Bioresource Technology 112 (2012) 51–60

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Application of a moving bed biofilm reactor for tertiary ammonia treatment in high temperature industrial wastewater

Jennifer L. Shore [a,b], William S. M'Coy [b], Claudia K. Gunsch [a], Marc A. Deshusses [a,*]

[a] Department of Civil & Environmental Engineering, Duke University, Durham, NC 27708, United States
[b] HDR Engineering, Inc., 5700 Lake Wright Drive, Suite 300, Norfolk, VA 23502, United States

ARTICLE INFO

Article history:
Received 7 November 2011
Received in revised form 6 February 2012
Accepted 8 February 2012
Available online 17 February 2012

Keywords:
Moving bed biofilm reactor
Industrial wastewater
Biological nutrient removal
Nitrification
qPCR

ABSTRACT

This study examines the use of a moving bed biofilm reactor (MBBR) as a tertiary treatment step for ammonia removal in high temperature (35–45 °C) effluents, and quantifies different phenotypes of ammonia and nitrite oxidizing bacteria responsible for nitrification at elevated temperatures. Bench scale reactors operating at 35 and 40 °C were able to successfully remove greater than 90% of the influent ammonia (up to 19 mg L^{-1} NH_3–N) in both the synthetic and industrial wastewater. No biotreatment was observed at 45 °C, although effective nitrification was rapidly recovered when the temperature was lowered to 30 °C. Using qPCR, *Nitrosomonas oligotropha* was found to be the dominant ammonia oxidizing bacterium in the biofilm for the first phases of reactor operation. In the later phases, *Nitrosomonas nitrosa* was observed and its increased presence may have been responsible for improved ammonia treatment efficiency. Accumulation of nitrite in some instances appeared to correlate with temporary low presence of *Nitrospira* spp.

1. Introduction

The Chesapeake Bay is the largest estuary in the United States. In recent years the rehabilitation and reduction of point source pollution to the Bay has been the focal point of many environmental conservation efforts. Wastewater treatment facilities (WWTF) are responsible for 19% of the nitrogen loading in the Chesapeake Bay watershed. In order to meet point source pollution reduction goals and stringent maximum loading regulations, many municipal WWTFs will be required to add enhanced nutrient removal to their existing operations (Chesapeake, 2009). Hopewell Regional Wastewater Treatment Facility (HRWTF; Hopewell, VA) is one of the many facilities facing new regulations. Wasteload allocations for HRWTF have been set at 830 tons of total nitrogen per year by 2011. Projected domestic growth in 2011 through 2015 will increase the total nitrogen loading above allocated amounts unless nutrient removal improvements are implemented. This poses some challenges in that the Hopewell facility receives a blend of 15% domestic wastewater and 85% industrial wastewater from various chemical and paper industries in the area. Waste streams from these industries have a high temperature and vary in chemical composition, both of which can inhibit biological nutrient removal (BNR) (Kelly et al., 2004; Grunditz and Dalhammar, 2001).

BNR is the preferred treatment method for reduction of organic materials and nutrients; however the limit of technology is quickly being reached. In recent years, the development and use of a moving bed biofilm reactor (MBBR) as an enhancement of BNR has evolved to treat a broad range of wastewaters. Advantages of MBBRs include the reduction in space as compared to traditional activated sludge system, ease in upgrade of existing facilities, reduction of headloss when compared to submerged filter configurations, fewer requirements for cleaning or backwash, increased solid retention time for slow growing organisms, and more rapid recovery from extreme loading conditions (Rusten et al., 1995; Odegaard et al., 1994; Jahren et al., 2002; Khan et al., 2011). Biofilm-based processes may also lessen the presence of non-flocculating filamentous bacteria which, in turn, mitigates problems with poor flocculation and settling in downstream treatment (LaPara et al., 2001; LaPara and Alleman, 1999). Bench scale MBBRs have been shown to be more stable for nutrient removal than membrane bioreactors under fluctuating loading conditions (Yang et al., 2009), and fixed microorganisms are more resilient to fluctuations in pH, nutrient concentration, toxic substances (Hosseini and Borghei, 2005; Lazarova and Manem, 1995; Jaroszynski et al., 2011).

Elevated temperature poses significant challenges for nitrification. In bench scale experiments, optimal temperatures for ammonia oxidizing bacteria (AOB) range from 30–35 to 35–40 °C for nitrite oxidizing bacteria (NOB) (Grunditz and Dalhammar, 2001; Bae et al., 2001; Jones and Hood, 1980). Recently, Zhang et al.

* Corresponding author at: Box 90287, Duke University, Durham, NC 27708-0287, United States. Tel.: +1 919 660 5480; fax: +1 919 660 5219.
E-mail address: marc.deshusses@duke.edu (M.A. Deshusses).

0960-8524/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2012.02.045

(2009) operated a 15 L sequencing batch biofilm reactor seeded with municipal wastewater to evaluate nitrification rates at five temperatures ranging from 15 to 35 °C. The sequencing batch reactor achieved an optimal ammonia oxidation rate of 4.7 mg NH_4^+–N L^{-1} h^{-1} at 31 °C, while at the maximum operating temperature tested (35 °C), a rate of 3.2 mg NH_4^+–N L^{-1} h^{-1} was achieved. The temperature sensitivity of nitrifying bacteria has stimulated detailed research on the microbial ecology of AOB and NOB. Quantitative polymerase chain reaction (qPCR) has emerged as the method of choice to evaluate and relate nitrifying bacteria community structure to nutrient treatment rates, because it can show functional diversity between specific phenotypes and may be applied to environmental samples. Methods have been developed to target the ammonia monooxygenase gene (*amoA)* sequences specific to AOB (Dionisi et al., 2002; Geets et al., 2007; Harms et al., 2003; Kuo et al., 2006; Layton et al., 2005; Lim et al., 2008a) and the specific 16S rDNA sequences conserved in the various clusters of AOB (Dionisi et al., 2002; Lim et al., 2008a,b). Because NOB are less phylogenetically diverse, primers for their quantification are more consistent in the literature (Juretschko et al., 1998; Gieseke et al., 2001). The primer set targeting *Nitrospira* spp., which are believed to be the dominant NOB in wastewater, is the most commonly used one (Kim and Kim, 2006).

The aim of this research was to evaluate nitrification in a bench scale MBBR at three elevated temperatures (35, 40, and 45 °C) with synthetic wastewater and with secondary treated effluent from the Hopewell facility. It was hypothesized that biofilm growth on the carrier media would provide some protection for AOB and NOB activity at those temperatures and allow continued treatment of ammonia despite the adverse operating conditions. Nitrifying bacteria were quantified by qPCR in an attempt to link nutrient treatment rates with specific bacterial population densities.

2. Methods

2.1. Reactor setup and operation

Three bench scale reactors were operated for 116 days. Each reactor had a 3.5 L operating volume, and a 200 min. hydraulic residence time. They were filled with 300 ± 5 pieces each of BioPortz™ media, a high surface area (580 m^2 m^{-3}) cylindrical support made of HDPE (ca 2 cm dia. × 2 cm tall, Entex Technologies, Chapel Hill, NC) which corresponds to a 50% fill. Fig. 1 shows a schematic of the experimental setup. Carrier media with a nitrifying biofilm was collected from a pilot study at South Durham Wastewater Treatment Facility (Durham, NC) described in Kim et al. (2010) and immediately transferred to the bench scale reactors. Synthetic wastewater (see composition below) was pumped into each reactor at a rate of 17.5 mL min^{-1}. This flow was selected because it provided a hydraulic residence time (200 min) comparable to that of a possible full scale basin at HRWTF. The synthetic wastewater nutrient concentrations were based on historical average secondary effluent concentrations at HRWTF and consisted of 23 mg L^{-1} acetic acid, 53 mg L^{-1} glutamic acid, 41 mg L^{-1} NH_4Cl, 21 mg L^{-1} $Na_2S_2O_3$, 5.7 mg L^{-1} KH_2PO_4 and a trace mineral mixture of 2.6 mg L^{-1} $MgSO_4$ and 0.75 mg L^{-1} of each $Fe_2(SO_4)_3$, $MnSO_4$, and $ZnSO_4$ in deionized water. The COD of the synthetic waste water was 120 ± 10 mg L^{-1} (all soluble) which is slightly lower, but still comparable to the COD of the HRWTF water (see below) The pH was adjusted to 6.7 with sodium bicarbonate on a daily basis. Compressed air was bubbled in the reactors at a rate of 800 mL min^{-1} which facilitated liquid mixing and media up/down motion in the reactors and maintained dissolved oxygen concentrations above 4 mg L^{-1} as recommended by the media manufacturer.

The reactors were initially operated at room temperature (~20–22 °C) with synthetic wastewater for 18 days. The temperature in each reactor was then increased using heating tape wrapped around each reactor connected to a constant voltage source until the desired operating temperature (i.e., 35, 40 and 45 °C) was reached (Table 1). After 84 days of operation the influent water in two reactors was changed from synthetic wastewater to secondary treated effluent collected on two occasions from HRWTF. The concentration of key species in the first batch of HRWTF water was 18.6 mg L^{-1} NH_3–N, <1 mg L^{-1} NO_2–N + NO_3–N, 6.9 pH, 153 mg L^{-1} COD_{total}, 122 mg L^{-1} $COD_{soluble}$, and the temperature at the time of collection was 41.6 °C. Key species concentration in the second batch of water was 15.6 mg L^{-1} NH_3 N, <1 mg L^{-1} NO_2–N +NO_3–N, pH 6.7, 158 mg L^{-1} COD_{total}, 130 mg L^{-1} $COD_{soluble}$ and the temperature was 37.9 °C. After transport the water was stored at 5.6 °C to prevent biological growth until it was pumped into the reactors.

2.2. Analytical methods

Influent and effluent water testing was conducted on a daily basis for the duration of the experiment. The following nutrient concentrations were measured colorimetrically with Hach kits and a spectrophotometer DR/2500 (Hach, Loveland, CO): NO_3^- -N (cadmium reduction method), NO_2^-–N (diazotization method), NH_3–N (salicylate method), and unfiltered COD (reactor digestion method). pH was measured with an Accumet liquid-filled, glass body single-junction combination pH Ag/AgCl Electrode (Fisher Scientific, Pittsburg, PA). Temperature was monitored and recorded continuously with a stainless steel temperature probe and Labquest interface (Vernier Software & Technology, Beaverton, OR). Microsoft Office Excel 2007 was used for all statistical analysis. For mean comparison of more than two data sets ANOVA analysis was employed, whereas a two-tailed Student's *t*-test was used to evaluate statistical differences for paired two sample means. A 95% confidence interval was used for all statistical analyses.

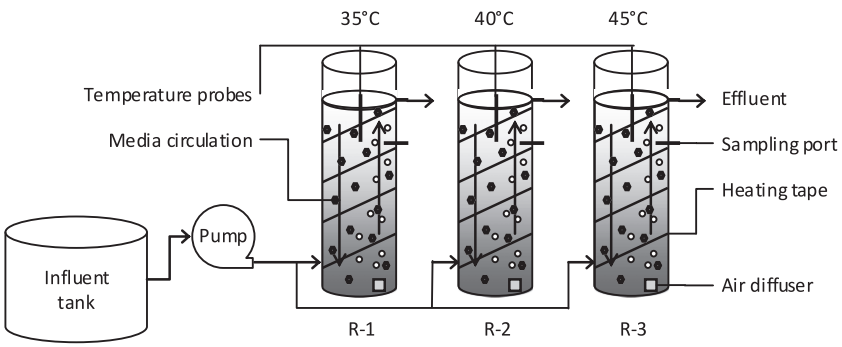

Fig. 1. Schematic of the bench scale experimental set-up.

Table 1
Reactor operating conditions.

Phase	Start-up	Phase 1	Phase 2	Phase 3	Phase 4	Phase 5
Operation days	1–17	18–32	32–59	60–83	84–100	101–116
Reactor 1	30 °C	30 °C	35 °C	35 °C	35 °C**	35 °C**
Reactor 2	30 °C	30 °C	40 °C	40 °C	40 °C**	40 °C**
Reactor 3	30 °C	30 °C	45–30 °C*	45 °C	45 °C	45 °C

* Failure of temperature control on day 38 resulting in overheating to 55 °C for 3 h, after which the temperature was lowered to 30 °C for two weeks to allow for recovery.
** Operation with HRWTF secondary effluent.

For dry-weight biomass quantification, three pieces of media were extracted from each reactor on selected dates. After each sampling event, three pieces of clean, new media were used to replenish the reactor. Upon extraction, each piece of media was placed in an aluminum pan and dried for 4 h at 105 °C. After weighing, each piece of media was placed in 100 mL of 5 N sulfuric acid and stirred for 24 h in an enclosed container. Following the acid digestion wash, each media piece was thoroughly rinsed with distilled water and dried at 105 °C for 4 h. The final mass was measured and subtracted from the initial mass to determine the dry weight of biomass per piece of media.

2.3. DNA extraction and quantitative polymerase chain reaction

DNA was extracted for analysis during each phase of reactor operation. A total of five samples for Reactors 1 and 2 and four samples from Reactor 3 were randomly taken for molecular analysis. Biomass was manually scraped off from inner walls of the Bioportz™ media and was stored at −80 °C until further processing. Prior to DNA extraction, biomass was thawed and 1 mL of sterile distilled water was added per 20 mg wet-weight biomass; biofilm flocs were manually homogenized by repeated passing through a 25 gauge syringe needle. Samples were centrifuged at 10,000*g* for one minute and DNA was extracted using the MOBIO UltraClean™ Microbial DNA isolation kit (Carlsbad, CA) following the manufacturer's protocols. DNA concentration and quality was determined by measuring absorbance at 260 and 280 nm using a NanoDrop Spectrophotometer ND-1000 (Fisher Thermo Scientific, Waltham, MA) and calculating the ratio of A_{260}/A_{280}. Samples with A_{260}/A_{280} ranging from 1.8 to 2.0 were used in subsequent analyses. Samples were diluted with PCR grade sterile water to a final concentration of 5 ng μL^{-1} and stored at −20 °C until further dilution for PCR amplification.

Total bacteria as well as AOB and NOB were quantified using published primer sequences and temperature cycling programs. The specific gene targets were *N. oligotropha amoA* and 16S *rDNA* from total bacteria, *N. europaea*, *N. nitrosa*, *Nitrospira* spp. and *Nitrosospira* spp. Table 2 lists the primers used in this study. All oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA). PCR amplicon lengths were verified by agarose gel electrophoresis. All qPCR experiments were performed using a M×3000P QPCR System (Stratagene, La Jolla CA) and iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories, Hercules, CA). At the end of each qPCR run, a dissociation curve analysis was performed with five serial dilutions of the target PCR amplicon to ensure the quality of the PCR products. The control was obtained through amplifications of phase 1 samples.

The qPCR samples and conditions were as described in the respective studies referenced in Table 2 with the following modifications. The final concentrations for each primer set were 300 nM for *N. oligotropha amoA*, 500 nM for *N. nitrosa* 16S *rDNA*, 600 nM for *Nitrospira* spp. 16S *rDNA*, and 200 nM for 16S *rDNA* for total bacteria. A final concentration of 1 ng μL^{-1} of total DNA was used for all reactions. All reactions were completed in duplicate. Standard curves for each target gene were generated by performing qPCR with DNA concentrations ranging from 0.1 to 1000 ng μL^{-1}. The efficiency (E) of each reaction was calculated as described in Livak and Schmittgen (2001); values were greater than 91% for all primers and except for the 16S rDNA reference primer (E = 66%). R^2 values for efficiency curves were greater than 0.985 for all primer sets.

Relative quantification was carried out by determining the relative gene abundance for a target population in comparison to a reference population (i.e., 16S *rDNA* for total bacteria) using the comparative threshold method (Pfaffl, 2001; Gunsch et al., 2006). Briefly, the threshold cycle numbers (C_t) which is a measure of a target gene concentration is determined as the number of PCR cycles for which a threshold fluorescence level is reached. C_t values can then be compared between treatments to determine the relative abundance of specific genes or specific microbial populations. In this study, the relative abundance was normalized at each time point relative to a calibrator sample. The calibrator sample was chosen as that collected on day 32 (last day of phase 1 operation). This time point was selected because all reactors had been operated under the same conditions up to this point and had similar ammonia treatment performance (87–88% of influent ammonia removed) and biomass concentrations. The relative gene target number (R) was calculated as shown in Eq. 1.

$$R = \frac{(1+E_{\text{target}})^{C_{t,\text{target,calibrator}} - C_{t,\text{target,sample}}}}{(1+E_{\text{total 16S}})^{C_{t,\text{total16S,sample}} - C_{t,\text{total16S,sample}}}} \quad (1)$$

Where E_{total16S} and E_{target} represent the amplification efficiency for total 16S rDNA (reference population) and for each target, respectively; $C_{t,\text{Total16S,calibrator}}$ and $C_{t,\text{target,calibrator}}$ are the threshold cycle numbers for total 16S rDNA and each target population at

Table 2
Oligonucleotide primers used in qPCR analysis.

Target	Primer	Sequence 5′–3′	Reference
N. oligotropha amoA gene	amoNo550D2f	TCAGTAGCYGACTACACMGG	Harms et al. (2003)
	amoNo754r	CTTTAACATAGTAGAAAGCGG	
Nitrosomonas nitrosa Cluster 16S rDNA	NSMnit-438f	TTCGG TCGGG AAGAW ATAG	Lim et al. 2008b
	NSMnit-633r	CTAGT YATAT AGTTT CAAAC GC	
Nitrospira spp. 16S rDNA	NSR1113f	CCTGCTTTCAGTTGCTACCG	Harms et al. (2003)
	NSR1264r	GTTTGCAGCGCTTTGTACCG	
16s rDNA Total bacteria	799F 16S rDNA	GGTAGTCYAYGCMSTAAACG	Bach et al. (2002)
	1063R 16S rDNA	GACARCCATGCASCACCTG	
Nitrosomonas europaea cluster 16S rDNA	NSMeur-828f	GTTGT CGGAT CTAAT TAAG	Lim et al. (2008a)
	NSMeur-1028r	TGTCT TGGCT CCCTT TC	

day 32; $C_{t,target,sample}$ and $C_{t,total16Ssample}$ are the threshold cycle numbers for total 16S *rDNA* and each target population at the selected sampling event.

3. Results and discussion

3.1. Effect of temperature on ammonia treatment

Average ammonia treatment efficiencies in Reactors 1, 2 and 3 are shown in Table 3. Ammonia treatment in the different bioreactors were not statistically different ($p > 0.05$) during Phase 1 when all reactors were operated at 30 °C. However, changes occurred after the temperature was increased and when the influent was switched from synthetic wastewater to HRWTF secondary effluent.

3.1.1. Reactor 1 (35 °C)

After the temperature was raised to 35 °C in Phase 2, Reactor 1 maintained nitrification rates which were not statistically different than those obtained in Phase 1 (Fig. 2A). Ammonia removal efficiency gradually increased in Phase 3, while a slight increase in the effluent nitrate concentration and a transient effluent nitrite concentration around 2–3 mg L^{-1} NO_2–N were observed. Ammonia effluent concentrations further decreased when influent water was switched from the synthetic wastewater to HRWTF water. Since HRWTF secondary effluent had a greater concentration of ammonia than the synthetic wastewater, Phases 4 and 5 also corresponded to an increase in ammonia loading. Ammonia influent concentrations were 18.6 and 15.6 mg L^{-1} NH_3–N in Phases 4 and 5, respectively, compared to 12.5 mg L^{-1} NH_3–N in Phases 1–3. Ammonia removal was significantly different when comparing to values when the synthetic wastewater was used in Phase 3 and the Hopewell water in Phases 4 and 5 (ANOVA, $p < 0.05$). The 5% increase in ammonia removal efficiency may be attributed to additional matrix chemicals in the Hopewell water, shifts in AOB population (discussed below), increased specific activity of the AOB or reduced competition with heterotrophic bacteria in the biofilm due to a possible decrease in bioavailable COD in the HRWTF water. During Phase 3, on days 67–83, an unexpected buildup of nitrite was observed (Fig. 2a), which peaked at roughly 3 mg L^{-1} NO_2^-–N. A priori, there was no particular reason for such a drastic change in treatment performance, but monitoring of the nitrifying community revealed that this partial upset coincided with a decrease in normalized NOB population during Phase 3. This is discussed later in Section 3.3. In Phases 4 and 5, nitrite concentrations remained less than 0.1 mg L^{-1} NO_2^-–N. Effective nitrification occurring at this temperature range was expected as optimal temperature for freshwater nitrifiers is approximately 35 °C (Grunditz and Dalhammar, 2001; Jones and Hood, 1980).

3.1.2. Reactor 2 (40 °C)

At the beginning of Phase 2, after the temperature was increased to 40 °C, nitrification was temporarily inhibited (Fig. 2b). The effluent nitrate concentration reached approximately 3 mg L^{-1} NO_3^-–N and ammonia treatment efficacy was less than 30%. However, within four weeks, the reactor recovered and, during the last eleven days of Phase 3, ammonia treatment efficacy was close to 90%, i.e., not significantly different than for operation at 30 °C. In Phase 3, the effluent nitrate concentrations increased to an average of 11.5 mg L^{-1} NO_3^-–N indicating that nitrite oxidation was well established. This corresponded with a relative increase in *Nitrospira* spp. population observed in Phase 3 (discussed in the next section). In Phases 4 and 5, ammonia treatment continued to improve, and was significantly increased as compared to treatment performance in Phases 1, 2, and 3 (ANOVA, $p < 0.05$). However, even though the ammonia loading was significantly different, there was no significant difference in ammonia treatment efficiency between Phases 4 and 5 (*t*-test, $p > 0.05$). Effluent nitrite concentrations remained stable during Phase 4 and 5 with an average concentration of 0.10 ± 0.04 mg L^{-1} NO_2-N. Effluent nitrate concentrations decreased slightly in Phase 5. This decrease was attributed to the lower ammonia concentration in the second batch of HRWTF secondary effluent. Surprisingly, within 6 h after the beginning of Phase 4 (switch to HRWTF secondary effluent), the movement of media in Reactor 2 changed drastically; roughly one third of the media floated to the top of the reactor, while the remainder continued to circulate in a pattern similar to previous operations. This was unchanged for the remainder of the experiments and was not observed in either Reactor 1 or 3. Based on effluent ammonia concentrations, this did not adversely affect the treatment performance of Reactor 2. It is unclear why this phenomenon was observed in this reactor only, and what or even if any parameter in the Hopewell water caused it. Slight variations in air bubbles distribution, possibly in bubble coalescence or differences in biofilm density could be the cause. Even so, treatment of ammonia remained unchanged because the mixing time in the bioreactor was well below the hydraulic retention time.

Table 3
Average ammonia treatment efficacy during reactor operation, values are calculated from the arithmetic mean of effluent concentrations measured from the final eleven days of operation in each phase.

	Temperature °C	Average % NH_3–N removal	Comments
Reactor 1			
Phase 1	30	87.7 (±7.6)%	Not significantly different from R-2, R-3 at 30 °C
Phase 2	35	–*	Acclimation period
Phase 3	35	92.6 (±6.8)%	Not significantly different from R-2 40 °C
Phase 4**	35	97.4 (±0.8)%	Removal % for different Hopewell samples are not significantly different.
Phase 5**	35	98.1 (±0.3)%	
Reactor 2			
Phase 1	30 °C	86.9 (±7.3)%	Not significantly different from R-1, R-3 at 30 °C
Phase 2	40 °C	–*	Acclimation period
Phase 3	40 °C	90.1 (±5.6)%	Not significantly different from R-1 35 °C
Phase 4**	40 °C	97.8 (±1.5)%	Removal % for different Hopewell samples are not significantly different.
Phase 5**	40 °C	98.3 (±0.2)%	
Reactor 3			
Phase 1	30	88.0 (±6.0)%	Not significantly different from R-1, R-2 at 30 °C
Phase 2	45–30	90.5 (±3.1)%	–
Phase 3	45	18.3 (±3.4)%	–
Phase 4	45	18.3 (±3.4)%	–

* Unsteady state, average removal not calculated
** Operation with HRWTF secondary effluent.

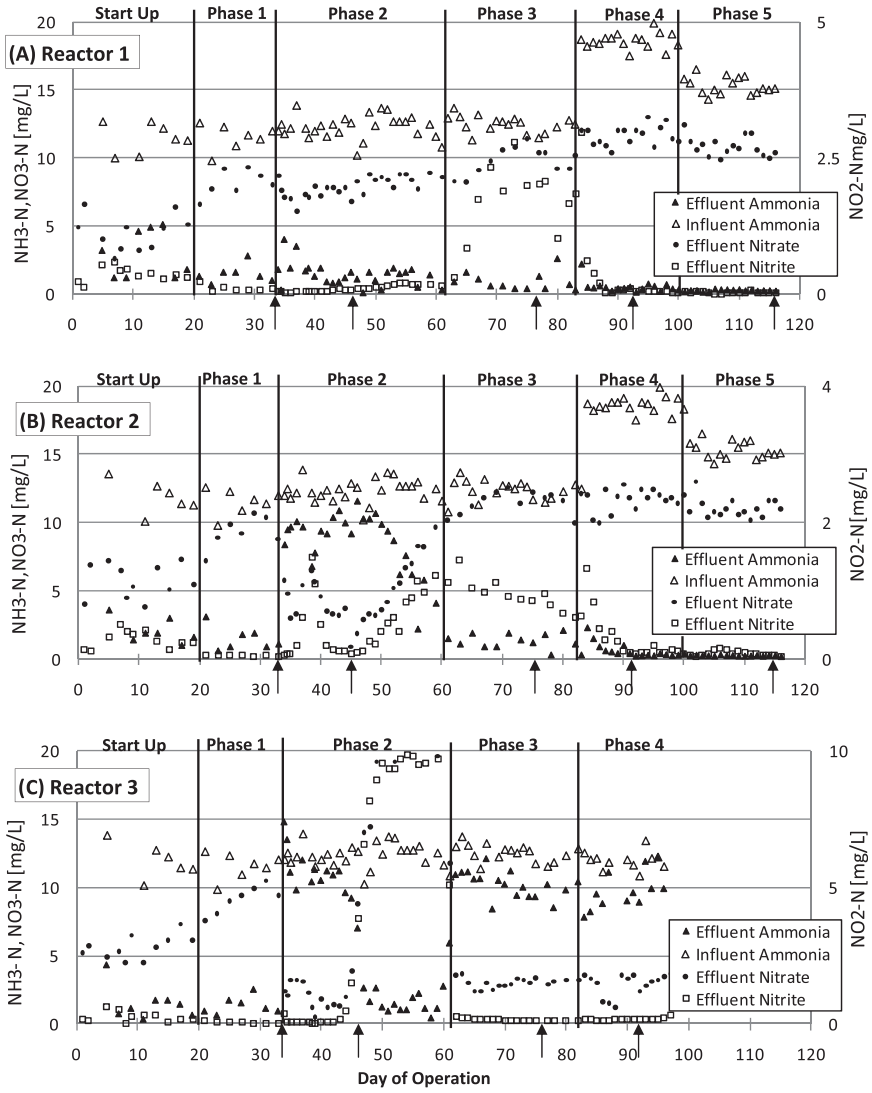

Fig. 2. Influent/effluent ammonia and nitrate concentrations (left axis), effluent nitrite concentrations (right axis) for the three bioreactors during the experiment. The dashed lines indicate the different phases and arrows indicate time of sampling for DNA analysis.

The data for Reactor 2 indicate that nitrification is possible at temperatures that exceed the documented optimal growth temperature for pure cultures of nitrifying bacteria operating in small scale batch reactors (Grunditz and Dalhammar, 2001; Bae et al., 2001; Jones and Hood, 1980). Batch reactor studies are useful in preliminary testing; however due to their short operation time and decreasing substrate concentrations, they are not optimal for bacterial adaptation. Results for Reactors 1 and 2 show that adaptation can range from several days to over a week. The similar nitrification rates observed at both 35 and 40 °C (Reactors 1 and 2) contradict previous findings by Zhang et al. (2009) whose recent study found that optimal ammonia treatment in a sequencing batch biofilm reactor occurred at 31 °C and decreased at 35 °C. The biofilm reactor was first operated at 35 °C for 14 days and at 31 °C for 28 days. In contrast, the reactors in this study were operated at elevated and constant temperatures for over 80 days to allow ample time for stabilization, as illustrated by the 97% ammonia removal efficiencies recorded in Phases 4 and 5. The difference between Zhang et al. and our findings suggests that extended acclimation time and time to build up dense biofilms are needed, when working with slow growing organisms such as nitrifiers, under significant environmental stress.

3.1.3. Reactor 3 (45 °C)

Reactor 3 was operated at 45 °C for five days (day 32–38) before failure of the temperature control resulted in overheating to 55 °C. After 3 h at 55 °C, the temperature was reduced to 30 °C to allow the microbial community to recover from the heat shock. Within two weeks, Reactor 3 was able to once again remove 90% of the influent ammonia without re-inoculation of nitrifying bacteria. During the two week recovery, the total inorganic nitrogen in the effluent was found to be double the amount of ammonia entering the reactor. This indicates that significant release of inorganic nitrogen occurred from the system, probably as a result of biomass hydrolysis at elevated temperature (Iranpour et al., 2002). The increase in the effluent nitrate and nitrite concentrations also correlated with an increase of ammonia removal (Fig. 2c). The recovery observed in Reactor 3 after the temperature shock indicates that a viable nitrifying biofilm was retained on the floating media. It also illustrates that the MBBR can successfully overcome inevitable process upsets without the added cost of biomass recycle, as in suspended biomass systems. Temperature was again increased to 45 °C on day 60 and the bioreactor was operated at this temperature for 34 days during which an average of 18.3 ± 4.5% ammonia was removed. Average influent and effluent nitrate concentrations

were $1.3 \pm 0.6\ mg L^{-1}$ NO_3^-–N and $3.0 \pm 0.6\ mg L^{-1}$ NO_3–N respectively, and average influent/effluent nitrite concentrations were $0.027 \pm 0.016\ mg L^{-1}$ NO_2^-–N and $0.164 \pm 0.04\ mg L^{-1}$ NO_2–N respectively indication minimal ammonia oxidation. The reactor's inability to effectively nitrify at 45 °C is consistent with previous studies (Grunditz and Dalhammar, 2001; Bae et al., 2001; Jones and Hood, 1980). Even so, Reactor 3 was able to remove approximately 44% of the influent COD at 45 °C (Figure S1, in Supplementary Information). This level of COD removal was not unexpected as thermotolerant and thermophilic heterotrophs are commonly reported for COD removal (LaPara and Alleman, 1999).

3.1.4. Nitrogen balance and distribution

An inorganic nitrogen balance was conducted by summing influent and effluent concentrations of NH_3–N, NO_2^-–N, and NO_3^-–N. If nitrification is the dominant ammonia removal process occurring in the reactor, then the total influent and effluent inorganic nitrogen concentrations should be nearly equal. Fig. 3 provides daily total influent and effluent inorganic nitrogen concentrations, while speciation of inorganic nitrogen can be found in Fig. 2, discussed earlier. In the early phases of operation, some discrepancy is observed between total influent and effluent inorganic nitrogen flux, most probably because of assimilation of nitrogen by bacteria, for cell growth, and partial stripping of ammonia before nitrification was well established. It is not clear why there were differences in the nitrogen balances through the beginning of Phase 3 between Reactor 1 and Reactor 2. Possibly greater growth occurred in Reactor 1 (which was operated at a lower temperature), a speculation supported by biomass measurements presented in Fig. 4 (discussed below). During the latter days of Phase 3, the average influent total inorganic nitrogen concentration was $13.9 \pm 0.7\ mg L^{-1}$–N and effluent values were 13.6 ± 0.9 and $13.5 \pm 0.1\ mg L^{-1}$–N for Reactors 1 and 2, respectively, indicating excellent closure (>99%) of the nitrogen balance. A different balance was observed when Reactors 1 and 2 were fed Hopewell water, as only between 60 and 70% of the total influent inorganic nitrogen was recovered (mainly as nitrate and nitrite, see Fig. 2) in the effluent. The 4–$8\ mg L^{-1}$ discrepancy in the total inorganic nitrogen entering and exiting the reactors during Phases 4 and 5 is indicative of nitrogen removal by a mechanism other than nitrification within the reactor. This finding is discussed in the next paragraph. The inorganic nitrogen balance in Reactor 3 generally showed excellent correspondence between influent and effluent nitrogen concentrations, except between days 43 and 60 during which very high nitrite concentrations were observed in the effluent. This was most likely caused by the upset of the culture and hydrolysis of organic nitrogen as a result of the temperature shock. This result shows that process failures can result in the temporary release of nitrogen concentrations that exceed the influent concentrations.

A detailed determination of nitrogen removal mechanisms other than nitrification was not conducted in this research, however several possibilities exist to explain the missing nitrogen fraction observed in

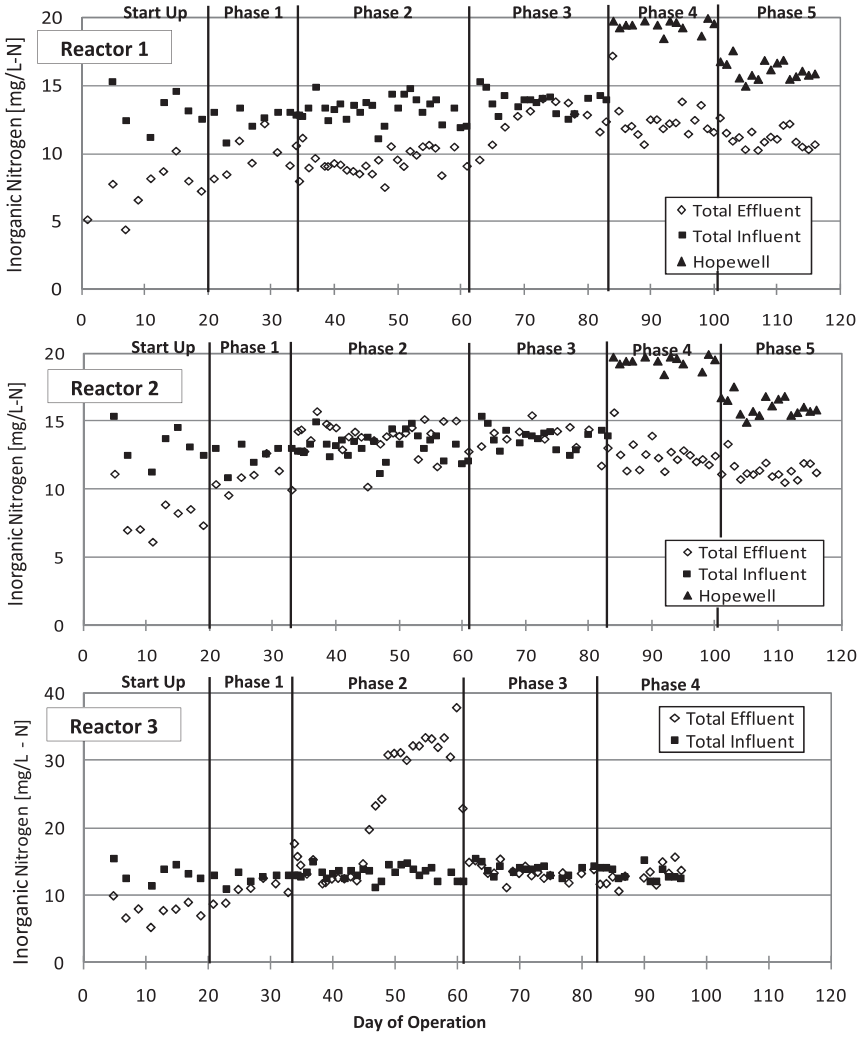

Fig. 3. Influent/effluent total inorganic nitrogen (NH_3–N + NO_2^- –N + NO_3^-–N) for the three bioreactors during the experiment.

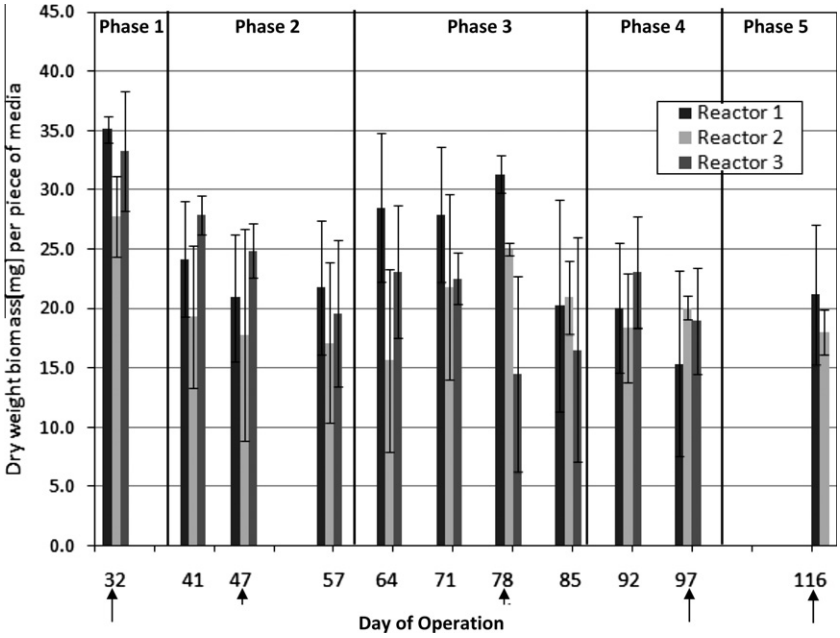

Fig. 4. Average dry weight of biomass ($n = 3$) in mg per piece of media collected at designated sampling days. Arrows indicate time of sampling for DNA analysis.

Phases 4 and 5 in Reactors 1 and 2. First, ammonia stripping may have occurred due to the continuous aeration; the extent of stripping is temperature dependent as the air/water partition coefficient of ammonia increases by a factor of 4 as the temperature increases from 15 to 40 °C. Calculations (not shown) based on the operating conditions in Reactor 2 indicate that approximately 8% of the influent ammonia could be removed by ammonia stripping. Stripping of ammonia can explain some of the differences in nitrogen balance closure between Reactor 1 and Reactor 2 through Phase 3. However, the extent of ammonia stripping would not have changed between the synthetic water and the HRWTF water and therefore is not the cause for the missing nitrogen fraction when the reactors were fed HRWTF water. Another possibility is the occurrence of simultaneous nitrification/denitrification by denitrifying bacteria in anoxic niches of the biofilm, a phenomenon which has been documented in a number of immobilized biofilm processes. In a recent study, Baek and Pagilla (2008) were able to achieve 16% total nitrogen removal by denitrification in a laboratory scale aerobic membrane bioreactor operating with relatively similar substrate conditions (DO of 3.5 $mg L^{-1}$, COD loading of 114 $mg L^{-1}$ and ammonia loading of 17.6 $mg L^{-1}$). Examination of the COD levels in Figure S1 indicates that COD removal decreased with HRWTF water and therefore, increased heterotrophic denitrification during Phases 4 and 5 was unlikely. The causes for the low closure of the nitrogen balance when feeding HRWTF water were not investigated further.

3.1.5. Dry weight biomass quantification

The dry weight of biomass attached to the MBBR media was measured to determine if reactor temperature affected the density of bacteria in the bioreactor. As shown in Fig. 4, the average dry weight of biomass ranged from 15 to 35 mg_{dw}/media piece with important variations from piece to piece as indicated by the relatively large error bars. Data appear to indicate a trend in biomass reduction with increasing temperature, however values were not statistically significantly different following the increase in reactor temperature. One problem in such analysis is the large standard deviation of attached biomass per media piece, which is an intrinsic property of such systems. A similar decreasing trend may exist after switching the feed to Hopewell water in Reactors 1 and 2 during Phases 1 and 2. This could be caused by lower bioavailability of the COD in the Hopewell water as compared to the synthetic feed. Previous analyses of the HRWTF wastewater composition by Linares et al. (2008) have shown that much of the COD in the secondary effluent is not bioavailable. This explanation is consistent with the decrease in COD removal efficiency in both Reactors 1 and 2 after the influent was switched from synthetic wastewater to HRWTF secondary effluent. Thus it is likely that the decrease in bioavailable substrates may have caused slower growth and some detachment of the heterotrophic organisms in the biofilm.

3.2. Temperature effects on nitrifying microbial community composition

Population dynamics of selected AOB and NOB in the three bioreactors were determined using qPCR (Fig. 5). Of the four primer sets tested, only those for the *amoA* gene of *N. oligotropha* and the 16S *rDNA* of *Nitrospira* spp. were detected in all samples, while positive detection of the 16S *rDNA* of *N. nitrosa* only appeared in Reactors 1 and 2, after Phase 4, and Phase 3 respectively. The *16S rDNA* gene of *N. europaea* cluster bacteria was never detected (although positive controls with ATCC 19718 *N. europaea* confirmed that the method was adequate); it came as a surprise as this organism is one of the most frequently detected ammonia oxidizers in wastewater treatment plants. But as shown in this research, it is not required for effective treatment.

Reactor 1 had a complex population dynamics. The relative abundance of microorganisms carrying the *amoA* gene representative of *N. oligotropha* increased up nearly two orders of magnitude following Phase 1. The important relative changes compared to the original values indicate that significant shifts in AOB community structure occurred, even long after a relatively steady treatment performance was observed. Over time, a generally increasing trend was noticed in the treatment efficacy of ammonia, although differences were not all significant, as was discussed in Section 3.1. Hence, a firm association between *amoA* gene abundance and ammonia treatment could not be established. Following the feeding of HRWTF secondary effluent, ammonia treatment increased

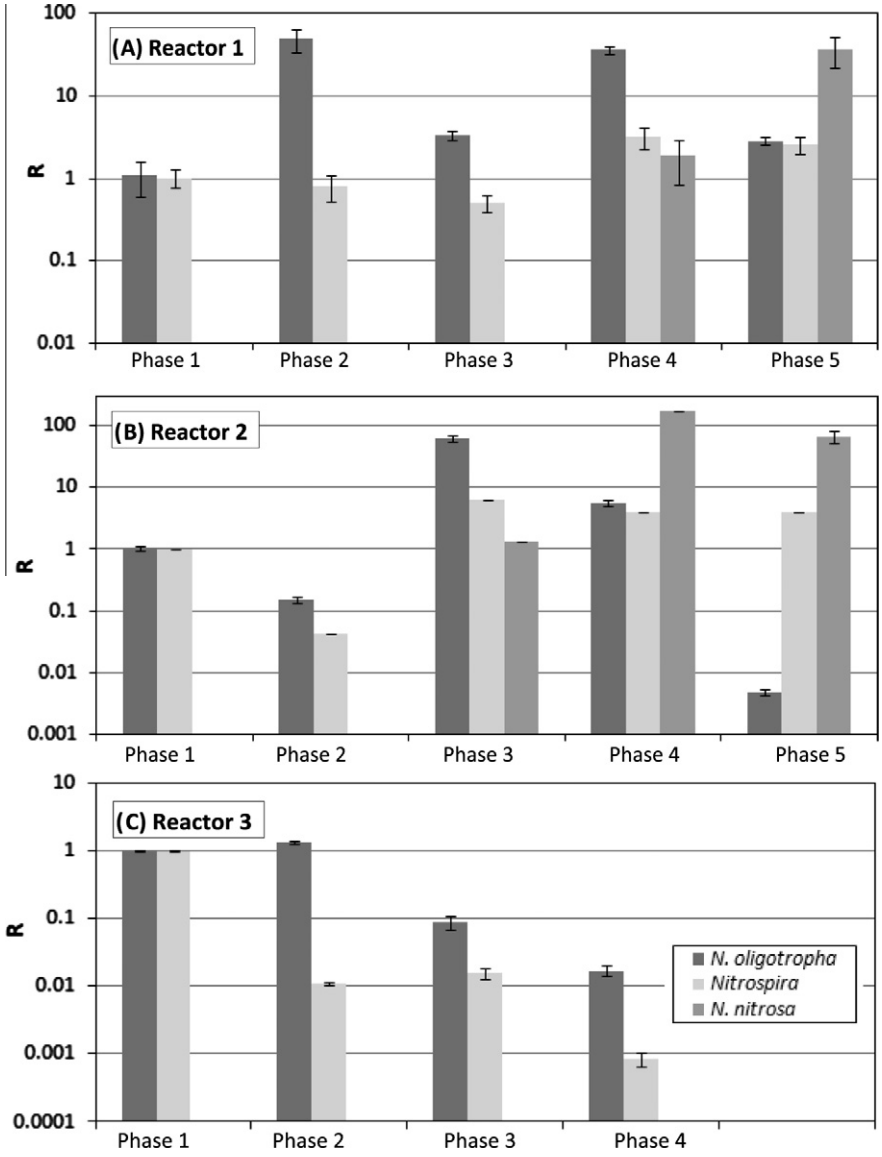

Fig. 5. Relative abundance ratio of target gene with respect to the control sample in Phase 1 and normalized to the reference total bacteria *16S rDNA*. Values represent the arithmetic average of ratios calculated for each phase and target primer set. Note the different *Y* scales.

to 97.4 (±0.9)%, a significant increase compared to earlier phases. At the same time, a new target AOB cluster corresponding to *N. nitrosa* was observed (Fig. 5a). No amplification had been obtained for this target in any sample obtained during Phases 1–3, and thus, *N. nitrosa* must have been introduced into the system via the Hopewell water, although no analysis was conducted to confirm this hypothesis. The relative *N. nitrosa* population numbers increased by about two orders of magnitude in Phase 5 while the *N. oligotropha* population returned to the original level observed during Phase 1. Fluctuations of AOB populations and the emergence of new nitrifying populations is not unusual. Kuo et al. alternated high (55 mg L^{-1} NH_3–N) and low (20 mg L^{-1} NH_3–N) ammonia concentrations in pilot-scale bioreactors and found that *N. nitrosa* populations decreased at lower nitrogen loading and increased with high nitrogen loading, while a high density of *N. oligotropha* correlated with very short solids retention times throughout the 120 day experiment. Van den Akker et al. (2010) also observed important fluctuations in AOB density over time in integrated fixed-film activated sludge (IFAS) bioreactors, however there was no correlation with treatment performance, which led to the conclusion that there was excess nitrifying capacity in their bioreactor. Furthermore, shifts from one dominant AOB species to another over extended time similar to that documented in the present study have been reported by others. Layton et al. (2005) reported on the ecology of an industrial wastewater treatment facility and showed dominance of *N. nitrosa* in the first 7 months, while beginning in month seven, *N. communis* was detected and dominated the AOB population for the remainder of the experiment (6 months).

The relative abundance of NOB decreased in comparison to its initial value after the reactor temperature was first increased (Phases 2 and 3, see Fig. 5a). Remarkably, this decrease also corresponded to the build-up of nitrite measured in Phase 3 (Fig. 2a). In Phases 4 and 5, the relative abundance of *Nitrospira* spp. in the bioreactor increased and nitrite concentrations decreased once again to levels below 0.1 mg L^{-1}. Thus, there appears to be some association between the relative abundance of *Nitrospira* spp. and nitrite oxidation.

Similar effects to that observed in Reactor 1 were found in Reactor 2 with respect to community dynamics (Fig. 5b). The relative

abundance of *N. oligotropha* and *Nitrospira* spp. decreased significantly in Phase 2 which corresponds to the first acclimation to elevated temperature, during which a transient deficiency in ammonia treatment occurred. This observation suggests that a temperature increase of 10 °C is capable of acutely disrupting the nitrifying microbial community, with visible impacts as far as treatment of ammonia and nitrite is concerned. In Phase 3, Reactor 2 was able to effectively remove ammonia and this corresponded to a rebound in population concentration for both *N. oligotropha* and *Nitrospira* spp. when compared to the Phase 1 control point. In Phase 3, the largest population of *N. oligotropha* was observed together with the emergence of *N. nitrosa*. Interestingly, this coincided with the detection of elevated levels of nitrite, indicating that AOB were able to produce nitrite at faster rates than NOB were able to oxidize it. In Phases 4 and 5, the balance of AOB and NOB was changed and effluent nitrite returned to low values (<2 mg L^{-1} NO_2^-–N).

The population dynamics results of Reactor 3 added further evidence that nitrifying populations, in particular *Nitrospira* spp., are especially sensitive to the high temperatures. During Phase 2, following the five day temperature shock (days 32–38), the relative abundance of *Nitrospira* spp. dropped by two orders of magnitude whereas the relative number of *N. oligotropha* microorganisms did not significantly change (Fig. 5c). The sampling event for Phase 2 occurred on day 47 when measured nitrite values were beginning to peak and the reactor was beginning to remove ammonia again at greater than 80% efficiency. These data indicate that *N. oligotropha* may be either less sensitive to temperature shock or that it recovered faster than *Nitrospira* spp. Later, as the temperature was increased to 45 °C the relative abundance of both *N. oligotropha* and *Nitrospira* spp. dropped by more than two orders of magnitude, while *N. nitrosa* was never detected in that reactor (but unlike Reactors 1 and 2, Reactor 3 was never fed Hopewell water). These observations are consistent with the quasi total loss in nitrification activity in Phases 3 and 4 (see Table 3).

Overall, the data in Fig. 5 and the above discussion illustrates that complex population dynamics were observed over time in all three bioreactors. These did not always translate into clear differences in ammonia or nitrite treatment. One limitation of the qPCR approach is that it cannot distinguish between active and inactive microbial fractions. Thus, here, the implicit assumption that gene abundance correlated with activity was made, although it is not necessarily valid. Further, in bioreactors that rely on biofilms, it is likely that some inactive organisms remain embedded deep in the biofilms, protected from shear by the outer layer of actively growing and continuously sheared organisms. This could possibly dampen the population dynamic patterns. Even so, certain distinct correspondences between population dynamics and treatment performance were identified which helped improve our understanding of the microbial ecology of MBBRs removing ammonia.

4. Conclusions

The MBBRs successfully treated high temperature secondary effluent. Nitrification was achieved at 35 and 40 °C with ammonia treatment exceeding efficacy of 95% and effluent concentrations below 1 mg L^{-1}-N. At 45 °C, nitrification could not be sustained for more than 24 h, however the MBBR recovered within two weeks once the temperature was decreased to 30 °C. Shifts in AOB from *N. oligotropha* to *N. nitrosa* were observed. In some cases, an increase in *N. nitrosa* was linked to improved ammonia treatment and decreases in *Nitrospira* spp. correlated with the build up of nitrite. These results demonstrate that some insight can be derived from correlating microbial population structure and bioreactor activity.

Acknowledgement

The funding for this study by HDR Engineering, Inc, Norfolk, Virginia is acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2012.02.045.

References

Bach, H.J., Tomanova, J., Schloter, M., Munch, J.C., 2002. Enumeration of total bacteria and bacteria with genes for proteolytic activity in pure cultures and in environmental samples by quantitative PCR mediated amplification. Journal of Microbiological Methods 49 (3), 235–245.

Bae, W., Baek, S., Chung, J., Lee, Y., 2001. Optimal operational factors for nitrite accumulation in batch reactors. Biodegradation 12 (5), 359–366.

Baek, S.H., Pagilla, K.R., 2008. Simultaneous nitrification and denitrification of municipal wastewater in aerobic membrane bioreactors. Water Environment Research 80 (2), 109–117.

Chesapeake Bay Program, 2009. Chesapeake Bay Program 2011 Milestones to Reduce Nitrogen and Phosphorus, Chesapeake Bay Program, Annapolis.

Dionisi, H.M., Layton, A.C., Harms, G., Gregory, I.R., Robinson, K.G., Sayler, G.S., 2002. Quantification of *Nitrosomonas oligotropha*-like ammonia-oxidizing bacteria and Nitrospira spp. from full-scale wastewater treatment plants by competitive PCR. Applied and Environmental Microbiology 68 (1), 245–253.

Geets, J., de Cooman, M., Wittebolle, L., Heylen, K., Vanparys, B., De Vos, P., Verstraete, W., Boon, N., 2007. Real-time PCR assay for the simultaneous quantification of nitrifying and denitrifying bacteria in activated sludge. Applied Microbiology and Biotechnology 75 (1), 211–221.

Gieseke, A., Purkhold, U., Wagner, M., Amann, R., Schramm, A., 2001. Community structure and activity dynamics of nitrifying bacteria in a phosphate-removing biofilm. Applied and Environmental Microbiology 67 (3), 1351–1362.

Grunditz, C., Dalhammar, G., 2001. Development of nitrification inhibition assays using pure cultures of Nitrosomonas and Nitrobacter. Water Research 35 (2), 433–440.

Gunsch, C.K., Kinney, K.A., Szaniszlo, P.J., Whitman, C.P., 2006. Quantification of homogentisate-1,2-dioxygenase expression in a fungus degrading ethylbenzene. Journal of Microbiological Methods 67 (2), 257–265.

Harms, G., Layton, A.C., Dionisi, H.M., Gregory, I.R., Garrett, V.M., Hawkins, S.A., Robinson, K.G., Sayler, G.S., 2003. Real-time PCR quantification of nitrifying bacteria in a municipal wastewater treatment plant. Environmental Science & Technology 37 (2), 343–351.

Hosseini, S.H., Borghei, S.M., 2005. The treatment of phenolic wastewater using a moving bed bio-reactor. Process Biochemistry 40 (3–4), 1027–1031.

Iranpour, R., Oh, S., Cox, H.H.J., Shao, Y.J., Moghaddam, O., Kearney, R.J., Deshusses, M.A., Stenstrom, M.K., Ahring, B.K., 2002. Changing mesophilic wastewater sludge digestion into thermophilic operation at Terminal Island Treatment Plant. Water Environment Research 74 (5), 494–507.

Jahren, S.J., Rintala, J.A., Odegaard, H., 2002. Aerobic moving bed biofilm reactor treating thermomechanical pulping whitewater under thermophilic conditions. Water Research 36 (4), 1067–1075.

Jaroszynski, L.W., Cicek, N., Sparling, R., Oleszkiewicz, J.A., 2011. Importance of the operating pH in maintaining the stability of anoxic ammonium oxidation (anammox) activity in moving bed biofilm reactors. Bioresource Technology 102 (14), 7051–7056.

Jones, R.D., Hood, M.A., 1980. Effects of temperature, pH salinity, and inorganic oxidation by nitrifiers isolated from wetland environments. Microbial Ecology 6 (4), 339–347.

Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K.H., Pommerening-Roser, A., Koops, H.P., Wagner, M., 1998. Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Applied and Environmental Microbiology 64 (8), 3042–3051.

Kelly, R.T., Henriques, I.D.S., Love, N.G., 2004. Chemical inhibition of nitrification in activated sludge. Biotechnology and Bioengineering 85 (6), 683–694.

Khan, S.J., Ilyas, S., Javid, S., Visvanathan, C., Jegatheesan, V., 2011. Performance of suspended and attached growth MBR systems in treating high strength synthetic wastewater. Bioresource Technology 102 (9), 5331–5336.

Kim, D.J., Kim, S.H., 2006. Effect of nitrite concentration on the distribution and competition of nitrite-oxidizing bacteria in nitratation reactor systems and their kinetic characteristics. Water Research 40 (5), 887–894.

Kim, H.S., Gellner, J.W., Boltz, J.P., Freudenberg, R.G., Gunsch, C.K., Schuler, A.J., 2010. Effects of integrated fixed film activated sludge media on activated sludge settling in biological nutrient removal systems. Water Research 44 (5), 1553–1561.

Kuo, D.H.W., Robinson, K.G., Layton, A.C., Meyers, A.J., Sayler, G.S., 2006. Real-time PCR quantification of ammonia-oxidizing bacteria (AOB): Solids retention time (SRT) impacts during activated sludge treatment of industrial wastewater. Environmental Engineering Science 23 (3), 507–520.

LaPara, T.M., Alleman, J.E., 1999. Thermophilic aerobic biological wastewater treatment. Water Research 33 (4), 895–908.
LaPara, T.M., Konopka, A., Nakatsu, C., Alleman, J.E., 2001. Thermophilic aerobic treatment of a synthetic wastewater in a membrane-coupled bioreactor. Journal of Industrial Microbiology & Biotechnology 26 (4), 203–209.
Layton, A.C., Dionisi, H., Kuo, H.W., Robinson, K.G., Garrett, V.M., Meyers, A., Sayler, G.S., 2005. Emergence of competitive dominant ammonia-oxidizing bacterial populations in a full-scale industrial wastewater treatment plant. Applied and Environmental Microbiology 71 (2), 1105–1108.
Lazarova, V., Manem, J., 1995. Biofilm characterization and activity analysis in water and wastewater treatment. Water Research 29 (10), 2227–2245.
Lim, J., Lee, S., Hwang, S., 2008a. Use of quantitative real-time PCR to monitor population dynamics of ammonia-oxidizing bacteria in batch process. Journal of Industrial Microbiology and Biotechnology 35, 1339–1344.
Lim, J., Do, H., Shin, S.G., Hwang, S., 2008b. Primer and probe sets for group-specific quantification of the genera Nitrosomonas and Nitrosospira using real-time PCR. Biotechnology and Bioengineering 99 (6), 1374–1383.
Linares, K.H.M., Grandstaff, E., Walker, H., Bailey, E., M'Coy, W., 2008. Troubleshooting plant performance upsets at the Hopewell Regional Wastewater Treatment Facility. Water Practice 2 (4), 1–18.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-[Delta][Delta]CT Method. Methods 25 (4), 402–408.
Odegaard, H., Rusten, B., Westrum, T., 1994. A new moving-bed biofilm reactor-applications and results. Water Science and Technology 29 (10), 157–165.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29 (9), 6.
Rusten, B., Hem, L.J., Odegaard, H., 1995. Nitrification of municipal waste-water in moving-bed biofilm reactors. Water Environment Research 67 (1), 75–86.
van den Akker, B., Beard, H., Kaeding, U., Giglio, S., Short, M.D., 2010. Exploring the relationship between viscous bulking and ammonia-oxidiser abundance in activated sludge: A comparison of conventional and IFAS systems. Water Research 44 (9), 2919–2929.
Yang, S., Yang, F.L., Fu, Z.M., Lei, R.B., 2009. Comparison between a moving bed membrane bioreactor and a conventional membrane bioreactor on organic carbon and nitrogen removal. Bioresource Technology 100 (8), 2369–2374.
Zhang, L.Q., Wei, C.H., Zhang, K.F., Zhang, C.S., Fang, Q., Li, S.G., 2009. Effects of temperature on simultaneous nitrification and denitrification via nitrite in a sequencing batch biofilm reactor. Bioprocess and Biosystems Engineering 32 (2), 175–182.