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Current perspectives of anoxic ammonia removal and blending of partial nitrifying and denitrifying bacteria for ammonia reduction in wastewater treatment

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ABSTRACT

Various anthropogenic activities and extensive urbanization are responsible for the generation of large amounts of wastewater containing toxic pollutants. Ammonia is one of the major pollutants in wastewater produced from households, industry and agriculture fields. Removal of ammonia during wastewater treatment is still a challenge. The conventional methods include two-step processes such as nitrification and denitrification. However, newly discovered processes of anammox and comammox seem to be more promising. This review elucidates on sources of ammonia contamination and its effects. Newly discovered process like anammox and comammox is discussed in detail. The habitat, abundance, the structure of bacteria and the chemical reactions involved in the processes are included in this review. Authors have brought out the analogy of the anammox process with the conventional nitrogen cycle and by coupling anammox with comammox to develop an efficient ammonia removal process.

1. Introduction

Water is one of the vital substances on the earth's surface for the survival of the living organism. The demand for pure water is increasing with the growth in the global human population and urbanization. Various natural water bodies like rivers, streams, coastal zones and estuaries are the most productive part of the ecosystems. Due to various anthropogenic activities, the volume of the wastewater is increasing proportionally [1]. Wastewater effluent contains pathogens, inorganic compounds, organic material and macroscopic pollutants. An increase in the nutrients leads to the growth of bacteria and algae, which increases the nitrogen and phosphorus levels in the aquatic ecosystem. This leads to decrease in the water clarity in water bodies causing threats to biodiversity, habitat loss, food chain disruption and eutrophication [2]. Eutrophication affects the zooplanktons and the entire food web. The nitrogen is present in different forms as ammonia, nitrites and nitrates into the water bodies. Presence of varying oxides of nitrogen and ammonia lead to the change in the pH and dissolved oxygen for aquatic life [2,3]. Ammonia is one of the major pollutants in freshwater, which contributes to water pollution. It is highly toxic in nature that is colourless alkaline gas and readily soluble in water [4]. Unionized ammonia is harmful to the organisms in the marine world [3]. Accumulation of the nitrogen content in the water bodies lead to nutrient pollution affecting the aquatic ecosystem. Nitrogen pollutants in the form of ammonia and nitrates pose various health risks if it is present in the drinking water, it causes suffocation, irritation of throat and eyes. So, it is necessary to reduce and remove the ammonia content in water [5]. Ammoniacal nitrogen (NH_3-N) is used in measuring the quantity of ammonia ions present in water or waste solvents [2]. Ammonia is found naturally in environment blended in air, soil and water. Ammonia is the second most manufactured chemical in quantity next to sulphuric acid in the world. There are two major sources of ammoniacal waste in water bodies: 1. *Anthropogenic activities*, which includes (a) Municipal Effluent discharge (b) Agricultural Runoff containing fertilizers (c) Industries like pharmaceuticals, plastics, explosives, textiles, pesticides, dyes and other chemical industries. 2. *Natural Sources* which includes (a) Nitrogen Fixation (b) Acid rain (c) Excretion of ammonia waste from animals [6,3,7–10]. Ammonia is highly soluble and reactive gas. Even at very low-level ammonia is toxic to aquatic animals. As per EPA (United States Environmental Protection Agency 2013) recommendation 1.9 mg/L TAN causes chronic toxicity and 17 mg/L TAN causes acute toxicity. Not only aquatic ammonia also affects terrestrial plants and animals including human being. Ammonia from wastewater is removed by the physical, chemical and biological

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methods. Physio-chemical technologies for nitrogen removal from wastewaters include various techniques like ion exchange, reverse osmosis, breakpoint chlorination and dechlorination using granular activated carbon [5]. The most traditional and cost-effective way of ammonia removal is by the microbial method. Traditional microbial method involves using microbes for nitrification and denitrification process. Nitrifying bacteria (example like Nitrosomonas and Nitrobacter) helps in the conversion of ammonia to nitrous and nitric oxides. Denitrifying bacteria like proteobacteria and paracoccus denitrificans convert the nitrogen oxides to dinitrogen. Anaerobic ammonia oxidation (ANAMMOX) and complete ammonia oxidation (COMAMMOX) are newly discovered processes of ammonia removal. In anammox process, microbes convert ammonia directly to nitrogen in the presence of nitrous oxide. It is a cost-effective and environmentally friendly process. Comammox is a two-step process involving chemolithoautotrophic bacteria. It includes nitrification of ammonia to nitrate and nitrite [11]. Nitrospira species are most common bacteria involved in comammox process. This review explains the ANAMMOX and COMAMMOX processes and detailed on bacteria involved in these processes. These processes can help in developing technology for faster reduction of ammonia from wastewaters by cost-effective methods. The main objective of this review article is to provide a comprehensive view on the development of anammox and comammox process for ammonia removal and the efforts on combining both the processes. This includes (a) the various sources of ammonia pollutants in wastewater (b) the natural distribution and abundance of anammox and comammox bacteria (c) their structure and physiological process (d) comparison of anammox and comammox process (e) the analogy of the anammox process with the nitrogen cycle (e) the coupling of anammox and comammox and its impact in faster ammonia reduction in wastewaters. This review will be useful for understanding various processes involved in ammonia reduction and development of novel technology by combining the anammox and comammox process for efficient ammonia reduction from wastewater.

2. Habitat and abundance of anammox and comammox

2.1. Habitat and abundance of anammox

The bacteria involved in anaerobic ammonia oxidation are ubiquitous in the environment. These bacteria belong to the phylum Planctomycetes [12–15]. There are 19 anammox species identified till now belonging to 5 genera (Table 1), among these only ten could be enriched.

Table 1
List of anammox bacterial species that are identified till date.

Sl no:	Genera	Species	Source
1.	Brocadia	*Candidatus Brocadia anammoxidans, Candidatus Brocadia fulgida and Candidatus Brocadia sinica*	Freshwater [24]
2.	Kuenenia	*Candidatus Kuenenia stuttgartiensis*	Anoxic membrane bioreactor [25]
3.	Anammoglobus	*Candidatus Anammoxoglobus propionicus and Candidatus Anammoxoglobus sulfate*	Anaerobic pond [26], Sequencing Batch reactor [27]
4.	Jettenia	*Candidatus Jettenia asiatica*	Mariculture wastewater treatment plants [20]
5.	Scalindua	*Candidatus Scalindua brodae, Candidatus Scalindua wagneri, Candidatus Scalindua sorokinii, Candidatus Scalindua arabica, Candidatus Scalindua sinooilfield, and Candidatus Scalindua zhenghei*	Seawater [24]

These bacteria grow in a pH range of 6.8–8.5, neither in an acidic or basic environment. Anammox bacteria are abundant in both marine and freshwater ecosystems. These are also observed in reactors used for wastewater treatment and paddy fields. In recent studies, it was observed that these bacteria could be enriched faster in groundwater than the demineralized water. Anammox bacteria are chemolithoautotrophic, which can utilize metals and trace elements present in the ground water. They are slow growing autotrophic bacteria whose doubling time varies from 11 to 22 days. Anammox bacteria from marine water and freshwater grow at different temperature range. In marine waters, these bacteria grow at a temperature range of 15 °C – 25 °C and in freshwater, these bacteria show maximum activation at 37 °C, usually ranging from 27 °C – 37 °C. [13] Five anammox genera listed, that have been discovered so far. (Table 1) Since anammox are un-culturable, techniques like qPCR and FISH are used in the quantification and abundance measurement of anammox bacteria [12,16]. Studies show that anammox population varies in the sediment samples from different places and it get affected by the pollution. Abundance data obtained by qPCR of anammox specific gene *hzo* and 16 s rDNA range from 1.19×10^4 to 5.9×10^6 copies/g of sediment [17,18]. There may be more than one copy of *hzo* gene in bacteria cell hence, the abundance calculated based on hzo gene may not be accurate but this has wider coverage for all the species of anammox. Scalindua, Jettenia, Brocadia, Anammoxoglobus bacterial species are abundant in the Yellow River Delta and Kuenenia East China [19]. While all five known species namely Brocadia, Kuenenia, Scalindua, Jettenia, and Anammoxoglobus spices are reported from East China [20]. Co-occurrence of anammox with methane oxidizing bacteria has been reported from the paddy fields. Due to water logging anoxic condition develops in paddy fields suitable for anammox bacteria [21]. Not only from mesophilic habitat, anammox are also reported from the thermophilic environment like oil fields in China [22,23].

2.2. Structure of anammox bacteria

Anammox bacteria possess a unique characteristic in their structure and biology. The cytoplasm in anammox bacteria is composed of three compartments that are separated by a single bilayer membrane. The three bound compartmental cells from inside outwards are anammoxosome, riboplasm and paryphoplasm [25]. Anammoxosome is a specialized organelle composed of a single bilayer membrane in anammox bacteria, providing a place for metabolism [12,28]. A proton motive force established over the anammoxosome membrane during an anammox reaction, help in ATP production in the riboplasm [29]. Anammoxosome membrane prevents the bacteria from the proton diffusion and transitional toxicity. Naturally, occurring ladderane lipids are present in the bacterial membranes, with an organic molecule containing two or more fused cyclobutane rings [12,28]. As Anammox could not be cultured as pure isolate, the information about genome were obtained in bits and fragments. Recently the genome of Candidatus *Jettina* is constructed from the metagenomic study of anammox granules. Genome is 3.9 mbp long containing 3970 putative protein coding genes involved in autotrophic carbon fixation and anammox metabolism. Genes for 16 s/23 s rRNA and 45 tRNA have also been identified. High diversity in nitrite reductase genes have been reported among anammox. For example, some anammox bacteria contain cytochrome cd type nitrite reductase (nirS) other have copper containing (nirK). Some anammox lack both nirS and nirK and use hydroxylamine dependent anammox mechanism using un-known nitrite reductase [30,31]. Anammox bacteria are enriched in bioreactors like sequencing batch reactor [32–34], fluidized-bed [35], fixed-bed, membrane reactor [36], fixed-film reactors [37,38], up-flow anaerobic sludge bed [39,33], rotating biological contactor [40], granular sludge-based reactor ([41]; [107] [42], moving bed biofilm reactor [43] and integrated fixed-biofilm activated sludge reactor [44,29]. Granular sludge-based reactor showed that the mixing of the reactor content was facile and the volumetric loading rate of the reactor is higher. With the slow growth of the anammox bacteria, biomass retention is efficient, which causes the natural growth of biofilm [42]. Anammox bacteria grow as consortia along with other microbes in granular form. In the microscopic observation, the anammox bacterial growth is seen in the middle of the granules and the smaller fraction of ammonia oxidizing bacteria (AOB) growth on the outer shell of the granules [45]. These granules are bright red, carmine, calamine to pale red or black in color. Anammox bacteria can be observed as red colour growth which is due to the high content of cytochrome and the colour fades away to black with the reduction of Heme C [29]. Anammox bacteria are slow growers. For every 11–20 days the cells double. *in situ* conditions reduce the doubling time and these microbes can thrive at deficient substrate concentration. Anammox are obligate anaerobes and oxygen-sensitive. Their metabolism is reversibly inhibited if the oxygen is above 2μM. These are also chemolithoautotrophic, which are capable of oxidizing ammonia to nitrogen gas using nitrate as an electron acceptor [46]. These bacteria require a period of 90–200 days for enrichment in a limiting condition when provided with a feed of ammonia, nitrite, nitrate and bicarbonate [28]. The anammox bacteria isolated and identified from different habitats are listed. (Table 2)

2.3. Habitat and abundance of Comammox

Comammox is a process involved in the complete oxidation of ammonia to nitrate by a single organism. It has higher growth yields compared with separate nitrification steps [57,58]. Comammox are widely spread in environment. These are abundant in terrestrial as well as aquatic ecosystems. These grow in the agricultural soils like rice paddy fields and forest soils. They are grown in freshwater habitats, brackish lake sediments, engineered systems like wastewater treatment plants and drinking water treatment systems [57]. Comammox organisms coexist with AOA, AOB and NOB. Division of labour between AOB and NOB is challenged by complete ammonia oxidizers like Nitrospira. Comammox are oligotrophic organisms which can grow in low nutrient conditions and convert ammonia to nitrite and nitrate. In drinking water treatment plants comammox outnumbered AOB and helped in ammonia oxidation [59]. The comammox microbes have more efficient carbon fixation pathway compared to AOB and hence require less organic carbon [60]. These are dominant ammonia oxidizers in the mainstream nitrification reactor with low dissolved oxygen [61]. Abundance of the bacteria is expressed as gene copy numbers quantified by qPCR per ng of DNA. Bacterial *amoA gene, comammox amoA* gene, and comammox and *nitrite-oxidizing Nitrospira nxrB* genes are quantified and expressed v. In the activated bioreactors, the samples ranged from 5.5×10^3 to 3.2×10^4 copies ng^{-1} DNA. The canonical AOB amoA genes varying from 9.2×10^1 to 5.6×10^3 copies ng^{-1} DNA [63] The abundances of the *ammonia monooxygenase subunit A (amoA)* genes for comammox, *AOA, AOB, and anammox 16S rRNA* were 2.43×10^8, 1.07×10^8, $3.24 \times 35\ 10^7$, and 3.21×10^{11} copies g^{-1} dry sediment, respectively [64]. The comammox bacteria varied from 1.0×10^8 copies ng^{-1} to 1.75×10^8 copies ng^{-1} are abundant in AOA amoA gene and AOB amoA genes mostly found in the sewage discharged areas of the bioreactor plant [65]. They grow in low dissolved oxygen conditions or partially oxic conditions [61]. To prevent the growth of the phototrophic microorganisms, they are grown in dark conditions [62]. The pure cultures of

Table 2
Anammox bacteria: The different habitats, enrichment and types of bacteria identified around the world and the areas where the research is done.

Sl no	Different habitats the anammox bacteria was isolated	Types of bacteria identified	Different areas around the world (research about anammox bacteria was carried out)	Enrichment and the growth of bacteria	References
1.	The Brazilian waterweed Egeria densa harvested from an artificial pond	*Bacteroidetes Proteobacteria, Firmicutes, Proteobacteria, Synergistetes, Spirochaetes, and* unclassified bacteria	Tokyo, Japan.	Completely stirred tank reactor (CSTR)	[47]
2.	The pharmaceutical wastewater collected from a colistin sulphate and kitasamycin manufacturing plant	NN	Zhejiang Province, China	Sequencing Batch reactor (seed sludge was collected from pilot scale nitrate reactor)	[48]
3.	Flooded rice paddy field with long-term fertilization	*Candidatus Anammoxoglobus propionicus, Candidatus Jettenia asiatica, Candidatus Kuenenia stuttgartiensis, Scalindua profunda and Brocadia fulgida and strain KSU-1*	Zhejiang Province, China	Column was constructed	[49]
4.	Municipal wastewater treatment plant	*Candidatus Brocadia*	Kanazawa, Japan	Laboratory scale up-flow anaerobic-anoxic biological filter reactor	[50]
5.	Dhandupura sewage treatment plant	*Candidatus Brocadia, Candidatus Jettenia Anammoxoglobus Propionicus*	Uttar Pradesh, India	Anaerobic hybrid reactor	[51]
6.	Estuaries, salty marshes, sand spit. These waters are the discharges of domestic and industrial waste which contain high concentration of metals.	Consortium of bacteria	North East England	Samples were directly collected and stored in 4 degrees.	[1]
7.	Anaerobic digested sewage sludge-wastewater treatment plant	Indigenous (naturally occurring) iron-oxidizing bacteria	Yuen Long district, Hong Kong	Samples were directly collected and stored in four degrees.	[52]
8.	Domestic wastewater treatment plant	*Methanomicrobiales and Methanosaetaceae*	Bennekom, Netherlands	Samples were directly collected and stored in four degrees.	[53,54]
9.	Mariculture wastewater treatment plant	*Ca. Scalindua, Ca. Scalindua clade I, Ca. Scalindua-clade II, Ca. Scalindua- clade III, Ca. Jettenia clade, novel anammox clade I, anammox clade II*	Baisha and Moshui rivers, Wastern Bay	Samples from wastewater treatment plant.	[108]
10.	Sewage treatment plant -sludge digester liquor,	*Candidatus Kuenenia and Nitrospira, Chitinophagaceae*	Beijing, China	Sequencing Batch Reactor	[55]
11.	Wastewater treatment plants	*Candidatus Nitrospira inopinata, Candidatus Nitrospira nitrificans, Candidatus Nitrospira nitrosa,Candidatus, Brocadia caroliniensis and Candidatus Scalindua profunda*	United States (California and Virginia), Denmark, and Singapore	conventional BNR, moving bed biofilm reactor (MBBR), DEMON deammonification	[56]

comammox bacteria that are isolated and identified so far are: 1. *Candidatus Nitrospira inopinata* 2. *Candidatus Nitrospira nitrosoa* 3. *Candidatus Nitrospira nitrificans*. These bacteria can grow in extreme conditions from 22 °C–56 °C at a pH ranging from 6 to 8.5 [11]. Comammox Nitrospira has been identified in many engineered systems. It was also found to be dominating the ammonia oxidizing community in a nitrification reactor of municipal wastewater treatment plant [61]. Physiological data including ammonia oxidation kinetics [66], metabolic versatility [11] and comparative genomic analysis [59] revealed that comammox organisms functionally outcompete other canonical nitrifiers under highly oligotrophic condition [57]. The anammox and the comammox process is differentiated (Table 4).

2.4. Structure of comammox bacteria

The pure cultures of comammox bacteria like Nitrospira *inopinata*, *ca.* N. *uzonensis* observed under the scanning electron microscope, are found to be slightly curved or spiral-shaped, and few were round-shaped in size with 0.2-0.7 μm in width and 0.7−2 μm in length. [59,66] These cells are enclosed by a multi-layered envelope, composed of a cytoplasmic membrane and an outer membrane. The membrane is composed of storage compounds like glycogen and polyphosphate as deposits. *Nitrospira inopinata* is enriched in autotrophic nutrient media, which is rich in ammonia content. They are usually chemolithoautotrophic [67]. These microbes grow well in aggregates as biofilms or in a suspended growth system. These biofilms produce a network of the extracellular polymeric surface, which is composed of 50–90 % of total organic matter [66]. Comammox bacteria are enriched in rotating biological contractors [68], sequencing batch reactor [55] and moving bed biofilm reactor [56]. The comammox bacteria isolated and identified from different habitats are listed. (Table 3)

3. Chemical reactions of Anammox and Comammox

3.1. Enzymes and the mechanism involved in the chemical reaction of Anammox

Nitrogen removal by in anaerobic condition includes denitrification and anammox processes. Anammox is a single step process involved in the conversion of ammonia to nitrogen in the presence of nitrite [28]. *Hydrazine synthase (HZS), hydroxylamine oxidoreductase (HAO) and hydrazine oxidoreductase* (HZO) are the enzymes involved in the energy metabolism of the anammox process [24]. Gene encoding, *hydrazine oxidoreductase (hzo)*, is isolated from several cultures belonging to the anammox enriched bacteria. It belongs to the octaheme cytochrome c hydroxylamine oxidoreductase protein family. It plays a vital part in the anammox biochemical process [22,23]. Fig. 1. explains the different processes and the enzymes involved in ammonia oxidation. The anammox pathway includes the following reactions [28]:

1. Reduction of nitrite to nitric oxide

$$NO_2^- + H_2O \xrightarrow{nxr} NO_3^- + 2H^+ + 2e^-$$

2. Condensation of ammonia and nitric oxide, along yielding hydrazine

$$NH_4 + NO + 2H^+ + 3e^- \xrightarrow{hzs} N_2H_4 + 2H_2O$$

3. Oxidation of hydrazine to produce dinitrogen gas

$$N_2H_4 \xrightarrow{hzo} N_2 + 4H^+ + 4e^-$$

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

Table 3
Comammox bacteria: The different habitats, enrichment and types of bacteria identified around the world and the areas where the research is done.

S. No.	Different habitats the comammox bacteria was isolated	Types of bacteria identified	Different areas around the world (research about comammox bacteria was carried out)	Enrichment and the growth of bacteria	References
1.	Household tap water-filtration units	*Candidatus Nitrospira inopinata, Ca. Nitrospira nitrificans, Ca. Nitrospira nitrosoa*	Hainan province, China	Filtration unit	[59]
2.	Aplysina aerophoba sample collected by scuba diving	*Nitrospira-like bacterium derived from the mesohyl of Aplysina aerophoba*	Ute Hentschel, France	Direct samples and stored in 4 degrees in batch cultures	[67]
3	Soil, sediment, and sludge samples	*Nitrosomonas, Nitrospira bacterial strains, Nitrospira inopinata*	Shanghai, China	Samples were directly collected	[69]
4.	Tertiary municipal wastewater treatment plant	*Ca.* Nitrosocosmicus and nitrospira species	Ontario, Canada	Full scale rotating biological contactors	[68]
5.	Sewage treatment plant -sludge digester liquor,	*Candidatus Kuenenia and Nitrospira, Chitinophagaceae*	Beijing, China	Sequencing Batch Reactor	[55]
6.	Wastewater treatment plants	*Candidatus Nitrospira inopinata, Candidatus Nitrospira nitrificans,Candidatus Nitrospira nitrosa, Candidatus Brocadia caroliniensis and Candidatus Scalindua profunda*	United States, Denmark, and Singapore.	conventional BNR, moving bed biofilm reactor (MBBR), DEMON deammonification	[56]
7.	Water Reclamation Plant (WRP)	*Ca. Nitrospira nitrosa*	Skokie, Illinois, USA	Sequencing batch reactor	[61]
8.	Ruttner Standard Water Sampler of River surface	*Nitrospira inopinata, Proteobacteria, Bacteroidetes, Actinobacteria, and Chloroflex*	(Hydro Bios Company)	Samples were stored in 4 degrees and collected directly	[70]
9.	The twelve drinking water treatment plants (DWTPs)	*Nitrospira species*	Denmark	Samples collected directly and stored at a lower temperature.	[71]
10.	Sediments from the tidal flat of the Yangtze Estuary	*Proteobacteria, Candidatus Nitrospira inopinata, Candidatus Nitrospira nitrosa, and Candidatus Nitrospira nitrificans*	Yangtze Estuary	A bioreactor in laboratory scales	[72]

Table 4
Comparison of anammox and comammox bacteria.

	Anammox bacteria	Comammox bacteria
Full form	Anaerobic ammonia oxidation	Complete oxidation of ammonia
Unique characteristic	Single-step process	The single organism with higher growth which performs two steps
Equation	$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$	$NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$
Process involved	Partial nitrification	Nitrification
Habitat	Seawater, anaerobic ponds, estuaries, sewage treatment plant, domestic wastewater and freshwater.	Freshwater, agricultural soils, drinking water systems and wastewater treatment plants
Source of Energy	Obligate anaerobes, autotrophic, chemolithoautotrophic	Chemolithoautotrophic
Light	Dark conditions	Dark conditions
Morphology	Coccid bacteria (Gram-negative)	Spiral shaped cells with a flagellum
Oxic / Anoxic	Anoxic	Partially anoxic, low DO
pH	6.8-8.5	6-8.5
Colour	Carmine or pale red	Few Nitrospira bacteria are green in color
Temperature	15 °C-37 °C	22 °C–56 °C
Doubling period	11-22 days	7-14 days
Location of the process involved in the cell	Anammoxosome	Periplasm and membrane-associated cytoplasm
Enzymes	Hydrazine hydrolase (HZO), Hydrazine oxidoreductase	Ammonia monooxygenase (amo), Hydroxylamine dehydrogenase(hao), nitrite oxidoreductase(nxr)
Identified bacteria	*Brocadia, Kuenenia, Anammoglobus, Jettenia, Scalindua*	*Ca. Nitrospira inopinata, Ca. Nitrospira japonica, Ca. Nitrospira nitrosoa, Ca. Nitrospira nitrificans*
Enrichment and growth (Process development)	Sequencing batch reactor, secondary wastewater treatment plants, anaerobic ponds, industrial wastewater, marshy coastal areas, estuaries.	Biofilms from groundwater, drinking wastewater system and fresh wastewater biofilters, hot springs, activated sludge process, rotating biological contractors, terrestrial and limnic habitats, sponge tissue, geothermal springs.
Advantages	Cost-effective, no aeration and carbon dioxide emission, low sludge production, environmentally friendly	Cost-effective, higher growth yields and performs both nitrification steps
Application	Wastewater treatment, sewage water treatment, domestic wastewater treatment plants, pharmaceutical industries and other industries rich in ammonia.	Drinking water treatment plants and small pilot-scale plants.

Anammox process is a chemiosmotic mechanism that helps in conserving the energy derived from conversion of ammonia to nitrite. Anammox bacteria exclusively synthesize hydrazine which acts as principal reductant in nature. The electron obtained in the hydrazine oxidation is transferred to the cytochrome bc1 complex. It carries the electrons towards nitrite reduction and hydrazine synthesis. A proton motive force is created when the protons are translocated across a membrane system, which is coupled by the electron transfer [28]. *Ca. Kuenenia stuttgartiennia* is an anammox bacteria has a remarkable property of generating its own electron acceptor and donor by converting nitrate into nitrite and ammonia [73]. Anammox process has been studied broadly from lab scale to industrial scale. The anammox method is widely used in sewage treatment plants and conventional wastewater treatment plants. In conservative wastewater treatment systems for the removal of nitrogen, a lot of energy is utilised to create an aerobic condition for bacterial nitrification. A less energy intensive substitute method involving anaerobic ammonia oxidation can be used [74–77]. The list of species involved in anammox process are listed. (Table 5)

3.2. Enzymes and the mechanism involved in the chemical reaction of Comammox

In 1890, Winogradsky discovered that nitrification includes two successive steps by two distinct groups of bacteria like ammonia-oxidizing bacteria and nitrite-oxidizing bacteria. Phylogenetically, the chemolithoautotrophic AOB and nitrite oxidizing bacteria (NOB) are not closely related [58]. Kinetic theory of the optimal design of the metabolic pathway explains that there has been maximum production of ATP with the division of labour of microorganisms. Reducing the pathway length, increases the growth yield. The higher growth yield of the bacteria would be beneficial for cultivation of bacteria in clonal clusters in the form of biofilms. Recently discovered process of complete ammonia oxidation shows that single organism is capable of performing both the reactions of converting ammonia to nitrite and nitrate. Due to the unique metabolic functionality and diverse presence, these bacteria are used in biological engineering design and process [56,79]. Energy yield for complete nitrification is (−349 kJ/mole) the same as that of two-step process of ammonia to nitrite and nitrite to nitrate oxidation (−275 kJ/mole and −74 kJ/mole) respectively. The growth and energy yield are more in using the organisms involved in complete nitrification than the incomplete ammonia-oxidizing bacteria and nitrite-oxidizing bacteria which could be due to the competition of the complete nitrifiers [11].

Ammonia oxidation

$$NH_3 \xrightarrow{amoA} NO_2^- \quad G^{o'} = -275\ kJ/mol$$

Nitrite Oxidation

$$NO_2^- \xrightarrow{nxr} NO_3^- \quad G^{o'} = -74\ kJ/mol$$

Nitrospira plays a critical role in nitrification. Few species of Nitrospira and their habitats are listed (Table 6) [80].

The comammox species can grow in highly lithotrophic conditions where the nutrient and DO supply is very low. [61] It is observed that in the drinking water systems comammox outnumbered the AOB. ([59]; [106]. Conventional nitrification process by AOB and NOB is aerobic oxidation and in anoxic/anaerobic condition enzymes of NOB get deactivated. [106] Therefore, energy intensive aeration is required. Comammox flourish in low DO/ anoxic condition hence nitrification becomes more efficient. Comammox enzyme has higher affinity for ammonia compare to AOB enzymes therefore it overtakes the nitrification and subsequently dominates the system [60,66].

Enrichment of Nitrospira is conspicuous. These are formed in the form of microbial biofilm. These are enriched in mineral media rich in ammonia and bicarbonate as the sole source of energy [80]. Among the species of Nitrospira identified till date only Candidatus *Nitrospira inopinata* has higher affinity for ammonia compared to other ammonia oxidizing bacteria and archaea. It enables them to perform significant ecological process in natural and engineered environment [81]. Comammox are considered to be unculturable thermophilic Nitrospira members. *Nitrospira inopinata* is the first cultured comammox species. They are even grown in anoxic denitrification tanks with very little dissolved oxygen content (below 0.2 mg O_2/L) [82]. These are present in microcolonies, flocs and biofilms. Biofilms are the clusters of microbial species growing on a surface area producing extracellular polymeric substance (EPS). EPS helps in the attachment to the surface and matrix formation. Biofilms are extensively used in the wastewater treatment and the improvement of nitrogen removal. The nitrogen removal is more

Fig. 1. Ammonia oxidation: Location of the reaction which is taking place in the microbial cell (either AOB, NOB, CMX, AMX bacteria) along with the enzymes involved. The different colour in the diagrams represents different processes- **Green- Comammox; Yellow- Anammox; Orange-Denitrification.**

Table 5
List of enzymes involved in the mechanism of anammox.

Sl no	Genus/Species Identified	Enzymes involved	Studies	Mechanism	References
1.	*Candidatus Scalindua brodae, Vibrio cholerae,Thalassomonas sp., Hyphomicrobium sp., Microbacterim laevaniformans, Chloroflexi, Verrucomicrobia,Proteobacteri, Gemmatimonadetes, and Actinobacteria phyla*	*hzo* genes	PCR and phylogenetic analysis	Anammox	[22]
2.	*Candidatus Brocadiafulgida, Nitrospira species, Nitrosomonaseuropea and Nitrosomonaseutropha*	*amoA genes, nxrA genes, pmoA genes, nirS genes*	PCR, cloning and phylogenetic analysis	AOB, NOB, denitrifiers and anammox	[78]
3.	*Ca. Scalindua and Candidatus Kuenenia stuttgartiensis*	*hzo genes*	qPCR	Anammox	[108]
4.	*Candidatus Brocadia anammoxidan, Nitrosomonas, Nitrospira, uncultured denitrifying bacteria, Aminobacteraminovorans, and Candidatus Anammoxoglobus propionius*	*nirK and amo gene*	PCR analysis	AOB, NOB and anammox	[41]

efficient in the biofilms rather than the activated sludge. *Candidatus Nitrospira nitrosoa* is enriched in sequencing batch reactors that were operated and sustained in cyclic anoxic or anaerobic and microaerobic conditions in various operational stages [83–85]. Comammox bacteria can be enhanced by batch scales and chemo statically. All the known comammox are the members of the genus Nitrospira possess a full genetic complement for both ammonia and nitrite oxidation [61]. Enzymes involved in the comammox process are (a) AMO- Ammonia monooxygenase a transmembrane enzyme plays a vital role in the conversion of ammonia-to-ammonia hydroxide. (b)HZO- Hydroxylamine oxidoreductase- converts ammonia hydroxide to nitrite in the periplasm. (c) NOR- Nitrate oxidoreductase- a membrane associated enzyme oxidizes nitrite to nitrate [28]. The species and enzymes involved in comammox process are listed. (Table 7). The growth and the

Table 6
Nitrospira species and the habitat.

Sl no:	Species of Nitrospira	Habitats
1.	*Nitrospira marina*	Ocean water
2.	*Nitrospira defluvii*	Activated sludge
3.	*Nitrospira japonica*	Activated sludge
4.	*Nitrospira moscoviensis*	Heating pipes
5.	*Nitrospira inopinata*	Hot groundwater

phylogenetic classification of the comammox bacterial species are analysed through metagenomic sequencing. The metagenomic evidence proved the presence of these enzymes in the comammox species. From a pilot scale to wastewater treatment plants, these comammox bacteria play a significant role in the ammonia reduction to nitrate.

4. Analogy of anammox process with the nitrogen cycle

$$NH_4^+ + NO \xrightarrow{HH} N_2H_4 \xrightarrow{HZO/HAO} N_2 \quad (1)$$

$$NO_3^- \xrightarrow{NarG} NO_2^- \quad (2)$$

$$NO_2^- \xrightarrow[NirS/NirK]{NirS/NirK} NO \quad (3)$$

$$NO \xrightarrow{NOR} N_2O \xrightarrow{NOS} N_2 \quad (4)$$

Eq. 1 and 3 represents the anammox process; Equations 2–4 represents the denitrification process.

HH – hydrazine hydrolase; HZO- hydrazine oxidizing enzyme; HAO-hydrazine oxidoreductase; Nar- membrane bound nitrate reductase; **Nir-nitrite reductase (anammox process)**; Nir-nitrite reductase (denitrifying process) NOR- nitric oxide reductase; NOS-nitrous oxide reductase

Anammox process involves ammonia reduction to nitrogen in the presence of nitrous oxide. It includes enzymes like HH, HZO, HAO, NirS/NirK. Denitrification process is a part of nitrogen cycle which includes reduction of nitrate and nitrite to nitrogen gas in the presence of various enzymes like NarG, NirS/NirK, NOR and NOS. Some of the enzymes like NirK/NirS involved in Anammox process are orthologous to enzymes involved in denitrification process. In Eq. 3 we can see the ortholog of anammox and denitrifying genes. In a combined anammox and partial denitrification processed shows a faster and major ammonia reduction than the separate anammox and partial denitrification process [86,87]. Co-existence of microorganisms like *Thauera, Candidatus, Anammoximicrobium* and *Pseudomonas* contribute to simultaneous sludge fermentation, denitrification and anammox [37,38]. *Candidatus Scalindua* is an anammox bacterial species. It has the *Nir S* gene which is involved in the denitrification process. Few other uncultured anammox bacterial species also have the presence of Nir K gene [88,89]. Enzymes like ammonia monooxygenase gene and nitrite reductase gene contain microbial species like *Nitrospira europaea/eutropha, Nitrospira oligotropha and Betaproteobacteria* help in faster ammonia reduction [78]. The current perspectives show the homology of nitrogen cycle and anammox process [90].

5. Coupling of anammox process with partial nitrification and denitrification processes

The conventional method of ammonia reduction includes nitrification and denitrification process. In nitrification process the ammonia is oxidized by AOB and NOB to nitrite and nitrate respectively. Nitrate is further reduced to nitrogen with the help of denitrifiers. The ammonia reduction can be enhanced by coupling of various processes with the anammox process. Anammox process was first identified in the denitrifying fluidized bed reactor [35,91]. Anammox and ammonia oxidizing bacterial species coexisted in the environment as well as in wastewater treatment reactors enriched with paddy field wastewater, sewage waters and tannery wastewaters [88]. In this process anaerobic

Table 7
Nitrospira species and their enzymes involved in the comammox process.

Sl no	Genus/Species involved	Enzymes involved	Studies	Mechanism	References
1.	*Nitrospira*	*AOB amoA*, and *comammox amoA* genes	qPCR, Metagenomic sequencing	Comammox	[68]
2.	*Nitrospira* like *comammox* *Candidatus Nitrospira sp. SG-bin1* *Candidatus Nitrospira sp. SG-bin2, Candidatus Nitrospira sp. HN-bin3,* *Candidatus Nitrospira sp. ST-bin4″ and Candidatus Nitrospira sp. ST-bin5*	*Ammonia monooxygenase* genes, *amoB, amoC, hao* and *NXR* genes (subunits nxrA and nxrB)	Metagenomic sequencing	Ammonia oxidizing process, Complete nitrification	[59]
3.	*Nitrospira inopinata*	*amoA* and *pmaoA* genes	Metagenomic sequencing, THDP-PCR method	Ammonia Oxidizing bacteria (AOB)	[69]
4.	*Candidatus Nitrospira nitrificans, Candidatus Nitrosomonas defluvii*	*amoA, haoB, nxrB, haoB* and *nxrB*	Metagenomic studies	AOB, NOB and AMX	[56]
5.	*Nitrospira, Brocadia*	*amoA*, and *nxrB* gene	PCR, qFISH	Comammox	[61]
6.	*Nitrospira inopinata*	*narG, nitrous oxide reductase(nosZ), nitrite reductase (nirS), nitrite oxidoreductase (nxrA), ammonia monooxygenase (amoA)* and *hydrazine oxidoreductase (hzo)*	qPCR studies	AOB, NOB, Anammox	[70]
7.	*Nitrospira, Pseudomonas putida KT2440, Clostridium thermocellum, Methanoculleus bourgensis and Meliobacter roseus*	*nxrB Nitrospira* and *amoA Nitrospira gene*	PCR studies	Comammox	[71]
8.	*Nitrosomonadaceae, Nitrososphaerales and Nitrosopumilales*	*COM-A amoA* gene	Metagenomic studies	COM-A, AOA, β-AOB, and Nitrospira	[72]
9.	*N. viennensis, N. gargensis, N. inopinata*	*amoA*	qPCR and metagenomic sequencing	AOB and comammox	[66]
10.	*Ca. Nitrospira nitrosa*	*ammonia monooxygenase (amoCAB)* and *hydroxylamine dehydrogenase (haoABcycAB) gene*	Metagenomic sequencing	Comammox	[83]

autotrophs converts ammonia directly to gaseous N_2 in presence of NO_2 without producing toxic intermediate NO_x. The nitrate produced from partial nitrification is used in both anammox and the denitrification process. Genes involved in both of these processes show partial similarity [92,93]. Coupling of denitrification process with anammox help in producing carbon source and substrates for the anammox bacteria. The partial denitrification engrosses low DO while consuming organic carbon which provides anoxic condition for anammox. The combined process helps in total nitrogen removal of 84 %–93 % [86]. Partial denitrification process coupled with anammox process helps in lowering the nitrite production, reduced oxygen consumption and minimal sludge production [11]. The comparison of various methods involved in the coupling of anammox process is explained. (Table 8)

6. Coupling of anammox and comammox – an innovative approach

NITROGEN CYCLE (NITRIFICATION AND DENITRIFICATION)

$$NH_3^+ \xrightarrow{1} NO_2^- \xrightarrow{2} NO_3^- \xrightarrow{3} NO_2^- \xrightarrow{4} N_2$$

CONVENTIONAL NITRIFICATION

$$NH_3^+ \xrightarrow{1} NO_2^- \xrightarrow{2} NO_3^-$$

COMAMMOX PROCESS

$$NH_3^+ \xrightarrow{5} NO_2^- \xrightarrow{5} NO_3^-$$

CONVENTIONAL DENITRIFICATION

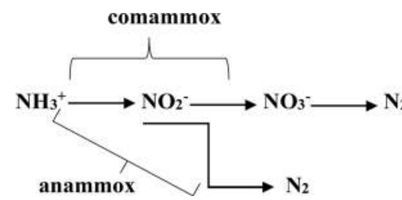
$$NO_3^- \xrightarrow{3} NO_2^- \xrightarrow{4} NO \xrightarrow{6} N_2O \xrightarrow{7} N_2$$

ANAMMOX PROCESS

$$NH_3^+ + NO_2^- \xrightarrow{8} N_2$$

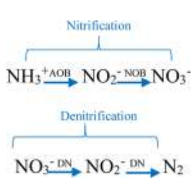
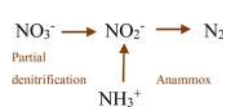
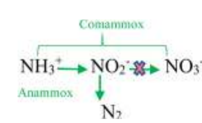
1-Ammonia oxidizing bacteria;2-Nitrite oxidizing bacteria;3-Nitrate reductase;4-Nitrite reductase;5-comammox; 6- Nitric oxide reductase ;7-Nitrous oxide reductase;8-Anammox

COUPLING ANAMMOX AND COMAMMOX



In biological wastewater treatment systems of nitrogen removal, there is exists a complex relation among ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), anammox (AMX) and comammox (CMX) bacteria. These bacteria utilize common substrates like ammonia, nitrite and inorganic carbon [56,97]. Anammox process has shown a significant reduction of ammonia when there is a selective enrichment of ammonia-oxidizing bacteria and anammox bacteria. Anammox enrichment is also done with the anammox pellets, which is used as a seed for inoculation. Metagenomic studies showed that in an anammox rich wastewater treatment plant system, there is a growth of complete ammonia oxidizers (Comammox – *Nitrospira*). These dominated the AOB, which were present in anaerobic conditions, and it was also found that comammox organisms helped in effective ammonia removal [98]. Comammox bacteria is homologous to the ammonia-oxidizing bacteria. In metagenomic studies, it was observed that *Ca. N. inopinata* possess enzymes of ammonia oxidation, AMO and hydroxylamine dehydrogenase. The *hao* gene, which is one of the vital enzymes, is involved in the anammox process [11]. In reference to paper [56], the combined growth of anammox and comammox bacteria promoted the growth of biofilms. These bacteria possessing genes like *amx*, *cmx, amoA* and *hao B* are involved in biological nitrogen removal [56]. A novel approach of combining the anammox and comammox process in a

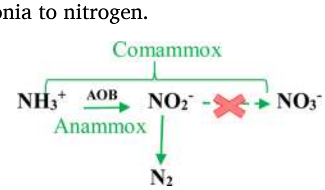
Table 8
Comparison of ammonia removal by traditional nitrification/denitrification and Anammox process coupled with partial denitrification or comammox.

	Traditional nitrification and denitrification process	Coupling of partial-denitrification and anammox	Coupling of comammox and anammox
Equation	 AOB-Ammonia oxidizing bacteria NOB-Nitrite oxidizing bacteria DN- Denitrifying bacteria		
Carbon Source	Inorganic /Organic compounds [102]	Acetate, ethanol and methanol. Low Carbon consumption [101]	Inorganic compounds/ bicarbonates.
Division of labour	Division of labour between AOB and NOB in nitrification step	–	Single organism oxidizes both ammonia and nitrite
Dissolved Oxygen	Yes	Low DO	Very low DO and partial anoxic condition
Aeration	Required [101]	Oxygen consumption is reduced [104]	Not required
Biomass	Accumulation of denitrifying sludge.	Minimal sludge production [94]	Minimal sludge production
Intermediate products	Accumulation of nitrate and other components which inhibits the growth of denitrifying bacteria	Stable production of nitrite produced by partial nitrification is utilised in the anammox process to form nitrogen. ;[103] [95])	Stable nitrite production by comammox is utilised in the anammox process to form nitrogen. [105]
Cost	In traditional method two different reactors (nitrification and denitrification) is required for the ammonia reduction.	Cost of the infrastructure is reduced to a single reactor setup compared to two stage process.	Cost of the infrastructure is reduced to a single reactor setup compared to two stage process.
Greenhouse gases	The greenhouse gas like nitrous oxide is produced as intermediate in ammonia reduction process.	Reduction of greenhouse gas emission in wastewater nitrogen removal. [101]	Reduction of greenhouse gas emission in wastewater nitrogen removal. ([101]; [96])
Nitrogen removal	Long process which includes nitrification and denitrification steps.	Enhanced nitrogen removal.	Enhanced nitrogen removal.

sludge digestor liquor has been a cost-effective process in nitrogen reduction. In a sequencing biofilm batch reactor (SBBR), the combination of the three processes as ammonia-oxidizing bacteria (*Chitinophaeae*), anammox bacteria (*Candidatus Kuenemia*) and comammox bacteria (*Nitrospira*) helped in the reduction of ammoniacal nitrogen. The average efficiency of nitrogen removal was 98 %, which included 55 % of partial nitrification, 18.28 % of anammox and 26.62 % comammox process. With comparison to traditional nitrification, anammox is efficient as there is no external carbon source and is cost effective [55]. The recent studies [56], shows that CMX has better growth yields than the specific growth rates of AOB and NOB. The combination of anammox and comammox species in the biofilm lead to quicker biological nitrogen removal [56]. It outcompetes the heterotrophic denitrifying bacteria under certain conditions [99]. The coupling of comammox (partial nitrification) with anammox helps significantly in the reduction of aeration, external carbon source and sludge formation. The presence of the biofilm induces the faster growth of anammox and ammonia reduction [99]. The blending of both the anammox and comammox process would be further developed from the laboratory scale to large scale wastewater treatment plants. Innovation method involved in coupling of two process is cost effective, conception and environmentally friendly to the scientific community in ammonia reduction from wastewater. Nitrogen cycle is one of the biogeochemical cycles which plays a major role in the ecosystem. Discovery of new processes like anammox and comammox made it clear that nitrogen cycle is more complex than it was thought. To maintain ecological balance, the excessive nitrogen pollution in water is removed to prevent eutrophication. Therefore, it is necessary to understand the relationship of the various process in nitrogen cycle. Anammox very effectively converts ammonia directly to gaseous N_2 in presence of NO_2 without producing toxic intermediate NO_x. Recent review on coupling of anammox and partial denitrification [98] has given updates of the process.

6.1. Advantages of coupling of Comammox and Anammox processes

The coupling of comammox with the anammox is explained in the below equation where the nitrite produced from the oxidation of ammonia by comammox is utilised by the anammox bacteria in converting the ammonia to nitrogen.



- Low DO favours comammox prevalence in reactor. Therefore, the aeration is not required as needed for ammonia removal with canonical AOB/NOB system. Hence the energy cost for aeration can be minimized [61].
- The solid retention time (SRT) is higher for comammox as they are prevalent in attached growth and suspended phase [100].
- Growth rate is slower and higher growth yield compared to AOB and NOB, therefore adapted to the environment with low substrate fluxes. The sludge formation will be less [60].
- Higher ammonia removal efficiency. Comammox has higher affinity for ammonia compared to AOB therefore influent with low ammonia concentration can also be treated [60,66].
- Emission of greenhouse gases like nitrous oxide (N_2O), nitrous acid (HNO_2^-), nitrogen dioxide (NO_2) and CO_2 can be reduced by use of comammox. These are obligate intermediates of aerobic ammonia oxidation [96].
- Comammox Nitrospira has a competitive advantage over canonical ammonia oxidizers as they completely oxidize ammonia by a single microorganism in comparison to two step oxidations by AOB and NOB. Comammox dominates in nutrient depleted environment like ammonia depleted biofilms, microbial aggregates, drinking water purification system and low nutrient waste water treatment systems [58].
- The kinetics studies of ammonia oxidation in comammox bacterium *Nitrosopumilus inopinata* showed higher ammonia affinity compared to AOB [66].
- The enriched comammox Nitrospira species [11] can use urea as an alternative to ammonia for energy source and biosynthesis enabling comammox organisms to thrive in habitats with low or fluctuating urea concentrations, such as fertilized agricultural soils, WWTPs and many aquatic and terrestrial systems [57].
- Stable nitrite produced by comammox process is utilised by the anammox to reduced ammonia to nitrogen.
- The coupling of anammox with comammox enhances the nitrogen removal process by reduced infrastructure cost and simple operation.

7. Conclusions

Ammonia is a major pollutant of environment; soil, water as well as air. Several microbial ammonia removal processes have been developed. Traditional understanding of ammonia removal process includes nitrification and denitrification. Nitrification is carried jointly by chemolithoautotrophic ammonia oxidizing bacteria (AOB) and nitric oxide oxidizing bacteria (NOB). Nitrification is followed by denitrification (NO_3 to N_2) through many intermediate steps carried out by chemo-organo-heterotrophic bacteria. Energetically this is very expensive process and produces several environment polluting gasses like N_2O, CO_2 and other nitrogen oxides. Since the discovery, anammox process has attracted many scientists to explore its utility in treating ammonia rich waste water. Anammox is unique and a single step process where a particular bacterium helps in the conversion of ammonia to nitrogen in anoxic conditions without an external carbon source. Slow growth, instability under variable inflow and availability of NO_2 are the limitations for use of anammox in water treatment plants. Combining other nitrite producing processes like canonical nitrification, denitrification with anammox seems to be more effective. An alternate shortcut can be made by combining comammox and anammox process. Comammox can do complete nitrification (NH_3 to NO_3) in a single organism, which is energetically favourable in comparison to canonical nitrifiers. Further the accumulation of NO_2 can be avoided as anammox will utilize it for ammonia oxidation. Coupling of comammox and anammox processes will save the cost of aeration energy for nitrifiers and external carbon source for denitrifiers. Coupling of anammox process with comammox can help in overcoming the problems like; influent composition, more flexible conditions, single reactor process, effluent composition (less toxic NO, N_2O NO_2). This mechanism also helps in the faster enrichment of the microbes involved in the ammonia reduction from wastewater. An eco-friendly, efficient and economical process can be developed by combining comammox and anammox processes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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