Sludge bed development in denitrifying reactors using different inocula-performance and microbiological aspects

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Abstract Aerobic and methanogenic consortia were evaluated as inocula for laboratory scale denitrifying reactors, fed with a synthetic wastewater with acetate as the main electron donor. The denitrifying microflora of inocula and reactors was evaluated by specific denitrifying activity, enumeration and isolation of denitrifiers, which were screened by amplified ribosomal DNA restriction analysis. Reactor performance was monitored by COD and nitrate removal efficiencies and granule size. The aerobic sludge failed to form granules, probably due to the development of a filamentous, nitrate-reducing organism which was characterised by 16SrDNA sequencing as Bacillus cereus. The methanogenic sludge showed denitrifying activity and adapted very rapidly to denitrifying conditions in the two reactors seeded with granules of different sizes. Denitrifiers grew around the granules, increasing the specific denitrifying activity of the sludge over 10-fold. Exopolymer-forming organisms, belonging to the same species, were isolated from both reactors. Granule size increased during operation, but flotation of the aggregates, related to gas retention was observed.

Keywords Denitrification; granulation; inocula; sludge bed anoxic reactor

Introduction
The need for the removal of nitrogen compounds from effluents before final disposal is being increasingly recognised, and should be considered when designing a biological treatment system. Although a biological denitrification step is widely used to lower nitrate concentrations to acceptable levels, the information about the microbiology and sludge behaviour of denitrifying reactors is still scarce. Recently, it has been shown that the denitrifying organisms isolated from an anoxic reactor treating leachate from a sanitary landfill could not be identified by conventional methods, requiring genetic characterisation based on 16SrDNA sequences (Etchebehere et al., 2001).

Anoxic UASB reactors have been successfully used for nitrate removal in an integrated system (anaerobic, aerobic, anoxic) treating effluents with high organic and nitrogen content (Borzacconi et al., 1999; Morgan-Sagastume et al., 1994). However, there are few reports on the parameters that affect granulation of the denitrifying biomass (Cuervo-López et al., 1999). Additionally, buoyancy problems have been reported in UASB anoxic reactors (Cuervo López et al., 2000a; Cuervo López et al., 2000b; Hendriksen and Ahring, 1996).

In the present work, we describe the behaviour of laboratory scale UASB denitrifying reactors fed with acetate and nitrate. The aim was to study the effect of the nature of the inoculum on granulation and reactor performance considering both microbiological and operational aspects.

Materials and methods
Three upflow reactors (4.6 L) were seeded with different inocula: i) aerobic sludge from a RBC reactor treating acetate. ii) intact granules from an anaerobic UASB reactor treating...
brewery wastewater (medium size: 0.8 mm) iii) ground granules (medium size: 0.4 mm)
from the same reactor. The reactors were fed with synthetic effluent with acetate as carbon
source and nitrate (COD/N-NO₃ = 4). The nitrate loading rate was gradually increased up to
values of 0.9 g NO₃-N/l.d at the end of the study.

COD, nitrate and nitrite were determined at the inlet and the outlet of the reactors. In
addition, sludge bed development was followed by measuring VSS, TSS and the average
granule size, throughout time. Analyses were carried out according to Standard Methods
(APHA, AWWA, WEF, 1995) and the granule size was measured using the technique
described by Jeison and Chamy (1998).

In order to test the ability of the sludge to aggregate, different up flow velocities were
applied to approximately 10 g of biomass in a glass column of 3 cm diameter and 2 m
height.

Reactor sludge samples were analysed for total exopolysaccharides (EPS), specific de-
nitrifying activity (SDA), most probable number (MPN) enumeration, isolation and char-
acterisation of denitrifiers. Total exopolymers were extracted from sludge samples by
alkaline lysis according to Judice (1991). Carbohydrates were analysed by the phenol-sul-
phuric acid colorimetric method. SDA was measured in sludge samples by N₂O production
rates by acetylene blockage of N₂O reduction to N₂, with acetate as electron donor
(Etchebehere et al., 2001). Denitrifiers MPN enumeration was performed as previously
described (Quevedo et al., 1998). Denitrifiers were isolated on TSA plates from the most
diluted positive tubes and a preliminary characterisation was performed by Gram stain,
catalase and oxidase tests.

Bacterial isolates were differentiated by amplified 16S rDNA restriction analysis
(ARDRA), carried out as previously described, with primers specific for the domain
Bacteria using Hae III and Hha I as restriction enzymes (Fernandez et al., 1999).
Amplification and 16S rDNA sequence analysis were performed as reported by
Etchebehere et al. (2001). Sequencing of the purified PCR product was done at the
University of Florida, DNA Sequencing Core Laboratory. DNA-DNA hybridisation was
performed at Institute of Microbiology, Russian Academy of Science.

In order to detect the persistence of methanogens from the inoculum in the sludge,
fluorescent in situ hybridisation (FISH) was performed on sludge samples using probes
for the domain Archaea (Arch 915) and Bacteria (Eub 338) as described by Amann
et al. (1990). For quantitative analysis total sludge cells targeted by a probe were
expressed as a percentage of total cells counterstained with 4′,6-diamidino-2-phenylindole
(DAPI).

**Results and discussion**

**Aerobic inoculum**
The aerobic sludge tested in the UASB reactor failed to form granules, with filament forma-
tion being observed macroscopically. Microscopic examination of the inoculum revealed
the presence of a filamentous organism, that was isolated and characterised. 16SrDNA
sequencing revealed high similarity to Bacillus cereus. The same morphology was
observed in the reactor, under denitrifying conditions. Filamentous organisms have been
reported to colonise aerobic sludges, with a negative effect on settling properties. Our
results suggest that although the aerobic sludge showed denitrifying capacity (SDA =
2.0 úmole N₂O (VSS g)⁻¹min⁻¹) the presence of filamentous organisms resulted in the
inability to form granules. Studies in pure culture showed that this organism could grow
with a wide range of electron donors, and had the ability to efficiently reduce nitrate to
nitrite, explaining the persistence in the denitrifying reactor.
Anaerobic inoculum

The methanogenic anaerobic inoculum showed the ability to denitrify, although at a low rate, as has been previously reported for other methanogenic ecosystems and the number of denitrifiers was relatively high (Table 1), increasing with reactor operation. Furthermore, as of week 20, a comparison of the number of aerobic organisms, as determined by plate count in TSA, and the MPN of denitrifiers showed that a very high proportion of the cultured bacteria were denitrifiers.

The SDA of the biomass increased over 10-fold during the first month of operation, as expressed per g of VSS (Table 1) indicating that the anaerobic sludge adapted very easily to efficient nitrate removal. The potential denitrifying activity of the sludges (SDA, Table 1) was approximately 10-fold larger than the rate of nitrate removal in the reactors, calculated using the nitrate removal efficiency and the loading rate. This indicates that the microflora might adapt to an increase in nitrate loading rate, at least in terms of nitrate removing ability.

Figure 1 shows the behaviour of the reactors seeded with intact granules (Reactor 1) and with ground granules (Reactor 2). Nitrate removal was detected in a short time of operation (about 1 week) in both reactors.

ARDRA profiles of the isolated denitrifying strains were performed to group the isolates. Strains 23 and 25, isolated from Reactor 2 after one month of operation, showed the same profile as strain 20, isolated from the methanogenic sludge, suggesting the adaptability of this organism to the new denitrifying conditions. The ARDRA profiles were similar to those of organisms of the genus *Pseudomonas*, a very frequently isolated genus that harbours denitrifiers (Figure 2).

### Table 1 Evaluation of sludge samples of the anaerobic inoculum and Reactors 1 and 2 over time

<table>
<thead>
<tr>
<th>Sample (date)</th>
<th>SDA a</th>
<th>MPN ml⁻¹</th>
<th>SDA gVSS⁻¹ b</th>
<th>EPS c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanogenic granules</td>
<td>0.022</td>
<td>4.3 × 10⁶</td>
<td>0.57</td>
<td>3.5</td>
</tr>
<tr>
<td>Disrupted methanogenic granules</td>
<td>0.006</td>
<td>N.D.</td>
<td>0.37</td>
<td>8.3</td>
</tr>
<tr>
<td>R1 (week 4)</td>
<td>0.040</td>
<td>2.4 × 10⁸</td>
<td>6.15</td>
<td>13.3</td>
</tr>
<tr>
<td>R2 (week 4)</td>
<td>0.092</td>
<td>2.4 × 10⁷</td>
<td>14.8</td>
<td>10.5</td>
</tr>
<tr>
<td>R1 (week 14)</td>
<td>0.047</td>
<td>1.5 × 10⁷</td>
<td>12.7</td>
<td>14.0</td>
</tr>
<tr>
<td>R2 (week 14)</td>
<td>0.096</td>
<td>4.3 × 10⁶</td>
<td>40.0</td>
<td>16.1</td>
</tr>
<tr>
<td>R1 (week 20)</td>
<td>0.122</td>
<td>&gt; 2.4 × 10⁹</td>
<td>38.2</td>
<td>39.8</td>
</tr>
<tr>
<td>R2 (week 20)</td>
<td>0.122</td>
<td>2.4 × 10⁸</td>
<td>17.7</td>
<td>11.4</td>
</tr>
<tr>
<td>R1 (week 42)</td>
<td>0.111</td>
<td>1.6 × 10⁹ d</td>
<td>17.6</td>
<td>N.D.</td>
</tr>
<tr>
<td>R2 (week 42)</td>
<td>0.120</td>
<td>9.5 × 10⁸ d</td>
<td>26.0</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

a. Expressed as µmoles N₂O. ml⁻¹ min⁻¹. Values are the mean of two replicates. The standard deviation was always less than 10% b. Expressed as µmoles N₂O/VSS gr⁻¹ min⁻¹ c. Expressed as µg exopolysaccharides (VSSmg)⁻¹ d. Performed as total plate count (aerobic conditions) N.D. Not determined

**Figure 1**  
COD, NO₃ removal efficiencies and granule size for Reactors 1 and 2 (anaerobic inocula)
Figure 3 shows that the percentage of organisms belonging to the domain *Archaea*, probably methanogens from the inoculum, decreased during operation of Reactor 2, as evaluated by FISH. However, *Archaea* were still detected after approximately one year of operation, suggesting that they were the support for the adhesion of denitrifiers. Similar results were obtained for Reactor 1.

After two months of operation, a white biomass grew around the anaerobic granules (Figure 4), in both reactors. In Reactor 1 it covered the individual granules whereas in Reactor 2 the ground granules were progressively aggregated.

The denitrifying organisms isolated from this white layer in week 12 (Strains 27 and 28, from Reactors 1 and 2, respectively) and Strain 30, isolated in week 14, showed ARDRA profiles, which were very similar to those of bacteria belonging to the genus *Thauera* (Figure 2). This result suggests that strains 27, 28 and 30 may be members of such genus, known to perform efficient denitrification. Furthermore, strains 27 and 28 showed a value of 81% for DNA-DNA hybridisation, indicating that the same species developed in both reactors. Both strains produced exopolysaccharides (EPS), when grown in pure culture, suggesting that adhesion of these organisms to the granules caused the observed increase of granule diameter. The ability to adhere may also explain the development of organisms of the same species in both reactors, even though a wide variety of denitrifiers are able to utilise acetate coupled to denitrification. The effect of EPS production on granule forma-
tion has been studied for anaerobic sludges, however, little is known about EPS production for denitrifying biomass. Table 1 shows that, for the reactors under study, an increase in EPS concentration was observed during operation, suggesting a correlation with the increase in the population of denitrifiers, as evidenced by the increase in SDA per g of VSS, and granule size.

Gas bubbles, observed inside the granules and also trapped in the sludge bed, led to granules and sludge flotation (Figure 4). Gas recycling prevented the sludge bed from rising but not granule flotation caused by inner gas retention. A black colour persisted in the centre of the granules, which turned into light brown when sludge samples were aerated. After aeration, the samples were incubated in serum vials under anoxic conditions, containing nitrate, acetate and nutrients, turning into black colour after complete depletion of nitrate. This suggests the persistence of sulphate reduction in the granules.

During the experience the FSS/VSS ratio increased from values of 0.17 at the start up to values of 1, probably due to the precipitation of calcium and magnesium as carbonate, as indicated by the calcium, magnesium and iron values detected in the sludge, which were 250 mg Ca gTSS⁻¹, 30 mg Mg gTSS⁻¹ and 6 mg Fe gTSS⁻¹.

Grounded sludge from Reactors 1 and 2 and a pure culture of strain 28 isolated were tested at different up-flow velocities. The three samples showed the same behaviour. High adhesion forces within the biomass were observed, even at high up-flow velocities. Applying velocities smaller than 10 m/h it was impossible to segregate the biomass. Using velocities higher than 10 m/h, aggregates could be detached. The former behaviour, could be related with the physical properties of the predominant bacteria, which could also affect nitrogen release from the granules, causing the observed sludge flotation.

**Conclusions**

The results indicate that both the aerobic and the anaerobic inocula were adequate in terms of adaptation to denitrifying conditions. The presence of a filamentous organism in the aerobic sludge probably prevented granule formation, whereas for the anaerobic sludge, an increase in the size of the aggregates was observed during reactor operation. However, this did not result in good settling properties of the biomass, as has been reported for other denitrifying reactors (Cuervo-López et al., 1999). The flotation of the aggregates seems to be related to gas retention, and needs to be further investigated in order to improve the operation of such reactors. Concerning the influence of the granule diameter at the inoculation, in Reactor 1 seeded with 0.8 mm anaerobic granules, the denitrifying biomass grew around the granules, causing the granules to became larger throughout time. In Reactor 2 the denitrifying biomass grew, trapping the grounded anaerobic granules. In spite of the fact...
that the sludge seeded in both reactors was of different granule size after some months the aggregates looked quite similar.

The ability to adhere explains that bacteria of the same species, probably belonging to the genus \textit{Thauera}, developed in both reactors, even though a large diversity of denitrifiers can perform denitrification under the operating conditions of this study. Members of the domain \textit{Archaea} persisted in the reactors, indicating that the methanogenic granules used for inoculation were the support for denitrifying biomass attachment and development during start-up.

\textbf{References}


