Monitoring of microbial diversity by fluorescence *in situ* hybridization and fluorescence spectrometry

V. Ivanov, S.T.-L. Tay and J.-H. Tay

School of Civil and Environmental Engineering, Nanyang Technological University, 50 Nanyang Ave., Singapore 639798

Abstract The goal of the research was the development of a simple method to quantify microbial groups in environmental samples. Fluorescence intensity was measured in the sample before and after whole cell fluorescence *in situ* hybridization with rRNA-targeted, fluorochrome-labeled oligonucleotide probes. To determine specific and non-specific binding of different oligonucleotide probes the following approaches have been used: (1) incubation of the sample with probes at two different temperatures; (2) hybridization of labeled probe in the presence of unlabeled probe; (3) incubation of the sample with labeled specific probe or labeled nonsense probe. Specific binding (hybridization) of the probe was calculated as the difference between total binding and non-specific binding of the probe. Specific binding was 40–50% of total binding in the environmental samples tested. The ratio of the specific binding of different probes may be used to quantify the ratio of different microbial groups in the environmental samples. This quantification is suitable for the microbiological monitoring of microbial aggregates because it is a simple technique and the results can be measured by a portable fluorometer.

Keywords Fluorescence *in situ* hybridization; fluorescence spectrometry; microbial aggregates; oligonucleotide probes

Water Science and Technology **Vol 47 No 5 pp 133–138** © IWA Publishing 2003

Introduction

Whole cell fluorescence *in situ* hybridization (FISH) with rRNA-targeted, fluorescent oligonucleotide probes is a popular approach to study the microbiology of complex microbial communities (Amann and Kuhl, 1998). Whole cell FISH is usually detected by confocal scanning laser microscopy (CSLM) (Tagawa *et al*., 2000) but it is complicated by the variability of the microscopic images and CLSM parameters. Information on the relative abundance of the rRNAs of the target microbial groups can be obtained by hybridization of ^{32}P-labeled oligonucleotide probes with extracted RNA (Hansen *et al*., 1999; Raskin *et al*., 1994). The disadvantages of this method are the extraction of RNA and the use of radioactive labels. In this research, the fluorescence of the probe was measured for the bulk sample before and after FISH and was not hampered by the physical heterogeneity of the sample. The main problem in the measurement of FISH by fluorescence spectrometry is in differentiating between specific binding of the probe with 16S rRNA and non-specific binding with cellular or extracellular components of the sample. The experimental data on the quantification of FISH using fluorescence spectrometry have been described elsewhere (Kim and Ivanov, 2000; Tay *et al*., 2001). Practical considerations relevant to the microbiological monitoring of microbial biofilms and aggregates by FISH and fluorescence spectrometry are described in this paper.

Materials and methods

The samples of microbial aerobic and anaerobic biomass were taken from an aerated tank and a mesophilic anaerobic digester in a municipal wastewater treatment plant, and from a nitrifying biofilm (Kim *et al*., 2000). The samples were fixed in 3% of paraformaldehyde in phosphate buffered saline (PBS). The set of 16S rRNA-targeted oligonucleotide probes

used, were synthesized with an aminolinker at the 5′-end and purified by HPLC. A Eub338 probe (S-D-Nbact-0338-a-A-18) with a sequence 5′-GCTGCCTCCCGTAGGAGT-3′ labeled by carboxytetramethyl rhodamine (TAMRA) was used for the detection of Bacteria (Alm *et al*., 1996). An Arc915 probe (S-D-Arch-0915-a-A-20) with a sequence 5′-GTGCTCCCCCGCCAATTCCT-3′ labeled by tetrachlorofluorescein (TET) was used to detect the cells of Archaea (Alm *et al*., 1996; Raskin *et al*., 1994). A Nsm156 probe (S-G-Nsm-0156-a-A-19) with a sequence 5′-TATTAGCACATCTTTCGAT-3′ labeled with TET was used for the detection of ammonium-oxidizing bacteria from the *Nitrosomonas* cluster (Wagner *et al*., 1998). The final concentrations in the hybridization solution were as follows: Eub338, 27 pmol/mL; Nsm156, 9 pmol/mL; Arc915, 18 pmol/mL; microbial biomass, 17 mg of dry mass/mL.

The final concentration of unlabeled probe in the hybridization solution was 10–25-fold higher than the concentration of the corresponding labeled probe. The unlabeled probe was used to competitively inhibit the hybridization of the labeled probe with the target. Another kind of control probe was the nonsense probe. This probe was labeled by the same fluorochrome as the main probe, but its sequence did not target rRNA. It was tested by the software of the Ribosomal Database Project (Maidak *et al*., 2001). The following "nonsense" probes were used in this study: TAMRA-labelled ns-Eub with the sequence 5′-CGACGGAGGGCATCCTCA-3′ and TET-labelled ns-Arc with the sequence 5′-CACGAGGGGGCGGTTAAGGA-3′.

The emission spectra for one-probe-applications and synchronous fluorescence spectra for multi-probe-applications were obtained using a Luminescence Spectrometer LS-50B (Perkin-Elmer, UK). The typical spectra for the determination of the probe binding by the synchronous scan are shown in Figure 1.

FISH was performed in the temperature range from 20°C to 70°C. An aliquot of 10 µL of the probe solution was added to a mixture of 0.5 mL of fixed microbial sludge and 0.5 mL of hybridization buffer (0.9 M NaCl, 0.01% sodium dodecyl sulfate, 20 mM Tris-HCl, pH 7.2). The mixture was placed in 2 mL polypropylene microtubes in the hybridization incubator with a rotational speed of 6 rpm for 4 hours at different temperatures. After the incubation, the suspension was cooled at 25°C for 30 min and then centrifuged. The solution was used for the measurement of the concentration of the non-hybridized probe (experiment) by emission fluorescence scanning. To determine the fluorescence of the probe

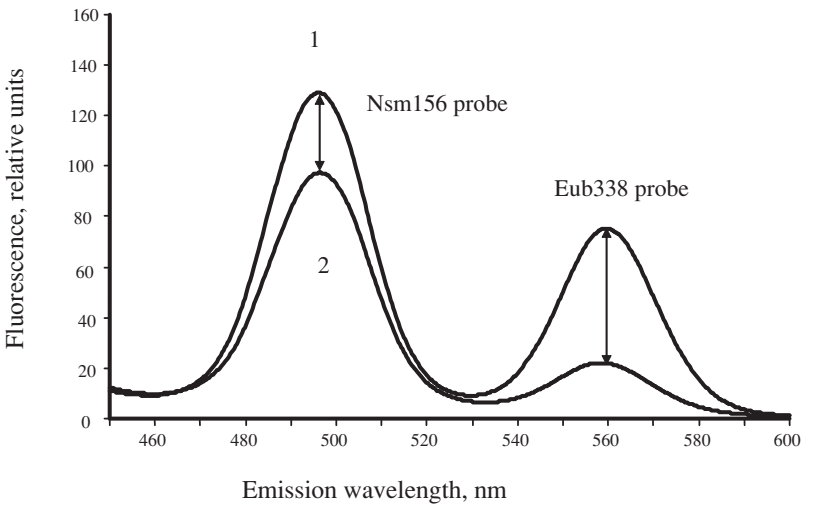

Figure 1 Synchronous fluorescence emission spectra of the fluorescent-labeled probes Eub338 and Nsm156: (1) before and (2) after incubation with a microbial biomass

before and after hybridization, two controls were used. In control A, 0.5 mL of the suspension of the fixed sludge was mixed with 0.5 mL of hybridization buffer and 1.0 mL of formamide. This mixture was incubated and treated as described above. However, the solution of the probe was added to the supernatant before the fluorescence measurement. This control A was used to account for the effect of substances extracted from the biomass during the incubation on the probe fluorescence. This is an important point of determination because the substances, which are extracted from the biomass during the incubation, can significantly change the fluorescence of the probe. Control B was used to measure the autofluorescence of the extracted substances. The total quantity of bound probe (Q, pmol/mg of biomass) was calculated from the fluorescence in control B, control A, and in the experiment by the equation:

$$Q_{total} = C\,(F_a - F_e)/(F_a - F_b)X \quad (1)$$

where C is the concentration of the probe in hybridization solution before FISH (in control A), pmol/mL; F_a, F_b, and F_e are the fluorescence of the probe in control A, control B, and experiment, respectively (based on the unit of measurement in the fluorescence spectrometer); X is the final concentration of the biomass in the incubation sample, mg/mL.

Results

Influence of temperature on the binding of labeled probe

The binding of the probes at temperatures significantly lower than the melting temperature (T_m) of the oligonucleotide probe was lower than the binding at temperatures close or higher than the T_m. The T_m of the applied probes was in the range from 50°C to 66°C according to the information given by the supplier. The unlabeled probe Arc915 did not inhibit the binding of the labeled probe Arc915 at temperatures of 20°C or 30°C but inhibited the binding of labeled probe Arc915 at temperature higher than T_m (Table 1).

Therefore, the binding of the probe at these temperatures was considered as non-specific binding. The coefficient of variation for the measurements of probe binding was approximately 30%. The specific binding of the probe (hybridization with rRNA) was calculated as the difference between total binding of the probe and its non-specific binding with the cellular and extracellular components of the microbial sludge (Tables 1 and 2). The specific binding of the oligonucleotide probes with aerobic microbial biomass after incubation at a temperature close to T_m was 69% of the total binding of Arc915 probe with anaerobic microbial biomass (Table 1), 68% of the total binding of the Eub338 probe with aerobic microbial biomass, and 48% of the total binding of the Nsm156 probe with aerobic microbial biomass (Table 2).

The ratio of the binding of nonsense probes ns-Eub and ns-Arc labeled probes at low temperature 10°C–30°C was close to 1.0 but dropped to 0.5–0.6 at the temperature 60°C–70°C. It additionally proves that the binding at low temperature was largely non-specific. The binding of labeled ns-Arc and ns-Eub probes was used for the evaluation of

Table 1 Influence of incubation temperature on specific and non-specific binding of TET-labeled Arc915 probe (T_m = 66°C) with anaerobic microbial biomass

Temperature	Total binding (pmol/mg)	Inhibition of total binding by unlabeled probe (%)	Specific binding of Arc915 probe (pmol/mg)
20°C	2.5	0	0
30°C	2.4	0	0
60°C	2.6	0	0
70°C	3.9	69	2.7

Table 2 Influence of incubation temperature on specific and non-specific binding of Eub338 probe (T_m = 60°C) and Nsm156 probe (T_m = 50°C) with aerobic microbial biomass

Temperature	Total binding of Eub338 probe (pmol/mg)	Specific binding Eub338 probe (pmol/mg)	Total binding Nsm156 probe (pmol/mg)	Specific binding Nsm156 probe (pmol/mg)
20°C	0.32	0	0.018	0
30°C	0.46	0	0.018	0
50°C			0.035	0.017
60°C	1.00	0.68	0.027	0.011
70°C	1.00	0.68		

non-specific binding of the fluorochrome attached to the probe. The values of non-specific binding of the labeled nonsense probes were close to the values determined as described above.

Influence of unlabeled probe on the binding of labeled probe

Specific and non-specific binding of the probes with the microbial sludge may be evaluated by the incubation of the cells with the probes at two temperatures or by the incubation of the cells with a mixture of labeled and unlabeled probe. If the concentration of unlabeled probe in the hybridization solution was higher than the concentration of labeled probe, the specific binding (hybridization) with labeled probes was inhibited (Table 3).

The quantity of specifically bound (hybridized) probes may be evaluated from the data as the part of the total binding of the labeled probe, which is inhibited by the corresponding unlabeled probe. The specific binding of an oligonucleotide probe with the microbial biomass used in anaerobic wastewater treatment was 50% of the total binding for the Arc915 probe and 55% of the total binding for the Eub338 probe.

FISH measurement by fluorescence spectrometry

Taking into consideration the above data, the following approaches may be used for the determination of specific and non-specific binding of an oligonucleotide probe: (1) incubation at two different temperatures ($T << T_m$ and $T \approx T_m$); (2) hybridization of labeled probe in the presence of unlabeled probe; (3) incubation of the sample with labeled specific and labeled nonsense probes. The procedures for the analysis are shown in Tables 4–6. The fluorescence at different wavelengths will be measured in the case of multi-probe application.

The specific binding of the probe can be determined by the following equation:

$$Q_{specific} = C\,(F_u - F_e)/(F_a - F_b)/X \qquad (2)$$

where F_u is the fluorescence of the supernatant from the tube where both labeled and unlabeled probes were added.

Table 3 Influence of unlabeled Eub338 and Arc915 probes on the total binding of corresponding labeled probes with microbial anaerobic sludge

Ratio of the concentrations of unlabeled and labeled probes	Percentage of inhibition for Arc915 probe	Percentage of inhibition for Eub338 probe
0	0	0
2	15	44
13	50	44
26	50	55

Table 4 The steps of the FISH quantification by fluorescence spectrometry using labeled and unlabeled probes

Tube	Operation							
	Addition of the sample	Addition of the hybridization buffer	Addition of the labeled probe(s)	Addition of the labelled and unlabeled probe(s)	Hybridization	Centrifugation (filtration)	Addition of the labeled probe(s)	Value of the fluorescence
1	+	+	+		+	+		F_e
2	+	+		+	+	+		F_u
3	+	+			+	+	+	F_a
4	+	+			+	+		F_b

Table 5 The steps of the FISH quantification by fluorescence spectrometry using low temperature ($T \ll T_m$) and high temperature ($T \approx T_m$) incubation

Tube	Operation							
	Addition of the sample	Addition of the hybridization buffer	Addition of the labeled probe(s)	Incubation at low temperature	Hybridization	Centrifugation (filtration)	Addition of the labeled probe(s)	Value of the fluorescence
1	+	+	+		+	+		F_h
2	+	+	+	+		+		F_l
3	+	+			+	+	+	F_a
4	+	+			+	+		F_b

Table 6 The steps of the FISH quantification by fluorescence spectrometry using specific labeled and nonsense labeled probes

Tube	Operation							
	Addition of the sample	Addition of the hybridization buffer	Addition of the labeled probe(s)	Addition of the nonsense labelled probe(s)	Hybridization	Centrifugation (filtration)	Addition of the labeled probe(s)	Value of the fluorescence
1	+	+	+		+	+		F_e
2	+	+		+	+	+		F_n
3	+	+			+	+	+	F_a
4	+	+			+	+		F_b

The specific binding of the probe can be determined by the following equation:

$$Q_{specific} = C\,(F_l - F_h)/(F_a - F_b)/X \quad (3)$$

where F_l and F_h are the fluorescence of the supernatant from the tubes incubated at low and high temperatures, respectively. The specific binding of the probe can be determined by the following equation:

$$Q_{specific} = C\,(F_n - F_e)/(F_a - F_b)/X \quad (4)$$

where F_n is the fluorescence of the supernatant from the tube where labeled nonsense probe was added.

Discussion

The quantification of microbial groups by FISH means the determination of the relative abundance of the rRNAs of the target microbial groups or absolute number of the targeted rRNAs in the sample. The ratio of specific binding of different probes with the microbial

cells reflects the ratio of 16S rRNAs in a microbial community. Therefore, the measurement of FISH by fluorescence spectrometry can be used as a simple method to quantify microbial groups in environmental samples and to monitor the microbial communities in biotechnological processes. It is most applicable for the quantification of such physiological groups as lithotrophs, oligotrophs, and obligate anaerobes. However, a major disadvantage of all quantitative methods based on rRNA-targeted probes is the lack of a constant correlation between the number of bacterial cells and the binding of the corresponding rRNA probe, because the number of rRNA molecules in the cells depends on the growth rate. Therefore, the quantification of microbial groups by FISH and fluorescence spectrometry is a conventional method. It is best suited to studying the changes in the microbial communities. An advantage of the above method is that an analysis may be performed by a simple procedure, without extraction of RNA or DNA, and the results can be measured by a simple portable fluorometer. The proposed method is simpler than many conventional microbiological and molecular–biological methods and is suitable not only for laboratory research but for the practical microbiological analysis of the biomass in the biofilms, sludges, bioflocs, and biogranules. The minimum amount of biomass for the analysis was 50 mg of dry weight. Coefficient of variations of the performed analysis was approximately 30%.

Conclusions

The measurement of specific binding of labeled oligonucleotide probes with an environmental sample can be used to quantify microbial groups. The following approaches may be used to determine the specific binding of oligonucleotide probe: (1) incubation of the sample at two different temperatures; (2) hybridization of labeled probe in the presence of unlabeled probe; (3) incubation of the sample with labeled specific or labeled nonsense probes. These analyses are suitable for the practical microbiological analysis of microbial biofilms and suspended aggregates because it is a simple technique and the results can be measured by a portable fluorometer.

Acknowledgements

This research was supported by Nanyang Technological University, Singapore.

References

Alm, E.W., Oerther, D.B., Larsen, N., Stahl, D.A. and Raskin, L. (1996). The oligonucleotide probe database. *Appl. Environ. Microbiol.*, **62**(10), 3557–3559.

Amann, R. and Kuhl, M. (1998). *In situ* methods for assessment of microorganisms and their activities. *Curr. Op. Microbiol.*, **1**(3), 352–358.

Hansen, K.H., Ahring, B.K. and Raskin, L. (1999). Quantification of syntrophic fatty acid-beta-oxidizing bacteria in a mesophilic biogas reactor by oligonucleotide probe hybridization. *Appl. Environ. Microbiol.*, **65**(11), 4767–4774.

Kim, I.S. and Ivanov, V.N. (2000). Detection of nitrifying bacteria in activated sludge by fluorescent *in situ* hybridization and fluorescence spectrometry. *World J. Microbiol. Biotechnol.*, **16**(5), 425–430.

Kim, I.S., Stabnikova, E.V. and Ivanov, V.N. (2000). Hydrophobic interactions within biofilms of nitrifying and denitrifying bacteria in biofilters. *Bioprocess Eng.*, **22**(4), 285–290.

Maidak, B.L., Cole, J.R., Lilburn, T.G., Parker, C.T., Saxman, P.R.Jr., Farris, R.J., Garrity, G.M., Olsen, G.J., Schmidt, T.M. and Tiedje, J.M. (2001). The RDP-II (Ribosomal Database Project). *Nucleic Acids Res.*, **29**(1), 173–174.

Raskin, L., Poulsen, L.K., Noguera, D.R., Rittmann, B.E. and Stahl, D. (1994). Quantification of methanogenic groups in anaerobic biological reactors by oligonucleotide probe hybridization. *Appl. Environ. Microbiol.*, **60**(4), 1241–1248.

Tagawa, T., Syutsubo, K., Sekiguchi, Y., Ohashi, A. and Harada, H. (2000). Quantification of methanogen cell density in anaerobic granular sludge consortia by fluorescence in-situ hybridization. *Water Sci. Technol.*, **42**(3–4), 77–82.

Tay, S.T.-L., Ivanov, V., Kim, I.-S., Lin, F. and Tay, J.-H. (2001). Quantification of ratios of *Bacteria* and *Archaea* in methanogenic microbial community by fluorescence *in situ* hybridization and fluorescence spectrometry. *World J. Microbiol. Biotechnol.*, **17**(6), 583–589.

Wagner, M., Noguera, D.R., Juretschko, S., Rath, G., Koops, H.P. and Schleifer, K.H. (1998). Combining fluorescent in situ hybridization (FISH) with cultivation and mathematical modeling to study population structure and function of ammonia-oxidizing bacteria in activated sludge. *Water Sci. Technol.*, **37**(4–5), 441–449.