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# Distinctive Microbial Ecology and Biokinetics of Autotrophic Ammonia and Nitrite Oxidation in a Partial Nitrification Bioreactor

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**ABSTRACT:** Biological nitrogen removal (BNR) based on partial nitrification and denitrification via nitrite is a cost-effective alternate to conventional nitrification and denitrification (via nitrate). The goal of this study was to investigate the microbial ecology, biokinetics, and stability of partial nitrification. Stable long-term partial nitrification resulting in $82.1 \pm 17.2\%$ ammonia oxidation, primarily to nitrite ($77.3 \pm 19.5\%$ of the ammonia oxidized) was achieved in a lab-scale bioreactor by operation at a pH, dissolved oxygen and solids retention time of $7.5 \pm 0.1$, $1.54 \pm 0.87$ mg $O_2$/L, and 3.0 days, respectively. Bioreactor ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) populations were most closely related to *Nitrosomonas europaea* and *Nitrobacter* spp., respectively. The AOB population fraction varied in the range $61 \pm 45\%$ and was much higher than the NOB fraction, $0.71 \pm 1.1\%$. Using direct measures of bacterial concentrations in conjunction with independent activity measures and mass balances, the maximum specific growth rate ($\mu_{max}$), specific decay ($b$) and observed biomass yield coefficients ($Y_{obs}$) for AOB were $1.08 \pm 1.03$ day$^{-1}$, $0.32 \pm 0.34$ day$^{-1}$, and $0.15 \pm 0.06$ mg biomass COD/mg N oxidized, respectively. Corresponding $\mu_{max}$, $b$, and $Y_{obs}$ values for NOB were $2.6 \pm 2.05$ day$^{-1}$, $1.7 \pm 1.9$ day$^{-1}$, and $0.04 \pm 0.02$ mg biomass COD/mg N oxidized, respectively. The results of this study demonstrate that the highly selective partial nitrification operating conditions enriched for a narrow diversity of rapidly growing AOB and NOB populations unlike conventional BNR reactors, which host a broader diversity of nitrifying bacteria. Further, direct measures of microbial abundance enabled not only elucidation of mixed community microbial ecology but also estimation of key engineering parameters describing bioreactor systems supporting these communities.

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**KEYWORDS:** microbial ecology; biokinetics; partial nitrification; qPCR; respirometry

## Introduction

Conventional biological nitrogen removal (BNR) is achieved by *complete* oxidation of ammonia to nitrate (nitrification) followed by the reduction of nitrate to dinitrogen gas (denitrification). However, if engineering based control of nitrification could be achieved to result in *partial* oxidation of ammonia solely to nitrite, 25% savings in aeration cost could be realized (Grady et al., 1999). Correspondingly, denitrification based on nitrite rather than nitrate could result in up to 40% savings on electron donor costs (Grady et al., 1999). These significant reductions in overall operating costs would thereby allow utilities to comply with stringent present and future *total*-N effluent concentration limits in a cost effective and sustainable fashion.

Partial nitrification results from selective proliferation of ammonia oxidizing bacteria (AOB) over nitrite oxidizing bacteria (NOB). Since AOB typically have higher affinity for oxygen than NOB (Grady et al., 1999), the oxidation of nitrite to nitrate can be limited by maintaining low dissolved oxygen (DO) concentrations during nitrification *coupled* with an operational solids retention time (SRT) that facilitates selective NOB washout (Garrido et al., 1997; Van Dongen and Van Loosdrecht, 2001). Additionally, high free ammonia (FA) concentrations (0.1–10 mg FA/L) (Anthonisen, 1974; Chandran and Smets, 2000b) in partial nitrification bioreactors not achieving complete ammonia oxidation can also selectively inhibit NOB. As such, partial nitrification is especially relevant for cost-effective nitrogen removal from waste streams containing high ammonia (or total Kjeldahl nitrogen, TKN) relative to biodegradable organic carbon, such as anaerobic digestion centrate or filtrate, landfill leachate or livestock liquid waste. Although the range of operating conditions required for successful partial nitrification have been described widely (Ciudad et al., 2005; Garrido et al., 1997; Jianlong and Ning, 2004), few studies have systematically evaluated the constituent dynamics of the microbial diversity and their

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biokinetics in terms of partial nitrification stability. Yet another limitation to our understanding of nitrifying communities in mixed microbial populations relates to the very estimation of their kinetic and stoichiometric coefficients. Most biokinetic estimation studies are based on mathematically approximated concentrations of the nitrifying communities in mixed culture (Chandran and Smets, 2000b). There are few, if any biokinetic descriptors of nitrifying bacteria in mixed communities that are based on *direct* measures of AOB or NOB abundance. In the absence of such direct measurements, the estimated coefficients are mere approximations and could lead to erroneous bioreactor design, operating and monitoring strategies.

Therefore, the objectives of this study were to:

1. Determine the dynamics of AOB and NOB populations, biokinetics, and performance in a partial nitrification bioreactor under steady-state and transient operation.
2. Employ ammonia and nitrite oxidation ascribed specific oxygen uptake rates (sOUR) in conjunction with direct measures of AOB and NOB concentrations to estimate key reactor biokinetic and stoichiometric parameters.

## Materials and Methods

### Bioreactor Operation

The partial nitrification bioreactor ($V = 11.18$ L) consisted of a custom built Plexiglas container with an internal settling chamber, which was physically isolated from the aeration chamber via a rectangular Plexiglas baffle. The bioreactor was seeded with nitrifying biomass kindly provided by Dr. Daniel Oerther (University of Cincinnati, Cincinnati, OH). Initially, the bioreactor was operated in fed-batch mode by first inoculating 100 mL of biomass into 250 mL feed medium and adding more medium in 500 mL–1 L increments whenever the pH rose higher than 8.5 (a qualitative indication of ammonia depletion). During this period, aeration was not provided and the biomass just mixed using a magnetic stir-bar. Fed-batch operation was conducted for 9 days by which point ammonia removal was 37.3% with 48.9% of the ammonia removed accumulating as nitrite. At this point, the bioreactor liquid volume was 5 L and continuous operation was initiated by feeding growth medium at a flow rate of 10 L/day. During continuous operation, the bioreactor was operated at room temperature at a hydraulic retention time (HRT) and target SRT of 1.118 and 3 days, respectively. pH was automatically controlled at $7.5 \pm 0.1$ using a 50 g/L solution of sodium bicarbonate. Aeration was provided using laboratory air at a flow rate of 3 L/min filtered through a 0.2 μm cartridge filter (Millipore®, Ann Arbor, MI). DO was maintained at $1.54 \pm 0.87$ mg $O_2$/L and monitored in real-time using YSI 5331A DO probes and YSI 5300 DO meter (Yellow Springs Instruments, Yellow Springs, OH) interfaced to a personal computer. The feed medium contained 500 mg-N/L ammonium and was devoid of organic carbon as previously described (Chandran and Smets, 2000b; Hockenbury and Grady, 1977). Bioreactor performance was monitored three times a week via ammonia (Fisher accumet® gas-sensing electrode, Waltham, MA), nitrite (diazotization and colorimetric detection) and nitrate (Fisher accumet® ion selective electrode) measurement (Eaton et al., 2005). Total reactor biomass concentrations were approximated using total chemical oxygen demand (tCOD) measurements using a commercially available assay (Hach Chemical Co., Loveland, CO; Eaton et al., 2005). After a sufficiently long period of undisturbed operation, the bioreactor was spiked with 500 mg nitrite-N/L on day 283. The objective of the spike was to determine the response of NOB to increased substrate availability and consequent impact on bioreactor stability in terms of performance, biokinetics and community dynamics.

### Biokinetics Estimation

Biokinetics of ammonia and nitrite oxidation were estimated via a previously described extant respirometric technique (Chandran and Smets, 2000b). Respirometric assays were initiated by a sequential spike of nitrite (4 mg-N/L) followed by ammonia (5 mg-N/L) and were performed under oxygen saturation (35–40 mg-$O_2$/L). Biokinetics were expressed as the maximum sOUR. sOUR was computed by dividing the maximum oxygen uptake rate ($dO_2/dt_{max}$, mg $O_2$/L/day), obtained by linear regression of the respirograms, by tCOD concentrations.

### DNA Extraction, Cloning, and Sequencing

Total DNA was extracted from bioreactor samples with a DNeasy Blood & Tissue kit (Qiagen, Inc., Germantown, MD). DNA extracts obtained on two independent operation dates (before and after the nitrite spike) were amplified against eubacterial 16S rRNA primers 11f (Kane et al., 1993) and 1492r (Weisburg et al., 1991). Amplicons were cloned (TOPO TA Cloning® for Sequencing, Invitrogen, Carlsbad, CA) and plasmid inserts were sequenced (Molecular Cloning Laboratories, San Francisco, CA) to obtain near complete sequence information of the inserts. Sequences were aligned, edited manually, and screened for chimera (CHIMERA_CHECK, http://rdp8.cme.msu.edu/html/). The closest matching sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov). ClustalX (Infor-Max, Inc., North Bethesda, MD) software was used to establish and bootstrap phylogenetic trees. The Neighbor Joining (NJ) method (Saitou and Nei, 1987) was used for tree construction and positions with gaps were excluded and multiple substitutions were corrected. The tree was subjected to 1,000 bootstrap trials. The rooted bootstrapped tree was rendered using TreeView® software (http://taxonomy.zoology.gla.ac.uk/rod/rod.html) with *Methanosarcina thermophila* as the outgroup.

### Quantification of AOB and NOB Concentrations Via Quantitative PCR

AOB concentrations were determined via qPCR using primers CTO 189A/B/Cf, and RT1r (Hermansson and Lindgren, 2001). Although not detected in either clone library (described in Results Section), quantification of *Nitrobacter* spp and *Nitrospira* related NOB was still pursued via more sensitive qPCR, using primer sets FGPS 872/1269′ (Cebron and Garnier, 2005) and NTSPAf/NTSPAr (Kindaichi et al., 2006), respectively. Standard curves for qPCR were constructed using genomic DNA from *Nitrosomonas europaea* ATCC 19718 (ATCC, Manassas, VA) and *Nitrobacter winogradskyi* NB 255 (kindly provided by Dr. Daniel Arp, Oregon State University, Corvallis, OR) and custom synthesized amplicon, from the partial sequence AB117711 (Kindaichi et al., 2006) (IDT, Coralville, IA). qPCR was performed on a BioRad *iQ5* system (BioRad, Hercules, CA) using SYBR Green chemistry as described previously (Cebron and Garnier, 2005; Hermansson and Lindgren, 2001; Kindaichi et al., 2006). Genomic DNA concentrations were converted to cell concentrations based on an average cell mass of $2.8 \times 10^{-13}$ g and genomic DNA content of 3.1% by mass (Madigan and Martinko, 2006). Cell concentrations were converted to AOB and NOB biomass COD concentrations ($X_{AOB}$ and $X_{NOB}$) by multiplying with a COD equivalence factor of 1.42 gCOD/g cell (Grady et al., 1999) and dividing by the measured DNA extraction efficiency, which is described next.

### Determination of DNA Extraction Efficiency

Losses in genomic DNA during extraction and propagation to measured AOB and NOB biomass concentrations were addressed by measuring the DNA extraction efficiency of each sample. Extraction efficiency was defined as the ratio of total bacterial DNA quantified by qPCR using eubacterial primers, BACT1369F and PROK1492R (Suzuki et al., 2000) to the theoretical DNA content of the samples used for extraction (assuming a cell mass of $2.8 \times 10^{-13}$ g/cell and genomic DNA content per cell of 3.1% by mass) (Madigan and Martinko, 2006). Additionally, one copy of the 16S rRNA operon per genome was assumed, which is true for *N. europaea* (Chain et al., 2003) and *Nitrobacter* spp. (Starkenburg et al., 2006).

### Estimation of $\mu_{max}$, $b$, and $Y_{obs}$

For each day of bioreactor operation, $\mu_{max}$ estimates were computed using independent measures of ammonia or nitrite oxidation rate and AOB or NOB abundance [Eqs. 1a and 1b, after (Grady et al., 1999)]. Estimates of $Y_{true,AOB}$ were experimentally determined previously (Chandran and Smets, 2000a,b) and a widely reported value of $Y_{true,NOB}$ was adapted from literature (Pirsing et al., 1996; Rittmann and McCarty, 2001; Sharma and Ahlert, 1977; Wiesmann, 1994) (Summarized in Table I).

$$\mu_{max,AOB} = \frac{Y_{true,AOB}}{(1 - Y_{true,AOB})} \frac{(dO_2/dt_{max,nh})}{X_{AOB}} \quad (1a)$$

$$\mu_{max,NOB} = \frac{Y_{true,NOB}}{(1 - Y_{true,NOB})} \frac{(dO_2/dt_{max,no_2})}{X_{NOB}} \quad (1b)$$

### Calculation of Observed Biomass Yield Coefficient

For each day of operation, observed biomass yield coefficients ($Y_{obs}$) for AOB and NOB were estimated based on respective biomass concentrations ($X_{AOB}$ and $X_{NOB}$), HRT ($\tau$), SRT ($\theta_C$), and extent of ammonia or nitrite oxidation (Eqs. 2a and 2b, after Grady et al., 1999). Both equations do not address potential nitrate and nitrite loss via denitrification, which were minimal in the partial nitrification bioreactor (Please see Results Section).

$$Y_{obs,AOB} = \frac{X_{AOB}\tau}{\theta_C(S_{no_2,eff} + S_{no_3,eff})} \quad (2a)$$

$$Y_{obs,NOB} = \frac{X_{NOB}\tau}{\theta_C S_{no_3,eff}} \quad (2b)$$

### Calculation of Autotrophic Biomass Decay Coefficient

Specific decay coefficients ($b$) for AOB and NOB were estimated based on their respective true yield and observed yield coefficients, SRT and $f_D$ [fraction of biomass decayed that results in biomass debris = 0.2 mg COD debris produced per mg COD active biomass decayed (Eqs. 3a and 3b, after Grady et al., 1999)].

$$b_{AOB} = \frac{(Y_{true,AOB}/Y_{obs,AOB}) - 1}{\theta_C[1 - f_D(Y_{true,AOB}/Y_{obs,AOB})]} \quad (3a)$$

$$b_{NOB} = \frac{(Y_{true,NOB}/Y_{obs,NOB}) - 1}{\theta_C[1 - f_D(Y_{true,NOB}/Y_{obs,NOB})]} \quad (3b)$$

## Results

### Partial Nitrification Performance

Initiation of steady-state was operationally defined as the first day of operation when the SRT averaged (3 days running average) ammonia removal and nitrite accumulation were both higher than 66%. Based on this criterion,

**Table I.** Summary of biokinetic parameter estimates describing the partial nitrification bioreactor.

| Parameter estimated in this study (average ± standard deviation) | | Additional parameters needed for estimation | Source | Type of reactor or biomass |
|---|---|---|---|---|
| $\mu_{max,AOB}$ (1/day) | 1.08 ± 1.03 | $Y_{true,AOB} = 0.24$ mg X COD/mg-N oxidized | Chandran and Smets (2000a) and Chandran and Smets (2000b) | *Complete* nitrifying enrichment culture |
| $Y_{obs,AOB}$ (mg X COD/mg N oxidized) | 0.15 ± 0.06 | None | | |
| $b_{AOB}$ (1/day) | 0.32 ± 0.34 | $f_D = 0.2$ mg debris COD/mg COD X, $Y_{obs,AOB}$: estimated above | Grady et al. (1999) | Activated sludge |
| $\mu_{max,AOB}^{(DO-limitation)}$ (1/day) | 0.73 ± 0.70 | $K_{S,O_2,AOB} = 0.74$ mg $O_2/L_B$ | Guisasola et al. (2005) | *Complete* nitrifying enrichment culture |
| $b_{AOB}^{(DO-limitation)}$ (1/day) | 0.20 ± 0.22 | | | |
| $\mu_{max,NOB}$ (1/day) | 2.6 ± 2.05 | $Y_{true,NOB} = 0.1$ mg X COD/mg-N oxidized | Pirsing et al. (1996), Rittmann and McCarty (2001), Sharma and Ahlert (1977), and Wiesmann (1994) | *Complete* nitrifying enrichment culture, including activated sludge |
| $Y_{obs,NOB}$ (mg X COD/mg N oxidized) | 0.04 ± 0.02 | None | Grady et al. (1999) | Activated sludge |
| $b_{NOB}$ (1/day) | 1.7 ± 1.9 | $f_D = 0.2$ mg debris COD/mg COD X, $Y_{obs,NOB}$: estimated above | | |
| $\mu_{max,NOB}^{(DO-limitation,FA-inhibition)}$ (1/day) | 0.12 ± 0.10 | $K_{I,FA,NOB} = 0.1$ mg $NH_3$-N/L | Chandran and Smets (2000b) | *Complete* nitrifying enrichment culture |
| | | $K_{S,O_2,NOB} = 1.75$ mg $O_2/L$ | Guisasola et al. (2005) | *Complete* nitrifying enrichment culture |
| $b_{NOB}^{(DO-limitation)}$ (1/day) | 0.75 ± 0.80 | $K_{S,O_2,NOB} = 1.75$ mg $O_2/L$ | Guisasola et al. (2005) | *Complete* nitrifying enrichment culture |

steady-state based on bioreactor performance was achieved within 47 days of continuous operation (Fig. 1). After reaching steady-state, long term stability of ammonia to nitrite oxidation was reflected in 82.1 ± 17.2% ($n = 110$) ammonia removal relative to influent ammonia concentrations (Fig. 1). The major fraction of ammonia oxidized was to nitrite (77.3 ± 19.5%, $n = 110$) and not to nitrate (19.7 ± 18.3%, $n = 110$). Based on a nitrogen mass balance around the reactor, minimal nitrogen losses of 7 ± 8.3% ($n = 109$) were observed. Bioreactor performance was transiently diminished by the nitrite shock load ($t = 283$ days) (Fig. 1).

## AOB and NOB Biokinetics

sOUR for ammonia oxidation ($sOUR_{nh}$) was 4–30 times the sOUR for nitrite oxidation ($sOUR_{no2}$) (Fig. 2). Considerable variability in $sOUR_{nh}$ and $sOUR_{no2}$ was also observed over the period of performance-based steady-state bioreactor operation. After the sodium nitrite spike at $t = 283$ days, $sOUR_{nh}$ decreased slightly (Fig. 2). In response, the SRT was increased to approximately 5 days (not shown), whereupon performance and $sOUR_{nh}$ recovered. However, $sOUR_{no2}$ also increased concurrently and remained at these elevated levels throughout the remainder of the study (Fig. 2).

## Microbial Population Diversity and Abundance

Based on clone libraries constructed from DNA extracts obtained on two independent sampling dates (before and after the nitrite spike), most bioreactor AOB were closely related to *N. europaea*. Clones related to AOB such as *Nitrosospira* spp. or NOB, *Nitrobacter* spp. or *Nitrospira* spp.




**Figure 1.** Ammonia removal and nitrite accumulation during the operation of the partial nitrification reactor. The reactor conditions were maintained at pH = 7.5 ± 0.1, target SRT = 3 days, operating HRT = 1.118 days, and T = 21°C. Error bars represent the standard deviation of duplicate measurements. The shaded boxes represent periods of initial fed-batch operation and continuous unsteady-state operation, preceding continuous steady-state operation.

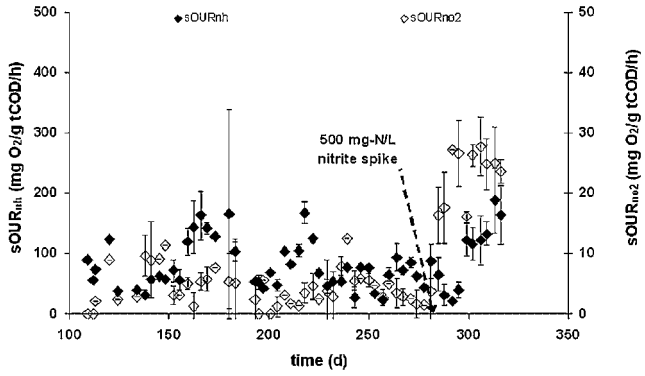


**Figure 2.** Specific oxygen uptake rates associated with ammonia oxidation ($sOUR_{nh}$, left hand axis) and nitrite oxidation ($sOUR_{no2}$, right hand axis). Error bars represent the standard deviation of duplicate measurements.

were not detected (Fig. 3). The dominance of AOB determined via clone library analysis was corroborated by routine qPCR results (Fig. 4). The measured DNA extraction efficiency varied in the range $15.1\% \pm 15.5\%$ (avg. $\pm$ SD, $n = 41$). As observed with sOUR measures, microbial abundance also varied quite dynamically, although bioreactor performance was at steady-state for most of the study period. During steady-state operation, AOB constituted $61 \pm 45\%$ ($n = 39$) of the total bioreactor population as COD. The corresponding steady-state *Nitrobacter* spp. related NOB fraction was much lower at $0.7 \pm 1.1\%$ ($n = 41$). The increased SRT (combined with the increased reactor nitrite concentrations) following the nitrite spike resulted in a rapid and transient increase in $X_{NOB}$ concentrations (Fig. 4). This trend suggested that select NOB populations remained viable and poised to proliferate in the partial nitrification bioreactor, when the optimal conditions

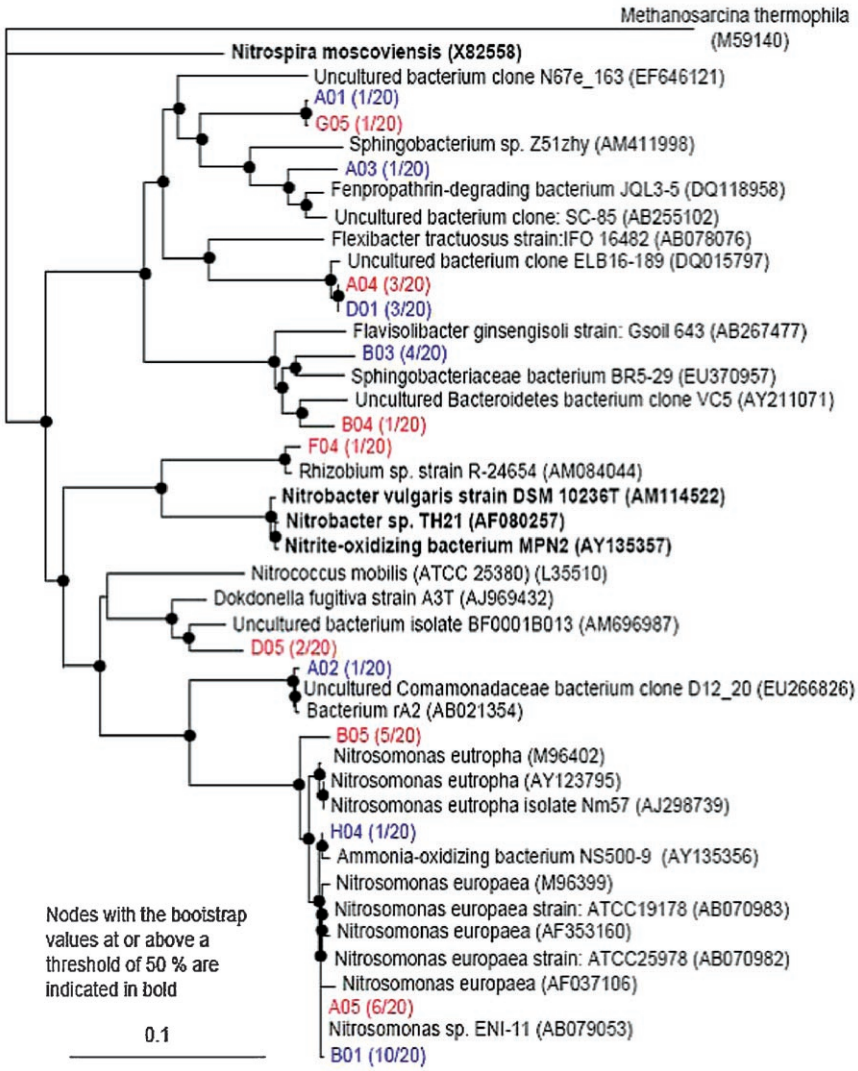


**Figure 3.** Phylogenetic tree depicting the dominant bacterial populations in the partial nitrification bioreactor from two independent samples (shown in red and blue). Numbers in parentheses represent fraction of clones most closely associated with a given phylogenetic lineage.

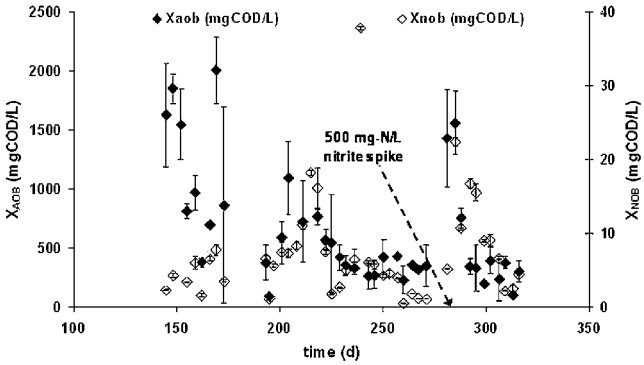


**Figure 4.** Significant preponderance of $X_{AOB}$ over $X_{NOB}$ in the partial nitrification bioreactor from qPCR measurements. Error bars represent standard deviation of duplicate qPCR based measurements.

arose (in casu, higher influent substrate load and higher SRT). *Nitrospira* spp. related NOB were detected exactly once during the entire study, immediately following the nitrite spike and were three orders of magnitude less abundant than *Nitrobacter* related NOB (not shown).

### Estimation of Parameters Using Combined Biomolecular, Biokinetics and Mass Balance Approaches

Estimates of $\mu_{max,AOB}$, $b_{AOB}$, and $Y_{obs,AOB}$ were $1.08 \pm 1.03$ $day^{-1}$, $0.32 \pm 0.34$ mg COD/mg-N oxidized and $0.15 \pm 0.06$ $day^{-1}$, respectively. Corresponding estimates of $\mu_{max,NOB}$, $b_{NOB}$, and $Y_{NOB}$ were $2.6 \pm 2.05$ $day^{-1}$, $1.7 \pm 1.9$ $day^{-1}$, and $0.04 \pm 0.02$ mg COD/mg-N oxidized, respectively. Thus, the extremely low NOB population abundance coupled with the low $sOUR_{no2}$ and extent of nitrite oxidation in the partial nitrification bioreactor directly contributed to the high $b_{NOB}$ and low $Y_{NOB}$ estimates, compared to $b_{AOB}$ and $Y_{AOB}$ estimates.

## Discussion

BNR strategies based on partial nitrification are more sustainable than those based on conventional nitrification owing to their lower operating costs (25% less oxygen and 40% less electron donor for denitrification). Using parameter estimates from this study and those documented in literature (Table I), we confirmed that sustained partial nitrification in this study was achieved by a combination of oxygen limitation and FA inhibition to NOB, coupled with a 3 days operating SRT by the following calculations. $\mu_{max,AOB}$ and $\mu_{max,NOB}$ measured via respirometry under oxygen saturation were translated to average reactor DO concentrations using a Monod type saturation function (Eqs. 4a and 4b; Grady et al. 1999). The same saturation functions were used to describe the impact of oxygen limitation on $b_{AOB}$ and $b_{NOB}$ (Eqs. 4c and 4d). $K_{S,O_2,AOB}$ and $K_{S,O_2,NOB}$ values of 0.74 mg $O_2$/L and 1.75 mg $O_2$/L, respectively, were adapted from a recent experimental study that used a nitrifying enrichment culture fed with a high nitrogen containing stream, similar to that in this study (Guisasola et al., 2005). FA inhibition to NOB was captured by a non-competitive inhibition model and coefficient (Eq. 4b, Chandran and Smets, 2000b). Nitrite concentrations in the range of this study were not expected to inhibit AOB activity, as previously determined (Chandran and Smets, 2000b).

$$\mu_{max,AOB}^{(DO-\ limitation)} = \mu_{max,AOB} \frac{S_{O2}}{K_{S,O_2,AOB} + S_{O_2}} \tag{4a}$$

$$\mu_{max,NOB}^{(DO-\ limitation,FA\text{-}inhibition)} = \mu_{max,NOB} \frac{S_{O2}}{K_{S,O_2,NOB} + S_{O_2}} \frac{1}{1 + S_{FA}/K_{I,FA}} \tag{4b}$$

$$b_{AOB}^{(DO-\ limitation)} = b_{NOB} \frac{S_{O2}}{K_{S,O_2,AOB} + S_{O_2}} \tag{4c}$$

$$b_{NOB}^{(DO-limitation)} = b_{NOB} \frac{S_{O2}}{K_{S,O_2,NOB} + S_{O_2}} \tag{4d}$$

Accounting for DO limitation to AOB and NOB and FA inhibition to NOB in the bioreactor, effective $\mu_{max,AOB}$ and $\mu_{max,NOB}$ estimates were reduced to $0.73 \pm 0.70$ and $0.12 \pm 0.10$ $day^{-1}$, respectively. $b_{AOB}$ and $b_{NOB}$ estimates were $0.20 \pm 0.22$ and $0.75 \pm 0.80$ $day^{-1}$, respectively. Based on these "in-reactor" $\mu_{max}$ estimates, the average minimum SRT ($\theta_{C,min}$) required to sustain AOB and NOB in the bioreactor were calculated (Eq. 5, Grady et al., 1999).

$$\theta_{C,min} = \frac{1}{\mu_{max} - b} \tag{5}$$

The average limiting SRTs for AOB and NOB were 1.9 days and infinity (reflecting net negative growth of NOB), respectively. Thus, the operating SRT of the partial nitrification bioreactor (3 days) was sufficient to sustain the presence of AOB but not of NOB therein. This suggested overall preponderance of AOB over NOB during partial nitrification using the above analysis was indeed confirmed by qPCR results.

It can be seen that for many parameters estimated in this study, the knowledge of additional parameter estimate values was needed (Table I). Most of these additional parameters were obtained from recently conducted experimental studies that most closely mimicked conditions similar to this reactor (Table I). $Y_{true,AOB}$ and $Y_{true,NOB}$ were taken from a previous study on nitrifying communities subject to an identical feed stream but performing *complete* nitrification (Chandran and Smets, 2000b) and a value widely reported across literature (Pirsing et al., 1996;

Rittmann and McCarty, 2001; Wiesmann, 1994), respectively. Half-saturation coefficients for oxygen were adapted from a recent study that actually differentiated between the two nitrification steps for these parameter estimates (Guisasola et al., 2005). However, it is acknowledged that half-saturation coefficient values might depend not only upon the true microbial affinity for their substrate but also physical transport into the biological flocs (Perez et al., 2005). Similarly, transport of ammonia in the floc might also govern the "apparent" FA inhibition coefficient ($K_{I,FA}$) and might differ from system to system. Notwithstanding these factors, the calculated $\theta_{C,min}$ values reflect that in general, the conditions in the partial nitrification bioreactor imposed a kinetic selection favoring AOB presence therein. On the other hand, the sensitivity of estimates of $b$ to variation in $f_D$ was determined to be negligible in the range 0–0.4 mg COD debris produced per mg COD active biomass decayed using sensitivity analysis techniques previously described (Chandran and Smets 2000a) (data not shown). Since most values of $f_D$ for activated sludge biomass are in a narrow range close to 0.2 mg debris COD/mg COD active biomass (Grady et al., 1999), the estimates of $b_{AOB}$ or $b_{NOB}$ determined via Equations 3a and 3b are not expected to vary significantly for different types of biomass samples.

The unique partial nitrification operating conditions, namely, low DO, limiting SRT, and high reactor FA and nitrite concentrations enriched for a narrow community of AOB and NOB, both ecologically and biokinetically. The bioreactor AOB community was distinctly less diverse than the seed from which it was developed (Smith and Oerther, 2006). AOB populations in the seed consisted of *N. europaea*, *N. marina*, *N. aestuarii*, and *N. mobilis* related organisms (Smith and Oerther, 2006). AOB and NOB populations in this study were mainly related to *N. europaea* (from clone libraries) and *Nitrobacter* spp. (from qPCR analysis), respectively. These results were in contrast to nitrifying activated sludge wherein a wide diversity of beta proteobacterial AOB has been shown (Purkhold et al., 2000). Dominant AOB in activated sludge bioreactors, where ammonia concentrations are continuously limiting, include not only *N. europaea*, but also members of the *N. eutropha*, *N. marina*, *N. oligotropha*, and *Nitrosococcus mobilis* clusters (Purkhold et al., 2000). It maybe speculated that the lower ammonia concentrations in completely nitrifying activated sludge may give rise to AOB that are able to scavenge ammonia more effectively. Interestingly, the dominance of *N. europaea* related bacteria in this study (abundant ammonia concentrations) as well as in nitrifying activated sludge (limiting ammonia concentrations) suggests that they could function ecologically as both *r*-strategists (higher specific growth rates, and low substrate affinity) and *K*-strategists (lower specific growth rates and high substrate affinity). In contrast, the remaining members of the AOB community in nitrifying activated sludge might be predominantly *K*-strategists, unable to compete effectively at high ammonia concentrations in partial nitrification conditions.

Among NOB, *Nitrospira* spp., which are phylogenetically distinct from *Nitrobacter* spp., are more prevalent in activated sludge bioreactors (Burrell et al., 1998; Daims et al., 2001a; Dionisi et al., 2002; Gieseke et al., 2005; Juretschko et al., 1998; Schramm et al., 1998). *Nitrobacter* spp. are more dominant in bioreactors with high standing nitrite concentrations such as during partial nitrification (Kim and Kim, 2006; Schramm et al., 2000). Such observations can be ecologically explained by the fact that *Nitrobacter* spp. are *r*-strategists that thrive under high nitrite concentrations and *Nitrospira* spp. are *K*-strategists that thrive under low nitrite concentrations, typical of activated sludge (Schramm et al., 2000). Indeed, in environments that experience a wide spectrum of nitrite concentrations, such as sequencing batch reactors, the coexistence of both *Nitrobacter* and *Nitrospira* spp. has been shown (Daims et al., 2001b).

Estimates of $\mu_{max,AOB}$ (uncorrected for DO limitation) from this study were in general close correspondence with not only those for a partial nitrification bioreactor, obtained using parameter estimation techniques (Pambrun and Spérandio, 2006) but also experimentally determined for *complete* nitrification bioreactors operated at an SRT of 20 days (Chandran and Smets, 2000b, 2005). There is limited information on $b_{AOB}$ estimates reported for partial nitrification systems, but the estimates from this study were within the range reported for AOB in general (Grady et al., 1999; Henze et al., 1995; Pambrun and Spérandio, 2006; Wiesmann, 1994). Thus, although the partial nitrification conditions enriched for a narrow spectrum of AOB, their biokinetics were quite similar to those from a broad spectrum of reactor operating conditions. On the other hand, estimates of $\mu_{max,NOB}$ and $b_{NOB}$ (uncorrected for DO limitation or FA toxicity) obtained in this study were higher than those reported in literature for partial as well as *complete* nitrification (Chandran and Smets, 2000b, 2005; Grady et al., 1999; Henze et al., 1995; Pambrun and Spérandio, 2006; Wiesmann, 1994). Such high estimates might be due to sustained exposure to high nitrite concentrations herein. Possible comparisons of affinity are precluded by the fact that we did not estimate $K_S$ in the present study. The high $\mu_{max}$ of the NOB in the partial nitrification bioreactor allowed them to rapidly adapt to changes imposed thereupon, which was, in turn, was manifest in the distinct increase in $X_{NOB}$ and $sOUR_{NOB}$ in response to the nitrite shock. Thus, although NOB maybe present in extremely low concentrations in partial nitrification bioreactors, they are capable of rapidly proliferating upon encountering favorable growth conditions thereby ultimately destabilizing partial nitrification bioreactor performance.

Recently, molecular tools targeting 16S rRNA (Egli et al., 2003; Gieseke et al., 2001; Juretschko et al., 1998; Mobarry et al., 1996; Schramm et al., 1998; Wagner et al., 1998), 16S rDNA (Harms et al., 2003; Kowalchuk et al., 1997), ammonia monooxygenase subunit A (*amoA*) gene DNA (Hoshino et al., 2001; Okano et al., 2004), and *amoA* mRNA

(Bollmann et al., 2005; Ebie et al., 2004) have emerged as powerful alternates to measure the presence and activity of AOB in natural and engineered systems. Similar characterization for NOB has also been conducted using 16S rRNA and 16S rDNA (Burrell et al., 1998; Daims et al., 2001a; Dionisi et al., 2002; Gieseke et al., 2005; Juretschko et al., 1998; Kim and Kim, 2006; Schramm et al., 1998) and more recently by targeting the nitrite oxidoreductase (*nxr*) gene (Poly et al., 2008). The results of this study and some recent ones (Blackburne et al., 2007; Kindaichi et al., 2006) highlight the additional immense utility of using molecular measures for the estimation of engineering parameters (such as $\mu_{max}$, $b$, and $Y_{obs}$). Equations 1a and 1b demonstrate that estimates of $\mu_{max}$ derived from respirometric methods are inversely proportional to the $X_{AOB}$ and $X_{NOB}$ concentrations. Therefore, it is critical to directly estimate $X_{AOB}$ or $X_{NOB}$ in order to avoid erroneous estimates of $\mu_{max}$ that might confound partial nitrification bioreactor design, operation and control (via Eq. 5). Additionally, such direct biomass concentration estimates should incorporate measures of DNA processing and extraction efficiency, preferably on a sample specific basis as described herein. Although estimates of specific biomass concentrations can be approximated via mass balances (Grady et al., 1999) or model based interpretation of respirograms (Brouwer et al., 1998; Spanjers and Vanrolleghem, 1995), such approximations require a priori knowledge of the observed biomass yield coefficient, which is typically system specific and recursively linked to the knowledge of the community specific decay constant "*b*" (Eqs. 2a, 2b, 3a, 3b, Grady et al., 1999).

## Conclusions

In sum, sustained partial nitrification was achieved by selective washout of NOB via a combination of FA toxicity, low DO concentration and operation at an NOB limiting SRT. The imposed bioreactor operating conditions enriched for distinct AOB (ecologically) and NOB (ecologically and biokinetically) populations compared to those in conventional activated sludge bioreactors. Using a combination of biomolecular estimates of protagonist AOB and NOB populations, biokinetic measurements and mass balances, we directly estimated parameters that are of key relevance in partial nitrification bioreactor design and operation.

## Nomenclature

| | |
|---|---|
| $\mu_{max}$ | maximum specific growth rate (1/day) |
| $K_S$ | half saturation constant (mg/L as N or $O_2$) |
| $b$ | specific decay coefficient (1/day) |
| $Y_{true}$ | true biomass yield coefficient, 0.24 mg COD biomass synthesized/mg N oxidized for AOB (Chandran and Smets, 2000a,b) and 0.1 mg COD biomass synthesized/mg N oxidized for NOB (Pirsing et al., 1996; Rittmann and McCarty, 2001; Sharma and Ahlert, 1977; Wiesmann, 1994) |
| $Y_{obs}$ | observed biomass yield coefficient (mg biomass COD synthesized/mg N oxidized) |
| sOUR | specific oxygen uptake rate (mg $O_2$/g tCOD/h) |
| $\frac{dO_2}{df_{max}}$ | maximum oxygen uptake rate computed from the slope of a given respirogram (mg $O_2$/L/day) |
| $X$ | biomass COD concentration determined via qPCR (mg COD/L) |
| $\tau$ | hydraulic retention time (day) |
| $\theta_C$ | solids retention time (day) |
| $\theta_{C,min}$ | minimum solids retention time required to sustain a given biomass population in a reactor (day) |
| $S_{no_2,eff} + S_{no_3,eff}$ | sum of effluent nitrite and nitrate concentrations in the effluent and refers to the extent of ammonia oxidized by AOB, accounting for oxidation of the produced nitrite by NOB (mg-N/L) |
| $S_{no_3,eff}$ | nitrate concentration in the effluent and refers to the extent of nitrite oxidized by NOB (mg-N/L) |
| $f_D$ | fraction of biomass decayed that results in biomass debris = 0.2 mg COD debris produced per mg COD active biomass decayed (Grady et al., 1999) |
| $K_{I,FA}$ | FA inhibition constant of NOB, 0.1 mg $NH_3$-N/L (Chandran and Smets, 2000b), physically defined as the FA concentration at which NOB activity is reduced to 50% of that in the absence of FA |
| $\mu_{max,AOB}^{(DO-limitation)}$ | $\mu_{max}$ of AOB accounting for DO limitation in the partial nitrification bioreactor |
| $\mu_{max,NOB}^{(DO-limitation,FA-inhibition)}$ | $\mu_{max}$ of NOB accounting for DO limitation and free ammonia inhibition in the partial nitrification bioreactor |
| $S_{O_2}$ | bioreactor dissolved oxygen concentration (average 1.54 mg $O_2$/L) |
| $K_{S,O_2,AOB}$ | DO half saturation coefficient of AOB, 0.74 mg $O_2$/L (Guisasola et al., 2005) |
| $K_{S,O_2,NOB}$ | DO half saturation coefficient of NOB, 1.75 mg $O_2$/L (Guisasola et al., 2005) |
| $S_{FA}$ | bioreactor free ammonia concentration [average 0.91 mg $NH_3$-N/L at pH = 7.5 and $T = 21^{\circ}C$ (Stumm and Morgan, 1996), $n = 97$] |

*Subscripts*

| | |
|---|---|
| AOB | ammonia oxidizing bacteria |
| NOB | nitrite oxidizing bacteria |
| nh | ammonia |
| $no_2$ | nitrite |
| $O_2$ | oxygen |
| FA | free ammonia |

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