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Nitritation via heat shock using immobilized active sludge aggregates

Guanghui Chen*, Jun Li, Jia Wei, Jinping Zeng, Yanzhuo Zhang, Wei Bian, Hailiang Deng

Key Laboratory of Beijing for Water Quality Science and Water Environment Recovery Engineering, Beijing University of Technology, Beijing 100124, China, Tel. +86 15652356918; emails: ghui0066@163.com (G. Chen), lijunbjut@163.com (J. Li), 84241337@qq.com (J. Wei), yufeng569@163.com (J. Zeng), 83995983@qq.com (Y. Zhang), yangzhoubw@126.com (W. Bian), 1210793384@qq.com (H. Deng)

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ABSTRACT

The nitritation process is considered to be one of the most energy-saving and efficient methods for treating polluted water. In this study, active sludge was immobilized with waterborne polyurethane (WPU), and a heat-shock method was employed to treat the immobilized aggregates. When environmental factors that adversely affect nitritation (pH, dissolved oxygen, temperature, etc.) were controlled, steady nitrite nitrogen accumulation was also successfully achieved. We investigated the effect of temperature and timing of the heat-shock method on the activity of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). Subsequently, temperatures of 60 and 70˚C were selected to evaluate the nitritation stability of the heat-shocked immobilized aggregates, and continuous-flow experiments were conducted. The results of preliminary experiments indicated that NOB can be completely deactivated at temperatures above 60˚C after 10 min, whereas AOB continued to exhibit some activity. In addition, the effect of temperature was more significant than the effect of heating time for NOB. The results of the nitritation stability assay indicated that the heat-shocked immobilized aggregates that formed at two temperatures remained in stable nitritation; however, the performance of the AOB was decreased at the highest temperature tested. A high temperature and long duration were required for stable nitritation. In the continuous-flow experiments, we discovered that nitrate nitrogen accumulation occurred after 65 d of operation. Therefore, the immobilized aggregates were heat shocked again, and nitrite nitrogen accumulation recurred in the reactor (results not shown). Heat shock is an effective method for stabilizing the nitritation of immobilized nitrifying sludge; this method also provides new ideas for a nitritation-based nitrogen removal process. Key innovations are: (1) a new nitration method based on heat shock has been proposed to generate steady nitritation using immobilized bacterial aggregates; (2) polymerase chain reaction (PCR) has been applied to analyze variations in the AOB and NOB populations of immobilized aggregates before and after heat shock; (3) optimal heat-shock conditions (including duration and temperature) have been identified.

*Corresponding author.

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Keywords: Immobilized aggregates; Nitritation; Heat shock; Activated sludge

1. Introduction

The traditional nitrification process can be divided into two steps. The first step involves a reaction that is referred to as "nitritation," in which ammonium is oxidized to nitrite by ammonium-oxidizing bacteria (AOB). In the reaction that occurs in the second step, which is referred to as "nitratation," nitrite is oxidized to nitrate by nitrite-oxidizing bacteria (NOB). Compared to traditional nitrification, nitritation requires less aeration and less energy consumption. Nitritation offers a reduction in organic carbon consumption in the denitrification stage and increases the nitrogen removal efficiency [1]; it is an economic and feasible technique for treating wastewater with high concentrations of ammonia and a low C/N ratio [2] and has prospects for global application [3–5]. However, AOB are autotrophic, their growth rate is low, their biomass is easily washed out of a reactor, and a stable nitrite accumulation rate (NAR) is difficult to maintain in continuous-flow conditions [6]; these characteristics limit the application of nitritation technology.

The embedded immobilization technique is a new technique for the immobilization of microbes in the biological engineering field. Free cells or enzymes are immobilized in a constrained area by immobilization materials such that the activity of the cells or the enzymes can be maintained, and the cells and enzymes can then be reused. The embedded immobilization technique has a suitable retaining effect on microbes [7,8]. Studies have shown that the production of immobilized aggregates using the embedded immobilization technique can effectively prevent the loss of bacteria, maintain the biomass in the reactor, and enhance shock-loading resistance [9]. Thus, this technique is conducive to maintaining stable nitritation.

Two methods can be employed to achieve stable and immobilized aggregates for nitritation. The first method involves the immobilization of a pure AOB culture; however, this method is not practical due to the high cost and substantial amount of time required to cultivate pure AOB cultures [10]. The second method involves the use of active sludge as seed sludge to produce immobilized aggregates; after culture activation, the immobilized aggregates may serve a nitritation function. Active sludge contains high concentrations of AOB and is available in large amounts from sewage treatment systems; however, NOB are also present. Because NOB are immobilized in the gel carrier, nitratation will also occur; thus, the nitritation process cannot be stabilized.

In this study, active sludge was utilized to produce immobilized aggregates that were treated with a novel "heat shock" method. We discovered that heat-shock treatments can be employed to suppress nitratation, while nitritation still occurs. Different heat-shock durations and temperatures were investigated. Molecular biological analysis (polymerase chain reaction-denaturing gradient gel electrophoresis, PCR-DGGE) was employed to investigate the changes in AOB and NOB in immobilized aggregates after heat-shock treatment. The stability of nitritation during long-term operation with immobilized aggregates was investigated to provide theoretical support for this novel nitrogen removal technique.

2. Materials and methods

2.1. Preparation of the immobilized aggregates

The embedding sludge that was utilized in this study was obtained from the active sludge in the aeration tank of a sewage disposal station in Beijing, China. The sludge was washed three times with phosphate-buffered saline (PBS, pH 7.4, 0.1 M) to remove any residual substrate on the sludge surface and then centrifuged for 10 min at 4,000 rpm. To begin polymerization, the sludge was mixed with waterborne polyurethane (WPU) in a beaker; an initiator (potassium persulfate) and a promoter (N,N,N′,N′-tetramethylethylenediamine) were then added to the beaker. After approximately 30 min, the mixed solution formed a gel, and this polymerized gel was cut into 3-mm cubes. The gel carrier contained 10% (w/v) WPU, 0.3% (w/v) initiator, 0.2% (w/v) promoter, and 4% (w/v) sludge. After one week in activation culture, the immobilized aggregates were extracted for subsequent use [11].

2.2. Water quality

The primary components of the water that was prepared for use in this study are listed in Table 1. The NH_4^+-N and NO_2^--N were provided with NH_4Cl and $NaNO_2$, respectively, in the required concentrations.

The concentrations of NH_4^+-N, NO_2^--N, and NO_3^--N were determined according to standard meth-

Table 1
Composition of artificial wastewater

Main components	Mass concentration (mg/L)	Main components	Mass concentration (mg/L)
$NaHCO_3$	585	NH_4Cl	191
$CaCl_2$	11	$NaNO_2$	246
$MgSO_4{\cdot}7H_2O$	42	NaCl	25
$NaH_2PO_4{\cdot}12H_2O$	58	KCl	34

ods [12]. The temperature and pH values were determined using the WTW/Multi 3,420 multiparameter.

2.3. Effect of heat shock on the immobilized bacteria

A total of 100 mL of active immobilized gel was added to a thermostatic water bath at temperatures of 40, 50, 60, and 70℃. A total of 25 mL of immobilized gel was obtained after 10, 20, 40, and 60 min from each thermostatic water bath.

To assess nitritation performance, a total of 25 mL of immobilized gel was added to a 300-mL triangular bottle with synthetic wastewater. The initial concentration of NH_4^--N was 50 mg/L, and the remaining ingredients are listed in Table 1 (except for NO_2^--N, which was not present). The aeration head was placed inside the bottle to achieve sufficient aeration. This fitting was placed in the thermostatic water bath at 22℃. A pH of approximately 8.0 was maintained via the addition of NaOH during the reaction process. The concentration of NH_4^--N in the sample was determined after 48 h.

The characterization of nitratation was as follows: the initial concentration of the NO_2^--N synthetic wastewater that contained $NaNO_2$ was 50 mg/L, while NH_4^--N was not present. An approximate pH of 7.1 was maintained in the triangular bottle; the other parameters were appropriate for the determination of nitritation. The concentration of NO_2^--N in the sample was determined after 48 h. Three parallel groups were established, and the average concentration was measured in the experiment.

2.4. Molecular biological analysis

Immobilized partials were heat shocked at 60℃ for 10 min, and PCR-DGGE was performed. First, the partials were stirred by a mortar and fractured by an ultrasonic instrument using an amplitude of 50% for 60 s; then, DNA was extracted. The AOB were assessed by a nested-PCR method. In the first step, the template DNA was extracted and amplified using the primer pairs CTO189fA/B/C and CTO654r, according to the reaction system described by Cébron et al. [13]. The PCR products from the first step were employed as the template and amplified with the universal bacterial primers F357-GC/R518, according to the reaction system described by Muyzer et al. [14]. NOB were assessed with the nested-PCR method. In the first step, the extracted DNA was employed as the template and amplified using the primer pair FGPS1269/FGPS872, according to the reaction system described by Degrange et al. [15]. In the second step of the PCR, the conditions were identical to the conditions in the second step of the AOB assessment. The sequence information of the PCR primers and the reaction procedures are listed in Table 2.

The DCode™ DGGE system (Bio-Rad, Hercules, California, USA) was applied to extract the PCR amplification products. The electrophoresis conditions were as follows. A linear gradient of 35–55% denaturant was electrophoresed for 8 h at a voltage of 130 V and 60℃. The electrophoresis buffer solution consisted of 1 × TAE. The PCR products were stained with Partials Red nucleic acid gel dye for 30 min after electrophoresis. Photographs were obtained via a gel imaging system (Partials Doc™ XR^+, Bio-Rad). After the major bands were excised from the gel, they were cloned and sent to a company for sequencing.

2.5. Continuous flow experiment

A 2-L polymethyl methacrylate (PMMA) reactor was employed in the continuous flow experiment, as shown in Fig. 1. An overflow weir was installed at the top of the reactor. Water entered from the bottom of the reactor and exited from an outlet on the top of the reactor. The water flow outlet was installed with grid structures to prevent the outflow of immobilized aggregates with the water. An aeration device was installed inside the reactor to provide sufficient aeration to maintain the level of dissolved oxygen (DO) above 4 mg/L. A water bath was installed outside the reactor to maintain the temperature inside the reactor at 21℃. The immobilized aggregates were heat shocked for 20 min at 60 and 70℃. A volume-filling fraction of 10% was added to the reactor. Immobilized

Table 2
Primers and PCR conditions employed in this study

Target bacteria	Primer	Sequence	Reaction program
AOB	CTO189fA	GGAGAAAAGCAGGGGATCG	94℃ pre-denaturation 5 min; 94℃ denaturation 45 s, 57℃ annealing 45 s, 72℃ elongation 90 s, total 35 cycles; 72℃ elongation 10 min
	CTO189fB	GGAGGAAAGCAGGGGATCG	
	CTO189fC	GGAGGAAAGTAGGGGATCG	
	CTO654r	CTAGCYTTGTAGTTTCAAACGC	
NOB	FGPS1269	TTTTTTGAGATTTGCTAG	94℃ pre-denaturation 3 min; 94℃ denaturation 1 min, 50℃ annealing 1 min, 72℃ elongation 2 min, total 30 cycles; 72℃ elongation 3 min
	FGPS872	CTAAAACTCAAAGGAATTGA	
Total bacteria	F357-GC	CGCCCGCCGCGCCCCGCGC	94℃ pre-denaturation 5 min; 94℃ denaturation 1 min, 65℃ annealing 1 min, 72℃ elongation 1 min, total 20 cycles, each cycle decreased by 0.5℃; 94℃ denaturation 1 min, 55℃ annealing 1 min, 72℃ elongation 1 min, total 10 cycles; 72℃ elongation 8 min
		CCGGCCCGCCGCCCCCGCC	
		CCCCTACGGGAGGCAGCAG	
	R518	ATTACCGCGGCTGCTGG	

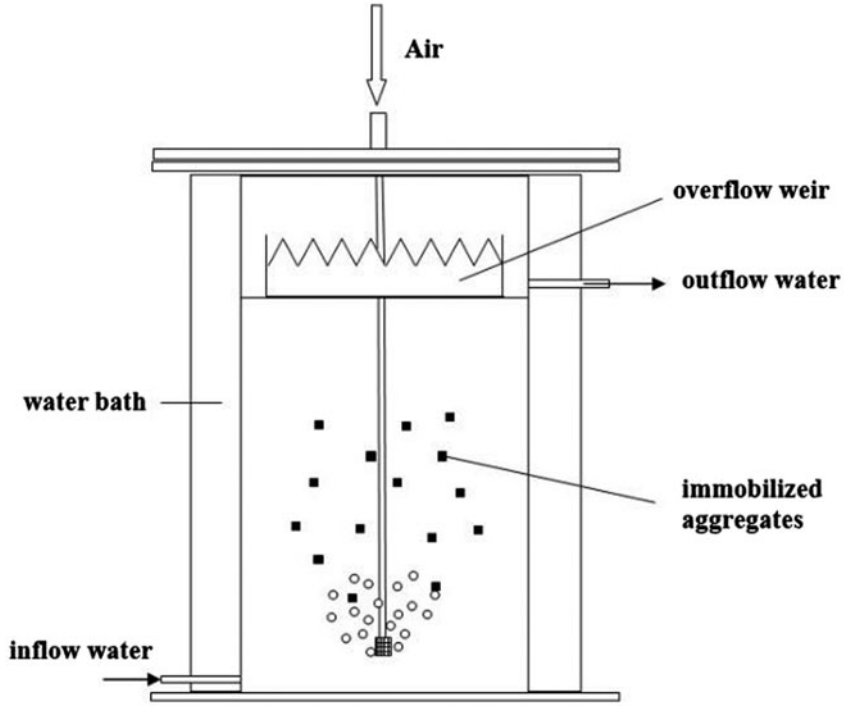

Fig. 1. Continuous tank reactor.

aggregates that had not been subjected to heat shock were employed as controls. The hydraulic retention time was 3 h, and synthetic wastewater was employed. The main components of the synthetic wastewater are listed in Table 1 (NO_2^--N was not added to the synthetic wastewater). The levels of NH_4^+-N, NO_2^--N, and NO_3^--N were measured for the inflow and outflow water, and the accumulation rate

of NO_2^--N was calculated from Eq. (1). In Eq. (1), η_{nit}: represents the accumulation rate of NO_2^--N; $\rho_{outflow}[NO_2^-\text{-N}]$: represents the mass concentration of outflow NO_2^--N; $\rho_{outflow}[NO_3^-\text{-N}]$: represents the mass concentration of outflow NO_3^--N.

$$\eta_{nit} = \frac{\rho_{outflow}[NO_2^-\text{-N}]}{\rho_{outflow}[NO_2^-\text{-N}] + \rho_{outflow}[NO_3^-\text{-N}]} \times 100\% \quad (1)$$

3. Experimental results

3.1. Effect of heat shock on the immobilized aggregates

Fig. 2 shows the effect of the heat-shock temperature and duration on the characteristics of nitratation using the immobilized aggregates. The figure demonstrates that NO_2^--N was almost completely removed from the control groups, which did not undergo heat shock, after 48 h. Conversely, the heat-shock treatment affected the nitratation process in the immobilized aggregates by decreasing the efficiency of NO_2^--N removal. The heat-shock temperature had a distinct effect on the nitratation of the immobilized aggregates. At an increased temperature, the efficiency of NO_2^--N removal decreased. At a temperature equal to or greater than 60℃, NO_2^--N removal was undetectable, which indicates that the immobilized aggregates lost their capacity for nitratation. The heat-shock duration affected the nitratation of the immobilized aggregates. After undergoing heat shock at 40℃ for 10 min, the efficiency of NO_2^--N removal was 85% after 48 h. However, an additional increase in the heat-shock duration decreased the efficiency of NO_2^--N removal. For example, the removal efficiency for NO_2^--N decreased to 64% when the heat-shock time was increased to 60 min. When the heat-shock temperature was increased above 60℃, NO_2^--N removal was undetected, regardless of the heat-shock duration, which suggests the complete inhibition of the nitratation process in the immobilized aggregates under these conditions.

The nitritation performance experiments showed that heat shock affected nitritation but did not completely inhibit the process. After heat shock at high temperatures for 60 min, the immobilized aggregates retained some capacity for nitritation. Fig. 3 shows the variation in the efficiency of NH_4^+-N removal by the immobilized aggregates when the aggregates were heat shocked at different temperatures for 60 min. Both the control and the aggregates that were heat shocked at 40℃ exhibited the complete removal of NH_4^+-N after 48 h. When the temperature exceeded 50℃, the removal efficiency decreased but remained above 50%. When the temperature exceeded 70℃, the immobilized aggregates retained a NH_4^+-N removal efficiency of 42.3%. These results demonstrate the preservation of nitritation when the temperature exceeds 60℃, while a complete inhibition of nitratation was realized by heat shock for 10 min; thus, nitritation occurred via the immobilized aggregates.

3.2. Molecular biological analysis

To investigate the effect of heat shock on AOB and NOB and the reason that heat shock produces steady nitritation, DGGE was employed to study the heat-shocked immobilized aggregates. Using this method,

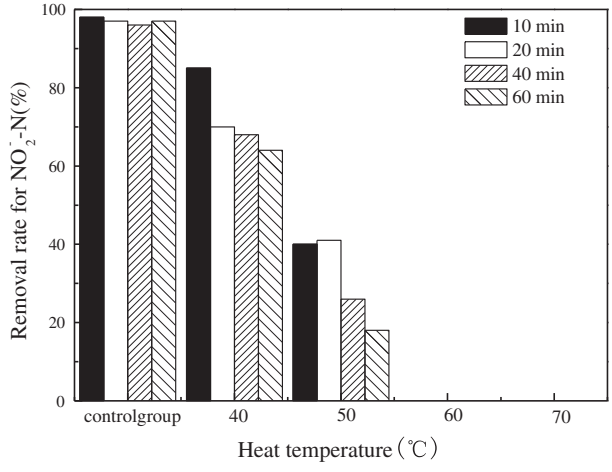

Fig. 2. Effect of heat shock on nitratation in the immobilized aggregates.

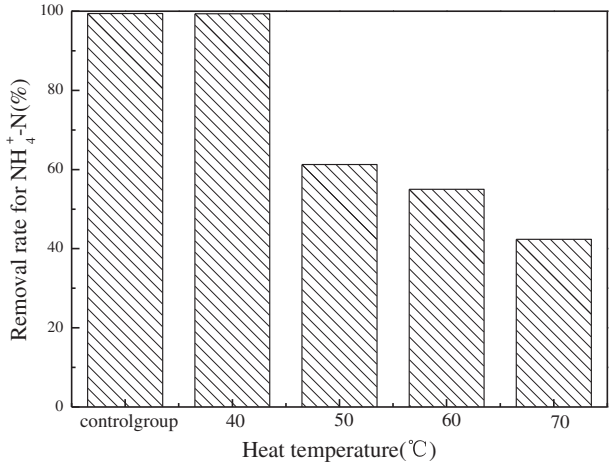

Fig. 3. Effect of treatment temperature on the nitritation of the immobilized aggregates (60 min).

we can observe the changes in AOB and NOB before and after heat shock directly in the lanes. The immobilized aggregates were subjected to heat shock at 60℃ for 10 min, and DGGE was performed on the aggregates before and after heat shock, as shown in Fig. 4.

Lanes H1 and H2 show the results before and after heat shock, respectively, for AOB, whereas lanes H3 and H4 show the results before and after heat shock for NOB. The types and biomasses of the aggregates can be distinguished via the distribution and intensity of the bands. From the two lanes shown in Fig. 4(a), six (1–6) different bands can be observed. A comparison of H1 and H2 shows that bands 1 and 3 disappeared after heat shock, whereas no intensity change was observed for bands 2, 4, 5, and 6. This result indicates that heat shock did not significantly affect the population diversity or the biomass of the AOB. In the two lanes shown in Fig. 4(b), seven (7–13) different bands were observed. A comparison of H3 and H4 shows that all bands in H4 darkened, and bands 7–12 almost disappeared, which suggests that the biomass of NOB decreased and the types of NOB changed following heat shock. After subjecting the bands in H2 and H4 to gel extraction, PCR, cloning and sequencing, the aggregates in H2 were found to be similar to uncultured *Nitrosomonas* sp. (HQ821461.1), *Pseudoxanthomonas* sp. (KJ666165.1), *Nitrosomonas* sp. HP8 (HF678378.1), and uncultured *Nitrosomonas* sp. (KJ023572.1), which indicates that these bacteria can retain a high activity at temperatures above 60℃. Sequencing of the aggregates in H4 showed the similarity of these aggregates to Candidatus *Nitrospira defluvii* (KP307153), with a similarity rate of 99%, which indicates that the majority of the NOB in the immobilized aggregates disappeared after heat shock; however, some NOB remained.

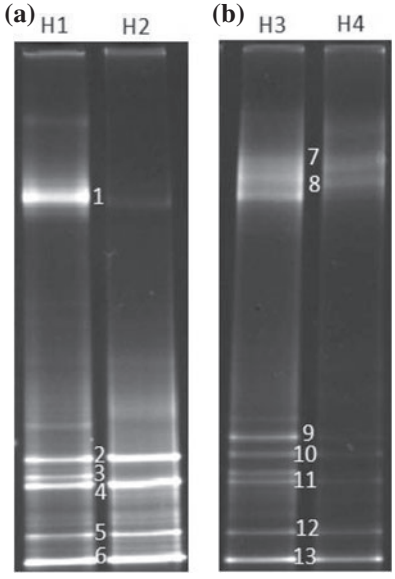

Fig. 4. DGGE results for (a) AOB and (b) NOB before and after heat shock.

3.3. Continuous flow experiments

The immobilized aggregates that were subjected to heat shock at 60℃ or 70℃ for 20 min and the untreated aggregates (as the control) were employed in the continuous flow experiments. The results are shown in Fig. 5. The nitrification process for the untreated immobilized aggregates is illustrated in Fig. 5(a); these aggregates exhibited excellent nitrification. The removal of NH_4^+-N occurred at 1 d and increased in efficiency over time. By 26 d, the NH_4^+-N concentration in the outflow water was 1.86 mg/L, and the ammonia nitrogen removal rate was 95.7%. In the beginning of the experiments, the untreated immobilized aggregates exhibited certain nitritation characteristics. At 7 d, the accumulation rate of NO_2^--N in the reactor was 65.1%. These results indicate that the presence of the immobilized aggregates affected the mass transfer efficiency of oxygen, which produced a low-oxygen concentration inside the immobilized aggregates, as well as the accumulation of NO_2^--N. With increased time, the mass transfer efficiency of the immobilized aggregates improved, which caused increased nitrification. After 17 d, the outflow water NO_2^--N concentration decreased to 0.845 mg/L, whereas the NO_3^--N concentration increased to 25.27 mg/L. Almost all NO_2^--N was converted to NO_3^--N.

For the aggregates that were heat shocked at 60℃, significant removal of NH_4^+-N occurred after 12 d, as shown in Fig. 5(b). The outflow water NO_3^--N concentration was almost 0, and the accumulation rate of NO_2^--N was steadily above 90%. The aggregates that were heat shocked at 70℃ also showed a similar steady NO_2^--N accumulation; however, the activation of the reaction required a much longer period, and significant removal of NH_4^+-N did not occur until 20 d. Compared to the untreated immobilized aggregates, the NO_2^--N removal efficiency of the aggregates that were treated with heat shock exceeded 90% and remained high. However, heat shock at high temperatures prolonged the period that was required to achieve steady nitritation. Fig. 5 also shows that the NH_4^+-N concentration of the outflow water was below 1 mg/L for the untreated immobilized aggregates; however, decreasing this concentration below 4 mg/L was difficult for the heat-shocked aggregates. This

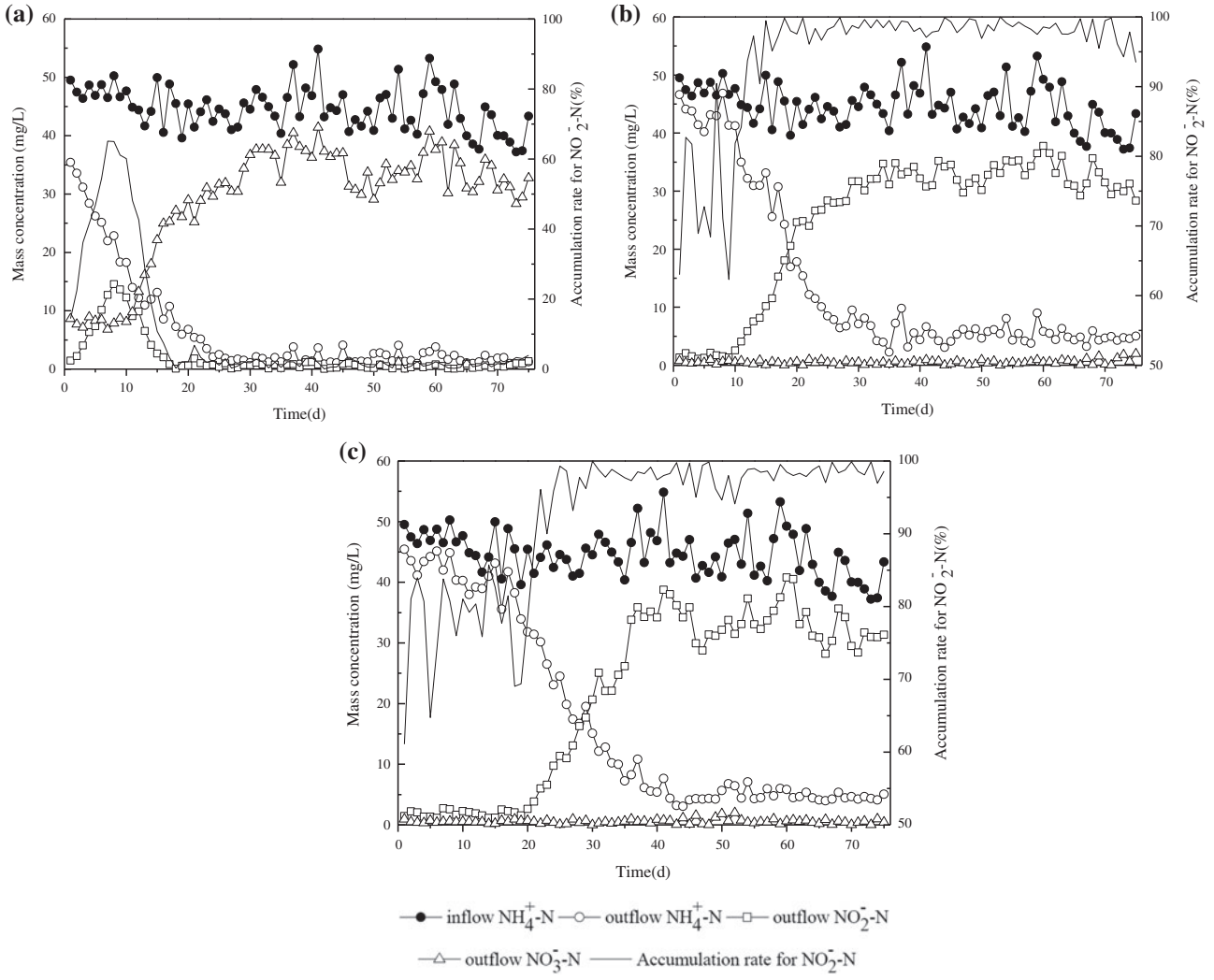

Fig. 5. Effect of heat shock on the nitrification process of the immobilized bacterial aggregates: (a) untreated, (b) 60℃ heat shocked, and (c) 70℃ heat shocked.

difficulty suggests that heat shock affected ammonia oxidation.

4. Discussion

PCR-DGGE can be employed to analyze the effects of heat shock on biomass composition in immobilized aggregates. Although two bands disappeared in the AOB lane after the immobilized aggregates were heat shocked at 60℃ for 10 min, as shown in Fig. 4(a), the remaining bands showed no change in brightness. These results suggest that the majority of the AOB aggregates maintained activity after heat shock; therefore, the immobilized aggregates retained their nitritation ability. However, the majority of the bands in the NOB control lane disappeared after heat shock. The remaining bands were determined to be Candidatus *N. defluvii* (KP307153) by sequencing, which has been shown to retain activity between 28 and 44℃, with an optimum temperature of 42℃ [16]. In this study, a small portion of Candidatus *N. defluvii* survived the 60℃ heat shock. Lücker et al. [17] discovered that Candidatus *N. defluvii* and anaerobic ammonium-oxidizing (anammox) bacteria have similar nitrite oxidoreductases (NCRs) and show evidence of gene transfer. These bacteria differ from other known NOB. An analysis of Candidatus *N. defluvii* revealed that this species is similar to anammox bacteria. The activity of these bacteria is only detectable when the biomass attains a certain value [18]. The heat-shocked and immobilized aggregate had a very low NOB biomass,

which did not show any nitratation characteristics. In total, the results demonstrated that heat shock caused nitritation via the immobilized aggregates.

In the continuous flow experiments, the NH_4^+-N concentration of the inflow water was low, approximately 50 mg/L; the corresponding free ammonia (FA) concentration ranged between 0.48 and 0.96 mg/L, which is significantly less than the concentration that is required to inhibit nitratation by NOB [19,20]. Steady nitritation was also realized, which implies that the heat-shocked immobilized aggregates were also adaptable to wastewater with low ammonia nitrogen concentrations. During the reaction, the pH in the reactor was not controlled and varied between 7.1 and 7.6. The temperature was maintained at 21℃. Sufficient aeration was maintained during the reaction by maintaining a DO level above 4 mg/L. These environmental factors were considered to be unfavorable for nitritation, as noted in previous studies [21–24]. In this study, these conditions did not prevent nitritation from occurring. Therefore, heat-shocked, immobilized aggregates are capable of adapting to different environments without strict process control and can be applied to different types of wastewater management. These features represent significant advantages over traditional methods. Heat-shocked, immobilized aggregates enable an expansive range of applications for the nitritation process.

However, the heat-shock method presents certain problems. First, as shown in Fig. 5(a), lag time was required for the recovery of ammonia oxidation by the immobilized aggregates. In the immobilized aggregates that were heat shocked at 60 or 70℃, significant removal of NH_4^+-N occurred after approximately 10 d or 20 d, respectively. Decreasing the NH_4^+-N concentration below 4 mg/L was difficult when heat-shocked aggregates were employed compared to the use of untreated, immobilized aggregates. This occurred because heat shock affected the AOB. Fig. 4(a) shows that bands 1 and 3 disappeared after the heat-shock method was employed. Sequencing revealed that these two bands exhibited the greatest similarity with uncultured beta *proteobacterium* (JF514815.1) and uncultured *Nitrosomonas* sp. (GU073372.1). These two types of bacteria have a high affinity for NH_4^+-N [10]; therefore, it can be assumed that ammonia oxidation in immobilized aggregates was affected by their disappearance. Second, in the experiments with aggregates that were heat shocked at 60 or 70℃, NO_3^--N accumulated after the steady accumulation of NO_2^--N for 65 d at a rate that increased over time (results not shown). This phenomenon occurred because the biomass of remnant NOB in the immobilized aggregates increased over time. When the NOB reached a certain concentration, the nitratation capabilities reemerged. Despite the difficulties, repeated heat shocking of the immobilized aggregates can result in a steady accumulation of NO_2^--N after a recovery period. In practice, multiple reactors can be set up in a temporally staggered pattern to address the restoration of the immobilized aggregates and the periodic activity problems that are associated with heat shock.

5. Conclusions

(1) Both the temperature and the duration of heat shock affected the AOB and NOB. However, the effect of temperature was more pronounced. The activity of the NOB was completely inhibited when the aggregates were heat shocked at temperatures higher than 60℃ for 10 min, whereas the AOB retained some ammonia oxidation activity. Therefore, heat shock can simultaneously result in the preservation of ammonia oxidation and the inhibition of NOB in immobilized aggregates, which causes nitritation.

(2) The remaining NOB in the immobilized aggregates after heat shock was Candidatus *N. defluvii* (KP307153). At low biomass levels, the remaining NOB did not show activity, and the biological activity only occurred when the biomass increased to a certain value. Although some NOB remained among the immobilized aggregates after heat shock, these bacteria did not display nitrite-oxidizing characteristics.

(3) Steady nitritation for 65 d occurred when the heat-shocked, immobilized aggregates were subjected to the described continuous flow conditions. The accumulation rate of NO_2^--N exceeded 90%. After this period, the accumulation of NO_3^--N reoccurred. Nitritation was restored by renewed heat shock. High temperatures were unfavorable for this restoration, and 60℃ was determined to be the ideal heat-shock temperature.

(4) The results demonstrated that heat shock can produce steady nitritation using immobilized aggregates in different environmental conditions via a simple method that does not require strict process control. This technique can be applied to different types of wastewater management and expand the applications of the nitritation process.

Acknowledgments

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