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Full Length Research Paper

Microbial morphology and community structure in a suspended carrier biofilm reactor as a function of substrate loading rates

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An aerobic suspended carrier biofilm reactor was efficient in simultaneous organic carbon and nitrogen removal, with COD removal efficiencies of 87.1–99.0% and simultaneous nitrification and denitrification (SND) efficiencies about 96.7-98.8%. The effects of substrate loading on microbial morphology and community structure were investigated by environmental scanning electron microscopy (ESEM), denaturing gel gradient electrophoresis (DGGE) and fluorescence *in situ* hybridization (FISH). Biofilms formed at different substrate loadings had different morphology and community structures. A higher substrate concentration resulted in denser and thinner biofilms, while a lower substrate concentration resulted in looser and thicker biofilms with significant presence of filamentous bacteria. Both sequence analysis of DGGE bands and FISH analysis indicated the dominance of β-Proteobacteria in the biofilm communities, especially Zoogloea. FISH analysis revealed that the relative abundance of β-proteobacteria ammonia oxidizing bacteria (AOB) was positively correlated with ammonium concentrations, whereas Nitrospira-like nitrite-oxidizing bacteria (NOB) were negatively affected by ammonia and nitrite concentrations. The presence of denitrifying bacteria, *Hydrogenophaga* spp., *Hyphomicrobium* spp. and *Rhizobium* spp. suggested that not only the oxygen microgradients within the biofilm but also aerobic denitrifiers may be responsible for SND in the aerobic biofilm.

Key words: Microbial community structure, microbial morphology, substrate loading, suspended carrier biofilm.

INTRODUCTION

Biological wastewater treatment is a benign biotechnology for the removal of environmental pollutants from wastewater. The removal of organic carbon and nutrients like nitrogen is of priority for limiting eutrophication of the receiving water bodies. This requires the combined or sequential activities of different groups of microorganisms. Conventional biological nitrogen removal involves aerobic nitrification by autotrophic nitrifing bacteria [ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB)] and anoxic denitrification by heterotrophic denitrifier. Therefore, two separate bioreactors under anoxic and aerobic conditions or sequencing batch reactor (SBR) exposed to alternating anoxic and aerobic conditions is required to provide suitable environments for nitrogen removal. Our previous study showed simultaneous nitrification and denitrification (SND) occurred in a aerobic biofilm reactor, and appropriate control of microbial community structure resulting from optimal carbon nitrogen ratio is beneficial to improve SND (Fu et al., 2010). The substrate loading rate is thought to be a key parameter in determining sludge or biofilm structure and function (Wijeyekoon et al., 2004; Li et al., 2008). Therefore, the effects of substrate loadings on microbial community in suspended carrier biofilm reactors for simultaneous organic carbon

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and nitrogen removal also needs to be further investigated.

The limitations of conventional culture-dependent techniques hindered the understanding of microbial ecology in a wastewater system. Fortunately, the recent development of the molecular biological techniques has enabled researchers to analyze the complex microbial communities involved in the wastewater (Sanz and Köchling, 2007). Environmental scanning electron microscopy (ESEM), a modified scanning electron microscopy (SEM), enables high magnification surface images of moist and non-conductive specimens, thus imaging of specimens can be conducted in their natural state without destructive sample preparation techniques associated with standard SEM imaging (Priester et al., 2007). The objectives of the current study were to (1) investigate morphology and community structure of suspended carrier biofilm as a function of substrate loadings by a combination of ESEM, DGGE and FISH, and to (2) compare the reactor performances of the aerobic biofilm reactor in terms of microbial diversity and nitrifying bacteria. The mechanism for nitrogen removal in the single biofilm reactor under aerobic condition was also discussed.

MATERIALS AND METHODS

Reactor operation

Details of the reactors and operation conditions are described elsewhere (Fu et al., 2010). The reactors were continuously fed with synthetic wastewater at three different substrate loadings with same COD:N:P ratios of 100:5:1 and airflow. The influent chemical oxygen demand (COD) was 500, 1000 and 1500 mg/l for the three reactors M1, M2 and M3, respectively. Organic carbon, nitrogen and phosphorus were added to the solution as glucose, NH_4Cl and KH_2PO_4. The influent was added with trace elements solution containing Fe, Co, Mn, Cu, Zn, Mo and Ni.

Analytical methods

The effluent samples were analysed immediately after filtering through 0.45 µm filter paper. The COD and NH_4–N concentrations were measured every day using the standard methods (APHA, 1998). The NO_2–N and NO_3–N concentrations were measured using the standard methods after steady state was achieved. The microbial morphology of suspended carrier biofilm without sample preparation was examined by a Quanta 200 FEG ESEM (FEI, Holand).

Biofilm sampling

Suspended carriers taken from the reactor were transmitted into 1.0 mM phosphate buffer solution (PBS). Biofilms were detached from carriers and homogenized by ultrasonication (120 s at 50 W) in PBS. The carriers were subsequently washed with PBS for three times to collect cells by centrifugation at 12,000 g for 10 min at 4°C. The wet samples were then freeze-dried, ground with liquid nitrogen, and stored at –20°C for DNA extraction. For FISH analysis, wet biofilm biomass were fixed in freshly prepared 4% paraformaldehyde at 4°C for 12 h immediately after sampling, and rinsed twice with PBS. After the fixation and washing steps, 0.01 g of each wet biofilm sample was stored in PBS-ethanol (1:1) at –20°C until further analysis.

DNA extraction, PCR and DGGE

The genomic DNA in dry samples was extracted following the protocol described by Miller et al. (1999). DGGE was performed as described by Fu et al. (2010). Specific bands were manually excised from the gel, and used as a template in a re-amplification using the primers Bac341F and Univ518R for sequencing. Gel images were analyzed to determine the microbial diversity using Quantity One Software Vers. 4.1 (Bio-Rad, Hercules, CA, USA). Community similarities in band patterns were calculated using the Dice coefficients and displayed graphically in a form of UPGMA dendrogram. The obtained sequences were compared with 16S rRNA gene sequences in NCBI database using the BLASTn search program.

FISH Analysis

Biofilm samples were spotted on gelatin-coated microscopic slide, air dried, dehydrated respectively in 50, 80 and 96% of ethanol solutions for 3 min, and then dried in the air. The sequences, specificities and hybridization conditions of the probes are listed in Table 1. The probes were labeled with cyanine 3 (Cy3) or carboxyfluorescein (FAM) at the 5' end (Genscript, Nanjing, China). The hybridization and washing procedures were carried out as the methods published by Amann et al. (1999). An AXIO IMAGER All fluorescent microscope equipped with FITC/DAPI specific filters (Zeiss, Oberkochen, Germany) was used for microscopic observation. The numbers of probe-hybridized cells were determined by direct counting of the minimum of ten randomly selected fields covering 36600 μm^2 in sample covering area of 400 mm^2 of slide. The hybridization experiments were carried out in duplicate, and the number was averaged.

RESULTS

Biofilm reactor performance

The measured influent and effluent COD and ammonia (NH_4–N) concentrations of the three reactors during 37 days are presented in Figure 1. The steady state between day 27 and 37 was characterized by stable bioreactor performance. The average COD removal rates during the steady state phase were 94.1, 93.4 and 95.6% for M1, M2 and M3, respectively (Figure 1a). The results show that all the biofilm reactors were efficient for the removal of organic substances despite different influent COD loadings. The effluent ammonia concentration decreased gradually with the operation time (Figure 1b). The ammonia oxidation rate was positively proportional to the influent NH_4–N concentration. The mean ammonia oxidation rate was 86.4% in M1 with the influent NH_4–N concentration being 25 mg/l, and that of M2 and M3 increased accordingly to 89.2 and 93.5% with the increase of influent NH_4–N concentration (approximately 50 for M2 and 75 for M3). The average nitrite

Table 1. FISH oligonucleotide probes employed in this study.

Probe	Sequence (5'-3')	Specificity	% FA[a] /NaCl concen[b] (mM)	Reference
EUB338	GCTGCCTCCCGTAGGAGT	Most *bacteria*	20/225	Amann et al. (1990)
ALF1b	CGTTCGYTCTGAGCCAG	Most alpha *proteobacteria*	20/60	Manz et al. (1992)
BET42a	GCCTTCCCACTTCGTTT	Most beta *proteobacteria*	35/80	Manz et al. (1992)
GAM42a	GCCTTCCCACATCGTTT	Most gamma *proteobacteria*	35/60	Manz et al. (1992)
NSO190	CGATCCCCTGCTTTTCTCC	Ammonia-oxidizing beta *proteobacteria*	55/20	Mobarry et al. (1996)
Ntspa662	GGAATTCCGCGCTCCTCT	Genus *Nitrospira*	35/80	Daims et al. (2001)

[a] FA, formamide concentration in the hybridization buffer.
[b] NaCl concen, NaCl concentration in the wash buffer.

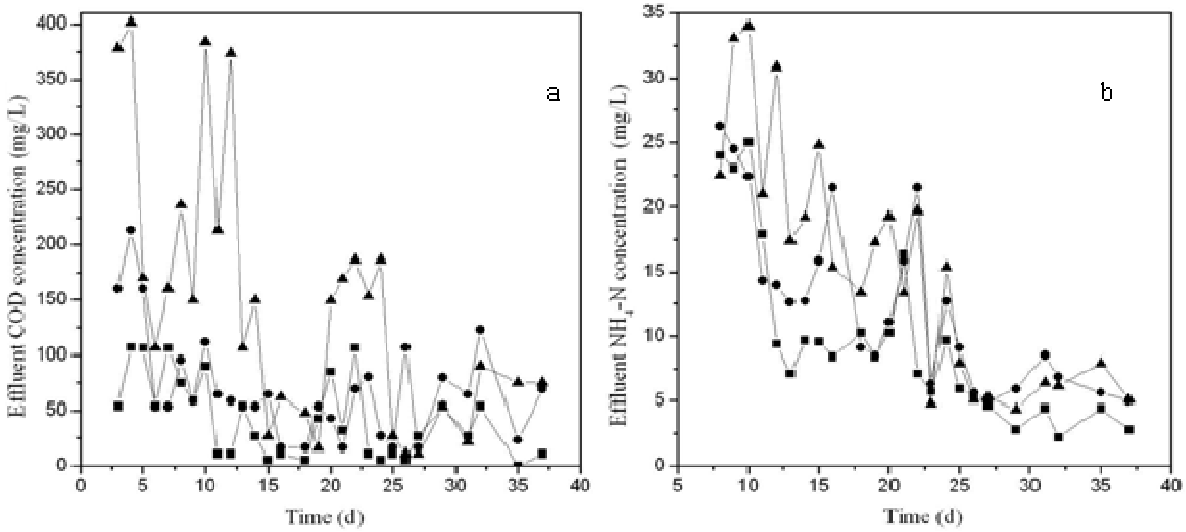

Figure 1. Effluent COD (a) and NH_4-N concentrations (b) of M1 (■), M2 (●) and M3 (▲). During the operation the influent COD was about 500, 1000 and 1500 mg/l for M1, M2 and M3, respectively, and the influent NH_4-N was about 25, 50 and 75 mg/l for M1, M2 and M3, respectively. Samples were collected on day 20 and 37.

concentrations in the effluent were around 0.18, 0.46 and 1.06 mg/l, respectively, in the bulk liquid of M1, M2 and M3 for the steady state. Additionally, the average nitrate concentrations were 2.28, 1.67 and 1.55 mg/l, respectively, showing that efficient simultaneous carbon and nitrogen removal based on SND occurred in the aerobic biofilm reactor under different substrate loadings. Gupta and Gupta (2001) also reported that an aerobic rotating biological contactor (RBC) biofilm achieves simultaneous carbon and nitrogen removal from high strength domestic wastewater.

Biofilm characteristics

Both visual and microscopic observations obtained at the end of experiment (Day 37) revealed that the biofilms formed at different substrate loadings had different morphological characteristics. All the internal surfaces and axial space of carriers from M1 were covered by the very hairy, thick and dark brown biofilm, with protrusions waving in the liquid. In comparison with M1, the biofilm attached to the carriers from M2 was thinner and light brown in colour with a sightly rough surface, while that of M3 was thinnest, dense, and light yellow in colour with a smooth surface profile. Visual observations showed that the thickness of suspended carrier biofilm decreased and the colour became light as the substrate concentration increased.

The morphology of the carrier biofilm developed under different substrate loadings as obtained by ESEM is illustrated in Figure 2. The biofilm formed in M1 was dominated by microsphere clusters coated by exopolysaccharide (Figures 2a and b), and much filamentous bacteria were visible (Figure 2c). Large numbers of various rod-shaped bacteria and cocci were present in the biofilm of M2 (Figure 2d and e), some filamentous bacteria were also present (Figure 2f).

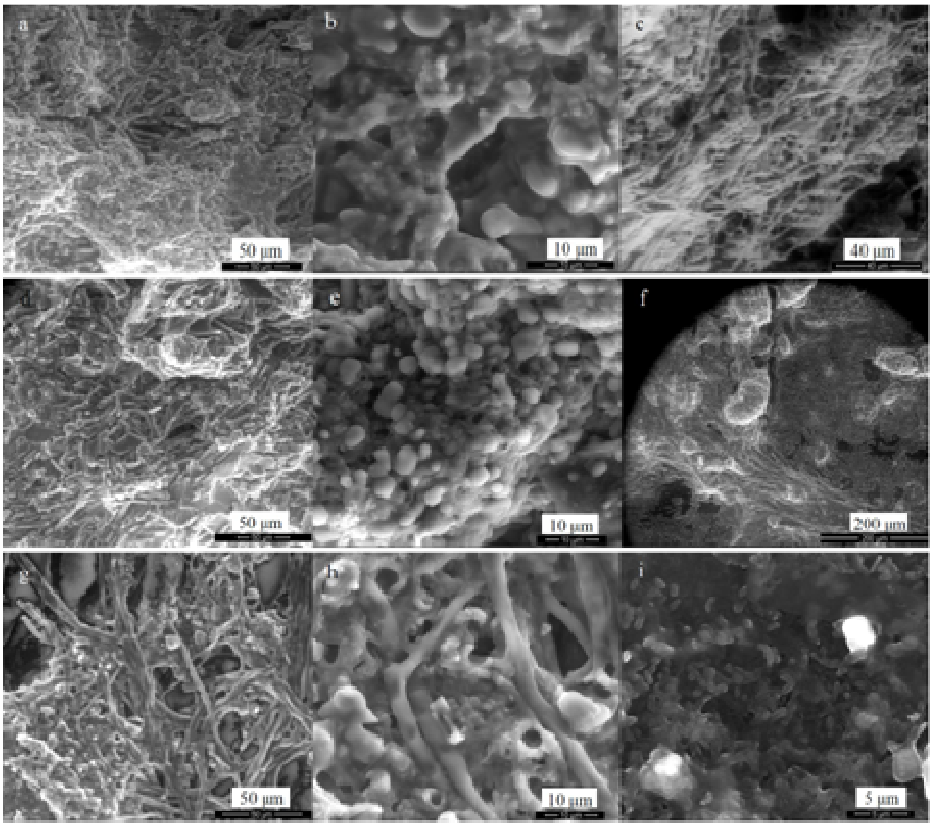

Figure 2. ESEM images of suspended carrier biofilm from M1 (a, b, c), M2 (d, e, f) and M3 (g, h, i).

Compared with M1 and M2, the biofilm structure of M3 was densely populated with the microorganisms with no visible pores. The outer layer mainly consisted of a massive accumulation of microspheres, which attached on the surface of filamentous bacteria (Figure 2g and h), and the internal biofilm growing on the surface of the carrier biofilm layer mainly consisted of rod-shaped bacteria with rounded ends and some cocci (Figure 2i).

DGGE profiles of microbial communities

Microbial community structures of the three reactors on two sample dates (Day 20 and 37) were analyzed by DGGE of the PCR-amplified variable V3 region of the 16S rRNA gene (Figure 3a). The microbial communities from the three reactors were only moderately similar to each other as Dice coefficient ranged between 0.60 and 0.71 on day 20, and the similarity index between two of the three bioreactors decreased to 0.49–0.52 on day 37 (Figure 3b). Apparently, the biofilm formed on the same feeding substrate with different concentrations had different bacterial population structures, and the difference gradually increased along with the prolonged operation time. The Dice coefficients of microbial community structures between day 20 and 37 were 0.54, 0.63 and 0.45 for M1, M2 and M3, respectively (Figure 3b), indicating that the microbial community structures varied during the biofilm formation and growth.

Sequence analysis of DGGE bands

The highest similarity results based on the partial 16S rRNA gene sequences of twelve DGGE bands (Figure 3a) are summarized in Table 2. The majority of the bacterial 16S rDNA sequences grouped with members of *Proteobacteria*, with seven in the β subdivision and two in the α subdivision. Two sequences were related to members of the *Firmicutes*, and the remaining one clustered with the *Bacteroidetes*.

Band 5 matched with uncultured microorganism *Zoogloea* sp., and sequences of band 1, 3 and 7 were closely related to *Zoogloea* sp., which is known to produce extracellular polymeric substances (EPS) and hold the cell aggregates together (Gerardi, 2006). Band 9 and 10 were identified as *Lactococcus* sp. (>98%), which are capable of producing lactic acid from a wide range of carbohydrates including glucose (Teuber and Geis, 2006). Band 11 had highest sequence similarities to *Spirosoma panaciterrae*, a novel species which can utilize D-glucose for growth but can not reduce nitrate to

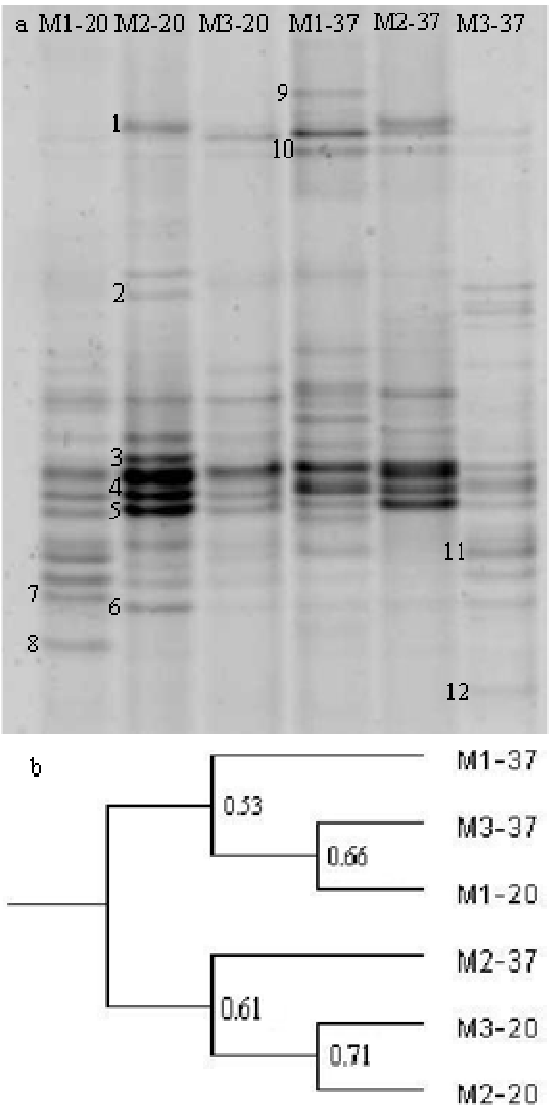

Figure 3. DGGE profile (a) and cluster analysis in the form of UPGMA dendrogram (b) of the microbial communities from the three reactors. Mn-20, Mn-37: samples were collected from Reactor n (1, 2 and 3) after 20 and 37 days of operation, respectively.

nitrite (Ten et al., 2009). According to the DGGE band patterns (Figure 3a), *S. panaciterrae* was observed in the biofilms of M1 and M3, and *Zoogloea* sp. and *Lactococcus* sp. were present in all the three reactors, indicating their important roles in the biofilm formation and stabilization. Band 8 had relatively low similarity (<86%) to reference sequences closely associated with *Rhodocyclaceae* bacterium, a kind of polyphosphate accumulating organism (PAO) found in several enhanced biological phosphorus removal (EBPR) sludges (Hesselmann et al., 2008). Rhodocyclaceae bacterium was detected only in M1 during 37 days of operation.

The unknown ammonia-oxidizing bacterium (Band 6) dominated in M1 after day 20, M2 and M3. The absence of this species may be attributed to the lower nitrification efficiency of M1 during the initial period (Figure 1). *Hydrogenophaga* sp. corresponding to band 4 was found as predominant bacteria in all the bioreactors throughout the study. Two clones affiliated with Alpha *Proteobacteria* were closely related to *Rhizobium* spp. (Band 12) and *Hyphomicrobium* spp. (Band 2). They were simultaneously present in M3 after biofilm formation. Band 2 and 12 was also observed in the initial 20 days of M2 and M1, respectively. Some members of the genera *Hydrogenophaga*, *Hyphomicrobium* and *Rhizobium* have been reported as heterotrophic denitrifiers (Daniel et al., 1982; Sperl and Hoare, 1971; Willems et al., 1989). Moreover, the *Hyphomicrobium* genus possess the potential capacity for aerobic denitrification (Meiberg et al., 1980).

Quantitative analysis of specific groups by FISH

FISH cell counts of specific groups are given in Figure 4 as percentages of the number of cells detected with the probe EUB338. FISH analysis reveals that the carrier biofilm was dominated by a great number of *proteobacteria* consisting of α, β and γ subclasses. The bacteria affiliated with β-*proteobacteria* (19.8–46.7%) followed by α-*proteobacteria* (16.59–41.15%) were numerically important populations. The dominance of β-*proteobacteria* is in agreement with the identification of DGGE band sequences (Table 2). This observation is consistent with that of Nogueira et al. (2002), who indicated the most abundance of β-*proteobacteria* in biofilm during combined organic carbon and ammonia oxidation. The AOB fractions relative to the total bacterial population in M1, M2 and M3 on day 20 were 3.04, 3.57 and 8.23%, respectively, and the data were 3.01, 6.01 and 8.37% at the end of experiment (Figure 4). The relative proportion of *Nitrospira* ranged from 7.48% in the bulk liquid of M1, through 5.91% in M2, and down to 4.31% in M3 on day 37 (Figure 4). On day 20, the proportions of nitrifier populations (AOB of β-*proteobacteria* plus *Nitrospira*-like NOB) in M1, M2 and M3 were 8.24, 9.45 and 14.95%, respectively, and those on day 37 were 10.49, 11.92 and 12.68%, respectively (Figure 4).

DISCUSSION

The explanations for the phenomenon of SND were given from two aspects (Münch et al., 1996). The conventional physical explanation is that SND occurs because of anoxic microzones in deeper layer of biofilm due to DO diffusion limitations which allows the occurrence of denitrification by heterotrophic denitrifiers. The biological explanations for SND are the existence of aerobic denitrifiers as well as heterotrophic nitrifiers. In this study, the presence of the denitrifying bacteria *Hydrogenophaga*

Table 2. Percentage similarity based on the partial 16S rRNA gene sequences of DGGE bands to the closest relatives in the NCBI nucleotide sequence database.

Band no.	Closest relative in NCBI database	Identity (%)	Phylogenetic division
1	*Zoogloea resiniphila* strain DhA-35	92	Beta *proteobacteria*
2	Uncultured *Hyphomicrobiaceae* bacterium clone Amb_16S_1766	97	Alpha *proteobacteria*
3	*Zoogloea resiniphila* strain DhA-35	98	Beta *proteobacteria*
4	*Hydrogenophaga* sp. EMB 85	96	Beta *proteobacteria*
5	Uncultured *Zoogloea sp.* clone N6	96	Beta *proteobacteria*
6	Uncultured ammonia-oxidizing bacterium clone B-12	91	Beta *proteobacteria*
7	*Zoogloea caeni* strain EMB 43	99	Beta *proteobacteria*
8	Uncultured *Rhodocyclaceae* bacterium	86	Beta *proteobacteria*
9	*Lactococcus* sp. YM05004	98	*Firmicutes*
10	*Lactococcus* sp. F116	98	*Firmicutes*
11	*Spirosoma panaciterrae* strain Gsoil1519	93	*Bacteroidetes*
12	*Rhizobium* sp. CC-CCM15-3	100	Alpha *proteobacteria*

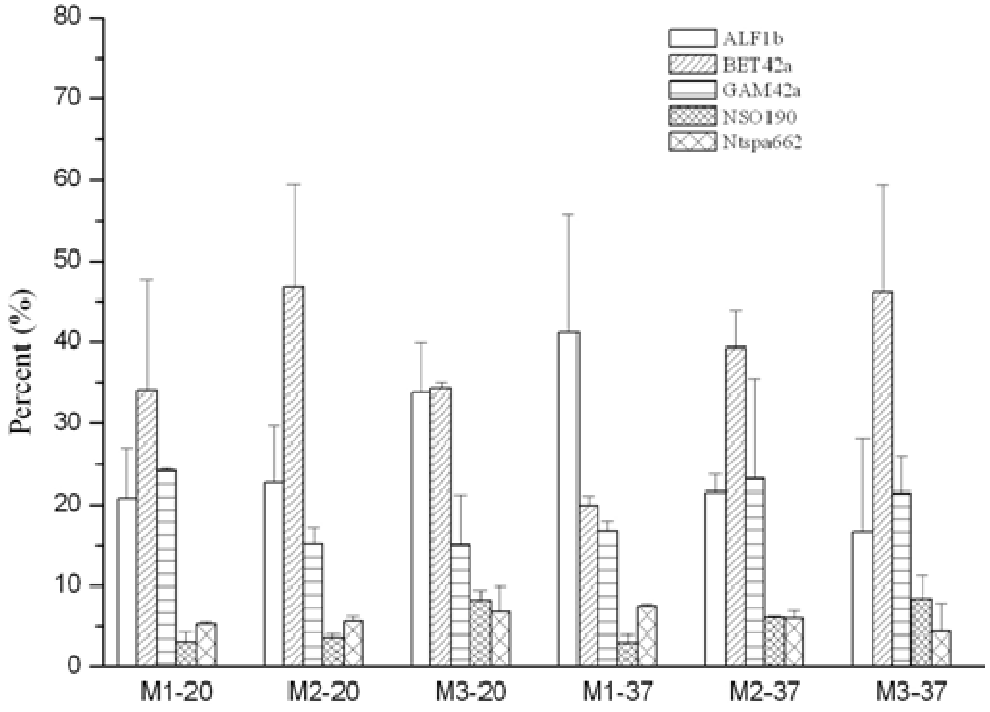

Figure 4. Bacterial community analysis of the biofilms in the three reactors as determined by FISH cell counts. The values obtained with the probes ALF1b, BET42a, GAM42a, NSO190 and Ntspa662 were expressed as percentages of the number of cells detected with the probe EUB338. Mn-20, Mn-37: samples collected from Reactor n (1, 2 and 3) after 20 and 37 days of operation, respectively.

spp., *Hyphomicrobium* spp. and *Rhizobium* spp. suggested the occurrence of both aerobic and anoxic denitrification. Consequently, not only the presence of DO micro-gradients within the biofilm but also aerobic denitrifiers might be the explanation for SND in the aerobic biofilm reactor.

Effects of substrate loadings on biofilm morphology can be explained in terms of substrate availability. Molecular diffusion is the predominant mechanisms by which the substrates are transported into the interior cells. According to Fick's first law, higher concentrations result in higher fluxes, making more substrate available for biofilm growth (Wijeyekoon et al., 2004). Availability of ample substrate for growth makes the region saturated with bacteria, leading to dense and smooth biofilms with low porosity. At lower substrate loadings, the filamentous

bacteria could easily penetrate outside the flocs, resulting in a higher substrate concentration than non-filamentous bacteria inside the floc (Martins et al., 2004). As a result, the lack of substrate retarded the growth of non-filamentous bacteria, and made the biofilm more filamentous and thick.

The ammonia and nitrite concentrations were mainly responsible for the shift in the relative abundance of nitrifying population. The positive effects of the initial ammonia concentration on the population size of AOB were in accordance with the published literature focused on the identity of AOB (Okano et al., 2004). Previous research showed that for low nitrite concentration dominant *Nitrospira* competed well with *Nitrobacter*, another key NOB in nitrifying bioreactors (Kim and Kim, 2006; Schramm et al., 2000). In addition, high free ammonia inhibited NOB in nitrifying biofilm process (Kim et al., 2005). Currently, the pure culture data are still unavailable for *Nitrospira* spp.. We speculate that *Nitrospira* is more inhibited with increasing nitrite and ammonia concentrations, which may be responsible for the decrease in relative proportion of *Nitrospira*. Although the decreasing *Nitrospira*-like NOB, the relative abundance of nitrifying populations (the sum of beta*proteobacteria* AOB and *Nitrospira*-like NOB) increased, resulting in the increasing nitrification performance (Figures 1 and 4).

In conclusion, simultaneous organic carbon and nitrogen removal achieved in the aerobic suspended carrier biofilm reactors with COD removal efficiencies of 87.1–99.0% and SND efficiencies about 96.7-98.8%. Ammonia oxidation rate was positively proportional to the influent ammonia concentration. DGGE analysis and ESEM observation reveals that biofilms formed at different substrate loadings had different morphology and community structures. Denser and thinner biofilms formed at a higher substrate concentration, while looser and thicker biofilms with significant presence of filamentous bacteria formed at lower substrate concentration. Both sequence analysis of DGGE bands and FISH analysis have indicated the dominance of β-*Proteobacteria* in the biofilm communities, especially *Zoogloea.* The proportion of AOB belonging to beta proteobacteria positively correlated with ammonia loadings, whereas the relative proportions of *Nitrospira*-like NOB were negatively affected by ammonia and nitrite concentrations.

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