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State of The Art Strategies for Successful ANAMMOX Startup and Development: A Review

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Abstract

ANAMMOX (Anaerobic ammonium oxidation) bacteria, leading proponent of autotrophic ammonia removal, requires dedicated enrichment and cultivation techniques due to its slow growth rate and low biomass yield. Sensitivity to inhibitory concentrations of NO_2^--N, O_2 etc., often present in industrial effluents makes ANAMMOX startup difficult to achieve. In this paper, significant parameters for ANAMMOX startup and development such as source of seed, type of reactors used, one and two stage ANAMMOX process, operational strategies and experimental conditions promoting long term enrichment, cultivation and quantitative analysis with biomass retention are discoursed in detail. Key chemical and molecular signatures including NO_2^--N, Alkalinity, O_2, Polymerase chain reaction (PCR), Fluorescence in situ hybridization (FISH), Scanning electron microscopy (SEM), inhibitors and affinity factors for ANAMMOX activity are deliberated. Summary of state of the art on ANAMMOX enrichment, recommendations for future research with specific contributions of this paper to scientific community is brought out in conclusion.

Keywords: ANAMMOX; Enrichment; Startup; Growth rate; NH_4^+-N removal

Introduction

Anaerobic ammonium oxidizers (ANAMMOX), the often overlooked key player in Nitrogen cycle, are involved in highly complex autotrophic microbial interactions to remove NH_4^+-N from nitrogen rich wastewaters. In this process NH_4^+-N is directly converted into dinitrogen gas using NO_2^--N as electron acceptor under anaerobic conditions. The ANAMMOX reaction was first observed in an anoxic denitrifying fluidized bed reactor (FBR) in Gist-Brocades, Delft, Netherlands, treating the effluent from a methanogenic reactor [1]. An ammonium loading rate of 0.4gNH_4^+-N/L/d was removed in this system. The stoichiometry of the ANAMMOX reaction is represented by the following Equation (1).

$$NH_4 + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_20 \quad (1)$$

The ANAMMOX reaction is carried out by members of deeply branched *Planctomycetes*, such as '*Candidatus brocadia anammoxidans*, *Candidatus kuenenia stuttgartiensis*, *Candidatus scalindua*, *Candidatus anammoxoglobus* and *Candidatus jettenia*' which makes use of NH_4^+-N as the electron donor (energy source) and NO_2^--N as the electron acceptor. These autotrophs utilize dissolved CO_2 or HCO_3^- for cell biosynthesis [2-5].

Due to the notorious slow growth rate and low biomass yield (0.13g dry weight/g NH_4^+-N oxidized) of ANAMMOX bacteria, dedicated enrichment and cultivation techniques are required [6-9]. The average doubling time of ANAMMOX bacteria is reported to be 14d [10]. Doubling times as low as 5.3 d and 8.9d were achieved by Park et al. (2010) [11], whilst 11d and 14d were reported by Strous et al. (1998) [12], Li et al. (2009) [9], Strous et al. (2006) [10] from ANAMMOX enriched laboratory scale reactors. The doubling time from the full-scale studies by Van der star et al. (2007) [13] were about 8.3 to 11 d, while Third et al. (2005) [8] achieved 12d. The main bottlenecks of the ANAMMOX process could be ascribed to the low yield (0.14gVSS/g NH_4^+-N) and slow growth rate of ANAMMOX bacteria (0.003 h^{-1}; 0.072d^{-1} at 32°C) and difficulty in isolating ANAMMOX bacteria in pure cultures [7,12]. Besides these rate limiting factors, presence of NO_2^--N, oxygen at critical concentrations can reversibly/irreversibly inhibit ANAMMOX process [1,12,14].

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 startup of the ANAMMOX process very difficult to achieve. The startup period can be minimized by selecting an appropriate seed biomass and running a suitable reactor configuration that promotes long term enrichment, cultivation and quantitative analysis with biomass retention. Proper control and monitoring of key parameters with no compromise on NH_4^+-N removal rates is essential. Some of the important parameters that play a significant role during ANAMMOX enrichment are the source of seed, type of reactors used, operational strategy and experimental conditions. In this paper, the different ANAMMOX enrichment procedure from seeds of different origin is evaluated and the key parameters (both chemical and molecular) that enable both quick growth of ANAMMOX bacteria and affect its growth rate are identified.

Key Parameters for ANAMMOX Enrichment

Source of seed

ANAMMOX bacteria could be developed and applied to startup new reactors from obtaining enriched seed from already operational ANAMMOX reactors which is already containing significant composition of ANAMMOX populations. The long startup period could be reduced and a number of ANAMMOX operational reactors could be initiated simultaneously. Full-scale ANAMMOX reactors are

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operational in Netherlands, Austria, and Germany and in other parts of Europe and USA. ANAMMOX process application can be developed by giving attention to application of enriched seed biomass for startup, since ANAMMOX process consumed long startup period (> 200d) [15]. But as of date, it has not been possible to achieve a pure culture of any ANAMMOX bacteria. It could be attributed to the fact that the activities or presence of other organisms such as AOBs and NOBs are required for them to grow in mixed cultures. ANAMMOX process could also be initiated from non enriched seeds that are availed locally when enriched seed is unavailable and/or exotic enriched seeds could be deemed unsuitable for local conditions.

Inoculation of enriched ANAMMOX seed can accelerate the startup operation within 2 months, while inoculation from non enriched locally available seed may take longer than 4 months [6,16]. Date et al. (2009) [16] carried out ANAMMOX enrichment using sewage, digester and nitrifying sludge as seed in non-woven fabric carrier for immobilization. Besides the simultaneous removal of NH_4^+-N and NO_2^--N and generation of NO_3^--N indicative of ANAMMOX activity, it was also reported that NH_4^+-$N_{removed}$: NO_2^--$N_{removed}$: NO_3^--$N_{produced}$ was 1:1.02:0.23, 1:1.19:0.25 and 1:1.31:0.33 for sewage, digester and nitrifying sludge respectively. The evaluation of efficacy of ANAMMOX process from both enriched and non enriched seeds are depicted in Table 1.

Experimental condition

Gas sparging: Sparging the feed tank and ANAMMOX reactor with inert gases such as Ar/CO_2 or N_2/CO_2 with 95/5% composition enables to maintain strict anaerobic condition for ANAMMOX enrichment. Oxygen leakage that could occur from recirculation operations would require strict deoxygenation steps by sparging. In any case, the gas mixture should contain about 5% CO_2 in order to buffer the medium. Although there are reports of ANAMMOX enrichment under aerobic conditions [17-19], anaerobic conditions at least during the first periods of enrichment is advisable due to the high susceptibility of ANAMMOX bacteria to even microaerobic conditions [17].

Light : Light is considered to be inhibitive to ANAMMOX bacteria since it leads to the undesirable growth of phototrophic algal growth [20]. Studies were conducted by Uyanik et al. (2011) [20] which compared the operation of ANAMMOX reactors with and without control over light penetration. The ANAMMOX reactors that were operated with control over light penetration achieved ANAMMOX enrichment within 50d of startup compared to 100d taken by ANAMMOX reactors that was exposed to light. The reactors that were covered and kept in dark room favored no development of photosynthetic O_2 production and algal development.

Addition of NO_3^--N, N_2H_4 and NH_2OH: During ANAMMOX startup from non enriched seeds, NO_3^--N concentration of about (10–50mg/L) is usually added [20]. Under anaerobic condition, the organic load found in the seed biomass and those produced from initial decomposition could drive the process towards denitrification against the favor of ANAMMOX bacteria. This is due to the autotrophic nature of ANAMMOX bacteria. The addition of NO_3^--N into the reactors was used to feed heterotrophic denitrification bacteria in order for them to consume the organic compounds. It has been reported that addition of NO_3^--N to the feed was effective on removal of organic load due to bacterial decomposition at the start of ANAMMOX enrichment [20]. In the startup phase, NO_3^--N is added to establish oxidative conditions, preventing the growth of other anaerobically respiring microorganisms such as sulfate reducers and methanogens. In response to the NO_2^--N consumption, its concentration in the feed is gradually increased [21]. The idea of supplying NO_3^--N was that, since the inoculum contained an active denitrifying population, NO_2^--N would be produced in the culture in low amounts by NO_3^--N reduction, using remaining organic compounds in the inoculum. The low amount of NO_2^--N production should be sufficient for any ANAMMOX bacteria present in the sludge. After the ANAMMOX reaction is clearly observed, NO_3^--N addition should be discontinued because of both production of nitrate by ANAMMOX reaction and lack of interest in enrichment of denitrification bacteria.

From the works of Third et al. [8], addition of 0.1mM final reactor concentration N_2H_4/NH_2OH to the ANAMMOX reactor was also reported to kick start the ANAMMOX process. The spiking of N_2H_4 and NH_2OH was attempted to take advantage of the cyclic nature of the ANAMMOX mechanism, since cells needed to invest reducing power to start their catabolism by producing NH_2OH from nitrite. This initial energy barrier can be overcome by the direct addition of NH_2OH or N_2H_4 [8,12].

Shear stress: Thorough mixing condition inside the ANAMMOX reactor is important so as to avoid accumulation of NO_2^--N that could inhibit ANAMMOX activity. The speed of stirrer which is used to mix contents in ANAMMOX reactors influences the stability of ANAMMOX

Sl No	Origin of seed	Highest Nitrogen Loading rate (kg N/m³/d)	Highest Nitrogen Removal Rate (kg N/m³/d)	Specific Nitrogen Removal Rate (g N/g VSS/d)	Reference
	Enriched ANAMMOX seed	0.28	0.08	0.13	Padin et al. (2009)
		1.2	0.75	0.18	Van Dongen et al. (2001)
		0.662	0.582	-	Guven et al. (2004)
		2.0	1.78	1.15	Dapena-Mora et al. (2004a)
		10.7	8.9	-	Sliekers et al. (2003)
	Activated sludge	2.6	2.4	0.30	Fux et al. (2002)
		1.6	1.57	0.92	Lopez et al. (2008)
		0.08	0.072	0.35	Wang et al. (2009)
	Anaerobic sludge digester	0.231	0.216	0.43	Bagchi et al. (2010)
	Anaerobic/Aerobic granular sludge	2.5	2.5	-	Yang et al. (2006)
	Anaerobic granular sludge	125-137.1	74.3-76.7	-	Tang et al. (2011)
	Full-scale UASB	0.015	0.009	0.64	Tran et al. (2006)
	Denitrifying reactor	58.5	26	1.6	Tsushima et al. (2007)
		1	1.8	180	Strous et al. (1997)
		1.2	1.5	150	

Table 1: Nitrogen removal efficiency achieved by ANAMMOX process from both enriched and non enriched seeds.

granules [22]. The stirring speed in the range of 60–250rpm was tested by Arrojo et al. [22]. It was reported that upto 180rpm (input power between 0.003 and 0.09kW/m^3) there was no significant effect on the performance of ANAMMOX process. ANAMMOX activity was reduced by 40% when the stirring speed was increased to 250rpm (input power 0.23kW/m^3). The biomass retention worsened due to the breakage of the granules and floatation caused by nitrite accumulation. This caused a loss of the system efficiency due to a combination of cellular lysis and granules breakage. The increase of stirrer speed which involves an increase of shear stress was found to provoke the changes on the properties of biomass aggregates and on the system efficiency [22]. Due to rise in shear stress the rupture of the granules or even the detachment of weak patches of biomass from the surface of the granules could result in increase of suspended solids washout of the reactor. This is due to the biomass flotation that is closely related to the NO_2^--N accumulation in the system [15].

Reactor Configurations

High NH_4^+-N removal rates could be obtained using ANAMMOX process in two ways: two reactors in series, with a partial nitrification reactor as a first step, and a separate unit for the anaerobic oxidation of NH_4^+-N as a second step. With this configuration, the two biological processes can be controlled separately [23]. The second option was to use biofilm systems where classical nitrification is developed by the ammonium oxidizers in the outer aerobic layers, and anaerobic oxidation takes place in the deeper zones of the biofilm [23]. Application of ANAMMOX process or coupling partial nitrification with ANAMMOX seemed promising. It could result in 60% savings in O_2 generation, 100% savings of external carbon source addition, less sludge production and CO_2 emission, with a total reduction in treatment cost by 90% [14,24-26].

One stage ANAMMOX process

Direct application of ANAMMOX process was adopted by Xu et al. [27] to treat NH_4^+-N rich leachate using Sequencing batch biofilm reactors (SBBR). The system was started up in 58d and stabilized in 33d, with DO of 1.2–1.4 mg/L, under alternate periods of aeration and anoxic condition. The leachate was used by spiking it with NH_4Cl to about 450 mg/L prior to feeding as influent to SBBR. The organic load was in the range of 1876 ± 547mg/L of COD and 1048 ± 436mg/L of BOD_5. NLR was optimized to 300mg/L/d, with pH around 7.3 to 7.8 without addition of alkali or acid. It was proposed that the repeated alteration between aeration and anoxic period made the acidity generated in the aeration phase neutralized in time by the alkalinity produced in the anoxic phase. The ratio of NH_4^+-N/ NO_2^--N was in the range of 1.058 to 1.074 in the aeration phase, and 0.558 to 0.776 in the anoxic phase, as compared to the theoretical value of 0.758 in ANAMMOX reaction [12]. It was proven that anoxic condition favored ANAMMOX activity when weighed against oxic condition.

While Guo and Qi [28] treated aged landfill leachate in an Upflow anaerobic sludge blanket (UASB) ANAMMOX bioreactor (HRT 24h) and achieved about 80% total nitrogen removal efficiency from influent containing 900mg TN/L and 88% NH_4^+-N removal from an influent of 350mg NH_4^+-N/L. During the study period for >200d, the mean COD removal was 24% from an influent of 1000mg/L. While alkalinity concentrations of both the influent and effluent during the steady phase of ANAMMOX activity were 1g/L and pH of influent and effluent were 8.3. This study indicated that alkalinity and pH could also be used to monitor the ANAMMOX activity. The ratio of NO_2^--N/ NH_4^+-N was in the range of 0.96 to 1.49, as compared to the stoichiometric value of 1.24 [12].

Application of ANAMMOX process to remove NH_4^+-N (1545mg/L) from wastewaters was performed by Sliekers et al. [29] in a gas lift reactor. The highest NLR reached during the ANAMMOX process was 10.3kgN/m^3/d with Ar/CO_2 sparged at flow of 200 mL/min. The ANAMMOX process was maintained in a gas lift reactor by maintaining anoxic condition during ANAMMOX process. NRR of 8.9 kgN/m^3/d was achieved for the ANAMMOX process.

For one stage ANAMMOX reactors, besides the NO_2^--N limitation, O_2 consumption by AOB plays a role in process design. O_2 transfer was indeed indicated as the limiting process for a lab-scale air-lift [29] and for a lab scale moving bed reactor [8]. The O_2 limitation could have originated from the slow diffusion into the biofilm or from the gas–liquid transfer. It was reported by Van der Star et al. [13] that O_2 penetration is limiting the rotating disk contactor and the moving bed reactor with conversions of 2.5 and 1.2 kg N/m^3/d, respectively. For the other reactors, gas–liquid oxygen transfer is potentially limiting as well. With a superficial gas velocity of 0.025 m/s the oxygen transfer is approximately 15 kg O_2/m^3/d (equivalent to a conversion of 8 kg-N/m^3/d [13,29].

Two stage ANAMMOX process

Application of coupling partial nitrification with ANAMMOX process was adopted by Liang and Liu [30] while treating landfill leachate (NH_4^+-N 1500 to 2500mg/L). An integrated Partial nitritation – ANAMMOX reactor – Underground soil infiltration system was applied. ANAMMOX operation was performed by upflow fixed bed biofilm reactor and achieved 67% NH_4^+-N and 77% NO_2^--N removal within 97d. The effluent of the partial nitritation process yielded a suitable influent for ANAMMOX process, by yielding 50% partial conversion of NH_4^+-N to NO_2^--N (ratio 1:1.3) favoring anaerobic ammonium oxidation. Nearly 60% of NH_4^+-N removal was achieved by the end of ANAMMOX process, and 97% removal was obtained at the end of the combined treatment train. From the initial COD of 1100-2500mg/L, nearly 89% COD was removed with the final effluent showing 30-250mg/L, where nearly 32% COD was removed by ANAMMOX process. The main limitation of the process could be ascribed to the low yield and slow growth rate of ANAMMOX bacteria resulting in slow removal of NO_3^--N (half the time taken for aerobic nitrification) [7,8,12].

The investigation of the aquatic humic substances (AHS) degradation by ANAMMOX process was conducted by Liang et al. [31] where the initial partial nitritation reactor was run for 166d continuously using raw leachate, with NH_4^+-N of 1430 to 2720mg/L and COD of 1170 to 2600mg/L. Upon removal of VFA and acquiring the proper mixture of NO_2^--N to NH_4^+-N ratio, this effluent with NH_4^+-N of 506 to 885mg/L and COD 303 to 954mg/L was further treated in ANAMMOX reactor. The pretreatment in partial nitritation enabled removal of biodegradable organics from raw leachate, resulting in higher content of AHS in the feed to ANAMMOX reactor (228mg/L). The effluent from ANAMMOX reactor is reduced to 91mg/L. The Dissolved organic carbon (DOC) was also reduced from 288 to 136mg/L in the ANAMMOX reactor.

Furukawa et al. [32] successfully demonstrated partial nitritation using nitrifying activated sludge entrapped in a polyethylene glycol (PEG) gel carrier, as a pretreatment to ANAMMOX process for treating supernatant of anaerobically digested sludge. The partial nitritation

reactor was operated at a NLR of 3.0 kgN/m^3/d and suspended solids (SS) concentration of 2000 to 3000 mg/L that resulted in effluent with a NO_2^--N/NH_4^+-N ratio between 1.0 and 1.4 that was suitable for ANAMMOX process. The ANAMMOX reactor achieved TN removal rates of 4.0 kgN/m^3/d under an applied nitrogen loading rate of 5.3 kgN/m^3/d. The authors reported that entrapping ANAMMOX bacteria in the gel carrier prevented inhibition from influent COD and SS concentration. The mean C/N ratio was 0.84 g TOC/g NH_4^+-N with no observed autotrophic ammonium oxidation inhibition.

Application of two stage partial nitritation and ANAMMOX process was also adopted by Fux et al. [33] for treating supernatant of anaerobically digested sludge. The partial nitritation was carried out in a continuous stirred tank reactor (CSTR) of working volume 2L resulting in 58% conversion of NH_4^+-N to NO_2^--N. The ANAMMOX process was conducted in a Sequencing batch reactor (SBR) of working volume 1.6L with nitrogen removal rate of 2.4 kgN/m^3/d, which constituted nearly 90% of the influent to ANAMMOX reactor. The nitrogen load to the ANAMMOX reactor was gradually increased based on the NO_2^--N concentration in the effluent, while the nitrogen removal was dependent on the ratio of NO_2^--N/NH_4^+-N in the influent.

Gali et al. [34] carried out studies to produce the influent for ANAMMOX process by partial nitrification process in SBR. The influent was the supernatant of anaerobically digested sludge of NH_4^+-N concentration of 700 to 800mg N/L with low HCO_3^-/ NH_4^+-N ratio favoring partial nitrification. Complete nitrification required 2mol of HCO_3^- for each mol of NH_4^+-N, whereas the HCO_3^-/ NH_4^+-N ratio was 1 for the influent, which bodes well for partial nitrification. The SBR was operated in 4 stages such as aerobic filling (5min), mixing (210min), settling (20min) and drawing (5min). By the end of 5 months the partial nitrification process yielded 50% NH_4^+-N removal efficiency with specific ammonium uptake rate of 42mg NH_4^+ -N/g VSS /h.

Partial nitritation–ANAMMOX process was applied for treating livestock manure by Yamamoto et al. [35]. The partial nitritation reactor was maintained for 32d under NLR of 1.6kgN/m^3/d resulted in mean conversion efficiency of 51%. The partial nitritation reactor achieved 1.65kgN/m^3/d of maximum NO_2^-- N production rate under NLR of 2.58 kgN/m^3/d. The ANAMMOX process was performed in a UASB reactor that achieved nearly 2.0 kgN/m^3/d under a NLR of 2.2 kgN/m^3/d. Another partial nitritation–ANAMMOX study was conducted by Yamamoto et al. [35] for treating livestock manure effluent of NH_4^+-N concentration in the range of 2000 to 4000 mg/L. The partial nitritation reactor was maintained at NLR of 1.0kgN/m^3/d for over 4 months which was followed by the ANAMMOX reactor. The conversion efficiency of NH_4^+-N to NO_2^--N and NH_4^+-N to NO_3^--N were estimated to be 58% and <5% respectively. The ANAMMOX process yielded consistent nitrogen removal rate of 0.22 kgN/m^3/d.

Leachate pretreatment prior to ANAMMOX reactor using SBR was studied by Ganigue et al. [36]. By adopting a step feed strategy, with alteration between aerobic and anoxic condition, leachate of 5000 mg NH_4^+-N/L was partially nitrified and obtained in the required ratio of 1:1.32. The effluent of 1500 to 2000 mg NH_4^+-N/L and 2000 to 3000 mg NO_2^--N/L was obtained. The reactor was operated for 450d with NLR around 0.85 kgN/m^3/d to 1.2 kgN/m^3/d. The suitable mixture of NH_4^+-N and NO_2^--N obtained was used as feed for ANAMMOX reactor. The presence of low concentrations of biodegradable organic matter (4357 ± 692 mg COD/L) in the leachate was used for denitrification. Supplementation of HCO_3^- externally to favor partial nitritation was adopted, thereby increasing NH_4^+-N conversion and NO_2^--N formation.

Type of Reactor Operation

It is important to comprehend the physical, chemical and the biological needs in the form of symbiotic relationships with key partners (AOBs, NOBs and ANAMMOX) in the mixed microbial community [37]. Hence the widespread strategy to obtain dense enrichments is by using batch cultures and different reactor types, both conventional and advanced configurations to facilitate the growth and development of ANAMMOX bacteria.

The slow growing ANAMMOX organism cannot be cultivated using conventional microbiological techniques [6]. Biological reactors are proven best for cultivating ANAMMOX bacteria [4,6,12,15,26,38-41]. Batch cultures were applied for enrichment purposes to confirm a wide array of samples from different seed origins, to reduce the startup duration needed to reduce trial and error in biological reactors and to decrease the number of reactors for simultaneous enrichment [37]. To apply the ANAMMOX process, the choice of reactor type is very important. It should be suited for long term enrichment, cultivation and quantitative analysis [12]. Full-scale application of ANAMMOX process can be achieved by choosing the appropriate seed sludge and an adequate reactor configuration for ANAMMOX enrichment [42].

ANAMMOX cultivation and enrichment in batch cultures

Applying enrichment in batch studies will enable obtaining successful inocula for starting up ANAMMOX biological reactors. The physiological pH and temperature ranges to be maintained are 6.7 to 8.3 and 20^{0}C to 43^{0}C [14]. Well mixing is vital to maintain redox potential in the denitrification zone and sulfide formation has to be avoided. Sludge retention is important owing to the slow growth rates of the bacteria [6-8]. Replenishment of both Nitrogen supplements (NH_4^+-N and NO_2^--N) and nutrients (enrichment medium) need to be performed to avoid substrate limitation.

In the batch study of ANAMMOX enrichment carried out by Sanchez – Melsio et al. [37], seed biomass were taken from natural environments and treatment plants, which included sediment samples from marine, brackish and freshwater system, seeds from WWTP digesters and from anoxic SBR systems. Enrichments were carried out in Erlenmeyer flasks, with 100mL sample and 300mL enrichment medium [43]. The enrichment flasks contained essential nutrients along with the presence of trace metals. NH_4^+-N and NO_2^--N were added as supplements step wise in order to increase the concentrations from 20mg/L. The enrichments were maintained in shaking incubator in the dark at 37^{0}C, with pH in the range of 6.7 to 8.3. The presence and activity of ANAMMOX was confirmed through the changes in nitrogen compound consumption and by application of molecular analyses. Kawagoshi et al. [44] performed ANAMMOX enrichment from marine sediments. *Candidatus Scalindua wagneri* was observed in the enrichment reactor that was operated at NLR of 0.4kgN/m^3/d. Mulder et al. [1] conducted batch studies with seed originating from FBR, with the NH_4^+-N conversion capacity of 2.7mg NH_4^+-N/g VSS/d. ANAMMOX activity was proved based on nitrogen and redox balances. It was concluded that NH_4^+-N conversion was NO_3^--N dependent [1].

While Bagchi et al. [45] carried out ANAMMOX enrichment from a non enriched locally procured seed in a completely stirred tank reactor (CSTR) with HRT of 2 d. Anaerobic digester sludge from a local STP with TSS and VSS of 38500 mg/L and 29800 mg/L was used as seed. The biomass was initially acclimatized in batch cultures incubated with synthetic medium and amoxicillin (250 mg/L). After 65d of incubation, the sludge with VSS of 78000mg/L was used as seed for

CSTR. The synthetic wastewater of total nitrogen concentration (NH_4^+-N+NO_2^--N) of 115 ± 5 mg/L was fed into CSTR. The NLR was increased as the ANAMMOX activity developed from 0.057 ± 0.003 kgN/m^3/d to 0.225 ± 0.014 kgN/m^3/d. The presence of AOB was identified on the surface and on the exterior layers of granules and the NOB and ANAMMOX bacteria inside. The NO_2^--N/NH_4^+-N was adjusted to enable ANAMMOX activity sustenance even in the presence of mixed community. The highest NRR obtained was 0.216 kgN/m^3/d and highest specific NRR was 0.434g N/g VSS/ d. It was also reported that the ANAMMOX activity in this system was not inhibited by NH_3 toxicity and the pH variations.

Batch tests were conducted by Chen et al. (2010) [46] in 500 mL flasks, for ANAMMOX enrichment, with temperature (35°C), pH (7–8) control and continuous mixing (150 rpm). The anoxic condition was maintained by flushing with a gas mixture of 95% Ar and 5% CO_2. The ANAMMOX enrichment was monitored by analyzing the initial and final substrate concentration for mass balance calculation. The initial NH_4^+-N and NO_2^--N concentrations were 60mg/L (each) and final substrate concentrations were 17 mg/L for NH_4^+-N and 0 mg/L of NO_2^--N with effluent NO_3^--N of 9.7 mg/L. The maximum Specific ANAMMOX activity (SAA) was determined to be 1.8 gN/g VSS/d. Enrichment of ANAMMOX using immobilized microbial consortia was carried out by Pathak et al. (2007) [47]. It was performed at low nitrogen level (<3 mg/L) at low temperature (20°C) in a laboratory scale upflow anoxic reactors. Nitrogen removal efficacy >92 % with the total nitrogen in the effluent <0.2 mg/L was achieved upon operating for 300d.

ANAMMOX process is not inhibited by NH_4^+-N or the byproduct NO_3^--N up to 1g of N/L [14]. The ANAMMOX process is completely inhibited by NO_2^--N>0.1g of N/L. The NO_2^--N inhibition could be overcome by addition of trace amounts of either of the ANAMMOX intermediates 1.4mg of N/L of N_2H_4 or 0.7 mg of N/L of NH_2OH, and enable restoration of the ANAMMOX activity [14]. NO_2^--N toxicity due to increase in substrate concentrations is addressed by recirculation of the effluent, thereby protecting the ANAMMOX sludge [46,48]. High NO_2^--N concentrations above 70mg/L for '*Candidatus Brocadia Anammoxidans*' and above 180mg/L *'Candidatus Kuenenia stuttgartiensis*' for prolonged periods is harmful to the process, so is O_2 concentration higher than 0.06 mg/L [24]. Complete irreversible inhibition of this process when CH_3OH concentration is ≥ 40 mg/L [23,49]. Since there was an existing competition for dominance between ANAMMOX bacteria, denitrifiers and AOBs, it might be a problem while treating wastewaters with high organic (Carbon) and nitrogen content [40].

ANAMMOX cultivation and enrichment in continuous systems

The development and growth of ANAMMOX bacteria in a number of conventional biological reactors such as FBR, SBR, UASB etc., has been undertaken to evaluate the apt configuration for ANAMMOX enrichment and application in treating NH_4^+-N rich wastewaters.

Fluidized Bed Reactor (FBR): The ANAMMOX activity was first reported in the denitrifying FBR treating effluent from a methanogenic reactor by Mulder et al. [1]. The FBR of 23L volume was operated at 36°C and pH 7 with HRT 4.2h. The anoxic liquid was recirculated to keep the bed fluidized of 255L/h flow with superficial liquid velocity 30 to 34m/h. Sand particles were used as biocarrier with biofilm concentration of 150 to 300 mg VSS/g carrier (14g VSS/L). During the initial 420d NH_4^+-N concentrations was similar to effluent concentrations (125 mg/L). From 420–560d the NH_4^+-N concentration started to decrease (from 50 mg/L to <5 mg/L). Highest NH_4^+-N removal rate of 0.4 kgN/m^3/d was reported. Gas composition analysis (v/v) performed during the ANAMMOX process showed 68 to 72% of N_2, 15 to 18% of CH_4, 13 to 18% of CO_2, while N_2O was below the detection limit of 65 ppm.

Sequencing Batch Reactor (SBR): SBR was considered for ANAMMOX enrichment as it held the following strong points such as simplicity, efficient biomass retention, homogenous distribution of substrates, products and biomass aggregates in the reactor, reliable operation for long period (>1 year), stable conditions under substrate limiting environment [4,12]. Using SBR, ANAMMOX process was successfully started in 4 months by Chamchoi and Nitisoravut [6] from sludge obtained from UASB, activated sludge and anaerobic digester. The initial biomass concentration in MLSS was 1500mg/L. The synthetic medium composition used was according to Van de Graaf et al. [43], with regular addition of NH_4^+-N and NO_2^--N (1:1.5) at neutral pH (7.7 to 8.4). The ratio of NH_4^+-N to NO_2^--N in the feed was higher than the stoichiometric ratio of 1:1.32 [12]. The reactors were operated under anaerobic conditions, with reaction (5 to 7h), settling (0.5h) and discharge periods (0.25h). The reactor was kept at anaerobic condition with Ar/CO_2 (95/ 5%) gas flushing. During the first 5 to 7 weeks after seeding, the effluent NH_4^+-N concentrations (75mg/L) were higher than influent (30mg/L). Significant removal of NH_4^+-N and NO_2^--N (80 and 100%) along with small amounts of NO_3^--N produced was observed in the initial three months of operation, indicative of ANAMMOX process [4]. Complete NO_2^--N removal was obtained in four months. The ratios obtained were 1:1.5, 1:1.53 and 1:1.5 for the three reactors containing seeds from UASB, activated sludge and anaerobic digester.

According to Liao et al. [42], granulation of ANAMMOX biomass is favored upon using SBR. Anaerobic methanogenic granular sludge was used (MLSS 72800mg/L and MLVSS 63400mg/L) to startup SBR (7L volume). About 1L of anaerobic granular sludge and 5L of enrichment medium [43] were added to SBR with N_2 gas sparging for fluidization of biomass with liquid and to maintain anaerobiosis of the reactor. The ANAMMOX activity showed improvement when the VSS concentrations reduced from 8.913 to 4.554mg/L and VSS/SS ratio also reduced from 94 to 84%.

Direct application of ANAMMOX process was adopted by Xu et al. [27] to treat NH_4^+-N rich leachate using Sequencing batch biofilm reactors (SBBR). The system was started up in 58d and stabilized in 33d, with DO level of 1.2–1.4mg/L, with alternate periods of aeration and anoxic condition. The leachate was used by spiking it with NH_4Cl and regulated to about 450mg/L with distilled water prior to feeding as influent to SBBR. The organic load was in the range of 1876±547mg/L of COD and 1048±436mg/L of BOD_5. Nitrogen loading rate (NLR) was optimized to 300mg/L/d, with pH around 7.3 to 7.8 without addition of alkali or acid. It was proposed that the repeated alteration between aeration and anoxic period made the acidity generated in the aeration phase neutralized in time by the alkalinity produced in the anoxic phase. The ratio of NH_4^+-N /NO_2^--N was in the range of 1.058–1.074 in the aeration phase, and 0.558–0.776 in the anoxic phase, as compared to the theoretical value of 0.758 in ANAMMOX reaction [12]. It was proven that anoxic condition favored ANAMMOX activity when weighed against oxic condition.

Rotating biological contactor (RBC): Liu et al. [39] developed ANAMMOX in rotating biological contactor (RBC) within 100d by increasing the NLR in the influent feed with gradually shortened HRT. Influent concentration of NH_4^+-N and NO_2^--N was raised from 100 to 350mg/L, with initial HRT of 1d and 99% and 97% of NH_4^+-N and NO_2^-

-N removal efficiency was observed. Upon varying the HRT during optimization, after 100d of operation, mean NH_4^+-N removal of 99%, NO_2^--N removal 100%, TN removal 84% was achieved at 350mg/L of NH_4^+-N and NO_2^--N with HRT 4h. Nearly 70% of nitrogen was removed in a nitrifying RBC for NH_4^+-N rich leachate (100 to 400mg/L) from a hazardous waste landfill [26]. With a pretreated inflow of leachate from RBC at 30-170m^3/d, the system was designed to nitrify 30kg NH_4^+-N/d (3.7gN/m^2/d). The nitrogen elimination without organic carbon was attributed to ANAMMOX process, where the NO_2^--N produced in the aerobic biofilm layer by *Nitrosomonas* near the surface, is diffused and reduced by anaerobic ammonium oxidation in the lower anoxic layer by the ANAMMOX bacteria. Thus spontaneously both aerobic and anaerobic ammonia oxidation was developed on the biofilm [50].

Similarly in another study on RBC [51] NH_4^+-N conversion efficiency of 79%, TN removal efficacy of 70% and COD removal of 94% was obtained at 0.69kgN/m^3/d and 0.34kg/m^3/d of nitrogen and COD loading rates, respectively. The ANAMMOX reactors used were capably mixed with mechanical stirrer and uncommonly with recirculation of the produced N_2 gas as well. Anoxic conditions were maintained by N_2/Ar/CO_2 sparging [4,6,15]. In these biofilm studies, long term establishment of AOB and ANAMMOX communities occurred with limited scope for NOBs [50].

Gas Lift reactor: Dapena-Mora et al. [15] used both SBR and Gas lift reactor under anaerobic conditions with steady increment in NH_4^+-N and NO_2^--N under maximum nitrogen load that could be treated by ANAMMOX. The SBR was operated in 330 min, 20 min, 10 min of reaction, settling and discharge phases. The mean ratio of NH_4^+-N utilization to generation of NO_2^--N was 1:0.26 and 1:0.2 in the gas lift and SBR as compared to 1:0.04 by Chamchoi and Nitisoravut [6]. Sliekers et al. [29] also used Gas lift reactor for ANAMMOX and CANON processes. The NH_4^+-N removal by ANAMMOX and CANON process attained were 8.9 and 1.5 kgN/m^3/d respectively. The reactor was initiated from an enriched seed from already operational ANAMMOX SBR. The gas lift reactor was operated as ANAMMOX mode and then followed by CANON mode. In the ANAMMOX mode, the NLR was 10.7 kgN/m^3/d at 6.7 h HRT that achieved nearly 80% NH_4^+-N removal. During the CANON mode of operation operated at 10h HRT, feed and effluent NH_4^+-N concentrations were 1545 and 899 mg/L, respectively.

Upflow Anaerobic Sludge Blanket Reactor (UASB): Besides SBR, Gas lift reactor and RBC, ANAMMOX bacteria was also developed using UASB reactor too [38]. Granular sludge from anaerobic digester (18.6g VSS/L) was used as potential seed source. Recycling of sludge at 3Q ratios was applied to accelerate mixing and induce dilution of influent thereby reducing the NO_2^--N toxicity to ANAMMOX bacteria. Onset of NH_4^+-N reduction was detected after 200d of sludge cultivation. The effluent NH_4^+-N concentration exhibited steadfast reduction with NO_2^--N consumption and low production of NO_3^--N as byproduct. The color of the sludge turned from black to reddish brown, which is a well-documented characteristic of ANAMMOX activity [6,7,38,41]. After 11 months of operation, the stoichiometric ratio of the ANAMMOX reaction was maintained at 1:1.32 (NH_4^+-N: NO_2^--N) with 60% of NH_4^+-N conversion.

In another study by Ahn et al. [40], ANAMMOX process was developed to treat piggery wastewater (56g COD/L and 5g TN/L), using UASB reactor at 35°C. The reactor was operated with 5d HRT, with NLR of 0.43 kg NH_4^+-N/ m^3/ d. To enable ANAMMOX process, Nitrogen loading rate was 0.36 kg NH_4^+-N/m^3/d and 0.5 kg NO_2^--N/m^3/d, using granular sludge of 18.6 g VS/L as seed biomass. The UASB was operated in semi continuous mode. At the end of the study, nitrogen conversion of 0.6 to 0.7g TN/L/d was achieved and the color of the biomass at the bottom of the reactor changed from dark gray to reddish brown, along with granulation of the ANAMMOX sludge in the lower part of reactor. The reddish brown color characteristic of the ANAMMOX enrichment was mainly attributed to the increase in cytochrome content [43]. Nearly 47 % of NH_4^+-N removal and 83% NO_2^--N removal by ANAMMOX process was reported with the mean specific nitrogen removal to be 0.064 to 0.08g TN/gVSS/d. UASB reactor was also used by Ni et al. [52], for quick ANAMMOX enrichment from enriched ANAMMOX seed as compared to Ahn et al. who used a locally available seed for startup. The NLR achieved by Ni et al. [52] was 0.28 kgN/m^3/d to 1 kgN/m^3/d with HRT<1d.

Upflow Biofilter (UBF): Chen et al. (2010b) [48] successfully started up ANAMMOX process in an Upflow Biofilter with effluent recirculation at high loading rates (34.5kgN/m^3/d). The high loading rates in ANAMMOX reactor was dependent on two parameters such as, the quantity and activity of functional biomass in the reactor. The seed used was taken from full-scale WWTP treating monosodium glutamate effluents of SS (33.3g/L) and VSS (14.6 g/L). The reactor was fed with synthetic water and trace metals solution [43]. The ANAMMOX reactor was started with (30 to 50 mg/L) of NH_4^+-N and (50 to 70 mg/L) of NO_2^--N with HRT of 9.6h. HRT and substrate concentration was adjusted to increase the NLR, with the highest substrate concentrations of 976 mg/L of NH_4^+-N and 1280 mg/L of NO_2^--N. The recirculation was adopted in UBF to decrease the influent substrates in the ratio of 1:1, 2:1 and 4:1, especially till the effluent NO_2^--N concentrations was below 50mg/L. The UBF operation resulted in granulation and biofilm formation of ANAMMOX bacteria. Tang et al. [25] also used UBF that was inoculated with anaerobic granular sludge of SS (51.2 g/L) and VSS (43.5 g/L) originating from an UASB reactor treating paper mill wastewater. The UBF was operated at a fixed HRT of 9.1h and upon occurrence of ANAMMOX activity with continuous removal of NH_4^+-N and NO_2^--N, the NLR was raised stepwise by adjusting the NH_4^+-N and NO_2^--N concentrations. The inhibitory effects of high pH and NH_3 on ANAMMOX bacteria were addressed in this study, since it affects the stabilization of the ANAMMOX process. The buffer concentration was increased from 0.5 g $KHCO_3$/L to 1.25 g $KHCO_3$/L to effectively reduce the pH variation, and to enhance the nitrogen removal performance of the reactor.

Membrane Bioreactor (MBR): Development of advanced biological system, such as MBR for startup of ANAMMOX process was considered as a better alternative for a quicker and stable system when compared to the other biological systems. Main limitation of SBR and other biofilm bioreactors which is the dependence of biomass retention on biomass settling was overcome by MBR. Cultivation of ANAMMOX bacteria as biofilms or cell aggregates in conventional biological systems can be beneficial from an applied, operation point of view. But based on microbiological, physiological and biochemical perspective, development of ANAMMOX bacteria as free cells is favored to avoid mass transfer and nutrient diffusion limitation experienced in biofilm bioreactors. Improvement of growth rate amongst free cells has been reported by Kartal et al. [53], with *K. stuttgartiensis* exhibiting doubling time of 11–20d in SBR compared to 7d in MBR. Successful cultivation of ANAMMOX bacteria with complete biomass retention, operated at high NLRs with production of ANAMMOX bacterial suspension as free cells or aggregates with high growth rate was achieved using MBR [7,41].

In a study conducted by Wang et al. [4], with mixed activated

sludge as seed source (MLSS–2.23g/L ; MLVSS–1.52g/L) and synthetic wastewater of composition prescribed by Van de Graaf et al. [43], the MBR was operated with <0.05mg/L of DO concentration to enable ANAMMOX growth and metabolism. Within 2 months of operation, successful enrichment of ANAMMOX population as the dominant climax community was achieved. Trigo et al. [7] developed ANAMMOX as aggregates in Membrane Sequence Batch Reactor (MSBR). Complete mixing was performed using mechanical stirrer from 40 to 160 rpm. The reactor was operated in 6h cycle comprising of 330 min of reaction, 9 min settling and 18 min discharge with permeate backwash for 3 min. At the end of 375d of operation, 1.22 mole of NO_2^--N and 0.22 mole of NO_3^--N were consumed for 1.0 mole of NH_4^+-N consumed.

ANAMMOX bacteria were cultivated as free cells in MBR in the study conducted by Van der Star et al. [41]. Enrichment medium and reduction of calcium and magnesium concentrations along with addition of yeast extract triggered the growth of ANAMMOX as free cells, reducing the appearance of flocs. Enriched granular ANAMMOX seed from a full-scale ANAMMOX reactor was used in this study. The reactor was operated for >250d. At the end of the study, the NO_2^--N:NH_4^+-N conversion ratio obtained was 1.1 to 1.3, and NO_3^--N:NH_4^+-N ratio was 0.10 to 0.25. In another study by Suneethi and Joseph [54] where 96 % NH_4^+-N removal efficiency was achieved with an influent Nitrogen loading rate of 5 kg NH_4^+-N/m^3/d within 129d. The performance in various biological systems applying ANAMMOX process is compared in Table 2.

Key Signatures for Anammox Activity/Enrichment

The prominent signatures applied for indicating the activity or enrichment of ANAMMOX bacteria includes both chemical and molecular nature. The details of which are entailed in 5.1 and 5.2.

Chemical signatures

pH: Changes in pH reversibly affect ANAMMOX activity. Optimal pH favorable for efficient ANAMMOX activity is in the range of 7 to 8 [12]. Instances of failures in pH control have resulted in unsuccessful attempts in ANAMMOX cultivation [12]. This usually leads to changes in concentrations of NH_4^+-N, NO_2^--N, NO_3^--N, which should be immediately addressed. Since increase in the growth of AOB and NOB instead of ANAMMOX will result in decrease in autotrophic ammonia removal by ANAMMOX activity.

N_2H_4, NH_2OH: The concentrations of N_2H_4 and NH_2OH in AnMBR [54] were around 0.03 to 0.001 mg/L (average of 0.011 ± 0.008 mg/L) and 0.08 to 0.33 mg/L (average of 0.055 ± 0.107 mg/L) respectively, confirming the AOB and ANAMMOX activity in the system [8,55]. The N_2H_4 generated from substrates NH_4^+-N and NO_2^--N during ANAMMOX activity with NO as a direct precursor [56]. Changes in N_2H_4 concentration is usually corresponded to changes in NO_2^--N concentration. This could be attributed to the fact that when NO_2^--N accumulation occurs inhibiting ANAMMOX activity, increased concentrations of N_2H_4 could be noticed [56]. Similarly the changes in the concentration of NH_2OH coincided with the change in feed NH_4^+-N concentration. NH_2OH was reported to be an intermediate of ammonia oxidation carried out by AOB, with increase in NH_2OH meant an inhibition of HAO enzyme activity [56]. Since NH_4^+-N is a common substrate for both AOB and ANAMMOX, change in feed NH_4^+-N concentrations might have led to NH_2OH buildup resulting in comparable tendencies of NH_2OH with NLR [56].

N_2O, NO_2, NO: In ANAMMOX enrichment units, production of N_2O, NO_2, and NO occurs. The changes in gas concentrations corresponded to subtle changes in N_2H_4 and NH_2OH, with NO classified to be one of the intermediate in ANAMMOX reaction [56]. NO could be produced from NH_2OH and NO_2^--N by AOB and ANAMMOX activity, respectively, while N_2O generated from NO by AOB activity [57]. NO produced can also revert to NO_2^--N and then NO_3^--N by NOB activity. Denitrification process was also claimed to produce NO and N_2O as well [57]. Not with standing such complexity in the sources of NO and N_2O concentrations, the ultimate harmless N_2 gas production could be resulted only by ANAMMOX and/or denitrification process.

Sl No	Type of Reactor	Working Volume (L)	Duration of operation (d)	HRT (d)	Influent Nitrogen concentration (mg/L)	Nitrogen removal efficiency (%)	Reference
	FBR	2.25	84	0.9-2	70 – 840 (NO_2^--N) 70 – 840 (NH_4^+-N)	83 – 85	Strous et al. (1997)
			150	0.1 – 11	70 – 840 (NO_2^--N) 1100 – 2100 (NH_4^+-N)	81 – 99	
	SBR	7	150	-	50 – 70 (NO_2^--N) 40 – 60 (NH_4^+-N)	100 80	Chamchoi and Nitisoravut (2007)
	Gas lift	7	200	1	1100 (NO_2^--N) 900 (NH_4^+-N)	>99 88	Dapena-Mora et al. (2004a)
	SBR	1		0.625	375 (NO_2^--N) 375 (NH_4^+-N)	100 78	
	Gas lift	1.8	-	0.42	1370 (NO_2^--N) 1360 (NH_4^+-N)	83	Sliekers et al. (2003)
	RBC	1.7	100	0.167	350 (NO_2^--N) 350 (NH_4^+-N)	100 99	Liu et al. (2008)
	UASB	6	325	3.5	90 (total inorganic nitrogen)	60	Tran et al. (2006)
	UBF	8	236	0.06 - 0.2	331.5 – 1280 (NO_2^--N) 204.6 – 976 (NH_4^+-N)	98.8	Chen et al. (2010a)
	MBR	4.8	60	2	50 (NO_2^--N) 50 (NH_4^+-N)	90 90	Wang et al. (2009)
	MBR	8	>250	2	552 (NO_2^--N) 552 (NH_4^+-N)	-	Van der Star et al. (2008)
	MSBR	5	375	1	390 (NO_2^--N) 390 (NH_4^+-N)	90 90	Trigo et al. (2006)
	AnMBR	15	129	2	100 (NO_2^--N) 10000 (NH_4^+-N)	96%	Suneethi and Joseph (2011)

Table 2: ANAMMOX process performance in conventional and advanced biological reactors.

Alongside these crucial elements, factors such as pH, HNO_2 and DO concentrations were also speculated to play a role in the NO, N_2O and NO_x gas emissions [57]. The production of N_2O and NO by AOBs at low O_2 conditions was reported, with especially a pure culture of *Nitrosomonas* generating N_2O and NO only in the absence of NO_2^--N consumer such as *Nitrobacter* [58].

NH_3 and HNO_2: The possibility of NOB inhibition by NH_3 was supported by studies conducted by Anthonisen et al. [59]. Occurrence of unionized NH_3 and HNO_2 is dependent on the pH and temperature in the biological system and leads to inhibition of NO_2--N conversion [60]. Presence of NOB (Nitrite Oxidizing Bacteria) inhibition is evident when NH_3 concentration is above 0.1 mg/L [32,59,60]. Likewise all nitrifying bacteria showed inhibition above 0.2mg/L of HNO_2 concentration as reported by other studies [32,59].

NH_4^+-N,NO_2^-, NO_3^-: The concentration of nitrite during the startup is of crucial importance for growth: a too low amount will result in substrate limitation and thus slower growth, while concentrations above 50–150 mg N/L can already lead to inhibition [14,50,61]. The stoichiometric ratio for NH_4^+-N removed: NO_2^--N converted: NO_3^--N produced indicating the ANAMMOX process was 1:1.32:0.26 [12]. During the buildup of Nitrogen Loading rate, low influent NO_2^--N/NH_4^+-N ratio could be observed with the overall obtained ratio was 1:0.84:0.02 [62]. Wyffels et al. [63] reported 0.20mol of production of NO_3^--N per mol of NH_4^+-N oxidized for ANAMMOX MBR system. Low degree but significant occurrence of ANAMMOX process, of approximately 1:1.15 for NO_2^--N consumption to NH_4^+-N consumption, was reported by Wang et al. [4] and about 1.05 by Wyffels et al. [63]. Denitrification process could also affect the effluent NO_2^--N to NH_4^+-N molar ratio [64]. The molar ratio of NO_2^--N conversion to NH_4^+-N removed of 1.22 while the NO_3^--N production to NH_4^+-N oxidized ratio of 0.22 was obtained by Trigo et al. [7].

Alkalinity and dissolved oxygen: Feed alkalinity along with DO concentrations are critical controlling parameters in a single-stage biological process for nitrogen removal. The control of alkalinity and dissolved oxygen (DO) concentrations in the feed to maintain an alkalinity to ammonia ratio of <8 and DO of <0.06 mg O/mg N/d, respectively, was necessary for inhibiting nitratation and enhancing partial nitritation and ANAMMOX [45].

To achieve a biological nitrogen removal in a single-stage, the activity of NOB has to be inhibited without affecting the activities of AOB and ANAMMOX bacteria. These three groups of microorganisms are closely interlinked with common electron donor and acceptors. From the works of Gong et al. [19] and Paredes et al. [23] it has been reported that by regulating the concentration of DO and NO_2^--N, partial control of NOB activity could be achieved. As NOB competes for DO and NO_2^--N with AOB and ANAMMOX bacteria, respectively in the absence of NO_2^--N in wastewater, NOB depends directly on AOB for their source of electron donor. By limiting DO concentration, AOB consume available DO for NO_2^--N production. Hence, under this condition, NOB experiences two-way limitations, initially in terms of electron donor (NO_2^--N) and later in terms of electron acceptor (O_2). AOB, NOB and ANAMMOX bacteria which are chemolithotrophic microorganisms also require inorganic carbon source for their cell growth [65]. By controlling bicarbonate alkalinity, the process of elimination of NOB can be further fine-tuned [45].

Molecular signatures

The common methodological approaches adopted to detect, identify and confirm ANAMMOX bacteria or their activity includes:

(i) Detection of the single ladderane lipids as biomarkers, which were reported to be unique structures, found in the intracytoplasmic organelle like structure of ANAMMOX bacteria by HPLC – Atmospheric Pressure Ionization – MS and GC – MS [66]. The compound specific stable carbon isotope ratios can also be monitored by GC – IR – MS system [67].

(ii) Chemical analyses of the Nitrogen compounds to detect the change in the concentrations of NH_4^+-N, NO_2^--N and NO_3^--N owing to ANAMMOX activity [60] along with determination of N_2H_4 and NH_2OH [67].

(iii) Analysis of gas composition of N_2, N_2O, NH_3, CO_2, CH_4 by GC – TCD [40,41], or only the gas composition analysis of N_2O carried out in real-time using gas-filter correlation (Teledyne API 320E).

(iv) Estimation of NO (nitric oxide) using a chemiluminescence detector (CLD) [41], or determination of NO and NO_2 by chemiluminescence method (Ecophysics CLD 64 monitor).

(v) Application of molecular techniques such as Polymerase Chain Reaction (PCR) or Fluorescence *In Situ* Hybridization (FISH), which are based on the nucleic acid analysis for identification. A number of specific sequences and primers were developed to amplify the 16S rRNA from the environmental and enrichment samples using the PCR based approach or by FISH analysis, as detailed in 5.2.2 [4,11,37,69].

(vi) Application of ISR FISH to assess the precursor rRNA concentration in a cell, the intergenic spacer region (ISR) between 16S rRNA gene and 23S rRNA gene by targeting using fluorescently labeled oligonucleotide probes [67].

(vii) Combination of Fluorescence *In Situ* Hybridization – Micro Autoradiography (FISH-MAR) that can directly link the uptake of radioactively labeled substrate (Eg: $NaH_{14}CO_3$) with uncultured organisms such as ANAMMOX [67].

(viii) Quantification of changes in microbial population of a mixed culture of nitrifiers, denitrifiers, NOBs, AOBs and ANAMMOX using quinone profiles, since this profile is usually represented as the mole fraction of each quinone type that is specific for a microbial community [70].

(ix) Observation of ANAMMOX cells using confocal micrographs or scanning electron microscope (SEM). The observations from SEM images are subject to confirmation using molecular analyses [4,615,22].

(x) Trace experiments with labeled $[_{15}N]NH_4^+$-N reacts uniquely, in a 1:1 ratio with unlabeled $[_{14}N]NO_2^-$-N to $N_2(_{14}N_{15}N)$ in the ANAMMOX reaction.

SEM observation of ANAMMOX seed biomass: Scanning electron microscope images were used to visualize the seed and membrane morphology in AnMBR by Suneethi and Joseph [54]. The seed cultivated in the AnMBR and the biomass on the membrane surface were mostly spherical and elliptical bacterial clusters with rough surface, interspersed with abundant aggregates of inorganic origin. This observation together with monitoring of the nitrogen transformations revealed the presence of ANAMMOX activity. There was also presence of few filamentous and short rod bacteria, indicating the harmonious coexistence of ANAMMOX bacteria with other microbial populations like AOBs, NOBs and denitrifiers in AnMBR [4,22]. The SEM photographs of the cultivated ANAMMOX sludge in AnMBR indicated

the various bacterial morphologies found in the sludge, though the cause of filamentous bacteria formation was unclear.

Biomass growth occurred as granules with an irregular cauliflower appearance, which was reported by Trigo et al. [7]. Dapena-Mora et al. [71] described the ANAMMOX biomass growth in SBR-ANAMMOX system as granular with an irregular cauliflower appearance. Presence of co–existence of cocci bacteria such as filamentous and short rod bacteria with spherical shaped bacteria in the seed sludges from activated sludge and anaerobic digestion sludge was reported by Chamchoi and Nitisoravut [6]. Negative role played by filamentous bacteria in system performance in UASB seed sludge was reported by Chamchoi and Nitisoravut [6]. Frequent appearance and development of *Chlorobi*– like filamentous bacteria in ANAMMOX reactors, was also reported by Li et al. [9]. The relation between the filamentous bacteria and ANAMMOX seed could be typically linked to unconfirmed metabolic connection with the involvement of the filamentous bacteria in bestowing structural integrity to the ANAMMOX in both granular and biofilm phase [11,69].

The SEM observations of the enriched ANAMMOX seeds were reported by Chamchoi and Nitisoravut [6] as spherical flocs of ANAMMOX sludge with smooth surfaces along with cocci and filamentous type bacteria. The seed sludge from UASB exhibited both filamentous and spherical shaped bacteria while the seed sludge from activated sludge and anaerobic digestion sludge, the main types of bacteria were spherical and short rod-shaped bacteria. The spherical cells were presumed to be ANAMMOX that coexisted harmoniously with AOBs and NOBs as observed from various bacterial morphologies. The cause for occurrence of filamentous bacteria was inconclusive in their study.

ANAMMOX identification: Usually the color of the ANAMMOX enriched sludge is reported to be reddish brown, which is a well-documented characteristic of ANAMMOX activity [6,7,38,41]. The recent detection methods and different molecular techniques available for the ANAMMOX organisms are fluorescence in situ hybridization (FISH), 16S rRNA or functional gene analysis, membrane lipids and tracer experiments with [$_{15}$N] labeled ammonia [3,72-78]. FISH can give both quantitative and qualitative figures of ANAMMOX bacterial population. In situ hybridizations with DNA probes for the detection of ANAMMOX bacteria are performed with fluorescent labeled compounds and fluorescent bacterial cells, which are detected by epifluorescent microscopy, confocal laser scanning microscopy or flow cytometry. The number of FISH probes targeting the specific ANAMMOX organisms is given in the Table 3. FISH signal intensity is directly proportional to the precursor rRNA concentrations in ANAMMOX cells and could be used as a direct measure of the growth rate (ribosome turnover rate) of the ANAMMOX organisms. The advantages of analyzing with FISH technique are reliable, with reduced misinterpret artifacts, conveys about the spatial location of bacteria and density in a limited region without the destruction of the sample. But if large amount of inert material was present and when the ANAMMOX bacterial cell was low in the sample (i.e. low numbers of rRNA) then the FISH technique may not be applied since the detection will not be possible in microscopy. In that case, the 16S rRNA or functional gene-based approach i.e. PCR amplification with 16S rRNA gene-targeted primers and phylogenetic analysis of the product is an excellent technique. Some ANAMMOX specific FISH probes are used as PCR primers for the specific amplification of the 16S rRNA genes of ANAMMOX organisms. The different PCR primer pairs for the ANAMMOX identification along with its PCR conditions are listed in the Table 4 are used for analyzing the entire group of ANAMMOX organisms. Disadvantages of PCR are low DNA extraction yield and the production of artifacts after PCR. Selection of an appropriate DNA extraction method should be carried out [76]. Real time quantitative PCR (RT-qPCR) and competitive PCR (cPCR) could also be used to find the ANAMMOX bacterial mass based on 16S rRNA or functional genes. qPCR is widely used as it is cheaper when compared to Competitive PCR which has lower accuracy. When compared to FISH, qPCR has higher throughput, more reliability and more sensitive quantification [9,74]. Functional genes like Hydrazine oxidoreductase (hzo) gene can be used as a biomarker in ANAMMOX detection, since it is reported to be an intermediate in ANAMMOX process to dehydrogenate N_2H_4 to convert it into N_2. The different primers targeting the hzo gene is given in the Table 4 along with their operating conditions and target length.

Inhibitor/Stimulator for ANAMMOX activity: The effects on the ANAMMOX activity due to addition of various inhibitors/stimulators are summarized in the Table 5. According to Strous et al. [14], 1gN/L for NH_4^+-N and NO_3^--N respectively, has no effect. Whereas 100 mg/L

Sl No	Probe	Specificity	Sequence	Target site	Formamide concentration (%)	Wash buffer NaCl (mM)	Reference
	Pla46	Planctomycetales	GACTTGCATGCCTAATCC	46-63 [a]	30	112	Neef *et al.* 1998; Kartal *et al.* 2007
	Pla886		GCCTTGCGACCATACTCCC	-	35	-	Neef *et al.* 1998
	Amx368	All ANAMMOX microorganisms	CCTTTCGGGCATTGCGAA	368-385 [a]	15	338	Schmid *et al.* 2003; Kartal *et al.* 2007
	Amx820	Candidatus "Brocadia Anammoxidans"	AAAACCCCTCTACTTAGTGCCC	820-841 [a]	25 40 25	159 56 56	Egli *et al.* 2001; Schmid *et al.* 2000; Mobarry *et al.* 1996
	Amx432		GTTAACTCCCGACAGTGG	-	40	-	Schmid *et al.* 2000
	Amx997		TTTCAGGTTTCTACTTCTACC	-	20	-	Schmid *et al.* 2000
	Amx1240		TTTAGCATCCCTTTGTACCAACC	-	60	14	Egli *et al.* 2001
	Apr 820	Candidatus "Anammoxoglobus propionicus"	AAACCCCTCTACCGAGTGCCC	820-840 [a]	40	56	Kartal *et al.* 2007
	Kst1273	Candidatus "Kuenenia stuttgartiensis"	TCGGCTTTATAGGTTTCGCA	-	25	159	Schmid *et al.* 2000; Egli *et al.* 2001

[a](16S rRNA position E.Coli numbering)

Table 3: Probes used in FISH technique for ANAMMOX identification.

Sl No	Probe	Specificity	Sequence	Condition (°C)	Formamide concentration (%)	Wash buffer NaCl (mm)	Reference
	Pla46F	Planctomycetales	GACTTGCATGCCTAATCC	58 46-63	25	159	Neef *et al.* 1998; Schmid *et al.* 2005
	Pla886	Isosphaera, Gemmata, Pirellula, Planctomyces	GCCTTGCGACCATACTCCC	-	30	112	Neef *et al.* 1998; Schmid *et al.* 2005
	First run PCR PLA46F& PLA886R Second run PCR P338F & P518R	Planctomycetales	-	56 63	-	-	Pynaert *et al.* 2003
	PLA40F & P518R P338F, P338-IIF & P518R		-	60 53	-	-	Pynaert *et al.* 2003
	Pla46F 1390R		GGATTAGGCATGCAAGTC ACGGGCGGTGTGTACAA	59	-	-	Li *et al.* 2009
	Pla46F Amx667R	16S rRNA ANAMMOX	GGATTAGGCATGCAAGTC ACCAGAAGTTCCACTCTC	57 56	-	-	Neef *et al.* 1998; Yapsakli *et al.* 2011
	Pla46F Amx368R	All ANAMMOX microorganisms	GACTTGCATGCCTAATCC CCTTTCGGGCATTGCGAA	59 (c300) 52 (c323)	-	-	Schmid *et al.* 2003; Li *et al.* 2010
	Amx368F/R		CCTTTCGGGCATTGCGAA	56	15	338	Schmid *et al.* 2003; Kartal *et al.* 2007
	Amx809F	ANAMMOX bacteria	GCCGTAAACGATGGGCACT	c809-826	-	-	Tsushima *et al.* 2007
	Amx818F		ATGGGCACTMRGTAGAGGGGTTT	c818–839	-	-	Tsushima *et al.* 2007
	Amx1066R		AACGTCTCACGACACGAGCTG	c1047–1066	-	-	Tsushima *et al.* 2007
	Amx808F Amx1040R	AnAOB 16S rRNA gene	ARCYGTAAACGATGGGCACTAA CAGCCATGCAACACCTGTRATA	60	-	-	Li *et al.* 2009
	Amx694F Amx960R	ANAMMOX bacteria	GGGGAGAGTGGAACTTCGG GCTCGCACAAGCGGTGGAGC	c694–713 c960–979	-	-	Jetten *et al.* 1999
	Brod541F Brod1260R		GAGCACGTAGGTGGGTTTGT GGATTCGCTTACCTCTCGG	60 (c720)	-	-	Li *et al.* 2010
	Brod541F Amx820R		GAGCACGTAGGTGGGTTTGT AAAACCCCTCTACTTAGTGCCC	59 (c280)	-	-	Li *et al.* 2010
	Pla46F Amx820R	Candidatus "Brocadia Anammoxidans"/ Candidatus Kuenenia	GACTTGCATGCCTAATCC AAAACCCCTCTACTTAGTGCCC	59 (c780) 52 (c775)	-	-	Schmid *et al.* 2000; Li *et al.* 2010
	Amx368F Amx820R		TTCGCAATGCCCGAAAGG AAAACCCCTCTACTTAGTGCCC	c368–385 c820–841	-	-	Schmid *et al.* 2003; Schmid *et al.* 2000
	Amx820 F/R		AAAACCCCTCTACTTAGTGCCC	56	25 40	159 56	Egli *et al.* 2001; Schmid *et al.* 2000
	Amx1900		CATCTCCGGCTTAACAA	-	30	112	Schmid *et al.* 2000; Schmid *et al.* 2001
	Kst0288	Candidatus "Kuenenia stuttgartiensis"	GCGCAAAGAAATCAAACTGG	-	10	450	Schmid *et al.* 2001
	Kst0193		CAGACCGGACGTATAAAAG	-	10	450	Schmid *et al.* 2001
	Kst0077		TTTGGGCCACACTCTGTT	-	10	450	Schmid *et al.* 2001
	Kst0031		ATAGAAGCCTTTTGCGCG	-	10	450	Schmid *et al.* 2001
	Kst1275		TCGGCTTTATAGGTTTCGCA	-	25	159	Schmid *et al.* 2000
	Kst0157		GTTCCGATTGCTCGAAAC	-	25	159	Schmid *et al.* 2001, Schmid *et al.* 2005
	Ban0389	Candidatus "Brocadia Anammoxidans"	GGATCAAATTGCTACCCG	-	10	450	Schmid *et al.* 2001
	Ban0222		GCTTAGAATCTTCTGAGGG	-	10	450	Schmid *et al.* 2001
	Ban0108		TTTGGGCCCGCAATCTCA	-	10	450	Schmid *et al.* 2001
	Ban0071		CCCTACCACAAACCTCGT	-	10	450	Schmid *et al.* 2001
	Amx1240		TTTAGCATCCCTTTGTACCAACC	-	60	14	Schmid *et al.* 2000
	Amx1154		TCTTGACGACAGCAGTCT	-	20	225	Schmid *et al.* 2000
	Amx1015		GATACCGTTCGTCGCCCT	-	60	14	Schmid *et al.* 2000
	Amx0997		TTTCAGGTTTCTACTTCTACC	-	20	225	Schmid *et al.* 2000
	Amx0613		CCGCCATTCTTCCGTTAAGCGG	-	40	56	Schmid *et al.* 2000
	Amx0432		CTTAACTCCCGACAGTGG	-	40	56	Schmid *et al.* 2000
	Amx0223		GACATTGACCCCTCTCTG	-	40	56	Schmid *et al.* 2000
	Amx0156		CGGTAGCCCAATTGCTT	-	40	56	Schmid *et al.* 2000
	Ban0162		CGGTAGCCCCAATTGCTT	-	40	56	Schmid *et al.* 2000
	BS820R	Candidatus "Scalindua wagneri / sorokinii"	TAATTCCCTCTACTTAGTGCCC	56	40	56	Schmid *et al.* 2000

	Scabr1114R	Candidatus "Scalindua brodae"	CCCGCTGGTAACTAAAAACAAG	56	20	225	Schmid *et al.* 2003; Schmid *et al.* 2005
	Sca1309R	Candidatus "Scalindua"	TGGAGGCGAATTTCAGCCTCC	56	5	675	Schmid *et al.* 2003; Schmid *et al.* 2005
	Targeting hzo gene						
	hzocl1F1 hzocl1R2	AnAOB hzo gene	TGYAAGACYTGYCAYTGG ACTCCAGATRTGCTGACC	50C (c470)	-	-	Schmid *et al.* 2008
	hzocl1F2 hzocl1R2		TGYAAGACYTGYCAYTGGG ACTCCAGATRTGCTGACC	53C (c470)	-	-	Schmid *et al.* 2008
	Ana-hzo 1F Ana-hzo 2R		TGTGCATGGTCAATTGAAAG ACCTCTTCWGCAGGTGCAT	53 (c1000)	-	-	Li *et al.* 2010
	hzoF1h hzoR1		TGTGCATGGTCAATTGAAAG CAACCTCTTCWGCAGGTGCATG	53 (c1000)	-	-	Li *et al.* 2010
	Ana-hzo1f Ana-hzo2r		TGTGCATGGTCAATTGAAAG ACCTCTTCWGCAGGTGCAT	53	-	-	Li *et al.* 2010

[c]PCR product length (-bp)

Table 4: Probes used in PCR for ANAMMOX identification.

Sl No	ANAMMOX activity inhibitor /stimulator	Mode of action	Concentration or period tested	Effect	Reference
	Ammonium	-	1gN/L	No effect	Strous *et al.* 1999; Bettazi *et al.* 2010
			55mM	50% inhibition	Dapena-Mora *et al.* 2007; Bettazi *et al.* 2010
			>70 mgN/L	Free ammonia inhibition	Jung *et al.* 2007; Bettazi *et al.* 2010
			13-90 mg NH_4^+-N/L	Negative effect	Waki *et al.* 2007; Bettazi *et al.* 2010
			90 mg NH_4^+-N /L	No effect	Bettazi *et al.* 2010
	Nitrate	-	1gN/L	No effect	Strous *et al.* 1999; Bettazi *et al.* 2010
			45 mM	50% inhibition	Dapena-Mora *et al.* 2007; Bettazi *et al.* 2010
			57 mg NO_3^--N/L	No effect	Bettazi *et al.* 2010
	Nitrite	-	100 mgNO_2^-N/L	Complete inhibition	Strous *et al.* 1999; Bettazi *et al.* 2010
			>13.2 mM	No activity	Egli *et al.* 2001; Bettazi *et al.* 2010
			25 mM	50% inhibition	Dapena-Mora *et al.* 2007; Bettazi *et al.* 2010
			70 mgNO_2^-N/L	Activity decrease	Jung *et al.* 2007; Bettazi *et al.* 2010
			>100 mgNO_2^-N/L	Inhibition	Lopez *et al.* 2008; Bettazi *et al.* 2010
			60 mgNO_2^-N/L (spiked)	Activity decrease	Bettazi *et al.* 2010
			>30 mgNO_2^--N/L (long exposure)	Activity decrease	Bettazi *et al.* 2010
	No biomass	None	0 mg/L	No activity	Jetten *et al.* 1999
	Sterilization at 121ºC	Denaturation	20-120 min	No activity	Jetten *et al.* 1999
	Gamma irradiation	Inactivation	60 min	No activity	Jetten *et al.* 1999
	Penicillin V	Inhibition of cell wall synthesis of bacteria	0-100 mg/L	None	Jetten *et al.* 1999
	Penicillin G	-	0-1000 mg/L	None	Jetten *et al.* 1999
	Bromoethane sulfonic acid	Inhibition of methanogenesis	0-20 mM	None	Jetten *et al.* 1999
	Na_2SO_4	Stimulation of sulphate reduction	20 mM	None	Jetten *et al.* 1999
	Na_2MoO_4	Inhibition of sulphate reduction	20 mM	None	Jetten *et al.* 1999
	Chloramphenicol	Inhibition of protein synthesis	0-400 mg/L	None	Jetten *et al.* 1999
	Hydrazine	Inhibition of NH2OH oxidation	0-3 mM	Activation	Jetten *et al.* 1999
	Acetone	Solvent for N-serve	10 mM	None	Jetten *et al.* 1999
	N-serve	Inhibition of nitrification	0-50 mg/L	None	Jetten *et al.* 1999
	Allylthiourea	Inhibition of nitrification	0-10 mM	None	Jetten *et al.* 1999
	Acetylene	Inhibition of nitrification and denitrification	6 mM	Inhibition	Jetten *et al.* 1999
	2,4-Dinitrophenol	Uncoupler	0-400 mg/L	Inhibition	Jetten *et al.* 1999
	Carbonyl cyanide m-chlorophenylhydrazone	Uncoupler	0-40 mg/L	Inhibition	Jetten *et al.* 1999
	$HgCl_2$	Cell damage	0-300 mg/L	Inhibition	Jetten *et al.* 1999
	Oxygen	Oxidative stress	0-0.2 mM	Inhibition	Jetten *et al.* 1999

	Phosphate	Chelating agent	<1 mM	None	Jetten *et al.* 1999; Dapena-Mora *et al.* 2007
			>2 mM	Inhibition	Jetten *et al.* 1999
			5 or 50 mM	Loss of activity	Van de Graaf *et al.* 1996; Dapena-Mora *et al.* 2007
			21 mM	50% inhibition	Dapena-Mora *et al.* 2007
	Sulphide	-	1 or 5 mM	Increase	Van de Graaf *et al.* 1996; Dapena-Mora *et al.* 2007
			0.3 mM	50 % inhibition	Dapena-Mora *et al.* 2007
	Chloride	-	50 mM	No effect	Van de Graaf *et al.* 1996; Dapena-Mora *et al.* 2007
			200 mM	50% inhibition	Dapena-Mora *et al.* 2007
	Acetate	-	1 or 5 mM	Increase	Van de Graaf *et al.* 1996; Dapena-Mora *et al.* 2007
			39 mM	50% inhibition	Dapena-Mora *et al.* 2007
			50 mM	70% of inhibition	Dapena-Mora *et al.* 2007
	Oxygen	-	0.5%	Reversibly inhibited	Van Dongen *et al.* 2001
			0.06 mg/L	Reversibly Inhibition	Paredes *et al.* 2007
			1 µM (>18 % oxygen saturation)	Irreversibly Inhibition	Zhang *et al.* 2008
	Organic matter	-	300 mg COD/L	Inactivation	Chamchoi *et al.* 2008
	Methanol	-	<1 mM	Inhibition	Guven *et al.* 2004 Kartal *et al.* 2004
	Ethanol	-	<1 mM	Inhibition	Guven *et al.* 2004; Kartal *et al.* 2004

Table 5: Inhibitor/Stimulator for ANAMMOX activity.

of NO_2^--N cause complete inhibition. Biomass plays an important role in the ANAMMOX process; if no biomass available then the activity was not observed [79]. When subjecting the biomass with gamma radiation or sterilization, no change in the NH_4^+-N or NO_2^--N was noticed showing no activity until the 60-120 min. Around 10-20 mM concentration of the compounds such as Penicillin V, Penicillin G, Bromoethane sulfonic acid, Sodium sulphate, Sodium molybdate, Chloramphenicol, Acetone and Allylthiourea has no effect on the ANAMMOX activity [79]. But with chemical compounds such as Methanol, Ethanol and sulphide even with very low concentrations of 1mM has inhibition over ANAMMOX activity. The Oxygen from 0.2 to 200mM has an oxidative stress on the ANAMMOX bacteria [21,80]. 50 mM of acetate resulted in 70% of inhibition in the ANAMMOX process [15]. Phosphate concentrations higher than 180 mg/L and NH_3 with higher concentration inhibit ANAMMOX activity [23]. Trace amounts of either of the ANAMMOX intermediates N_2H_4 (>1.4 mgN/L) and NH_2OH (>0.7 mgN/L) can activate the ANAMMOX process [14,18,45,81].

Conclusions

Some of the challenges facing successful ANAMMOX startup and development include slow growth rate, operational difficulty and long startup time. With dedicated enrichment and cultivation techniques the sensitivity of ANAMMOX bacteria to inhibitory concentrations of NO_2^--N, Alkalinity, O_2 etc., could be minimized and ANAMMOX bacteria could be successfully developed to yield sustained NH_4^+-N removal. With the key parameters such as the source of seed, type of reactors used, operational strategy, experimental conditions and molecular signatures such as PCR, FISH, SEM and the inhibitors and affinity factors being monitored and optimized, ANAMMOX startup and development could be deemed successful.

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