Eco-physiology of autotrophic nitrifying biofilms

S. Okabe*, T. Kindaichi, Y. Nakamura* and T. Ito***

*Department of Urban and Environmental Engineering, Graduate School of Engineering, Hokkaido University, Kita-13, Nishi-8, Kita-ku, Sapporo 060-8628, Japan (E-mail: *sokabe@eng.hokudai.ac.jp; kitishi@eng.hokudai.ac.jp; tsukasa@eng.hokudai.ac.jp*)

**Department of Social and Environmental Engineering, Graduate school of Engineering, Hiroshima University, 1-4-1 Kagamiyama, Higashihiroshima 739-8527, Japan (E-mail: *tomokin@hiroshima-u.ac.jp*)

Abstract Microautoradiography combined with fluorescent in situ hybridization (MAR-FISH), a powerful tool for linking physiology with identification of individual cells, was applied to investigate microbial interactions between nitrifying bacteria and coexisting heterotrophic bacteria in an autotrophic nitrifying biofilm community fed with only ammonia as the sole energy source and bicarbonate as the sole carbon source. First, nitrifying bacteria were radiolabeled by culturing the biofilm samples with [^{14}C]bicarbonate for 6 h, and then the transfer of radioactivity from nitrifying bacteria to heterotrophic bacteria was monitored by using MAR-FISH. MAR-FISH revealed that the heterotrophic bacterial community was composed of bacteria that were phylogenetically and metabolically diverse. We could obtain direct evidence that organic matter derived from nitrifiers was subsequently utilized by mainly filamentous bacteria belonging to the *Chloroflexi* (green non-sulfur bacteria) group or CFB group in the biofilm, which was clearly visualized by MAR-FISH at single cell resolution for the first time. On the other hand, the members of the α- and γ-*Proteobacteria* were specialized to utilize low-molecular-weight organic matter. This community represents functionally integrated units that assure maximum access to and utilization of metabolites of nitrifiers.

Keywords Microautoradiography; fluorescent in situ hybridization; nitrifying biofilms

Water Science & Technology **Vol 52 No 7 pp 225–232** © IWA Publishing 2005

Introduction

Almost all bacteria in natural environments are present in the form of biofilms attached to surfaces. Microbial life in such biofilms is mostly characterized by multiplicity (many species together), nutrient limitation, changing microenvironment, and a structured distribution of the biomass. In addition, most members of microbial communities in natural and engineered biofilm systems cannot be cultured and characterized. Even when a microorganism can be cultivated, it is therefore not too surprising that traditional investigations of bacteria grown in the laboratory as pure culture with excess nutrients under constant and well controlled conditions in liquid suspensions do not really contribute directly to an understanding of the eco-physiology of microorganisms in natural environments. As a consequence, our understanding of the structure, function, and eco-physiological interactions of microbial populations in biofilm ecosystems is generally limited.

The community organization and eco-physiological interaction between nitrifiers and heterotrophs in the autotrophic nitrifying biofilm have been investigated by using advanced molecular and electrochemical approaches. Combination of fluorescence in situ hybridization (FISH) and microsensor technology is a powerful and reliable tool to link the community structure and function of nitrifying biofilms at community level (Okabe *et al.*, 1999, 2004; Schramm *et al.*, 1996). The MAR-FISH can be also used to simultaneously examine the 16S rRNA-based phylogenetic identity and the relative or actual specific metabolic activity of cultivable or uncultivable microorganisms within a complex microbial community at a single-cell level (Cottrell and Kirchman, 2000; Daims *et al.*, 2001; Ito *et al.*, 2002; Kindaichi *et al.*, 2004; Lee *et al.*, 1999; Nielsen *et al.*, 2000; Nielsen *et al.*, 2003; Ouverney and Fuhrman, 1999). The resolution level of these new

approaches ranges from single-cell level to entire biofilm ecosystems. Therefore, information obtained at both levels must be combined to draw a clear picture of the autotrophic nitrifying biofilm ecosystem.

In this study, to understand spatial organization of microbial communities and their in situ functions as members of biofilm communities, we have cultured carbon-limited autotrophic nitrifying biofilms fed only NH_4^+ as a simple and carefully controlled model biofilm ecosystem and applied MAR-FISH, and microsensor measurements.

Materials and methods

Biofilm samples

Autotrophic nitrifying biofilms were cultured with synthetic medium containing NH_4^+ (3.6 mM) as the sole electron donor and $NaHCO_3$ (17.8 mM) as carbon source in partially submerged rotating disk reactor (RDR) consisting of five polymethyl methacrylate disks. The autotrophic nitrifying biofilms were first cultured with the primary settling tank effluent from the Sousei-gawa municipal wastewater treatment plant (Sapporo, Japan) for 2–3 days and then cultured with synthetic nutrient medium as described elsewhere (Okabe *et al.*, 1996, 1999).

Phylogenetic analysis of 16S rRNA gene

DNA was extracted from the biofilm sample (approximately 0.2 ml) with the Fast DNA spin kit (BIO101, Qbiogene Inc., Carlsbad, CA). 16S rRNA gene fragments were amplified using bacterial primer sets 11f and 1492r as described elsewhere (Kindaichi *et al.*, 2004). The purified PCR products were ligated and transformed into *Escherichia coli* JM109 competent cells (Promega, Tokyo, Japan). Nucleotide sequencing was performed with an automatic sequencer (Prism 310 Genetic Analyzer, Applied Biosystems). All partial sequences (approximately 500 bp) were checked for chimeric artifacts by the Chimera Check program from the Ribosomal Database Project and compared with similar sequences of the reference organisms by BLAST search. Sequences with 97% or higher sequence similarity were grouped into operational taxonomic units (OTUs). Nearly complete sequencing of 16S rRNA gene of each representing OTU was performed and the sequences were aligned with the CLUSTAL W package. The phylogenetic tree was constructed by the neighbor-joining method. Details of the phylogenetic analysis have been described elsewhere (Kindaichi *et al.*, 2004).

Incubation with radioactive compounds

The following inorganic and organic substrates, labeled with a radioisotope, were used: (i) sodium [^{14}C]bicarbonate (specific activity, 58 mCi mmol^{-1}) to radiolabel nitrifying bacteria; (ii) [1-^{14}C]acetic acid (sodium salt; specific activity, 61 mCi mmol^{-1}) as low-molecular weight organic substrates produced through decomposition of organic compounds; (iii) L-amino acid mixture, [U-^{14}C] (amino acids; specific activity, 50 mCi mmol^{-1}) as low-molecular weight organic substrates produced through decomposition of proteins; and (iv) *N*-acetyl-D-[1-^{14}C]glucosamine (NAG; specific activity, 57 mCi mmol^{-1}) as a constituent of cell wall. Radioactive chemicals were obtained from Amersham Biosciences (Little Chalfont, UK) and ICN Biomedical Inc. (Irvine, CA).

The homogenized biofilm samples were diluted to the final concentration of 3 g of volatile suspended solids (VSS) per litre with a basal medium (0.4 mM K_2HPO_4, 0.41 mM $MgSO_4 \cdot 7H_2O$, 1.25 mM NaCl, pH 8.0). For each experiment, 1.9 ml portions of diluted biofilm sample were transferred to 10 ml serum bottles. Each bottle was supplemented with a radioactive substrate (final activity of 10 μCi) and nonradioactive substrates (ammonium and/or bicarbonate) and sealed with a gas-tight rubber stopper. The final

volume of each sample was 2 ml. During the oxic incubation at 25 °C, the bottles were shaken at 100 rpm to maintain oxic conditions. Biofilm samples pasteurized at 80 °C for 15 min were incubated with the radioactive and nonradioactive substrates in parallel as a control for possible adsorption phenomena.

Substrate cross-feeding experiment

The autotrophic biofilm samples were incubated in a batch mode with [^{14}C]bicarbonate (86 μM) and 2 mM of NH_4^+ as the sole carbon and energy source for 1 day, 5 days, 9 days, and 25 days, respectively. Samples were taken from each culture for MAR-FISH to determine which phylogenetic group(s) can uptake radiolabeled organic matter derived from nitrifiers that originally incorporated [^{14}C]bicarbonate.

Sample fixation

The samples were fixed for 3 h at 4 °C by adding 2 ml of 8% paraformaldehyde, giving the final concentration of 4% paraformaldehyde. Subsequently, the samples were centrifuged at 10,000 × *g* for 8 min, washed three times with 1 ml of PBS to remove excess soluble radioactive compounds, and stored in 50% ethanol in PBS at −18 °C. After the fixation and washing steps, the samples were spotted on gelatin-coated cover glass as described elsewhere (Ito *et al.*, 2002; Lee *et al.*, 1999).

Liquid scintillation counting

The uptake of radioactive substrates by heterotrophic bacteria was confirmed by liquid scintillation counting in all experiments before MAR-FISH analysis. The ^{14}C content was directly measured in the culture sample (biomass plus culture medium). The ^{14}C contents in both biomass fraction and liquid medium fraction were determined as described previously (Kindaichi *et al.*, 2004). To evaluate the uptake of radioactive compounds, the percentage of radioactive substrate incorporated into the biomass was calculated.

Oligonucleotide probes and FISH

The 16S and 23S rRNA-targeted oligonucleotide probes and the hybridization conditions used in this study are listed in Table 1. The probes were labeled with fluorescein isothiocyanate (FITC) and tetramethylrhodamine 5-isothiocyanate (TRITC). Dehydration and FISH were performed by using the procedure described previously by Amann (1995) and Okabe *et al.* (1999).

Autoradiographic procedure

MAR was performed directly on the cover glass as described by Lee *et al.* (1999). After the FISH procedure, autoradiographic liquid film emulsion (LM-1, Amersham Biosciences, Little Chalfont, UK) was used. The optimal exposure time was adjusted to 2 days, under which condition formation of silver grain around the heterotrophic bacterial cells was negligible.

Microscopy and enumeration by MAR-FISH

A model LSM510 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) equipped with an Ar ion laser (458 and 488 nm) and two HeNe ion lasers (543 nm) was used. The formation of silver grains in the autoradiographic film was observed by using the transmission mode of the system. A MAR-positive cell was defined as a cell covered with more than 5 silver grains. The numbers of MAR-positive cells and total probe-hybridized cells were enumerated by directly counting on a minimum of 500 silver-grain-covered cells in randomly chosen microscopic fields.

Table 1 16S and 23S rRNA-targeted oligonucleotide probes used for in situ hybridization

Probe	Probe sequence (5′ to 3′)	FA (%)[a]	Specificity	Reference
EUB338	GCTGCCTCCCGTAGGAGT	–[b]	Most bacteria	Amann *et al.* (1990)
BET42a[c]	GCCTTCCCACTTCGTTT	35	Beta-subclass of *Proteobacteria*	Manz *et al.* (1992)
Nso190	CGATCCCCTGCTTTTCTCC	55	Ammonia-oxidizers	Mobarry *et al.* (1996)
Nsm156	TATTAGCACATCTTTCGAT	5	*Nitrosomonas* cluster	Mobarry *et al.* (1996)
GAM42a[d]	GCCTTCCCACATCGTTT	35	Gamma-subclass of *Proteobacteria*	Manz *et al.* (1992)
ALF1b	CGTTCGYTCTGAGCCAG	20	Alpha-subclass of *Proteobacteria* including *Nitrospira*	Manz *et al.* (1992)
CF319 a/b	TGGTCCGTRTCTCAGTAC	35	Members of CFB[e] cluster	Manz *et al.* (1996)
S-*-CFB-0655-a-A-18	CGCTCACCTCCACAACAT	20	Members of CFB cluster	Kindaichi *et al.* (2004)
S-*-CFB-0730-a-A-18	TACAGKCTAGYAAGCTGC	20	Members of CFB cluster	Kindaichi *et al.* (2004)
Ntspa1026	AGCACGCTGGTATTGCTA	20	*Nitrospira moscoviensis*	Juretschko *et al.* (1998)
S-*-GNS-0667-a-A-18	CACCCSGAATTCCACRTT	20	Members of *Chloroflexi*	Kindaichi *et al.* (2004)

[a]FA, formamide concentration in the hybridization buffer
[b]Usable at any formamide concentrations
[c]When BET42a was used, unlabeled probe GAM42a was used as a competitor to enhance specificity
[d]When GAN42a was used, unlabeled probe BET42a was used as a competitor to enhance specificity
[e]CFB, Cytophaga/Flavobacterium/Bacteroides

Results and discussion

Microbial community structure

Phylogenetic differentiation (identification) of heterotrophic bacteria was performed by 16S rRNA gene sequence analysis. Based on the phylogenetic analysis, FISH was performed with group specific probes to determine the community structure and the spatial organization, i.e., niche differentiation in the biofilm. FISH analysis showed that this autotrophic nitrifying biofilm comprised 60% of nitrifying bacteria (ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB)) and 40% of heterotrophic bacteria, where member distribution was as follows: α-*Proteobacteria* (15%), *Chloroflexi* (green non-sulfur bacteria) (10%), γ-*Proteobacteria* (9%), *Cytophaga-Flavobacterium-Bacteroides* (CFB) division (3%), and unidentified (could not be hybridized with any other probes except for EUB338) (3%). This result indicated that a pair of nitrifiers (AOB + NOB) supported a heterotrophic bacterium via production of soluble microbial products (SMP).

Substrate uptake patterns of different phylogenetic groups

To evaluate substrate uptake patterns of seven different phylogenetic groups of bacteria (i.e., AOB, NOB, γ-subclass *Proteobacteria*, α-subclass *Proteobacteria*, *Chloroflexi*, *Cytophaga-Flavobacterium-Bacteroides* (CFB), and unidentified groups), MAR-FISH was performed with three radioactive substrates (i.e., [^{14}C]bicarbonate, [^{14}C]acetic acid, [^{14}C]amino acids and [^{14}C]NAG) and various sets of FISH probes. Uptake of organic compounds differed greatly among the phylogenetic groups (Table 2). The nitrifying bacteria (AOB + NOB) took up only [^{14}C]bicarbonate, underlining the autotrophic growth of these bacteria. No uptake of [^{14}C]bicarbonate by any other groups of heterotrophic bacteria was observed for 4-h incubation. [^{14}C]acetic acid was utilized by the α-*Proteobacteria*, the γ-*Proteobacteria*, and unidentified group that was detected with the Eub338 probe but not with any other specific probes (Table 2). The α- and γ-*Proteobacteria* were main [^{14}C]acetic acid-utilizing bacteria. [^{14}C]amino acids were utilized by all groups except nitrifiers (Table 2). The members of the CFB and *Chloroflexi* groups predominantly utilized *N*-acetyl-D-[1-^{14}C]glucosamine (NAG), even though their abundance was relatively low in this biofilm. These results might indicate that members of the α- and γ-*Proteobacteria* are specialized to utilize low-molecular weight organic matter, whereas the members of the CFB and *Chloroflexi* groups are considered to be specialists for degrading and scavenging structural cell components in this biofilm.

Table 2 Summary of uptake of radiolabeled substrates by various phylogenetic groups of heterotrophic bacteria

	Radiolabeled substrate			
Phylogenetic group	**Acetic acid**	**Amino acids**	**NAG**	**Bicarbonate**
α-*Proteobacteria*	+++[a]	+++	−	−
γ-*Proteobacteria*	+++	+++	−	−
Chloroflexi	−	+	++	−
CFB	−	+	+++	−
AOB	−	−	−	+++
NOB	−	−	−	+++
Unidentified	++	++	+	−

[a]MAR + probe-hybridized cells/MAR + Eub338 probe-hybridized cells (%):
+++, more than 30%; ++, 15–30%; +, 0–15%; −, MAR-negative

Uptake of organic matter derived from nitrifiers by heterotrophs

The autotrophic biofilm samples were incubated in a batch mode with [^{14}C]bicarbonate and 2 mM of NH_4^+ as the sole carbon and energy source for 25 days. Subsamples were taken at 1 day, 5 days, 9 days, and 25 days for MAR-FISH to determine which phylogenetic group(s) can uptake radiolabeled organic matter derived from nitrifiers that originally incorporated [^{14}C]bicarbonate. Since NH_4^+ was depleted within one day, uptake of [^{14}C]bicarbonate ceased. Thereafter the nitrifiers might decay and release radiolabeled organic matter derived from mainly biomass decay (i.e., biomass-associated products (BAP)). We, however, could not measure the compositions and concentrations of the organic matter in this study. It could be speculated that the organic matter derived from nitrifiers biomass contains humic and fulvic acids, polysaccharides, amino acids, structural cell components, and low-molecular-weight fatty acids, some of which could be utilized by coexisting heterotrophic bacterial groups.

The nitrifiers (AOB + NOB) accounted for about 60% of total bacteria detected with the probe mixed-Eub338 probes and constituted almost 100% of MAR-positive bacteria after 1 day of incubation with [^{14}C]bicarbonate (Figures 1A and 1C), underlining the

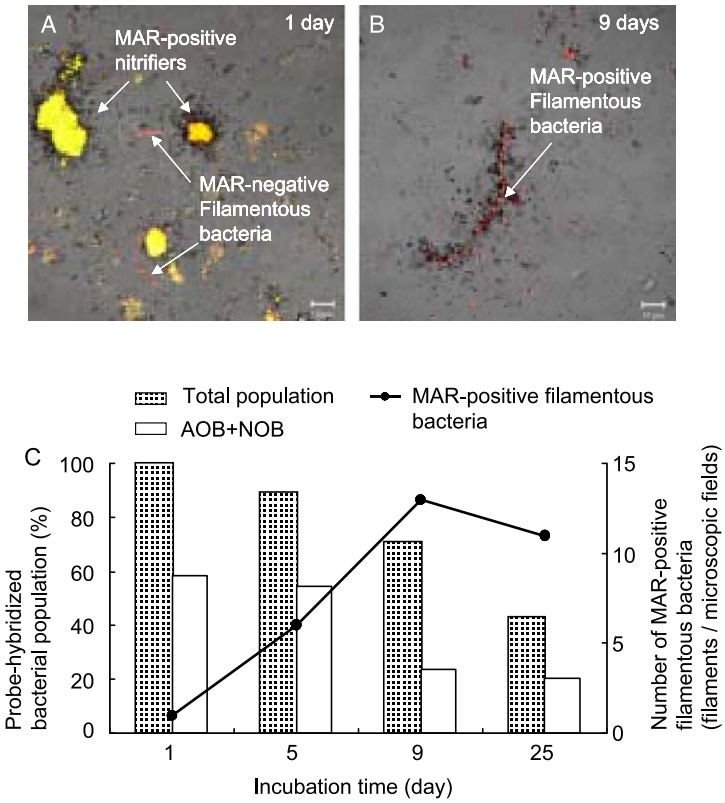

Figure 1 Time-dependent shift of radiolabeled (MAR-positive) microbial communities, showing [^{14}C]bicarbonate originally incorporated in nitrifying bacterial cells was subsequently utilized by mainly filamentous heterotrophic bacteria. (A), MAR-FISH image after 1 day of incubation with [^{14}C]bicarbonate, showing that nitrifiers were all MAR-positive, but filamentous bacteria were MAR-negative. (B), after 9 days of incubation, some MAR-positive filamentous heterotrophic bacteria were clearly found. FISH was performed with FITC-labeled Nso190 and Ntspa1026 probes (green) and TRITC-labeled Eub338 probe (red). The bars represent 10 μm. (C), Population dynamics of the total bacterial population (Eub338-hybridized cells), nitrifiers (AOB + NOB that hybridized with Nso190 and Ntspa1026 probes), and MAR-positive filamentous heterotrophic bacteria. The number of probe Eub338-hybridized cells at day 1 was defined as 100% of total bacterial population. Subscribers to the online version of *Water Science and Technology* can access the colour version of this figure from http://www.iwaponline.com/wst.

autotrophic growth of these bacteria. No uptake of [^{14}C]bicarbonate by any other groups of heterotrophic bacteria was observed at this stage. After 1-day incubation, filamentous bacteria belonging to the *Choroflexi* group and the unidentified group (bacteria hybridized with probe Eub338 but not with any other probes used in this study) did not utilize radiolabeled substrates yet, and therefore no silver grain was formed around cells (Figure 1A). However, significant accumulation of silver grains around these filamentous bacteria cells was first detected after 9-day incubation (Figure 1B). Thereafter, the population of MAR-positive filamentous bacteria significantly increased with incubation time, whereas the population of nitrifiers and Eub338-hybridized cells decreased by approximately 50% after 25 days of incubation due to cell decay (Figure 1C). This result clearly suggested that [^{14}C]bicarbonate originally incorporated in nitrifying bacterial cells was preferentially utilized by mainly filamentous heterotrophic bacteria belonging to the *Chloroflexi* group and the unidentified group. The phylogenetic identification of unidentified filamentous bacteria was not yet completed in this study. The identification is now in progress.

Conclusions

MAR-FISH revealed that heterotrophic bacterial community was composed of bacteria that were phylogenetically and metabolically diverse and to some extent metabolically redundant, which ensured the stability of integrated ecosystems as a biofilm. We could show direct evidence that organic matter derived from nitrifiers could be subsequently utilized by heterotrophic bacteria coexisting in the autotrophic nitrifying biofilm, which was visualized by MAR-FISH at single cell resolution for the first time. Based on these results, we concluded that [^{14}C]bicarbonate was first incorporated in nitrifying bacteria, and then the radiolabeled organic matter derived from nitrifiers was utilized by mainly filamentous bacteria belonging to the *Chloroflexi* (green non-sulfur bacteria) group and the unidentified group in the biofilm. Furthermore, the members of the α- and γ-*Proteobacteria* were specialized to utilize low-molecular-weight organic matter, whereas the members of the CFB division and *Chloroflexi* group were considered to be specialists for degrading structural cell components. This evidenced that an efficient food web (carbon cycle) existed in the autotrophic nitrifying biofilm community to assure the maximum utilization of metabolites of nitrifiers.

Acknowledgements

This research has been partly supported by Grant-in Aid (No. 09750627) for Developmental Scientific Research from the Ministry of Education, Science and Culture of Japan.

References

Amann, R.I., Krumholz, L. and Stahl, D.A. (1990). Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J. Bacteriol.*, **172**, 762–770.

Amann, R.I. (1995). *In situ* identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In *Molecular microbial ecology manual*, Akkerman, A.D.L., van Elsas, J.D. and de Bruijn, F.J. (eds), Kluwer Academic Publishers, Dordrecht, The Netherlands.

Cottrell, M.T. and Kirchman, D.L. (2000). Natural assemblages of marine proteobacteria and members of the *Cytophaga-Flavobacter* cluster consuming low- and high-molecular-weight dissolved organic matter. *Appl. Environ. Microbiol.*, **66**, 1692–1697.

Daims, H., Nielsen, J.L., Nielsen, P.H., Schleifer, K.-H. and Wagner, M. (2001). *In situ* characterization of *Nitrospira*-like nitrite-oxidizing bacteria active in wastewater treatment plants. *Appl. Environ. Microbiol.*, **67**, 5273–5284.

Ito, T., Nielsen, J.L., Okabe, S., Watanabe, Y. and Nielsen, P.H. (2002). Phylogenetic identification and substrate uptake patterns of sulfate-reducing bacteria inhabiting an oxic-anoxic sewer biofilm determined

by combining microautoradiography and fluorescent *in situ* hybridization. *Appl. Environ. Microbiol.*, **68**, 356–364.

Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K.-H., Pommerening-Roser, A., Koops, H.-P. and Wagner, M. (1998). Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominate populations. *Appl. Environ. Microbiol.*, **64**, 3042–3051.

Kindaichi, T., Ito, T. and Okabe, S. (2004). Eco-physiological interaction between nitrifying bacteria and heterotrophic bacteria in autotrophic nitrifying biofilms as determined by MAR-FISH. *Appl. Environ. Microbiol.*, **70**, 1641–1650.

Lee, N., Nielsen, P.H., Andreasen, K.H., Juretschko, S., Nielsen, J.L., Schleifer, K.-H. and Wagner, M. (1999). Combination of fluorescent *in situ* hybridization and microautoradiography—a new tool for structure-function analyses in microbial ecology. *Appl. Environ. Microbiol.*, **65**, 1289–1297.

Manz, W., Amann, R., Ludwig, W., Wagner, M. and Schleifer, K.-H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. *Syst. Appl. Microbiol.*, **15**, 593–600.

Manz, W., Amann, R., Ludwig, W., Vancanneyt, M. and Schleifer, K.-H. (1996). Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. *Microbiology*, **142**, 1097–1106.

Mobarry, B.K., Wagner, M., Urbain, V., Rittmann, B.E. and Stahl, D.A. (1996). Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Appl. Environ. Microbiol.*, **62**, 2156–2162.

Nielsen, J.L., Christensen, D., Kloppenborg, M. and Nielsen, P.H. (2003). Quantification of cell-specific substrate uptake by probe-defined bacteria under *in situ* conditions by microautoradiography and fluorescence *in situ* hybridization. *Environ. Microbiol.*, **5**, 202–211.

Nielsen, P.H., Aquino de Muro, M. and Nielsen, J.L. (2000). Studies on the in situ physiology of *Thiothrix* spp. present in activated sludge. *Environ. Microbiol.*, **2**, 389–398.

Okabe, S., Hirata, K., Ozawa, Y. and Watanabe, Y. (1996). Spatial microbial distributions of nitrifiers and heterotrophs in mixed population biofilms. *Botechnol. Bioeng.*, **50**, 24–35.

Okabe, S., Satoh, H. and Watanabe, Y. (1999). *In situ* analysis of nitrifying biofilms as determined by *in situ* hybridization and the use of microelectrodes. *Appl. Environ. Microbiol.*, **65**, 3182–3191.

Okabe, S., Kindaichi, T., Ito, T. and Satoh, H. (2004). Analysis of size distribution and areal cell density of ammonia-oxidizing bacterial microcolonies in relation to substrate microprofiles in biofilms. *Biotechnol. Bioeng.*, **85**, 85–95.

Ouverney, C.C. and Fuhrman, J.A. (1999). Combined microautoradiography-16S rRNA probe technique for determination of radioisotope uptake by specific microbial cell types *in situ*. *Appl. Environ. Microbiol.*, **65**, 1746–1752.

Schramm, A., Larsen, L.H., Revsbech, N.P., Amann, R.I. and Schleifer, K.-H. (1996). Structure and function of a nitrifying biofilm as determined by *in situ* hybridization and the use of microelectrodes. *Appl. Environ. Microbiol.*, **62**, 4641–4647.