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ORIGINAL PAPER

Start-up and stabilization of an Anammox process from a non-acclimatized sludge in CSTR

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Abstract Development of an Anammox (anaerobic ammonium oxidation) process using non-acclimatized sludge requires a long start-up period owing to the very slow growth rate of Anammox bacteria. This article addresses the issue of achieving a shorter start-up period for Anammox activity in a well-mixed continuously stirred tank reactor (CSTR) using non-acclimatized anaerobic sludge. Proper selection of enrichment conditions and low stirring speed of 30 ± 5 rpm resulted in a shorter start-up period (82 days). Activity tests revealed the microbial community structure of Anammox micro-granules. Ammonia-oxidizing bacteria (AOB) were found on the surface and on the outer most layers of granules while nitrite-oxidizing bacteria (NOB) and Anammox bacteria were present inside. Fine-tuning of influent NO_2^-/NH_4^+ ratio allowed Anammox activity to be maintained when mixed microbial populations were present. The maximum nitrogen removal rate achieved in the system was 0.216 kg N/(m^3 day) with a maximum specific nitrogen removal rate of 0.434 g N/(g VSS day). During the study period, Anammox activity was not inhibited by pH changes and free ammonia toxicity.

Keywords Anammox · CSTR · FISH · SEM · Nitrogen removal

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Introduction

Nitrogen removal is an important aspect in wastewater treatment and is mostly carried out by multi-step microbial processes. The anaerobic ammonium oxidation (Anammox) process is one such microbial process that has changed the traditional concept of biological nitrogen removal. In this process ammonia is directly converted into dinitrogen gas by using nitrite as an electron acceptor under anaerobic conditions. This new process was first detected in a fluidized bed reactor in Delft, the Netherlands [1]. It was later converted into Anammox technology by workers at Delft [2, 3]. The existence of a bacterium capable of carrying out the Anammox reaction was predicted long ago by Broda [4] based on thermodynamic calculations. With the discovery of this 'missing lithotroph' in 1995, most of the studies that followed have been carried out by this single group at Delft [5].

Anammox activity is now believed to be present in many ecosystems including in biological units of wastewater treatment plants [6–8]. In spite of this, till now very few full-scale Anammox plants have been successfully implemented [9]. Even in bench-scale experiments, the maximum nitrogen loading rate (NLR_{max}) varies widely from 0.015 to 58.5 kg N/(m^3 day) (Table 1). Extremely slow growth rates and difficulty in isolating the Anammox bacteria in pure cultures are the two bottlenecks of the Anammox process [10]. Apart from these two major rate-limiting factors, the presence of nitrite, sulphide, and oxygen at critical concentrations can also inhibit the process [11]. The low growth rate combined with the sensitivity of the microorganisms to inhibitory concentrations of some compounds that are often present in industrial effluents makes the start-up of the Anammox process very difficult to achieve [12, 13]. The start-up period can be

Table 1 Comparison of maximum NLR, NRR and SNRR of different Anammox reactors developed from different sludge sources

Sludge source	Reactor type	NLR_{max} [kg N/(m^3 day)]	NRR_{max} [kg N/(m^3 day)]	SNRR [g N/(g VSS day)]	References
Enriched Anammox biomass[a]	SBR	0.28	0.08	0.13	[33]
Enriched Anammox sludge[a]	SBR	1.2	0.75	0.18	[34]
Activated sludge[b]	SBR	2.6	2.4	0.30	[35]
Aerobic activated sludge[b]	MBR	0.08	0.072	0.35	[18]
Anaerobic sludge digester[b]	SGSR	0.231	0.216	0.43	Present work
Full-scale UASB[b]	UASB	0.015	0.009	0.64	[26]
Enriched Anammox sludge[a]	CSTR	0.662	0.582	VSS not reported	[36]
Activated sludge[b]	SBR	1.6	1.57	0.92	[16]
Enriched Anammox sludge[a]	Gas lift	2	1.78	1.15	[14]
Enriched Anammox sludge[a]	Gas lift	10.7	8.9	VSS not reported	[37]
Denitrifying basin[b]	Biofilm reactor	58.5	26	1.6	[22]

UASB up-flow anaerobic sludge bed, *SBR* sequential batch reactor, *MBR* membrane aerated bioreactor, *SGSR* stirred gas solid reactor, *CSTR* continuous stirred tank reactor, NLR_{max} maximum nitrogen loading rate, NRR_{max} maximum nitrogen removal rate, *SNRR* specific nitrogen removal rate, *VSS* volatile suspended solids

[a] Enriched Anammox sludge from Delft, the Netherlands, was used

[b] Conventional sludge was used for development of the Anammox process

minimized by selecting an appropriate type of reactor configuration that allows high sludge retention such as a fluidized bed reactor, sequential batch reactors, membrane bioreactor, etc. [2, 14, 15]. Granulation has been observed to be another essential factor in the Anammox process [16–19].

In this paper, an approach for developing an Anammox system in a well-mixed continuous stirred tank reactor (CSTR) using a non-acclimatized anaerobic digester sludge is described. The check points needed for the faster start-up period and maintenance of Anammox activity over a long period of time are also discussed. A simple method is described for characterizing the granular sludge in terms of chemolithotrophic activities, besides confirming the presence of nitrifying and Anammox bacteria by using a fluorescence in situ hybridization (FISH) assay.

Materials and methods

Experimental setup

The schematic of the experimental setup is presented in Fig. 1. The 3.3-l glass reactor had a working volume of 2.75 l. It had detachable dissolved oxygen (DO) and pH probes for monitoring DO and pH (Multiparameter PCD 650, Eutech Instruments, Singapore) respectively, of influent and effluent. The feed was introduced by using a peristaltic pump (Sci Q 323 pump, Watson Marlow) at a predetermined and constant flow rate. The content in the reactor was stirred continuously at moderate speed of

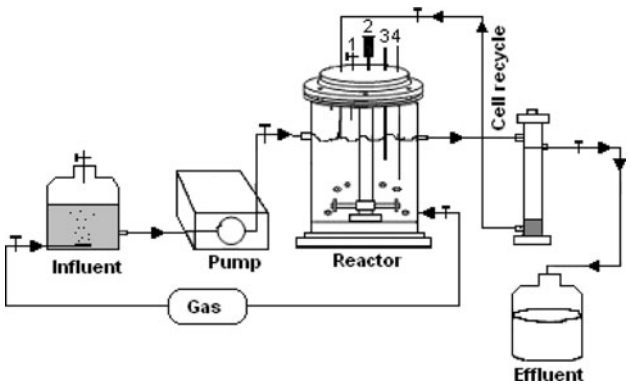

Fig. 1 Schematic diagram of experimental setup of continuous stirred tank reactor (CSTR). *1* Gas vent, *2* motor, *3* pH probe and *4* DO probe

30 ± 5 rpm. A settler was provided prior to the collection tank and the biomass was recycled manually from the settler to the reactor. Provision was made for nitrogen gas purging in the feed tank as well as in the reactor. The units, namely the influent tank, the reactor, settler and the effluent collection tank were made of glass and were tightly sealed by butyl rubber stoppers.

Seed culture

The anaerobic biomass for seeding the Anammox reactor was obtained from an anaerobic sludge digester operating at a municipal sewage treatment plant (STP), located at Nagpur, India. The anaerobic seed culture was black in colour, granular in appearance with a mean diameter of

1–1.5 mm and has total suspended solids (TSS) and volatile suspended solids (VSS) values of 38.5 g/l and 29.8 g/l, respectively.

Synthetic wastewater

The composition of the synthetic wastewater is (in g/l): $NaHCO_3$ 1.25, KH_2PO_4 0.025, $CaCl_2{\cdot}2H_2O$ 0.3, $MgSO_4{\cdot}7H_2O$ 0.2, $FeSO_4$ 0.00625, EDTA 0.00625 and 25 ml/l of trace element solution. The trace element solution contained (in g/l): EDTA 15, $ZnSO_4{\cdot}7H_2O$ 0.43, $CoCl_2{\cdot}6H_2O$ 0.24, $MnCl_2{\cdot}4H_2O$ 0.99, $CuSO_4{\cdot}5H_2O$ 0.25, $NaMoO_4{\cdot}2H_2O$ 0.22, H_3BO_4 0.014. Ammonium (NH_4^+) and nitrite (NO_2^-) were added to the mineral media in the form of $(NH_4)_2SO_4$ and $NaNO_2$, respectively. The concentrations of nitrite and ammonia were varied during the experiment (Fig. 2). The influent pH ranged between 7.5 and 8.0. Fresh synthetic wastewater was prepared daily.

Process operation

Initial acclimatization of biomass was carried out in two tightly closed 1-l-capacity glass bottles. Seed biomass (1.86 g VSS/l) in one of the bottles was incubated in synthetic medium containing 250 mg/l of amoxicillin, while in the other a low seed concentration (0.56 g VSS/l) was used. Samples were withdrawn every alternate day. Filtered samples were analysed for pH, NH_4^+-N, NO_2^--N, NO_3^--N, alkalinity and DO concentrations. Initially, one-third of the supernatant in each bottle was replenished once in a week. As ammonia reduction progressed, feeding gap was reduced to every alternate day. During sampling and feeding, care was taken to avoid direct contact of atmospheric air with the contents of the bottle. After 65 days of incubation under batch mode, sludge from both the bottles was mixed and seeded into the bench-scale reactor (CSTR) with initial VSS of 0.78 g/l. After seeding, the CSTR was fed with synthetic wastewater containing total nitrogen (ammonia + nitrite) concentration of 115 ± 5 mg N/l. Influent nitrite to ammonia (NO_2^-/NH_4^+) ratio in the feed was maintained at around 1.43 to support Anammox activity. The nitrogen loading rate (NLR) was maintained at 0.057 ± 0.003 kg N/(m^3 day) during this period (from 1st day to 97th day). Once Anammox activity was initiated in the reactor, ammonia and nitrite concentrations in the reactor were gradually increased from 50 ± 4 to 200 ± 6 mg NH_4^+-N/l and 65 ± 4 to 287 ± 35 mg NO_2^--N/l, respectively. The NLR consequently increased from 0.057 ± 0.003 to 0.225 ± 0.014 kg N/(m^3 day). The highest average NLR of 0.225 ± 0.014 kg N/(m^3 day) was maintained during the last 30 days (280th to 310th day) of reactor operation.

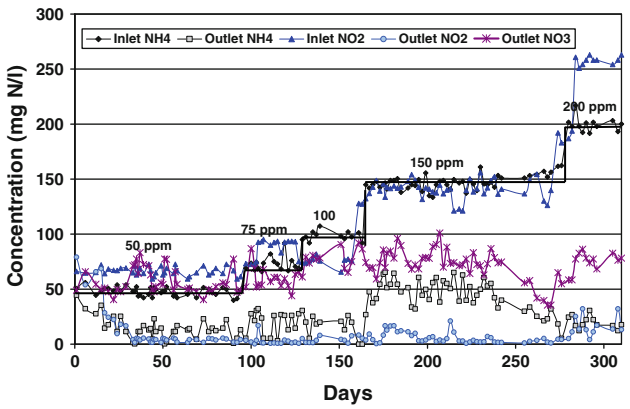

Fig. 2 Inlet and outlet nitrogen concentrations showing performance of CSTR

The reactor was operated with an hydraulic retention time (HRT) of 48 h at ambient temperature. During the period of study, the liquid temperature of the reactor varied from 25°C in winter to 38°C in summer. The biomass in the reactor was slowly mixed with a slow-speed single blade stirrer, rotating at 30 ± 5 rpm. Nitrogen gas purging into the reactor was initiated from the 201st day on continuous basis at 2–5 l/min to maintain anaerobicity. During this period, the synthetic wastewater fed into the reactor was also purged with nitrogen gas at a high flow rate (50 l/min) for 5 min to maintain dissolved oxygen (DO) concentration at less than 0.05 mg/l in the influent feed.

Activity tests

Activity tests were conducted to characterize the granular sludge and to understand the spatial distribution of various chemolithotrophic populations within the micro-granular structures developed in the CSTR. Sludge taken from the reactor was allowed to settle for 10 min. The settled granular biomass and the supernatant were separated. An aliquot of sample from both the fractions was then inoculated into three sets of 250 ml mineral medium, maintaining a cell concentration of 125 ± 20 mg/l as dry weight. The first set contained only ammonia as substrate, the second contained only nitrite and the third contained both ammonia and nitrite as substrates. The experiments were conducted in 250 ml mineral medium, contained in butyl-rubber-stoppered 500-ml conical flasks. The flasks were incubated anaerobically at 37 ± 1°C in a shaker incubator (Remi, India). The rotational speed of the shaker incubator was set at 30 rpm to avoid mechanical disruption of granules due to shearing force. Samples were withdrawn once in every 24 h. Each conical flask was purged with nitrogen just before and after inoculation and after withdrawal of every sample for analysis. Purging with N_2 gas was continued until DO concentration in the medium

dropped to less than 0.05 mg/l. All the experiments were conducted in duplicates to confirm the results.

Analytical methods

Ammonia (NH_4^+-N) was estimated by a colorimetric method as per the standard methods [20]. Nitrate (NO_3^--N) and nitrite (NO_2^--N) were analysed by an ion chromatography system using conductivity detection (Metrohm, comprising five separate modules: 818 IC pump, 819 IC detector, 820 IC separation centre, 830 IC interface and 833 IC liquid handling unit) [20]. Separation and elution of anions were carried out on an IonPac 250A anion column, utilizing carbonate/bicarbonate eluent and autosuppressor technology. Dissolved oxygen (DO), temperature and pH were measured by using a multimeter (Multiparameter PCD 650, Eutech Instruments, Singapore). The nitrogen loading rate [NLR, kg N/(m^3 day)] was calculated as the sum of mass fluxes of ammonia (NH_4^+-N) and nitrite (NO_2^--N) (mg N/day), applied per maximum reactor volume (m^3), per day. The nitrogen removal rate [NRR, kg N/(m^3 day)] was calculated as the sum of masses of NH_4^+-N and NO_2^--N removed per maximum reactor volume (m^3), per day. The specific nitrogen removal rate [SNRR, g N/(g VSS day)] was calculated as the sum of NH_4^+-N and NO_2^--N removed per gram of biomass measured as gram VSS and per day. Nitrogen removal (ΔN) and mass balance were calculated as follows [21]:

$$\Delta N = \left(\left(NH_4^+ - N_{inf}\right) + \left(NO_2^- - N_{inf}\right)\right) - \left(\left(NH_4^+ - N_{eff}\right) + \left(NO_2^- - N_{eff}\right) + \left(NO_3^- - N_{eff}\right)\right)$$

All data are presented as mean value of measurements made on three replicate samples. The statistical analysis of the experimental results and the linear regressions of the experimental data were analyzed by using Minitab 15.

Scanning electron microscopy (SEM)

The biomass from CSTR was observed under a scanning electron microscope (JEOL JXA-840) for detection of granular structures. Diameter of the granules was measured by inbuilt software in the electron microscope. For SEM analysis the samples were pretreated by fixing overnight with 2.5% gluteraldehyde in a 0.1 M phosphate buffer. The samples were then washed and dehydrated sequentially in 30, 50, 70, 90 and 100% ethanol solutions, respectively.

Fluorescence in situ hybridization analysis

FISH assays of the biomass were carried out to detect Anammox bacteria. Cell fixation and FISH analysis were performed according to the standard hybridization protocol for environmental samples (FISH Protocol© Natuschka Lee, Environmental and Geomicrobiology, Department of Microbiology, TUM, Germany). The 16S rRNA gene probes used were Pla 46 [22], Amx820 [22], NSO1225 [23] and NIT3 [23]. All 5′-fluorescein-tagged probes were purchased from Bangalore Genei (Bangalore, India). Hybridizations were performed on sludge samples fixed with 4% (w/v) paraformaldehyde. Negative controls were used to eliminate false positive results due to autofluorescence. The images acquired through an epifluorescence microscope (Nikon H600L, Japan) were resolved by using standard software (Nikon DS-U2, version 5.03).

Results and discussion

Biomass from an anaerobic sludge digester was expected to be rich in strictly anaerobic microorganisms like Anammox bacteria and hence was used for developing the Anammox system. To enrich the growth of Anammox bacteria, two strategies were employed. In the first, amoxicillin was added at a concentration of 250 mg/l. During enrichment, addition of antibiotic ensured inhibition of cell wall forming bacteria, thereby promoting growth of microorganisms having no cell walls. Anammox bacteria belonging to the class *Planctomycetes* do not posses a cell wall. In the second strategy, low inoculum size was used for selective enrichment of slow-growing but dominating anaerobic populations in the biomass [24]. When inoculum size is kept low, fast-growing microbial populations which compete with slow-growing bacteria are diluted in number. A strategy based on this principle is often employed for isolating slow-growing and dominating bacteria from mixed cultures through higher serial dilutions [25]. During this period of batch incubation in anaerobic glass bottles, ammonia reduction was observed. The biomass characteristics also changed from black granular sludge to brown loosely aggregated flocs. After 65 days of incubation, the biomass from both the bottles was seeded into CSTR for continuous mode of operation. The CSTR seeded with this biomass was operated for more than 310 days under varied ammonia and nitrite concentrations (Fig. 2).

Start-up of Anammox activity

Figure 2 depicts performance of CSTR at increasing feed ammonia and nitrite concentrations throughout the experimental period and the data presented in Table 2 depict the molar ratio between the various nitrogen compounds in the influent and effluent of the CSTR.

Figure 2 shows that from the 17th day simultaneous removal of ammonia and nitrite started, indicating initiation of Anammox activity. Since NO_2^--N accumulation

Table 2 Molar ratios of influent (NO_2^-/NH_4^+), consumed (NO_2^-/NH_4^+) and nitrate generated to ammonium oxidized (NO_3^-/NH_4^+) during different periods of reactor operation

Period (days)	Influent NO_2^-/NH_4^+ ratio	Consumed NO_2^-/NH_4^+ ratio	Nitrate generated to ammonia oxidized ratio
1–96	1.43	1.56	0.85
97–128	1.19	1.63	0.66
129–164	0.91	1.02	0.62
165–280	0.98	1.37	0.60
281–310	1.25	1.31	0.22

was not observed, both influent ammonia and nitrite concentrations were gradually increased in a stepwise manner. At the highest total feed nitrogen concentration of 487 ± 45 mg N/l comprising 200 ± 6 mg NH_4^+-N/l and 287 ± 35 mg NO_2^--N/l, the effluent was characterized with 20 ± 5 mg NH_4^+-N/l, 15 ± 7 mg NO_2^--N/l and 70 ± 5 mg NO_3^--N/l. The NRR_{max} of 0.216 kg N/(m^3 day) was obtained at NRL of 0. 231 kg N/(m^3 day). The range of NRR reported in the literature varies widely from 0.08 to 26 kg N/(m^3 day) (Table 1). Since biological nitrogen removal is a function of biomass concentration, it is more accurate to compare results in the literature using the values of SNRR [14]. The $SNRR_{max}$ reported in the literature ranges from 0.13 to 1.6 g N/(g VSS day) (Table 1), while that obtained in the present study is 0.434 g N/(g VSS day).

The time required for simultaneous removal of ammonia and nitrite by Anammox sludge is considered as the start-up period for Anammox activity. In the present study, this start-up took 82 days, including the enrichment period of 65 days in anaerobic glass bottles. The actual period required for acclimatization of slow-growing Anammox bacteria depends on various factors, namely the source of seed culture, initial concentration of Anammox bacteria in the seed culture, species of Anammox bacteria being enriched and cultural conditions. A start-up time of 120–200 days is reported for developing Anammox activity from non-conventional sludge [12, 26].

Activity test and characterization of biomass

Within 100 days, the visual appearance of the biomass changed from blackish-brown to reddish-brown granular sludge with time and evolved into micro-granules of 10- to 20-μm diameter (Fig. 3). Granular biomass is reported to be desirable in the Anammox process [10, 17, 24].

The Anammox granules were harvested from reactor on the 100th day and were tested for ammonia-oxidizing activity. The results of activity tests are presented in Fig. 4. Activity test with suspended cells showed positive

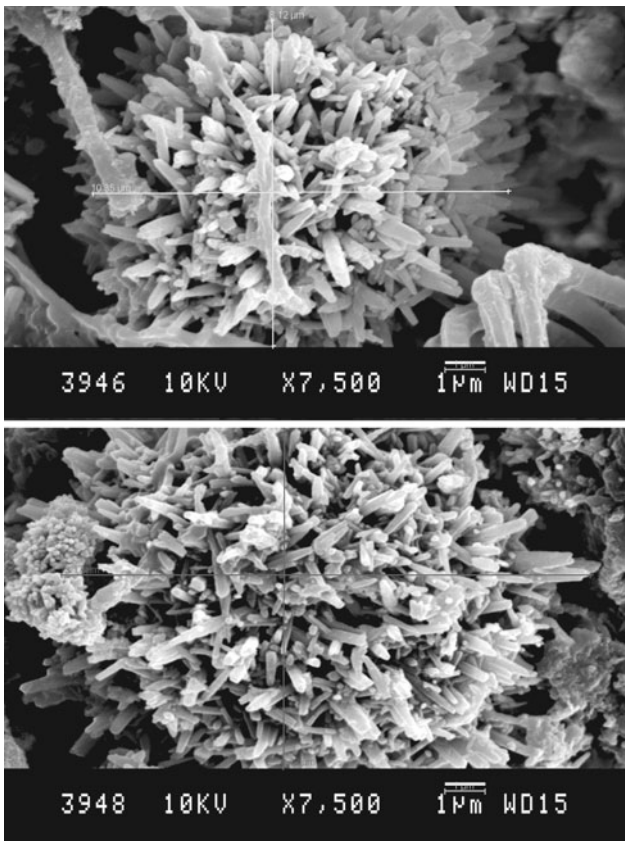

Fig. 3 Scanning electron micrograph of granular Anammox sludge in CSTR

ammonia-oxidizing activity but nitrite-oxidizing activity was totally absent. Granular biomass showed positive test for both ammonia-oxidizing activity and nitrite-oxidizing activity. The results of supernatant clearly indicated the presence of ammonia-oxidizing bacteria (AOB), while the results with the granular biomass indicated mixed activities of AOB, nitrite-oxidizing bacteria (NOB) and/or Anammox bacteria. The experiment also indicated that AOB that were present on the surface of the micro-granule escaped easily into the matrix and also existed as free cells, while the Anammox bacteria proliferated in obligately anaerobic niches within the granule. *Candidatus "Brocadia anammoxidans"* and *Candidatus "Kuenenia stuttgartiensis"* species were detected in the enriched Anammox sludge through FISH analysis with 5′-fluorescein-tagged Amx 820 probe. Positive hybridization with 5′-fluorescein-tagged Pla 46 probes indicated the presence of the broad group of *Planctomycetales* in the biomass (Fig. 5). The AOB and NOB were also detected in the granular sludge through FISH assay (Fig. 5). Wang et al. [18] reported similar observations in a membrane bioreactor where Anammox bacteria grew in a cluster, while a few aerobic AOB existed freely in suspended condition, surrounding the Anammox

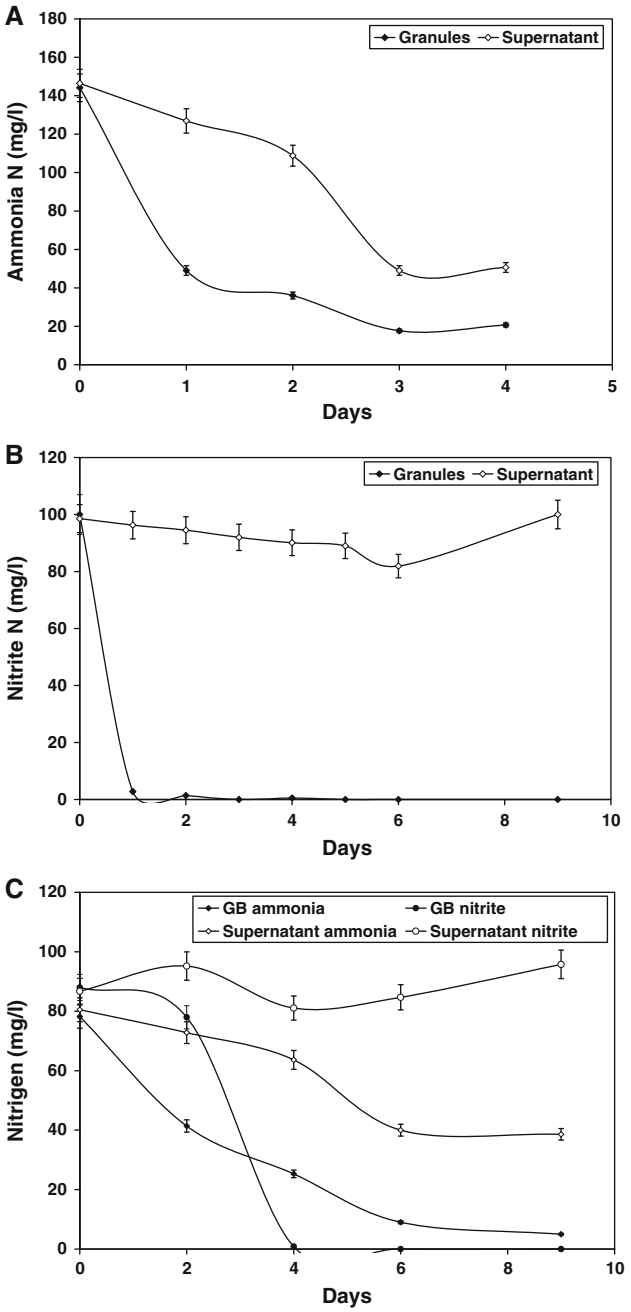

Fig. 4 Results of activity tests of granular biomass (GB) and cell supernatant in batch studies. *Vertical lines* depict *error bars*. **a** Depicts the decrease in ammonia concentration in 'ammonia-only media', while **b** depicts decrease in nitrite concentration in 'nitrite-only media'. **c** Shows both ammonia and nitrite concentration decrease in media containing ammonia and nitrite as substrates

cluster. Further studies are necessary to understand the complete community structure of such micro-granules.

Evolution of Anammox activity

The nitrogen mass balance (ΔN) revealed a gradual increase in the amount of unaccounted nitrogen over time (Fig. 6). The amount of unaccounted ΔN termed as 'loss of total nitrogen' evolved from less than 4 mg N/l to 404 mg N/l during 310 days of operation. This loss could be imparted partly or in combination to either biological denitrification or to Anammox activity. Since organic carbon was not added in the synthetic medium, denitrification cannot take place. Thus, nitrogen loss could be attributed to Anammox activity only. Increase in the value of ΔN over time indicates evolution of Anammox activity (Fig. 6). With time the molar ratio of nitrate formation to ammonia consumption (NO_3^-/NH_4^+) decreased from 0.85 to 0.22, and the consumed NO_2^-/NH_4^+ ratio reached the value of 1.37 (Table 2). This supports Anammox activity as per Eq. 1 [27]:

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

Interestingly, the nitrate generation was still on the high side when the consumed NO_2^-/NH_4^+ ratio reached the desired ratio of 1.37 for Anammox activity (Table 2). This may be due to the presence of some novel species of Anammox bacteria in the bioreactor besides those confirmed by FISH assay.

Maintenance of Anammox activity

The influent and the consumed molar ratio of NO_2^-/NH_4^+ throughout the experimental period are depicted in Fig. 7. As shown, the consumed NO_2^-/NH_4^+ratio during the period from the 50th to 94th day was above the stoichiometric ratio in the Anammox system (indicated by a horizontal line in the Fig. 7). This indicated greater nitrite consumption than ammonia consumption, indicating the dominating role of nitrite-consuming microorganisms. Since organic carbon was not added in the synthetic medium, denitrification cannot take place and hence the presence of NOB is strongly indicated. Batch test of the biomass had already confirmed nitrifying activities. The dominance of NOB activity can be attributed to the presence of excess substrate (NO_2^--N) and also due to availability of residual dissolved oxygen (DO) concentrations (4.2 ± 0.3 mg/l) in the feed. Prior to this period, DO in the feed was not stripped out using inert gas. For industrial applications, stripping out DO from the feed or from the reactor to maintain anaerobicity is uneconomical. DO in the bioreactor and in the effluent was always below detectable limits, indicating the consumption of residual DO. Although DO loading of 0.05 mg O/(mg N day) in the bioreactor was negligible as compared to the NLR, our previous experiments showed that aerobic nitrifying bacteria have the ability to survive under such a limiting DO environment [28]. To decrease the NOB activity, a strategy

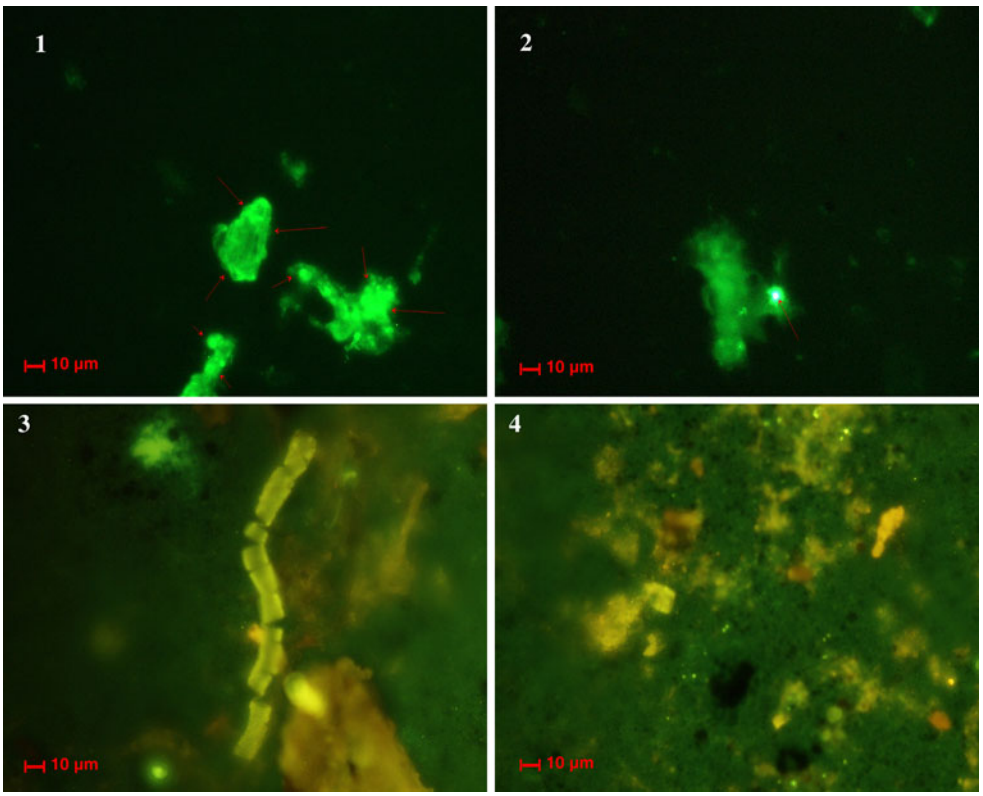

Fig. 5 Fluorescent images of Anammox granules hybridized with fluorescently labeled gene probes, namely **1** Pla 46, **2** Amx 820, **3** NSO 1225 and **4** NIT 3. Images are taken after 100 days of reactor operation under a fluorescein isothiocyanate (FITC) filter (excitation at 465–495 nm). *Scale bar* 10 µm

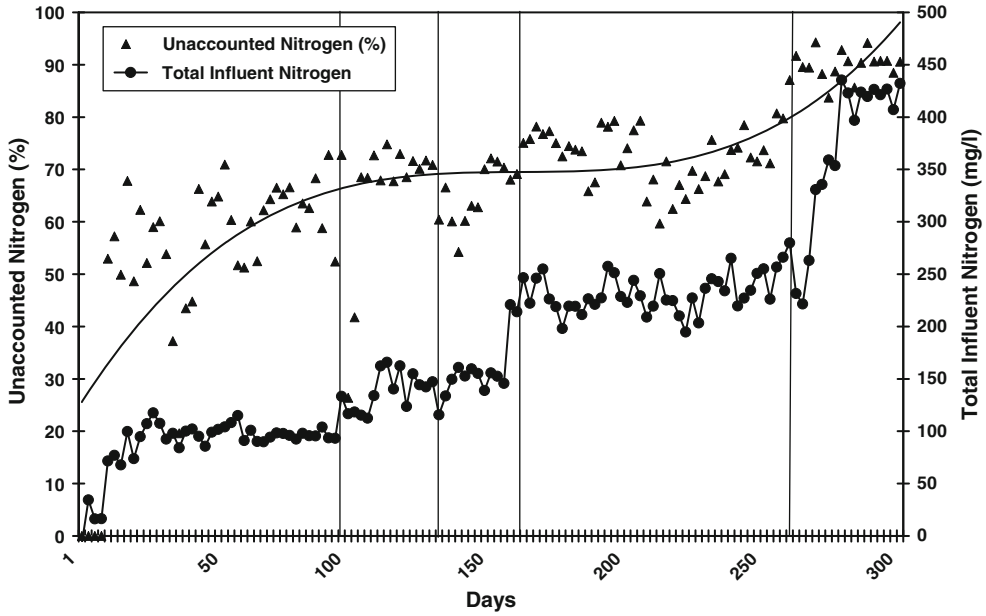

Fig. 6 Nitrogen mass balance indicates rise in the percentage of unaccounted nitrogen fraction implying evolution of Anammox activity in CSTR. The *fitted curvilinear plot* depicts increase in per cent unaccounted nitrogen with increase in influent total nitrogen concentration (mg/l)

of 'limiting substrate' was adopted. From the 97th day, influent NO_2^-/NH_4^+ ratio was decreased from 1.43 to 1.2. However, the NOB activity did not show any decrease until the 200th day. From the 201st day purging with 99.99% N_2 gas stripped out residual DO present in the influent and achieved oxygen-free conditions in the

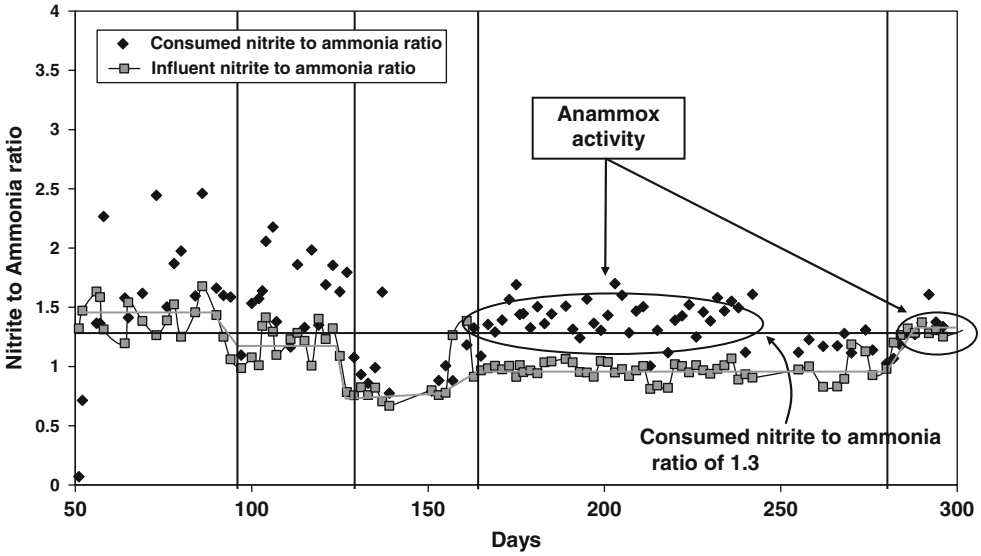

Fig. 7 Changes in nitrite to ammonia ratio with time. As per Eq. 1, Anammox activity results in a consumed NO_2^-/NH_4^+ ratio of 1.3, which is represented as a *horizontal line*. For biomass having mixed populations of nitrifying and Anammox bacteria, constant fine-tuning of feed NO_2^-/NH_4^+ ratio is needed in order to drive the process towards Anammox activity which is represented by nearing of the consumed NO_2^-/NH_4^+ ratio towards the *horizontal line*. All data in this figure are based on molar ratio

bioreactor. Gas purging did not have any effect on molar consumption of NO_2^-/NH_4^+ ratio as DO values were already negligible as compared to NLR, and further decrease in feed DO concentrations had no effect on reactor performance (Fig. 2).

As NOB activity did not decrease at an influent NH_4^+/NO_2^- ratio of 1.2, it was further decreased to 0.8 from the 129th day (Fig. 7). As a result, the consumed molar ratio of NO_2^-/NH_4^+ also dropped below the stoichiometric ratio of 1.3. This phase indicated dominance of AOB. To prevent this condition, the influent NO_2^-/NH_4^+ ratio was immediately increased to 1.0 from the 160th day onwards. By maintaining an influent NO_2^-/NH_4^+ ratio of 1.0, the consumed molar ratio of NO_2^-/NH_4^+ approached the stoichiometric value of 1.3. This period was maintained for the next 120 days. Anammox activity again declined slightly from the 242nd day, and the consumed molar ratio of NO_2^-/NH_4^+ decreased below 1.3 and fluctuated in the range of 1.16–1.2. This indicated competition from AOB and not inhibition of Anammox bacteria. The linear regression between applied ammonia load and ammonia removal confirms that inhibition of ammonia removal did not occur (Fig. 8). Increasing the influent ratio of NO_2^-/NH_4^+ to 1.25 resulted in reinstating Anammox activity as reflected by the consumed NO_2^-/NH_4^+ molar ratio of 1.3.

This study indicated that in a mixed population, severe competition exists between AOB, NOB and Anammox bacteria. To maintain conditions in favour of Anammox bacteria, constant tuning of influent NO_2^-/NH_4^+ ratio is essential. NOB can be kept out of the competition by reducing their share of substrate i.e. nitrite and oxygen. Although AOB also requires oxygen as substrate, studies have revealed that many AOB species are able to adapt their metabolism even in acute absence of oxygen [28, 29].

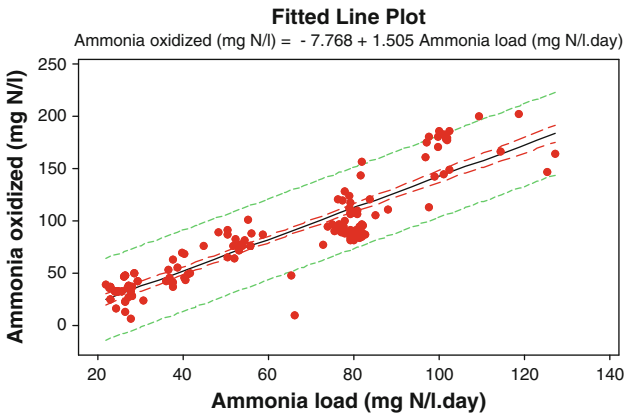

Fig. 8 Linear regression (*continuous line*) of concentration of ammonia oxidized (mg N/l) (*filled circles*) in CSTR against applied ammonia load [mg N/(l day)]. The *inner dashed lines* represent confidence intervals that span the range of observed values. The prediction intervals represented by *outer dashed lines* illustrate the range of likely values for new observations

Once NOB is out of the competition, the only competition is between AOB and Anammox bacteria. Contrastingly, AOB are also essential for Anammox activity as they not only scavenge oxygen molecules but also provide nitrite for Anammox activity. In the present case, since nitrite was already present in the feed, AOB posed as a competitor to Anammox bacteria instead of having a synergistic effect on them. By maintaining an influent NO_2^-/NH_4^+ ratio of 1.0, it was possible to keep AOB out of the completion. However, after the 242nd day AOB reverted back and their activity started dominating the Anammox bacteria from the 242nd to 276th day. This is because AOB tend to adapt to an increasingly difficult environment. Hence, constant tuning of feed conditions is essential for maintaining the Anammox activity.

pH analysis

In Anammox reactors, pH plays a very important role. The optimal pH range of Anammox bacteria is within 7.7–8.2 [30]. Hence, the pH of the influent was maintained at 7.6 ± 0.3. The effluent pH was 7.4 ± 0.3. Table 3 represents the influent and effluent pH variations during days 129–310. During this period, the Anammox and AOB populations were highly competitive. As can be seen from Table 3, the effluent pH was always lower than the inlet pH except during the period of days 280–310 when the inlet and outlet pH were the same. Similar observations were also reported by Chamchoi and Nitisoravut [12] and Strous et al. [27]. However in contrast to this, various workers have reported an increase in effluent pH during Anammox activity [19, 31, 32]. As per the stoichiometry in Eq. 1, 1.3 mol of protons is consumed per mole of ammonia oxidized. Hence pH should increase during Anammox activity. Tang et al. [19] also observed very high effluent pH in the range of 8.7–9.1 during Anammox activity and also reported inhibition of Anammox activity due to free ammonia (FA) concentrations at higher pH values. However, they attributed their observation to lower buffering capacity of the medium. When they increased K_2HCO_3 dose in the medium from 0.5 to 1.25 g/l, they reduced pH variation and further improved Anammox activity. In the present study, $NaHCO_3$ dose was maintained at 1.25 g/l which imparted 600 ± 50 mg/l of alkalinity to the medium. The buffering activity of $NaHCO_3$ can be considered as one of the reasons for preventing increase in pH and thereby toxicity to Anammox bacteria due to FA concentration at higher pH.

Ammonia oxidation by AOB and nitrite oxidation by NOB also causes a drop in alkalinity. As discussed in the previous section, a strong competition exists between AOB and Anammox bacteria in the present system. Hence, another reason for the lower effluent pH can be attributed to competition from AOB activity. With the stabilization of Anammox activity for a period of time, the difference between inlet and outlet pH also decreased (Table 3). Thus, in a mixed population predominated by AOB and *Anammox* species, pH of the medium is buffered by the antagonistic effects of ammonia oxidation through partial nitrification and Anammox reaction.

Table 3 Influent and effluent pH during different periods of reactor operation

Period (days)	Influent pH	Effluent pH	Difference in pH
129–164	7.75 ± 0.26	7.55 ± 0.3	0.2
165–242	7.5 ± 0.25	7.3 ± 0.25	0.2
242–280	7.67 ± 0.3	7.57 ± 0.2	0.1
281–310	7.4 ± 0.18	7.4 ± 0.18	0.0

Conclusion

In this study, successful start-up of an Anammox process was carried out using conventional sludge in a continuous stirred tank reactor within a very short period of 82 days. The methodology adopted to achieve this shorter start-up period was initial enrichment with low sludge concentration and addition of amoxicillin, a cell wall inhibiting antibiotic, in the growth medium. By maintaining low stirring speed in the suspended bioreactor, granulation was achieved which was important for development of the Anammox process. The presence of Anammox bacteria in the anaerobic niches within a granule was confirmed by FISH analysis. Activity test indicated spatial distribution of AOB on the outer surface of the micro-granules, while NOB and Anammox bacteria existed within the core layers of the granules. As a result of the granular structure, the DO concentration in the bulk liquid had no effect on Anammox activity and has practical implications in biological nitrogen removal in wastewater with low C/N ratio. The study also revealed that stability of the process depends on maintaining an appropriate influent NO_2^-/NH_4^+ ratio. The NRR_{max} achieved under stable conditions in the system was 216.1 mg N/(l day) with an $SNRR_{max}$ of 0.434 g N/(g VSS day).

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