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Enrichment of anaerobic ammonium oxidation (anammox) bacteria for short start-up of the anammox process: a review

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ABSTRACT

The application of the anaerobic ammonium oxidation (anammox) reaction in a biological nitrogen removal system to treat wastewater has become of great interest since its discovery. The anammox reaction is performed by anammox bacteria that belong to the Planctomycete phylum. The reaction occurs in the presence of ammonium using nitrite as the substrate under anaerobic conditions. However, the bacteria have an extremely slow growth rate and stringent metabolic conditions that cause difficulty in culturing and applying the system for wastewater treatment. Anammox enrichment has a long start-up period for the anammox process that hinders researchers using laboratory and full-scale systems for the first time. Many attempts have been made to culture anammox to establish a successful anammox culture with a shorter start-up period for the anammox reaction and high nitrogen removal activity. This paper reviews previous studies on anammox enrichment with emphasis on (i) inoculum selection, (ii) enrichment techniques and (iii) factors influencing anammox enrichment. This review will assist researchers in planning and designing an appropriate anammox enrichment. The findings should widen the application of anammox in biological nitrogen removal systems for nitrogenous wastewater.

Keywords: Anaerobic ammonium oxidation (anammox); Enrichment; Start-up; Biological nitrogen removal; Nitrogenous wastewater

1. Introduction

The excess amount of ammonium in wastewater (e.g. landfill leachate, wastewater from seafood processing industries, slaughterhouse wastewater, fertilizer manufacturing wastewater and tannery wastewater [1–3]) is a potential hazard to the environment. The discharge of untreated wastewater with a high ammonium concentration into the receiving waters can harm aquatic life because the presence of the substance may lead to serious problems, including oxygen depletion and eutrophication [4,5]. Therefore, treating wastewater by removing ammonium from

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landfill leachate is considered to be an environmentally crucial step before the water can be safely discharged. During the nitrogen removal process, ammonium is removed through either biological or physicochemical processes. The conventional biological treatment process includes two steps of nitrification and denitrification that are performed by ammonia oxidizing and nitrite oxidizing bacteria. Nitrification is the oxidation of ammonium to nitrite and, subsequently, nitrate under aerobic conditions, whereas the denitrification process involves the conversion of nitrite and nitrate into free nitrogen under anoxic conditions.

At the end of the 1990s, a novel process for treating ammonium in wastewater was discovered in the Netherlands. The process involved the anaerobic conversion of ammonium into nitrogen using nitrite as the electron acceptor [6]. The process was performed by anaerobic bacteria termed anaerobic ammonium oxidizing bacteria (anammox). The anammox reaction was found to be more cost effective than the conventional nitrification–denitrification system due to lower sludge production and no requirement for aeration [7]. Its prominent potential for wastewater treatment has stimulated research interest in studying and developing anammox for biological nitrogen removal from wastewater.

However, anammox involves a slower growing bacterium that is difficult to culture, thereby limiting its application in wastewater treatment systems. Due to the extremely slow growth rate of the microorganism, the start-up of anammox activity in a reactor takes a long time [8]. Several studies reported that the doubling time of this microorganism is approximately 10–12 d under optimal conditions [9–12]. The slow growth rate of anammox is an advantage for the lower production of sludge; nevertheless, it causes difficulties in enrichment and contributes to the long anammox start-up period [7]. The start-up of the anammox process in the bioreactor is usually time-consuming and may take from months to years [13].

Among the major challenges contributing to the long start-up of the anammox process are (i) inappropriate selection of seed sludge, (ii) biomass washout during reactor operation and (iii) substrate inhibition and toxicity. Therefore, many studies have focused on developing techniques for culturing the bacteria with a shorter start-up period (either in a serum bottle or in a laboratory scale bioreactor) to establish a successful anammox culture. The duration of the start-up period of the anammox process is influenced by the proper selection of seed sludge, reactor type and optimal operational conditions [14]. This paper discusses previous studies on anammox enrichment with an emphasis on the appropriate selection of inocula, enrichment techniques and factors that influence the process. This paper will benefit researchers in planning and designing an appropriate anammox enrichment with a short start-up period and high anammox activity.

2. Anaerobic ammonium oxidation (anammox) process

2.1. The discovery of anammox

The anammox process was first revealed in Delft, the Netherlands, when the ammonium in denitrifying fluidized bed reactor (FBR) effluent was found to be reduced following an increase in the production of nitrogen gas during reactor operation [6]. This process was performed by an anaerobic ammonium oxidation (anammox) bacteria belonging to the Planctomycetes phylum. Following the first discovery, more findings of anammox activity were reported worldwide. Most of the anammox were identified in wastewater treatment plants that operate using nitrogenous wastewater [15].

Anammox bacteria occupy a deep branching group within phylum Planctomycetales [16]. To date, 14 species of anammox have been successfully identified; these species are divided into six *Candidatus* genera. As shown in Table 1, all of the genera belong to the *Brocadiales* order. Nevertheless, none of the species have been successfully cultivated in pure culture. The "*Candidatus*" status corresponds to prokaryotes that have not yet been isolated in pure culture; therefore, a phenotypic study and sequence analysis were used to separate the bacteria from other prokaryotic organisms [17]. The *Brocadia, Kuenenia, Jettenia, Anammoxoglobus* and *Anammoxomicrobium* genera were discovered in both wastewater treatment plants and freshwater [18–24]. The genus *Scalindua* has commonly been identified in marine environments worldwide [23], as well as in some wastewater treatment plants [25] and freshwater [26].

2.2. Cell structure and physiology of anammox

The anammox cell morphology observed under scanning electron microscopy (SEM) is a highly compact sphere. The sphere has a diameter in the range of 0.6–1.0 μm when observed under a transmission electron microscope (TEM) [27,28]. The compartmentalization of anammox bacteria has been studied in detail. The anammox cytoplasm comprises three separate compartments: (i) paryphoplasm (outermost), (ii) riboplasm (surrounded by an intracytoplasmic membrane)

Table 1
Taxonomy of anammox bacteria

Genus	Species	Source	Ref.
Brocadia	'*Candidatus*Brocadia anammoxidans'	Wastewater	[18]
	'*Candidatus* Brocadia sinica'	Wastewater	[113]
	'*Candidatus* Brocadia fulgida'	Wastewater	[19,131]
Kuenenia	'*Candidatus* Kuenenia stuttgartiensis'	Wastewater freshwater	[20,23,132]
Jettenia	'*Candidatus* Jettenia asiatica'	Wastewater	[21,133,134]
	'*Candidatus* Jettenia caeni'	Wastewater	[135]
Anammoxoglobus	'*Candidatus* Anammoxoglobus propionicus'	Synthetic wastewater	[22]
Scalindua	'*Candidatus* Scalindua brodae'	Wastewater (marine)	[129]
	'*Candidatus* Scalindua wagneri'	Wastewater (marine)	[129]
	'*Candidatus* Scalindua sorokinii'	Sea water	[129,136]
	'*Candidatus* Scalindua marina'	Marine sediments	[137]
	'*Candidatus* Scalindua profunda'	Marine	[23]
	'*Candidatus* Scalindua arabica'	Marine (Arabian sea)	[138]
Anammoxomicrobium	'*Candidatus* Anammoxomicrobium moscowii'	Wastewater sludge	[24]

and (iii) anammoxosome (tubule-like structure) [28]. Based on electron microscopy, it has been observed that the cell wall of an anammox cell lacks peptidoglycan; this characteristic is common for all Planctomycetes and differentiates them from other archaeal wall types [17]. For this reason, the anammox bacteria are neither Gram positive nor Gram negative in terms of structural cell wall types.

The anammoxosome is a unique ultrastructural compartment surrounded by a single membrane that acts as the reaction site of anammox catabolism for ATP synthesis [28]. The single membrane surrounding the anammoxosome contributes to the uniqueness of the anammox bacteria. The membrane is incorporated with impermeable ladderane lipids that prevent diffusion and confine anammox intermediates (hydrazine and hydroxylamine) in the anammoxosome [29], thereby protecting the cell from toxic intermediates [30]. The anammoxosome occupies approximately 51–85% of the total cell volume of an anammox cell [27,28].

The anammox bacteria perform a chemolithoautotrophic bioconversion process that involves the oxidation of ammonium (NH_4^+) in the presence of nitrite (NO_2^-) as an electron acceptor and produces dinitrogen gas as the end product under anaerobic conditions (Eq. (1)) [10,31].

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (1)$$

The stoichiometry of the anammox reaction that involves the conversion of NH_4^+ and NO_2^- into free nitrogen (N_2) and nitrate (NO_3^-) is shown in Eq. (2) [32]. The ratio of ammonium consumption to nitrite consumption to nitrate production is 1:1.146:0.161.

$$1NH_4^+ + 1.146NO_2^- + 0.071HCO_3^- + 0.057H^+ \rightarrow 0.986N_2 + 0.161NO_3^- + 0.071CH_{1.74}O_{0.31}N_{0.20} + 2.002H_2O \quad (2)$$

The anammox bacteria have been observed in both sessile (aggregated biofilm) and planktonic lifestyles (single cells) [33]. However, the reason for the change between these two transitional states is unknown. During anammox bacteria enrichment, a very low concentration of nitrite (<10 mM) is added during the starting process because nitrite concentrations >10 mM are capable of reducing anammox metabolism; indeed, 20 mM nitrite will completely inhibit the process [34]. The sensitivity and tolerance of anammox bacteria to a particular inhibitor is dependent on the species type and exposure time. The physiological characteristics of anammox bacteria are tabulated in detail in Table 2. The operating temperature, pH, nitrite concentration, dissolved oxygen and phosphate concentration are among the factors that may affect anammox activity during the enrichment process; hence, they will determine the start-up period required for anammox activity. Anammox activity is also negatively affected by light exposure; a decrease from 30 to 50% in anammox activity was observed when light was available [35].

2.3. Application of anammox

The application of the anammox process in biological nitrogen removal systems in wastewater treatment

Table 2
Physiological characteristics of *Brocadia anammoxidans*, *Brocadia sinica*, *Kuenenia stuttgartiensis*, *Scalindua* sp., and *Jettenia caeni* modified from Ref. [33]

Physiological characteristics	*Brocadia anammoxidans*	*Brocadia sinica*	*Kuenenia stuttgartiensis*	*Scalindua* sp.	*Jettenia caeni*
Temperature (℃)	20–43	25–45	25–37	10–30	20–42.5
pH	6.7–8.3	7–8.8	6.5–9	6.0–8.5	6.5–8.5
Growth rate	0.0027	0.0041	0.0026–0.0035	0.0020	0.002
Doubling time (days)	10.7	7	8–11	–	–
Tolerance					
Nitrite (mM)	7	<16	13, 25	7.5	11
Dissolved oxygen (uM)	<1	<63	<200	–	–
NaCl (mM)	–	<500	200	–	–
Phosphate (mM)	–	–	20	–	–
Ref.	[10,18,99,139]	[113]	[69,77]	[115]	[135]

is developing rapidly. The system has been successfully applied for the treatment of various types of nitrogenous wastewater, such as landfill leachate [36], digester liquor [37], pig manure effluents [38] and turtle breeding wastewater [39]. From the environmental and economic perspectives, the anammox process has several advantages over the conventional nitrification and denitrification system. This process demands lower cost and no additional organic carbon and is effective in reducing sludge production. According to previous studies, the anammox process can lower aeration by 63% and sludge production by 80–90% [40,41]. Furthermore, the application of the anammox process to nitrogen removal systems is believed to be capable of reducing the operational costs by up to 90% [16].

Anammox is a substrate-dependant process in which sufficient amounts of ammonium and nitrite must be provided. Ammonium and nitrite are utilized in a ratio of 1:1.146 in a single anammox reaction [32]. In contrast to ammonium, nitrite is not common in wastewater. Therefore, a nitrification step is required to first convert ammonium into nitrite to obtain a proper ammonium-to-nitrite ratio. Alternatively, nitrite can be produced by the partial nitrification process. The partial nitritation–anammox system can be operated either in one reactor or in two separate reactors that run in series. Due to the presence of both aerobic ammonium oxidizing bacteria (AOB) and anammox bacteria, the combination of partial nitrification and the anammox process in a single reactor has been termed the CANON (completely autotrophic nitrogen removal over nitrite) process [42]. This process is very sustainable because it does not require organic carbon, it can lower the energy required for aeration by 40%, and it produces almost no sludge [42]. The stoichiometry of the process is described in Eq. (3):

$$NH_4^+ + 0.85O_2 \rightarrow 0.435N_2 + 0.13NO_3^- + 1.3H_2O + 1.4H^+ \quad (3)$$

The SHARON (single reactor system for high rate ammonium removal over nitrite) process also applies a system involving a partial nitrification process. In this process, AOB convert ammonium into nitrite rather than nitrate (Eq. (4)). SHARON is commonly used to treat high ammonium-containing wastewater.

$$NH_4^+ + 0.75O_2 + HCO_3 \rightarrow 0.5NO_2^- + 0.5NH_4^+ + CO_2 + 1.5H_2O \quad (4)$$

The end product of SHARON (nitrite) will be subsequently fed to anammox in the SHARON-anammox combined process. The combination of the SHARON-anammox process is believed to result in a simple process with low operational costs because it demands low oxygen, has lower alkalinity consumption and does not need organic carbon [43]. For successful anammox application, the aerobic step in which the partial oxidation of ammonium is performed to produce nitrite is necessary prior to the anammox reaction, where nitrite serves as the electron acceptor and reacts with the remaining ammonium to produce free nitrogen [44]. The combination of the partial nitritation/anammox system in a single bioreactor was accomplished by suppressing nitrate formation by controlling the operating pH. The pH was controlled by maintaining the alkalinity <8 and the dissolved oxygen <0.06 mg O/(mg N d) to enhance nitritation and inhibit nitratation [45].

3. Selection of inocula for anammox enrichment

3.1. Inoculum source

Selecting the appropriate seeding sludge or culture starter as the inoculum is the crucial step for the prompt establishment of a successful enrichment reactor with a rapid start-up of anammox activity [21,46,47]. Anammox bacteria have been reported to survive in a habitat that can provide a concurrent supply of ammonium and nitrite. This type of environment is usually found in the aerobic–anaerobic interface of sediments or water bodies [48]. Various types of seeding sludge have been used as the inoculum for fast start-up of the anammox reactor. These include nitrifying sludge [11], denitrifying sludge [35], activated sludge [14,46,49], anammox biomass [13], [50], mature granular anammox sludge [51], nitritation sludge [52], a mixture of more than one type of seeding sludge [14,53,54] and marine sediments [47,55,56]. Table 3 shows the source of the inocula used as culture starters during the enrichment process in fast anammox start-ups. Reportedly, the inoculation of mature anammox granules for anammox enrichment in an up-flow anaerobic sludge blanket (UASB) reactor successfully accelerated the start-up period of the anammox reaction, resulting in the start of the anammox process within two weeks of enrichment [51]. Anammox granules have an advantage over flocculent anammox sludge in terms of organic matter tolerance, sludge retention and the creation of a favourable habitat for anammox [57,58]. These factors might represent reasons for the fast anammox start-up following the use of sludge as the inoculum. The most common anammox reactor start-up practice uses active anammox biomass from operating anammox reactors as the culture starter. However, this process limits the application of the anammox system for the full-scale treatment of nitrogen-rich wastewater [59].

3.2. Molecular detection of anammox for inoculum selection

Due to the extremely slow growth rate of anammox, none of the anammox species have been successfully isolated and cultivated in pure cultures. As a consequence, culture-independent methods (specifically molecular techniques) have become the most widely used approaches for anammox identification [15]. The techniques used to detect anammox include (i) polymerase chain reaction-based methods (PCR) [60], (ii) denaturing gradient gel electrophoresis (DGGE) [61] and (iii) fluorescence *in situ* hybridization (FISH) [21,60].

3.2.1. Polymerase chain reaction (PCR)

PCR is a molecular method in which million copies of new DNA strands that are complementary to the template DNA are synthesized by amplification. The amplification step relies on the use of a specific primer to which the first nucleotide can bind. The amplified region is based on the primers used because they determine the binding site for the first nucleotide during the initial reaction. The 16S rRNA gene is the most frequent and widely used phylogenetic biomarker in microbial community studies [62]. Anammox identification has been performed by PCR using specific primers for the 16S rRNA region of the anammox bacteria. More recently, a phylogenetic analysis was conducted to identify the bacterial species. Analysis of the 16S rRNA

Table 3
The fast start-up of the anammox process in anammox enrichment according to type of inoculum and reactor used

Reactor type[a]	Seed sludge	Start-up period (d)	TN[b] Removal efficiency (%)	NRR[c] ($kgm^{-3}\ d^{-1}$)	Ref.
UASB	Anammox granule	14	99.29	–	[51]
MBR	Mixed aerobic activated sludge with nitrifying sludge	16	>90	–	[53]
UASB	Anammox biomass	18		0.0183	[50]
SBR	Mixed partial nitritation sludge with anammox sludge	35	–	6.2	[14]
FBR	Mixed aerobic activated sludge with nitrifying sludge	39	89	9.2	[54]
MSBR	Anammox biomass	80	73.6	0.71	[13]

[a]UASB: Up-flow anaerobic sludge blanket, MBR: membrane bioreactor, SBR: Sequencing batch reactor, FBR: Fixed bed reactor, MSBR: Membrane sequencing batch reactor.
[b]TN: Total nitrogen.
[c]NRR: Nitrogen removal rate.

genes together with a phylogenetic study of anammox bacteria revealed that they formed a monophyletic branch in the Planctomycete phylum [62]. A list of primers used in previous studies for anammox identification is provided in Table 4. The PCR method promises a rapid, inexpensive and simple technique for the detection of the presence of anammox bacteria in various types of seeding sludge, especially when the anammox cell counts are too low for FISH. However, the use of the PCR reaction for anammox identification has disadvantages due to PCR biases (i.e. non-specific primer annealing) that can affect the product.

3.2.2. Real-time polymerase chain reaction (rt-PCR)

The molecular method rt-PCR can be used to quantitatively measure uncultured bacteria with fast and reliable results. The principle of rt-PCR is based on continuous monitoring of fluorescence intensity during the PCR reaction. rt-PCR has been successfully used to estimate the doubling time of enriched anammox bacteria from a rotating disk reactor (RDR) biofilm (approximately 3.6–5.4 d) [21]. This method has been reported to be effective for screening anammox in environmental samples due to its sensitivity and speed, particularly in samples with a low abundance of anammox bacteria. The limitation for this application is the demand for special and expensive instruments and reagents [63].

3.2.3. Denaturing gradient gel electrophoresis (DGGE)

A PCR product from an uncultured bacterial sample that targets the 16S rRNA gene may represent different populations of bacteria that exist in the samples. DGGE is a molecular fingerprinting method in which PCR products are separated by agarose gel electrophoresis based on sequence differences that result in many single DNA bands with differential denaturing characteristics. DGGE is an effective technique to determine microbial structural differences in PCR products to study the broad microbial phylogeny of a community. PCR–DGGE uses a GC-clamp primer (GC-clamp added to the 5′ terminus of the forward primer) for amplification prior to gel electrophoresis. DGGE was used in previous studies of anammox samples (i) to detect key microorganisms that contributed to the loss of ammonium and nitrite in batch assay [64], (ii) to analyse bacterial population in the biofilm of the UASB reactor [39] and (iii) to study the microbial population in the anammox reactor [65]. DGGE has been commonly chosen to define phylogenetic relationships among bacteria because the technique can supply precise and abundant information concerning genetic diversity by separating different base-pair sequences (approximately 200–700 base pairs) [66]. However, the sensitivity of DGGE was found to be limited because the produced DGGE bands are usually less than 500 base pairs [66], thereby limiting the DNA sequence information as well as phylogenetic identification.

3.2.4. Fluorescent in situ hybridization (FISH)

Anammox identification by FISH applies the concept of hybridization of a labelled specific oligonucleotide probe with its target DNA sequence prior to detection under fluorescence microscopy. Another

Table 4
List of primers for PCR amplification of the 16S rRNA genes for anammox identification, modified from Ref. [140]

Primer name	Specificity (16S rRNA)	Annealing temperature (˚C)	Sequence (5′–3′)	PCR primer	Ref.
Brod 541	*Scalindua*	60	GAGCACGTAGGTGGGTTTGT	Forward	[20]
Brod 1260	*Scalindua* sp.	60	GGATTCGCTTCACCTCTCGG	Reverse	[20]
Pla46	Planctomycetes	58	GGATTAGGCATGCAAGTC	Forward	[21,141]
Amx 820	*Brocadia, Kuenenia*	56	AAAACCCCTCTACTTAGTGCCC	Forward/Reverse	[21,142]
Amx 368	Anammox	56	CCTTTCGGGCATTGCGAA	Forward/Reverse	[129]
Amx 694[a]	Anammox	60	GGGGAGAGTGGAACTTCGG	Forward	[57]
BS 820	*Scalindua*	56	TAATTCCCTCTACTTAGTGCCC	Reverse	[136]
AMX1066[a]	Anammox	60	AACGTCTCACGACACGAGCTG	Reverse	[21]
AMX809[a]	Anammox	60	GCCGTAAACGATGGGCACT	Forward	[21]
AMX818[a]	Anammox	60	ATGGGCACTMRGTAGAGGGGTTT	Forward	[21]
Amx960[a]	Anammox	60	GCTCGCACAAGCGGTGGAGC	Reverse	[57]

[a]Designed for real-time PCR.

technique in FISH (quantitative fluorescent *in situ* hybridization) allows the quantification of anammox through fluorescent microscopy and the use of an analysis software. FISH is known to be a highly sensitive, simple and rapid molecular technique for bacterial detection because it uses specific fluorochrome-labelled DNA oligonucleotide probes. Its efficiency in identifying environmental microorganisms at any taxonomical level makes it more advantageous over other molecular identification techniques [66]. FISH has been widely applied (i) to determine the microbial community structure of anammox [46,67], (ii) to confirm the presence of anammox bacteria in enriched biomasses [50] and (iii) to define anammox bacteria from an environmental sample in a quantitative or qualitative study [15]. In previous studies, the quantification of anammox via application of the FISH technique was successfully performed for an enriched

Table 5
List of commonly used probes for FISH targeting of the 16S rRNA gene in anammox identification, modified from Ref. [140]

Probe name	Specificity	OPD[a] designation	Sequence (5′–3′)	FA (%)[b]	Ref.
Pla46	Planctomycetales	S-P-Planc-0046-a-A-18	GACTTGCATGCCTAATCC	25/159	[22,69,143]
Amx 368	All anammox organisms	S-*-Amx-0368-a-A-18	CCTTTCGGGCATTGCGAA	15/338	[129]
Amx 820	*Brocadia anammoxidans* *Kuenenia stuttgartiensis*	S-*-Amx-0820-a-A-22	AAAACCCCTCTACTTAGTGCCC	40/56	[69,129]
Apr 820	*Anammoxoglobus propionicus* *Jettenia asiatica*	S-*-Apr-0820-a-A-21	AAACCCCTCTACCGAGTGCCC	40/56	[131]
Amx 1240	*Brocadia anammoxidans*	S-*-Amx-1240-a-A-23	TTAGCATCCCTTTGTACCAACC	60/14	[69,142]
Kst 157	*Kuenenia stuttgartiensis*	S-S- Kst-0157-a-A-18	GTTCCGATTGCTCGAAAC	25/159	[132]
BS 820	*Scalindua wagneri* *Scalindua sorokinii*	S-*-BS-820-a-A-22	TAATTCCCTCTACTTAGTGCCC	40/56	[136]
Sca 1309	*Scalindua*	S-G-Sca-1309-a-A-21	TGGAGGCGAATTTCAGCCTCC	5/675	[129]
Ban 162	*Brocadia anammoxidans*	S-S-Ban-0162 (B.anam.)-a-A-18	CGGTAGCCCCAATTGCTT	40/56	[142]
Amx 156	*Brocadia anammoxidans*	S-*-Amx-0156-a-A-18	CGGTAGCCCCAATTGCTT	40/56	[142]
Bfu 613	*Brocadia fulgida*	S-*-Bfu-0613-a-A-24	GGATGCCGTTCTTCCGTTAAGCGG	30/112	[131]
Scabr 1114	*Scalindua brodae*	S-*-Scabr-1114-a-A-22	CCCGCTGGTAACTAAAAACAAG	20/225	[129]
Kst 1275	*Kuenenia stuttgartiensis*	S-*-Kst-1275-a-A-20	TCGGCTTTATAGGTTTCGCA	25/159	[142]
Amx 223	*Brocadia anammoxidans*	S-*-Amx-0223-a-A-18	GACATTGACCCCTCTCTG	40/56	[142]
Amx 432	*Brocadia anammoxidans*	S-*-Amx-0432-a-A-18	CTTAACTCCCGACAGTGG	40/56	[142]
Amx 997	*Brocadia anammoxidans*	S-*-Amx-0997-a-A-21	TTTCAGGTTTCTACTTCTACC	20/225	[142]
Amx 1015	*Brocadia anammoxidans*	S-*-Amx-1015-a-A-18	GATACCGTTCGTCGCCCT	60/14	[142]
Amx 1154	*Brocadia anammoxidans*	S-*-Amx-1154-a-A-18	TCTTGACGACAGCAGTCT	20/225	[142]
Amx 613	*Brocadia anammoxidans*	S-*-Amx-0613-a-A-22	CCGCCATTCTTCCGTTAAGCGG	40/56	[142]

[a]Oligonucleotide Probe Database.
[b]Formamide concentration for washing buffer.

anammox biomass [15,67,68]. The FISH method was also successfully applied for anammox identification from enrichment cultures based on 16S rRNA-targeting probes [69]. The oligonucleotide probes used for anammox detection in previous studies are provided in Table 5. Despite its advantages, FISH also has limitations in its application. Among the limitations are (i) the reduction of the probe penetration problem that may affect probe assessment, (ii) the difficulty of the use of the method in environmental sampling with low numbers of rRNA molecules per microbial cell and (iii) the formation of dense microbial clusters [70].

4. Anammox enrichment technique

4.1. Anammox enrichment in batch experiments

Anammox enrichment in batch experiments applies the concept of a partially closed system where all of the required nutrients are supplied to the culture at the start of the enrichment. In this system, the gas and pH control solutions are the only materials added and removed during the enrichment process. Typically, anammox batch assays are performed during inhibition studies of anammox activity [71–74] and to determine specific anammox activity (SAA) [75]. However, the batch system is also applicable for anammox culture because '*Candidatus* Kuenenia stuttgartiensis' was reported to have been successfully enriched using the batch method [2]. It is recommended that a batch assay be performed for seeding sludge to test several parameters that indicate the existence of anammox activity. This analysis confirms whether the culture starter performs the anammox reaction and is suitable for the following enrichment process in the bioreactor [21]. During anammox enrichment, a batch experiment is usually conducted on a small scale that is specifically designed for anaerobic culture. It is crucial to maintain the culture under an anaerobic environment during the inoculation as well as throughout the enrichment period. To avoid oxygen contamination at the start, the inoculation work can be performed in an anaerobic chamber and the medium should be sparged using nitrogen or argon gas in advance. The technique of sparging anammox cultures using argon or nitrogen at the start of the culture process has been applied to remove oxygen from batch experiments [2,71,74,75]. The initial concentration of nitrite that is added to the culture needs to be below the inhibition level because bacterial activity is inhibited at nitrite concentrations ≥15.1 mM [72]. The operating conditions for the batch experiments applied in previous studies are summarized in Table 6.

4.2. Anammox enrichment in bioreactor systems

Cultivation of anammox can be successfully established by the enrichment technique using a bioreactor [76]. Indeed, it is possible to produce a dominant anammox culture in which anammox dominates 95% of all organisms in the biomass [77]. However, the frequent challenge in anammox enrichment is the long start-up of anammox activity due to their long doubling time and slow metabolism; hence, they demand a well-designed bioreactor with an effective biomass retaining ability at a low substrate concentration to be suitable for long-term operations [16,33,77]. The reactor configuration during anammox enrichment has been reported to be one of the factors that influence the anammox start-up process [54,67]. An efficient anammox reactor should also provide a highly specific surface area for the reaction to occur. The use of carrier material can increase the total surface area for biomass retention, and thereby increase the volumetric loading rates of anammox activity [11]. In contrast, a reactor with a lower specific surface area can limit the mass transfer of nitrite into the biofilm and lower the capability of anammox to perform the nitrogen removal process. In previous studies, researchers successfully cultivated anammox bacteria in suspended and attached growth types of bioreactors that were specifically designed to address the slow growing bacteria. The suspended-type bioreactors includes the sequencing batch reactor (SBR) [10], [78–81], UASB [49,52,58,76], membrane bioreactor (MBR) [13], anaerobic membrane bioreactor (AnMBR) [5] and the gas-lift reactor [82,83]. The attached growth-type bioreactors, including the FBR [35], rotating biological contactor (RBC) [84] and upflow biofilter (UBF) [85], use supporting material to retain the anammox microbial population. Typical examples of bioreactor configurations used during anammox enrichment are discussed below, and the operating conditions applied for anammox enrichment in bioreactor systems in previous studies are summarized in Table 7.

4.2.1. Sequencing batch reactor (SBR)

SBR is a variation of treatment for activated sludge; it is operated in batch mode equipped with mixing properties, and all of the treatment steps are conducted in a single tank. SBR was reported to be a good system for anammox enrichment because it promotes a homogenous distribution of the biomass in the reactor and prevents nitrite accumulation [10]. A homogenous mixture of substrate and biomass in the

Table 6
Operational conditions of anammox batch experiments

Culture holder	Total volume (mL)	Working volume (mL)	Inoculum volume/culture volume (v/v)	Temperature (℃)	pH	Shaking speed (rpm)	Inoculum	Ref.
Vial	38	25	n.s[a]	30	7.8	150	Enriched anammox biomass	[71,73]
Serum flask	160	100	n.s[a]	30 ± 2	7.4–7.5	115	Enriched anammox biomass	[72]
Serum bottle	140	120	n.s[a]	35 ± 1	7.4–7.6	180	Enriched anammox biomass	[75]
Serum bottle	120	n.s[a]	75%	37	u.c[b]	n.s[a]	Wastewater	[2]
Serum bottle	160	120	12.50%	35 ± 1	7.5 ± 0.2	180	Enriched anammox biomass	[74]

[a]n.s: not stated
[b]u.c: uncontrolled.

reactor is important to prevent substrate inhibition of anammox because the bacteria are negatively affected by high nitrite concentrations [79]. Anammox enrichment has been successfully established using SBR with a fast anammox start-up activity of 60 d and a high removal efficiency of nitrogen (82%) [80]. An experimental set-up of an SBR system that applied biomass retention and a biofilm system provided a stable substrate-limiting enrichment condition and was suitable for long-term cultivation (>1 year) [10]. The use of zeolite particles as carrier material improved the SBR system due to a reduction in biomass washout in the effluent to 3 mg VSS L^{-1} with a specific anammox activity (SAA) of 0.5 g N (g VSS d)$^{-1}$ [86]. A simultaneous denitrification and anammox process in SBR resulted in an efficient nitrogen removal process in which a nitrogen removal efficiency of 97.47% was recorded [81]. The most commonly applied pH and temperature ranges in the SBR operational system were 7.0–8.0 and 30–35℃, respectively. An applied nitrogen loading rate (NLR) of 0.1 to 0.7 kg-N m^{-3} d^{-1} in an SBR operating with anammox allowed the complete removal of nitrite, while a rate of 0.75 kg-N m^{-3} d^{-1} of NLR started to show biomass flotation [87]. A shear effect analysis on anammox revealed that a high SBR stirring speed (approximately ≥ 250 rpm) disturbed anammox activity, contributing to a decrease in anammox granular size and disrupting the efficiency of the system [79]. To avoid the shear stress effect, the SBR stirring speed applied needs to be maintained lower than the level of the maximum mechanical stress that is tolerated by anammox to create an optimum condition during the enrichment process.

4.2.2. UASB reactor

The UASB reactor is an advanced anaerobic technology used to treat wastewater by applying an anaerobic microorganism to the suspended growth system; the system is designed with appropriate separation of gas, liquid and solids to retain granular sludge. The UASB reactor is among the most effective and stable set-ups for culturing anammox. The upflow reactor configuration is a reliable system that provides a good sludge settling capability and produces a high nitrogen removal efficiency, with the achievement of total nitrogen removal of 91.82% [88]. A super high-performance rate of anammox with a total NLR of 74.3–76.7 kg m^{-3} d^{-1} was accomplished in a treatment that applied high-loaded anammox to a UASB reactor [89]. A study was conducted to determine the optimal condition for an efficient anammox process in granular UASB. This study confirmed that an influent substrate concentration of 644 to 728 mg-N L^{-1} with an optimum hydraulic retention time (HRT) of 0.90–1.25 h and upflow velocity of 0.60–1.79 m h^{-1} was the best working conditions for the operation [90]. The optimal conditions of pH and temperature (7.5–7.8 and 32–34℃, respectively), were reported to be responsible for the fast start-up of

Table 7
Operational condition of anammox enrichment in bioreactor systems

Bioreactor configuration	Reactor type[a]	Working volume (L)	Inoculum[b]	HRT (d)	NLR (kg-N m^{-3} d^{-1})	pH	Temperature (°C)	Stirring speed (rpm)	SAA	NRR	Biomass carrier/ membrane material[c]	Ref
Suspended	SBR	15	Anammox biomass	27	–	7.0–8.0	32–33	80 ± 10	–	–	–	[10]
	SBR	1	Anammox biomass	1	0.3	7.5–8.0	30 ± 1	60–250	0.4 g N (g VSS d)$^{-1}$	–	–	[79]
	SBR	5	Anammox biomass	1	0.6–0.6	7.0–8.0	33	150	0.5 g N (g VSS d)$^{-1}$	–	Zeolite	[86]
	SBR	1	Anammox biomass	0.625	0.042–2.0	7.8–8.0	35	70	0.65 g g^{-1} d^{-1}	–	–	[87]
	SBR	2	Anammox biomass	2	0.4	7.0–8.0	33	100	–	–	–	[86]
	UASB	3.2	Activated sludge	–	–	7.5–7.8	32–34	–		–	–	[88]
	UASB	1.1	Anammox biomass	0.00416–0.00667	137.1	6.8–7.0	35 ± 1	–	76.7 ± 4.5 kg-N m^{-3} d^{-1}	–	–	[89]
	UASB	2.8	Anammox biomass	0.026–0.068	–	–	35 ± 1	–	–	10.0 kg m^{-3} d^{-1}	–	[90]
	UASB	3	Return activated sludge	1.167	0.09	–	24	–	–	–	–	[76]
	UASB	6.25	Full-scale UASB granular sludge	1–3.5	–	–	–	–	–	–	–	[91]
	Submerged AnMBR	4	Anammox biomass	0.5–1	–	7.50 ± 0.05	35.0 ± 0.5	50–150	0.56 kg-N (kg VSS)$^{-1}$ d^{-1}	–	Hollow PVDF	[94]
	Submerged MBR	5	Anammox biomass	1	–	~8.0	35	–	–	700 mg L^{-1} d^{-1}	Hollow ultrafiltration fibre	[13]
	Submerged MBR	220	Activated sludge	0.3667	–	–	25 ± 2	–	–	–	Hollow PVDF	[95]
	RFMBR	13	Anammox biomass	–	–	–	33	20	–	–	PVDF	[67]
	MBR	15	Anammox biomass	2	–	7.1–7.5	38	160	–	–	Microfiltration module type Zeeweed	[77]
	AnMBR	15	Anammox biomass	1–3	0.025–5	5.88–8.53	–	100	–	–	–	[5]
	Gas-lift	7	Anammox biomass	–	0.042–2.0	8.0 ± 0.1	30	–	0.9 g g^{-1} d^{-1}	–	–	[87]
	Gas-lift	1.8	Anammox biomass	10	10.7 ± 3	–	–	–	–	8.9 kg N (m^3 reactor)$^{-1}$ d^{-1}	–	[83]
Attached	FBR	2.5	Anammox biomass	0.175	–	7	30–36	–	1,500 nmol $NH_4^+h^{-1}$ (mg VS)$^{-1}$	–	Sand particles	[35]
	FBR	6.0	Anammox biomass	0.063–0.333	–	–	35	30–80	0.32 kg-N (kg VSS)$^{-1}$ d^{-1}	6.6 kg-N m^{-3} d^{-1}	Non-woven material	[12]
	FBR	2.5	Anammox biomass	0.916–1.75	–	8	36	–	–	0.18 kg N (kg VSS)$^{-1}$ d^{-1}	Sand particles	[99]
	RBC	6.2	Anaerobic activated sludge	–	–	–	35 ± 5	–	–	–	Polyvinyl chloride discs	[84]
	UBF	1.2	Activated sludge	0.126–0.863	0.14–0.56	7.5–8.0	30 ± 1	–	–	–	Three dimensional plastic media	[102]
	UBF	8.0	WWTP sludge	0.4–236	34.5	7	35 ± 1	–	–	–	Hollow bamboo balls	[103]

[a]SBR: Sequencing batch reactor; UASB: Up-flow anaerobic sludge blanket; MBR: Membrane bioreactor; RFMBR: rotating flat-sheet membrane bioreactor; AnMBR: Anaerobic membrane bioreactor; RBC: Rotating biological disc; FBR: Fluidized bed reactor; UBF: Upflow biofilter; NRBC: Net-like rotating biological contactor.
[b]WWTP: Wastewater treatment plant.
[c]PVDF: Polyvinylidene fluoride.

anammox enrichment (within two months) with a high nitrogen removal efficiency (91.82%) [88]. The most commonly applied temperature for the UASB operating system ranges from 32 to 36˚C; however, anammox bacteria have been successfully cultivated in an UASB within 4.5 months with a slightly lower temperature of 24˚C (average room temperature) [76]. HRT during the UASB operation can also affect anammox performance, with a longer HRT promising better performance of the anammox depending on the reactor volume and operating NLR. The best performance of 1.1 L in a UASB reactor with an ammonium removal efficiency of 90% was recorded with an HRT longer than 1.58 h and NLR of 10.5 kg-N m^{-3} d^{-1}; however, the performance dropped as the HRT was further decreased to 0.21 h [89]. As a strategy to prevent nitrite accumulation during the initial operation, a 6.25 L UASB was operated at HRTs of 1–5 d and subsequently maintained at 3.5 d when stable anammox activity was achieved [91]. The high volumetric removal rate of anammox in UASB was reported to be the result of the granulation of the anammox biomass in the reactor [92]. The stable configuration of the UASB prevents substrate inhibition due to nitrite concentration shock, and thus, the reactor was capable of contributing to a high biomass concentration [88].

4.2.3. Membrane bioreactor (MBR)

A MBR is another type of suspended bioreactor used for wastewater treatment, particularly in combination with a membrane process. MBRs are categorized into submerged MBRs [93] and external MBRs according to the location of the membrane component. During the membrane process, a membrane material that is impermeable to microbial cells is used for microfiltration to efficiently retain biomass. The most commonly used membrane for the MBR operating system is a fibre membrane with a pore size of 0.2–0.4 μm; the fibre can be either a hollow membrane fibre [13,94,95] or a flat sheet type of membrane [67] according to the reactor configuration. MBR operation via the application of an anaerobic technique also has been reported (AnMBR or Anaerobic Membrane Bioreactor) [5]. A rotating flat sheet membrane bioreactor (RFMBR) is another alternative of the MBR system that was introduced for the anammox process [67]. The RFMBR recorded a stronger shear stress effect at a stirring speed of 20 rpm compared to a conventional MBR with a stirring speed of 60 rpm, most likely due to its rotatable multi-flat sheet structure. A fast anammox start-up (one week) with an enrichment purity of 97.6% was successfully achieved by employing a 15 L MBR and applying a sludge retention time (SRT) of 12 d, stirring speed of 160 rpm and control of the influent nitrite between 100 and 120 mM [77]. The use of MBRs has served as an alternative technique to obtain full biomass retention during anammox enrichment [13]. One study claimed that an MBR yielded high concentrations of mixed liquor suspended solids (MLSS) and reduced excess sludge production [96]. However, the limitation of the application of the MBR systems is the higher cost for membrane material and the membrane fouling action of the submerged MBR [13,67,96]. The membrane fouling problem is probably due to solute-membrane adsorption and the deposition of sludge flocs onto the membrane [96], which promote the loss of membrane permeability [97]. The accumulation and deposition of micro-organisms, colloids solutes, and cell debris on the membrane material leads to an increase in transmembrane pressure or reduction in the permeate flux [96]. The particle and distribution size of the biomass determine the fouling action problem because smaller particles are more likely to cause a much more severe fouling action [93]. A backwashing strategy was applied to the operation of a submerged MBR to resolve the membrane fouling problem by flushing the membrane regularly during backwashing [95].

4.2.4. Gas-lift reactor

A gas-lift reactor is a type of suspended biofilm reactor system that uses an artificial lift technique (i.e. compressed air, gas bubbles, water vapour or other vaporous bubbles) for the purpose of either liquid raising, biomass mixing, maintaining the biomass fluidization state or maintaining the anaerobic condition during reactor operation. An attempt to develop the anammox process in a gas-lift reactor system was performed using a tube (riser) inside the reactor for the bubbling process to provide the biomass with enough turbulence to produce a circular flow of biomass and liquid. Then, 100% argon was used to flush the reactor to maintain the fluidization of the biomass and keep the reactor under anaerobic conditions [87]. The anammox process in the gas-lift reactor with an NLR of 10.7 ± 3 kg-N m^{-3} d^{-1} and HRT of 1 d has contributed to a high nitrogen removal rate (NRR) of 8.9 kg N (m^3 reactor)$^{-1}$ d^{-1}, thereby demonstrating that the reactor configuration can maintain a very good gas–liquid transfer capability and suitable operational conditions for the maintenance and culture of anammox bacteria [83]. Nitrogen gas accumulation caused biomass flotation problems in a gas-lift reactor because the flotation of large clusters of flocs and granules was observed during the operation [87]. Biomass flotation would

subsequently cause biomass washout due to the reduced performance of biomass settling; this is the real problem facing the reactor operation because the upflow and gas-lift reactor system rely on the continued presence of settling granules. Hence, biomass retention is the most important aspect in the operation of these reactors, including the gas-lift type.

4.2.5. Fluidized bed reactor (FBR)

The FBR is an attached growth bioreactor system that employs a fluidization process in which the biomass is passed through a granular solid material (commonly the catalyst) supported by a porous plate (distributor). This is accomplished using sufficient velocity to suspend the solid and allow it to act as a fluid. Controlling the velocity will maintain the biomass particles in a fluidized state and hence promote better mass transfer characteristics compared to the other attached growth system [98]. An operating system of an anammox process in a 2.5 L FBR was conducted under the preferred anammox operational conditions confirmed by the batch test: 36˚C and pH 7 [35]. In a similar study, the recirculation of anoxic fluid from the reactor top was achieved during reactor operation with a flow rate of 47 L h^{-1} to keep the bed fluidization state at a superficial liquid velocity of 24 m h^{-1}. Microorganisms that grow as a biofilm attached to small carrier particles remain fluidized in the reactor. Commonly, the employment of FBR for the anammox process has used sand particles with a diameter of 0.3–0.6 mm [35,99] and non-woven fabric material [12] as the carrier materials for biomass attachment. The FBR is commonly designed in large volumes to provide the reactor with a sufficient flow rate to suspend the catalyst particles. It was reported that a FBR could fasten the formation of anammox granules and thus reduce suspended biomass washout during the operation [12]. Anammox granule formation serves as a complete mixed-bulking biomass and hence could enhance the substrate transfer process for anammox activity [12]. The system is suitable for the application of slow growing bacteria to speed the reactor start-up, especially in the anammox case. Following treatment with anammox in a 2.5 L FBR, the sludge digestion effluent recorded a specific total NRR of 0.18 kg-N (kg VSS)$^{-1}$ d^{-1} [99]. However, an unstable fluidization process may contribute to excessive microorganism loss and subsequently lead to a reduction in the nitrogen removal efficiency [12,99]. The cost of constructing and maintaining the system is high and thus limits its application.

4.2.6. Rotating Biological Contactor (RBC)

The RBC system is an alternative to the activated sludge process that has been called a disc, surface, media and biofilm reactor [100]. RBCs consist of parallel packs of rotating discs with attached microorganisms on their surfaces in the form of biofilms. These microorganisms will react with the wastewater during the reactor operation. The employment of RBCs for anammox enrichment has been successfully performed in a 6.2 L reactor using 13 polyvinyl chloride discs with a total surface area of 0.32 m^2, resulting in 87% submersion of the disk surface [84]. A reactor start-up that used conventional anaerobic activated sludge as the inoculum and operated at 35˚C recorded a high influent nitrogen removal of 92.1% at the highest influent surface load of 12 g m^{-2} d^{-1}. In the biofilm system, the compound mass transfer is the major variable that determines the microbiology of the RBC; it is influenced by operational parameters, biofilm structure, biofilm attachment/detachment mechanisms and the boundary layer thickness of the biofilm [101]. The RBC system has been widely used for anaerobic wastewater treatment; however, laboratory-scale experiments involving the anammox process are limited.

4.2.7. Upflow Biofilter (UBF)

The UBF is another bioreactor system used in wastewater treatment with an operating system that involves the flow of wastewater through a sediment basin of packing material/medium inside the reactor in an upward motion. The packing material provides surface area for biofilm attachment to perform the nitrogen removal process and promotes biomass retention of the slowly growing bacteria [102]. The UBF system has been successfully applied for the anammox process using conventional wastewater treatment plant sludge as the inoculum; the system was operated at pH 7.0–8.0 and 30–36˚C [85,103]. The anammox process in the UBF system showed a high NH_4^+-N removal efficiency of 89% at the optimum HRT (>4.6 h); however, further decreases in the HRT showed a drop in the removal efficiency [102]. The formation of anammox granules and biofilms, the appropriate effluent recirculation and the relatively high operating temperature (35˚C) in the UBF system with hollow bamboo balls as the biomass carriers contributed to the anammox enrichment process and promoted higher SAA at a high NLR of 34.5 kg-N m^{-3} d^{-1} [103]. In a comparative study of the SBR and UBF systems in the anammox enrichment process, UBF was demonstrated to be a better reactor configuration in terms of anammox start-up time and

stability against substrate loading shocks during the cultivation process [102]. Nevertheless, the UBF results in a lower biomass production than SBRs according to a similar study [102].

4.3. Carrier material for the bioreactor

The application of a bioreactor system with carrier material enhances biofilm attachment during anammox enrichment, and therefore speeds up the start-up process. Polyethene sponge carriers and bamboo charcoal are among the suitable carrier materials that are capable of preventing biomass washout and retaining stable immobilization of anammox bacteria [54,104,105]. According to previous studies, nonwoven materials have been demonstrated to be effective carrier materials for biomass retention in biological nitrogen removal through anammox [21,106]. The nonwoven material is proficient in retaining free bacterial cells and preventing a membrane fouling problem when operated for long enrichment periods (up to 400 d) [96]. Nonwoven fabric is porous and contains many small hollow areas; it aids in the attachment of biomass to prevent washout, thereby enhancing the performance of the anammox reactor [54]. Biomass retention ability was also accomplished through biofilm development on the interior surface of a nonwoven material via flocs formation in the reactor [57]. An efficient anammox reactor with a super high NRR of 26 kg-N $m^{-3}d^{-1}$ was successfully established using a nonwoven fabric as the biomass carrier [21]. In another study, the attachment of biomass to a nonwoven carrier was demonstrated in a hybrid reactor (a combination of a fixed-bed and FBR), resulting in improvement of the NRR by 8% [12]. The use of bamboo charcoal has also been effective in facilitating the anammox reaction start-up during the anammox enrichment process. The bamboo charcoal carrier has a large specific area for the adhesion of anammox bacteria and thus supports the growth of biofilms

Table 8
Performance of anammox enrichments in different reactor systems

Reactor type[a]	Carrier material	Maximum NLR[b] kg-N $m^{-3}d^{-1}$	Start-up period	Highest NRR[c] kg-N m^{-3} d^{-1}	N removal performance	Ref.
Biofilm reactor	Non-woven material	–	–	~0.041	60 ± 6.3%	[106]
DHS	Sponge material	5.96	–	2.27	95%	[109]
SBR	–	–	within 54 d	–	97.47%	[81]
EGSB	–	27.31	–	25.86	–	[89]
UASB	Bamboo charcoal	–	85 d	–	98%	[105]
MBR	Submerged hollow fibre membrane	–	within 80 d	0.71	–	[13]
Up-flow column reactor	Polyethene sponge strips	4 ± 0.1	within 56 d	3.6	>85%	[104]
UASB	–	137.1	–	74.3–76.7	–	[110]
Fixed bed reactor	Non-woven fabric rings	10.3	39 d	9.2	~90%	[54]
Hybrid reactor	Non-woven fabric	8.9	38 d	6.6	>70%	[12]
Biofilm reactor	Non-woven material	–	–	1.6	–	[144]
MBR	–	–	16 d	–	90%	[53]
SBR	–	1.4	60 d	–	80%	[80]
Up-flow fixed-bed reactor	Polyethene sponge	8.4	–	7.6	90%	[65]
Anammox reactor	Non-woven membrane	1.263	8 months	1.048	90.9%–NH_4^+ 95.0%–NO_2^-	[57]
UASB	–	24	18 d	0.0183	–	[50]
UASB	–	~0.26	56 d	–	83.10%	[9]
Fixed bed biofilm reactor	Non-woven membrane	58.5	50 d	26	–	[21]

[a]DHS: Down-flow hanging sponge reactor; SBR: Sequencing batch reactor; EGSB: Expanded granular sludge bed; MBR: membrane bioreactor; UASB: Up-flow anaerobic sludge blanket.
[b]NLR: Nitrogen loading rate.
[c]NRR: Nitrogen removal rate.

[105]. Moreover, bamboo charcoal is able to support the growth of the anammox biofilm due to (i) a good NH_4^+ adsorption capacity [107], (ii) a microporous structure that prevents oxygen penetration into the inlet zone [105] and (iii) by increasing the settling velocity of anammox and improving anammox retention [13,86,104,108]. The use of a down flow hanging sponge (DHS) carrier for anammox enrichment was reported to be applicable for retaining the high biomass and minerals in the sponge material. It is also capable of maintaining a long sludge retention time, making it suitable for the enrichment of slow growing bacteria [109]. However, part of the DHS will float in a liquid medium, resulting in the accumulation of insoluble precipitated inorganic matter on the dry area [109]. Table 8 summarizes the anammox enrichment performance of different systems.

4.4. Monitoring anammox performance during enrichment

Anammox performances can be determined by monitoring several indicators, including changes in pH, biomass granulation, nitrogen removal efficiency and sludge colour of the anammox biomass [14]. Anammox activity can initially be indicated by a higher effluent pH than influent pH because the anammox consumption reaction involves acid [104]. Therefore, an increase in effluent pH under influent conditions and a constant HRT represents good progress of the anammox performance [14]. The presence of anammoxosome and ladderane lipids, the rate of substrate conversion (ammonium and nitrite) and the production of hydrazine from hydroxylamine represent specifications that could be taken into consideration to confirm the existence of anammox in culture [22]. A higher NRR with a precise ammonium to nitrate conversion ratio in an anammox system indicates the presence of a higher capacity anammox population in a reactor. Moreover, the progress of anammox enrichment can be roughly measured by observing the changes in colour and gradual granulation of the culture. The changes in colour of the anammox culture during enrichment were explained by the increase in cytochrome content, with the cytochrome spectra gradually increasing to a peak at 470 nm during enrichment [35]. A stable anammox population in a bioreactor will show an orange-red colouration, especially when anammox has dominated the culture. The red colour of anammox has been proposed to be due to the presence of Heme c-containing enzymes involved in the anammox pathways [89,110]. However, this visual observation does not completely signify anammox activity during enrichment because only a rough evaluation has been performed [14].

5. Factors influencing anammox enrichment in bioreactors

5.1. pH

A stable performance of anammox cultures can be ensured by treating the enriched culture to achieve the optimal environmental conditions of pH and temperature [111,112]. The inhibition of anammox activity was reported to occur at pH values <5, while high anammox activity was observed between pH values of 6 and 8 [112]. Similarly, another study reported that anammox worked best in a pH range of 7.8–8.0 [78]. This finding is also supported by other researchers who observed anammox activity only between pH values of 6.5 and 8.8, with an optimum pH of 8 [111,113].

5.2. Temperature

Based on previous studies, good anammox activity levels were detected at temperatures ranging from 35 to 40°C [69,113,114]. The maximum activity of the anammox reaction was observed from 35 to 40°C; when the temperature was raised gradually, the anammox activity showed an irreversible decrease at 45°C due to biomass lysis [114]. At a very low temperature, the anammox system became unstable due to nitrite accumulation, thereby affecting the anammox activity [114]. However, the marine anammox bacteria '*Candidatus* Scalindua' favour lower temperatures for their growth compared to the wastewater anammox species [55,115,116]. It was also reported that the anammox '*Candidatus* Brocadia fulgida', enriched from wastewater treatment plant sludge, survived at 10°C and produced a biomass yield of 0.046 g biomass/g and an N conversion that was similar to that observed at high temperatures [117]. Another study that used a moving bed biofilm reactor showed the inhibition of anammox activity at a temperature of 10°C [60]. The tolerance of anammox activity to temperature has been reported to be dependent on the species of the anammox bacteria [118]. The increase in temperature is directly proportional to the increase in the percentage removal of both ammonium and nitrite until the process reached its maximum percentage removals at 35°C [112].

5.3. Inhibitor and stimulator

There are several types of inhibitors and stimulators that can affect anammox activity during anammox enrichment. These may include the substrates (ammonium and nitrite), organic matter, anammox metabolites and the common wastewater constituents themselves [72]. To establish a successful anammox culture with a fast start-up and stable operation in a bioreactor, it is

important to ensure that the anammox biomass is in the optimum condition for anammox growth and is free from any inhibitor that is capable of limiting the anammox activity. A long enrichment period demands an effective culture technique by which the anammox biomass can be retained at a low concentration of substrate to mimic the anammox natural habitat [33].

Hydrazine (H_2N_4) was reported to be an intermediate of the anammox reaction [28,119] and has been proposed to be capable of enhancing the nitrogen removal activity of anammox. The presence of a low concentration of hydrazine (0.03 mM) in an anammox culture was reported to have a slightly stimulatory effect on anammox activity [72]. The addition of 0.1 mM hydrazine to an anammox culture recovered the anammox activity from cultures previously inhibited by nitrite [18]. Nevertheless, a higher concentration of hydrazine (>0.3 mM) can either stimulate or cause moderate inhibition of anammox activity [72].

The addition of low organic matter can enhance the nitrogen removal efficiency by promoting the heterotrophic denitrification process simultaneously with the anammox process. The addition of low organic matter does not have much of an effect on ammonia and nitrite removal but does affect total nitrogen removal through denitrification [58]. Denitrification has also been used to treat excess NO_3^--N accumulation during anammox enrichment in SBR; this process will occur simultaneously with the presence of organic carbon [78,120]. A high nitrogen removal efficiency of 97.47% was achieved with simultaneous anammox and denitrification at an influent COD to nitrite (C/N) ratio of 2 [120]. However, a high concentration of organic matter will inhibit anammox activity due to competition between the anammox population and the excessive growth of heterotrophic bacteria, thus resulting in a decrease in the nitrogen removal efficiency.

The mineralization of organic matter and sulphate reduction in an anaerobic reactor produces H_2S sulphide, which causes sulphide inhibition of anammox activity [35,58,72]. A complete inhibition of anammox activity has been proposed to occur at an H_2S concentration of 0.65 mM [71]. In another study, the anammox biomass was severely inhibited at a very low sulphide concentration of 0.03 mM [72]. The inhibitory effect of sulphide on the anammox process might be due to its interaction with the haeme centres of cytochrome oxidase that result from haeme iron reduction in cytochrome c; this interaction can potentially disrupt anammox metabolism [121]. The inhibitory effects of sulphide on anammox activity depend on the exposure time and sulphide concentration in the biomass because long-term exposure to sulphide at a concentration of 32 mg L^{-1} inhibited anammox activity [122].

Nitrite and ammonium are anammox substrates that inhibit their activity at high concentrations. However, the inhibition threshold value of nitrite was reported to be lower than the threshold of ammonium because anammox activity was observed to be more susceptible to high concentrations of ammonium than to high concentrations of nitrite [118,122]. However, inhibition by nitrite was reported to be irreversible [69,123]. Nitrite accumulation is capable of decreasing anammox catabolic activity and thus damaging the microorganism structures [50]. Therefore, it is crucial to avoid nitrite inhibition for successful anammox enrichment. The inhibitory effect of nitrite on anammox activity was reported to depend on the reactor configuration, physical protection of the anammox bacteria within the sludge [124] and the time of exposure to nitrite [125]. The anammox activity started to show inhibition effects when exposed to nitrite at concentrations ≥15.1 mM [72]. Anammox granules from SBR were found to be capable of tolerating high nitrite concentrations up to 500 mg NO_2–N L^{-1} within 3–4 h of exposure. In contrast, a total loss of anammox activity was observed after a prolonged exposure of 24 h with the IC_{50} (half maximal inhibitory concentration) set at 173 ± 23 mg NO_2–N L^{-1} [125]. It was suggested that influent and effluent nitrite respective concentrations of 280 and 100 mg L^{-1} should be considered to be the cautionary values for the anammox process [126]. The inhibition of the anammox process in response to a moderate concentration of nitrite can be overcome by stopping the influent flow, thereby reducing the accumulation of nitrite in the reactor system [125].

The occurrence of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) in the reactor system can also inhibit the anammox process. This is due to the competition for the ammonium and nitrite substrates between these bacteria and the anammox population. This competition can be avoided by applying a short HRT to promote the washout of the AOB and NOB [50].

Phosphate is commonly used in detergents and fertilizers; hence, it has also been found in wastewaters [127]. The tolerance of anammox to phosphate differs between suspended anammox biomasses and anammox biofilms, with a higher tolerance observed in the latter [118]. In a gas-lift bioreactor, the anammox biofilms tolerated phosphate at an IC_{50} (half maximal inhibitory concentration) of 20 mM [35]. Exposure to 5 mM phosphate resulted in the complete inhibition of the suspended anammox biomass [35]. Thus, it was suggested that the tolerance of anammox bacteria to phosphate is greatly influenced by anammox biofilm and floc formation [72].

Various types of industrial effluents contain high salinity levels that may affect anammox activity [72]. Microorganisms tend to become plasmolysed, dormant or die in high salinity environments. No effect of sodium chloride (NaCl) on anammox activity was observed at concentrations <150 mM (8.8 kg m^{-3}). In contrast, anammox activity was affected by potassium chloride (KCl) and sodium sulphate (Na_2SO_4) at concentrations > 100 and 50 mM, respectively [71]. The anammox bacterial tolerance to salinity is believed to depend on their adaptability. Freshwater anammox bacteria were found able to tolerate 30 kg m^{-3} salinity when gradually exposed to salt concentrations [128]. The marine anammox bacteria prefer high salinity conditions for their growth, resulting in conditions that are similar to their natural habitat. '*Candidatus* Scalindua sp.' requires salinity in a range of 0.8–4.0% for growth [115], while '*Candidatus* Scalindua sorokinii/brodae' has been successfully enriched at a high salt concentration of 33 g L^{-1} [55]. Reduction of salinity from 3.5 to 1.75% and 0.875% has been observed to slightly affect the '*Candidatus* Scalindua' anammox process [116].

5.4. Dissolved oxygen

Anammox bacteria are obligate anaerobes; thus, they are very sensitive to the presence of oxygen. However, the inhibitory effect of oxygen is reversible once oxygen is completely removed from the reactor [129]. An earlier study showed that anammox activity was completely inhibited at an oxygen concentration of 0.04 mg L^{-1} (0.5% of air saturation) [99]. However, the inhibitory effect of dissolved oxygen varies according to the type of anammox system used during the enrichment. In a UASB reactor, there was no apparent effect of exposure to approximately 0.30 mg L^{-1} of dissolved oxygen on anammox activity [14]. In another study, the complete inhibition of anammox activity was observed in anammox batch experiments when oxygen was deliberately introduced to the cultures [130]. The absence of oxygen was also believed to promote the dominance of the anammox bacteria because anaerobic conditions completely inhibit the nitrification activity performed by the oxygen-dependent AOB.

6. Conclusion

To ensure successful anammox enrichment, it is crucial to design an anammox system that can endure the long start-up period required for the anammox reaction. This can be achieved by considering several operational strategies, such as the selection of appropriate inoculum, selection of suitable bioreactor and medium carrier, favouring the operational conditions for optimal anammox growth and monitoring the reactor performance. This review guides researchers in the consideration of anammox enrichment operational strategies for the application of anammox to the removal of ammonium from wastewater.

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