Anammox – A novel microbial process for ammonium removal

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Anammox[1,2], a process of anaerobic ammonium oxidation, is an innovative technological advancement in the removal of ammonia nitrogen in waste water. This new process combines ammonia and nitrite directly into dinitrogen gas[3]. The anammox reaction can be represented as $NH_4^+ + NO_2^- = N_2 + 2H_2O$. This reaction is carried out by anammox bacteria belonging to the planctomycete group. Bacteria capable of anaerobically oxidizing ammonia were not identified earlier and were known as 'lithotrophs missing from [4]. Subsequently, this missing lithotroph was discovered[1] and identified as a new autotrophic member of the order Planctomycetales. It appears that they might be the most primitive group of bacteria, at the very root of the bacterial tree. Two of the anammox bacteria have been named provisionally[5]: *Candidatus* 'Brocadia anammoxidans' and *Candidatus* 'Kuenenia stuttgartiensis'. The former bacterium was responsible for anaerobic oxidation of ammonia observed in the Netherlands, while the latter organism has been shown to be responsible for ammonia oxidation anaerobically in several wastewater treatment plants in Germany and Switzerland. Interestingly the two species of the anammox bacteria which have been characterized are rarely found together in a single anammox reactor, they must have different, as-yet unknown, ecological niches[5].

It is well known that the biological nitrogen cycle plays an important part in the maintenance of nitrogen balance in global biosphere. The conventional biological nitrogen cycle involves microbial fixation of nitrogen gas to ammonia symbiotically and non-symbiotically which is subsequently converted to organic nitrogen. The ammonia released from organically bound nitrogen and from man-made activities, is biologically oxidized aerobically to nitrite and then to nitrate. The resultant nitrate and nitrite are reduced to nitrogen gas by a denitrifying group of bacteria using some electron donors (organic or inorganic compounds). It has been indicated[6] that anaerobic ammonia oxidation also could be a major biological activity to be included in the nitrogen cycle, thus necessitating its modification. Recently[7] it was discovered that anammox makes a significant (up to 70%) contribution to nitrogen cycling in the world's oceans. Figure 1 shows the involvement of anammox process in the biological nitrogen cycle including metabolic pathway for anaerobic ammonium oxidation.

Initially anammox research[5] was focused on the basic properties of the process and on providing evidence for its microbial nature and the principles of the nitrogen and carbon metabolism. It appears that the anammox process is based on energy conservation from anoxic ammonium oxidation with nitrite as the electron acceptor and hydrazine and hydroxylamine as the intermediates. Carbon dioxide is used as the main carbon source for growth. One of the key enzymes of anaerobic ammonium oxidation is hydroxylamine oxidoreductase[8]. Its importance is illustrated by the fact that it constitutes 10% of the total cell protein. It catalyses the oxidation of hydrazine and hydroxylamine. The enzyme has been located in a membrane-bounded, 'organelle' named anammoxasome, in the cytoplasm of the anammox cells[9]. This 'organelle' appears to be the center of anaerobic ammonium oxidation. It is quite likely that its function could be containment of hydrazine. The anammoxasome is believed to be surrounded by a bilayer membrane that consists of unique 'ladderane' lipids[5].

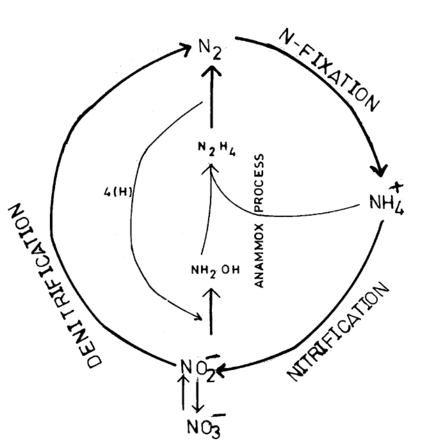

Figure 1. Involvement of anammox process in biological nitrogen cycle including metabolic pathway for anaerobic ammonium oxidation (adapted from refs 3 and 5).

The structure and function of anammox lipids has been published recently[10]. Lipid membranes are essential to the functioning of cells facilitating the existence of concentration gradients of ions and metabolites. Microbial membrane lipids are known to contain three-, five-, six-, and even seven-membered aliphatic rings, but four-membered aliphatic cyclobutane rings have been reported for the first time, in the dominant membrane lipids of two anammox bacteria. These lipids contain up to five linearly fused cyclobutane moieties with *cis* ring junctions. These 'ladderane' moieties, rigid and dense ladders of concatenated cyclobutane rings, occur in the membrane of the anammoxasome, the intracytoplasmic compartment, where anammox catabolism takes place. A membrane with ladderanes in its core is highly impermeable to passive diffusion of chemicals. Such a membrane is required to maintain concentration gradients during the very slow anammox metabolism. It also protects the remainder of the cell from toxic anammox intermediates, viz. hydroxylamine and hydrazine. Hydrazine and hydroxylamine were observed to diffuse in and out of anammox cells as free molecules[3]. The containment of these chemicals inside the anammoxasome was considered impossible, since both compounds diffuse through biomembranes. The discovery[10] of the unprecedented molecular structure of the anammox membrane lipids shows that the anammoxasome membrane is much less permeable than normal biomembranes because of the presence of unique 'ladderane' lipids. From a bioenergetic perspective, the energy loss associated with loss of one molecule of hydrazine from the anammox cell is equivalent to at least 10 full catabolic cycles, leading to 50% decrease in biomass yield at 10% hydrazine loss. For these reasons, limitation of diffusion is very important for these bacteria which are slow growers. One of these lipids was identified as a sn-2-glycerol monoether with a C_{20} alkyl chain containing four rings. The second lipid was found to be a methyl ester of a C_{20} fatty acid containing five rings. From an evolutionary perspective we may note that part of the ladderane tails is ether linked to the glycol backbone. As of now, only a minority of bacteria (either thermophiles or sulphate reducers) have ether-linked lipids. Ladderane lipids can be employed as molecular labels for tracing anammox activity in natural ecosystems. They are promising building blocks in opto-electronics. Since the ladderanes are difficult to be synthesized chemically, presently the anammox process is the only natural source.

Due to stringent wastewater discharge limits for ammonia (50 mg/l NH_4–N) and nitrate (10 mg/l NO_3–N) for inland surface waters, it is essential to remove them to the permissible level. Biotreatment, being cost effective, is normally adopted for their removal in wastewater. Conventional biotreatment for ammonia removal involves nitrification and denitrification. Biological nitrification is generally carried out by autotrophic nitrifying bacteria that oxidize ammonia to nitrite and nitrite to nitrate with molecular oxygen as electron acceptor. Nitrite and nitrate are subsequently reduced to nitrogen gas by denitrifying bacteria, under anoxic conditions. However, this conventional biological nitrification/denitrification process is associated with the following requirements. Nitrification process demands very efficient oxygen supply coupled with adjustment of alkalinity of the wastewater due to the formation of nitrate ions. Denitrifying bacteria essentially need a carbon source as the electron donor. These two requirements demand additional recurring expenditure.

The anammox process based on the application of anammox bacteria reduces the requirements of oxygen for biological ammonia oxidation to nitrate and eliminates the need for external organic carbon for denitrification. Potential benefits that acrue from the application of this novel process would be further substantial reduction in capital and operating costs over the conventional biotreatment process. In order to remove ammonia, it is necessary to partly convert it microbially into nitrite only under aerobic conditions and subsequently allow this nitrite and the remaining ammonium to react anaerobically using anammox bacteria, thereby converting it into dinitrogen gas. For efficient conversion of ammonia to dinitrogen gas via nitrite intermediate, oxygen supply needs to be controlled.

Thus, under oxygen limitation (< 0.5% air saturation) a coculture of aerobic and anaerobic ammonium oxidizers has been obtained. These cultures converted ammonia directly to dinitrogen gas with nitrite as the intermediate. Application of this concept in wastewater treatment could lead to complete ammonia removal in a single autotrophic reactor[5]. This concept has been named 'CANON', meaning 'completely autrophic nitrogen removal over nitrite', and referring to the way the two groups of microorganisms interact performing two sequential reactions simultaneously[5]. While studying a two-stage biological treatment system for ammonium nitrate laden wastewater, it was observed that after nitrate removal in the first stage fixed bed anoxic denitrifying unit, there was substantial reduction in ammonia in a second stage pond system. Gas bubbles were seen generated from the bottom of the pond. A possible mechanism for ammonia removal has been suggested analogous to the above CANON concept[11].

Very recently, a sequencing batch reactor (SBR) has been evaluated using a mix of anammox bacteria and aerobic nitrifying bacteria (predominantly bacteria oxidizing ammonia to nitrite). In order to obtain the right blend of these bacteria, the reactor was initially started anoxically after inoculating with anammox bacteria. Subsequently oxygen was supplied to the reactor and a nitrifying population developed. The SBR was then operated under oxygen-limiting conditions. Under this condition, completely autotropic ammonia removal to dinitrogen gas was achieved. This process was very stable and easy to maintain during the period tested (3 months). Nitrosomonas-like bacteria converted partially ammonia to nitrite and anammox bacteria converted the mixture of ammonia and nitrite mainly to dinitrogen gas. In the reactor, no aerobic nitrite oxidizing bacteria were detected. The denitrifying potential of the biomass was below the detection limit. 85% of ammonia was converted to dinitrogen gas and the remainder was recovered as nitrate[12]. Development of such a biotreatment system for ammonia removal would benefit industries which discharge ammonia-containing effluents, viz. meat processing, milk processing, petroleum refining, fertilizer manufacture, certain synthetic fiber plants, explosive and latex processing industry, low temperature and high temperature carbonization process and ammonium nitrate manufacturing unit in steel plants.

The research findings on this unique group of anammox bacteria have not only brought to light the missing lithotroph involved in the nitrogen cycle, but also have given scope for developing a low cost biotechnology for control of ammonia nitrogen pollution.

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