NITRIFICATION AT COLD TEMPERATURES USING BIOAUGMENTATION

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ABSTRACT

Bioaugmentation of nitrifying bacteria is an attractive alternative for wastewater treatment plants in cold climates or for those in the process of upgrading to include nitrification. One possible source of ammonia for the production of these nitrifying bacteria is the liquor generated during the dewatering of anaerobically digested sludges. In this research, biomass produced during nitrification of dewatering liquors at 10°C and 20°C were seeded into cold (10°C) sequencing batch reactors (SBRs) at various hydraulic and solids retention times. Full NH_3-N removal was achieved in SBRs with an SRT of 12 d and HRT of 8 h when seeded with nitrifying bacteria acclimated to 10°C (NB10) and 20°C (NB20). Partial NH_3-N removal was achieved in the SBR seeded with NB10 when the SRT was 4 d and HRT was 12 h. No removal was achieved in the reactor seeded with NB20 with the same operating conditions. Fluorescence *in situ* hybridization (FISH) analysis was used to examine the ammonia oxidizing bacteria in the seeded systems. Seeded SRTs were estimated and showed that nitrifying biomass acclimated to 20°C required a longer seeded SRT to achieve the same level of treatment as nitrifying biomass acclimated to 10°C.

Key words- wastewater, bioaugmentation, nitrification, hydraulic retention time, solids retention time, temperature, sequencing batch reactors, fluorescence *in situ* hybridization

INTRODUCTION

Centrate Treatment for Short-SRT Nitrification

Nitrification of cold wastewater requires a long solids retention time (SRT) due to the slow growth rates of nitrifying bacteria. Maintaining a long SRT requires large tanks and clarifiers to accommodate the accumulation of solids inventory. A significant source of ammonia (NH_3) in wastewater treatment plants with sludge processing is from the dewatering of anaerobically digested sludge (centrate). Some facilities receive solids from other facilities thus generating a disproportionate amount of NH_3 in the return liquors. Such is the case in Winnipeg, Manitoba where the North End plant receives sludge from two other plants. This results in a centrate stream contributing 30% of the total nitrogen load to the plant.

The North End plant needs to remove approximately 30 to 50% of the incoming NH_3-N load to mitigate potential toxicity of un-ionized NH_3 to the Red River. The plant is a high purity oxygen activated sludge (HPOAS) facility with a short hydraulic retention time (an HRT of 2 to 3 h) and has limited real estate for expansion. Extending the SRT at this plant creates settlability problems in retrofitted final clarifiers, therefore any method of

decreasing the SRT and solids inventory while improving effluent quality would be beneficial. Additionally, the clarifiers cannot handle solids concentrations above 2500 mg/L at existing flows.

Sludge liquor treatment in a dedicated side-stream reactor has particular relevance to the North End plant. One of the options for the first stage of the plant upgrade is to include centrate nitrification with the production of a nitrifying biomass that can be recycled into the main-stream reactors. Considerable bench-, pilot- and full-scale research has been directed to utilization of centrate for the production of nitrifying bacteria for bio-augmentation, or seeding (e.g. Kos, 1998; Plaza *et al.*, 2001; Katehis *et al.*, 2002; Berends *et al.*, 2003). It has been shown with modeling that bio-augmentation can significantly decrease the SRT required for nitrification in the treatment system while still maintaining NH_3 removal (Rittmann, 1996; Kos, 1998).

Cold Shock and Seeding

To minimize the size of the side-stream nitrification tank, the warm temperature of the centrate should be maintained. In the temperature range found in centrate (30 to 35°C), nitrification growth rates are maximized. However, the effect of sudden change in temperature on nitrification rates are of particular importance here because the centrate is much warmer than the main-stream process which in Winnipeg, can run at 10°C. Head and Oleszkiewicz (2003) have already shown that nitrification continues despite a sudden decrease in temperature down to 10°C but at a decreased rate. The nitrification rates decreased by 58%, 71% and 82% for nitrifying biomass acclimated to 20, 25 and 30°C, respectively.

Determining Seeded Sludge Age

Seeding nitrifying bacteria into a wastewater treatment system changes the way that SRT is usually calculated. Typically, the SRT of a system without seeding is defined by Equation 1 and is termed "apparent SRT" or θ_x^a. This calculation ignores influent solids and is an expression of the proportion of the total solids wasted daily.

$$\theta_x^a = \frac{X_r V_r}{Q^e X_e + Q^w X_w} \qquad [1]$$

In a seeded system the nitrifying bacteria are treated as a separate entity from the other solids in the system. The first step in determining the SRT of a seeded system is to determine the mass of nitrifying bacteria can be generated from centrate. The NH_3 available to produce nitrifiers is actually a limited resource; *i.e.*, as the mass of NH_3 available in the centrate is limited, so too is the mass of nitrifying bacteria that can be generated from it.

When nitrifying bacteria are supplemented from an external source, the SRT of the nitrifying bacteria ("seeded SRT" or θ_x^s as defined by Equation 2) becomes less than the

apparent SRT (Rittmann and McCarty, 2001). Only the nitrifiers entering ($Q^s X_a^s$) and leaving the system ($Q^e X_a^e + Q^w X_a^w$) are considered rather than the total solids inventory.

$$\theta_x^s = \frac{X_a V_r}{Q^e X_a^e + Q^w X_a^w - Q^s X_a^s} \quad [2]$$

Because the seeded SRT is longer than the apparent SRT, the need to increase the solids inventory is reduced. A smaller tank can then be used for nitrification without overloading the final clarifiers.

Tracking seeded nitrifiers

Microbial techniques now exist such that specific types of microorganisms can be observed *in situ*. Fluorescent *in situ* hybridization uses an oligonucleotide probe with a fluorescent marker that binds to a specific sequence of a cell's ribosomal RNA (rRNA). When observed with a fluorescence microscope, the target organism can be observed and quantified in relation to all other organisms present in the sample. This method has been used by a number of researchers (e.g. Daims *et al.*, 2001; Biesterfeld *et al.*, 2001; Morgenroth *et al.*, 2000) to observe ammonia oxidizing bacteria (AOB) in nitrification studies and has been shown in some cases to correlate with nitrification rates.

OBJECTIVES

The objective of this research was to determine the differences in seeding potential between nitrifying seed acclimated to 10 and 20°C. This study will investigate the use of FISH for tracking ammonia oxidizing bacteria in seeded SBR to better estimate seeded SRT.

METHODS

Substrates

Centrate was delivered from the North End plant in Winnipeg, MB, Canada every 3 weeks in 60 L batches and stored in a closed container at 4°C. The North End plant receives sludge from two other plants in the City: South End is a HPOAS non-nitrifying plant (ADWF 60 ML/d; SRT 3.5 d) and West End is a non-nitrifying, coarse bubble air activated sludge plant (ADF 30 ML/d; SRT = 3.5 d). The North End plant is a HPOAS and treats 230 ML/d (ADWF) with approximately 40% of the drainage area served by combined sewers; with some food and garment industry. The two smaller plants are serviced by separate sewer system and carry mainly domestic wastewater. Sludge treatment at North End consists of blending of primary and waste activated sludges, gravity co-thickening, anaerobic digestion at 38°C for 17 d, with dewatering of digested solids by centrifuge. Centrate characteristics varied somewhat over the course of this research but it typically contained 650 mg NH_3-N/L, 200 mg SCOD/L and had an alkalinity between 2000 and 3000 mg/L as $CaCO_3$.

Synthetic wastewater was used as feed for the SBRs operating at 10°C. The feed was prepared in 40 L batches and stored in a closed container at 4°C. The feed recipe contained 150 mg/L beef extract, 150 mg/L yeast extract, 556 mg/L $NaHCO_3$, 150 mg/L NH_3Cl and a mixture of microelements. The concentrations of NH_3-N and TCOD in the mixed feed were 25 mg/L and 350 mg/L respectively.

Seed Source Reactor Operation

The warm nitrifying seed was obtained from a well established nitrifying biomass maintained in a 2.4 L reactor operated at 20°C (NB20). The reactor was operated under continuous aeration with an SRT and HRT equal to 5 d. Centrate (160 mL) was fed to the reactors 3 times per day with wasting once per day by removing one fifth of the reactor volume after the third cycle. Each cycle consisted of feed (5 min) and react (8 h). The pH was automatically controlled at or above 7.2 using concentrated $NaHCO_3$ that was metered by peristaltic pump.

The cold nitrifying seed was generated from centrate at 10°C (NB10). The SRT and HRT of this reactor was 12 d. The reactor was operated under continuous aeration with feeding and wasting once per day. The pH was monitored continuously and adjusted manually once per day immediately before feeding by adding a volume of concentrated $NaHCO_3$ such that the pH was raised to at least 8.0.

Seeded SBR Operation

SBRs (2 L working volume) were operated in a walk-in environmental chamber set at 10°C. The feed source for these reactors was synthetic wastewater. All of the SBRs were operated with an apparent SRT near 4 or 12 days which was controlled by wasting directly from the mixed liquor daily during aeration.

The SBRs with an SRT of 4 d had 3 cycles per day while the reactors with HRT 8 h had 4 cycles per day. The cycles consisted of: feed (50 min), aerate, settle (60 min) and decant (50 min). The biomass in the SBRs was acclimated for at least 3.5 apparent SRTs before sampling commenced. After establishing a baseline of effluent quality, daily seeding of 100 mL of nitrifying bacteria from the seed source reactors began. Samples for influent and effluent NH_3-N were taken at least 5 days per week and TSS, VSS, NO_3-N and COD were measured 3 times per week. The reactor configuration is shown in Figure 1.

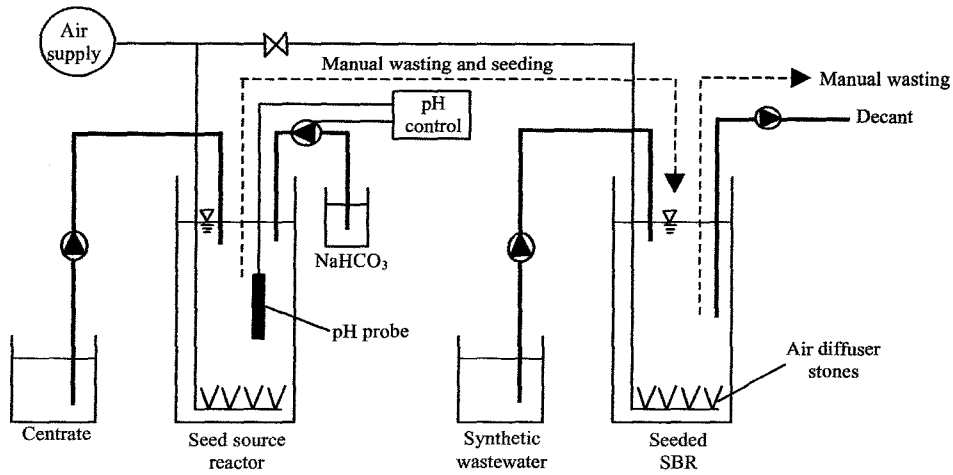

Figure 1. Reactor configuration for seeding nitrifiers into cold SBRs.

Biomass sampling and cell fixation

Grab samples of mixed liquor suspended solids were collected from the seed source reactors and the seeded reactors over the course of the research. The samples were fixed and hybridized according to the procedure described by Oerther *et al.* (2002). The probe and DNA stain are listed in Table 1.

Table 1. FISH probes used for preliminary visualization of biomass.

Target	Probe	Sequence (5' to 3')	Label	Formamide (%)	NaCl (M)
All DNA			DAPI	-	-
Ammonia oxidizing ß Proteobacteria[1]	S-*-Nso-1225-a-A-20	CGC CAT TGT ATT ACG TGT GA	Cy3	40	0.056

1) Schramm, 1999, Ballinger *et al.*, 1998, Guschin *et al.* 1997

Microscopy and image analysis

Photographs of the biomass were taken with a high resolution digital camera with image analysis and quantification done with the UTHSCSA ImageTool™ program (2002) (developed at the University of Texas Health Science Center at San Antonio, Texas and available free from the Internet by anonymous FTP from ddsdx.uthscsa.edu).

Quantification was done by relative area quantification against the total biomass stained by DAPI. The percent of FISH signal was normalized against the concentration of biomass in the reactor at the time of sampling by multiplying the percent signal by the concentration of VSS.

RESULTS

Seed Characteristics

During steady state operating conditions (consistently greater than 98% NH_3-N removal), the seed source reactors were sampled to determine the proportion of ammonia oxidizing bacteria (AOB) in the biomass. Using the probe Nso1225, an average of 17.9 % of the biomass in NB10 was labelled while an average of 9.3 % of the biomass was labelled in NB20 (Figure 1). This corresponds to a "normalized concentration" of approximately 21 mg VSS/L and 10.4 mg VSS/L of AOB in NB10 and NB20, respectively.

Alternately, estimates of nitrifier concentrations using Equation 1 were:

$$X_{a10C} = \frac{0.24(631 - 5.7 mg\, NH_3 - N/L)}{1 + 0.04 d^{-1} \bullet 12 d} = 99.8 mg/L$$

$$X_{a20C} = \frac{0.24(631 - 3.5 mg\, NH_3 - N/L)}{1 + 0.1 d^{-1} \bullet 5 d} = 100.4 mg/L$$

The calculated concentrations using Equation 1 correspond to 80% and 33% of the VSS concentration in the reactors NB10 and NB20, respectively.

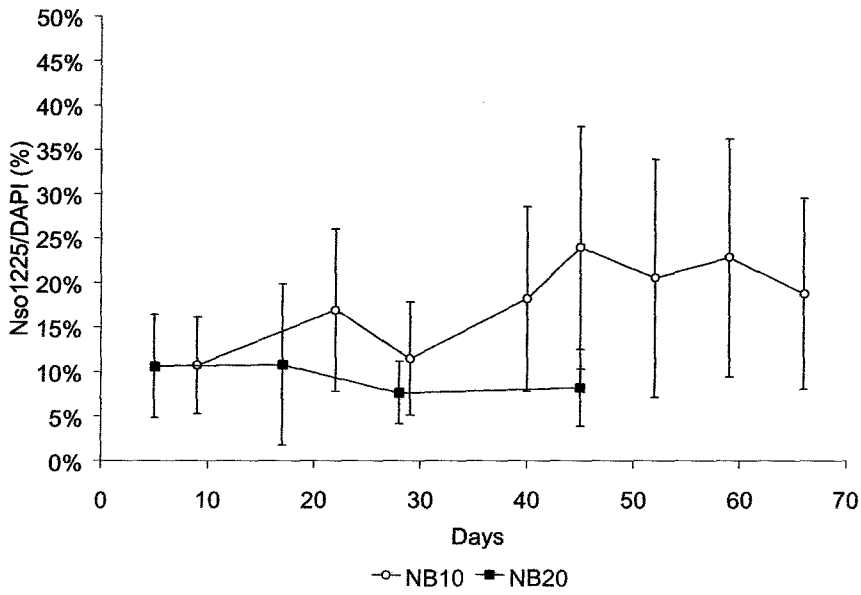

Figure 2. Percent Nso1225 against total area stained by DAPI for NB10 and NB20.

Results of SBRs with SRT 12 d and HRT 8 h

Nso1225 signal corresponded well with NH_3-N decreases and NO_3-N increases in the effluent for both reactors (Figures 3 and 4). The reactor seeded with NB10 had an increase in AOB with the initiation of seeding as shown by the increase in area labelled

by the probe Nso1225 (Figure 3). When seeding was stopped there was a slight decrease in AOB concentration but effluent NH_3-N remained low. After seeding was stopped for 30 days effluent NH_3-N concentrations did not increase and the Nso1225 signal remained high indicating that AOB washout did not occur. The normalized AOB concentrations shown in Figure 3 correspond with relative area percentages of 1.7 % before seeding up to 7.6 % on day 38.

For the SBR seeded with NB20, increases and decreases in Nso1225 signal corresponded with the start and stop of seeding (Figure 4). The normalized AOB concentrations shown in Figure 4 correspond with relative area percentages within the range of 0.7 % to 7.5 % of the total area labelled by DAPI.

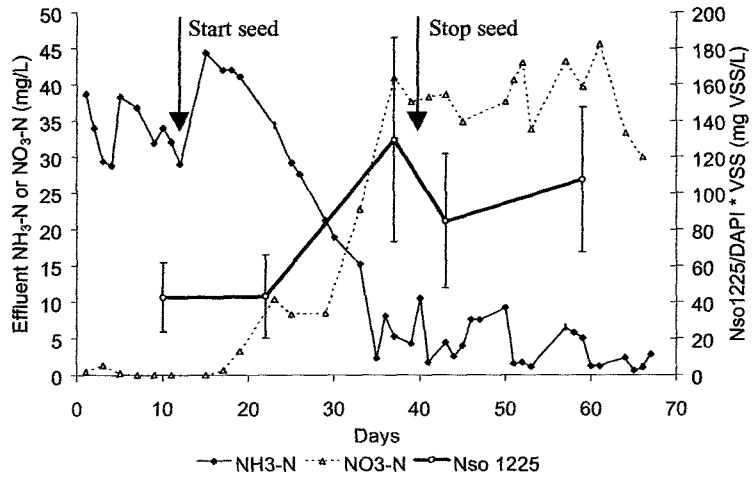

Figure 3. Effluent NH_3-N and mixed liquor AOB normalized concentration for an SBR seeded with NB10.

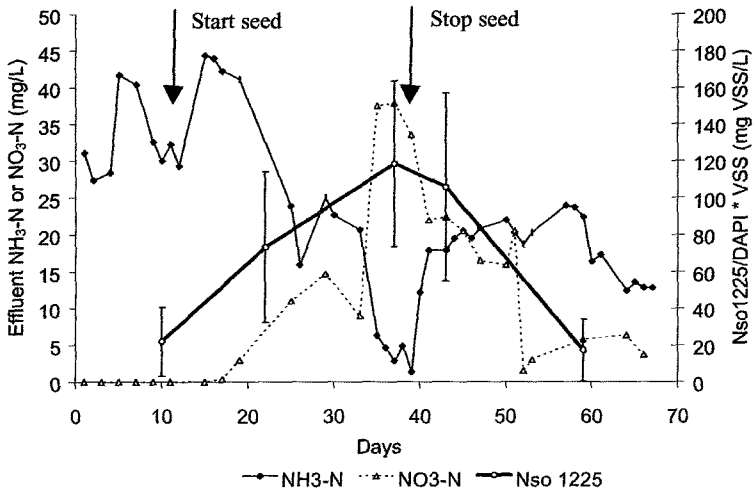

Figure 4. Effluent NH_3-N and mixed liquor AOB normalized concentration for SBR seeded with NB20.

Results of SBRs with SRT 4 d and HRT 12 h

The SBR seeded with NB10 was able to achieve approximately 50% NH_3-N removal and effluent NH_3-N rose after seeding was stopped (Figure 5). The SBR seeded with NB20 failed to achieve a significant level of NH_3-N removal despite the presence of AOB in the reactor at all times (Figure 6). FISH analysis showed that the effluent from the reactor seeded with NB10 had up to 5 times more AOB in the effluent than in the reactor biomass (Figure 4) while NB20 had up to 4 times more AOB in the effluent than in the reactor biomass (Figure 5).

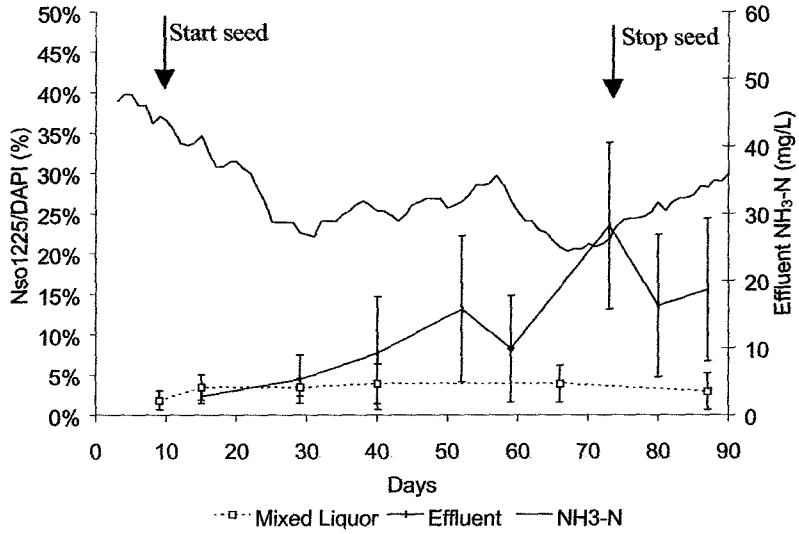

Figure 5. Percentage of biomass labelled with Nso1225 in the reactor mixed liquor and effluent solids for the SBR seeded with NB10.

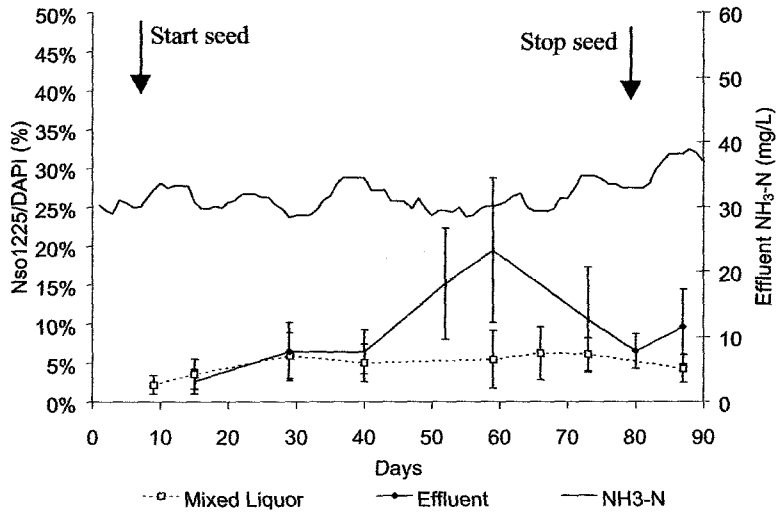

Figure 6. Percentage of biomass labelled with Nso1225 in the reactor mixed liquor and effluent solids for the SBR seeded with NB20.

DISCUSSION

FISH analysis showed that the reactor treating centrate at 10°C (NB10) could produce a higher proportion of AOB than the reactor treating centrate at 20°C (NB20) even though NB20 was treating an NH_3-N load 2.5 times greater than NB10. The degree of difference in AOB proportion and concentration between these reactors was not expected. The only parameters that affect the concentration of AOB in this case are the yield, decay rate and SRT. Because yield is not affected by temperature (Abyesinghe *et al.*, 2002) and the SRT is known, the differences must result from a greater change than expected in decay rate with temperature.

The calculated values for X_a were much larger than that predicted by FISH analysis. There are a number of possible reasons for this:

- The assumed values for Y and b could be incorrect; i.e. Y was overestimated or b was underestimated.

- It is possible that some AOB were not labelled by the Nso 1225 probe. Simultaneous labelling with another AOB probe might have achieved a better representation of the total AOB population.

- The area stained by DAPI may not have accurately represented the VSS concentration in the reactor. Our underlying assumption in the FISH analysis was that the area of DAPI stain is directly related to the VSS concentration in the reactor where VSS is made up of both dead and living cells (total biomass). VSS is a recognizable and frequently measured parameter in wastewater treatment. The Nso 1225 probe used in FISH analysis, however, is only indicative of metabolically active cells (Mudaly *et al.*, 2000, 2001).

  The relative area determination of the Nso1225 probe in this study was normalized against the concentration of biomass in the reactor on the day of sampling and expressed as "normalized concentration". In this way, the variations in biomass concentration in the reactor could be accounted for since the proportion of biomass labelled is meaningless in systems that have any variation in biomass concentration.

Full nitrification was achieved in the SBRs seeded with NB10 and NB20 then the SRT was 12 days and the HRT was 8 hours. After seeding was stopped in the reactor seeded with NB20 nitrification failed but nitrification continued in the reactor seeded with NB10. It is thought that initially the reactors did not contain AOB that were suitable for growth in the conditions that were provided. But NB10 did contain the "right type" of AOBs that could grow and survive in the new, seeded environment thus allowing nitrification to continue after seeding was stopped. NB20 did not contain the "right type" of AOBs thus nitrification failure resulted once seeding was stopped.

AOB loss with the decant liquor could not be anticipated without further microbial analysis. A simple solids balance could not accurately predict the seeded SRT of the

nitrifiers. Based on the results of FISH analysis, an additional term was incorporated into the seeded SRT determination. The term P acknowledges that the proportion of nitrifiers in the effluent may be different from that in the reactor. The seeded SRTs of the SBRs were then recalculated with the results listed in Table 2

Table 2. Comparison of apparent SRT and seeded SRT for seeded SBRs.

			$P = 1$*		$P = 2$†		$P = 4$†	
Reactor	Seed source	Apparent SRT (d)	θ_x^s (d)	μ (d^{-1})	θ_x^s (d)	μ (d^{-1})	θ_x^s (d)	μ (d^{-1})
HRT 12 h	NB10	3.8	8.2	0.12	6.2	0.16	4.2	0.23
HRT 8 h	NB10	11.9	15.0	0.07	10.0	0.1	6.00	0.16
	NB20	12.4	19.6	0.05	12.6	0.08	7.36	0.14

*Assumes that the proportion of nitrifiers in the effluent solids is the same as that in the reactor mixed liquor

†Compensates for the disproportionate amount of AOB lost with the decant liquors as shown by FISH analysis

In some cases, compensating for the nitrifiers lost with the effluent caused the seeded SRTs to be shorter than the apparent SRTs (Table 2). This could only occur if more nitrifiers were being wasted than were being added to the reactor. Obviously, it is not possible for nitrification to continue if nitrifiers were continually being lost at this high rate. It is possible, though, to occasionally lose more nitrifiers than that added if the seeded nitrifiers were multiplying within the seeded system. Figures 5 and 6 demonstrate that the amount of nitrifiers lost with the decant liquor was variable over the course of seeding.

Determination of growth rates ($1/\theta_x^s$) showed that the growth rates of the nitrifiers were greater in the reactors seeded with NB10 than in those seeded with NB20. Additionally, as the amount of nitrifiers lost with the decant liquor increases, the seeded SRT decreases and the growth rate must increase to achieve the same level of treatment.

With FISH analysis it was shown that poor seeding results when the apparent SRT was approximately 4 d was due to inadvertent AOB wash out with the decant liquors and a disproportionate amount of AOB in the effluent solids. The loss of AOB was likely due to the poor settling properties of that biomass and failure of the AOBs to be incorporated into, or captured by, the sludge floc during settling. In order for seeding to succeed, the AOBs would have to be maintained in the reactor by using a physical barrier such as membrane filtration or by the use of a carrier material.

CONCLUSIONS

- Bioaugmentation with nitrifying biomass acclimated to 10 and 20°C was effective in initiating full nitrification in SBRs at 10°C with an apparent SRT of approximately 12 days and HRT of 8 hours. Nitrification continued in the SBR seeded with NB10 after seeding was stopped while nitrification failed in the reactor seeded with NB20.

- Normalizing the FISH data against total VSS resulted in a rise and decline in Nso1225 signal that mirrored effluent NH_3-N concentration and corresponded well with effluent NO_3-N.

- FISH analysis showed that the proportion of AOB in the effluent solids was greater than that in the reactor. Calculating seeded SRT based on a solids balance would not take this into consideration and would overestimate the seeded SRT.

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LIST OF SYMBOLS

μ	growth rate of ammonia oxidizers, d^{-1}
θ_x^a	apparent SRT, d
θ_x^s	seeded SRT, d
P	ratio of ammonia oxidizers in the effluent to that in the reactor, %/%
Q^e	flow rate of effluent, L/d
Q^s	flow rate of seed stream, L/d
Q^w	flow rate of waste stream, L/d

V_r	volume of reactor contents, L
X_a	concentration of ammonia oxidizers in the reactor, mg VSS/L
X_e	concentration of volatile suspended solids in the effluent stream, mg/L
X_r	concentration of volatile suspended solids in the reactor, mg/L
X_w	concentration of volatile suspended solids in the waste stream, mg/L
X_a^e	concentration of ammonia oxidizers in the effluent, mg VSS/L
X_a^s	concentration of ammonia oxidizers in the seed source, mg VSS/L
X_a^w	concentration of ammonia oxidizers in the waste stream, mg VSS/L