ANALYSIS OF MICROBIAL COMMUNITY STRUCTURE AND IN SITU ACTIVITY OF NITRIFYING BIOFILMS

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ABSTRACT

Wastewater biofilms are very complex multispecies biofilms, displaying considerable heterogeneity with respect to both the microorganisms present and their physicochemical microenvironments. To understand the eco-physiology of individual microorganisms in the biofilm, techniques and tools with a high spatial and temporal resolution are required for direct detection of the spatial distributions of microbial species and their activities in minimally disturbed their natural habitats (e.g., biofilms). In this paper, we will, therefore, address the great potential of the combined use of the current FISH technique and microelectrodes to study the microbial ecology of complex microbial communities such as biofilms. The combination of these two techniques will provide reliable and direct information about relationships between in situ microbial activity and the occurrence of specific microorganisms in biofilms. As an example of the combined study, we will illustrate the in situ spatial organization of ammonia-oxidizing and nitrite-oxidizing bacteria on fine scale in autotrophic nitrifying biofilms by applying the full-cycle of 16S rRNA approach followed by fluorescence in situ hybridization (FISH), which is linked to their in situ activity distributions at a similar resolution determined by use of microelectrodes. The combination of these techniques allows relating in situ microbial activity directly to occurrence of nitrifying bacteria population.

KEYWORDS : Nitrifying biofilms, 16S rDNA-cloning analysis, fluorescent in situ hybridization (FISH), microsensors, nitrification, population dynamics.

INTRODUCTION

In wastewater treatment bioreactors, microorganisms are present and active in biofilms and aggregates. Besides conventional culture dependent techniques, modern molecular biological techniques have been used to study the diversity and ecology of microorganisms in wastewater treatment processes since the mid-1980s. Since that time many new insights into aerobic and anaerobic microbial wastewater treatment processes have been gained, which significantly expanded our understandings of process design and control. Based on fundamental knowledge of microbial community composition and the metabolic properties of microorganisms, wastewater treatment systems must be developed and operated to maximize microbial activities. Further insights into the factors affecting structure and function of mixed microbial communities in bioreactors are essential for advancing wastewater treatment.

Although microbial nitrification processes for nitrogen removal are becoming more important due to strict regulations on nitrogen discharge, nitrification is recognized as being difficult to maintain in wastewater treatment systems. Since the diverse nitrifying bacterial populations are expected to be present in wastewater biofilms, different species of NH₄⁺- and NO₂⁻-oxidizing bacteria exhibit different in situ growth kinetics, substrate affinities, and sensitivities to various environmental factors (e.g., pH, temperature, O₂ concentration and substrate concentrations). Thus, a better understanding of microbiology, ecology, and population dynamics of nitrifying bacteria in wastewater biofilm systems is essential for improving process performance and control.
Therefore, we investigated successional development of nitrifying bacterial community structure and in situ nitrifying activities in biofilms when the biofilms were grown on rotating disk reactors (RDR) with domestic wastewater and a synthetic nutrient medium. To achieve this goal, we have combined molecular techniques (i.e., 16S ribosomal DNA (rDNA)-cloning analysis and fluorescent in situ hybridization (FISH) with a set of fluorescently labeled 16S rRNA-targeted DNA probes and microsensor measurements for NH$_4^+$, NO$_2^-$, NO$_3^-$, and O$_2$. The combined use of these techniques made it possible to relate in situ nitrifying activity directly to the occurrence of nitrifying bacterial populations. FISH visualized successional development of nitrifying bacterial community within an autotrophic nitrifying biofilm. After reaching the steady-state condition, microprofiles of NH$_4^+$, NO$_2^-$, NO$_3^-$, and O$_2$ in the biofilms were measured by use of microsensors, and the spatial distributions of in situ nitrifying activities were determined. The relationship between the spatial organization of nitrifying bacterial populations and the in situ activity of these populations within the biofilms was discussed.

**Microsensors**

For analysis of microbial structure and function (activity) of such complex microbial communities, classical microbiological techniques like isolation and physiological characterization have limitations. Therefore, appropriate methods with sufficiently high spatial resolution are needed for (1) in situ identification, localization, and quantification of microbial populations, (2) the determination of physicochemical microenvironment, and (3) the measurement of their in situ activity. Combination of FISH and microsensor technology became a powerful and reliable tool during the last two decades. The structure and principle of commonly used amperometric and potentiometric microsensors are shown in **Fig.1**. The spatial resolution of microsensors is about two times the tip diameter of the sensors as long as analyte consumption by the sensor is negligible and the sensor is small enough to cause minimum disturbance. The tip diameter of microsensors applied for biofilms and aggregates is about 10 µm (**Fig.1**), indicating the spatial resolution of about 20 µm. This resolution is good enough to characterize the concentration gradients across the biofilms, microbial mats and sediments and to calculate the net rates (areal and volumetric) of production and consumption at a certain depth or of whole microbial community. During the last decade, microelectrode measurement was nicely combined with FISH to relate microbial community structure and function of SRB (Ito et al., 2002; Kuhl and Jorgensen, 1992; Okabe et al., 1999b; Okabe et al., 2003; Ramsing et al., 1993) and nitrifying bacteria (Gieseke et al., 2001; Okabe et al., 1999a; Okabe et al., 2001; Satoh et al., 2003; Schramm et al., 1996; Schramm et al., 2000) in biofilms. The combination of two methods allows relating in situ microbial activity directly to occurrence of specific microorganisms within complex microbial consortia. Microelectrodes, however, measure only net chemical profiles, and the spatial resolution is also above a single-cell level. To address the question of the higher abundance and activity of SRB in oxic zones of biofilms (Okabe et al., 1999b; Ito et al., 2002), for example, the resolution of microelectrode measurements is not high enough. In

![Figure 1](image_url). The structure and principle of the amperometric and potentiometric (LIX) microsensors.
addition, when the resources used by an uncultured microorganism are unknown or the abundance of the targeted microorganism is low in complex and heterogeneous habitats, the chemical profiles and fluxes cannot correlate to the abundance of the specific bacterial populations. Therefore, an analytical method at a single-cell level that allows us to more directly correlate the identity (16S rRNA-based phylogeny) and specific metabolic activity of individual cells has been desired.

16S rRNA Approach

Microbial community structure analysis, primarily based on 16S ribosomal RNA (rRNA) gene sequencing, is becoming the most powerful tool to study nitrifying bacterial populations present in biofilm reactors (Amann et al., 1995; Head et al., 1998; Olsen et al., 1986). An overview of a microbial community analysis with 16S rRNA approach is given in Figure 2. Nucleic acids from an environmental sample including multispecies microorganisms are extracted, selectively amplified by the polymerase chain reaction (PCR) using a diverse set of primers and separated by several fingerprinting techniques including denaturing gradient gel electrophoresis (DGGE) (Muyzer and Smalla, 1998), temperature gradient gel electrophoresis (TGGE) (Muyzer and Smalla, 1998), and terminal restriction fragment length polymorphisms (t-RFLP) (Liu et al., 1997). The amplified 16S rRNA gene fragments are “shotgun cloned”, and the different types of cloned rRNA genes are then sorted and are subsequently subjected to sequence analysis. The retrieved sequences can be phylogenetically analyzed by comparing with existing 16S rRNA gene database and used for probe and primer designing. The fingerprinting techniques can be used to monitor microbial community changes in natural environments and bioreactors. Different hybridization analyses are methods to quantify and identify the RNA genes of different microbial populations. Fluorescent in situ hybridization (FISH) (Amann, 1995) is used for identification and quantification of microbial species and to observe the spatial distribution of specific microbial populations in biofilms and aggregates. This rRNA approach does not include cultivation, which circumvents the biases associated with culture-dependent techniques. It must be noted that this approach enables identification and quantification of “as yet unknown and/or uncultivated microorganisms”.

![Figure 2. Flow diagram of the different steps in the full- cycle rRNA approach.](image-url)
EXPERIMENTAL MATERIALS AND METHODS

Biofilm Samples

Two types of biofilms, a domestic wastewater biofilm and an autotrophic nitrifying biofilm, were studied. Both biofilms were cultured in partially submerged rotating disk reactors (RDR) consisting of 5 poly-methyl-methacrylate disks. Eight removable slides (1×6 cm) were installed in each disk for sampling biofilms (Okabe et al., 1996). The autotrophic nitrifying biofilms were first cultured with the primary settling tank effluent from the Shoseigawa municipal wastewater treatment plant, Sapporo, Japan for a few days and then were cultured with synthetic nutrient. The nutrient medium was composed of the followings (mM): NH₄Cl, (3.6); NaHCO₃, (17.8); K₂HPO₄, (0.03); MgSO₄·7H₂O, (0.41); NaCl, (1.25); pH=7.0±0.2. The reactor volume was 1370 cm³, and total biofilm area was 2830 cm². Temperature was maintained at 20°C. Disk rotational speed was fixed at 14 rpm. Dilution rate in the reactors was kept at 0.2 h⁻¹.

Nucleic Acid Extraction and PCR Amplification

Approximately, 1 g of each wet biofilm sample was mixed with 1 ml of AE buffer (20 mM sodium acetate [pH 5.5], 1 mM EDTA) in clean 15-ml tubes. After ultrasonic treatment (10 min) and lysis (5 mg of lysozyme per ml for 40 min at 37°C and 2 mg of protease K per ml for 30 at 55°C), bacterial DNA was extracted from biofilm samples by a combined freeze-thaw (three cycles of freezing in liquid nitrogen and heating at 37°C), 1% (wt/vol) sodium dodecyl sulfate (SDS) treatment, and hot phenol-chloroform-isoamyl alcohol treatment (Teske et al., 1996). The 16S rRNA genes (rDNA) from mixed bacterial DNA were amplified by PCR with the primer set of CTO189f and CTO654r as described by Kowalchuk et al. (1997). For general bacteria, almost-full length bacterial 16S rDNA fragments were amplified using the primer set of GM3f (Escherichia coli 16S rDNA positions 8 to 24) and GM4r (E. coli positions 1492 to 1507) as described by Muyzer et al. (1995). To minimize nonspecific annealing of the primers to nontarget DNA, a hot-start and touch-down PCR program was used for all amplification (Muyzer et al., 1997). The PCR products were evaluated on a 1 % (w/v) agarose gel.

Cloning of 16S rDNA

One microliter of the amplified bacterial 16S rDNA fragments (465 bp including variable V3 region) was directly ligated into the pGEM-T vector cloning system (Promega) and transformed into competent cells (high-efficiency E. coli JM109 [Promega]) as described in the manufacturer’s instruction.

Sequencing and Phylogenetic Analysis

Plasmids were extracted and purified from clones with the Wizard Plus Minipreps DNA purification system (Promega) in accordance with the manufacturer’s instructions. To avoid redundant sequencing, PCR-amplified rDNA fragments of all clones were analyzed by RFLP (Restriction fragment length polymorphism) after digestion with restriction enzymes of cfoI or haeIII as described in the manufacturer’s instruction. The PCR fragments digested were loaded on a 2.0 % (w/v) agarose gel. Similar fragment migration patterns were defined as identical recombinants, and one representative of each group of recombinants was selected for comparative sequence analysis. Partial sequencing (ca. 465 bp) of the 16S rDNA inserts was performed with an automatic sequencer (HITACHI). All sequences were checked for chimeric artifacts by the CHECH_CHIMERA program in the Ribosomal Database Project (RDP)(Maidak et al., 1997) and compared with similar sequences of the reference organisms by BLAST search (Altschul et al., 1990). Sequence data were aligned with the CLUSTAL W package (Thompson et al., 1994). Phylogenetic trees were constructed by the neighbour-joining method (Saito and Nei, 1987) with Tree Explore. Bootstrap resampling analysis for 100 replicates was performed.

Fixation and Cryosectioning of Biofilm Samples

Biofilm samples were taken at regular time intervals during the periods of the biofilm development. The biofilm samples were fixed with freshly prepared paraformaldehyde solution (4% in phosphate buffered saline (PBS), pH=7.2) for 4 to 8 h at 4°C and embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN) overnight to
infiltrate the OCT compound into the biofilm. After rapid freezing at –21°C, 10 to 20-µm-thick vertical slices were cut with a cryostat (Reichert-Jung Cryocut 1800, Leica) and placed on a gelatin-coated slide (Cell-line, USA, 0.1 % gelatin and 0.01 % chromium potassium sulfate). After air-drying overnight, the slices were dehydrated by successive passage through 50, 80, and 98 % ethanol washes (for 3 min each), air-dried, and stored at room temperature.

Oligonucleotide Probes

The following oligonucleotide probes were used: Nso190 (Mobarry et al., 1996), NEU (Wagner et al., 1995), Nsm156 (Mobarry et al., 1996), Nsv443 (Mobarry et al., 1996), NIT2 (Wagner et al., 1996), NIT3 (Wagner et al., 1996), Ntspa454 (Hovance et al., 1998), Ntspa685 (Hovance et al., 1998), and Ntspa1026 (Juretschko et al., 1998). Probes were labeled with fluorescein isothiocyanate (FITC) or tetramethylrhodamine-5-isothiocyanate (TRITC). Unlabelled competitor CNIT3 (Wagner et al., 1996) and CTE (Schleifer et al., 1992) probes were added to an equimolar amount of NIT3 and NEU probes, respectively.

In Situ Hybridization

The previously published optimal hybridization conditions were used for each probe. All *in situ* hybridizations were performed according to the procedure described by Amann (1995) in hybridization buffer (0.9 M NaCl, 20mM Tris hydrochloride (pH=7.2), 0.01% sodium dodecyl sulfate (SDS), x% formamide) at 46°C for 2-3 hours. The final probe concentration was approximately 5 ng µl⁻¹. Subsequently, a stringent wash step was performed at 48°C for 20 min in 50 ml of pre-warmed washing solution (x mM NaCl, 20 mM Tris hydrochloride (pH=7.2), 0.01 % SDS). The stringency of the washing step (at 48°C) was adjusted by lowering the sodium chloride concentration to achieve the appropriate specificity. The slides were then rinsed briefly with ddH₂O and allowed to air dry. Simultaneous hybridization with probes requiring different stringency was performed by a successive hybridization procedure: hybridization with the probe requiring higher stringency was performed first, and then hybridization with the probe requiring lower stringency was performed (Wagner et al., 1994). Slides were mounted in SlowFade™-light antifade kit (Molecular Probes, Eugene, OR).

Microelectrode Preparation and Measurements

For determination of concentration profiles in the biofilms, cathode type oxygen microelectrodes with a tip diameter of about 10 µm was prepared and calibrated as described previously by Revsbech and Jorgensen (1986). Liquid ion-exchanging membrane (LIX) microsensors for NH₄⁺, NO₂⁻, and NO₃⁻ were prepared according to deBeer et al. (1997). The LIX microsensors were calibrated in dilution series (10⁻³-10⁻⁶ M) of NH₄⁺, NO₂⁻, and NO₃⁻ in the medium used for the measurements. All measurements were performed as described previously (deBeer and Heuvel, 1988) in a flow cell reactor at 20°C, with an average liquid velocity of 2-3 cm s⁻¹ by blowing air on the liquid surface. The composition of the medium used for microprofile measurements was described previously by deBeer et al. (1993). The biofilm samples taken from the reactor was acclimated in the medium a few hours before the measurement, to ensure that steady state profiles were obtained.

Estimation of Consumption and Production Rate Profiles

Net specific consumption and production rates (R; µmol cm⁻³ h⁻¹) of NH₄⁺, NO₂⁻, and NO₃⁻ were estimated from the measured microprofiles by using the Fick's second law of diffusion as previously described by Lorenzen et al. (1998). Molecular diffusion coefficients of 1.38×10⁻⁵ cm² s⁻¹ for NH₄⁺, 1.23×10⁻⁵ cm² s⁻¹ for NO₂⁻, and 1.23×10⁻⁵ cm² s⁻¹ for NO₃⁻ at 20°C were used for the calculations (Andrussow, 1969).

RESULTS AND DISCUSSION

Spatial Distributions of Nitrifying Bacteria

In the autotrophic nitrifying biofilm, spherical clusters of densely packed probe Nso190-stained NH₄⁺-oxidizing bacterial cells were detected throughout the oxic biofilm strata, indicating more or less a homogeneous spatial
distribution of NH$_4^+$-oxidizing bacteria (Fig. 3B). In contrast, clusters of the Ntspa 454 probe-stained Nitrospira-like cells were primarily detected in the deeper part of the oxic region. No hybridization signal was observed when Nitrobacter-specific probes NIT2 and NIT3 were used with any of the samples. The sequential oxidation of NH$_4^+$ and NO$_2^-$ found by microelectrode measurements coincides with locations where higher abundance of NH$_4^+$- and NO$_2^-$-oxidizing bacteria were detected. The lower NO$_2^-$ oxidation activity in the surface zone can be explained by the absence (or lower abundance) of NO$_2^-$-oxidizing bacteria. This is a good example showing a correlation between the distribution of microbial species and accompanying expected activity. However, it is not always to find such correlation, because the spatial distributions of microbial species can be a result of previous stages of biofilm development, rather than an optimal adaptation to the actual microenvironments.

Figure 3. Fluorescence in situ hybridization result combined with microsensor measurements. An autotrophic nitrifying biofilm was cultured with synthetic medium (the substrate C/N ratio was 0). In situ hybridization of a vertical biofilm thin section with TRITC-labeled Nso190 probe specific for NH$_4^+$-oxidizing bacteria of the beta subclass of the Proteobacteria (red stain clusters) and FITC-labeled Ntspa454 and Ntspa1026 specific for Nitrospira moscoviensis and some environmental clones (green stain clusters) (A). Corresponding steady-state microprofiles of O$_2$, NH$_4^+$, NO$_2^-$, and NO$_3^-$ in the autotrophic nitrifying biofilm (B). The distribution and magnitude of the estimated specific rates of net consumption and production of NH$_4^+$, NO$_2^-$, and NO$_3^-$ (C). The solid lines are the best fits from the model to calculate the specific consumption and production rates of NH$_4^+$, NO$_2^-$, and NO$_3^-$ in this zone. The values are means of triplicate measurements.

Microelectrode Measurements

Steady-state concentration profiles of O$_2$, NH$_4^+$, NO$_2^-$, and NO$_3^-$ within the nitrifying biofilm were measured by microsensors (Fig. 3B). All measurements were performed in a flow cell reactor at 20°C, with an average liquid velocity of 2-3 cm s$^{-1}$ by blowing air on the water surface. The medium used for microprofile measurements contained following ingredients: 200 µM NH$_4$Cl, 50 µM NaNO$_2$, 300 µM NaNO$_3$, 570 µM Na$_2$HPO$_4$, 84 µM MgCl$_2$·6H$_2$O, 200 µM CaCl$_2$, and 270 µM EDTA (pH=7.0). Oxygen penetrated approximately 200 µm into the biofilm (biofilm thickness = ca. 250 µm). The NH$_4^+$, NO$_2^-$, and NO$_3^-$ profiles showed that the consumed NH$_4^+$ was primarily converted to NO$_2^-$ in the upper 75 µm with a NO$_2^-$ peak of 64 - 73 µM at 50 - 75 µm and no significant NO$_3^-$ production in this zone. The produced NO$_2^-$ was eventually converted to NO$_3^-$ in the deeper oxic layer.
Microbial diversity of ammonia-oxidizing bacteria clones sequenced were closely related to members of respectively. 16S rDNA sequence analysis revealed that about 62% of the total domestic wastewater biofilm taxonomic units: OTUs) were found in the domestic wastewater biofilm and autotrophic nitrifying biofilm libraries, was conducted for phylogenetic analysis. Among the clones analyzed, 10 and 7 different sequences (operational (CTO189f and CTO654), and partial sequencing (465-bp) including variable V3 region of the clonal 16S rDNAs

When biofilms were cultured with domestic wastewater and synthetic media containing organic carbon, the active NH4+ oxidation zone was vertically separated from the active NO2- oxidation zone. That is, the active NH4+ oxidation zone was located in the outer part of a biofilm, whereas the active NO2- oxidation zone was located just below the NH4+ oxidation zone. We will discuss more about the relationship between the in situ activity of NH4+ and NO2- oxidation and the spatial distributions of NH4+- and NO2-oxidizing bacteria within biofilms grown in different media.

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Micro-scale Spatial Organization of Nitrifying Bacteria

Although the biofilm was cultured in the synthetic medium containing no organic carbon, the NH4+-oxidizing bacteria clusters were surrounded by a number of heterotrophs including filamentous bacteria, which may suggest these heterotrophs could utilize soluble organic compounds secreted from NH4+-oxidizing bacteria (Kindaichi et al., 2004). NH4+-oxidizing bacteria formed densely packed spherical clusters and closely associated with NO2-oxidizing bacteria, demonstrating the sequential metabolism of ammonia via nitrite to nitrate. By such close association, the diffusion path from NH4+-oxidizing bacterial clusters to the surrounding NO2-oxidizing bacteria is short and facilitates an efficient transfer of the intermediate NO2-. It should be noted that these micro-scale spatial organizations of two phylogenetically unrelated species can only be obtained by use of the FISH technique.

Microbial diversity of ammonia-oxidizing bacteria

The phylogenetic microbial diversity of two types of nitrifying biofilms, a domestic wastewater and an autotrophic nitrifying biofilm, were determined by 16S rDNA-cloning and compared (data not shown). 16S rDNA clone libraries were constructed by PCR with a beta-subdivision ammonia-oxidizing bacteria-specific primer set (CTO189f and CTO654), and partial sequencing (465-bp) including variable V3 region of the clonal 16S rDNAs was conducted for phylogenetic analysis. Among the clones analyzed, 10 and 7 different sequences (operational taxonomic units: OTUs) were found in the domestic wastewater biofilm and autotrophic nitrifying biofilm libraries, respectively. 16S rDNA sequence analysis revealed that about 62% of the total domestic wastewater biofilm clones sequenced were closely related to members of Nitrosomonas ureae with more than 97% sequence similarity. These clones were closely related to each others. We also detected three clones affiliated with Nitrosomonas europaea, Nitrosomonas eutropha, and Nitrosococcus mobilis, respectively. One clone was affiliated with a deeply branched group of Nitrosovibrio and Nitrosococcus. In the autotrophic nitrifying biofilm library, the most
dominant sequence was affiliated with *Nitrosomonas eutropha* with more than 95% sequence similarity. Six clones were closely related to *Nitrosomonas europaea*. This result indicated that although the strains affiliated with *N. ureae* were numerically dominant NH$_4^+$-oxidizers in the domestic wastewater biofilm, *N. eutropha* and *N. europaea* who have higher growth rates became dominant populations in the autotrophic nitrifying biofilm after switching to the synthetic nutrient medium.

**Development of NH$_4^+$-oxidizing Bacterial Populations in Biofilms**

To visualize the population dynamics of NH$_4^+$-oxidizing bacteria in the autotrophic nitrifying biofilm, fluorescent *in situ* hybridization (FISH) with a set of 16S rRNA-targeted oligonucleotide probes (i.e., Nso190, Nsm156 and NEU) were performed. *In situ* hybridization of vertical biofilm thin sections clearly indicated that the numbers of probe NEU-stained NH$_4^+$-oxidizing bacteria (i.e., *Nitrosomonas marina*-lineage, *Nitrosomonas europaea*-lineage, *Nitrosomonas eutropha*, and *Nitrosomonas halophila*) were very low in the young autotrophic nitrifying biofilm, and other *Nitrosomonas*-lineages which hybridized with probe Nsm156 but did not hybridized with NEU were numerically dominant populations. As the biofilm grew, probe NEU-stained NH$_4^+$-oxidizing bacteria became the dominant populations in the autotrophic nitrifying biofilm. This population shift might be attributed to the inhibitory effect of NO$_2^-$ accumulated up to approximately 1.5 mM during the biofilm growth and higher growth rates.

In contrast, *Nitrosomonas* spp. which hybridized with probe Nsm156 but did not hybridized with NEU were the numerically dominant species in the domestic wastewater biofilm. According to the results of 16S rDNA-cloning analysis, NH$_4^+$-oxidizing bacteria which hybridized with probe Nsm156 but did not hybridized with NEU could be a member of *Nitrosomonas ureae*. The FISH result reflected the results of 16S rDNA-cloning analysis. The NO$_2^-$-oxidizing bacteria belonging to the genus *Nitrobacter* could not be detected; instead, *Nitrospira* were found to be the main NO$_2^-$-oxidizing bacteria in both types of biofilms.

**CONCLUSIONS**

16S rRNA-based fluorescence *in situ* hybridization (FISH) and microsensor techniques have a high spatial (at a single-cell resolution) and temporal resolution and great potential, and provide reliable and direct information about the occurrence of specific microorganisms and their *in situ* microbial activity in biofilms, respectively. Such information cannot be obtained by conventional culture-dependent techniques. These observations have considerable significance to our understandings of microbial nitrification occurring in wastewater biofilm processes.

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