The effect of C/N ratio on ammonia oxidising bacteria community structure in a laboratory nitrification-denitrification reactor

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Abstract A laboratory scale reactor operated as a single sludge, denitrification-nitrification bioreactor (DNB), was fed a synthetic wastewater. The effect of the C/N ratio of the influent on the structure of β-proteobacterial autotrophic ammonia-oxidizing bacterial (AOB) communities was determined by DGGE analysis of 16S rRNA gene fragments amplified using a range of AOB-selective primers. Fluorescence in situ hybridisation (FISH) was used to determine quantitative changes in the AOB communities. When operated at a C/N ratio of 2 the DNB was effective in nitrogen removal and nitrification was measured at approximately 1.0 mg NH₄⁺N/g dry wt/h. Altering the C/N ratio to 5 resulted in a 50% reduction in nitrification rates. Nitrification was restored to its original level when the C/N ratio was returned to 2. AOB were detected by DGGE analysis of samples from the DNB under all operating conditions but the changes in C/N ratio and nitrification rates were accompanied by changes in the community structure of the AOB. However, quantitative FISH analysis indicated that β-proteobacterial AOB were only present in high numbers (ca. 10⁸ cells/ml) under the original operating conditions with a C/N ratio of 2. β-Proteobacterial AOB could not be detected by FISH when the C/N ratio was 5. When nitrification activity was restored by returning the C/N ratio to 2, β-proteobacterial AOB were still not detected and it is likely that either β-proteobacterial AOB were not responsible for ammonia oxidation or that β-proteobacterial AOB that did not contain the target sites for the range of 4 AOB selective probes used, were present in the reactor.

Keywords Ammonia-oxidising bacteria, nitrification, rRNA

Introduction Refinery wastewater treatment plants (and many other industrial treatment plants) must cope with large changes in C/N ratios in comparison to the domestic treatment plants. Theoretical and practical experience suggests that these changes may have a profound effect on nitrification. It has been known for some time that changing the C/N ratio of wastewater can affect nitrification. Reduced levels of nitrification are associated with an increased proportion of carbon and vice versa. However, the effect of fluctuation in C/N ratio on community structure in AOB populations has remained unknown.

Previous workers have found AOB community structure to be on the whole rather resistant to environmental change (Princic et al., 1998). However, in that study the perturbation was in a system fed only mineral medium with ammonium salts as the sole energy source. Industrial wastewater treatment plants are less well defined and may be subject to long periods of profound perturbation. We have examined the effect of C/N ratio on AOB community structure in a laboratory denitrification-nitrification (DNB) system, treating a synthetic wastewater containing a mixture of organic electron donors and ammonium salts, using denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments, in conjunction with quantitative fluorescence in situ hybridisation (FISH) and detailed characterization of the AOB populations by sequencing of 16S rRNA gene fragments.
Methods

The DNB reactor

The laboratory DNB system consisted of a first stage 2.5 litre, anoxic continuously stirred tank (CST) reactor, a second stage 5 litre aerobic CST reactor and a 1 litre cell settler. The temperature of both reactors was maintained at 25°C. The aerobic reactor was maintained at pH 7.4 and the anoxic reactor at pH 7.0 through auto-titration with 1 M NaOH. The sludge in both reactors was mixed by six-bladed Rushton impellers (300 r.p.m). The anoxic reactor feed, aerobic reactor and recycle loop flow rates were 0.21, 0.84 and 0.21 l/h respectively, with an overall hydraulic retention time of 24 hours. The mixed liquor concentration was maintained between 3–4 g/l through periods of increased wastage from the aerobic reactor. The anoxic reactor was gassed with argon at 400 ml/min. and the aerobic reactor with air at 1,000 ml/min. The synthetic wastewater contained butanol (30 mg/l), sodium acetate (30 mg/l), glycerol (30 mg/l), (NH₄)₂SO₄ (106 mg/l), H₃PO₄ (31 mg/l), MgSO₄.7H₂O (6 mg/l), CaCl₂ (6 mg/l), 0.1 M FeSO₄ (0.2 ml/l) and trace elements solution (1 ml/l; Watson, 1971). The aerobic reactor was seeded with nitrifying sludge from a wastewater treatment plant treating oil refinery process effluents. Molar C/N ratios of 2 or 5 were obtained by adjusting the concentration of the organic carbon compounds. The DNB reactor was operated for a period of 775 days. The C/N ratio of the influent was changed from 2 to 5 on day 446 and returned to a value of 2 on day 578.

Samples from the feed, the anoxic and aerobic reactors and the effluent were stored at 4°C for ammonium, nitrite and nitrate determination, and at –20°C for nucleic acid extraction. Aerobic reactor samples for analysis by in situ hybridisation were either stored in ethanol (1:1) before fixing or were fixed directly in 4% paraformaldehyde.

DNA extraction and polymerase chain reaction

Samples of DNB mixed liquor (1 ml) or AOB culture (200 ml) were pelleted (13,000 r.p.m, 5 minutes, MSE Microcentaur) and washed (twice) with 0.12 M sodium phosphate buffer (pH 8.0). The supernatant was discarded and the pellet was resuspended in 100 µl of sterile TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). This was added to a 2 ml screw-cap tube containing 0.5 g sterile glass beads (0.17–0.18 mm diameter; B. Braun Ltd, Germany). Sodium phosphate buffer (1 ml; 0.12 M, pH 8.0) containing 6% sodium dodecylsulfate (SDS) and 0.5 ml phenol/chloroform (1:1; pH 8.0) were added and the sample vortexed for 1 minute. Lysis of cells was carried out by bead beating for 1 minute in a Mikrodismembrator U (B. Braun Ltd, Germany) set to 2,000 r.p.m. The solution was centrifuged (13,000 r.p.m, 2 minutes) to break the emulsion formed. The upper aqueous phase was transferred into a fresh microcentrifuge tube and extracted twice with an equal volume of phenol/chloroform (1:1; pH 8.0). The upper aqueous phase was transferred to a fresh tube and an equal volume of 30% polyethylene glycol (PEG) and one-tenth volume of 5 M sodium chloride were added. Samples were vortexed for 1 minute and placed on ice for 15 minutes before centrifugation (13,000 r.p.m, 10 minutes). The supernatant was removed and the pellet redisolved in 50 µl sterile TE buffer. Ice-cold ethanol (2 volumes) was added and incubated overnight at –20°C. The precipitated DNA was pelleted (13,000 r.p.m, 10 minutes) and the supernatant removed. Residual ethanol was allowed to evaporate and the pellet was dissolved in 50 µl sterile TE buffer.

Template DNA (4 µl) was added to 96 µl of a standard PCR reaction mix in a 0.75 ml microcentrifuge tube. The reaction mixture contained 20 pmol of each primer, 10 nmol of each deoxynucleoside triphosphate (Pharmacia, UK), 50 µl of 10 × PCR reaction buffer (Finnzymes Oy, Finland), 3 µl (6 U) Dynazyme DNA polymerase (Finnzymes Oy, Finland) and 417 µl of filter-sterile MilliQ water. Samples were overlaid with 100 µl sterile mineral oil. PCR was done using an Omnigene thermal cycler (Hybaid, UK) using the
following cycling parameters: Initial denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 1 min, primer annealing for 1 min and primer extension at 72°C for 1 minute. A final primer extension step at 72°C for 10 minutes was included at the end of the PCR. Annealing temperatures were different for each primer set used and are given in Table 1.

**Denaturing gradient gel electrophoresis**

DGGE profiles were obtained from PCR amplified 16S rDNA sequences from replicate samples from the DNB (n = 3) and from cloned-sequences (Figures 1–2). Three ammonia-oxidiser selective primer sets Nso190 and Nso1225, Nsv443 and Nso1225, and Nsm156 and Nso1225 (Mobarry et al., 1996; Table 1), were used to amplify sequences from the beta-proteobacterial AOB and from the genera *Nitrosospira* and *Nitrosomonas*, respectively. The PCR products obtained from these initial amplifications were subsequently re-amplified using DGGE primers targeting sequences found in the 16S rRNA genes of the majority of bacteria (Table 1). The DGGE primers used, amplified a fragment of ca. 500 bp. DGGE was conducted using the methods of Muyzer et al. (1993) with a D-Gene denaturing gel electrophoresis system (BioRad, Hemel Hempstead, UK). Polyacrylamide gels (10% [w/v] polyacrylamide, 37:1 acrylamide:bisacrylamide) were prepared in TAE buffer (40 mM Tris, 2 mM EDTA, pH 7.4) containing a 40–70% linear gradient of denaturant (100% denaturant is 40% [v/v] formamide plus 7.0 M urea). Gels were run at 60°C for 4.5 hours at a constant 200V. Gels were stained with ethidium bromide (0.5 µg ml–1) for 4 minutes, washed in MilliQ water (2 × 5 minutes) and the DNA fragments visualised using a UV transilluminator (UVP, San Gabriel, California, USA) and photographed using a Polaroid cam-

### Table 1 Oligonucleotides used as PCR primers in the current study

<table>
<thead>
<tr>
<th>Oligonucleotide Primer</th>
<th>Primer Sequence 5’–3’</th>
<th>Specificity</th>
<th>Annealing Temperature(˚C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nso190</td>
<td>GGA GAAA AGC AGG GGA TCG</td>
<td>16S rRNA gene of the β-subgroup ammonia oxidising bacteria.</td>
<td>58</td>
<td>Mobarry et al., 1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Forward primer (189–207)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nso 1225</td>
<td>CGC CAT TGT ATT ACG TGT GA</td>
<td>16S rRNA gene of β-subgroup ammonia oxidising bacteria.</td>
<td>58</td>
<td>Mobarry et al., 1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse primer (1224–1243)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nsm 156</td>
<td>ATC GAA AGA TGT GCT AAT A</td>
<td>16S rRNA gene of the genus <em>Nitrosomonas</em>.</td>
<td>54</td>
<td>Mobarry et al., 1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Forward primer (155–173)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nsv 443</td>
<td>CGG AAC GAA ACG GTC ACG G</td>
<td>16S rRNA gene of the genus <em>Nitrosospira</em>.</td>
<td>58</td>
<td>Mobarry et al., 1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Forward primer (443–461)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pD&lt;sup&gt;GC&lt;/sup&gt;</td>
<td>CAG CAG CCG CGG TAA TAC</td>
<td>16S rRNA gene of eubacteria.</td>
<td>Touchdown Edwards et al., (55)</td>
<td>1989</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Forward primer (518–536)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pE&lt;sup&gt;+&lt;/sup&gt;</td>
<td>CCG TCA ATT CCT TTG AGT TT</td>
<td>16S rRNA gene of eubacteria.</td>
<td>Touchdown Edwards et al., (55)</td>
<td>1989</td>
</tr>
</tbody>
</table>

Numbers shown correspond to the positions in the *E. coli* 16S rRNA (Brosius et al., 1979)

§ When used as FISH probes, the reverse complement of the sequence shown was employed

GG<sup>DGGE</sup> primer with a 5’ GC-clamp:

(5’-GCACCGCGCGCGCGCGCGCGGGGGGGGGGCACGGGGGG-3’)

Numbers shown correspond to the positions in the *E. coli* 16S rRNA (Brosius et al., 1979)
era (CU-5, GRI, Essex, UK). To check for co-migration and incomplete specificity of the diagnostic oligonucleotide primers, DNA fragments were excised from DGGE gels and sequenced.

**Fluorescence in situ hybridisation**

Fluorescent *in situ* hybridisation was done essentially as described by (Gray *et al.*, 1999). Samples of fixed biomass were hybridised with a range of probes selective for all AOB (Nso 190; 5′-CGATCCCTGCCTTTCTCC-3′), *Nitrosospira* spp. (Nsv443; 5′-CCGTGACCGTTTCGTTCCG-3′), *Nitrosomonas* spp. (Nsm156; 5′-TATTAGCATCTTTCGAT-3′), a *Nitrosomonas*-like cloned sequence recovered from the DNB (Nsm641; 5′-TGCCGACCTAGCTCTGCAGTT-3′) and the domain *Bacteria* (Eub338; 5′-GCTGCCGTAAGGAGT-3′). The reverse complement of Eub338 was used as a negative control to determine levels of fluorescence due to non-specific probe binding in all FISH assays. All ammonia-oxidiser probes were labelled with tetramethylrhodamine (TRITC) and Eub338 was labelled with fluorescein isothiocyanate (FITC). Hybridization was conducted in liquid suspension in microcentrifuge tubes. After hybridization samples were pelleted (12,000 r.p.m, 3 minutes) and resuspended in 100 µl of hybridisation buffer and incubated at the hybridisation temperature for a further 15 minutes. This washing procedure was repeated twice and the pellet was washed in 100 µl of sterile Milli-Q water. Cell suspensions were centrifuged and the supernatant discarded. The cells were resuspended in 10 µl of sterile MilliQ water and pipetted on to a gelatin-coated microscope slide. Preparations were allowed to dry before being mounted in Citifluor (Citifluor Ltd, Canterbury, UK). A cover-slip was placed over the preparation and sealed with clear nail varnish. Slides were stored at 4°C in the dark until they were examined microscopically. Slides were viewed at 600× magnification using a confocal laser scanning microscope (CLSM; MRC 600, Biorad, UK) which incorporated a Nikon Optiphot 2 epifluorescent microscope. Images were captured and probe conferred fluorescence was measured using the COMOS program (Biorad, UK). Quantitative determination of AOB cell numbers was determined based upon a calibration of AOB microcolony diameter and the number of cells present in the microcolony. Microcolonies were counted and measured in 46 random fields of view and the number of AOB per millilitre of sample was determined.

**Results and discussion**

**Nitrification in the DNB**

For the first 446 days of operation the DNB system was fed an influent with a C/N ratio of two. During this period all of the influent NH$_4^+$ (~106 mg N/l) was removed. The NH$_4^+$ concentration within the anoxic reactor was stable between 20 – 30 mg NH$_4^+$-N/l. On average, 0.3 mg N/g dry wt/h was removed through assimilation into new cell biomass in the anoxic reactor. The remaining NH$_4^+$ passed directly into the aerobic reactor where it was either oxidised to nitrate, by nitrification, or assimilated for cell growth. The mean specific rates for NH$_4^+$ consumption (1.04 mg N/g dry wt/h) and NO$_x^-$ production (1.03 mg N/g dry wt.h$^{-1}$) in the aerobic reactor, were comparable, indicating that virtually all of the NH$_4^+$ in the aerobic reactor was consumed during nitrification. The maximum nitrification rate (NO$_x^-$ + NO$_3^-$ production rate) observed during this period was 1.69 mg N/g dry wt/h. The oxygen consumption rates for this period were relatively stable with a mean value of 30.8 mg O/h. However, the amount of oxygen consumed through nitrification, estimated from the nitrate production rates (Sharma and Ahlert, 1977) was less (~3.8 mg O/h) indicating that most of the oxygen (> 70%) within the aerobic reactor was consumed by heterotrophs.

On day 446 the C/N ratio was increased to five (C/N = 5). As a consequence there was an...
immediate reduction in the specific rates of \( \text{NH}_4^+ \) consumption and \( \text{NO}_x^- \) production. However, nitrification was not totally lost with the mean rates of ammonium consumption and \( \text{NO}_x^- \) production decreasing from 1.04 to 0.52, and from 1.03 to 0.38 mg N/g dry wt/h, respectively, representing an overall reduction in the rate of approximately 50% compared to the rates observed when the influent C/N ratio was 2.

On day 578 the influent C/N ratio was changed from 5 back to 2 and there was a rapid increase in the total amount of nitrogen removal. Although ammonium was still present in the effluent, the concentration gradually decreased until, on day 633, it could no longer be detected. This suggests that the autotrophic nitrifier populations had recovered and were re-established within the aerobic reactor with activities similar to those observed during the original conditions (C/N = 2). The specific rate of ammonium consumption in the aerobic reactor steadily increased with time, until it reached a maximum value, on day 633, of 2.32 mg N/g dry wt/h. The specific rate of \( \text{NO}_x^- \) production in the aerobic reactor increased in a similar manner, indicating that most of the ammonium was consumed by nitrification. The mean rates of \( \text{NH}_4^+ \) consumption and \( \text{NO}_x^- \) production were 1.10 and 1.27 mg N/g dry wt/h, respectively which were up to 4 times greater than those obtained during the period of high carbon loading but were comparable to the values obtained during the original conditions.

Culture-independent analysis of AOB communities in the DNB

Samples were removed from the aerobic reactor while the DNB was operating with a C/N ratio of 2, following the change in C/N ratio to 5 and when the C/N ratio had been restored to a value of 2. These were analysed qualitatively using DGGE and quantitatively by FISH.

Assessing the validity of the AOB primer sets

The identities of individual bands in the DGGE profiles could not be reliably inferred through comparisons of sequence mobility to reference cloned sequences previously recovered from this reactor (Ballinger et al., 1998). Therefore, bands were excised from the gel, re-amplified using PCR and sequenced (Figure 1 and 2.). Comparative phylogenetic analysis of these sequences showed that all the ammonia oxidiser-selective primers sets used during this study also amplified other non-ammonia oxidiser sequences even at stringent annealing temperatures indicating that the primers were poorly selective in this environment. Only sequences S13, S21, S24, S25 and S27 (Figure 1 and 2) were related to AOB (Figure 3). In addition, DNA fragments with very different sequences (< 90% identity) co-migrated. Considerable caution must, therefore, be exercised in the analysis of specific bacterial groups using DGGE alone, even with primers that on the basis of database searches appear to be quite specific (Purkhold et al., 2000). Nevertheless, comparison of DGGE profiles does provide a valid means to assess broad-scale changes in microbial communities with the following caveats. It is not accepted that bands that co-migrate are identical and no assumptions are made regarding the identity of the taxa represented by bands in the DGGE gel.

Qualitative and quantitative changes in AOB community with C:N

The DGGE profiles obtained with primers Nso190 and Nso1225 (data not shown) that target all known \( \beta \) proteobacterial AOB were relatively simple containing only a few bands. The DGGE patterns remained stable over time indicating that the bacterial populations represented by the bands, were not affected by the changes in influent C/N ratio. The predominant bands within the profile, co-migrated with a \textit{Rhodocyclus}-like and a \textit{Nitrosopiria}-like sequence previously recovered from the DNB in a 16S rRNA gene clone library (Ballinger et al., 1998). Sequencing of the DGGE bands confirmed that they
represented a *Rhodocyclus*-like bacterium. No bands corresponding to the *Nitrosomonas*-like sequences obtained from the clone library were identified in these DGGE profiles.

Nevertheless DGGE analysis using *Nitrosomonas*-selective primers (Nsm156 and Nso1225) for each influent C/N ratio indicated that the populations of bacteria detected with these primers changed significantly following the changes in influent C/N ratio (Figure 2). Furthermore, different *Nitrosomonas*-like sequences were identified before (band S21), during (band S24 and S25) and after (band S27) the period of high carbon loading (Figure 1). The mobilities of these sequences were similar, but not identical to each other or to the *Nitrosomonas*-like clone sequences previously recovered from the reactor (Figure 1). This was reflected in their phylogeny that indicated the bacteria detected were related to the *Nitrosomonas*-like sequences obtained from the clone library (Figure 3; Ballinger et al., 1998).

DGGE profiles were also obtained for the *Nitrosospira* populations in the reactor using the *Nitrosospira*-selective primers, Nsv443 and Nso1225 (Figure 2). The predominant bands in these DGGE profiles co-migrated with the *Rhodocyclus*-like clone sequences previously recovered from the reactor (Figure 3; Ballinger et al., 1998) and sequence analysis confirmed that they were closely related to these sequences. Only one *Nitrosospira*-like sequence (S13) was identified which was present during the original conditions (C/N = 2).

Although the mobility of this sequence differed from reference cloned *Nitrosospira*-like sequences it was closely related to them (Figure 3).

The disappearance of the *Nitrosospira* band from the profiles following the change in influent C/N ratio to five suggests that this group of ammonia-oxidisers was probably washed out of the system. This was supported by data from FISH analysis, which indicated that members of the genus *Nitrosospira* were only present in appreciable numbers in the first phase of the experiment. The remaining sequences amplified with this primer set were all identified as non-*Nitrosospira* sequences however, all were members of the β-Proteobacteria.

DGGE analysis indicated that *Nitrosomonas* sp. were present and presumably growing even at unfavourably high C/N values. The DGGE data contrast with the results of FISH analysis with a suite of AOB selective probes which showed that AOB-like cells were only...
detected in appreciable numbers \((1.29 \times 10^8 \text{ cells/ml})\) in the first phase of the experiment when the C/N ratio was maintained at 2. When the C/N ratio was raised to 5, AOB cells could not be detected in the reactor, even though DGGE analysis showed them to be present. However, FISH is much less sensitive than PCR and it is likely that AOB persisted throughout the experiment but were not present in sufficient numbers to be detected by FISH. This would also account for the reduction, but not total loss of nitrifying activity observed following the change of influent C/N ratio to 5. When the C/N ratio was restored to 2, the rate of nitrification returned to the pre-perturbation level. However, different AOB were detected by DGGE, and FISH analysis using a suite of AOB-selective probes did not detect any AOB suggesting that the organism(s) responsible for ammonia oxidation in the reactor were either, not \(\beta\)-proteobacterial AOB or \(\beta\)-proteobacterial AOB that did not contain the target sequence for probe Nso190.

**Conclusions**

In summary, it is clear that the nitrifying community present in the DNB responded to changes in the C/N ratio of the influent feed. The initial community was eliminated when the C/N ratio was increased to 5 and was replaced by a new predominant *Nitrosomonas*-like organism when the C/N ratio was restored to 2. However, the qualitative information provided by DGGE obscured important features of the DNB reactor. Substantial amounts of nitrate were observed in the final phase of the experiment when the C/N ratio was restored to 2. This clearly indicated that nitrification was occurring. Nevertheless, despite detecting *Nitrosomonas*-like bacteria by PCR and DGGE, it proved impossible to detect \(\beta\)-proteobacterial AOB using FISH. This implies that the reactor had been colonised by novel ammonia-oxidizing organisms that could not be detected with the oligonucleotide probes.
targeting the β-proteobacterial AOB. We conclude that important and undiscovered ammonia-oxidizing organisms, which may not be β-proteobacterial AOB must have been responsible for nitrification in this phase of the experiment. Moreover, the restoration of the C/N to 2 did not result in the restoration of the original AOB community, suggesting that a single set of reactor operating conditions does not necessarily select for a unique AOB community.

Acknowledgements
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References


