Structure and microbial composition of nitrifying microbial aggregates and their relation to internal mass transfer effects

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Abstract This paper presents an analysis of the structure and microbial composition of nitrifying aggregates, formed as either flocs or granules, in sequencing batch reactors (SBR) operated with a high ammonium load. The aggregate structure and size was related to mass transfer limitations observed by measurements of OURs measured by either a titrimetric and off-gas analysis sensor (TOGA) or by microsensors. The FISH analysis showed that the spatial arrangement of the microbial consortia correlated well with the oxygen gradients inside the aggregates. In the larger aggregates, the ammonium- and nitrite-oxidising bacteria were mainly concentrated to the outer 100–200 μm, whereas in the floc system, the bacteria were distributed throughout the entire aggregate. This indicates that the internal mass transfer resistance is considerably larger when the aggregate size increases which is directly supported by TOGA measurements.

Keywords Fluorescence in situ hybridisation; mass transfer effects; nitrifying aggregates

Introduction In microbial aggregates both external and internal mass transfer processes can be significant. Substrates are transported through the bulk liquid to the external aggregate surface (external mass transfer), and then further through the aggregates (internal mass transfer) to be consumed by the microorganisms. Because the steps are sequential in nature, each one is important for determining the overall substrate reaction rate within the aggregate. The mass transfer leads to concentration gradients of substrates around and inside the aggregates. This may lead to stratification of the spatial distribution of the different microorganisms and consequently also the microbial processes. Aggregate size is an important factor since the potential for both internal and external mass transfer limitations increases with it. The effect of aggregate size on the external mass transfer limitation can be seen by interpretation of the dimensionless Sherwood (Sh) number correlation as detailed by Gapes (2003).

Previously, external and internal mass transfer effects of the same nitrifying systems as described in this paper were assessed (Wilén et al., 2004). The oxygen uptake rate was measured on both a macro-scale with a respirometric reactor using off-gas analysis (TOGA sensor) (Gapes and Keller, 2001; Pratt et al., 2003) and on a micro-scale with microsensors (Revsbech, 1989) at different DO concentrations and turbulence levels. Both methods were used since together they provide accurate measurements of the reaction rates and concentration profiles around the granules. The results showed that external mass transfer effects were mainly observed at low dissolved oxygen (DO) concentrations in the bulk liquid at the lower range of turbulence levels investigated. Despite the relatively limited effect of hydrodynamics on the overall transfer and reaction rates, there was a large drop in DO concentration from the bulk liquid to the aggregate surface as measured by microsensors. It was also
found that there was a significant internal mass transfer effect. This was concluded from the maximum OURs attained at a much higher DO concentration for larger aggregates than for smaller ones. The largest aggregates studied, which were formed as granules, had significantly larger internal mass transfer limitations than the smaller floccular aggregates.

Fluorescence in situ hybridisation (FISH) using 16S or 23S rRNA-targeted oligonucleotide probes has developed into a powerful technique for the in situ identification of individual microbial cells (Amann et al., 2001; DeLong et al., 1989). Confocal laser scanning microscopy (CLSM) enables a detailed determination of the exact position of the various bacterial cells in a microbial aggregate. By combining the microsensor technique with FISH and CLSM, information about the nutrient concentration profiles in microbial aggregates can be related to the spatial distribution of individual microbial cells or clusters within the aggregate (Amann and Kuhl, 1998). In a few previous studies the microsensor technique has been combined with FISH to study nitrite oxidation in a nitrifying fluidised bed reactor (Schramm et al., 1999a; Schramm et al., 1998); the occurrence of anoxic and anaerobic zones inside aerobic activated sludge (Schramm et al., 1999b); and the spatial distribution of ammonium oxidizers in a nitrifying biofilm (Okabe et al., 1999; Schramm et al., 1997). The combined application of different micro-scale techniques together with macro-scale respirometry studies in reactors can be very powerful in determining and confirming the mass-transfer and biological reaction processes around and within microbial aggregates. The two approaches are highly complementary, since the microscopic methods can provide detailed and direct evidence of the effects of concentration gradients on population distributions within individual aggregates, while the reactor-based methods give an overall assessment of aggregate phenotype.

The aim of this study was to determine the internal structure, microbial composition and spatial distribution of the cells of nitrifying microbial aggregates, formed as either flocs or granules, of different size and morphology and to relate it to the internal mass transfer limitation.

Materials and methods

Biomass

Nitrifying aggregates, formed as flocs or granules, were studied. The nitrifying biomass was taken from two laboratory-scale sequencing batch reactors (SBRs), each producing a biomass with different aggregate size distribution, as detailed by Gapes (2003). The SBRs were fed with synthetic wastewater and operated at high nitrogen loading (0.8–1.2 g N/L/day). The operational conditions for the two SBRs are given in Gapes et al. (2004). The main difference in operation was the settling time that was substantially shorter in the granular system. The ratio of volatile suspended solids (VSS) to total suspended solids (TSS) concentration was consistently between 0.77–0.79 for both floccular and granular systems. The size of the aggregates was measured by a laser diffraction instrument (Malvern Sizer for flocs), and by image analysis (100 images per sample for granules).

Microbial analyses

The microbial community structures of the aggregates were analysed by FISH (Amann, 1995; Manz et al., 1992). Aliquots of microbial aggregates were collected from the reactor and fixed in 4% paraformaldehyde (Manz et al., 1992) and embedded in OCT. The sample was dehydrated prior to embedding by placing it in different mixtures of OCT:15% sucrose solution (1:3, 1:1, 3:1) for at least 2 h. Thin slices (12 µm) of the embedded sample were cut with a cryotome and placed on microscopic slides. The samples were hybridised as reported by Amann (1995) for 1.5 h. Previously published probes for ammonium-oxidising and nitrite-oxidising bacteria (Table 1) were synthesised and 5′-labelled with the fluorochrome...
<table>
<thead>
<tr>
<th>Probe</th>
<th>Probe sequence (5' - 3')</th>
<th>rRNA target site (E. coli)</th>
<th>Specificity</th>
<th>% Formamide</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338</td>
<td>GCTGCCCTCCGCTAGGAGT</td>
<td>16S, 338–355</td>
<td>Most but not all bacteria</td>
<td>20</td>
<td>(Amann et al., 1990)</td>
</tr>
<tr>
<td>EUB338-II</td>
<td>GCAGCCACCCGTAGGTGT</td>
<td>16S, 338–355</td>
<td>Bacterial groups not covered by EUB338 and EUB338-III</td>
<td>20</td>
<td>(Daims et al., 1999)</td>
</tr>
<tr>
<td>EUB338-III</td>
<td>GCTGCCACCCGTAGGTGT</td>
<td>16S, 338–355</td>
<td>Bacterial groups not covered by EUB338 and EUB338-II</td>
<td>20</td>
<td>(Daims et al., 1999)</td>
</tr>
<tr>
<td>ALF1b</td>
<td>CGTTCG(C/T)TCTGAGCCAG</td>
<td>16S, 19–35</td>
<td>Many Alphaproteobacteria and many other bacteria¹</td>
<td>20</td>
<td>(Manz et al., 1992)</td>
</tr>
<tr>
<td>BET42a</td>
<td>GCCTTCCACTCTCGTT</td>
<td>23S, 1027–1043</td>
<td>Betaproteobacteria – used with unlabelled competitor</td>
<td>35</td>
<td>(Manz et al., 1992)</td>
</tr>
<tr>
<td>NSO190</td>
<td>CGATCCCTGCTTTTCTCC</td>
<td>16S, 190–208</td>
<td>Ammonium-oxidising Betaproteobacteria</td>
<td>55</td>
<td>(Mobarry et al., 1996)</td>
</tr>
<tr>
<td>NEU</td>
<td>CCCCTCTGCTGCACTCTA</td>
<td>16S, 653–670</td>
<td>Halophilic and halotolerant members of Nitrosomonas</td>
<td>40</td>
<td>(Wagner et al., 1995)</td>
</tr>
<tr>
<td>NIT3</td>
<td>CCTGTCGCTCAGCTCCG</td>
<td>16S, 1035–1048</td>
<td>Nitrobacter spp.</td>
<td>40</td>
<td>(Wagner et al., 1996)</td>
</tr>
<tr>
<td>NSR1156</td>
<td>CCCGTMTCTCGGGCAGT</td>
<td>16S, 1056–1173</td>
<td>Freshwater Nitrospira spp.</td>
<td>30</td>
<td>(Schramm et al., 1998)</td>
</tr>
</tbody>
</table>

¹Loy et al., 2002
fluorescein isothiocyanate (FITC) or one of the sulfoindocyanine dyes Cy3 and Cy5 (Thermohybaid Interactiva, Ulm, Germany). All bacteria were detected by hybridising with a mixture of EUB338, EUB338-II and EUB338-III (called EUBMIX). The FISH slides were viewed with a BioRad Radiance 2000 CLSM equipped with 40× and 60× objectives (oil immersion Nikon Corp, Tokyo, Japan). Excitation of FITC, Cy3 and Cy5 was done at 488 nm (Ar laser), 543 nm (HeNe laser) and 637 nm (red diode laser), respectively. Emissions were collected with filters 500–530 nm BP for FITC, 550–625 nm BP for Cy3 and 660 nm LP for Cy5. The collected images were finally evaluated using Adobe Photoshop (Adobe Systems Inc., USA). The aggregates were examined by light and phase contrast microscopy and digital images were captured.

Results and discussion

Internal mass transfer limitation in nitrifying aggregates

The volume based median size of the flocs was in the range 100–247 µm, whereas for the granules it was 500–690 µm. The microsensor work showed that in all profiles, an external concentration boundary layer (CBL) was observed (Wilén et al., 2004). A rapid decrease in DO concentration was observed within the aggregate, indicating that under a given condition, only a fraction of the granule was aerobic and hence capable of nitrification. The size and high activity of the granules clearly creates significant external and internal mass transfer effects. This together with the observation that the VSS-specific OUR was lower for the granular system compared to the floccular system for any given bulk DO concentration in the range 0.5–6 mg/l, confirms that the internal mass transfer limitation is a significant factor determining the substrate consumption rates in microbial aggregates (Gapes et al., 2004).

Due to the restricted oxygen supply to the granules, the main microbial activity and hence oxygen uptake, took place in the outer 50–100 µm of the granules under all conditions investigated, indicating a very high activity in this zone (Wilén et al., 2004). The DO concentration fell rapidly inside the granules to values that were much lower than the half rate concentrations, Kₜ (≤0.5 mg/L). In most cases a maximal fraction of only 60% of the granular volume was used, i.e. had DO concentration higher than 0.5 mg/l, even at a bulk DO of 8 mg/L, which is close to saturation with air at 25°C. At a DO concentration of 20 mg/L, the granules were fully oxygen penetrated and showed virtually immediate activity similar to that found in the outer zone of the granule. This is quite surprising given the fact that the internal volume of the granules is usually not supplied with both ammonium and oxygen at the same time, which is necessary for the growth of nitrifiers. Since the reactors were operated at DO concentration of 4–6 mg/L, it seems that these bacteria are able to remain viable and are very quickly activated as soon as both substrates are provided, as was the case in the experiment with DO = 20 mg/L.

Microbial investigation of the aggregates

Phase contrast light microscopy showed that there was a clear difference in morphology between the flocs and granules (Figure 1). The granules were found to be spherical to elliptical and very dense whereas the flocs were more irregularly shaped and more open. It was, however, observed that in both aggregate types there was a variation in morphology between individual aggregates. In the floc system, the small and medium sized aggregates were composed of microbial colonies uniformly distributed in a matrix of extracellular polymeric substances (EPS), whereas the larger flocs appeared to contain more EPS (interpreted through the microbial investigation as large and slightly autofluorescent areas without bacterial cells). In the granular sludge, the larger aggregates were well-defined granules while the smaller ones were more irregular, similar to flocs. Granules can be con-
sidered as an intermediate between flocs and surface-based biofilms (Beun et al., 2002). Aerobic granules have previously been observed in heterotrophic and nitrifying systems (de Beer et al., 1993; Beun et al., 1999; van Benthum et al., 1996; Morgenroth et al., 1997). Aerobic granules seem to be most readily formed in an intermittently operated system, such as SBRs. The system selects for granules when the settling time is drastically reduced so that only particles that settle rapidly are retained in the reactor.

In this work, nitrifying systems were studied to obtain a high OUR. Nitrification is a sequential transformation of $\text{NH}_4^+$ via $\text{NO}_2^-$ to $\text{NO}_3^-$. This reaction is catalysed by two distinctly different groups of microorganisms: the ammonium-oxidizing bacteria and the nitrite-oxidizing bacteria (Mobarry et al., 1996). The population in both the granules and flocs consisted mainly of BET42a- and ALF1b-positive cells. Cells binding to the BET42a probe formed large colonies with a characteristic morphology whereas the cells binding to the ALF1b probe seem as more spread out individual cells in the floc-matrix (Figure 2 d–h).

The CLSM images show that the ammonium oxidizing bacteria (indicated by binding to probes NSO190 and NEU) were mainly found at the outer edges of the dense granules whereas more open aggregates (flocs) had ammonium oxidising bacteria evenly distributed in the aggregates (Figure 2 a, b, d, f and g). The nitrite oxidisers (indicated by binding to probe NIT3) were also found mainly at the outer parts of the dense granules but they also grew in the centre of the flocs (Figure 2 c, e and h). No cells bound probe NSR1156, targeting *Nitrospira* sp. A large fraction of the bacteria that bound probe BET42a, also bound probes NSO190 and NEU. Most cells that bound NSO190 also bound NEU, but probe NEU gave better signals. From this it can be concluded that the ammonium-oxidising bacteria were identified as *Betaproteobacteria* in the genus *Nitrosomonas*. Most bacteria that bound probe ALF1b also bound probe NIT3, indicating that the nitrite-oxidisers were *Alphaproteobacteria*, in the genus *Nitrobacter* (Figure 2 e and h).

The flocs and granules appeared to contain similar types of bacteria but the main difference was the way bacteria grew. In the granules, the large characteristic microbial colonies grew mainly around the outer parts while the centre contained no active cells, concluded from the absence of cells binding probe EUBMIX. In the flocs, the colonies grew evenly distributed throughout. Most of these large colonies bound probe BET42a. The fact that not all *Betaproteobacteria* (BET42a-targeted) also bound the broad *Nitrosomonas*-specific probe NSO190 (Mobarry et al., 1996) might imply that some other ammonium-oxidising bacteria also existed in the aggregates. This was, however, not further investigated in this study. In the microsensor studies it was found that the smaller granules had a higher OUR per volume of active biomass (i.e. the fraction where the $\text{DO} > 0 \text{ mg/L}$). The microsensor measurements showed that a large fraction of the oxygen consumption took place within the first 50–100 µm (Wilén et al., 2004). However, in many granules ammonium oxidising cells were located deeper than 50–300 µm into the granule, where the DO concentration was zero at about 4–5 mg/L bulk DO. The “feast–famine” situation created by SBRs, could provide one explanation for this granule microbial architecture since near the end of the

![Figure 1](https://iwaponline.com/wst/article-pdf/50/10/213/419302/213.pdf)

**Figure 1** Micrographs of (a) floc; (b) granule; and (c) stereo-microscope image of granule.
Figure 2  CLSM images (a–h); overlay between red and green is shown as yellow, overlay between green and blue is shown as cyan, overlay between red and blue is shown as magenta, overlay between red, green and blue is shown as white: (A) granule: double labelling with NEU-FITC (green) and EUBMIX-Cy3 (red); (B) floc: double labelling with NEU-FITC (green) and EUBMIX-Cy3 (red); (C) granule: double labelling with EUBMIX-FITC (green) and NIT3-Cy3 (red); (D) granule: triple labelling with EUBMIX-Cy5 (blue), BET42a-Cy3 (red) and NEU-FITC (green); (E) granule: triple labelling with EUBMIX-Cy5 (blue), ALF1b-FITC (green) and NIT3-Cy3 (red); (F) floc: triple labelling with EUBMIX-Cy5 (blue), NEU-FITC (green) and BET-Cy3 (red); (G) same as D, but when the granule is very large, most binding to NEU was observed at the outer edge; (H) floc: triple labelling with EUBMIX-Cy5 (blue), ALF1b-FITC (green) and NIT3-Cy3 (red)

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react cycle, the ammonium and DO could have penetrated deeper into the granules. For the larger granules, it was, however, observed that there were fewer cells in the centre part.

The flocs and the granules contained similar microbial consortia, but the flocs were smaller and less dense, leading to a smaller internal mass transfer limitation. This further suggests that the differences in OUR between the granular and the floccular system was due to mass transfer limitation as the maximum OURs obtained for both systems were quite similar (Gapes et al., 2004) which further indicates that the overall makeup of the biomass was similar for the two systems. As opposed to other studies of nitrifying bacteria, the nitrite-oxidising bacteria belonged to the genera *Nitrobacter* (Alphaproteobacteria) (Wagner et al., 1996) and not to *Nitrospira* (Burrell et al., 1998; Juretschko et al., 1998; Okabe et al., 1999; Schramm et al., 1998; Wagner et al., 1996). Previous work, where FISH was combined with microelectrode measurements, suggested that *Nitrospira*-like nitrite oxidisers represent K-strategists which are adapted to low nitrite and oxygen concentrations, whereas *Nitrobacter* sp. are r-strategists that thrive at high nitrite and oxygen concentrations (Schramm et al., 1999a). The granules in this study were taken from a reactor operated as an SBR at very high loading of ammonium, which gave high variations in the concentrations of ammonium, nitrite and nitrate during the cycle. The appearance of significant concentrations of nitrite in the reactor may have selected for the *Nitrobacter* sp. favoured by high nitrite concentrations.

The ammonium-oxidizing bacteria and the nitrite-oxidizing bacteria were growing close to each other, which reflects the close interaction between the two groups of bacteria. Similarly, Juretschko et al. (1998) found that *Nitrospira*-like nitrite oxidisers and ammonium oxidisers were growing in the vicinity of each other. The ammonium-oxidising bacteria grew in large dense clusters that should experience larger diffusion resistance compared to the nitrite-oxidising bacteria that grew as single cells. This may also provide a reason for the observation that the nitrite oxidisers were generally observed further inside the granules.

**Conclusions**

- The spatial arrangement of the microbial consortia correlated well with the oxygen gradients observed inside the aggregates.
- In the larger granules, the ammonium- and nitrite oxidising bacteria were mainly seen in the outer 100–200 µm, whereas in the floc system they were distributed throughout the aggregates. This indicates that the mass transfer resistance is considerably more pronounced when the aggregate size increases. Nitrifying bacteria are only respiring at their maximum rate when the local DO concentration is above 2–3 mg/L. Due to the large DO drop in the external boundary layer, the bulk liquid DO concentration needs to be considerably above these levels to supply sufficient oxygen to the bacteria below the surface of the granules.
- Increasing the DO also allows oxygen penetration further into the granule. Although FISH does not detect many active nitrifiers near the centre of the granules, there might be dormant bacteria present there that are reactivated rapidly by sufficient supply of nutrients.

**References**


