

Effects of F/M ratio and Feast-Famine Condition on NO₂ Accumulation During Denitrification

Mehran Andalib¹, Amanda Ford², Art Umble¹, Mark van Loosdrecht³

¹Stantec Inc., Edmonton AB T5K 2L6, Canada

²Hazen & Sawyer Virginia Beach VA USA

³TU Delft Netherlands

Introduction:

Nitrite is one of the intermediates of the nitrate reduction process or denitrification. It is generally considered that the reduction rate of nitrite is higher than that of nitrate, therefore, nitrite accumulation will not occur during the denitrification process [1]. However, nitrite accumulation, in denitrification, is frequently observed. Recently, nitrite accumulation via denitrification was investigated in the literature as a means to provide substrate for anammox, an efficient and cost-saving process for nitrogen removal from wastewater [2]. However, the general operational approach is to prevent or reduce the amount of nitrite accumulation during conventional denitrification. In addition to elevated usage of chlorine as disinfectant, nitrite accumulation at threshold concentrations may cause toxic and inhibitory effects on other biological processes.

Several factors including organic carbon amount and type [3], low temperature [4], carbon to nitrogen ratio [5], dissolved oxygen, and phosphorus deficiency [6] have been linked to nitrite generation and accumulation from heterotrophic denitrifying bioreactors; however, the systematical impact of the engineered operational factors on resulting inefficient nitrate removal and accumulation of intermediates during denitrification in wastewater treatment processes has received limited attention.

Carbon sources and the subsequent C to N ratios have been analyzed extensively in laboratory batch experiments; however, the results are often in conclusive or contradictory. Batch cultures of *Paracoccus denitricans* cultivated on either methanol, acetate or ethanol accumulated different amounts of nitrite at 68-70%, 72% and 48%, respectively [7]. Another study investigated the effect of methanol, ethanol, sodium acetate, sodium propionate, and glucose on the nitrite accumulation in denitrification. They found that methanol, ethanol, sodium acetate,

and sodium propionate led to a peak nitrite accumulation followed by subsequent denitrification; however, when glucose is used as carbon source, both nitrate reduction and nitrite accumulation are not observed [8]. This is different from the conclusions that previously reported significant nitrite accumulation in a medium with glucose as a carbon source [7] and [9]. It was postulated that this phenomenon could be due to the presence of *Alcaligene* species, previously reported as not been able to utilize glucose as electron [10].

The effect of C/N ratio in batch experiments using methanol as the sole carbon source with C/N ratios 2.25 to 12 found a minimum ratio of 3.75 is sufficient for the reduction of nitrate to nitrite but is deficient for further reduction of nitrite to nitrogen gas [8]. The impact of the carbon sources citrate, acetate, and glucose with C/N ratios of 6.3 through 6.8 on isolated *Pseudomonas stutzeri* D6 was investigated and concluded that *Pseudomonas stutzeri* D6 preferred citrate and acetate for denitrification and that other carbon sources highly increased the nitrite accumulation rate [11].

The complexity of microbial communities mediate unique microbial pathways for the completion of respiration and cell production and those pathways may occur at different rates. In wastewater practice, however, there is an enormous lack of knowledge in the exact kinetic role of individual bacterium in the overall kinetic rate of a biological process such as denitrification. The availability, reliance and cost of different microbial ecology analysis in the plant operation level have significant roles in the insufficiency of the effect of microbial community in the nitrite accumulation during denitrification. In one rare study, nitrite and nitrous oxide accumulation was examined using three isolated pure denitrifying species of *Alcaligenes odornas*, *Pseudomonas fluorescens* and *Flavobacterium sp.*, and the observed accumulated nitrite when reducing nitrate in the first two (100% molar and 53% molar respectively) and not with the latest specie [12]. From the microbial pathway perspective, nitrite accumulation may result from a lag in synthesis of nitrite reductase (NiR) [13], inhibition on NiR or even from nitrate inhibition on nitric oxide reductase (NoR) [14]. It was hypothesized that if nitrate had inhibited nitrite reduction by acting on the nitrite reductase (NiR) itself, such inhibition should have been observed in cells incapable of nitrate reduction [12]. In a series of tests with *Flavobacterium sp.* and *P. fluorescens* cells, both inherently capable of complete denitrification, grown with nitrite alone reduced nitrate as rapidly as those grown with nitrate but cells grown with nitrite and tungstate, to prevent

formation of an active nitrate reductase, were unable to reduce nitrate. Nitrate concentrations as high as 8 mM did not show any adverse effect on the rate of nitrite reduction in both *Flavobacterium sp.* and *P. fluorescens*, thus concluded that accumulation of nitrite seemed to depend on the relative rates of nitrate and nitrite reduction with different species. In addition, each isolate rapidly reduced nitrous oxide even when nitrate or nitrite had been included in the incubation mixture. Based on observations in the literature the accumulation of nitrite is affected by the species composition within the denitrifying community and the sludge source. With respect to their capability to reduce nitrate and nitrite, four different groups of nitrate reducing bacteria are proposed. Group A is only capable of reducing NO_3 to NO_2 without further reduction of NO_2 to N_2 and caused NO_2 accumulation. These incomplete denitrifying bacteria lacked the key NO_2 reductase enzymes. *Acidovorax facilis*, *Citrobacter diversus*, and *Enterobacter agglomerans* were mentioned are examples of this group of bacteria [15]. Group B represented facultative anaerobic bacteria which reduce nitrate to nitrite, nitric oxide and nitrous oxide. Group B cannot grow solely on nitrous oxide (N_2O) and perhaps lack nitrous oxide reductase (Nos) or inhibited by NO_2 or NO_3 elevated concentrations and can only produce N_2O from nitrate and nitrite. Group B bacteria have shown in some cases to reduce N_2O at a very slow rate. *Pseudomonas fluorescens*, *Pseudomonas chlororaphis*, *Pseudomonas aureofaciens*, *Azospirillum lipoferum*, *Azospirillum itersonii*, *Corynebacterium nephridia*, *Pseudomonas aeruginosa*, *Staphylococcus sp.* are proposed to be among Group B bacteria [16]. Group C contained bacteria capable of reducing nitrate and nitrite without any nitrite accumulation. The nitrite reduction rates of these bacteria were reported higher than the corresponding nitrate reduction rates. Group D contained bacteria capable of reducing nitrate as well as nitrite but nitrate reduction was associated with a transient accumulation of different amounts of nitrite. In a quantitative study using different combination of *Staphylococcus sp.*, *Pseudomonas pseudoalcaligenes* and *Bacillus niacini* as Group B, C and D denitrifiers respectively at different carbon to nitrogen ratios (C/N) when Group C was dominant (>50%) in the denitrifier biocommunity, high denitrification rates were found with no nitrite accumulation, even at low C/N ratio of 2.5 (excluding the cell growth). When the Group C ratio fell below 25%, significant nitrite accumulation occurred with incomplete nitrogen elimination (<38%) [17]. In the study conducted by Du et al., where nitrite accumulation was observed at different C/N ratios the most abundant genus was identified as *Thauera* (67.25 %) which was a member of β -Proteobacteria,

mostly identified as denitrifiers. There were some strains of *Thauera* capable of reducing NO_3 to NO_2 under anaerobic condition Liu et al., 2013, which seemed consistent with the results of this study, in which when nitrate was added mid-way through the nitrite reduction phase, the nitrite reaction rate decreased by $\sim 85\%$. It was assumed that the dominant *Thauera* genus in the partial denitrification with high NO_2 accumulation was possibly related to the NO_2 reduction inhibition in the presence of NO_3 , assumed by the authors to be caused by the asynchronism of denitrifying enzyme synthesis for different electron acceptors (NO_3 and NO_2). If *Thauera* is classified as a Group D denitrifiers, the decrease in nitrite reduction rate when nitrate was added could be because of electron acceptor preference due to a higher kinetic rate.

It should be noted that in the majority of the studies summarized, the trend of COD reduction along with the reduction of nitrate to nitrite and nitrite to nitrogen gas were not reported, thus it is not clear whether sequestration of carbon occurred prior to completion of denitrification. As stated the literature results are inconsistent and furthermore the effect of feast and famine condition on the nitrite accumulation in the denitrification process has not been understood thoroughly.

Mathematical Model Development

In this study, a model-based approach, calibrated on multiple batch tests, demonstrates a correlation between initial F/M ratio and electron donor half saturation constant as surrogate for feast-famine conditions and nitrite accumulation in denitrification process. The model was developed to address the internalization of carbon (whether provided as an external source or influent) at feast and famine conditions when the concentrations of influent COD or added external carbon source (S_{ExC}) in the bulk liquid is significantly higher than the internalization half-saturation constants (denoted as K_{ExC} in Table 1, equations 7-9). This model has three new state variables and ten (10) new process rate equations, shown in Table 1. The stoichiometric matrix of this model is not included in the paper. The model includes process rates for the degradation of a non-methanol external carbon source under aerobic and anoxic conditions. To project the internalization ability of biomass, it is postulated that a specialist group of heterotrophic bacteria are capable of storing electron donor, hence the model introduces a new heterotrophic biomass, called OHO2 in addition to the ordinary heterotrophic organism (OHOs), used in the Activated Sludge Model (ASM). The new model structure has three critical

components: (1) the stoichiometric matrix which describes the mass balances; (2) the rate equations that describe the rates at which the model component conversions occur; and (3) the model parameters. The add on model rate equations were incorporated into Model Builder in the BioWin® simulator to demonstrate the feast and famine phenomenon based on literature data.

Aerobic behavior of the model

As shown in Table 1, based on experimental results reported in the literature on aerobic utilization of an organic matter (external carbon source in this case) by a non-enriched biomass, it is hypothesized for this model that normal heterotrophs can similarly utilize the external carbon source aerobically the same way as the specialist group of microorganisms (OHO₂), expressed as Eq (2).

Table 1- Summary of process rate equations for all processes

NO	Biological process	Reaction rates
1	Aerobic growth of Z _{OHO2} on internalized COD with NH ₃	$\mu_{m,OHO} \cdot \theta_1^{T-20} \cdot \frac{COD_{st}}{K_{ExcOHO} + COD_{st}} \cdot \frac{DO}{K_{ohet} + DO} \cdot \frac{NH_3N}{K_{na} + NH_3N} \cdot \frac{PO_4P}{K_{pgro} + PO_4P} \cdot Z_{OHO2}$
2	Aerobic growth of Z _{OHO2} and Z _{OHO} on external carbon with NH ₃	$\mu_{Exc} \cdot \theta_1^{T-20} \cdot \frac{C_{Exc}}{K_{Exc} + C_{Exc}} \cdot \frac{DO}{K_{omc} + DO} \cdot \frac{NH_3N}{K_{na} + NH_3N} \cdot \frac{PO_4P}{K_{pgro} + PO_4P} \cdot (Z_{OHO2} + f_{ExcHet} \cdot Z_{OHO})$
3	Anoxic growth of Z _{OHO2} and Z _{OHO} on external carbon with NH ₃ (NO ₃ →NO ₂)	$\psi_{Exc} \cdot \mu_{Exc1} \cdot \theta_2^{T-20} \cdot \frac{C_{Exc}}{K_{mExc} + C_{Exc}} \cdot \frac{K_{omc}}{K_{omc} + DO} \cdot \frac{NH_3}{K_{na} + NH_3N} \cdot \frac{NO_3N}{K_{no3Exc} + NO_3N} \cdot \frac{PO_4P}{K_{pgro} + PO_4P} \cdot (Z_{OHO2} + f_{MChet} \cdot Z_{OHO})$
4	Anoxic growth of Z _{OHO2} and Z _{OHO} on external carbon with NH ₃ (NO ₂ →N ₂)	$\psi_{Exc} \cdot \mu_{Exc2} \cdot \theta_2^{T-20} \cdot \frac{C_{Exc}}{K_{mExc} + C_{Exc}} \cdot \frac{K_{omc}}{K_{omc} + DO} \cdot \frac{NH_3}{K_{na} + NH_3N} \cdot \frac{NO_2N}{K_{no2MC} + NO_2N} \cdot \frac{PO_4P}{K_{pgro} + PO_4P} \cdot (Z_{OHO2} + f_{MChet} \cdot Z_{OHO})$

5	Anoxic growth of Z_{OHO2} on internalized COD with NO_3 ($NO_3 \rightarrow NO_2$)	$\psi_{Exc} \cdot \mu_{Exc1} \cdot \theta_2^{T-20} \cdot \frac{COD_{st}}{K_{mExc} + COD_{st}} \cdot \frac{K_{omc}}{K_{omc} + DO} \cdot \frac{K_{na}}{K_{na} + NH_3N}$ $\cdot \frac{NO_3N}{K_{no3MC} + NO_3N} \cdot \frac{PO_4P}{K_{pgro} + PO_4P} \cdot (Z_{OHO2})$
6	Anoxic growth of Z_{OHO2} on internalized COD with NO_2 ($NO_2 \rightarrow N_2$)	$\psi_{Exc} \cdot \mu_{Exc2} \cdot \theta_2^{T-20} \cdot \frac{COD_{st}}{K_{mExc} + COD_{st}} \cdot \frac{K_{omc}}{K_{omc} + DO} \cdot \frac{K_{na}}{K_{na} + NH_3N}$ $\cdot \frac{NO_2N}{K_{no2MC} + NO_2N} \cdot \frac{PO_4P}{K_{pgro} + PO_4P} \cdot (Z_{OHO2})$
7	Aerobic internalization of S_{Exc} by Z_{OHO2}	$\mu_{sto,Exc} \cdot \theta_3^{T-20} \cdot \frac{DO}{K_{ohet} + DO} \cdot \frac{S_{Exc}}{K_{OExc} + S_{Exc}} \cdot Z_{OHO2}$
8	Anoxic internalization of S_{Exc} by Z_{OHO2} ($NO_3 \rightarrow NO_2$)	$\mu_{sto,Exc} \cdot \theta_3^{T-20}$ $\cdot \eta_{sto} \cdot \frac{K_{ohet}}{K_{ohet} + DO} \cdot \frac{NO_3N}{K_{no3Exc} + NO_3N} \cdot \frac{S_{Exc}}{K_{stExc} + S_{Exc}} \cdot (Z_{OHO2} + f_{ExChet} \cdot Z_{OHO})$
9	Anoxic internalization of S_{Exc} by Z_{OHO2} ($NO_2 \rightarrow N_2$)	$\mu_{sto,Exc} \cdot \theta_3^{T-20}$ $\cdot \eta_{sto} \cdot \frac{K_{ohet}}{K_{ohet} + DO} \cdot \frac{NO_2N}{K_{no2Exc} + NO_2N} \cdot \frac{S_{Exc}}{K_{stExc} + S_{Exc}} \cdot (Z_{OHO2} + f_{ExChet} \cdot Z_{OHO})$
10	Decay of Z_{OHO2}	$d_{Exc} \cdot \theta_4^{T-20} \cdot Z_{Exc}$

Under aerobic condition and in parallel to the biological aerobic utilization of the external carbon source by OHOs and OHO_2 , the external carbon is predominantly internalized with an internalization yield of η_{sto} , and thereafter is used for biomass and CO_2 production. An amount of $(1-\eta_{sto})$ external carbon is directly used by OHOs and OHO_2 without any internalization. As shown in Eq (7), the aerobic internalization of carbon is postulated as a Monod function term of $\frac{S_{Exc}}{K_{OExc} + S_{Exc}}$. Based on this term if the $S_{Exc} \gg K_{OExc}$, the rate of internalization maximizes. In this model, the value of COD internalization yield (η_{sto}) is hypothesized not to be one, meaning a

portion of the external carbon source is used as exogenous substrate for cell growth. In support of the hypothesis, Moralejo-Gárate et al. (2013) observed that at high concentrations of electron acceptors (DO in that case), most of the assimilatory ammonium uptake (75% of the total) was established during the famine phase, indicating that growth occurred on the endogenous stored substrates (PHA and glycogen) [20]. For the low electron acceptor case, 70% of the assimilatory ammonium consumption occurred during the feast phase, indicating that growth was predominantly supported by the exogenous substrate, i.e. external carbon source. The substrate was directly used for growth rather than for production of storage polymers. During the famine phase, which lasted much longer than the feast phase (22 h versus 2 h), glycogen and PHB were degraded as the only carbon and energy sources and supported growth and cell maintenance.

In this model, the specialist group of bacteria (OHO_2) are the only non-PAO heterotrophs that can perform respiration using stored polymers. Equation (1) expressed the utilization rate of the internal stored carbon by OHO_2 .

Anoxic behavior in the model

The model behaves very similarly anoxically when it comes to internalization of the external carbon source, Eq (9). In addition, the following assumption were made for the anoxic behavior of both OHO s and OHO_2 with nitrate and nitrite as electron acceptors:

- (1) nitrate or nitrite are used as electron acceptors, with nitrate being reduced sequentially to nitrite and then to nitrogen gas;
- (2) OHO s and OHO_2 are both able to use external carbon source directly in two sequencing stages of denitrification (Equations 3 & 4)
- (3) The OHO_2 can perform both stages of denitrification using internalized carbon (Equations 3 & 4)

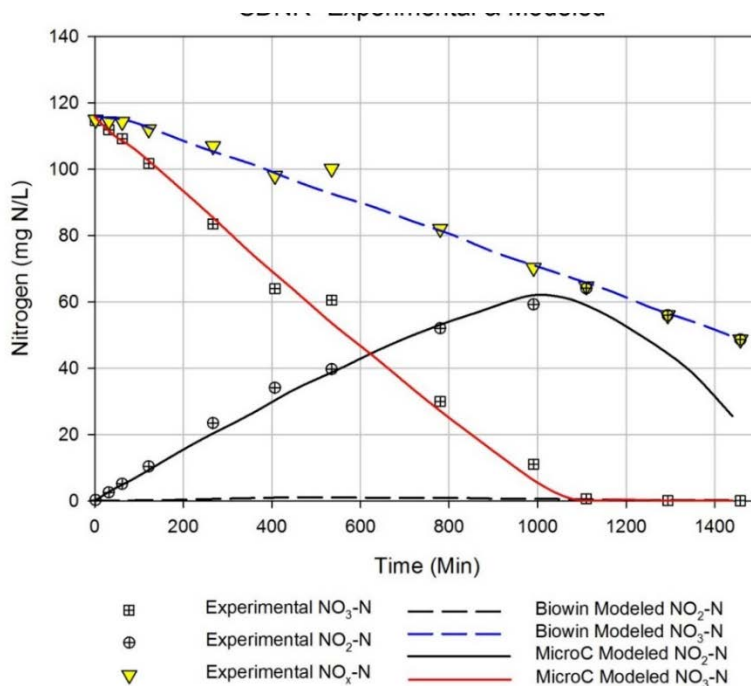
The kinetic value and stoichiometric constants are not included in this manuscript.

Results & Discussion:

Multiple batch experiments used in a previous study [18] were simulated to evaluate the model accuracy as well as conduct sensitivity analyses around the kinetic parameters. Figure 1 shows a sample result. The figure depicts an NUR test using glycerol-based MicroC[®] and the modeled

nitrate/nitrite concentration profile calculated by BioWin® using the AS/AD model with the model extension. The figure clearly shows the accumulation of nitrite during denitrification which was accurately predicted by the proposed model. The existing simulation platforms were not able to predict the nitrite accumulation phenomena at both high and low F/M ratios.

Figure 1: NUR Experimental and Modeled



In general, based on any two-stage denitrification mathematical model, the degree of nitrite accumulation is assumed to be the result of the kinetics of the denitrification and denitrification (which vary with different electron donors) and temperature. However, in practice, it has been observed that the degree of the nitrite accumulation during denitrification process is also a function of how an external carbon source is added into a denitrification zone (the value of anoxic F/M ratio) as well as the gradient of the F/M ratio along the denitrification reactor (PFR versus CSTR), herewith denoted as feast-famine conditions. This is necessary information for design.

In the proposed model, this phenomenon is described by utilizing Monod-type equations for sequestration of the electron donor, which varies with different carbon sources and substrate half-saturation constants. The rates of the sequestration of the electron donor differ based on the F/M ratio and the half saturation constants of the external carbon source. As shown in Table 1, equations (7), (8) and (9) internalization of the carbon source via both heterotrophic microorganisms and the specialist group of bacteria, which internalize COD at feast-famine condition, occurs at both aerobic and anoxic conditions. Based on the above equations, the degree of substrate sequestration and internalization, is a monod function of the substrate concentration in the bulk liquid and the electron donor half-saturation constant. As mentioned earlier, when the concentration of electron donor in the liquid is significantly higher than the half saturation constant, carbon sequestration and internalization will occur. Such a condition may happen both at high and low F/M ratio. Figure 2 depicts a conceptual graph of the $\text{NO}_2\text{-N}/\text{NO}_x\text{-N}$ ratio (or nitrite accumulation) at different F/M ratios and substrate half-saturation constants based on different electron donors.

Figure 2: Effect of K_s and F/M on Nitrite Accumulation

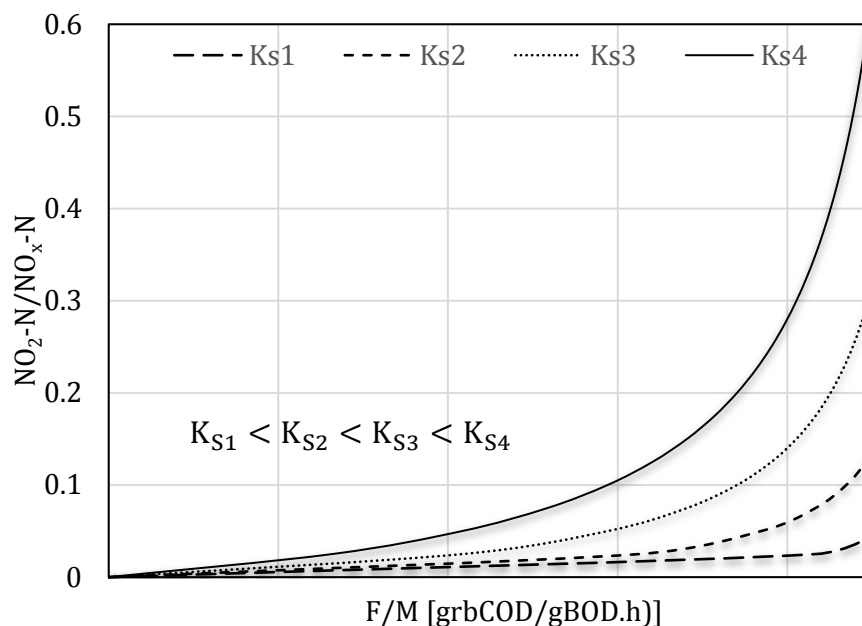


Figure 2 is based on assumed K_s values for different electron donors. One of the challenges with demonstrating this model is a very poor knowledge of the K_s values for different electron donors in the literature. In addition, half saturation constant is a function of environmental factors such as mass transfer coefficient and therefore subjected to variation in different processes. However, the proposed model introduces a modeling concept to mimic feast-famine phenomenon, independent of F/M ratio and mixed liquor concentrations and based on substrate concentration and sequestration kinetics. This model can be calibrated and utilized for process design and optimization, specially for waste stream with a much better understood K_s values such as municipal waste streams. However, extensive research on K_s values of different electron donors is required to be able to leverage this model with different electron donors.

Conclusion

In summary based on the model at low substrate half saturation constant (K_s) and/or low anoxic F/M ratio the rate of nitrite reduction to nitrogen gas is greater than the rate of nitrate reduction to nitrite and is greater than the sequestration rate. Therefore, insignificant nitrite accumulation will be observed. At high substrate half saturation constant (K_s) and/or high anoxic F/M ratio or feast-famine condition, the rate of substrate sequestration was greater or equal to the rate of nitrate reduction to nitrogen gas, therefore different degree of nitrite accumulation is expected. This study evaluates the degree of nitrite accumulation using the developed model and bench scale data.

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