2 h in the dark. The chamber included saturated filter paper with excess hybridization buffer. After the hybridization step, all slides were gently rinsed with pre-warmed washing buffer in the dark at 48 °C for 20 min. All slides were air dried in the dark. For total cell staining, DAPI was added in a final concentration of

1 μg/cm$^3$. Subsequent slides were closed with cover slides and sealed. Slides were examined using an epifluorescence microscope (Laborlux S, E. Leitz, Wetzlar, Germany) equipped with a mercury short-arc photo optic lamp (HBO 103 W/2, OSRAM Sylvania) at 10× and 1000×. The following light filter sets were used: Chroma #31000 for DAPI (excitation, 365 nm, dichroic mirror, 395 nm, suppression, 397 nm) and Chroma 41007A for CY3 (excitation, 546 nm, dichroic mirror, 580 nm, suppression, 590 nm) (Chroma Technology Corp., Brattleboro, Vt.). Pictures were taken with a charge-coupled device camera (Photometrics Coolsnap, Roper Scientific, Trenton, N.J.) and the Cool Snap software (version 1.0.0, Roper Scientific).

*Short discussion*. Figures 1–3 depict the results obtained for the bacteria isolated from the membrane bioreactor (MBR11). The bar represents the distance of 10 μm applied to all FISH pictures. Figure 1 shows the result of using EUB338 probe (Fig. 1a) which covers 90% of bacteria from bacteria domain, and ALF968 probe (Fig. 1b) for alpha-*Proteobacteria.* It can be noticed that the bioflocs mostly consist of alpha-*Proteobacteria.* Figure 2 presents microorganisms in the activated sludge biomass. The most microorganisms identified in the sample with the use of DAPI (Fig. 2c) are eubacteria (Fig. 2a) among which ammonia oxidizing bacteria (Fig. 2b) constitute a significant part. Unlike the amount of ammonia oxidizing bacteria shown in Fig. 2b, it is visible that in Fig. 3b after using THAU464 probe, there are few *Thauera spp*. bacteria found in the sample. This results confirm that FISH method provides information about the microbial composition in the sample and enables to access which microbes are dominant in the sample. However, despite many publications on this subject we are still not able to receive reliable information on the biomass of microorganisms in the wastewater. It is advisable to explore this issue in the future.

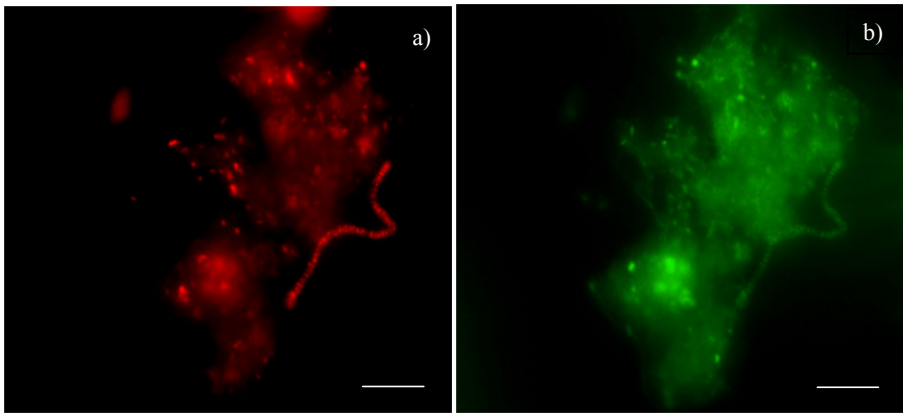


Fig. 1. FISH analysis for determination of alpha-*Proteobacteria* in the bioflocs taken from MBR11. Hybridization with the universal oligo probes EUB338 (a) and ALFA968 (b). Scale bar, 10 μm

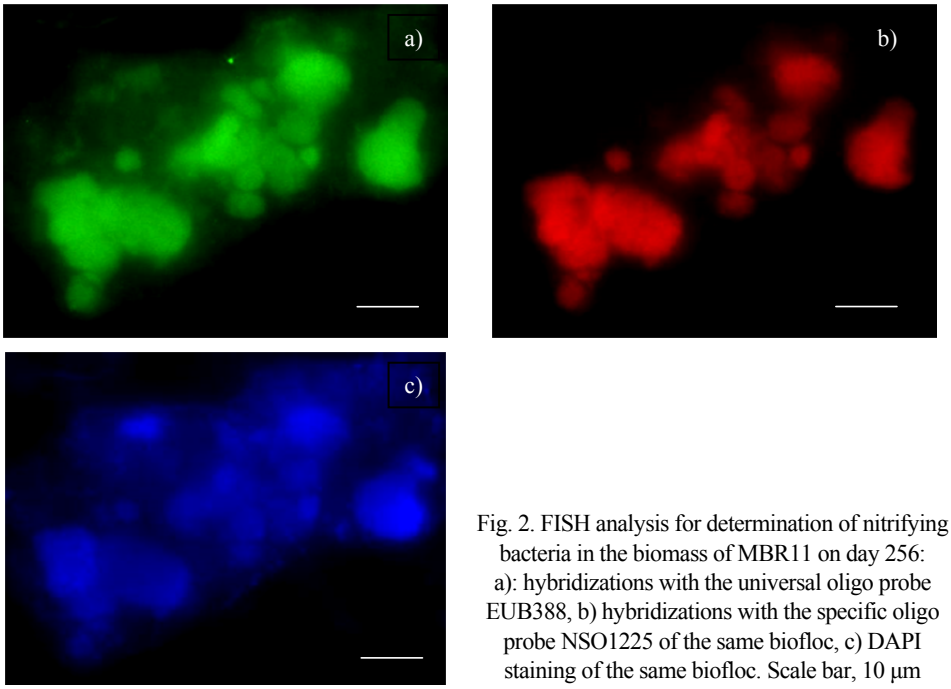


Fig. 2. FISH analysis for determination of nitrifying bacteria in the biomass of MBR11 on day 256: a): hybridizations with the universal oligo probe EUB388, b) hybridizations with the specific oligo probe NSO1225 of the same biofloc, c) DAPI staining of the same biofloc. Scale bar, 10 µm

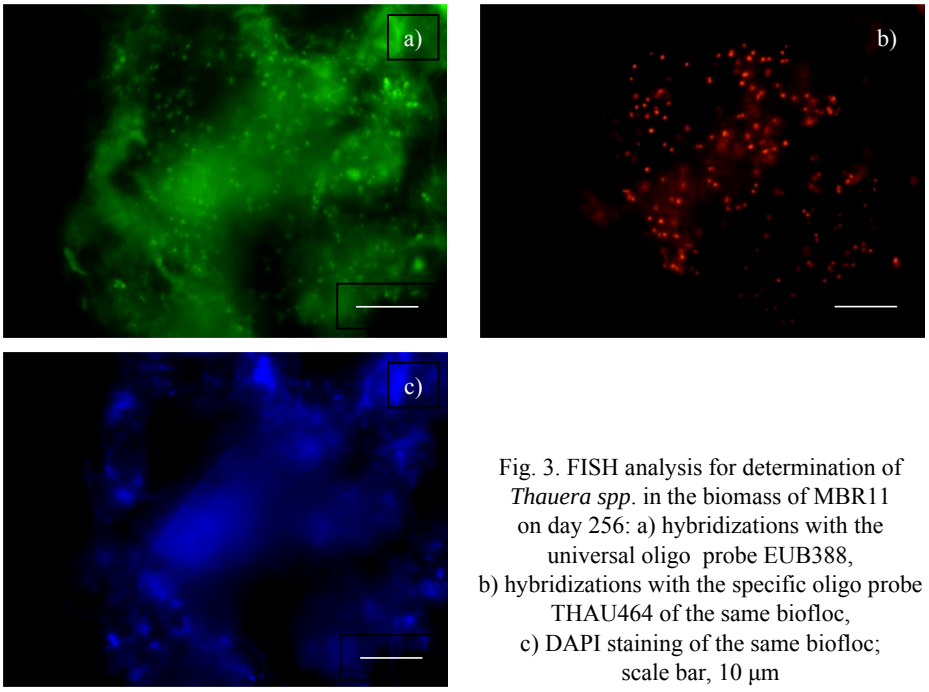


Fig. 3. FISH analysis for determination of *Thauera spp*. in the biomass of MBR11 on day 256: a) hybridizations with the universal oligo probe EUB388, b) hybridizations with the specific oligo probe THAU464 of the same biofloc, c) DAPI staining of the same biofloc; scale bar, 10 µm

## 5. CONCLUSIONS

• The advantage of the FISH is its simple and rapid performance as well as high sensitivity and reproducibility. The method had a great contribution to the research on the internal structure of the flocs, which enabled to put into the practice aerobic and anaerobic granular sludge, which has excellent settling ability.

• Apart from many advantages, FISH-based techniques are cost intensive thus their widespread use is seriously hampered by the inability of many laboratories to carry the high cost of fluorescence microscopes and molecular probes. Moreover, existing studies are merely focused on qualitative identification of bacteria in aerobic and anaerobic granular activated sludge and the biomass of bacteria cannot be determined in a practical way. It seems advisable, however, to initiate close cooperation between biotechnologists and engineers responsible for the operation of wastewater distribution systems in the field of implementing FISH-based methods in the future, especially in the area of practical quantitative usage.

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