Chapter 4

Development of a simultaneous partial nitrification, anaerobic ammonia oxidation and denitrification (SNAD) bench scale process for removal of ammonia from effluent of a fertilizer industry
Development of a simultaneous partial nitrification, anaerobic ammonia oxidation and denitrification (SNAD) bench scale process for removal of ammonia from effluent of a fertilizer industry

ABSTRACT

A simultaneous partial nitrification, anaerobic ammonia oxidation (anammox) and denitrification (SNAD) process was developed for the treatment of ammonia laden effluent of a fertilizer industry. Autotrophic anaerobic ammonia oxidizing (anammox) biomass was enriched which removed ammonia and nitrite in the ratio similar to the expected stoichiometry of anammox reaction carried out under anaerobic conditions. Anoxic removal of ammonia by the enriched anammox biomass using nitrite produced by autotrophic Ammonia Oxidizing Bacteria (AOB) was confirmed with synthetic effluent system. A seed consortium was developed by mixing two of the enriched anammox biomass and AOB biomass (as per chapter 2) having maximum activity in 1:1 proportion. This was applied in the treatment of ammonia laden effluent of a fertilizer industry in an oxygen limited bench scale SNAD type reactor (1 L) run at ambient temperature (~30°C). Around 98.9% ammonia removal was achieved with ammonia loading rate of 0.35 kgNH₄⁺-N/m³day in the presence of 46.6 mg/L COD with 2.31 days hydraulic retention time. Qualitative and quantitative analysis of the biomass from upper (oxic) and lower (anoxic) zone of the reactor revealed presence of AOB, Planctomycetes and denitrifiers as the dominant bacteria carrying out anoxic oxidation of ammonia in the reactor. Physiological and molecular studies strongly indicated presence of anammox bacteria in the anoxic zone of the SNAD reactor.
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4.1 Introduction

Ammonia released in the effluent of agriculture based industries has become a prime concern with the increasing awareness of pollution in water bodies leading to eutrophication and acidification of the aquatic systems. Conventional ammonia removal from wastewater accomplished through the combination of nitrification (aerobic) and denitrification (anaerobic) is expensive, energy and space requiring process that generates high amount of sludge (Bagchi et al., 2012). Attention therefore has been focused on the development of novel cost-effective processes for the treatment of high ammonia containing wastewater with no/low level of organic carbon.

New technologies developed over the time for efficient removal of ammonia from wastewaters towards meeting the rising demand of environment protection include, SHARON (single reactor system for high activity ammonia removal over nitrite), SND (simultaneous nitrification denitrification), SNAP (single stage nitrogen removal using anammox and partial nitritation), CANON (complete autotrophic nitrogen removal over nitrite), OLAND (oxygen limited autotrophic nitrification and denitrification), DEMON (DEamMONification), and BABE (Bio-Augmentation Batch Enhanced), each having its own advantages and disadvantages (Bagchi et al., 2012). These processes involve partial nitrification (oxidation of ammonia to nitrite) followed by anoxic oxidation of the remaining ammonia by the anammox bacteria in presence of nitrite as electron acceptor. These processes are operated in a single reactor unit with reduced aeration (1 kWh/kgN) and without external organic load requirements; saving 90% of the operation costs (Wang et al., 2010). Although significant nitrification is not expected at DO below 0.3 mg/L, treatment processes that promote simultaneous nitrification-denitrification can reach up to 80% of the total nitrification under anoxic conditions (Stenstrom and Poduska, 1980). In agreement with this, nitrifiers, denitrifiers and anammox bacteria (having optimum activities under completely different conditions) are reported to coexist in the same environment (Xiao et al., 2008).
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Of the methods mentioned for ammonia removal, OLAND and CANON are more suitable for the treatment of wastewaters with high ammonia concentration and without organic carbon, but have limited application for wastewaters containing organic carbon (Lan et al., 2011). Simultaneous partial Nitrification, Anammox and Denitrification (SNAD) described by Chen et al., (2009) was developed to solve the problem of organic carbon in CANON process. This recently developed process has been used efficiently at the full scale land fill leachate treatment plant for nitrogen removal (Wang et al., 2010).

Present study reports application of SNAD for the treatment of high ammonia containing effluent of a fertilizer industry with low levels of COD (46.6 mg/L). The process involved partial nitrification of ammonia by aerobic autotrophic Ammonia Oxidizing Bacteria (AOB) that convert ammonia to nitrite, the remaining ammonia and nitrite so formed are converted to molecular nitrogen by anammox bacteria which in turn release low levels of nitrate in the process. The nitrates so formed could be reduced to N\textsubscript{2} by denitrifiers at the expense of organic carbon in the effluents. Levels of COD and nitrate in the system would tend to limit the growth of denitrifiers such that, less sludge is developed during the process. Activity and growth of one kind of microorganism seems to provide substrate for the next establishing a succession of bacteria that maintain harmony and cooperation in the reactor to effectively remove ammonia. The kinetics of biological ammonia removal from the ammonia laden wastewater is discussed together with the characterization and quantification (by RT-PCR) of AOB, anammox and denitrifiers developed in the reactor during the process.

4.2 Materials and Methods

4.2.1 Enrichment of anaerobic ammonia oxidizers (anammox) biomass

Several soil and activated sludge samples from municipal wastewater treatment plant and fertilizer industries were used for the enrichment of anammox biomass (Table 4.1). Enrichment for anammox biomass was carried out with samples (5%) inoculated in inorganic medium (100 ml)
containing ammonium sulphate (3 mM) as the nitrogen source, sodium nitrite (6 mM) as the electron acceptor, potassium bicarbonate (25 mM) as the carbon source and other trace metals as described by Egli et al., (2001). Anaerobic conditions were created by flushing helium (He) gas (99.99% purity) through the medium. Anammox activity of the enriched biomass was measured by monitoring the amount of ammonia and nitrite removed. Gas produced during the process was confirmed to be nitrogen through gas chromatography (GC) analysis (Model: Varian 3600) using He as the carrier gas. Molecular sieve column made of stainless steel with 5 Å diameter and 3 m length was used. Gas tight syringe (SGE, Australia) was used to inject sample. To avoid air contamination the tubes were kept in a closed beaker in which He atmosphere was created. Two of the enriched biomass so developed showing higher anammox activity were used in the further study and were designated as PF-anammox and N4-anammox. Effect of hydrazine was checked on its anammox activity by adding of 0.25 mg/ml hydrazine. Amplification of Planctomycetes specific and anammox specific regions in the 16S rRNA gene was carried out as per following: Primers - Pla46F (forward primer) 5'GACTTGCATGCCTAATCC 3' and 1392R (reverse primer) 5'GACGGGCGGTGTG ACAA 3' were used for Planctomycetes specific amplification and Pla46F (forward primer) and Amx820 (reverse primer) 5'CCTTTCCGGGCATTGCGAA3' for anammox specific amplification according to Tal et al., (2006).

4.2.2 Maintenance of the enriched anammox biomass (PF-anammox and N4-anammox)

The enriched biomass was inoculated into small rubber tube with 3 mm diameter and 4 ft length containing inorganic medium (composition as described in 4.2.1) for growth and maintenance. Both ends of the tube were sealed and kept at 37°C for one month. Care was taken to exclude air intrusion by removing all air bubbles from the rubber tube. The rubber tubes being transparent facilitated monitoring of biomass development and generation of gas.
4.2.3 Development of seed consortium for SNAD bioreactor

Closed systems (500 ml) having synthetic effluent were used in this study to check the ability of PF-AOB and N4-AOB (developed as per chapter 2a) to survive and oxidize ammonia under anoxic conditions and to check the ability of PF-anammox and N4-anammox to utilize nitrite produced by AOB. The composition of synthetic effluent was same as that of the inorganic medium used for the enrichment of AOB and anammox biomass respectively without and with nitrite as mentioned below. The different reactor conditions and controls, used were as follows: i) AOB control- this constituted uninoculated synthetic effluent containing ammonium sulphate as the nitrogen source without sodium nitrite ii) Anammox control – this constituted uninoculated synthetic effluent containing ammonium sulphate and sodium nitrite and was made anaerobic by flushing He gas. iii) PF-AOB and N4-AOB (experimental sets) constituted of synthetic effluent containing only ammonium sulphate under anoxic condition inoculated with enriched PF-AOB and N4-AOB biomass (5% inoculum size) respectively. iv) PF-anammox and N4-anammox (experimental sets) constituted of synthetic effluent containing both ammonium sulphate and sodium nitrite under anaerobic conditions created by flushing He gas and inoculated with enriched PF-anammox and N4-anammox biomass (5% inoculum size) V) PF-AOB+PF-anammox and N4-AOB+N4-anammox (experimental sets) contained synthetic effluent with only ammonium sulphate under anoxic condition inoculated with mixture of PF-AOB and PF-anammox (ratio 1:5) and N4-AOB and N4-anammox (ratio 1:5). The reactors in which anoxic condition was to be created were not flushed with He, but were tightly closed such that external air could not enter. All the reactors were incubated at 30°C for three months. Concentration of ammonia, nitrite and nitrogen were measured after 3 months of incubation.

Final seed culture for the 1 L SNAD type reactor comprised of N4-AOB + N4-anammox and PF-AOB + PF-anammox mixed in 1:1 ratio.
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4.2.4 Operating conditions for removal of ammonia from effluent of a fertilizer industry in a 1 L SNAD type reactor

A cylindrical reactor with 1 L effective volume was designed with 45 cm height, internal diameter of 7 cm and outlet at 35 cm height. Schematic diagram of the reactor is shown in Fig 4.1. The reactor was operated in the up-flow mode as the influent was introduced from the bottom. Operating temperature was 30°C and was run in the batch mode for initial 15 days. Inlet and outlet ammonia, nitrite and nitrate were measured continuously every two days for 125 days while pH and dissolved oxygen was measured (YSI200 portable DO meter, USA) in the upper and lower zone of the reactor every 15 days. Total solids (TS), total dissolved solids (TDS), total suspended solids (TSS), mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS) and sludge volume index (SVI) were estimated at the commencement and termination of the reactor. Flow rate of the reactor was maintained at 0.3 ml/min and hydraulic retention time (HRT) was 2.31 days.
4.2.5 Ammonia conversion efficiency

In the SNAD process, AOB would partly convert ammonia to nitrite. The remaining ammonia would be anaerobically oxidized with the help of nitrite to form molecular nitrogen along with nitrate. This conversion of ammonia was calculated throughout the run according to Davery et al., (2012) by the equation

$$\frac{Y_{(NO_2^-+NO_3^-)/NH_4^+}}{\text{Eff.} \frac{\{(NO_2^- - N) + (NO_3^- - N)\}}{\text{Inf.} (NH_4^+ - N) - \text{Eff.} (NH_4^+ - N) x 100\%}}$$

Theoretically, 100% ammonia has been reported to produce 88% nitrogen and 11% nitrate (Davery et al., 2012). However, presence of heterotrophic denitrifiers have been reported to reduce ammonia conversion efficiency from its theoretical value (11%) because of the reduction of nitrate to N₂ by the heterotrophic denitrifiers. Presence of nitrite oxidizing bacteria would
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increase it due to oxidation of nitrite to nitrate, inhibiting anammox activity in the presence of high DO concentration (Davery et al., 2012).

4.2.6 Analytical methods

Ammonia consumed and nitrite released was measured according to Scheiner (1976) and Griess-Romijn (1996) respectively. Brucine sulfate method was used to determine nitrate according to Jenkins and Medsker (1964). Hydrazine and hydroxylamine were estimated from the seed cultures according to Watt (1952) and Frear and Burrel (1955) respectively. TS, TDS, TSS, MLSS, MLVSS and SVI were measured according to standard protocols (APHA, 1995).

Calculation for $N_2$% formed by the enriched anammox biomass (carried out in triplicate): For calculating $N_2$ produced in the sample, contaminating $N_2$ from air was subtracted from the sample $N_2$. For this area of $N_2$ and $O_2$ in air calculated from the GC chromatogram were 2297852 and 569121 respectively for two. Ratio of $O_2$ to $N_2$ in air is 1:3.7. Percentage of $O_2$ in the sample =

\[ \text{[area of sample } O_2 \times 21]/569121. \]

Percentage of contaminating $N_2$ in the sample =

\[ \text{[Percentage of sample } O_2\times 3.7]. \]

Therefore, percentage of $N_2$ produced in the sample = \[
\text{[(area of sample } N_2 \times 78.08) / 2297852 (area of } N_2 \text{ in air]} - \text{percentage of contaminating } N_2 \text{ in the sample.}
\]

4.2.7 Scanning Electron Microscopy (SEM) from the reactor

Morphology of the bacteria present in the lower anoxic zone of the reactor was studied using Joel Scanning Electron Microscope with Oxford EDS system model No. JSM-5610LV. Biomass from the lower zone was taken and fixed on a grease free glass slide using gluteraldehyde and dehydrated in a...
series of increasing acetone concentration as mentioned in Chapter 3. Preparations were dried sputter-coated with silver and examined in SEM.

4.2.8 Qualitative analysis of the biomass generated in reactor

Effluent and sludge (1 ml) from the upper oxic zone and lower anoxic zone of the reactor respectively were used to extract genomic DNA. The effluent and sludge were centrifuged and washed with N-saline. These were mixed with 450 μl of extraction buffer (100 mM Tris-HCl pH 8.0, 100 mM sodium EDTA pH 8.0, 100 mM sodium phosphate pH 8.0, 1.5 M NaCl, 1% CTAB) for half an hour at 37°C and were passed through French press at 1000 psi pressure. Extraction of genomic DNA was carried out according to Schmidt et al. 1991. Amplification of amoA (Rotthauwe et al., 1997), nirS (Throback et al., 2004), nosZ (Henry et al., 2006), genes and planctomycetes and anammox specific 16S rRNA (Tal et al., 2006) gene regions were carried out using respective primers to demonstrate the presence of AOB, denitrifiers and anammox bacteria. 16S rRNA gene was amplified using 27F and 1541R (universal primers) with following PCR program: initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation 95°C for 30 s, annealing 58°C for 45 s and elongation at 72°C for 1.5 min and a final elongation at 72°C for 10 min. The amplified fragment was cloned in pTZ57R/T vector using INSTA cloning kit (Fermentas, Inc.). Amplified ribosomal DNA restriction analysis (ARDRA) was carried out using Ahal restriction enzyme and the distinct patterns obtained were sent for commercial sequencing through single pass analysis from Xcelris Labs (Ahmedabad, India). The sequences determined in the study submitted in GenBank under accession numbers JX143764 – JX143801.

4.2.9 Quantitation of biomass generated in the reactor by Real-time PCR analysis

Absolute quantification of the genes was carried out by Real-time PCR analysis in Step One Real-Time PCR system (Applied Biosystems, USA) by the standard curve method. Bacterial 16S rRNA [amplified according to Quan et al. (2008)], ammonia monooxygenase (amoA), nitrite reductase
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(nirS) and nitrous oxide reductase (nosZ) genes were quantified using SYBR Green master mix (Applied Biosystems, USA) in 10 µl reaction system. Standard curves for all the genes were constructed with plasmids containing individual genes (16S rRNA, amoA, nirS and nosZ) prepared by cloning the genes in pTZ57R/T vector using INSTA cloning kit (Fermentas Inc.). The specificity of the PCR amplification was determined by the melt curve analysis and R² values obtained were greater than 0.98 for all the curves. PCR protocol used for 16S rRNA gene was: an initial denaturation temperature of 95°C for 10 min, 40 cycles of PCR with denaturation at 95°C for 15 s, annealing at 43°C for 45 s, and extension at 72°C for 30 s. Same protocol was followed for amoA and nirS genes, with annealing temperature 54°C and extension time 1 min for amoA and 45 s for nirS whereas for nosZ touchdown PCR protocol used was according to Henry et al. (2006).

4.2.10 Phylogenetic analysis

Phylogenetic analysis of the microorganisms present in the reactor was carried out using 16S rRNA gene sequences by MEGA version 4.0 software (Tamura et al., 2007). 16S rRNA gene sequence obtained by universal primers 27F and 1541R and anammox specific primes Pla46F and Amx820 were used to find the types of the microorganisms present in the reactor. Phylogenetic tree was constructed using the Neighbor-Joining method. All positions containing gaps and missing data were eliminated from the data set by complete deletion option. There were a total of 520 positions in the final data set.

4.3 Results and Discussion

4.3.1 Biomass development of anammox bacteria

Amongst the samples enriched for anammox biomass (Table 4.1), PF-anammox and N4-anammox biomass showed higher anammox activity under anaerobic conditions.

Ratio of ammonia:nitrite removed was 1:1.89 for PF-anammox and 1:1.08 for N4-anammox, indicating that higher nitrite was utilized by the enriched
Chapter 4: Bench scale SNAD type reactor for the removal of ammonia biomass. The observation was as per the expectation as nitrite in anammox reaction was used not just as an electron acceptor for oxidizing ammonia but also was used as electron donor for biomass formation in CO₂ fixation as per that reported by Kuenen and Jetten (2001). Stoichiometry obtained in the present study was almost similar to the reported stoichiometry for anammox bacteria (1:1.33) by Strous et al., (1999). Gas formed in the process was identified to be nitrogen through gas chromatography (44% by PF-anammox and 39% by N4-anammox) (Fig 4.2A and B).

Table 4.1 Anammox activity by enriched samples under anaerobic conditions.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Nitrite removed (mM)</th>
<th>Ammonia removed (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1 (CEPT, Nandesari)</td>
<td>0.64</td>
<td>1.08</td>
</tr>
<tr>
<td>A1 (Alembic sludge)</td>
<td>0.50</td>
<td>1.2</td>
</tr>
<tr>
<td>N4 (Municipal waste water treatment plant, Nandesari)</td>
<td>1.18</td>
<td>1.09</td>
</tr>
<tr>
<td>D1 (effluent, DNR A reactor, GNFC, Bharuch)</td>
<td>0.8</td>
<td>2.85</td>
</tr>
<tr>
<td>D2 (effluent, DNR B reactor, GNFC, Bharuch)</td>
<td>0.10</td>
<td>2.64</td>
</tr>
<tr>
<td>DnrA (Solid sludge, DNR A reactor, GNFC, Bharuch)</td>
<td>0.46</td>
<td>0.26</td>
</tr>
<tr>
<td>GS (Garden soil, Vadodara)</td>
<td>0.03</td>
<td>6.35</td>
</tr>
<tr>
<td>CP (Model farm soil, growing cereals and pulses in rotation, Vadodara)</td>
<td>0.31</td>
<td>6.38</td>
</tr>
<tr>
<td>PF (Paddy field soil, Mandya)</td>
<td>2.27</td>
<td>1.2</td>
</tr>
</tbody>
</table>
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Fig 4.2 Gas chromatography confirming production of $N_2$ by A) N4-anammox and B) PF-anammox biomass.

Hydrazine as an intermediate has been reported to strongly stimulate anammox activity (Strous et al., 1999). Presence of hydrazine and hydroxylamine were not detected during the enrichment process. Being intermediates in the anammox process, hydrazine and hydroxylamine would be used as substrates in the further reaction and hence they presumably do not accumulate to detectable levels. However, addition of hydrazine in the system led to 1.5 and 1.7 fold increase in ammonia removal, 1.09 and 1.23 fold increase in nitrite removal and 0.98 and 1.1 fold increase in nitrogen formation for N4-anammox and PF-anammox respectively. The enriched biomass also showed remarkable increase in red biomass in the presence of hydrazine in case of PF-anammox (Fig 4.3).

Fig 4.3 Effect of hydrazine on the growth of enriched anammox biomass. A) with hydrazine and B) without hydrazine
Amplification of planctomycetes specific and anammox specific regions of the 16S rRNA gene confirmed presence of anammox bacteria in the enriched biomass (Fig 4.4).

Fig 4.4 Confirmation of anammox bacteria in the enriched biomass A) Planctomycetes specific amplification (1350bp); Lane 1. 500bp ladder, Lane 2. PF-anammox, Lane 3. N4-anammox B) Anammox specific amplification (750bp) Lane 1. 100bp ladder, Lane 2. PF-anammox, Lane 3. N4-anammox.

4.3.2 Growth and maintenance of anammox biomass in rubber tubes

Both the enriched anammox biomass (PF-anammox and N4-anammox) were maintained in rubber tubes with very small diameter (3 mm) but 4 ft in length where in increase in growth and gas formed in the process could be visually monitored. Within 14 days of incubation in dark at 37°C temperature, bright red colored biomass developed (Fig 4.5C and D) indicating that optimum conditions were being provided for the growth of the anammox bacteria. Ammonia and nitrite removal by the enriched biomass together with generation of N\textsubscript{2} gas (Fig 4.5E) showed consistency in anammox activity by the enriched biomass. Gas produced could be visually observed as bubble formed in the tube and was confirmed to be nitrogen through gas chromatography (Fig 4.5A and B). Consistency in the detection of anammox activity substantiated earlier observation of Planctomycetes/anammox specific amplification and validated presence of anammox group of bacteria in the enriched anammox biomass.
Fig 4.5 Growth and maintenance of anammox bacteria in rubber tubes A) Nitrogen formed by N4-anammox B) Nitrogen formed by PF-anammox C) growth observed in N4-anammox D) growth observed in PF-anammox E) anammox activity observed in PF and N4-anammox enriched biomass.
4.3.3 Development of AOB-Anammox seed consortium for SNAD type bench scale (1 L) laboratory bioreactor

Anoxic removal of ammonia requires mainly two groups of the organisms, AOB, carrying out partial nitrification reaction and anammox bacteria carrying out anammox reaction leading ultimately to the removal of ammonia in the form of molecular nitrogen. Both groups of organisms are slow growers, therefore, for effective anoxic ammonia removal from ammonia laden effluent at a bench scale reactor would require sufficient biomass to be generated of both groups of the organisms to be used as seed in the reactor. Small reactors (500ml) were designed so as to make it run under both anoxic and anaerobic conditions for optimizing growth and nitrifying activity of AOB and growth and anammox activity of anammox bacteria. PF-AOB + PF-anammox (1:5) and N4-AOB + N4-anammox (1:5) were seeded in these reactors containing synthetic effluent. Red colored growth appeared in the reactors after 90 days of incubation as shown in Fig 4.6.

Fig 4.6 Bioreactor design for monitoring anammox activity of enriched biomass using synthetic effluent A) AOB control B) Anammox control C) PF-anammox D) N4-anammox E) PF-AOB F) N4-AOB G) PF-AOB + PF-anammox H) N4-AOB + N4-anammox.

Ammonia removing ability of the consortium was checked under anoxic conditions by keeping appropriate control as described in methods sec
4.2.2. As expected, decrease in ammonia and nitrite concentration was not observed in AOB and anammox control reactors (Table 4.2). PF-AOB and N4-AOB showed 79.8 (60.7%) and 81.8 (66.7%) mg/L utilization of ammonia with marginal increase in nitrite concentration, without accumulation of nitrate, with 69.78% and 66.79% nitrogen gas formation implying that simultaneous nitrification and denitrification activity was being carried out by the enriched AOB biomass. AOB belonging to *Nitrosomonas* genus has been reported to show this activity under anoxic conditions (Bock et al., 1995; Schmidt and Bock, 1997). PF-anammox and N4-anammox biomass showed decrease in both ammonia and nitrite under anaerobic conditions, with increased nitrogen gas formation (1.06 to 1.1 times) compared to PF-AOB and N4-AOB biomass (Table 4.2). The PF-AOB + PF-anammox and N4-AOB + N4-anammox system showed 97.6 (88.73%) and 108.4 (92.5%) decrease in ammonia without accumulating nitrite and nitrate (Table 4.2). Removal of ammonia by the combined AOB-anammox biomass was marginally less than anammox bacteria (Table 4.2). Nitrite removed by the system cannot be measured as the nitrite produced by the AOB would be simultaneously utilized by anammox bacteria present in the system (Table 4.2). Gas produced in all the system was confirmed to nitrogen through gas chromatography (Fig 4.7).

Gas formed by PF-AOB + PF-anammox consortium was 1.09 times higher than only PF-AOB but 0.98 times less than only PF-anammox (Table 4.2). Similar results were also obtained for N4-AOB + N4-anammox consortium (Table 4.2). Results emphatically confirmed ammonia removing ability of PF-AOB + PF-anammox and also N4-AOB + N4-anammox consortia under anoxic conditions. N4-AOB + N4-anammox biomass was mixed in 1:1 proportion with PF-AOB + PF-anammox and this PF-N4 AOB-anammox bacterial biomass was used as a seed consortium for the 1 L SNAD type reactor study.
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Fig 4.7 Gas chromatography showing $N_2$ in all the system A) AOB control B) N4-anammox C) PF-anammox D) N4-AOB E) PF-AOB F) N4-AOB+N4-anammox G) PF-AOB+PF-anammox
Table 4.2 Removal of ammonia from synthetic effluent under anoxic conditions.

<table>
<thead>
<tr>
<th>Ammonia Removed (mg/L)</th>
<th>Nitrite Removed (mg/L)</th>
<th>Gas formed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOB control</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Anammox control</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PF AOB</td>
<td>79.8±8.4</td>
<td>-1.55±1.39</td>
</tr>
<tr>
<td>N4 AOB</td>
<td>81.8±5.7</td>
<td>-1.08±2.7</td>
</tr>
<tr>
<td>PF anammox</td>
<td>107.8±7.7</td>
<td>133.5±2.67</td>
</tr>
<tr>
<td>N4 anammox</td>
<td>109.5±4.5</td>
<td>130.1±1.8</td>
</tr>
<tr>
<td>PF AOB + anammox</td>
<td>97.6±4.4</td>
<td>*</td>
</tr>
<tr>
<td>N4 AOB + anammox</td>
<td>108.4±3.4</td>
<td>*</td>
</tr>
</tbody>
</table>

*cannot be measured.

4.3.4 Ammonia removal performance of the SNAD type bioreactor from the effluent of a fertilizer company

An up flow, cylindrical SNAD type reactor with a working volume of 1 L was used for the removal of ammonia from the effluent of a fertilizer industry, seeded with 100 ml PF-N4 AOB-anammox consortium (1.0 g/L VSS) developed as described above. Proximate composition of the effluent to be treated is depicted in Table 4.3 for the 0th day.

It had high NH$_4^+$-N concentration with COD / NH$_4^+$-N ratio 0.066 whereas the same in the SNAD processes reported earlier ranged from 0.2 to 0.87 (Chen et al., 2009; Wang et al., 2010; Lan et al., 2011; Davery et al., 2012). The reactor was run in the batch mode for the first 15 days during which DO in the lower zone of the reactor reduced to 0.37 ppm and thereafter fluctuated between 0.1 to 0.4 ppm (conditions favorable for the growth of anammox bacteria) whereas in the upper region of the reactor, DO fluctuated between 2.9 to 3.9 ppm (Fig 4.8C). Nitrite present in the reactor, at any time was < 100 mg/L, in the range that does not inhibit the anammox activity according to Strous et al., (1999). During the batch mode, VSS reached to 41 mg/L with 52.84% removal in the ammonia concentration.
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(Fig. 4.8A and D). A significant reduction in the nitrite concentration was also observed during the batch mode (Fig 4.8B). pH of the reactor reduced from 9.2 to 7.5 and thereafter fluctuated between pH 7.5 to 8.1 (Fig 4.8D). The reactor was made continuous after 15 days with flow rate of 0.3 ml/min and HRT of 2.31 days. Inlet ammonia concentration varied from 700 to 800 ppm (Fig 4.8A). The concentration of ammonia in the wastewater reduced to permissible limits (32.38 ppm) within 64 days and was stably maintained at that level for the next 60 days with a development of 11.5 g/L VSS. The concentration of inlet ammonia (725-760 ppm) and ammonia removed (700-750 ppm) became identical (Fig 4.8A), leading to development of steady state in the reactor. COD concentrations in the reactor reduced to 24 mg/L at the end of the run. Ammonia conversion efficiency at the start of the reactor was higher than the theoretical value (Fig 4.8E). On the 14th day it reached 11.8%, indicating optimum anammox activity in the reactor (Fig 4.8E). The ammonia conversion efficiency fluctuated between 0.4 to 9.0% during the continuous mode (Fig 4.8E). Between 52 - 74th day, the process was run with ammonia conversion efficiency reaching near 0.4% to 0.02% suggestive of higher denitrifying activity than anammox activity (Fig 4.8E).
Table 4.3 Measurement of the effluent parameters at the start and end of process.

<table>
<thead>
<tr>
<th>Days</th>
<th>NH₄⁺-N (ppm)</th>
<th>NO₂⁻-N (ppm)</th>
<th>NO₃⁻-N (ppm)</th>
<th>DO (ppm)</th>
<th>COD</th>
<th>Total Solid (mg/L)</th>
<th>Suspended Solids (mg/L)</th>
<th>MLSS (mg/L)</th>
<th>MLVSS (mg/L)</th>
<th>MLVSS/MLSS</th>
<th>pH</th>
<th>SVI</th>
<th>MLSS (mg/L)</th>
<th>Dissolved Solids (mg/L)</th>
<th>Total</th>
<th>Total</th>
<th>NH₄⁺-N</th>
<th>NO₂⁻-N</th>
<th>NO₃⁻-N</th>
<th>DO</th>
<th>COD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>46.66</td>
<td>700-800</td>
<td>detectable</td>
<td>4300</td>
<td>40</td>
<td>1.65</td>
<td>1.46</td>
<td>0</td>
<td>9.2</td>
<td>0.88</td>
<td>14</td>
<td>114</td>
<td>7.5</td>
<td>5.8</td>
<td>700</td>
<td>700</td>
<td>5.8</td>
<td>0.39</td>
<td>detectable</td>
<td>4750</td>
<td>0.39</td>
</tr>
<tr>
<td>125</td>
<td>24</td>
<td>26</td>
<td>detectable</td>
<td>4030</td>
<td>40</td>
<td>1.65</td>
<td>1.46</td>
<td>0</td>
<td>9.2</td>
<td>0.88</td>
<td>14</td>
<td>114</td>
<td>7.5</td>
<td>5.8</td>
<td>700</td>
<td>700</td>
<td>5.8</td>
<td>0.39</td>
<td>detectable</td>
<td>4750</td>
<td>0.39</td>
</tr>
</tbody>
</table>
Beyond this period, the efficiency steadily increased reaching 4.9% by the end of the reactor, indicating anammox activity along with denitrifying activity in the reactor (Fig 4.8E). The overall efficiency of the process was similar to reactors designed earlier for the removal of ammonia using the SNAD process (Chen et al., 2009; Lan et al., 2011; Daverey et al., 2012). A highly efficient and stable system was thus developed for the treatment of ammonia laden effluent from a fertilizer industry without addition of external carbon or nitrite source and was operated at ambient temperature (30°C).

**Fig 4.8** Nitrogen removal performance and other parameters in the reactor during the process A) Ammonia B) Nitrite C) Dissolved oxygen D) pH and MLVSS E) Ammonia conversion efficiency.
4.3.5 Molecular analysis of the biomass developed in the reactor

Visually red colored biomass developed at the base of the reactor and red colored film developed on the upper wall of the reactor by the end of the run. SEM of the biomass from the anoxic zone showed dominance of coccoidal shaped cells forming aggregates with pear shaped cells and flagellated microorganisms (Fig 4.9A, B and C). Short rods were also observed through SEM. Budding pear and coccoidal cells in the anoxic zone of the reactor could be Planctomycetes (Fig 4.9B, C, D marked by arrow).

**Fig 4.9** SEM images of biomass taken from lower anoxic zone of reactor showing presence of A) cell aggregates formed, B, C and D are diluted biomass. Budding cells are marked by arrow in B, C and D. D) different shaped cells: tear shaped cells, flagellated cells, coccoidal cells, short rods.

The observation was supported by the amplification of Planctomycetes specific (1350bp) and anammox specific (750bp) regions of 16S rRNA gene from the biomass obtained from lower anoxic zone of the reactor (Fig 4.10 A and B). Cloning and sequencing of the anammox specific gene revealed clones having similarity with Planctomycetes (uncultured-93% similarity).
Chapter 4: Bench scale SNAD type reactor for the removal of ammonia

Fig 4.10 Amplification of anammox, nitrifiers and denitrifiers specific gene fragment from upper and lower zone of the reactor. A) Planctomycetes specific 16S rRNA gene (1350bp) B) anammox specific 16S rRNA gene (750bp) C) 16S rRNA gene (102bp) for quantification of total bacteria D) nirS gene (425 bp) E) amoA gene (491bp) F) nosZ gene (267bp) for quantification of respective group of organisms. Lane 1 A 500bp ladder, B-F 100bp ladder. Lane 2 A-F amplification of the respective genes from the upper zone of the reactor. Lane 3 A-F amplification of the respective gene from lower zone of the reactor.

Twenty eight distinct sequences were obtained by cloning 16S rRNA gene from both oxic and anoxic zones of the reactor (Fig 4.11). Phylogenetic tree was constructed by Neighbor-Joining method using MEGA version 4.0 software (Fig 4.12) representing relationship between the nitrifiers, denitrifiers and Planctomycetes present in the reactor. Mainly these could be grouped as aerobic and anaerobic ammonia oxidizers, aerobic and anaerobic denitrifiers and bacteria capable of simultaneous nitrification and denitrification (Fig 4.12). Specific enrichment for denitrifiers was not addressed in the study however; they must have got enriched due to anoxic conditions and presence of SMP in the reactor. Coexistence of denitrifiers along with anammox and AOB sharing nutrient metabolites is well documented (Kindaichi et al., 2004; Xiao et al., 2008), hence detection of denitrifiers with AOB and anammox bacteria was not unusual.
Fig 4.11 Representative ARDRA gel showing different patterns of 16S rRNA gene differentiating the different clones. The different patterns are marked by arrow.
Eight distinct species of AOB showing similarity to *Nitrosomonas* genus were identified. Amongst the microorganisms identified, AOB exhibited maximum diversity as they were the sole providers of nitrite to denitrifiers and anammox bacteria. This observation was in congruence with the earlier report by Xiao et al., (2008), who showed higher diversity of AOB amongst coexisting nitrosifiers, denitrifiers and anammox bacteria in sequencing batch biofilms reactor treating ammonia rich landfill leachate. Denitrifiers identified belonged to alpha (*Rhodopseudomonas* sp.), beta (*Thauera* sp., *Pusillimonas* sp., *Acidovorax* sp., *Comammonas* sp.) and gamma (*Thermomonas fusca, Xanthomonas* sp.), proteobacteria. Other heterotrophic key players possibly involved in the treatment process in the present study were *Rhodospesudomonas* sp., *Diaphorobacter* sp., *Acidovorax* sp. and *Comamonas* sp. reported to carry out simultaneous heterotrophic nitrification-denitrification (Satoh et al., 2006; Khardenavis et al., 2007; Heylen et al., 2008; Xiao et al., 2008). High temperature (30°C), high flow rate and low dissolved oxygen concentration prevalent in the reactor were not favorable for the growth of nitrite oxidizing bacteria (NOB) as reported by Jianlong and Ning (2004) and therefore they could have got washed out and hence not detected.
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Fig 4.12 Phylogenetic Neighbor-Joining tree showing relationship between the microorganisms present in the reactor based on the 16S rRNA gene
sequences obtained using universal primers and anammox specific primers. Bar indicates 2% sequence divergence. Values shown next to the branches indicate bootstrap values. Accession numbers of the sequences are given in parenthesis.

Quantification of biomass carried out from upper (oxic) and lower (anoxic) regions of the bioreactor using RT-PCR with group specific genes revealed predominance of AOB (carried out by \textit{amoA} gene amplification) (Fig 4.10E) in the upper oxic region (61.2%) whereas just 6% of the total population belonged to AOB in the lower anoxic region (Table 3). Denitrifiers (quantified using \textit{nirS} and \textit{nosZ} genes) constituted 10% of the total population in the upper oxic zone and 22% in the lower anoxic layer of the reactor (Table 3). The results suggested dominance of AOB in the upper region of the reactor whereas that in the lower zone of the reactor, showed dominance of neither AOB (6%) nor denitrifiers (22%). Anammox may constitute major population in this part of the reactor as evidenced by the detection of uncultured Planctomycetes (93% similarity) in the biomass amplified using Planctomycetes and anammox specific primers (Fig 4.10A and B). The bacteria identified in the system showed less similarity with the other reported members of this group and hence may be a novel organism belonging to Planctomycetes. However, this group of bacteria could not be quantified due to the presence of nonspecific amplicons obtained along with the required 750bp amplicon. Another proof for the presence of Planctomycetes in the lower zone of the reactor was given by SEM showing presence of budding coccoidal cells (Fig 4.9D), a characteristic for this group of organisms.

The bacterial community developed in the reactor could remove ammonia with high efficiency during the process. Oxidation of ammonia to nitrite in the upper oxic portion of the reactor could mainly be contributed by AOB followed by aerobic heterotrophic nitrifiers as the former being predominant population in this part of the reactor. Nitrite in the lower anoxic zone of the reactor would be utilized either by anaerobic denitrifiers or anammox bacteria. Competition for nitrite would always be there between the
anammox bacteria and denitrifiers. Owing to the low COD content in the reactor, denitrifiers would also need to depend on SMP released by AOB for fulfilling their organic carbon requirement. Dependence of denitrifiers on SMP released by AOB has been reported by Kindaichi et al., (2004). Conditions in the lower region of the reactor therefore favored growth of anammox bacteria capable of autotrophic growth as compared to denitrifiers. The observation justified Real-Time PCR results showing only 22% population in the lower layer constituted of denitrifiers (Table 4.4). However, lack of quantitation data for anammox bacteria failed to conclude this result. Bacteria capable of anammox activity in the reactor were mainly Planctomycetes sp. and Nitrosomonas sp. (Fig 4.12) as these organisms have been shown to exhibit anammox activity (Schmidt and Bock 1997; Strous et al., 1999). The molecular phylogenetic analysis of the biomass from the reactor revealed coexistence of AOB, heterotrophic nitrifiers, denitrifiers and anammox bacteria, also reported earlier by Xiao et al., (2008) and Kumar and Lin (2010).

Table 4.4 Quantification of the organisms present in the upper oxic and lower anoxic zones of the reactor by Real-Time PCR.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Size (bp)</th>
<th>Upper oxic zone of the SNAD Type Reactor</th>
<th>Lower anoxic zone of the SNAD Type Reactor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gene copy No./16S rRNA</td>
<td>Ratio of gene copy No./16S rRNA</td>
<td>Gene copy No./16S rRNA</td>
</tr>
<tr>
<td>16S</td>
<td>102</td>
<td>6.46 X 10^9 ± 1.0</td>
<td>1.31 X 10^11 ± 1.0</td>
</tr>
<tr>
<td>rRNA</td>
<td></td>
<td>6.15 X 10^8</td>
<td>2.5 X 10^9</td>
</tr>
<tr>
<td>amoA</td>
<td>491</td>
<td>3.96 X 10^9 ± 0.612</td>
<td>7.92 X 10^8 ± 0.006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.52 X 10^9</td>
<td>3.97 X 10^7</td>
</tr>
<tr>
<td>nirS</td>
<td>425</td>
<td>6.39 X 10^8 ± 0.099</td>
<td>3.19 X 10^10 ± 0.244</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.82 X 10^8</td>
<td>2.15 X 10^9</td>
</tr>
<tr>
<td>nosZ</td>
<td>267</td>
<td>3.35 X 10^8 ± 0.052</td>
<td>2.1 X 10^10 ± 0.161</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.39 X 10^7</td>
<td>6.65 X 10^9</td>
</tr>
</tbody>
</table>
An adequate balance between the different types of bacteria is required in activated sludge systems to have good settling ability of the sludge, such that low suspended solids level prevails in the effluent. Sludge volume index (SVI) is commonly used in wastewater treatment plants to analyze the separation of solids in the effluent and for characterizing the sludge settling ability. SVI provides just macroscopic evaluation of the activated sludge; therefore, microscopic characteristic of the sludge has been recently used where filamentous bacterial content in microbial aggregates is measured (Mesquita et al., 2009). Most of the denitrifiers detected in the present study (Fig. 4.9) are reported to be flagellated (Hougardy et al., 2000; Shen et al., 2001; Mergaert et al., 2003; Heylen et al., 2008). These flagellated bacteria assist in forming microbial aggregates (Sjoblad et al., 1985). In congruence with this, in the present study too, filamentous bacteria observed formed aggregates (Fig. 4.9A) which improved settling ability of the sludge in the reactor and prevented entangled cells from getting washed off from the reactor. Food to microbe ratio calculated was found to be 0.62 day⁻¹ indicating endogenous growth of microorganisms which was reported to have better settling ability and is more stable in nature (Rao and Datta, 1987).

To conclude, a SNAD type bioreactor was developed for efficient removal of ammonia from effluent of a fertilizer industry and was run continuously for 125 days wherein 98.9% ammonia removal was achieved. Coexistence of nitrosifiers, anammox bacteria and denitrifiers was confirmed in the reactor without supplementation of external organic carbon and without accumulation of nitrite or nitrate. Molecular phylogenetic analysis of the biomass generated revealed dominance of AOB in the upper oxic zone of the reactor while that of anammox followed by denitrifiers dominated in the lower anoxic zone of the reactor.