Microbiology/Biochemistry of the Nitrogen Cycle
Innovative Process Applications

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Abstract: SHARON®, InNitri R, and ANAMMOX® are very cost-effective treatment processes for the total removal of nitrogen components, thru nitrification/denitrification, from wastewater flow streams containing high concentrations of nitrogen. The processes are used in the treatment of municipal wastewater side streams with high ammonia concentrations to achieve high total overall nitrogen removal.

SHARON® is a high rate process for the removal of total nitrogen operating with minimal sludge retention time. Due to differences in growth rates of the bacterial species at the process design temperature (30-40°C) a selection can be made wherein the nitrite oxidizing bacteria can be washed out of the system while ammonia oxidizing bacteria are retained along with denitrifying bacteria. Using this metabolic mode of operation allows for a 25% reduction in aeration energy required for ammonia removal, a 40% reduction in the amount of BOD addition needed for denitrification, and a 33% percent reduction in sludge production. In addition since the process is accomplished in a side stream there are savings in mainstream reactor costs.

InNitri® is a high rate process for the conversion of ammonia to nitrate operating with a short sludge retention time allowable due to the temperature of the recycle stream. It converts the ammonia load in the side stream to nitrifying bacteria and nitrates. The nitrifying bacteria produced are used to seed the main stream reactors. The combined effect of this conversion and seeding of the main stream reactor is to reduce the reactor costs necessary for nitrification by 50%.

The ANAMMOX® process is in development it involves taking the effluent from a SHARON® reactor which is designed to produce a 50%/50% molar effluent of nitrite and ammonia and treating it with anammox bacteria to bring about an anaerobic oxidation of ammonia to nitrogen gas. The potential cost savings are the reduced costs for aeration by going with this metabolic pathway. To be cost effective the value of the ANAMMOX® reactor plus the savings for reduced aeration has to be less than the value of the reduced size SHARON® reactor plus the marginal cost of aeration to achieve a full oxidation of ammonia.

The pertinent biochemical principles of both nitrification and denitrification that apply to the processes are developed and illustrated. This involves unique biochemistry and metabolic pathways that may not be familiar to many consulting engineers.

The process design parameters for the five SHARON® plants in the Netherlands (Utrecht, Rotterdam, Zwolle, Beverwijk, and Groningen) are listed and compared to actual operating data. A description of the SHARON® project for New York New York's Ward Island plant with process design parameters is given. The process design parameters for the InNitri R pilot plant system at Tuscon AZ are listed and compared to the actual demonstration program operating results. The latest research results on the development of ANNAMOX® are also given.

SHARON® acronym Single reactor system for High activity Ammonium Removal Over Nitrate
InNitri® acronym Inexpensive Nitrification
ANAMMOX® acronym Anoxic Ammonium Oxidation

Keywords: nitrification and denitrification metabolic pathways, nitrification and denitrification biochemistry, growth rates of Nitrosomonas and Nitrobactor, Nitrification Bioaugmentation

INTRODUCTION

Three relatively new processes have been developed to remove nitrogen in high concentration from wastewater, especially recycled side streams from biosolids processing- SHARON®, ANAMMOX®, and InNitri®. They each have unique characteristics. SHARON® incorporates a different metabolic pathway than is usually implemented in wastewater treatment, InNitri® uses the concept of bioaugmentation in a new way, and ANAMMOX® uses an entirely new group of bacteria to oxidize ammonia anaerobically; all to remove nitrogen more cost effectively. What we will find is that the processes, while unique, do fit into our understanding of the bacteria and biochemistry that propel the nitrogen cycle. Further we will see that, while unique, the processes use the same principles of biological design that we have known for years.
NITROGEN CYCLE

The nitrogen cycle is the continuous series of natural and man made processes which passes nitrogen thru its various oxidation-reduction states from nitrogen gas thru ammonia, carbon bound nitrogen, nitrogen oxides and back to nitrogen gas.

Many of the natural steps are biologically mediated, with plants, fungi, or bacteria conducting the chemical reactions. The bacteria are of special interest in that they can be used to remove nitrogen from waste water. The process of growing biomass in waste water treatment is based on ammonium and nitrate assimilation from the wastewater to make the carbon bound nitrogen compounds in the protoplasm of the biosolids. Then when we breakdown those biosolids in their disposal we release back nitrogen in the form of ammonium by ammonification to the waste water. To remove ammonium from the waste water other bacteria can be used as oxidizers to provide nitrification and nitrification and as reducers to provide denitrification and denitrification; returning, if necessary nitrogen back to the atmosphere. Recently a new group of bacteria have been found that increased our knowledge of the nitrogen cycle. They are the so called anammox bacteria which can accomplish an anaerobic ammonium oxidation and release as a product nitrogen gas back into the atmosphere. While originally predicted by Broda (1), then “discovered” by Mulder (2)(3)(4), these bacteria are finally being uncovered in oceanic anaerobic zones such as the Baltic North Sea (5), the Black Sea (6), and the Thames Estuary (7). It is becoming known that these bacteria play a significant role in recycling large quantities of nitrogen from the sea beds of the world into the atmosphere.

CHEMICAL THERMODYNAMICS

The driving force of life is energy. The chemical compounds in wastewater contain the energy that bacteria use to synthesize the complex chemical compounds that is their own cellular protoplasm, which provides the force to transport nutrients into and within the cell that allows them to move and to reproduce themselves. A look at the free energies, ΔG, of the nitrogen compounds in waste water shows that:

\[
\begin{align*}
\text{NH}_3 + \text{O}_2 + 2\text{e}^- + 2\text{H}^+ &\rightarrow \text{NH}_2\text{OH} + \text{H}_2\text{O} \\
\text{NO}_2^- + 0.5\text{O}_2 &\rightarrow \text{NO}_3^- \\
\Delta G &= -287 \text{ kJ/mole} \\
\text{NO}_3^- + 2\text{e}^- + 2\text{H}^+ &\rightarrow \text{NO}_2^- + \text{H}_2\text{O} \\
\Delta G &= -358 \text{ kJ/mole} \\
\text{NH}_4^+ + \text{NO}_3^- &\rightarrow \text{N}_2 + \text{H}_2\text{O} \\
\Delta G &= -260.9 \text{ kJ/mole}
\end{align*}
\]
Thus while not providing large amounts of energy to the bacteria both the oxidation sequence of ammonia to nitrite and nitrate and the reduction of nitrate/nitrite to nitrogen can occur spontaneously; provided there is a means of transporting the electrons and protons involved in the reactions. The cellular material in the membranes of the bacteria do provide for this transport.

**ENZYMES**

Enzymes are very complex proteins; they function as biochemical catalysts. The thermodynamics of the nitrogen reactions of interest in waste water treatment have been shown to be spontaneous. However they would occur very slowly if left to themselves within the bacterial cell because a certain amount of activation energy is necessary to bring the reacting molecules together in the proper orientation to form a transition state leading to the product molecules of the reaction. Of particular note in regard to enzymes is the fact that they have metal atoms incorporated into their structures called metal cofactors at their physical centers. The metals in the enzymes used in nitrogen processing are iron, molybdenum, and copper. It seems that the iron enzymes are more common than molybdenum and copper; that is fortunate because iron is usually prevalent in waste water although there are cases of plants with iron shortages. Methods are just being developed in this area to determine if that is iron is indeed prevalent. However nitrification, nitrafication, denitrification, and denitrification could falter in waste waters lacking sufficient metals for proper enzyme development (8). The cytochromes used to transport electrons in many of the metabolic pathway are iron based.

![Figure 3 Cytochrome C](image)

**Figure 3 Cytochrome C**

**BACTERIA**

The bacteria of the nitroso-group oxidize ammonium to nitrate, those of the nitro-group finish the oxidation yielding nitrate. There are many bacteria that reduce nitrite and nitrate to nitrogen gas. The newly discovered group of anammox bacteria anaerobically oxidizes ammonium to nitrogen gas by means of oxygen from nitrite.

![Figure 4 Nitrosomonas, Nitrobactor, Denitr- and Denit- fying Bacteria, and Anammosox Bacteria](image)

**Figure 4 Nitrosomonas, Nitrobactor, Denitri- and Denitra- fying Bacteria, and Anammosox Bacteria**

**METABOLIC PATHWAY OXIC AMMONIA OXIDATION**

Nitrosomonas bacteria are illustrated (Figure 4). The first step in the metabolic path way is accomplished by the enzyme ammonia monooxygenase (AMO) the second step is accomplished by hydroxyl-amine oxidoreductase (HAO). Both enzymes are co-depandent because they generate the substrate and electrons, respectively, for each other. AMO catalyzes the oxygenation of ammonia to hydroxylamine. The two electrons required in this process are derived by form the oxidation of hydroxylamine to nitrite by (HAO). One of the oxygen atoms in nitrite derives from oxygen, the other comes from water. Two of the four electrons generated by HAO are transferred via the tetra-heme cytochrome \(c_{554}\) either to AMO or diverted into an electron transport chain, where many of the carriers involved and the intermediates have not been completely characterized. The overall reaction converts ADP to ATP an energy rich compound, see Figure 5.
METABOLIC PATHWAY OXIC NITRITE OXIDATION

A Nitrobactor bacterium is illustrated (Figure 4); note the folds in the membrane where the enzymes that catalyze the reaction are located. Nitrite is catalytically oxidized to nitrate by the enzyme nitrate oxidase. Again the overall reaction converts ADP to ATP, see Figure 5.

METABOLIC PATHWAY ANOXIC NITRITE/NITRATE REDUCTION

Typical bacteria that accomplish denitrification and denitrafication are illustrated (Figure 4); again note the folds in the membrane where the catalytic enzymes are located. To say denitrification and denitrafication is complex is almost an understatement. Let us start with an overview of a complete metabolic pathway (Figure 5); the diagram illustrates the reduction of either nitrite or nitrate in the cell by the enzymes and the lower diagram is for a gram negative bacterium, the model is based on research of the species pseudomonas stutzeri. Nitrate moves thru the cell membrane's anti porter to the cytoplasm and is reduced catalytically at a nitrate reductase (NAR) site in the plasma membrane to nitrite. The nitrite then flows back thru the plasma membrane into the periplasm where it is reduced catalytically by nitrite reductase in the periplasm to nitric oxide. The nitric oxide is then catalytically reduced at a nitric oxide reductase site in the membrane to nitrous oxide and finally a nitrous oxide reductase in the periplasm reduces the nitrous oxide to nitrogen, which leaves the cell thru its outer membrane and enters the mixed liquor.

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METABOLIC PATHWAY ANAEROBIC AMMONIA OXIDATION

At present, five bacteria have been identified as having an anammox metabolic pathway: candidatus brocadia anamnoxidans, candidatus kuenenia stuttgartiensis, candidatus scalindua wagneri, candidatus scalindua brodae, and candidatus scalindua sorokinii. An anammox bacterium is illustrated (Figure 4), note the unique structures of a dense fibrillar nucleoid, ribosome like particles, and crateriforms on the surface. A plausible metabolic pathway has been proposed to account for the facts discovered by the research done so far. Ammonia and hydroxylamine, generated from the reduction of nitrite by nitrite reductase, are converted to hydrazine by the catalytic enzyme HZF. Subsequently the hydrazine is oxidized to nitrogen gas by the catalytic enzyme hydroxylamine oxidoreductase, which generates four electrons that can be utilized in the reduction of nitrite.
AMBIENT ENVIRONMENTAL EFFECTS ON BACTERIAL GROWTH

TEMPERATURE All chemical processes are affected by temperature, especially biochemical processes. The effect of temperature on the ammonia nitrogen utilization coefficient of the bacterium nitrosomonas is parabolic in shape. The optimum temperature for this bacterium is 26-28°C. Its growth rate will decline on either side of that optimum. In practical terms this means that for that bacterium to proliferate and establish a representative population at lower or higher temperatures a long sludge retention time is needed, or the nitrosomonas population will be washed out of the reactor. With InNitri®, a new process concept, bioaugmentation of nitrifying biomass from one process reactor to another is proposed. That biomass transfer might be accomplished with a temperature differential between the reactors of as much as 20°C. Thus there is concern that the transferred biomass would be “shocked” and not function at the expected nitrification removal rate. This fear has been assuaged with a recent study. The study (11) was done at the University of Manitoba by Melanie Head PhD to determine the impact of a sudden temperature decrease on the nitrification rates of a nitrifying biomass grown at warm temperatures. The results indicate that nitrifying biomass grown at 20°C, 25°C, and 30°C and put into an environment of 10°C respond with decreases in nitrification rates that are less than the USA EPA factor of e\(^{-0.098(T-15)}\). The observed decreases in nitrification rates were 58%, 71%, and 82% for nitrifying biomass grown at 20°C, 25°C, and 30°C and cooled to 10°C. The USA EPA equation predicts nitrification rate reductions of 62.5%, 77%, and 85.9%.

DISSOLVED OXYGEN The level of dissolved oxygen (DO) impacts the ability of the nitrifiers and nitrifiers to attain good removal of ammonia as illustrated (Figure 12). The optimum DO is dependent on floc size which in turn depends up on the amount of turbulence in the reactor. The larger the flocs the higher the DO that will be needed to fully penetrated it and provide a minimum of 0.1mg/l DO to the individual bacteria. It can be seen that good performance can be obtained with a DO of 3-4. The opposite effect is needed for denitrification, a low DO. This is effect is illustrated (Figure 6). The catalytic enzyme nitrogen oxide reductases will operate only at low oxygen levels of DO. Figure 6 shows that with DO drop the bacteria start generating the enzymes to accomplish denitrification. Once a level of low threshold level of DO is occurs the bacteria utilize these enzymes to derive energy from the oxides of nitrogen.
The growth rate of the nitrifiers and nitrafiers is influenced by the pH of the mixed liquor. Growth occurs at low pH but it is slow, nitrification can be accomplished at low pH, but only at extended sludge retention times. This is attested to by the 20 oxygen activated sludge plants that accomplish complete nitrification at a pH range of 5.6-6 on a continuous basis.

One of the limiting nutrients in nitrogen removal systems that sometimes are over looked is carbon, many systems are carbon limited and the addition of carbon source is required. A popular commercial source is methanol; however high methanol prices have changed this and many plants are switching to other carbon sources. As pointed out the catalytic enzymes that are required to accomplish nitrogen removal are metal based and the waste water must have a minimum amount of Fe, Cu, and Mo for those enzymes to be synthesized.

The key to the development of the SHARON® process was the recognition that there was a substantial difference in growth rates between the two genera of bacteria that perform nitrogen oxidations, the nitroso- and nitro- bacteria, over the range of temperatures used in the design of waste water treatment plants. At temperatures below about 12°C the nitro- bacteria grow at a faster rate than nitroso- bacteria. At temperatures above about 12°C the nitroso- bacteria grow at a faster rate than nitro- bacteria. So at the temperatures that can readily be attained in a SHARON® reactor (25-40°C) if the sludge retention time (SRT) and hydraulic detention time (HRT) are set properly (0.5- 1.5 days) a situation results wherein nitroso- bacteria are preferentially grown; of course nitro- bacteria are present but are washed out of the reactor more rapidly than they can proliferate. This allows a different metabolic pathway to be used to convert ammonia to nitrogen gas.

Bioaugmentation of biological waste water treatment reactors has been known for a considerable period of time. Its most common form is the addition of bacteria or their enzymes to dysfunctional biological treatment reactors to improve their performance. In addition there was work done on moving a nitrifying biomass from one activated sludge reactor to another to observe if that would reduce the apparent sludge age required for nitrification(a), the results were positive in that the biomass in the reactor which was bioaugmented nitrified at a lower apparent sludge age than would be predicted. This method of bioaugmentation was never reduced to commercial practice; probably because main stream reactors do not have enough exportable nitrifying biomass to sustain that type of operation during cold weather due to the slow growth rates of both nitroso- and nitro- bacteria at low temperatures. Peter Kos recognized a special situation in regard to recycled side streams, especially those generated during biosolids processing; there the temperature of the waste water is warm and ammonia concentrations are high. Under these conditions a biological reactor can be designed and built to produce an exportable nitrifying biomass that can be used to lower the apparent sludge retention time necessary for nitrification in the main stream reactors. The main stream reactors necessary for nitrification can be reduced by 40-50% using this process(15).
NOVEL BACTERIAL SPECIES (ANAMMOX®)
The recent research on anammox bacteria shows us that the window is not closed on new processes for wastewater treatment. There are now several commercial anammox reactors in operation; now to be proved is that the savings due to reduced aeration provided are greater than the cost of installation and operation of the reactor.

FIGURE 7 SHARON, ANAMMOX, InNitri Processes

SHARON®'s CONTINUING OPERATION @ UTRECHT (13)

A review of a full year’s operating data for 1998 shows very stable operation. The influent ammonia varied from 400 to 750 mg/l during the period with a highly variable flow ranging from 0 to 1,375 m³/day, which is 0 to 363,275 gallons/day. During this period the effluent ammonia averaged less than 5 mg/l, albeit with some peaks in the period from 0 to 400 days. The peaks were caused by insufficient pH control. The NOX-N concentration in the effluent was generally under 100mg/l. Flows vary as a result of the variability of output from biosolids processing. The process was not upset by high influent suspended solids which at times reached concentrations of 30-50,000 mg/l and were often above 10,000 mg/l. The control of pH was done through recovered alkalinity from denitrification/denitrification caused by methanol addition in anoxic conditions. Occasionally supplemental sodium hydroxide, a backup pH control, is used in the case where insufficient methanol was dosed. The influent temperature varied over the year between 20 and 30°C. The reactor temperature was raised to 38°C by means of biochemical heat production, there is no need to provide supplemental heat. The installed supplemental heat exchange unit operation has been discontinued as the required reactor temperatures are maintained thru heat released by the reactions and the insulation of the reactors. It was the choice of the operations group to maximize ammonia removal so there was partial denitrification via nitrate. The ammonia removal efficiency aimed for can only be achieved with anoxic retention time of more than 2 days and therefore the conversion is partially to nitrate and partially to nitrite. Thus there is only partial nitrogen removal via nitrite in this SHARON®. This is confirmed by the average ratio of COD/N-denitrified which had a value of 3.3 g COD/g N. In theory the minimal stoichiometric demand would amount to 2.86 g COD/g NO₃-N denitrified or 1.72 g COD/g NO₂-N denitrified. Considering biomass yield, the demand is expected to be between 3.5 and 2.2 g COD/g N removed. Since installation of the SHARON® process the main stream plant has lowered the total nitrogen content in the effluent by 30%. During the first half of 1998 the total nitrogen content in the plant effluent dropped on average from 16 to 11 mg/l.

SHARON®'s CONTINUING OPERATION @ ROTTERDAM (14)
The results for 2002 show continued stable efficient operation. The large fluctuations in feed nitrogen continued ranging from 200-750 kg/day, which is 441-1653 lbs/day in a random pattern. During the second half of 2002 denitrification efficiency was raised from 60-80% to approximately 90% by increasing the methanol dosage. During that period ammonia removal was on average above 95%. The influent ammonia nitrogen varied from 700-1200 mg/l and was above 1000 mg/l the majority of the time. Effluent concentrations stayed in the range targeted by the operations staff. Depending on the process settings, effluent ammonia nitrogen concentrations ranged from 10-90 mg/l and averaged below 50 mg/l. The Rotterdam SHARON® system is controlled to remove nitrogen via nitrite which requires anoxic hydraulic retention below 2 days. At anoxic hydraulic retention time of approximately 1.5 days the COD/N ratio as methanol consumed illustrates clearly the metabolic pathway from
ammonia via nitrite to nitrogen. From 1999 thru 2000 the SHARON® process had a very beneficial effect on the
main stream effluent lowering the effluent ammonia from an average 6.2 mg/l to 2.1 mg/l and total nitrogen
from an average 7.5 mg/l to 3.9 mg/l.

OTHER SHARON® OPERATIONS
Sharon has been successfully implemented at Zwolle, Beverwijk, Groningen, and Haag Houttrust in the
Netherlands. All facilities are getting 90-95% nitrogen removal. A SHARON® System is in construction at New
York, NY, DEP’s Wards Island Plant.

ANAMMox® PROCESS (16)
An ANAMMOX® reactor was installed at Rotterdam to process the full flow from the SHARON® reactor, the plant
has had a difficult startup never achieved more than 30% of its design capacity for several years. The problems
were over come in early 2006 and it is now processing 100% of its design flow. Further testing is on going.

InNitr® TUCSON, ARIZONA DEMONSTRATION PLANT
Tucson, Arizona and Mixing and Mass Transfer Technologies LLC conducted a demonstration program at its Ina
Road plant that started in the fall of 2003 to simulate the effect of an InNitr® system on its UNOX®
carbonaceous removal system. The results of the pilot study have been presented at WEFTEC 2004(17). The
operating data indicates that central nitrafication was demonstrated, nitrafication was demonstrated in the
UNOX® system at a shorter apparent SRT than would have occurred with out bioaugmentation, and
denitrafication was demonstrated. Bioaugmentation allowed the UNOX®-InNitr® system to provide
significantly more nitrafication and denitrafication than would be expected at the apparent SRTs.

CONCLUSIONS
- Both SHARON® is cost effective relative to other means of treating recycled side streams with high
  concentrations of ammonia. SHARON® has been fully commercialized with five plants running and one in
  construction.
- Anammmox® is being commercialized with three plants running, one municipal and two industrial.
- InNitr® has been demonstrated and work is being done on its commercialization.

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