# CHAPTER 2

# THEORIES AND LITERATURE REVIEW

## 2.1 Biological Nitrogen Removal

Biological nitrogen removal is the most efficient method to reduce nitrogen from the wastewater. The nitrification and denitrification are the principal processes for biological nitrogen removal as shown in Figure 2.1. The nitrification occurs under aerobic conditions. Ammonium is oxidized to nitrate by autotrophic bacteria. The denitrification occurs under anaerobic conditions in which nitrate is reduced to nitrogen gas by heterotropic bacteria and organic carbon in wastewater is utilized as electron donor. Another one is ammonification that ammonia converted from organic nitrogen. It is an important process that ultimately allows organic nitrogen to be removed from wastewaters through hydrolysis to amino acids later be broken down to produce ammonium or directly incorporated into biosynthetic pathways in support of bacterial growth.

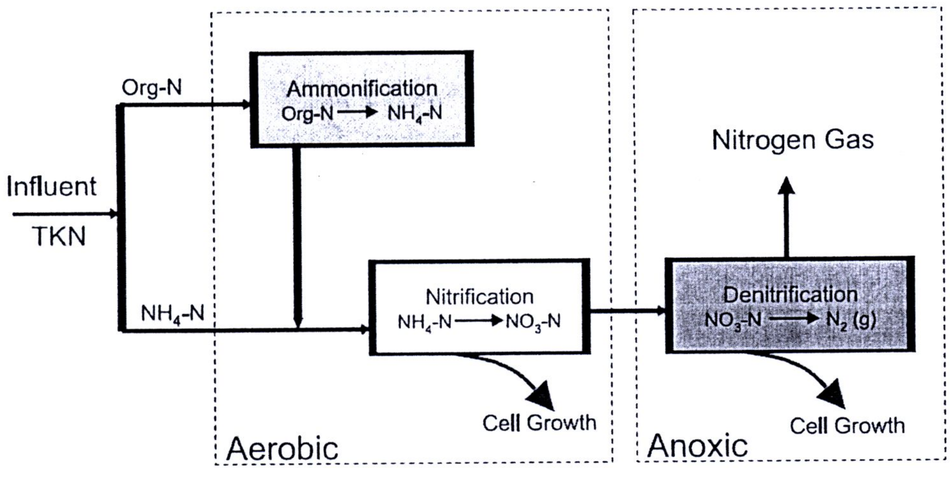


**Figure 2.1.** Biological Nitrogen Removal [8]

## 2.2 Nitrification

The nitrification process occurs in two steps: (1) ammonium is oxidized to nitrite by *Nitrosomonas* spp., and (2) nitrite is oxidized to nitrate by *Nitrobactor* spp.

The energy reaction of ammonium oxidized by *Nitrosomonas* spp. is as follows.

$$2NH_4^+ + 3O_2 \longrightarrow 2NO_2^- + 4H^+ + 2H_2O \qquad (1)$$

The energy reaction of nitrite oxidized by *Nitrobactor* spp. is as follows.

$$2NO_2^- + O_2 \longrightarrow 2NO_3^- \qquad (2)$$

The overall of energy reaction of both equation (1) and (2) is as follows.

$$NH_4^+ + 2O_2 \longrightarrow NO_3^- + 2H^+ + H_2O \qquad (3)$$

In nitrification process, both *Nitrosomonas* spp. and *Nitrobactor* spp. are a homogenous group and it is called nitrifying bacteria. The nitrifying bacteria use carbon dioxide ($CO_2$) as the carbon source for cell synthesis. The overall equation of nitrification that combines between the energy process and the cell synthesis process [9] is shown in the following:

$$NH_4^+ + 1.86O_2 + 1.98HCO_3^- \longrightarrow 0.02C_5H_7NO_2 + 0.98NO_3^- + 1.88H_2CO_3 + 1.04H_2O \qquad (4)$$

## 2.3 Denitrification

The conventional denitrification is heterotrophic denitrification that reduces nitrate to nitrogen gas by utilizing organic carbon. Organic carbon is a carbon source for cell growth and an electron donor in energy reaction under the absence of oxygen. The energy reaction of heterotrophic denitrifying bacteria is as follows:

$$0.014C_{18}H_{19}O_9N + 0.2NO_3^- + 0.2H^+ \longrightarrow 0.1N_2 + 0.24CO_2 + 0.014HCO_3^- + 0.014NH_4^+ + 0.2H_2O \qquad (5)$$

The overall equation of denitrification that includes energy and the cell synthesis reaction [9] is shown in the following:

$$0.65C_{18}H_{19}O_9N + 4.89NO_3^- + 4.89H^+ \longrightarrow C_5H_7NO_2 + 2.27N_2 + 6.70H_2CO_3 + 5.12H_2O \quad (6)$$

### 2.3.1 External carbon requirement

The performance of a biological nutrient removal (BNR) system is strongly affected by the characteristics of the wastewater influent. The biological nitrogen removal and the biological phosphorus removal are accomplished at sufficient biodegradable organic carbon as measured in form of COD or BOD. These processes are accomplished by heterotrophic bacteria and they require organic carbon for their metabolism. Therefore, the nitrogen and phosphorus are removed completely by BNR process, if the influent wastewater contains sufficient organic carbon.

Then, the suitable compositions of wastewater such as COD/N and COD/P ratios are important for biological nutrient removal processes. It is necessary to add an external carbon source if these ratios are too low.

### 2.3.2 The C/N ratio

In denitrification plant, organic carbon in wastewater will be used for three purposes: conversion of nitrite to nitrogen gas, sludge production, and respiration with oxygen.

Then, the consumption of organic carbon in operation is more than the consumption of organic carbon in theory. The optimum C/N ratio is stated for different types of organic carbon as shown in Table 2.1.

**Table 2.1.** The optimum C/N ratio for different types of organic carbon to be used for denitrification. [9]

| Organic carbon | (C/N) optimum | Unit |
|---|---|---|
| Organic carbon in wastewater | 3 – 3.5 | kg BOD/kg N |
| | 4 – 5 | kg COD/kg N |
| Organic carbon in sludge | 1.5 – 2.5 | kg BOD/kg N |
| | 2.9 – 3.2 | kg COD/kg N |
| Methanol | 2.3 – 2.7 | kg $CH_3OH$ /kg N |
| | 3.5 – 4.1 | kg COD/kg N |
| | 1.0 – 1.2 | mol $CH_3OH$ /mol N |
| Acetic acid | 2.9 – 3.5 | kg $C_2H_4O_2$/kg N |
| | 3.1 – 3.7 | kg COD/kg N |
| | 0.9 – 1.1 | mol $CH_3OH$ /mol N |

If the COD/N ratio in wastewater is too low, it means that the denitrification process will occur at a low rate which results in low efficiency of nitrogen removal.

With methanol ($CH_3OH$) as the carbon source, the stoichiometry of the COD/N ratio in denitrification process can be calculated from the following equation.

$$NO_3^- + 1.08CH_3OH + 0.24H_2CO_3 \longrightarrow 0.056C_5H_7NO_2 + 0.4N_2 + HCO_3^- + 1.68H_2O \qquad (7)$$

From Equation (7), 1 mole of nitrate-nitrogen ($NO_3$-N) consumes 1.08 mole of methanol ($CH_3OH$). Then the methanol consumption is 2.47 g $CH_3OH$/g N. The COD value of methanol can be calculated from the following equation.

$$CH_3OH + 1.5\ O_2 \longrightarrow CO_2 + 2H_2O \qquad (8)$$

The COD value of methanol is 1.5 g COD/g $CH_3OH$. Then, the COD/N ratio in denitrification can be calculated to be 2.47 x 1.5 = 3.7 g COD/g N.

## 2.4 Anaerobic ammonium oxidation (anammox) process

Normally, nitrogen is removed via the two step process. In the first step, ammonium is oxidized to nitrite and nitrate by autotrophic bacteria in aerobic condition, and in the second step, nitrate is reduced to nitrogen gas by heterotrophic bacteria under anoxic conditions. However, ammonium in wastewater can reduced to nitrogen gas via anoxic conditions known as the ANAMMOX process.

In the ANAMMOX process, ammonium is oxidized to nitrogen gas by utilizing nitrate as an electron acceptors and ammonium as an electron donor. The reaction of the anammox process is shown in the following equations.

The energy reaction of oxidized ammonium and nitrite to nitrogen gas is as follows.

$$NH_4^+ + NO_2^- \longrightarrow N_2 + 2H_2O \quad (9)$$

The synthetic reaction is as follow.

$$CO_2 + 2NO_2^- + H_2O \longrightarrow CH_2O + 2NO_3^- \quad (10)$$

The overall reaction is as follow.

$$NH_4^+ + 1.32\, NO_2^- + 0.066HCO_3^- + 0.13H^+ \longrightarrow$$

$$0.066CH_2O_{0.5}N_{0.15} + 1.02N_2 + 0.26NO_3^- + 2.03H_2O \quad (11)$$

### 2.4.1. Metabolic Pathway

Strous et al. (1997) [10] studied the feasibility of ammonium removal with the anammox process. In an experiment, the anammox was maintained in fluidized bed with volume of 2.5 liters. Temperature and pH value were controlled at 36°C and 8 respectively. The liquid from the top of reactor was returned to the boost at flow rate 47 L/h. Sand particles (diameter 0.3-0.6 mm) were used as the carrier material. A synthetic wastewater was used in this experiment; it contained ammonium and nitrite concentrations of 0.84 kg $NH_4$-N/$m^3$ and 0.84 kg $NO_2$-N/$m^3$, respectively. The result of this experiment showed that the efficiencies of anammox process for ammonium and nitrite removal were 82 and 99 percent, respectively.

The anammox process is a novel metabolic pathway in which nitrogen in wastewater is almost completely removed by autotrophic bacteria. In this mechanism, ammonium ($NH_4^+$) is together with hydroxylamine ($NH_2OH$) were converted to hydrazine ($N_2H_4$). Subsequently, hydrazine is oxidized to nitrogen gas and four electrons as shown in Figure 2.2. These electrons are used for reducing nitrite to hydroxylamine [15].

For instance, these metabolically versatile bacteria are capable of oxidizing short chain fatty acids with nitrate [11], co-oxidizing propionate and ammonium in the presence of nitrite and nitrate [12], and performing dissimilatory nitrate reduction to ammonium [13]. In addition, anammox bacteria were recently shown to be able to tolerate higher $O_2$ concentrations than originally established by Strous et al. (1997) [10].

Anammox bacteria have an internal ribosome-free compartment, or anammoxosome, where the catabolism of anammox is assumed to take place. This physical feature result sinaring- shaped fluorescence in situ hybridization (FISH) signal, which aids in cell recognition [14].

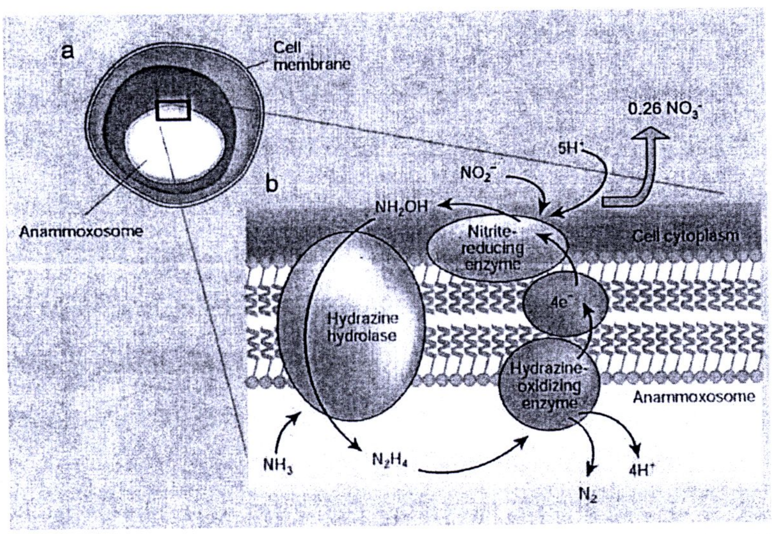


**Figure 2.2.** Mechanism of anaerobic ammonium oxidation [15]

### 2.4.2. Nitrite inhibition to Anammox

Many researchers who have reported that the inhibition range of nitrite concentration to anammox activity is as follows.

- Kimura et al. (2010) [16] investigated the effects of nitrite on anammox bacteria entrapped in gel carriers by using batch and continuous feeding tests. The results showed that the nitrite concentration in a batch reactor must be less than 274-mg N/L in order to prevent a decrease in the anammox activity. The anammox activity at nitrite concentration of 305 mg-N/L seems to decrease; however, this point is within standard division of the value at 100 mg-N.L. Nitrite inhibition might be started around 300 mg-N/L.
- Dapena-Mora et al. (2007) [17] reported the effect of substrates and product on the maximum specific anammox activity SAA. Experiments with different initial concentrations of ammonium, nitrite and nitrate were individually performed at concentrations between 5 and 200mM. Nitrite exerted the highest inhibitory effect on the maximum SAA. Concentrations of nitrite of 25mM corresponded to the 50% inhibition concentration (IC50). This result differs considerably from that obtained. Since these authors found that at concentrations of nitrite higher than 7mM the anammox activity was completely inhibited.
- Tang et al. (2009) [18] found that nitrogen removal performance deteriorated when influent $NH_4^+$–N and $NO_2^-$–N concentrations were further increased to 320 and 380 mg $L^{-1}$, respectively. Effluent $NH_4^+$–N and $NO_2^-$–N concentrations significantly increased to 179 and 149 mg $L^{-1}$. Ammonium, nitrite and total nitrogen removal efficiencies were decreased to 44, 61 and 53%, respectively. Ammonium, nitrite and total nitrogen removal rates sharply declined to 50, 66 and 59% of the corresponding removal rates obtained in the stationary phase.
- Nitrite is known to inhibit anammox activity irreversible at $NO_2$-N concentrations ranging from 70-100 mgN/L [9], [19].

## 2.5 Environmental factors

### 2.5.1. Dissolved oxygen

Nitrifying bacteria needs dissolved oxygen through ammonia and nitrite oxidizing mechanism. Hence, both denitrification and anammox are inhibited by dissolved oxygen. Denitrification has been observed to be inhibited at DO concentrations above 0.2 mg/L [2] and anammox organisms are reversibly inhibited by DO concentrations as low as 2 μmole/L or 0.032 mg/L [20].

### 2.5.2. Temperature

The temperature influences the composition of the microbial community and is importance for growth and reaction rates in the system. Microbial reactions are often dependent on enzyme-catalysed reactions that increase in velocity at higher temperatures, hence, the microorganism is allowed to grow faster [21]. Temperature does also influence non-viable factors like settling characteristics, gas solubility and transfer rates [22].

Nitrification can be operated in a temperature interval of 0-40 °C with a temperature optimum between 30-35 °C [22]. Denitrifying bacteria are less sensitive to temperature than nitrifiers and denitrification can take place in a temperature interval from 2-75 °C with an optimum around 30 °C [23]. Anammox bacteria are active in optimum temperature at 30°C [24].

### 2.5.3. pH and alkalinity

pH, which is the measurement of a solutions acidity or alkalinity, is another important environmental factor that impacts the growth rate of the microbial activity. Each bacteria species have a pH growth range and optimum.

Nitrification was favoured by light alkaline conditions with pH optimum in the range of pH 8.0-8.4 [22]. The nitrification rate was significantly declined by low pH values less than 6.8. A neutral pH of around 7 is normally maintained to achieve satisfactory nitrification rates [2].

Denitrification produced alkalinity and pH was generally raised by the process. pH optimum was ranging 7-9 depending on local conditions [9]. Cell synthesis in the anammox reaction was active in a pH range 6.5-9 with an optimum around 8 [25].

### 2.5.3. Substrate

Nitrifying, denitrifying and anammox bacteria were on different substrates for energy and synthetic reactions. The ability to utilise their substrate varies between bacterial species.

Denitrification rate and capacity was very dependent on available organic carbon source. External carbon sources like methanol, ethanol and acetic acid were readily biodegradable and give much higher denitrification rates than denitrification with organic compounds found in the wastewater [26].

Anammox bacteria had high affinity for their substrates of ammonia and nitrite. Common substrate for nitrifying and anammox bacteria is ammonia. Nitrifier and anammox microorganism utilize ammonia in energy reaction as electron donor. Common substrate for heterotrophic denitrifiers and anammox microorganism is nitrite. Heterotrophic and anammox microorganism ultilize nitrite in energy reaction as electron acceptor [27].

**Table 2.2**. The oligonucleotide probes

| **Probes** | **Sequence (5'-3')** | **Target groups** | **% Formamide** | **Lable** | **Refernces** |
|---|---|---|---|---|---|
| Nsm156 | TAT TAG CAC ATC TTT CGA | Ammonia oxidizing bacteria (*Nitrosomonas)* | 40 | Cy3 | Mobarry *et al.*(1996) [28] |
| Amx820 | AAA ACC CCT CTA CTT AGT GCC | Anammox bacteria (Brocadia anammoxidans) | 25 | Cy3 | Strous (2000) [29] |
| EUB338 | GCT GCC TCC CGT AGG AGT | Most bacteria | 15 | FITC | Amann *et al.*(1990) [30] |
| AMX368 | CCT TTC GGG CAT TGC GAA | all Anammox organism | 15 | FITC | Schmidt *et al.*(2003)[31] |

## 2.6 Fluorescence *In Situ* Hybridization (FISH) Technique

According to several types of microorganisms involved in nitrogen removal, the investigation on type and population of microorganisms is useful for understanding the mechanisms of the system. There are several methods for identification of these microorganisms in the biological nutrient removal (BNR) process. At this time, the method of fluorescence *in situ* hybridization (FISH) technique was applied widely. The FISH technique applies technology of DNA (deoxyribonucleic acid) for identifying specific types of microorganisms. It is a highly effective method for the complex microbial community, due to the possibility of detecting specific bacterial cells at the single cell level by *in situ* hybridization using phylogenetic markers (oligonucleotide probes) labeled with a fluorescent compound. The FISH technique includes four steps: first is the fixation of sample, second is the hybridization of fixed sample with oligonucleotide probes, third is washing sample for removed unbound probe out of the sample and the final is the detection of labeled cells by fluorescent microscopy [32]. The oligonucleotide probe used in the FISH technique is generally between 15 to 30 nucleotides long. The examples of the oligonucleotide probes are used in the FISH technique, as shown in Table 2.3.

## 2.7 Anammox applied wastewater

### 2.7.1. Municipal wastewater

Most municipal wastewater was treated by septic tank which could remove only organic matter. However, it is not enough to remove nitrogen in order to reach to the standard before release into environment. Moreover, the primary treatment decreases the organic matter until not enough for microorganism to remove nitrogen in the conventional denitrification process. Therefore anammox is one of the promised processes. Characteristics of municipal wastewater are shown in Table 2.3.

**Table 2.3.** The Compositions of wastewater from buildings in Thailand [33]

| Source | Composition (mg/L) | | | |
|---|---|---|---|---|
| | BOD | TSS | Oil and Grease | TKN |
| Condominium and Dwelling House | 151* | 63* | 473* | 33* |
| Hotel | 190 | 84 | 563 | 23 |
| Dormitory | 723** | 660** | 377** | 329** |
| Hospital | 238 | 87 | 631 | 15 |
| Restaurant | 1759 | 913 | 1570 | 63 |
| Market | 1172 | 660 | 897 | 76 |
| Department Store | 81 | 61 | 577 | 66 |
| Office | 180 | 158 | 450 | 44 |

Note: *some treated
**from septic tank

### 2.7.2. Seafood canning wastewater

The wastewater from seafood industries, such as canning food and of frozen seafood, contains high nitrogen concentration.

- *Canning food industries*

The wastewater from canned food industry divides into two types that are precooked wastewater and combined wastewater. Actually, precooked wastewater was separated from combined wastewater because it is more concentrated and perhaps can be reused. The characteristics of combined wastewater from canning tuna are shown in Table 2.4.

**Table 2.4. The compositions of wastewater from canning tuna industry** [34]

| Parameter | Concentration | | |
|---|---|---|---|
| | Factory 1 | Factory 2 | Factory 3 |
| pH | 5.6-6.1 | 5.5-6.2 | 5.7-6.8 |
| COD (mgCOD/L) | 6,298-16,452 | 3,685-12,143 | 2,533-6,469 |
| $BOD_5$ (mg/L) | 3,325-14,400 | 2,625-6,300 | 1,443-4,385 |
| TKN (mgN/L) | 221-714 | 148-1,613 | 268-612 |
| TSS (mg/L) | 1,810-12,905 | 1,810-6,620 | 707-2,536 |
| Oil and Grease (mg/L) | 4,083-9,550 | 5,017-13,183 | 637-1,889 |

Note: Factory 1 Canning tuna factory in Songkhla 140 tons/day
Factory 2 Canning tuna factory in Songkhla 50 tons/day
Factory 3 Canning tuna factory in Samutprakarn 50 tons/day

- *Frozen food industries*

Manufacturing produces the wastewater with average values COD and nitrogen of 3,395 mg-COD/L and 346 mg-N/L, respectively.

### 2.7.3. Port and pier wastewater

The port and jetty wastewater mostly are discharged from fish processing and washing that some fish around in the handling area was cleaned by spraying the sea water. The washing water finally flows back to the sea without treatment. In addition, there are other processing not only selling fish such as icehouse and cold storage located around that area of which these washing water also flows directly to the river and sea without treatment. This wastewater contains high salinity as shown in Table 2.5.

**Table 2.5. The compositions of port and pier wastewater** [35]

| Parameter | Range of concentration | Average concentration |
|---|---|---|
| pH | 6.38-8.01 | 6.9 |
| BOD (mg/L) | 46.2-12,780 | 3,875.52 |
| SS (mg/L) | 60-5,875 | 1,569.53 |
| TKN (mgN/L) | 3.1-1,779 | 556.7 |
| Salinity (g/L) | 3-32 | - |
| Oil and Grease (mg/L) | 0.3-1,059.2 | 501.6 |

### 2.7.4. Swine wastewater [36]

The pig farm waste is divided into two types, which are (1) solid waste from excrement and food scraps around stable, and (2) is waste cleaning water and urine, which is the wastewater. Normally, the amount of polluted substances is measured by BOD (Biochemical Oxygen Demand), depends on cleaning behavior at stable and the pig size. The ordinary wastewater contains BOD and nitrogen in the range of 4,000-7,000 mg-COD/L and 400-800 mg-N/L respectively. The example of swine wastewater before anaerobic treatment has average BOD of 6,372±1,639 mg-COD/L and nitrogen of 918±164 mg-N/L but after anaerobic treatment BOD and nitrogen reduced to 1,229±397 mg-COD/L and 739±136 mg-N/L respectively [37].

Another study reported that nitrogen removal by traditional nitrification and denitrification is difficult due to too low organic to nitrogen ratio in anaerobically treated swine wastewater. This study attempted to modify the baffled reactor utilizing the fixed-film autotrophic bacteria which are nitrifying and anammox bacteria to remove nitrogen. The studied variable was the nitrogen loading which was increased from the initially value of 100 mgN/L by increasing ammonia concentration whereas maintained the COD/NH4-N ratio of 4:1. The results showed that the total nitrogen removal was 56 percent. As the nitrogen loading increased in a step-wise manner of 100 mgN/L up to 500 mgN/L which was equivalent to 0.25 gN/L.day with aeration in the first two chambers to maintain the dissolve oxygen at 1.0 mgO2/L, the total nitrogen removal was 89 percent [38].

### 2.7.5. Seasoning sauce wastewater [39]

The main raw material in seasoning sauce processing results in COD and nitrogen in the discharged wastewater. The wastewater from seasoning sauce factory contains COD and nitrogen around 3,000-8,000 mg-COD/L and 200-1,000 mg-N/L respectively, and after being treated by the anaerobic system the COD and nitrogen were reduced to average of 292 mg-COD/L and 368 mg-N/L respectively. The anaerobically treated effluent of a seasoning sauce processing factory contained C/N ratio of 0.76 which was lower than the requirement of the ordinary microbes which removing nitrogen via denitrification process. This study aimed to remove nitrogen by using autotrophic bacteria via partial nitrification coupling with anaerobic ammonium oxidation (ANAMMOX) in fixed-film baffled reactor (FFBR). There were two types of media. It was found that total nitrogen removal efficiencies were 94.80±3.87% and 95.63±2.75% for FFBR1 and FFBR2, respectively, at the hydraulic retention time of 48 h and nitrogen loading of 194.75±10.31 g $N/m^3$-d and was found that microbial characterization by FISH technique revealed that anammox bacteria and *nitrosomonas* were coexisting in both FFBRs.

## 2.8 Literature Reviews

Arrigo (2005) [40] investigated the anammox activity in the anoxic ocean sediments using $^{15}$N-labelled $NH_4^+$. The approach is based on the fact that because anammox combines N from $NO_2^-$ with $NH_4^+$ to form $N_2$, the anammox reaction can be recognized by the production of singly labelled $N_2$ gas (15N14N, with the 14N coming from $NO_2^-$ and the 15N coming from $NH_4^+$).

Op den Camp et al. (2006) [41] suggested that in principle, anammox bacteria could make use of the regular denitrification pathway to produce dinitrogen gas. Anammox bacteria first reduced nitrite to ammonium and subsequently oxidized ammonium in the anammox reaction. In wastewater treatment, anammox would have the potential to replace the conventional denitrification step, if preceded by partial nitrification to nitrite.

Mao et al. (2006) [42] reported that in biological nitrogen removal systems, most of the $N_2O$ gas was emitted during the aeration phase. Any measures designed to reduce $N_2O$ emissions should focus on this phase. A problem with anammox process was that it required $NO_2^-$ as a substrate, and as nitrification itself produced $N_2O$ gas, if the $NO_2^-$ source were provided by nitrification, it would introduce an $N_2O$ source.

Alinsafi et al. (2008) [43] investigated that at low COD/N, the $NO_2^-$ was accumulated which inhibits the $N_2O$ reductase and leading to $N_2O$ gas emission because not all denitrifiers can utilize $NO_2^-$, the accumulation of $NO_2^-$ is probably also affected by theconsortium composition within the denitrifying community and the nature of carbon sources used in the study. The estimation of the $NO_2^-$ accumulation and the $N_2O$ emission may be used as indicators for the denitrification efficiency and for an optimisation of the denitrifying bacterial population.

Marlies et al. (2007) [44] studied a full-scale two-reactor nitritration-anammox process at the Dokhaven-Sluisjesdijk municipal WWTP of the Netherlands and found that during apply anaerobically treated sludge supernatant water treatment to the process, NO and $N_2O$ are emitted. Due to the strong fluctuations in NO and $N_2O$ level in both the nitritation as well as in the anammox reactor, only time-dependent measurements will yield a reliable estimate of the NO and $N_2O$ emissions. The emission of the total full-scale nitritation–anammox system (based on time-dependent measurements) consisted mainly of $N_2O$ (2.3% of the nitrogen load). The larger part of the $N_2O$ (78%) and NO (99%) was produced in the aeration reactor. Denitrification by ammonia-oxidizing bacteria in anammox process was considered the main source of NO and $N_2O$ emission that was stripped from the nitritation reactor due to aeration. Even anammox bacteria have not been shown to produce $N_2O$ on lab scale, it is also suspected that ammonia-oxidizing bacteria contribute most to $N_2O$ production in the anammox reactor.

Marlies et al. (2008) [44] analyzed literature data and showed that the most important operational parameters leading to $N_2O$ emission in wastewater treatment plants are: (i) low dissolved oxygen concentrations in both nitrification and denitrification stages, (ii) increased nitrite concentrations in both nitrification and denitrification stages, and (iii) low COD/N ratio in the denitrification stage.