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Detection of nitrifying bacteria in activated sludge by fluorescent *in situ* hybridization and fluorescence spectrometry

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Summary

16S rRNA-targeted oligonucleotide probes for eubacteria (EUB338), ammonium-oxidizing bacteria (Nsm156) and nitrite-oxidizing bacteria (Nb1000) were used for the rapid detection of nitrifying bacteria in the activated sludge of a pilot nitrifying reactor by whole-cell, fluorescent *in situ* hybridization (FISH). Emission scanning and synchronous scanning fluorescence spectrometry were used to measure the hybridization. The binding of the probes at a temperature significantly lower than the melting temperature of the hybrids was conventionally considered as non-specific. Total binding of the probes at a temperature significantly higher than the melting temperature of the hybrids was conventionally considered as the sum of non-specific and specific binding (hybridization). Non-specific binding of the oligonucleotide probes with a biomass of activated sludge was 37% of the total binding of the EUB338 probe, 54% of the total binding of the Nsm156 probe, and 69% of the total binding of the Nb1000 probe. The ratio of the specific binding of the Nsm156 and Nb1000 probes was 2.3:1. The ratio of the numbers of ammonium-oxidizing bacteria to nitrite-oxidizing bacteria, determined by microbiological methods, was 2.4:1. Measuring fluorescent *in situ* hybridization by fluorescence spectrometry appears to be a practical tool for monitoring the microbial communities that contain nitrifying bacteria. However, a method that accounts for the non-specific binding of the probes more easily and reliably should be developed for practical application.

Introduction

Nitrifying bacteria are used in environmental biotechnology in the bioremoval of nitrogen from wastewater, in the co-metabolic degradation of chlorinated solvents, and as an element of biosensors. The accumulation and diversity of a nitrifying microbial community in the activated sludge or in the biomass of biofilm play a crucial role in the performance of nitrification in wastewater treatment. However, to monitor and control the content of nitrifying bacteria in the biomass of a microbial community, the routine bacteriological enumeration by spread-plate or most-probable-number count is not suitable because several weeks of incubation are necessary before these bacteria can be enumerated.

A promising approach is the use of whole cell fluorescent *in situ* hybridization (FISH) with rRNA-targeted, fluorescent oligonucleotide probes (Amann & Kuhn 1998; Amann *et al.* 1996). Taxon-specific short nucleotide sequences of bacterial 16S and 23S rRNA genes are widely used in environmental microbiology and biotechnology as molecular probes for identifying species, genus, family, and higher taxa (Alm *et al.* 1996; Maidak *et al.* 1999). Many successful applications of FISH are known to detect nitrifying bacteria in the biomass of activated sludge (Guschin *et al.* 1997; Hovaneck & DeLong 1996; Juretschko *et al.* 1998; Mobarry *et al.* 1996; Roske *et al.* 1998; Schramm *et al.* 1997, 1998, 1999; Waarde *et al.* 1998; Wagner *et al.* 1998).

The FISH was detected by confocal laser microscopy with computer image analysis. However, the variability of the microscopic images obtained for one sample and the presence of non-transparent particles in the environmental sample complicated the enumeration of the cells by whole-cell FISH when analysed by fluorescent microscopy and image analysis. Therefore, the enumeration of nitrifying bacteria in the activated sludge, obtained by FISH measured by fluorescent microscopy, has thus far yielded no statistically reliable data. The idea of our research was that fluorescence spectrometry would probably be applicable to quantify the FISH because the fluorescence measures the bulk of the sample unhampered by the heterogeneity of the sample's

physical structure. Therefore, the applicability of fluorescence spectrometry to the measurement of FISH with rRNA-targeted oligonucleotide probes for nitrifying bacteria was examined in this study.

Materials and Methods

Samples

Experimental samples of activated sludge were collected from a 10-l pilot nitrifying reactor, which was used for the simultaneous bioremoval of ammonium and organic matter from wastewater (Kim & Kim 1999). Some samples of the activated sludge were taken for microbiological analysis, and other samples were fixed in 50% ethanol (v/v, final concentration) immediately after the sampling and stored at 2 °C for the FISH. To determine the concentration of suspended matter (biomass), 1.5 ml of the sample was centrifuged in an Eppendorf tube at 11,000 × *g* for 10 min in an Eppendorf 5415C centrifuge and dried at 60 °C until it reached a constant weight.

Microbiological enumeration

The samples were diluted by a factor of either 10^{-4} or 10^{-5}. An amount of 0.1 ml of the suspension was spread onto a solid medium for the growth and enumeration of the colonies of ammonium- and nitrite-oxidizing bacteria. The solid medium used to cultivate the ammonium-oxidizing bacteria contained the following ingredients per liter of distilled water: 0.5 g $(NH_4)_2SO_4$, 0.04 g $MgSO_4 \cdot 7H_2O$, 0.04 g $CaCl_2 \cdot 2H_2O$, 0.2 g KH_2PO_4, 20 g Noble Agar (Difco), and 0.1 ml solution of metals '44'. The medium was adjusted to pH 8.0 with 10 N NaOH. The composition of the solution of metals '44' per 100 ml of distilled water was as follows: 1.1 g $ZnSO_4 \cdot 7H_2O$, 0.5 g $FeSO_4 \cdot 7H_2O$, 0.25 g EDTA, 0.15 g $MnSO_4 \cdot 7H_2O$, 0.04 g $CuSO_4 \cdot 5H_2O$, 0.02 g $Co(NO_3)_2 \cdot 6H_2O$, 0.02 g $Na_2B_4O_7 \cdot 10H_2O$, and a pH adjusted to 4 with 10 M H_2SO_4. The inoculated Petri dishes were incubated in the dark at 28 °C for at least two weeks before counting the colony-forming units (c.f.u.). The solid medium used to cultivate the nitrite-oxidizing bacteria contained the following ingredients per liter of distilled water: 1.5 g $KHCO_3$, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.3 g KNO_2, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g NaCl, 0.01 g $CaCl_2 \cdot 2H_2O$, 20 g Noble Agar (Difco), and 0.2 ml solution of metals '44'. The inoculated Petri dishes were incubated in the dark at 28 °C for at least two weeks before counting the c.f.u.

Oligonucleotide probes

Two 16S rRNA-targeted oligonucleotide probes were used to compare the nitrifying community in the aerobic sludge and in the biomass of a nitrifying reactor. The probes were synthesized with an aminolinker at the 5′-end and purified by HPLC. The Nsm156 and Nb1000 probes were supplied from Synthegen, TX, USA, and the EUB338 probe was supplied from MWG-Biotech AG, Germany.

The EUB338 probe is a sequence 5′-GCTGCCTCCCGTAGGAGT-3′, and is used to detect most eubacteria. It is also known under the name S-D-Nbact-0338-a-A-18 (Christensen *et al.* 1999; Guschin *et al.* 1997; Schramm *et al.* 1998). According to the manufacturer's data, the melting temperature for this probe is 60.5 °C. The probe was labelled with rhodamine. The Nsm156 probe was used for hybridization with the cells of the *Nitrosomonas* group of the ammonium-oxidizing bacteria, namely *Nitrosomonas europea*, *Nitrosomonas eutropha*, and *Nitrosococcus mobilis*. It was a sequence 5′-TATTAGCACATCTTTCGAT, also known as S-G-Nsm-0156-a-A-19 (Juretschko *et al.* 1998; Mobarry *et al.* 1996; Roske *et al.* 1998; Schramm *et al.* 1998; Wagner *et al.* 1996). The probe was labeled with FITC (Isomer I). The Nb1000 probe was used for the hybridization with the cells of the *Nitrobacter* group of the nitrite-oxidizing bacteria. It was a sequence 5′-TGCGACCGGTCATGG, also known as S-G-Nit-1000-b-A-15 (Guschin *et al.* 1997; Mobarry *et al.* 1996). The probe was labeled with 6-TET (6-tetrachlorofluorescein). The solutions of probes were stored frozen at −18 °C.

Whole-cell hybridization

The FISH was performed at temperatures from 20 to 80 °C; the optimal temperature was between 46 and 53 °C. The final concentration of formamide in the hybridization solution was 40% v/v. These conditions and the contents of the hybridization solution were described for the FISH with Nsm156 and Nb1000 probes (Mobarry *et al.* 1996; Schramm *et al.* 1998).

The final concentrations of the probes in the hybridization solution were as follows: 27 pmol/ml EUB338, 4 pmol/ml Nsm156, and 19 pmol/ml Nb1000. The final concentrations in the hybridization solution with the mixture of the probes were as follows: 27 pmol/ml EUB338, 9 pmol/ml Nsm156, and 19 pmol/ml Nb1000. The final concentration of the biomass was 17 mg of dry weight/ml.

The solution of a single probe or the mixture of probes was added to 1.5 ml of the suspension of the biomass in the formamide solution at a final concentration of 40% v/v and incubated for 2 h at different temperatures. After the hybridization, the suspension was incubated at 4 °C for 0.5 h, then centrifuged at 11,000 × *g* for 10 min in an Eppendorf 5415C centrifuge, and 1.5 ml of water was added to the supernatant. This solution was used to measure the concentration of the unhybridized probes by emission scanning or synchronous scanning fluorescence spectrometry.

To determine the fluorescence of the probe before the hybridization (in the control), 1.5 ml of the suspension of biomass in the formamide solution (final concentra-

tion was 40% v/v) was incubated and treated as described above, but the solution of the probe was added to the supernatant before the fluorescence measurement. This was done to take into account the effect of the substances extracted from the biomass on the fluorescence of the probe. The quantity of the hybridized probe was calculated by the difference between the concentrations of the probe before (in the control) and after hybridization (in the experiment). An example of the spectra used for this calculation is shown in Figure 1.

Fluorescence spectrometry of the probes

To determine the concentration of the probe in the solution before and after the hybridization, emission scanning or synchronous fluorescence spectrometry was performed with a Luminescence Spectrometer LS-50B (Perkin-Elmer). The fluorescence emission scanning of the EUB338 probe was made from 575 to 600 nm with an excitation wavelength of 561 nm, and the maximum emission was obtained at 583 nm (Figure 1a). The fluorescence emission scanning of the Nsm156 probe fluorescence was performed from 500 to 550 nm with an excitation wavelength of 490 nm, and the maximum emission was obtained at 519 nm (Figure 2a). The fluorescence emission scanning of (the) Nb1000 probe fluorescence was performed from 500 to 580 nm with an excitation wavelength of 480 nm, and the maximum emission was at 541 nm.

The optical slits were 10 nm for the excitation and emission wavelengths. Synchronous fluorescence spectrometry was performed by changing the excitation and emission wavelengths with interval of 20 nm. The excitation wavelength was increased from 380 to 580 nm with a scan speed of 240 nm/min during the synchronous scanning. Correspondingly, the wavelength of the monochromator of the emission light was increased synchronously from 400 to 600 nm. The excitation and emission optical slits were 5 nm. The maximum emissions for the labelled probes were as follows: 496–500 nm for Nsm156, 523–526 nm for Nb1000, and 556–558 nm for EUB338. The height of the peaks of (the) fluorescence emission correlated linearly (coefficient of correlation was 0.99) with the concentration of the probes in the hybridization solution.

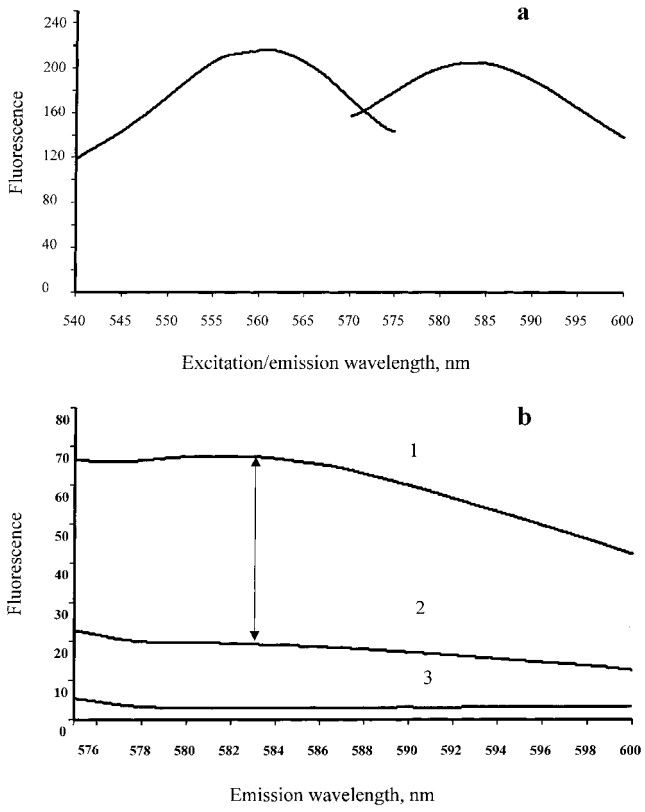

Figure 1. (a) Fluorescence excitation/emission spectra of the EUB338 probe dissolved in the water, and (b) emission spectra showing the binding of the EUB338 probe with a biomass of activated sludge. (1) Spectrum for the solution of the probe in the biomass extract; (2) spectrum for the same solution after hybridization; (3) spectrum for the substances extracted from the biomass during the hybridization. A difference between the spectra at 561 nm was used to calculate the bound probe. The final concentration of the probe in the hybridization solution was 27 pmol/ml. The final concentration of the biomass was 17 mg of dry mass/ml.

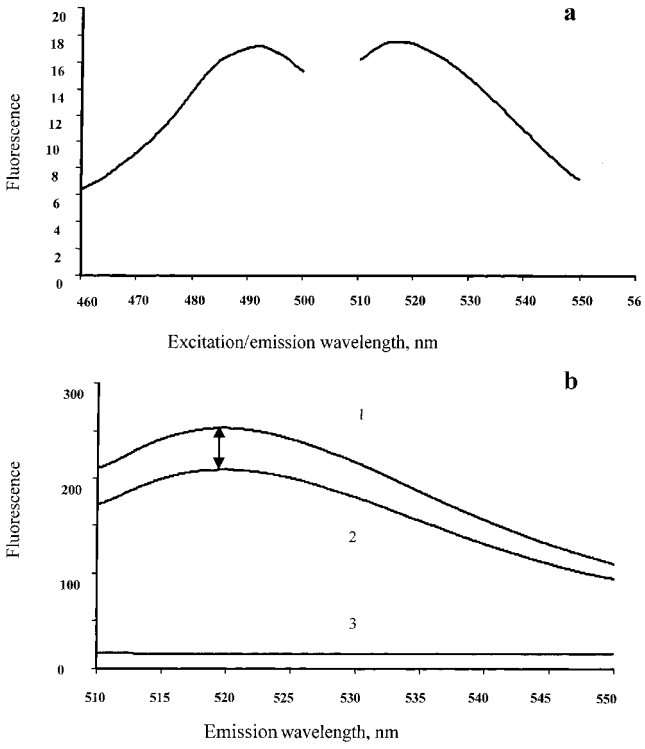

Figure 2. (a) Fluorescence excitation/emission spectra of the Nsm156 probe dissolved in the water, and (b) emission spectra showing the binding of the Nsm156 probe with a biomass of activated sludge. (1) Spectrum for the solution of the probe in the biomass extract; (2) spectrum for the same solution after hybridization; (3) spectrum for the substances extracted from the biomass during the hybridization. A difference between the spectra at 519 nm was used to calculate the bound probe. The final concentration of the probe in the hybridization solution was 4 pmol/ml. The final concentration of the biomass was 17 mg of dry mass/ml.

Results

Effect of temperature on FISH

The binding of all the probes with the biomass of aerobic sludge was analysed in triplicate by emission scanning fluorescence spectrometry. The excitation/emission spectra of the EUB338 and Nsm156 probes dissolved in water are shown in Figures 1(a) and 2(a). The examples of the emission scanning spectra of the EUB338 and Nsm156 probes used to calculate the probe binding are shown in Figures 1(b) and 2(b).

There was minimal binding of the probes at temperatures lower 40 °C, but when the temperature was increased to 60 °C the binding was increased step-wise. This pattern of binding was similar for the curve of the hybridization of oligonucleotides and reflects the melting of hybrids at temperatures that are higher than the melting temperature (T_m). The T_m for all probes were between 40 and 60 °C. The binding of the EUB338 probe with the suspended nitrifying biomass at temperatures lower than T_m was 0.40 ± 0.03 pmol/mg of dry biomass of activated sludge. At 1.08 ± 0.04 pmol/mg of dry biomass after the step-wise increase, the temperature was higher T_m (Figure 3a). Similar values for the binding of the Nsm156 probe were 0.019 ± 0.002 pmol/mg of dry biomass of activated sludge and 0.035 ± 0.04 pmol/mg of dry biomass, respectively (Figure 3b). These values for the binding of the Nb1000 probe were 0.016 ± 0.003 pmol/mg of dry biomass of activated sludge and 0.023 ± 0.003 pmol/mg of dry biomass, respectively (Figure 3c).

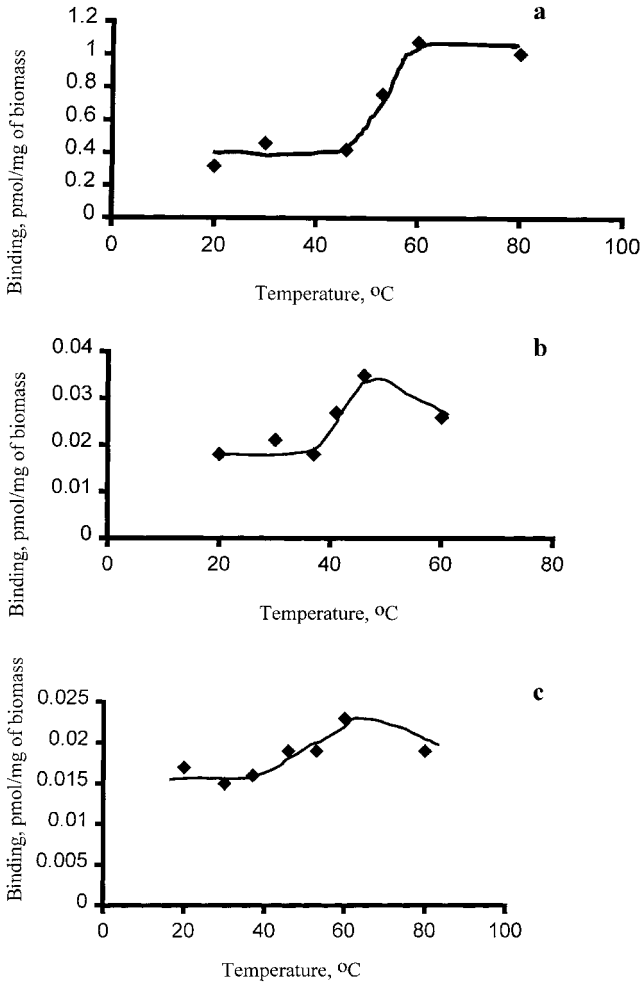

Figure 3. Melting curves of the oligonucleotide probes used for the whole-cell FISH. (a) EUB338 probe; (b) Nsm156 probe; (c) Nb1000 probe.

Evaluation of non-specific binding of the probes

To evaluate the non-specific binding of the probes, certain assumptions were made. It was considered that the stoichiometry of the non-specific binding of the oligonucleotide core and fluorescent label of the probes does not significantly depend on maintaining a temperature within the range of 20–80 °C. However, the specific binding (i.e. the hybridization between the probe and sequence of rRNA) does depend on temperatures that occur in steps.

It is well known that the hybridization is absent when the temperature is below T_m, but the hybridization is maximized when the temperature is slightly higher than T_m. Therefore, the binding of the probe at a temperature lower than T_m may be conventionally considered as the non-specific binding of a probe with cell components. The values of T_m were within the range of 40–60 °C, so the binding of the probe at a temperature lower than 40 °C was conventionally considered as a non-specific one. It was conventionally considered that the same value of non-specific binding was at a temperature higher than T_m, but the additional binding at this temperature was specific binding of the oligonucleotide probe (hybridization). To calculate this specific binding (level of hybridization), the value of the non-specific binding at a temperature lower than T_m was subtracted from the value of the total binding at a temperature higher than T_m.

The non-specific binding of the oligonucleotide probes with the biomass was 37% of the total binding of the EUB338 probe, 54% of the total binding of the Nsm156 probe, and 69% of the total binding of the Nb1000 probe. The specific binding of the probes was as follows: 0.68 ± 0.04 pmol of the EUB338 probe/mg of dry biomass, 0.016 ± 0.002 pmol of the Nsm156 probe/mg of dry biomass, and 0.007 ± 0.003 pmol of the Nb1000 probe/mg of dry biomass of the activated sludge. The ratio of the specific binding of the Nsm156 and Nb1000 probes was 2.3:1.

Simultaneous FISH with some probes

Another technique, synchronous scanning fluorescence spectrometry, was used to measure the simultaneous FISH with some probes. Mixing the EUB338 and Nsm155 probes may be used to simultaneously characterize the ratio between the eubacteria and ammonium-oxidizing bacteria in the suspended nitrifying biomass

(Figure 4a). The mixture of the EUB338, Nsm156, and Nb1000 probes may be used to simultaneously characterize the ratio between the eubacteria, ammonium-oxidizing bacteria, and nitrite-oxidizing bacteria in the suspended nitrifying biomass (Figure 4b). The data are shown in the Table 1. The ratio of the specific binding of the Nsm156 and Nb1000 probes was 2.0:1.

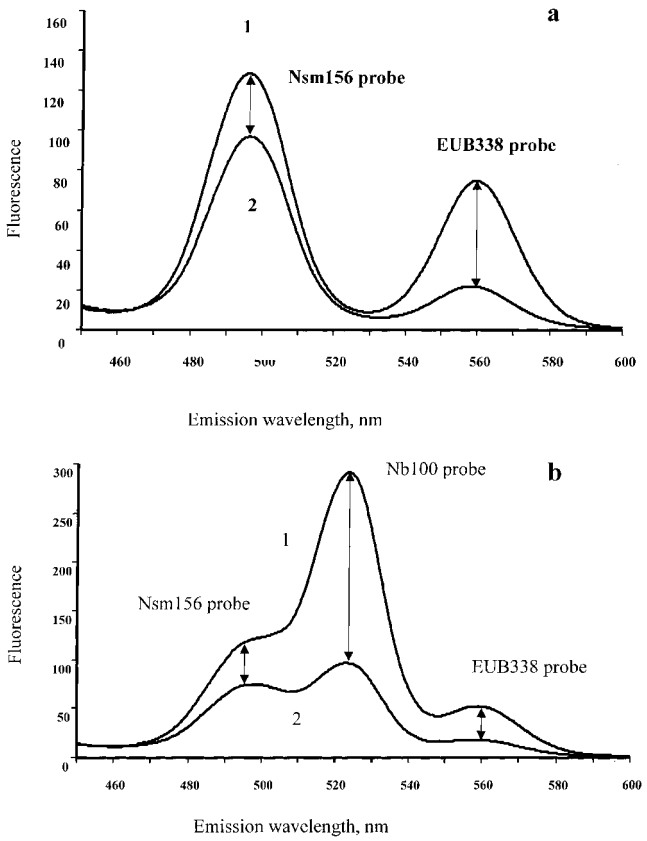

Figure 4. Synchronous fluorescence emission spectra of the fluorescent-labelled probes (1) before and (2) after incubation with a biomass of activated sludge. (a) Mixture of the EUB338 and Nsm156 probes; (b) mixture of (the) EUB338, Nsm156, and Nb1000 probes. Excitation and emission wavelengths were changed synchronously, and the difference between the excitation and emission wavelengths was 20 nm. The final concentrations in the hybridization solution with the mixture of the probes were as follows: EUB338, 27 pmol/ml; Nsm156, 9 pmol/ml; Nb1000, 19 pmol/ml. The final concentration of the biomass was 17 mg of dry mass/ml.

Table 1. Whole-cell FISH with the mixtures of three probes measured by synchronous fluorescence spectrometry (average value ± standard deviation are shown).

Binding of biomass (pmol/mg)	Probe		
	EUB338	Nsm156	Nb1000
Total	0.75 ± 0.08	0.04 ± 0.01	0.05 ± 0.01
Specific[a]	0.47 ± 0.05	0.02 ± 0.00	0.01 ± 0.00

[a] Specific binding of the probe was calculated as the difference between the total binding and the binding at a temperature lower than T_m. Ratio of specific bindings is 47:2:1.

Enumeration of ammonium-oxidizing and nitrite-oxidizing bacteria

The content of the ammonium-oxidizing bacteria was $(55 \pm 7) \times 10^6$ c.f.u./mg of dry biomass, and the content of the nitrite-oxidizing bacteria was $(23 \pm 2) \times 10^6$ c.f.u./mg of dry biomass. The ratio of the number of ammonium- and nitrite-oxidizing bacteria in the biomass was 2.4:1.

Discussion

An important procedure during the whole-cell FISH measured by fluorescent microscopy and image analysis is washing out the probe, which is non-specifically bound with the cells. Fluorescent-labelled probes very often have significant non-specific binding during whole-cell FISH (Loge *et al.* 1999). We did not wash out the probe because the quantification of the FISH was based on the measurement of the fluorescence before and after hybridization. We propose in this paper to determine the non-specific binding of the probes by analysing the melting curve of the hybrids.

The binding of the probe at a temperature lower than T_m was considered to be non-specific. The non-specific binding of the oligonucleotide probes with the biomass was on average about 50% of the total binding. The specific binding of the probe can be calculated as the difference between the total binding and the non-specific binding. However, this approach is only suitable for laboratory research. A simpler method of determining the specific binding must be developed for the practical application of the FISH measured by fluorescence spectrometer or fluorometer.

The data showed that the ratio of the specific binding of the probes that are specific for the *Nitrosomonas*/*Nitrosococcus* group and the *Nitrobacter* group was 2.3:1 and 2.0:1, respectively. The real ratio between the numbers of the ammonium- and nitrite-oxidizing bacteria, determined by the microbiological method, was 2.4:1. Therefore, it is probable that the ratio of the *Nitrosomonas*- and *Nitrobacter*-specific probes, which were bound with the biomass specifically, may be used to monitor the structure of the bacterial nitrifying communities.

However, the direct transformation of the data obtained by the fluorescence spectrometry into the number of the cells of nitrifying bacteria is not possible at present because more data are needed to check the linearity of the correlation, influence of the growth conditions, and minimal detectable number. In every case, there will be no constant correlation between the number of some bacterial cells and the binding of the corresponding probe. This proved to be a disadvantage of the proposed method of whole-cell FISH measurement. However, it is clear that measuring the FISH by fluorescent spectrometry can be applied in environmental microbiology to compare and monitor the diversity of microbial communities.

The variability of the microscopic images of one sample, or the presence of bacterial aggregates and non-transparent particles in the environmental samples, complicates the measurement of the FISH by fluorescent microscopy and image analysis. For example, the nitrifying bacteria in the activated sludge grow in the form of microcolonies embedded in a flock of heterotrophic microorganisms (Wagner *et al.* 1998), making the heterogeneity of the microscopic images high. Therefore, the main advantage of the FISH measured by fluorescent spectrometry is that the data are obtained for the bulk of the sample and do not depend on the presence of aggregates or particles in the sample. It is especially suitable for monitoring the growth of the nitrifying bacteria because the duration of a routine microbiological enumeration is at least two weeks.

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