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ABSTRACT

In this study, the effects of the ammonium loading rate (ALR) and inorganic carbon loading rate (ILR) on the nitrification performance and composition of a nitrifying bacterial community were investigated in a moving bed biofilm reactor, using poly(vinyl alcohol) (PVA) sponge cubes as a supporting carrier. Between the two ALRs of 0.36 and 2.16 kg-N m^{-1} d^{-1}, stable partial nitration was achieved at the higher ALR. Inorganic carbon was dosed at high levels: 33.1, 22.0, 16.4, 11.0, and 5.4 times the theoretical amount. Nonetheless, nitrification efficiency was not affected by the ILR at the two ALRs. Quantitative PCR analysis of ammonia- and nitrite-oxidizing bacteria revealed that ALR is an important determinant of partial nitration by accumulating ammonia-oxidizing bacteria in the nitrification system. In comparison, two nitrite-oxidizing bacterial genera (*Nitrobacter* and *Nitrospira*) showed almost the same relative abundance at various ALRs and ILRs. Terminal restriction fragment length polymorphism targeting the gene of ammonia monooxygenase subunit A revealed that *Nitrosomonas europaea* dominated under all conditions.
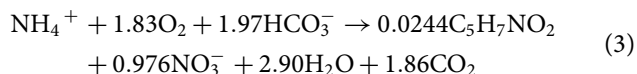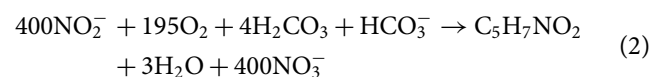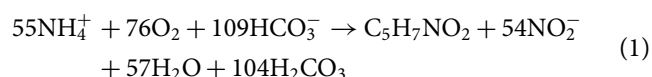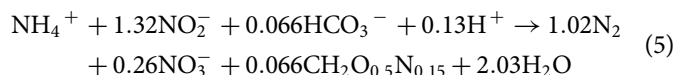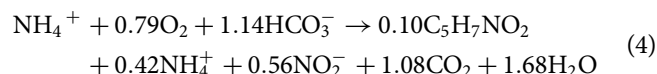
Introduction

Biological nitrogen removal (BNR), which involves nitrification and denitrification, is considered as a practical solution because of its high efficiency and cost-effectiveness.[1] In BNR processes, ammonia (NH_4^+) is oxidized to nitrate (NO_3^-) via nitrite (NO_2^-) by two kinds of bacteria, ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), under aerobic conditions (Eqs. (1)–(3)).[2] At the final stage of BNR, denitrification is performed by heterotrophic bacteria, which convert oxidized nitrogenous compounds such as NO_3^- and NO_2^- to dinitrogen gas (N_2) under anoxic conditions.

$$55NH_4^+ + 76O_2 + 109HCO_3^- \rightarrow C_5H_7NO_2 + 54NO_2^- + 57H_2O + 104H_2CO_3 \quad (1)$$

$$400NO_2^- + 195O_2 + 4H_2CO_3 + HCO_3^- \rightarrow C_5H_7NO_2 + 3H_2O + 400NO_3^- \quad (2)$$

$$NH_4^+ + 1.83O_2 + 1.97HCO_3^- \rightarrow 0.0244C_5H_7NO_2 + 0.976NO_3^- + 2.90H_2O + 1.86CO_2 \quad (3)$$

Compared to conventional BNR processes, combined partial nitration (PN)–anaerobic ammonium oxidation (ANAMMOX) processes are advantageous because of their low operational costs. The PN reaction consumes less oxygen (O/N = 0.79 in

Eq. (4)) than the full nitrification process (from NH_4^+ to NO_3^-, O/N = 1.83 in Eq. (3)).[3,4] Thus, large amounts of energy for oxygen supply are theoretically reduced by 56.8%, according to Eqs. (3) and (4). In the autotrophic ANAMMOX reaction, NH_4^+ as an electron donor is converted to N_2 with NO_2^-, and the byproduct NO_3^- is formed (Eq. (5)). Therefore, addition of an exogenous electron donor is not required as compared to the denitrification process, which consumes substantial amounts of external carbon sources (C/N = 1.08 for denitrification).[5] Furthermore, the PN–ANAMMOX process produces 50–90% less sludge waste,[6] and CO_2 emission is reduced by 85%.[7]

$$NH_4^+ + 0.79O_2 + 1.14HCO_3^- \rightarrow 0.10C_5H_7NO_2 + 0.42NH_4^+ + 0.56NO_2^- + 1.08CO_2 + 1.68H_2O \quad (4)$$

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (5)$$

The key requirements of the PN process are the maintenance of the molar NO_2^-/NH_4^+ ratio of 1.31:1 in the effluent and the absence of NO_3^- production by NOB. NOB activity is selectively inhibited by means of operational factors, such as the ammonium loading rate (ALR) and high concentrations of free ammonia (FA) and dissolved oxygen (DO). ALR at different hydraulic retention time (HRT) values in the range of 1.04–

1.81 and 2.15–4.06 kg-N m^{-3} d^{-1} leads to PN at an NH$_4^+$-N to NO$_2^-$-N ratio (NH$_4^+$-N/NO$_2^-$-N) of 1:1.2 in an activated sludge reactor and a moving bed biofilm reactor (MBBR), respectively.[8] FA, whose concentration depends on total NH$_4^+$ concentration, temperature, and pH, effectively provides an inhibitory environment for control over NOB activity.[9] The NO$_3^-$ production rate begins to decline above an ALR of 0.5 kg-N m^{-3} d^{-1} because of the high FA concentration (>10 mg L^{-1}) at pH 7.8.[10] With respect to DO, AOB outcompete NOB at low DO concentrations because of their lower half-saturation constant ($K_{s,DO}$), i.e., higher affinity (0.033–1.45 mg L^{-1}) than that of NOB, which is 0.3–1.1 mg L^{-1}.[11,12] However, these inhibitory strategies involving FA and DO are difficult to properly implement because of similar properties between AOB and NOB. For example, oxygen-limiting conditions result in low activities of both AOB and NOB at many wastewater treatment plants.[13] Furthermore, the wide range of $K_{s,DO}$ for AOB covers a subset of $K_{s,DO}$ for NOB. This situation makes it difficult to determine the optimal DO concentration for specific suppression of NOB.[9,14] The presence of high concentrations of FA is also hazardous to AOB,[15] particularly for susceptible species in the *N. europaea–Nitrosococcus mobilis* cluster.[16]

Bicarbonate concentration is considered as alternative selective pressure for nitration (from NH$_4^+$ to NO$_2^-$).[17] Bicarbonate, an inorganic carbon (IC) source for chemolithotrophic bacteria, shows an equilibrium between the liquid and gas phases. Solubilized CO$_2$ in the liquid phase is converted to carbonic acid (H$_2$CO$_3$), bicarbonate (HCO$_3^-$), and carbonate (CO$_3^{2-}$), depending on their dissociation constants (Eq. (6)).[18]

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \\ \leftrightarrow 2H^+ + CO_3^{2-} \tag{6}$$
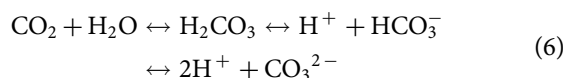
HCO$_3^-$ is the major source of alkalinity in biological wastewater treatment processes at neutral pH. HCO$_3^-$ has a critical role in buffering the pH reduction during nitrification, thereby maintaining an optimal pH range of 7.9–8.5 and 6.5–7.9 for AOB and NOB, respectively.[19–21] Theoretically, AOB activity largely depends on the HCO$_3^-$ concentration because NH$_4^+$ oxidation leads to a dramatic decrease in pH, whereas NO$_2^-$ oxidation hardly changes pH. According to Eqs. (1) and (2), 104 moles of HCO$_3^-$ per 55 moles of NH$_4^+$ is consumed for buffering acidification (HCO$_3^-$/NH$_4^+$ = 2), whereas NOB require no buffering agent. In addition, IC is a prerequisite for the growth of chemolithotrophic bacteria including AOB and NOB. A higher biomass yield over nitrogen of AOB (0.14–0.21 g-COD g-N^{-1}) than that of NOB (0.084–0.11 g-COD g-N^{-1}) requires a sufficient IC supply for the high level of bacterial growth during NH$_4^+$ oxidation.[22,23] AOB require high concentrations of bicarbonate for optimal growth; the half-saturation constant of bicarbonate for AOB (K_{HCO3^-} AOB) is 28.49 mg-C L^{-1}, whereas this value for NOB is as low as 0.1 mg-C L^{-1}.[24]

In summary, a sufficient supply of HCO$_3^-$ can selectively promote AOB growth during a PN process. In actual nitrification processes, alkalinity is increased by means of sodium hydroxide (NaOH), sodium bicarbonate (NaHCO$_3$), or sodium carbonate (Na$_2$CO$_3$). In previous research, the addition of Na$_2$CO$_3$ as a buffering agent improved nitrification performance by 51% as compared to that in the presence of NaOH.[25] This finding suggests that IC-based alkalinity offers better growth conditions for nitrifiers. In addition, IC concentration in the range of 100–160 mg-C L^{-1} in the form of Na$_2$CO$_3$ results in stable nitration during a long-term period (i.e., NO$_2^-$ production).[21] To improve a NO$_2^-$ yield, NaHCO$_3$ can be employed to enhance the nitratation performance by selectively supporting AOB activity, as compared to NaOH in a continuous nitrifying reactor.[26] Limited IC concentrations below 36 mg-C L^{-1} have a stronger negative effect on nitration than on nitratation (from NO$_2^-$ to NO$_3^-$ by NOB).[24] In contrast, when the IC concentration is increased to 0.3 mg-C L^{-1}, the NO$_2^-$ production rate gradually increases at an ALR of 1 kg-N m^{-3} d^{-1}, with HCO$_3^-$ as an alkalinity source.[27] Stable nitration was also implemented in a high-IC environment (approximately 7.2 g-C L^{-1}, HCO$_3^-$/NH$_4^+$ = 4.2).[26] Thus, IC concentration is involved in controlling the activities of AOB and NOB.

On the other hand, there is limited information regarding the comparison of IC concentration. In this respect, different IC loadings were applied under low and high ammonium loading conditions. The two ALRs were utilized to distinguish the presence and absence of an inhibitory FA concentration. Real-time quantitative PCR (qPCR) and terminal restriction fragment length polymorphism (T-RFLP) were also performed to evaluate the population dynamics of all bacteria, AOB, and two genera of NOB (*Nitrobacter* spp. and *Nitrospira* spp.).

Materials and methods

Experimental setup

An MBBR with a working volume of 2 L was operated for 190 d (Fig. 1). A PVA sponge (5 × 5 × 5 mm) served as the biomass carrier, and the packing ratio of sponge volume to total reactor volume was 28% (v/v). The seeding inoculum was obtained from a bench scale nitrifying reactor with an ammonium conversion rate (ACR) of 0.8 kg-N m^{-3} d^{-1}, and most NO$_2^-$ was converted to NO$_3^-$ (above 99% nitrifying efficiency). The inoculum was initially added to the reactor at a concentration of 0.5 g-volatile suspended solid (VSS) L^{-1}. During the startup period of 0–49 d, nitrifying biomass was recovered from the effluent and recycled to form a stable nitrifying biofilm on PVA sponge carriers.[28] The average suspended solid (SS) concentration in the effluent was 29.16 ± 7.28 mg L^{-1} throughout the operation period. Temperature and agitation speed were maintained at 35°C and 200 rpm, respectively, by an automated control system.

Operational strategy

Ammonium in the influent was supplied in the form of (NH$_4$)$_2$SO$_4$. Sodium bicarbonate (NaHCO$_3$) was used as the IC source. The mineral components consisted of 6 mg-P L^{-1} KH$_2$PO$_4$, 12 mg-Mg L^{-1} MgSO$_4$·7H$_2$O, and 48 mg-Ca L^{-1} CaCl$_2$·2H$_2$O. Additionally, 1 mL L^{-1} of each trace element solution was added to the influent. Trace element solution I
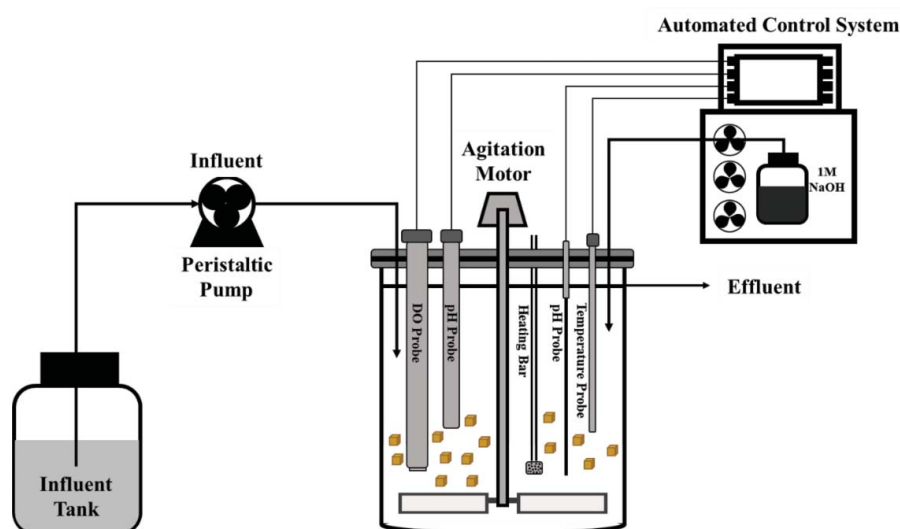
Figure 1. An outline of the moving bed biofilm reactor.

consisted of 5 g L^{-1} EDTA and 5 g L^{-1} FeSO$_4$·7H$_2$O. Trace element solution II consisted of 5 g L^{-1} EDTA, 0.43 g L^{-1} ZnSO$_4$·7H$_2$O, 0.24 g L^{-1} CoCl$_2$·6H$_2$O, 0.99 g L^{-1} MnCl$_2$·4H$_2$O, 0.25 g L^{-1} CuSO$_4$·5H$_2$O, 0.22 g L^{-1} Na$_2$MoO$_4$·2H$_2$O, 0.19 g L^{-1} NiCl$_2$·6H$_2$O, 0.21 g L^{-1} Na$_2$SeO$_4$·10H$_2$O, and 0.014 g L^{-1} H$_3$BO$_3$.[29]

The operational conditions were divided into four phases (Table 1). Hydraulic retention time was maintained at 3.3 h and DO was kept at less than 0.5 mg L^{-1}.[30] Phases I and III were startup periods for different ALRs (0.36 and 2.16 kg-N m^{-3} d^{-1}) at ammonium concentrations of 50 and 300 mg-N L^{-1}, respectively. The effects of different IC loading rates (ILRs) were tested for ALRs of 0.36 and 2.16 kg-N m^{-3} d^{-1} in Phases II and IV, respectively. The ILR was determined by means of the stoichiometric balance of IC to support chemolithotrophic growth in accordance with the ALR based on Eqs. (1) and (2) (Table 2). The theoretical minimal ILRs for nitrifiers were 0.028 and 0.17 kg-C m^{-3} d^{-1} for ALRs of 0.36 and 2.16 kg-N m^{-3} d^{-1}, respectively. The actual ILR levels were adjusted to 33.1, 22.0, 16.4, 11.0, and 5.4 times the theoretical ILR. Accordingly, ILR was reduced from 0.93 to 0.15 kg-C m^{-3}

d^{-1} in four steps in Phase II. Similarly, in Phase IV, ILR was decreased from 5.30 to 0.87 kg-C m^{-3} d^{-1}. During all phases, pH was maintained at 8.3 with 1N NaOH to exclude the effects of pH variation and buffering capacity.

Bacterial-community analysis

Three sponge samples were collected from the reactor for DNA extraction. Sample codes at different ALRs and ILRs are summarized in Table 3. Genomic DNA of samples was extracted with a Power Soil^{TM} DNA Kit (Mo Bio Laboratories, Carlsbad, CA, USA). The yield and purity of extracted DNA for each sponge samples were evaluated using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The DNA yield ranged from 46.5 to 99.1 ng μL^{-1}. In case of DNA purity represented by optical density (OD) 260/280 ratio, the value from 1.8 to 2.0 is considered as acceptable range. In this study, the average value of OD 260/280 ratio was 1.88 ± 0.03. T-RFLP analysis of the bacterial *amoA* gene was performed to investigate the community structure of AOB. Two primers, amoA1F (5′-GGGGTTTCTACTGGTGGT-3′)

Table 1. Operational conditions of the moving bed biofilm reactor.

Phase	Period (d)	Feeding conditions		HRT (h)
		Ammonium Conc. (mg-N L^{-1})	Bicarbonate conc. (mg-C L^{-1})	
I (stabilizing period)	50 (0–49)	50	85	3.3
II	50 (50–99)	50	128/85/64/43/20	
III (stabilizing period)	33 (100–132)	100/300	169/485	
IV	59 (132–190)	300	730/485/360/254/120	

Table 2. Inorganic carbon conditions in the moving bed biofilm reactor.

Phase	Period (d)	Ammonium loading rate (kg-N m^{-3} d^{-1})	Theoretical inorganic carbon loading rate (kg-C m^{-3} d^{-1})	Actual inorganic carbon loading rate (kg-C m^{-3} d^{-1})	Ratio of actual to theoretical inorganic carbon loading rate
I	50 (0–49)	0.36	0.028	0.62	22.1
II	50 (50–99)			0.93/0.62/0.46/0.31/0.15	33.1/22.0/16.4/11.0/5.40
III	33 (100–132)	0.73/2.16	0.056/0.16	1.23/3.53	22.0
IV	59 (132–190)	2.16	0.16	5.30/3.52/2.62/1.85/0.87	33.1/22.0/16.4/11.0/5.40

Table 3. A summary of sample codes for qPCR and T-RFLP.

Sample code	Ammonium loading rate (kg-N m^{-3} d^{-1})	Ratio of actual to theoretical inorganic carbon loading rate
L1	0.36	33.1
L2		22.0
L3		16.4
L4		11.0
L5		5.40
H1	2.16	33.1
H2		22.0
H3		16.4
H4		11.0
H5		5.40

Table 5. qPCR amplification conditions.

Target	Thermal cycling conditions	Reference
All bacteria	1 cycle of 2 min at 50°C; 1 cycle of 10 min at 95°C; and then 40 cycles of 15 s at 95 °C and 1 min at 60°C	[31]
AOB	1 cycle of 2 min at 50°C; 1 cycle of 10 min at 95°C; and then 30 cycles of 15 s at 95°C and 1 min at 60°C	[34]
Nitrobacter spp.	1 cycle of 2 min at 95°C; and then 40 cycles of 20 s at 95°C and 40 s at 68°C	[35]
Nitrospira spp.	1 cycle of 2 min at 95°C; and then 40 cycles of 20 s at 95°C and 40 s at 60°C	

and amoA2R (5′-CCCCTCKGSAAAGCCTTCTTC-3′), labeled with 6-carboxyfluorescein (FAM) and 5-hexachlorofluorescein (HEX), respectively, were used. The PCR samples were composed of 12 μL of deionized water, 15 μL of PCR pre-Mix (SolGent, Daejeon, Korea), 1 μL (10 μM) of each primer, and 1 μL of a DNA template. PCR was conducted as follows: 1 cycle of 2 min at 95°C; 30 cycles of 20 s at 95°C, 40 s at 57°C, and 40 s at 72°C; and then 1 cycle of 5 min at 72°C on a MyCycler™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). PCR products were purified with a gel extraction and purification kit (Qiagen, Hilden, Germany) and digested with endonuclease *Taq*I (10 U; Takara, Shiga, Japan) at 65°C for 3 h. Samples were analyzed using GeneScan 3.7 software (Applied Biosystems, Foster City, CA, USA).

qPCR was conducted to quantify the populations of all bacteria, AOB, and two genera of NOB. The reaction mixture (20 μL) was combined with the TaqMan Universal PCR Master Mix Kit (10 μL, Applied Biosystems) with each primer and probe in sterile deionized water prior to addition of a DNA template (1 μL). The mixture was then thoroughly mixed on a vortex shaker and briefly centrifuged in a microcentrifuge. The total concentrations of primers and probe and the detailed PCR amplification process for all bacteria, AOB, and NOB are described in Tables 4 and 5.[31–35] qPCR analysis was carried out on a thermal cycler (ABI Prism 7300 sequence detection system, Applied Biosystems). All qPCR procedures were performed in triplicate. Amplification efficiencies of all bacteria, AOB, *Nitrobacter* spp., and *Nitrospira* spp. were 84.5, 99.5, 85.8, and 104.4%, respectively. The correlation coefficients (R^2 value) for four kinds of bacteria were above 0.99.

Sample analysis

NH_4^+-N was analyzed by the Kjeldahl method (Kjeltec 2300, Foss Tecator, Hilleroed, Denmark). NO_2^--N and alkalinity were measured by standard methods.[36] NO_3^--N was analyzed with a HACH kit (program 351 for nitrate, HACH, Loveland, CO, USA). pH was monitored with a pH meter (Accumet® Basic AB15 Plus pH Meter, Fisher Scientific, Waltham, MA, USA). The DO concentration and temperature were determined using auto fermenter sensors (InPro 6850i and Mettler Toledo™ 51343310, Mettler Toledo, Columbus, OH, USA).

Results and discussion

Nitrification performance

Profiles of nitrogenous compounds, ALR, and the nitrogen conversion rate of the lab scale MBBR are presented in Figure 2. In Phase I (0–49 d), ALR and ILR were maintained at 0.36 kg-N m^{-3} d^{-1} (i.e., 50 mg-NH_4^+-N L^{-1}) and 0.62 kg-C m^{-3} d^{-1} (i.e., 85 mg-C L^{-1}), respectively. After 12 d, the dominant nitrogenous compound was NO_3^- N, while the effluent NH_4^+-N and NO_2^--N concentrations were under 2 mg-N L^{-1}. On days 12–49, the average effluent NH_4^+-N, NO_2^--N, and NO_3^--N concentrations were 1.69 ± 1.16, 0.83 ± 0.96, and 46.50 ± 2.80 mg-N L^{-1}, respectively, with average efficiencies of NH_4^+-N conversion (from NH_4^+ to NO_2^-) and NO_2^--N conversion (from NO_2^- to NO_3^-) of 96.84% and 98.27%, respectively.

ILR was decreased from 0.93 to 0.15 kg-C m^{-3} d^{-1} in Phase II to evaluate the effects of IC on the process at a low ALR (Table 1). However, the nitrogen pattern did not change with changes in ILR, and full nitrification persisted until the end of

Table 4. Primer and probe sets and concentrations for qPCR.

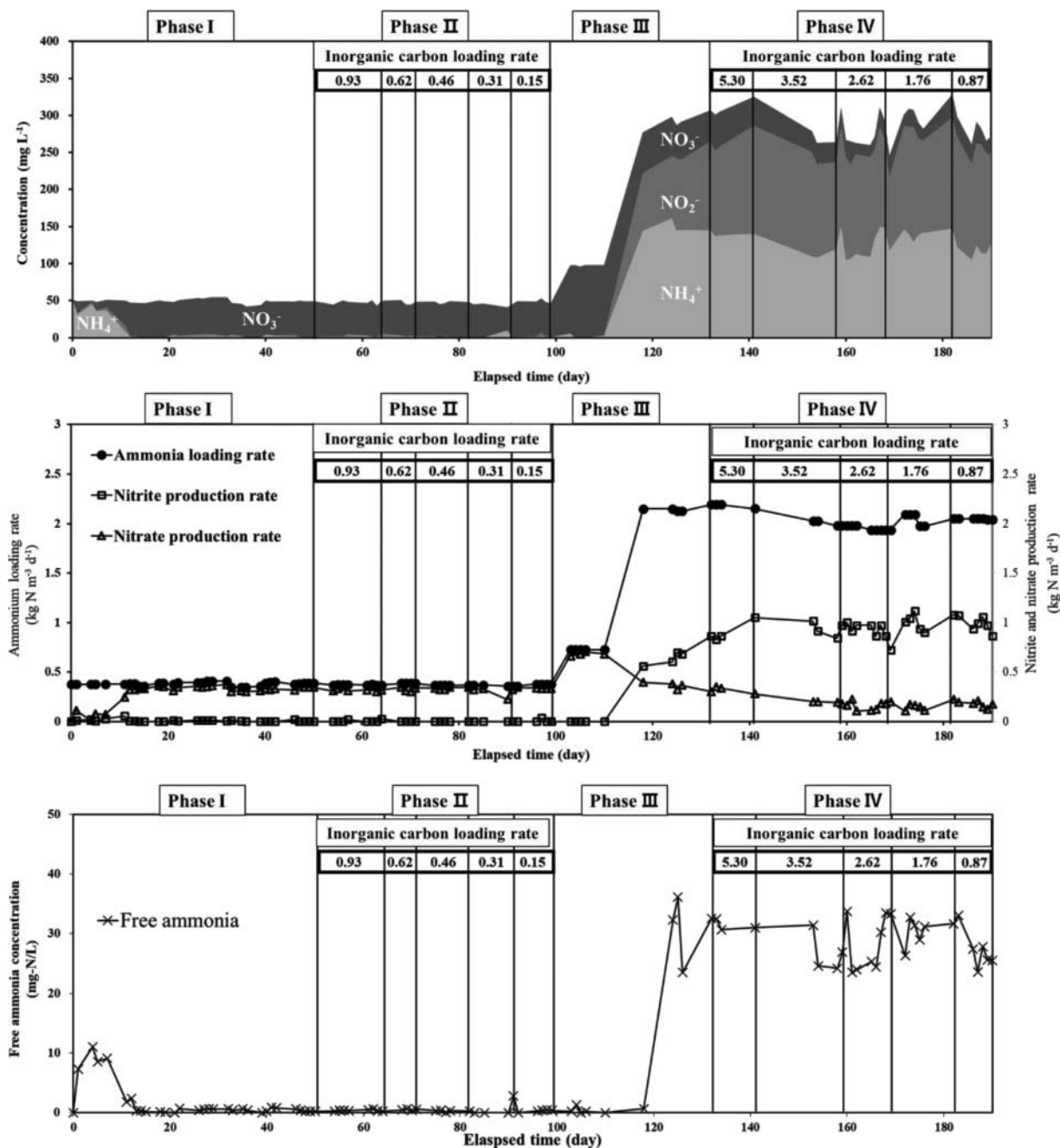| Target	Primer /probe set	Concentration in mixture (nM)	Sequence (5′–3′)	Reference
All bacteria	331F	100	5′-TCCTACGGGAGGCAGCAGT-3′	[31]
	772R	100	5′-GGACTACCAGGGTATCTAATCCTGTT-3′	
	515Taq	100	5′-CGTATTACCGCGGCTGCTGGCAC-3′	
AOB	CTO 189fA/B	150	5′-GGAGRAAAGCAGGGGATCG-3′	[32]
	CTO 189fC	150	5′-GGAGGAAAGTAGGGGATCG-3′	
	RT1r	300	5′-CGTCCTCTCAGACCARCTACTG-3′	
	TMP1Taq	125	5′-CAACTAGCTAATCAGRCATCRGCCGCTC-3′	
Nitrobacter spp.	Nspra-675f	600	5′-GCGGTGAAATGCGTAGAKATCG-3′	[33]
	Nspra-746r	600	5′-TCAGCGTCAGRWAYGTTCCAGAG-3′	
	Nspra-723Taq	250	5′-CGCCGCCTTCGCCACCG-3′	
Nitrospira spp.	Nitro-1198f	600	5′-ACCCCTAGCAAATCTCAAAAAACCG-3′	
	Nitro-1423r	600	5′-CTTCACCCCAGTCGCTGACC-3′	
	Nitro-1374Taq	250	5′-AACCCGCAAGGAGGCAGCCGACC-3′	

Figure 2. Temporal profiles of nitrogen concentration (top), ammonia loading rate, nitrite production rate, and nitrate production rate (middle) and FA concentration (bottom).

this phase. The average effluent concentrations of NH_4^+-N, NO_2^--N, and NO_3^--N were 1.67 ± 1.84, 0.44 ± 1.31, and 45.22 ± 3.51 mg-N L^{-1}, respectively, during Phase II. In Phase II, the average NH_4^+-N conversion efficiency was 96.76%, and most NO_2^--N was oxidized to NO_3^-.

ALR was gradationally increased to 2.16 kg-N m^{-3} d^{-1} (i.e., 300 mg-NH_4^+-N L^{-1}) via 0.72 kg-N m^{-3} d^{-1} (i.e., 100 mg-NH_4^+-N L^{-1}) in Phase III (Table 1). Excess ILR was added to the reactor (Table 2). Along with this increased ALR, NO_3^--N production dramatically decreased, while NH_4^+-N and NO_2^--N concentrations increased compared to those in Phases I and II. At 118–132 d, the average effluent NH_4^+-N, NO_2^--N, and NO_3^--N concentrations were 148.12 ± 7.30, 94.80 ± 11.12, and 49.20 ± 5.50 mg-N L^{-1}, respectively.

ILR was controlled from 5.30, 3.52, 2.62, and 1.76 to 0.87 kg-C m^{-3} d^{-1} (Phase IV; Table 2). Nevertheless, the residual NO_3^- concentration was maintained below 50 mg-NO_3^--N L^{-1}, regardless of the bicarbonate dose. In Phase IV (days 132–190), the PN reaction was stable. The average effluent NH_4^+-N, NO_2^--N, and NO_3^--N concentrations were 128.00 ± 15.71, 132.00 ± 12.73, and 26.41 ± 8.94 mg-N L^{-1}, respectively. Compared to Phase II, the NH_4^+-N conversion efficiency decreased to 54.63% and average NO_2^--N conversion efficiency decreased to 16.60%. As a result, the average NH_4^+-N, NO_2^--N, and NO_3^--N ratio in Phase IV was maintained at 1:1.05:0.21.

Based on these results, ALR is a crucial factor for successful implementation of PN. In another study, low ALR only minimally suppressed NOB activity, even at a low DO

concentration, and consequently full nitrification occurred.[37] In the present study, complete nitrification also occurred at low ALR: 0.36 kg-N m^{-3} d^{-1} (50 mg-NH_4^+-N L^{-1}). Nonetheless, a higher ALR of 2.14 kg-N m^{-3} d^{-1} (300 mg-NH_4^+-N L^{-1}) resulted in the accumulation of NH_4^+ and NO_2^-. Aside from the dominant effect of ALR, varying the levels of ILR had negligible effects on the PN reaction at high and low ALRs. In this study, FA concentration was maintained below 1.0 mg-N L^{-1}, which is not in the range of NOB inhibition (0.1–1.0 mg L^{-1} < FA < 10–150 mg L^{-1}) in Phase II.[9] In contrast, in Phase IV, the average FA concentration increased to 28.58 ± 3.45 mg L^{-1}. Only this range of FA concentrations led to a stable PN

reaction by inhibiting NOB in this study. In previous research, high ALR (>1.0 kg-N m^{-3} L^{-1}) and pH (>8.0) also resulted in stable PN mainly because FA concentration as a function of NH_4^+-N concentration, pH, and temperature led to inhibition of NO_3^- production.[10]

In the present study, excess IC was introduced at different ALRs from 0.36 to 2.16 kg-N m^{-3} d^{-1}. At an ALR of 0.36 kg-N m^{-3} d^{-1} (Phase II), IC concentration did not significantly affect the nitrifying characteristics; accordingly, full nitrification was achieved at high IC concentrations. Likewise, the PN reaction was not influenced by the IC dose at an ALR of 2.16 (Phase IV). These results indicate that PN was highly dependent on
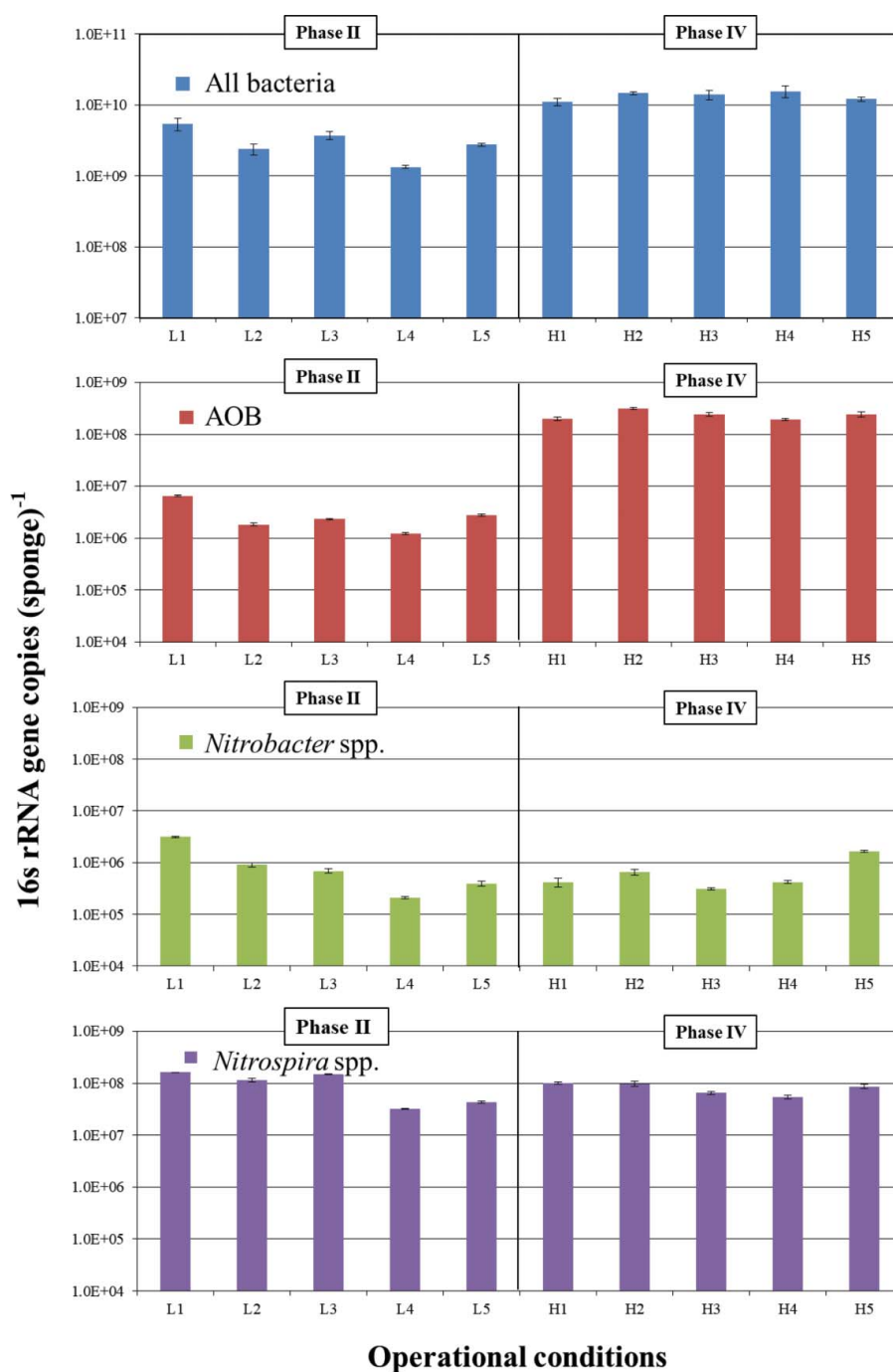

Figure 3. qPCR results on all bacteria (blue), AOB (red), *Nitrobacter* spp. (green) and *Nitrospira* spp. (purple) in Phases II and IV.

ALR rather than on IC concentration. However, the role of IC remains controversial. Lee et al. demonstrated that nitration in a sequencing batch reactor-type nitrifying reactor is stable at high ammonium concentrations (458-1650 mg-NH_4^+-N L^{-1}), and NO_2^- oxidation efficiency was maintained at a low level of 0.73% at an NH_4^+-N to IC ratio of 2.[38] By contrast, ACR decreased with a decrease in the IC to NH_4^+-N ratio to less than 2 because of the decrease in pH. Tokutomi et al. also observed that a high IC concentration leads to stable PN.[26] The NO_2^--N conversion rate is increased, whereas the ACR is decreased by a reduction in the IC dose.[26]

From a practical perspective, the application of PN at a high ILR has some limitations. For example, at a full-scale treatment plant, maintaining high ILR will be costly. The IC concentration, which was taken from a previous study, was extremely high as compared to the presence of general anaerobic digesters. Bae et al. reported that the molar ratio of $CaCO_3$-C to NH_4^+-N was only 0.55 ± 0.075 (8 cases from anaerobic digester and land fill leachates).[28]

qPCR analysis

To quantify the bacterial community, qPCR analysis was performed on all bacteria, AOB, and two genera of NOB (*Nitrospira* and *Nitrobacter*) in the MBBR (Fig. 3). The ratios of AOB, *Nitrobacter* spp. and *Nitrospira* spp. to all the bacteria are shown in Figure 4.

At a low ALR (conditions L1–L5, Table 3), the average 16S rRNA gene copy number of all bacteria (corresponds to the number of bacterial cells) was quantified and found to be $3.1 \pm 1.5 \times 10^9$ copies per sponge. After applying relatively high ALRs (conditions H1–H5, Table 3), the average all bacterial abundance increased to $1.3 \pm 0.18 \times 10^{10}$ copies per sponge. Nonetheless, there was no significant change in the all bacterial count, in accordance with ILR at both low and high ALRs. In the case of AOB, the concentration of the 16S rRNA gene was $2.9 \pm 2.1 \times 10^6$ copies per sponge at a low ALR. The average AOB abundance sharply increased after high ALR was applied (conditions H1–H5, Table 3), by approximately 2 orders of magnitude, to $2.1 \pm 0.48 \times 10^8$ copies per sponge. In contrast, varying the ILR conditions had a weaker effect on the AOB

abundance. The *Nitrobacter* spp. abundance under conditions L1–L5 (Table 3), on average, was $1.1 \pm 1.2 \times 10^6$ copies per sponge. Despite the increased ALR, 16S rRNA gene copy numbers decreased slightly to $6.9 \pm 5.6 \times 10^5$ copies per sponge for *Nitrobacter* spp. Thus, *Nitrobacter* spp. persisted at a constant abundance regardless of ALR and ILR. The average 16S rRNA gene copy number of *Nitrospira* spp. was $1.0 \pm 0.60 \times 10^8$ copies per sponge at low ALR. The copy number at a high ALR was maintained at an average value of $8.1 \pm 2.1 \times 10^7$ copies per sponge. As was the case for *Nitrobacter* spp., varying the ALR and ILR conditions had minimal effects on *Nitrospira* spp. abundance.

As shown in Figure 4, AOB dominance was easily influenced by ALR, while the relative abundance of major NOB, *Nitrospira* spp., stayed the same regardless of the increase in ALR. The increase of ALR, as described above, also attenuated NOB suppression by over 1.1 kg-N m^{-3} d^{-1}. Despite the inhibitory FA concentration and ALR conditions, the 16S rRNA gene copy numbers of *Nitrobacter* spp. and *Nitrospira* spp. stayed relatively high, and the dominant genus persisted without a washout in all phases. These results were attributed to the characteristics of the biofilm nitrifying reactor. Li et al. suggested that the bacterial quantitative ratio of AOB to NOB in an attached-growth system is stabler than that in a suspension culture system.[39] Similarly, the NOB population attached to the sponges in this study was sustained at constant abundance by preventing a washout from the MBBR reactor.

NOB are subdivided into two groups based on substrate affinity (K_s): k-strategists and r-strategists. *Nitrospira* spp. are representative k-strategists (lower K_s value with higher affinity), whereas *Nitrobacter* spp. are known as r-strategists (higher K_s value with lower affinity). In low-strength ammonium wastewater, slowly growing *Nitrospira* spp. are predominant, whereas fast-growing NOB, such as *Nitrobacter* spp., are suppressed because of differences in substrate affinity.[40] DO concentrations also differentiate members of the NOB niche in the reactor. For example, *Nitrospira* spp. are dominant at relatively low DO concentrations (under 2 mg-O_2 L^{-1}), whereas *Nitrobacter* spp. are predominant at DO concentrations higher than 2 mg-O_2 L^{-1}.[41] In the present study, favorable low substrate and DO concentrations for *Nitrospira* spp. increased the
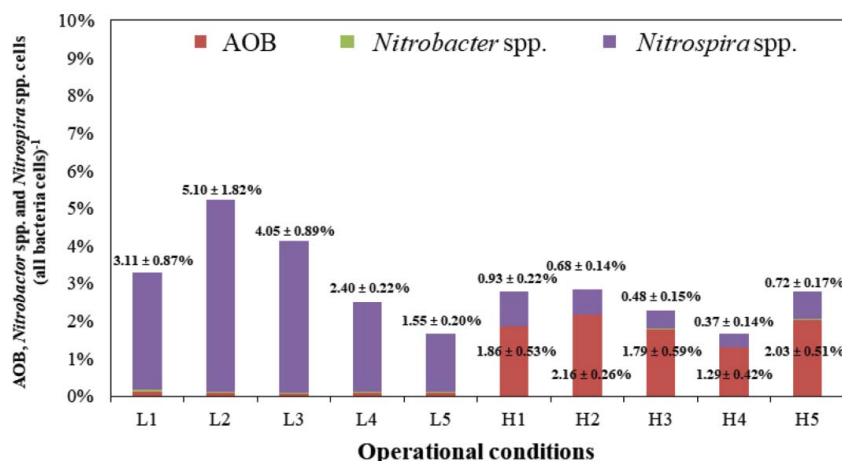

Figure 4. Community composition of AOB (red), *Nitrobacter* spp. (green), and *Nitrospira* spp. (purple) as a proportion (%) of all bacterial cells quantified by qPCR.
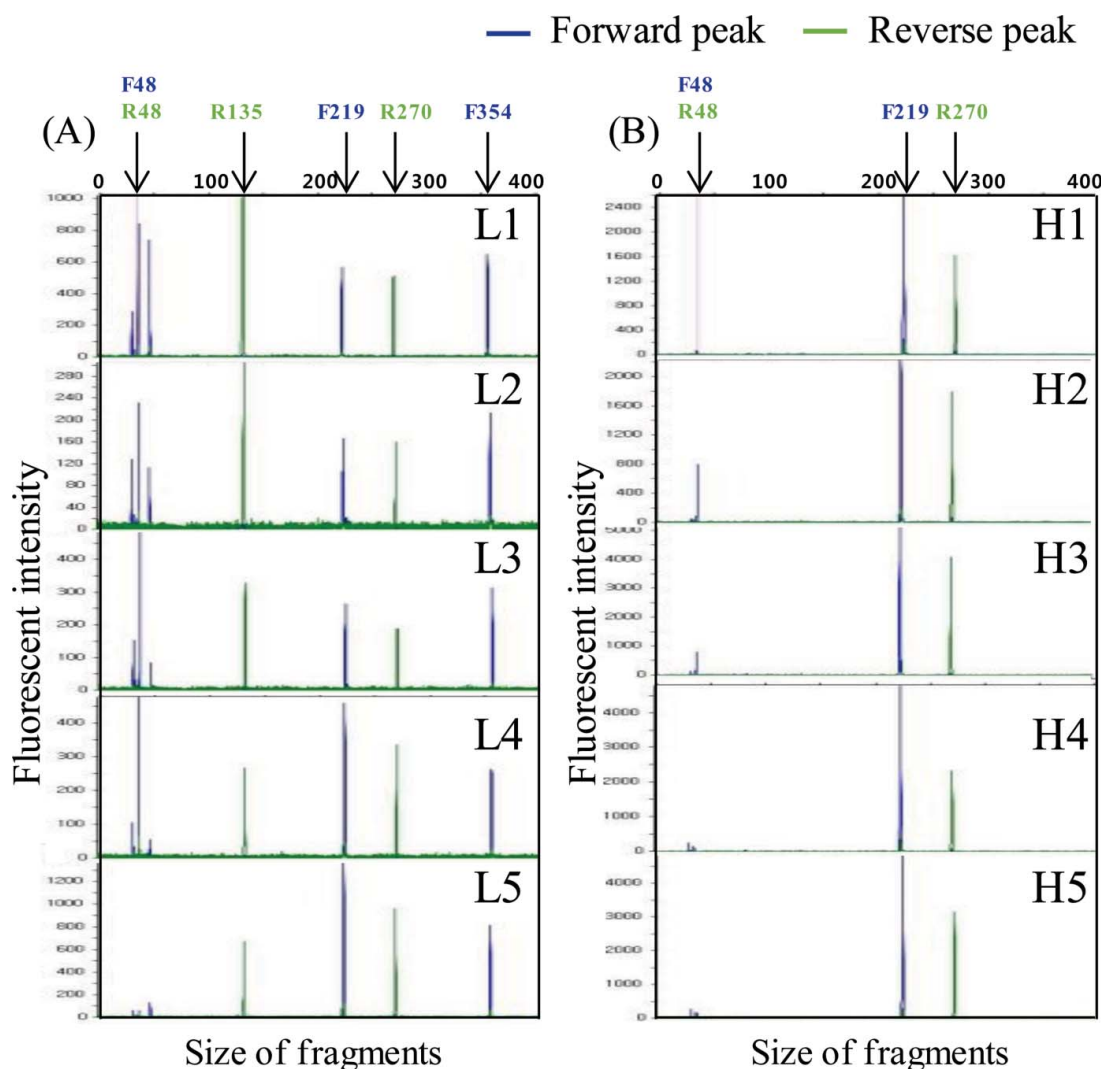
— Forward peak — Reverse peak

Figure 5. T-RFLP profiles of the AOB population in Phase II (A) and IV (B).

abundance of *Nitrospira* spp. to more than 100 times that of *Nitrobacter* spp.

T-RFLP analysis

AOB communities were studied in terms of *amoA*-based T-RFLP patterns at different ALRs and ILRs. The results of the T-RFLP electropherograms are depicted in Figure 5. The forward (i.e., F48, F219, F354, and F491) and reverse (i.e., R48, R135, R270, and R491) terminal fragments (TFs) were relatively abundant. The possible combination of forward and reverse TFs for corresponding AOB was examined in terms of sequence information on the *amoA* gene in the same manner as in a previous study.[42] The TF pairs of T-RFLP results revealed dominant AOB species of *Nitrosomonas oligotropha* (F48/R135, F219/R135, and F354/R135) and *N. europaea* (F219/R270).

Their relative dominance did not significantly change under different operational conditions in terms of ILR but clearly changed at different ALRs. For example, at low ALR (Phase II), the AOB community structure was dominated by F219, F354, R135, and R270, which was a mixture of *N. oligotropha* and *N. europaea*, at all ILRs, while F48 for one *N.*

oligotropha showed negligible dominance at ILR of 0.93 kg-C m^{-3} d^{-1}.

The selective pressure of high ALR (Phase IV) enhanced the dominance of *N. europaea* (F219/R270). *N. europaea* prefers eutrophic conditions because of the high K_s for free ammonia (0.42–0.85 mg L^{-1}). In contrast, *N. oligotropha* was previously shown to have remarkably weak affinity for free ammonia (0.027–0.059 mg L^{-1})[43] and consequently is highly competitive under nutrient-limited conditions such as oligotrophic freshwater[44,45] and wastewater treatment systems.[46] Compared with *N. oligotropha*, it is well-known that *N. europaea* has high affinity for NH$_3$ as the dominant AOB population at various ALRs. Nevertheless, the dominance of *N. europaea* (F219/R270) was not affected by ILR.

Conclusion

In this study, a nitrifying MBBR, using a PVA sponge, was evaluated to determine the influence of ALR and ILR on the nitrification process and changes in the nitrifying bacterial community. The concentration of IC was maintained high as compared to the stoichiometric demand. Relatively low ALR (0.36 kg-N m^{-3} d^{-1}) resulted in full nitrification, whereas a

stable PN process was achieved by increasing ALR to a higher level: 2.16 kg-N m^{-3} d^{-1}. In contrast, ILR appeared to have little influence on the nitrification process. Based on the qPCR results, AOB was greatly influenced by ALR, whereas the abundance levels of two genera (*Nitrobacter* spp. and *Nitrospira* spp.) stayed at a constant level regardless of ALR and ILR. T-RFLP analysis revealed that *N. oligotropha* and *N. europaea* coexisted at low ALR, whereas *N. europaea* became the dominant AOB at high ALR.

Funding

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