Microbial community structure in autotrophic nitrifying granules characterized by experimental and simulation analyses

Shinya Matsumoto,1 Mayu Katoku,1 Goro Saeki,1 Akihiko Terada,2 Yoshiteru Aoi,3 Satoshi Tsuneda,1* Cristian Picioreanu4 and Mark C. M. van Loosdrecht4
1Department of Life Science and Medical Bioscience, Waseda University, Wakamatsu-cho 2–2, Shinjuku-ku, Tokyo 162-8480, Japan.
2Institute of Environment and Resources, Technical University of Denmark, DK-2800 Lyngby, Denmark.
3Waseda Institute for Advanced Study, Waseda University, Wakamatsu-cho 2-2, Shinjuku-ku, Tokyo 162-8480, Japan.
4Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 bc Delft, the Netherlands.

Summary
This study evaluates the community structure in nitrifying granules (average diameter of 1600 μm) produced in an aerobic reactor fed with ammonia as the sole energy source by a multivalent approach combining molecular techniques, microelectrode measurements and mathematical modelling. Fluorescence in situ hybridization revealed that ammonia-oxidizing bacteria dominated within the first 200 μm below the granule surface, nitrite-oxidizing bacteria a deeper layer between 200 and 300 μm, while heterotrophic bacteria were present in the core of the nitrifying granule. Presence of these groups also became evident from a 16S rRNA clone library. Microprofiles of NH4+, NO2−, NO3− and O2 concentrations measured with microelectrodes showed good agreement with the spatial organization of nitrifying bacteria. The models described well the bacterial distribution obtained by fluorescence in situ hybridization analysis, as well as the measured oxygen, nitrite, nitrate and ammonium concentration profiles. Results of this study are important because they show that a combination of simulation and experimental techniques can better explain the interaction between nitrifying bacteria and heterotrophic bacteria in the granules than individual approaches alone.

Introduction
The low growth rate and low fraction of autotrophic bacteria in activated sludge processes make nitrification the rate-limiting step in most wastewater treatment processes. Thus, a simpler and effective method of immobilizing nitrifying bacteria within wastewater treatment processes is desired. Granulation without using any carriers has been proposed for immobilizing nitrifying bacteria in inorganic wastewater treatment processes (De Beer et al., 1997; Tay et al., 2002; Tsuneda et al., 2003). Our group successfully produced nitrifying granules using an aerobic upflow fluidized bed (AUFB) reactor (Tsuneda et al., 2003). Furthermore, the time required for producing nitrifying granules could be greatly reduced by use of pre-aggregating seed sludge with hematite (Tsuneda et al., 2004). However, detailed information on granule formation and nitrogen conversion in primarily autotrophic systems is still lacking.

To date, there are reports indicating ecophysiological interaction between nitrifying bacteria and heterotrophic bacteria in autotrophic nitrifying suspended cultures (Rittmann et al., 1994) and biofilms (Kindaichi et al., 2004; Okabe et al., 2005) grown without an external organic carbon source. In such systems, nitrifying bacteria may consume inorganic carbon not only to form cell mass but also to excrete organic carbon (soluble microbial products, SMP), which will support heterotrophic bacterial growth (Rittmann et al., 2002). Although very much needed, a detailed analysis on microbial ecology of nitrifying granules including nitrifying and heterotrophic bacteria has never been reported. Better understanding on the formation of nitrifying granule’s structure (especially...
the microbial community distribution) and microscale distribution of activities of each bacterial species is necessary for improving process performance and further application of the nitrifying granules to wastewater treatment.

In this study, we used experimental and simulation analyses in combination to study the microbial community of nitrifying granules. Since community structure in the granules is determined by a complex interplay of various factors including the concentration of chemical species, presence of other bacteria and their physiology, mathematical modelling provides a logical framework for the exploration of processes within granules. A large variety of mathematical models have been developed and widely applied to assess the multi-species biofilm dynamics on support materials (see a review in Wanner et al., 2006). Traditional models consider the dominant one dimensional (1-d) gradients of solutes and biomass concentrations (e.g. Wanner and Gujer, 1985; Okabe et al., 1996). More complex two- or three-dimensional (2-d and 3-d) models describe the dynamics of multiple microbial populations by various approaches: grid-based biomass (e.g. cellular automata based by Noguer et al., 1999; Picioreanu et al., 1999; Bell et al., 2005), gridless individual-based models (IbM, by Kreft et al., 2001; Picioreanu et al., 2004), continuum (Alpkvist and Klapper, 2007) or a hybrid individual/continuum (Alpkvist et al., 2006). Extracellular polymeric substances (EPS) were also included in all approaches (in cellular automata: Laspidou and Rittmann, 2004; in IbM: Kreft and Wimpenny, 2001; Xavier et al., 2005a,b, Xavier and Foster, 2007; in hybrids: Matsumoto et al., 2007). Some of these biofilm models have been adapted to represent granular biofilms (e.g. Xavier et al., 2007 for aerobic granular sludge and Picioreanu et al., 2005; Batstone et al., 2006 for anaerobic granular sludge) or autotrophic/heterotrophic growth of immobilized biomass in gel beads (Vogelsang et al., 2002). We used in this study an IbM to describe the microbial ecology of the granules (following directly the approach reported in Kreft et al., 2001; Picioreanu et al., 2004; Xavier et al., 2005a; 2007).

This study is new and relevant because it combines three different research approaches, usually applied alone in the investigation of microbial biofilms and granules. First, community structure and spatial organization in nitrifying granules were analysed by molecular (clone libraries of 16S rRNA genes) and microscopic imaging techniques (lectin-binding analysis and fluorescence in situ hybridization, FISH). Second, concentration profiles of essential chemical species (NH$_4^+$, NO$_2^-$, NO$_3^-$ and O$_2$) along the granule radius were measured by microelectrodes. Finally, 1-d and 2-d numerical biofilm models focusing on eco-physiological interaction between nitrifying and heterotrophic bacteria in the nitrifying granules were developed in order to evaluate and interpret the experimental measurements.

**Results**

**Phylogenetic analysis**

A 16S rRNA gene clone library of bacteria was constructed from the nitrifying granules with a diameter of 1600 µm (Fig. 1). Totally, 93 clones were analysed, approximately 600 bp per clone was sequenced and the clones were grouped into 39 operational taxonomic units (OTUs) on the basis of more than 97% sequence similarity within an OTU. Clone sequences analysed were distributed over seven major lineages of the domain Bacteria: members of the Cytophaga-Flavobacterium-Bacteroides division (CFB), genus of Nitrosomonas of the β-subclass ammonia-oxidizing bacteria (AOB), member of the phylum Verrucomicrobia, Ϝ-Proteobacteria, Chloroflexi, δ-Proteobacteria, genus Nitrobaeter of the α-subclass nitrite oxidizing bacteria (NOB) and α-Proteobacteria except for Nitrobacter. Nitrospira-associated sequences were not detected in the nitrifying granule. Almost all of the clones belonging to putatively heterotrophic bacterial groups had a similarity of less than 96% to sequences determined in other environmental diversity surveys.

**Microbial community structure in nitrifying granules**

The in situ spatial organization of heterotrophic bacteria, nitrifying bacteria and EPS was visualized by FISH with group-specific probes and lectin staining. The samples used for the experiment were the nitrifying granules cultured with a diameter of 1600 µm. Both groups of nitrifying bacteria, i.e. AOB and NOB, were detected with the Nso190 and NIT3 probes respectively (Fig. 2A and B). The AOB created clusters, whereas NOB were present mostly in the form of single scattered cells near the AOB [Fig. 2C; the 3-d image was constructed by the software Daime (Daime et al., 2006)]. Although CFB division in the granule was not stained with the extensively used probe CF319a/b, the CFB719 probe successfully detected bacteria related to CFB division. CFB719-stained bacteria were thin filamentous shaped (Fig. 2D). GNSB-941-stained bacteria were long, rod-shaped (Fig. 2E). Bacteria related to phylum Verrucomicrobia were not clearly detected with probe EUB338II due to low signal intensity and low abundance. Figure 2F shows the spatial distribution over a section cut through the middle of the granule of bacterial cells and EPS stained by propidium iodide (PI) and fluorescein iso thiocyanate (FITC)-labelled lectin respectively. The main habitat of bacteria stained with PI was at the edge of the granule, whereas the inner part of the granule was dominantly occupied by EPS. Moreover,

Fig. 1. Phylogenetic trees based on 16S rRNA gene sequences for Proteobacteria (A) and Verrucomicrobia, Chloroflexi and the CFB group (B). The trees were constructed using the neighbor-joining method. The number on the nodes indicates the number of times the species (shown on the right) grouped together in 1000 bootstrap samples. Bootstrap values below 500 are not shown. The root of the tree was determined using the 16S rRNA gene of *Aquifex pyrophilus* as an outgroup. Scale bar indicates the 10% estimated difference in nucleotide sequence position.
it appears that the bacteria and EPS formed neat concentric layers along the granule radius.

The microbial nitrifying community composition in the granules with a diameter of 1600 μm was determined by quantitative FISH with various sets of probes (Table 1 and Fig. 3). Members of the AOB (detected with probe Nso190) and NOB (the genus Nitrobacter belonging to the α-Proteobacteria) accounted for 68.0% of the total bacteria detected with EUB338 probe mix. The ratio of all nitrifiers (AOB plus NOB) to all heterotrophs detected was approximately 3:1.

The spatial distributions of populations in the nitrifying granule were determined by results from quantitative 2-d image analysis of confocal image series (Fig. 4). Most bacteria detected with EUB338 probe mix at the outer edge of the granule were nitrifying bacteria; AOB
dominated within first 200 μm below the granule surface, whereas NOB were most abundant in the layer between 200 and 300 μm below the granule surface. Bacteria related to CFB division and Chloroflexi were detected mainly in the zone where nitrifying bacteria exist and in the inner part of the granules respectively. Proteobacteria were present throughout the granules. It should be noted that absolute abundance of bacteria (shown by open circle in Fig. 4) at the inner part of granules is much lower than at the surface of granules. Thus, even though Chloroflexi occupy inner part of the granules, absolute amount of Chloroflexi is much lower than that of AOB.

Concentration profiles in nitrifying granules

Local concentrations of NH$_4^+$-N, NO$_2^-$-N, NO$_3^-$-N and dissolved oxygen (DO) in the nitrifying granules with a diameter of 1600 μm were measured under steady-state conditions (Fig. 5). The profiles of NH$_4^+$-N, NO$_2^-$-N and NO$_3^-$-N indicated that NH$_4^+$ was oxidized to NO$_2^-$ mainly down to 250 μm from the granule surface. Little nitrite accumulation detected shows that NO$_2^-$ was immediately oxidized to NO$_3^-$. The active ammonium- and nitrite-oxidizing zones were in good agreement with the spatial distribution of AOB and NOB detected by FISH (Fig. 4). The DO concentration at the granule surface...
was 7 mg l \(^{-1}\) and oxygen penetrated about 300 µm into the granule.

**Numerical simulation analyses**

One-dimensional (with gradients along the granule radius only) and 2-d numerical models were developed to describe the measured concentration profiles and microbial distribution within the granules (see Experimental procedures). The 1-d models combine the advantages of simplicity and faster computations, while the 2-d models give a more detailed granule structure and solutes spatial distribution. Both types of models used identical parameters (see Tables S1–5) and were compared in terms of their predictions of: (i) solutes evolution in time in the reactor bulk liquid and (ii) solutes and microbial distributions in the granule. Figure 6 shows 2-d simulation results of the microbial distribution in the nitrifying granule developing in time. The comparison of dynamics of solute concentrations in the bulk liquid provided by 1-d simulation and three 2-d simulations is shown in Fig. 7. Figure 8 shows the comparison of steady-state solute concentrations of solutes in the biofilm (day 100) from 1-d and 2-d simulations, and experimental microelectrode data of NH\(_4\)-N, NO\(_2\)-N, NO\(_3\)-N and DO. Figure 9 shows the comparison of the steady-state biomass concentrations in the biofilm (day 100) from 1-d and 2-d simulations. The animations of simulated granule formation in time are given in Supporting information and can also be downloaded from: http://www.biofilms.bt.tudelft.nl/NitrifyingGranules/index.html.

In the early stages of granule formation, due to sufficient supply of ammonia and oxygen from the bulk liquid, the granule is mainly constituted of nitrifiers (Fig. 6A). As the granule size increases, an anoxic zone is created at the inner part of the granule. That condition is preferable for heterotrophic bacteria to denitrify consuming the excreted organic material (SMP) as an electron donor. Therefore, in the later stage of granule formation, heterotrophic bacteria exist at the inner part of the granule.

### Table 1. Probe sequences and formamide concentrations in the hybridization buffer required for specific in situ hybridization.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Specificity</th>
<th>Sequence of probe (5′ to 3′)</th>
<th>FA (%)(^{\text{a}})</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338</td>
<td>Most bacteria</td>
<td>GCTGCCCTCCGTTAGGAGT</td>
<td>__________</td>
<td>Amann et al. (1990)</td>
</tr>
<tr>
<td>EUB338 II</td>
<td>Planctomycetales</td>
<td>GCAGCCACCCGGTAGGAGT</td>
<td>__________</td>
<td>Daims et al. (1999)</td>
</tr>
<tr>
<td>EUB338 III</td>
<td>Verrucomicrobiales</td>
<td>GCTGCCACCCGTTAGGAGT</td>
<td>__________</td>
<td>Daims et al. (1999)</td>
</tr>
<tr>
<td>ALF1b</td>
<td>(\alpha)-Proteobacteria, including Nitrobacter</td>
<td>CGTTGGYCTTGCAGGCA</td>
<td>20</td>
<td>Manz et al. (1992)</td>
</tr>
<tr>
<td>BET42a</td>
<td>(\beta)-Proteobacteria</td>
<td>GCCTCCCCATTCGTGT</td>
<td>35</td>
<td>Manz et al. (1992)</td>
</tr>
<tr>
<td>GAM42a(^{\text{a}})</td>
<td>(\gamma)-Proteobacteria</td>
<td>GCCTCCCCACATCTGT</td>
<td>35</td>
<td>Manz et al. (1992)</td>
</tr>
<tr>
<td>GNSB-941</td>
<td>Phylum Chloroflexi</td>
<td>AAACACACGCTCCGCT</td>
<td>35</td>
<td>Gich et al. (2001)</td>
</tr>
<tr>
<td>CF319a/b</td>
<td>CF cluster</td>
<td>TGGTGGCTTCTAC</td>
<td>35</td>
<td>Manz et al. (1996)</td>
</tr>
<tr>
<td>CFB719</td>
<td>Groups of the Bacteroidetes phylum</td>
<td>AGCTGCTTCGCAATCG</td>
<td>30</td>
<td>Weller et al. (2000)</td>
</tr>
<tr>
<td>Nso190</td>
<td>Ammonia-oxidizing (\gamma)-Proteobacteria</td>
<td>CGATCCCCCTGCTTTCTCC</td>
<td>35(^{\text{d}})</td>
<td>Moberly et al. (1996)</td>
</tr>
<tr>
<td>NIT3(^{\text{e}})</td>
<td>Nitro bacter spp.</td>
<td>CCTGTGGCTCAGTGCCTCCG</td>
<td>40</td>
<td>Wagner et al. (1996)</td>
</tr>
<tr>
<td>Comp NIT3</td>
<td>Competitor for NIT3</td>
<td>CCTGTGGCTCAGGCTCCG</td>
<td>40</td>
<td>Wagner et al. (1996)</td>
</tr>
<tr>
<td>Ntspa1026</td>
<td>Nitrospira moscoviensis</td>
<td>AGCAGCGCTGTTATTGCTA</td>
<td>20</td>
<td>Juretschko et al. (1998)</td>
</tr>
</tbody>
</table>

\(^{\text{a}}\) Formamide concentration in the hybridization buffer.

\(^{\text{b}}\) Used at any formamide concentration.

\(^{\text{c}}\) Unlabeled probe BET42a was used as a competitor to enhance specificity.

\(^{\text{d}}\) Although originally 55% formamide should be used, we referred to Pynaert et al., 2003.

\(^{\text{e}}\) Unlabelled probe comp NIT3 was used as a competitor to enhance specificity.

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**Fig. 3.** Microbial community composition for the nitrifying granule as determined by quantitative FISH. The \(\alpha\)-Proteobacteria is the bacterial group that hybridized with probe ALF1b, excluding the genus *Nitrobacter* that hybridized with probe NIT3.
while nitrifying bacteria dominate at the outer edge. In this later stage, the inner part of the granule becomes dominated by EPS produced by the heterotrophic bacteria (Fig. 6C and D).

Because the 2-d model contains stochastic elements (i.e. the initial spatial distribution of microbial types and the direction of cell division and EPS production), results obtained with identical sets of parameters are not identical, but only very similar. This is why three 2-d simulations were performed in the same conditions. The concentrations of N-containing species in the bulk liquid in time calculated in three 2-d simulations shown in Fig. 7 are comparable with 1-d simulation results (which are fully deterministic). For granules smaller than the maximum size imposed (until day 20), the concentrations computed with 1-d and 2-d models are identical. However, although the calculated steady state values of ammonium, nitrite and nitrate concentrations in the bulk liquid are similar with 1-d and 2-d models (from approximately day 70 on), the 2-d results show more oscillations (noise) around the average value. This is because unlike the microbial distribution in concentric shells inherent in 1-d models, in 2-d there are radially developed colonies or cell clusters of one microbial type (see Fig. 6C and D). After the granule has reached the maximum imposed size, in 2-d whole colonies can be quickly detached from the granule surface, while in 1-d this detachment process is completely smooth. For example, if a large patch of NOB is eliminated from the granule in a short interval of time, the nitrite concentration will suddenly increase while the nitrate will decrease. Because of this fluctuations, a true steady state is never reached in 2-d simulations, neither for solutes nor for microbial compositions. We can speak about steady values only by comparing values averaged over an interval of time of about 5–10 days.

The steady-state solute concentrations along the granule radius calculated with the 1-d and 2-d models describe well the experimentally obtained microelectrode data (Fig. 8). Moreover, the concentration profiles obtained by averaging on concentric shells the 2-d distributions of solutes match the 1-d calculated profiles. These results show that for a description of the main solute concentrations and fluxes in the biofilm (ammonium, nitrite, nitrate and oxygen), a 1-d model is sufficient. However, for low concentration solutes (the organic substrates UAP and BAP), the 2-d profiles are more dependent on the spatial distribution of different microbial groups, which leads to different profiles from those calculated with a 1-d model (Fig. 9). Nevertheless, this is the first direct quantitative comparison between solute concentrations in the biofilm calculated by a multidimensional model and those measured by microelectrodes (for \( \text{NH}_4^+ \), \( \text{NO}_2^- \), \( \text{NO}_3^- \) and DO).

**Discussion**

**Bacterial community structure of nitrifying granules**

The phylogenetic analysis revealed that AOB present in nitrifying granules are affiliated with *Nitrosomonas europaea* and *Nitrosococcus mobilis*. *N. mobilis* is known to be a halophilic bacteria easily overgrown by *N. europaea* in low-salt environments (Juretschko et al., 1998). The
synthetic wastewater supplied to nitrifying granule reactor in this study was high in salinity (42.6 mM as Na₂SO₄). Thus, *N. mobilis* and *N. europaea* would coexist in nitrifying granules. Nitrite-oxidizing bacteria detected in nitrifying granules were affiliated with *Nitrobacter* sp. Although *Nitrospira* sp. are widespread in nature and earlier known from, for example, freshwater, brackish waters, marine waters, soils and wastewater treatment plants, *Nitrobacter* outcompete *Nitrospira* under halophilic condition (Cebron and Garnier, 2005). Heterotrophic bacteria such as *Chloroflexi*, CFB and *Proteobacteria* were detected from nitrifying granules cultured with inorganic substrate. These results are consistent with the previous studies demonstrating that heterotrophic bacteria are present in biofilms cultivated with inorganic substrate (Nogueira et al., 2002; Kindaichi et al., 2004; Okabe et al., 2005). Nitrifying bacteria are known to release soluble products (SMP) from substrate metabolism and biomass decay, which can provide a supplementary organic substrate for heterotrophic bacteria (Rittmann et al., 1994; Barker and Stuckey, 1999). A large amount of SMP could be accumulated within nitrifying granule because sludge retention time of nitrifying granule is extremely long (Tsuneda et al., 2003). Since organic carbon was not added to the substrate for nitrifying granule reactor, the SMP released by nitrifying bacteria would be the sole organic substrates for heterotrophic bacteria. Although there are several considerations with regard to EPS production by nitrifiers, we hypothesized that EPS production by nitrifying bacteria is extremely poor following the report that polysaccharides

production was not confirmed from the supernatant of *N. europaea* and *Nitrobacter winogradskyi* (Tsuneda et al., 2001).

Two different types of AOB affiliated with *N. europaea* and *N. mobilis* (more than 98% similarity) were detected by phylogenetic analysis. Thus, oligonucleotide probe Nso190 that can detect both types of AOB was applied to FISH analysis to determine the bacterial distribution within nitrifying granules. Considering the FISH results with direct comparison with microelectrode results, AOB produce nitrite while consuming ammonia and NOB produce nitrate while consuming nitrite at the outer layer of granules (up to 250 μm from the surface). Sufficient excess ammonia was measured all along the granule depth. Therefore, oxygen supply appeared to be the limiting factor for nitrification in nitrifying granules. Since only small amount of nitrite was detected by microelectrode measurements, nitrite is promptly oxidized to nitrate. The first reason for the rapid oxidation of nitrite to nitrate is that, in the nitrifying granule, NOB exist close to AOB (Fig. 2C), which prevents accumulation of nitrite produced by AOB. Furthermore, it was reported that the specific nitrite oxidation rate of *Nitrobacter* is at most 40 times higher than the specific ammonia oxidation rate of *N. europaea* (Prosser, 1989). Regarding heterotrophic bacteria, CFB and γ-Proteobacteria were present at outer layer of granule, Chloroflexi were inner granule and α-Proteobacteria were throughout the granule. At the inner parts of the granule (deeper than 250 μm from the surface), oxygen is depleted and nitrate produced by NOB is present. Furthermore, as the profile of nitrate is not perfectly linear, denitrification relying on SMP would occur. The measured loss of total N-species (NH₄⁺-N, NO₂⁻-N and NO₃⁻-N) in the reactor liquid also indicated that denitrification occurred (Fig. 3). Nucleic acid and lectin staining revealed that inner parts of a granule are occupied with EPS, which is produced by heterotrophic bacteria present throughout the granule. The similar EPS distribution is reported in the aerobic granular sludge cultured by substrate containing organic carbon (McSwain et al., 2005).

**Uptake patterns for organic substrates derived from nitrifying bacteria**

There is a limited number of studies reporting about organic substrate uptake pattern of heterotrophic bacteria detected in nitrifying granules. O’Sullivan and colleagues (2002) reported that some members of CFB group uptake various biomacromolecules, such as cellulose, chitin, DNA, lipids, and proteins, which may accumulate in biofilms. Sekiguchi and colleagues (1999) reported that yeast extract-like nutrient is essential for growth of Chloroflexi. Cottrell and Kirchman (2000) reported that the

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**Fig. 7.** Comparison of dynamics of solute concentrations in the bulk liquid calculated by 1-d (thick lines) and 2-d (thin lines) models with identical sets of parameters. Three different 2-d simulation results are shown here for comparison, because the stochastic elements included in the 2-d model lead to slightly different results each run.

**Fig. 8.** Comparison of the steady-state solute concentrations in the biofilm calculated in 1-d (thick lines) and 2-d (thin lines) simulations, with the experimental microelectrode data (open circles for NH₄⁺, NO₂⁻, NO₃⁻ and O₂). The model results are at day 100. To obtain the comparable profiles along the radius, the 2-d concentration distributions were averaged in concentric shells with different radii.
α- and γ-Proteobacteria in marine environments utilize amino acids. Furthermore, Okabe and colleagues (2005) reported that the member of the Chloroflexi utilize the decaying nitrifying bacteria cell materials (i.e., biomass associated products; BAP), the member of the CFB utilize substrate utilization-associated products (UAP), and the members of the α-Proteobacteria and γ-Proteobacteria utilize low-molecular-weight organic matter (Org) produced by hydrolysis of EPS. In the present model, based on those studies, we integrated three types of heterotrophic bacteria: as HetU, HetB and HetO that utilize UAP, BAP and Org respectively. Although those hypotheses are not reflecting all of the bacterial uptake patterns for organic substrates derived from nitrifying bacteria, the results of this study can be plausibly explained on the basis of hypotheses. Detailed 2-d simulated distribution of heterotrophs in the granule is shown in Fig. 10. HetU is present at the surface of nitrifying granule where sufficient UAP is produced by active nitrifying bacteria (Fig. 10A). HetB is mainly present in the inner parts of granule where BAP is produced from inactivated nitrifying bacteria because of depletion of oxygen (Fig. 10B). HetO are present throughout the granule because EPS produced by heterotrophic bacteria spread broadly in granule (Fig. 10C). Although the model simulations performed in this study were not intended to provide quantitative matches to the experimental data, the simulation results indicated that distributions of HetU, HetB and HetO are consistent with those of CFB, Chloroflexi and Proteobacteria, respectively, obtained by FISH analysis (1-d profiles of biomass are shown in Fig. 9). Thus, it can be concluded that the implemented organic substrate uptake patterns of heterotrophic bacteria were plausible.

The model simulations used in this study reveal interactions leading to the properties of biofilm systems that are generally not tractable by experimental approaches. So far, there have been only a very few reports of experimental verification of the 2-d or 3-d multi-species biofilm model predictions (Xavier et al., 2005a; Matsumoto et al., 2007). As far as we know, this paper is the first report that evaluated biofilm phenomena concerning microbial ecology by combination of experimental data such as...
FISH and microelectrode, and 2-d multi-species biofilm model.

In conclusion, we have combined experimental analyses using molecular techniques (i.e. 16S rRNA-cloning analysis and FISH) and microelectrode measurements for \( \text{NH}_4^+ - \text{N}, \text{NO}_2^- - \text{N}, \text{NO}_3^- - \text{N}, \) and \( \text{O}_2 \) concentration profiles with a 2-d biofilm model of granule formation. It is now clear that the concerted use of these techniques helps more understanding the development of microbial populations in nitrifying granules than the use of individual techniques alone. The experimental analyses make it possible to understand the spatial organization of bacteria and in situ bacterial activity. The multidimensional (here 2-d, but directly extensible to 3-d) biofilm model can be used to more accurately describe microbial interactions such as syntrophy and competition for space and substrates, all of which are more difficult to be explained by previously developed 1-d models. However, 1-d models seem to be sufficient for the prediction of solute concentrations and overall nutrient fluxes, and preferable in this case because of their simplicity and computational efficiency.

**Experimental procedures**

**Wastewater composition**

Ammonia-rich synthetic inorganic wastewater based on that discharged from a thermal power station was used in this study. Synthetic wastewater used as influent consisted of \( 2375 \text{ g m}^{-3} (\text{NH}_4)_2\text{SO}_4 \) (as \( 500 \text{ g N m}^{-3} \)), \( 6044 \text{ g m}^{-3} \text{ Na}_2\text{SO}_4 \), \( 0.5 \text{ g m}^{-3} \text{ FeSO}_4\cdot7\text{H}_2\text{O} \) and \( 0.4 \text{ g m}^{-3} \text{ KH}_2\text{PO}_4 \). Besides these components, \( \text{NaHCO}_3 \) (1511 \text{ g m}^{-3} ) was added to the influent both for \( \text{pH} \) adjustment and as an inorganic carbon source for autotrophic nitrifying bacteria. Total dissolved solid was \( 9930 \text{ g m}^{-3} \). Details are described elsewhere (Tsuneda et al., 2003).

**Reactor configuration and operating conditions**

The granules used in this study were produced in an AUFB reactor with a diameter of 50 mm and a height of 3.2 m (effective volume: 6.3 l) where the synthetic wastewater had been continuously fed for approximately 600 days (Tsuneda et al., 2006). Granules were then inoculated to a continuous stirred tank reactor with an effective volume of 1.0 l (Tsuneda et al., 2003), 2006) and had been cultured for another 600 days to ensure that the granules attain steady state. The average diameter of mature granules reached about 600 days (Tsuneda et al., 2003). The hydraulic retention time of the reactor was 1 day.

**DNA extraction, PCR amplification, cloning and sequencing of the 16S rRNA gene**

DNA was extracted from a granule sample (approximately 0.5 g wet weight granule) with a DNA extraction kit (ISOPLANT II, NIPPON GENE, Tokyo, Japan), as described in the manufacturer’s instructions. Then, 16S rRNA gene fragments from isolated total DNA of the granule sample were amplified using bacterial PCR primer sets 11f (Kane et al., 1993) and 1492r (Weisburg et al., 1991). Purified PCR products were cloned using a Qiagen PCR cloning kit (Qiagen, Valencia, CA, USA). The DNA insert was amplified and used as template DNA in a cycle sequencing reaction with a Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) by using bacterial PCR primer sets 341f and 907r (Muyzer et al., 1997). Nucleotide sequencing was performed with an automatic sequencer (Prism 2100 avant genetic analyser, Applied Biosystems). Sequences were aligned by ChromasPro version 1.4.1 (Technelysium, QLD, Australia). Partial sequences (approximately 600 bp) were compared with similar sequences of the reference organisms by a BLAST search (Altschul et al., 1990). In the next step, all clones having a 16S rRNA sequence similarity of more than 97% with each other were grouped into an OTU. Phylogenetic trees were constructed with ClustalW using the neighbor-joining method, 1000 times bootstrap and distance model Kimura. All partial 16S rRNA gene sequences were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databank under the accession numbers AB505163 to AB505201.

**Fixation and cryosectioning of granule samples**

The granule sample was fixed with freshly prepared 4% paraformaldehyde solution for 18 h at 4°C. The sample was embedded in Tissue-Tek OCT compound (Sakura Finetek, Tokyo, Japan) overnight to infiltrate the OCT compound into the granular biofilm, as described earlier (Okabe et al., 1999). After rapid freezing at −20°C, 20 \( \mu \text{m} \) thick slices through the middle of the granule were prepared with a cryostat (CM1850, Leica, Heidelberg, Germany) and placed on a gelatin-coated slide (Matsunami, Osaka, Japan). After 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 (Aoi et al., 2000).

**In situ hybridization**

The sequences of all oligonucleotide probes used in this study are summarized in Table 1. The detailed information about all these probes is found in probeBase (Loy et al., 2003). All in situ hybridizations were performed according to the protocol (Amann et al., 1990) in hybridization buffer at 40°C for 2–3 h. The buffer contained 0.9 M NaCl, 20 mM Tris hydrochloride (pH 7.2), 0.01% sodium dodecyl sulfate and formamide with concentrations listed in Table 1. Subsequently, a stringent washing step was performed at 48°C for 15 min in 50 ml of washing solution (NaCl dependent on formamide concentration, 20 mM Tris hydrochloride at pH 7.2
and 0.01% sodium dodecyl sulfate). The slides were then rinsed briefly with ddH₂O and allowed to dry. After slides were mounted in FluoroGuard Antifade Reagent (Bio-Rad, CA, USA), probe-stained cells were detected and recorded by a confocal laser scanning microscope (IX71, Olympus, Tokyo, Japan) equipped with an Ar ion laser (488 nm) and a HeNe laser (543 nm).

The spatial distributions of bacteria were quantitatively analysed by using probes specific to the several phylogenetic groups of bacteria. The quantitative analysis was conducted along radial transects with the constant z position through the granules by scanning adjacent fields of about 140 μm × 1 mm. Six radial transects (about 40 confocal laser scanning microscope images) from different sections were analysed for each probe. For determination of bacterial distributions, the average surface areas of the specific-probe-hybridized cells and EUB338 probe mix hybridized cells were quantified by simultaneous in situ hybridization with various probe sets. Threshold values were defined to exclude background fluorescence and the probe-positive cell areas were quantified by the software daime (Daims et al., 2006).

Nucleic acid and lectin staining

Propidium iodide was used to stain the nucleic acids of bacterial cells in the granules. Concanavalin (ConA) and WGA lectin labelled with FITC were used to stain most glycoconjugate fractions of EPS (Bockelmann et al., 2002; McSwain et al., 2005). Since these stains may also bind with protein and glycoconjugate groups associated with cell walls, a counter stain with PI was used to distinguish EPS from cells.

Microelectrode measurements

The DO profiles were obtained with a Clark-type microelectrode with a tip diameter of 10–15 μm (Unisense, Aarhus, Denmark). The DO microelectrode was calibrated according to previous reports (De Beer et al., 1997; Okabe et al., 1999). An intact whole granule was fixed in a loop of a nylon thread connected to a loader tip and placed in the flow cell (Hille et al., 2005). All the measurements were performed in a water chamber containing 300 ml of medium taken from the reactor at 20°C as described elsewhere (Matsumoto et al., 2007).

Biofilm model description

The general structure of the granule models used in this study is based on biofilm models previously reported. Therefore, only a few specific details newly introduced here will be described. The 1-d model is based on the work of Wanner and Gujer (1985), and implemented to the well-established AQUASIM simulation software (Reichert, 1998). The 2-d ibM follows the principles described in Piccioreanu and colleagues (2004), and Xavier and colleagues (2005a; 2007).

A schematic diagram of the 2-d model spatial scales is shown in Fig. 11. The development of a single granule is simulated in a square computational domain defined in Cartesian coordinates, with size of 1800 μm. The computational domain consists of three spatial subdomains: bulk liquid, diffusion boundary layer and the granular biofilm. The bulk liquid subdomain is considered completely mixed and, thus, has a uniform distribution of the component concentrations. The boundary layer is defined at constant thickness from the granule surface. In the granule and the boundary layer subdomains, the time-dependent concentrations of solute components are calculated from diffusion-reaction equations. Bulk concentrations of solute and particulate components are calculated from overall mass balances in the system, including inlet-outlet flows, reactions in the bulk and mass transfer to/from the granules. These mass balances are explained in detail in Piccioreanu and colleagues (2004) and Xavier and colleagues (2005a).

The granule structure is represented by a collection of discrete non-overlapping hard cylinders of solid (also called 'particulate') components (see Piccioreanu et al., 2004; Bat-
stone et al., 2006). The initial biomass particles are distributed within a small circle with the centre in the middle of the square computational domain. Each biomass particle contains active biomass of a single microbial type and inert material biomass and is surrounded by an EPS capsule produced by the biomass within the particle (see Xavier et al., 2005a). Each biomass particle grows and produces EPS according to a rate defined by concentrations of solutes and particulates. The biomass particles are divided into two daughter particles when their size exceeds a critical size. An EPS-only particle is excreted when the mass fraction of EPS relative to total particle mass reaches a critical value. After division and EPS excretion, particles are redistributed (‘shoved’; Kref et al., 2001) not to overlap each other. In this sequence of steps the whole granule size develops. A maximum granule diameter is set to be 1600 µm, which is a typical average value found in the AUFB reactor. All biomass particles growing outside this size are removed from the computational domain, a computational operation similar to biomass detachment.

The kinetic model for the biological processes assumes that organic carbon for the growth of the heterotrophic bacteria was derived from three reactions: autotrophic growth (UAP), biomass lysis (BAP) (Rittmann et al., 2002) and hydrolysis of EPS (Org) (Laspidou and Rittmann, 2002) (see Supporting information). Although Laspidou and Rittmann (2002; 2004) defined that BAP is produced from the hydrolysis of EPS, in this study, to distinguish organic carbon derived from biomass lysis and EPS lysis, we called them BAP and Org respectively. In this particle-based biomass description, all microbial groups (i.e. AOB, NOB, UAP-uptaking heterotrophic bacteria HetU, BAP-uptaking heterotrophic bacteria HetB and Org-uptaking heterotrophic bacteria HetO) and EPS as well are particles defined by an internal composition (mass of one or more particulate substances), by size and by location in space. EPS is excreted by HetU, HetB and HetO, but not by either AOB or NOB.

All model parameters are presented in the Supporting information (Table S1–4) together with the complete description of all microbial processes considered. The source code of the 2-d model was written in C++ and the model was executed on a personal computer with Intel(R) Xeon CPU 3.0 GHz processor.

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References


**Supporting information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Scheme of biological transformations taken into account in the model.

**Table S1.** Stoichiometric matrix for microbial reactions.

**Table S2.** Kinetic rate expressions for microbial reactions.

**Table S3.** Stoichiometric parameters for microbial reactions.

**Table S4.** Kinetic parameters for microbial reactions.

**Table S5.** Other parameters used in the simulations.

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