



# Enhancement of Anammox performances in an ABR at normal temperature by the low-intensity ultrasonic irradiation

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## ABSTRACT

A lab-scale ultrasound enhancing Anammox reactor (ABR<sub>U</sub>) was established and irradiated once a week by ultrasound with the optimal parameter (frequency of 25.0 kHz, intensity of 1.00 W cm<sup>-2</sup> and exposure time of 36.0 s) obtained by response surface methodology (RSM). ABR<sub>U</sub> and the controlled Anammox reactor (ABR<sub>C</sub>) without ultrasonic treatment were operated in parallel. The start-up time of Anammox process in ABR<sub>U</sub> (59 d) was shorter than that in ABR<sub>C</sub> (69 d). At the end of the nitrogen load-enhancing period, NLR (0.500 kg N m<sup>-3</sup> d<sup>-1</sup>) and NRR (0.430 kg N m<sup>-3</sup> d<sup>-1</sup>) in ABR<sub>U</sub> were both higher than NLR (0.400 kg N m<sup>-3</sup> d<sup>-1</sup>) and NRR (0.333 kg N m<sup>-3</sup> d<sup>-1</sup>) in ABR<sub>C</sub>. The results of RTQ-PCR demonstrated that the specific low-intensity ultrasound irradiation improved the enrichment levels of AnAOB in mature sludge. SEM images and the observation of the macroscopic morphology of mature sludge showed that the ultrasound irradiation strengthened the formation of Anammox granular sludge, thereby improved the interception capacity and impact load resistance of the reactor, and enhanced the nitrogen removal performance in ABR<sub>U</sub>. The ultrasonic enhanced Anammox reactor based on an ABR with the optimal parameters can promote the rapid start-up and efficient and stable operation of the Anammox process at normal temperature (around 25.0 °C).

## 1. Introduction

Nowadays, degradation of water-environmental quality is becoming a global issue which leads to a series of negative effects on human's production and life. Nitrogenous pollutants in industrial wastewater and municipal sewage can cause eutrophication of water bodies and even water-environmental problems such as algal blooms and red tides. To deal with these problems, several biological nitrogen removal processes have been developed, including nitrification, denitrification, anaerobic ammonia oxidation (Anammox), etc. In recent years, Anammox process has attracted increasing attentions home and abroad as it is a highly efficient and consumption-saving biological nitrogen removal process. Anammox process is a biological process, in which Anammox bacteria uses ammonia as an electron donor and nitrite as an electron acceptor to convert ammonia into dinitrogen gas under anaerobic conditions so as to achieve nitrogen removal [1]. Compared with traditional biological nitrogen removal processes, Anammox has many advantages, e.g., no addition of organic carbon sources, no consumption of dissolved oxygen (DO), no requirement of acid-base neutralization reagents, less sludge output, less carbon dioxide emissions and smaller footprint [2].

Anaerobic ammonia-oxidizing bacteria (AnAOB) are the functional bacteria responsible for Anammox process. The quite slow specific growth rate and the relatively long multiplied time of AnAOB lead a long start-up period of Anammox process and affect its stability so as to limit the wide applications of the process [3,4]. To shorten the start-up period of this process and elevate its operational stability, the activity of AnAOB can be enhanced through adding chemical agents and physical field [5-7]. However, the method of adding chemical agents may irreversibly inhibit microorganisms if overdosed, and may also cause toxins in the effluent bringing about the ecological risk. The ultrasound wave belongs to the physical field and has been applied to strengthen biological wastewater treatment performances as a bioaugmentation method. Furthermore, the low intensity ultrasonic irradiation is an advantageous method to enhance the activity of AnAOB so as to strengthen performance of Anammox process [8,9]. The proper strength and frequency ultrasound wave can enhance the activity of AnAOB through improve the permeability of cell membrane and promote the electron transport process efficiency as well as cell motility.

Up to now, there have been some studies on the preliminary mechanism of ultrasonic enhancement for the activity of AnAOB or Anammox

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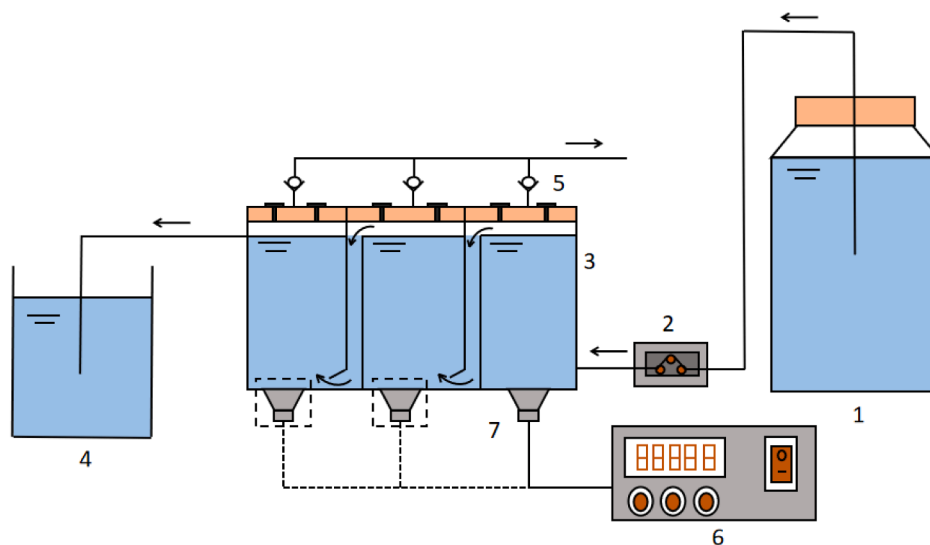
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1. influent tank; 2. influent pump; 3. ABR; 4. effluent; 5. check valve; 6. ultrasound generator; 7. the horn-shaped vibrator

Fig. 1. Scheme of the ABR for the start-up and operation of Anammox process.

consortium. Zhang et al. (2015) [10] showed that, in the strategy of restoring the Anammox reactor in which activity was inhibited by copper ions, ultrasonic irradiation at an interval of 8 days could stimulate dehydrogenase and promote kinetic behavior and thereby accelerate the restoration of the reactor performance. Yu et al. (2014) [11] found the ultrasound irradiation (the intensity of  $0.700 \text{ W cm}^{-2}$ , the frequency of 25.0 kHz, the duration of 1.90 min) at  $35.0 \text{ }^\circ\text{C}$  decreased the sludge granule diameter and thickness of cell membrane and extracellular polymer substrate (EPS) which contributed to substrate mass transfer efficiency and increase the specific Anammox activity (SAA). Duan et al. (2011) [12] demonstrated that the low intensity ultrasound produced the positive effect on Anammox consortium by increasing extracellular substances to form porous structure and creating a stable cavitation to enhance mass transfer and fluid mixing. They also demonstrated that the shearing stress caused by ultrasound energy could make the cell wall and cell membrane thinner to improve their permeability so as to promote mass transfer. Given this, it is feasible to improve the start-up and operation performance of Anammox process by applying low-intensity ultrasound. However, the present researches mainly focus on the short-term enhancing effects of Anammox process or its activity under ultrasonic irradiation with proper parameters. There are limited reports on the long-term enhancing effects of Anammox process under specific low-intensity ultrasound irradiation, and the enhancing mechanism of ultrasonic treatment on this process also needs more in-depth study.

Ultrasonic irradiation will produce different enhancement effects when acting on different reactors. For quick start-up and stable operation of Anammox process, selection of reactor configuration is necessary. The selected reactor configuration should be suitable for the enrichment of AnAOB with the extremely slow growth rate. ABR is a suitable reactor to start up and operate Anammox process from the activated sludge. The multi-stage retention capacity of ABR enables it to maintain a high internal retention of biomass. In addition, ABR has excellent solid-liquid separation ability, sludge granulation ability and load shock resistance ability, which is conducive to improve the stability of Anammox process [13]. However, there is no literature involving the long-term enhancing effects and mechanism of low-intensity ultrasound on Anammox process in an ABR.

In our study, an ABR was employed to start up and operate Anammox

process and the low-intensity ultrasound with an optimal parameter was introduced to enhance the reactor performances. The Anammox performances in the ABR was investigated and the mechanism of the low-intensity ultrasound was discussed. The experiment was conducted under the normal temperature condition ( $25.0 \text{ }^\circ\text{C}$ ). The temperature of actual wastewater is often lower than the optimum temperature (about  $30.0 \sim 35.0 \text{ }^\circ\text{C}$ ) for AnAOB. Therefore, using low-intensity ultrasonic irradiation to strengthen the Anammox process at the normal temperature is meaningful for applications of this process on actual wastewater.

Based on the above description, the objectives of this study were as follows: (i) to optimize the parameters of ultrasonic irradiation using the response surface methodology (RSM) for enhancement of Anammox performances; (ii) to construct a robust ultrasound enhancing Anammox reactor on the basis of an ABR; (iii) to use the ultrasound irradiation to accelerate the start-up and to improve operational performances of Anammox process in the ABR at normal temperature ( $25.0 \text{ }^\circ\text{C}$ ); (iv) to probe the enhancing mechanism of ultrasonic treatment on Anammox performances by analyzing microbial community of the cultivated sludge and microbial morphology of the mature Anammox sludge.

## 2. Materials and methods

### 2.1. Batch tests

In order to gain the optimal intensity and exposure duration of ultrasound irradiation for improvement of Anammox activity, batch tests were performed using rectangular jars (length 110 mm, width 100 mm, height 200 mm, bottom area  $110 \text{ cm}^2$ ). Each jar corresponded to one compartment of the ABR utilized in next continuous experiment and was added 1.00 L of Anammox sludge, whose concentration was adjusted to  $0.100 \text{ g VSS L}^{-1}$ . The Anammox sludge was harvested from a lab-scale Anammox reactor for 3 months operation. In each jar, the temperature was controlled at  $25.0 \text{ }^\circ\text{C}$ , the pH was kept at 7.80 and DO was lower than  $0.200 \text{ mg L}^{-1}$ . An ultrasound generator was applied for ultrasound irradiation. The Anammox sludge was treated by ultrasound wave through a horn-shaped vibrator on the bottom of the jar. Ultrasound power could be adjusted from 0 to 100% with a maximum power of 180 W. In this study, the two ultrasonic parameters (exposure time and

**Table 1**  
The applied specific primers for Anammox bacteria and eubacteria.

Primer name	Target position (5'-3')	Target organism	Analytical method
AMX809F	GCCGTAAACGATGGGGCACT	ANAMMOX	RTQ-PCR
AMX1066R	AACGTCTCACGACACGAGCTG	bacteria	
Eub341F	CCTACGGGAGGCAGCAG	eubacteria	
Eub534R	ATTACCGGGCTGCTGGC		

ultrasound intensity) influencing the specific Anammox activity (SAA) were considered. The ultrasonic parameters used in the batch tests were as follows: the frequency was invariable at 25.0 kHz, the exposure time was from 10.0 to 60.0 s with a gradient of 10.0 s per step, and a series of ultrasound intensity was 0.300, 0.500, 0.800, 1.10, 1.40, 1.60 W cm<sup>-2</sup>. Each SAA obtained from different implement conditions described above were measured. A quadratic polynomial model was established using the RSM to optimize the exposure time and ultrasound intensity and gain the optimal ultrasonic parameters.

## 2.2. Reactors and inoculated sludge

The schematic diagram of the experimental device is shown in Fig. 1. Two ABRs were operated in parallel for Anammox process. One was irradiated by the periodical low frequency ultrasound at the optimal ultrasonic parameters gained from the batch tests and defined as ABR<sub>U</sub> while the other was run as a contrast system without ultrasound defined as ABR<sub>C</sub>. Both reactors were made of plexiglass and had a work volume of 6.00 L with a length of 380 mm, a width of 112 mm and a height of 210 mm. Each reactor had three equal compartments with a baffle diversion angle of 45°. Both reactors were wrapped by a shading cloth to avoid the adverse effect of photosynthetic microorganisms on AnAOB. At the top of each compartment, a check valve was fixed for facilitating nitrogen gas emission and preventing oxygen from leaking into the reactor. An ultrasonic generator was applied as the ultrasonic source which power could be adjusted from 0 W to 180 W. Previous studies

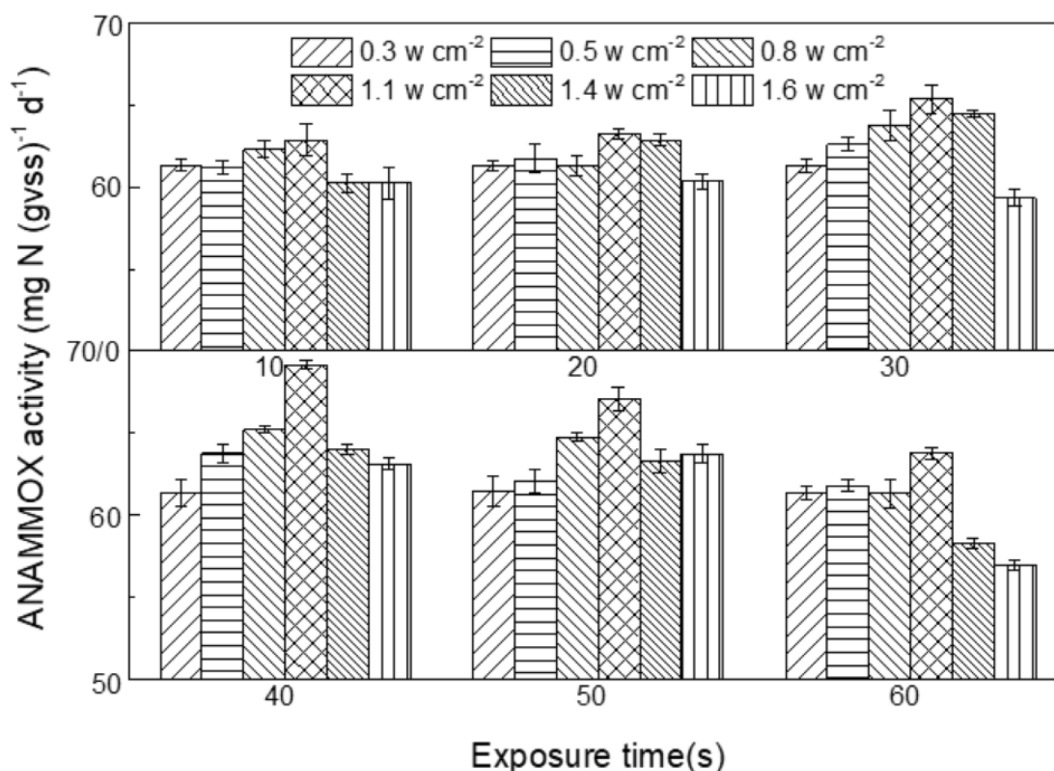
have shown that the enhanced effect of ultrasound on the activities of AnAOB could last 6 ~ 8 days [12,14]. Therefore, the horn ultrasonic vibrator was installed under each compartment of ABR<sub>U</sub> to provide ultrasound irradiation once a week.

Aerobic activated sludge from a local full-scale domestic sewage treatment plant was used as the inoculated sludge in the continuous experiment. Each compartment was inoculated with 1.00 L of the inoculated sludge. The sludge characteristics were as follows: mixed liquid suspended solids (MLSS) concentration was 2.68 g L<sup>-1</sup>, volatile suspended solids (VSS) concentration was 1.95 g L<sup>-1</sup> and MLVSS/MLSS was 72.8%.

## 2.3. Feeding media and operation strategy

The reactors (ABR<sub>U</sub> and ABR<sub>C</sub>) were feed with the same synthetic wastewater containing 1250 mg L<sup>-1</sup> of KHCO<sub>3</sub>, 25.0 mg L<sup>-1</sup> of KH<sub>2</sub>PO<sub>4</sub>, 300 mg L<sup>-1</sup> of CaCl<sub>2</sub>·2H<sub>2</sub>O, 200 mg L<sup>-1</sup> of MgSO<sub>4</sub>·7H<sub>2</sub>O, 25.0 mg L<sup>-1</sup> of FeSO<sub>4</sub>·7H<sub>2</sub>O, 1.80 mg L<sup>-1</sup> of EDTA-2Na, 50.0 mg L<sup>-1</sup> of yeast extract, and 1.50 ml L<sup>-1</sup> of trace element solution. The trace element solution was prepared according to the Anammox nutrient medium reported by Strous et al. [15]. Ammonia and nitrite were added as required to the feeding media in the form of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and NaNO<sub>2</sub>, respectively. Blowing nitrogen gas into the synthetic wastewater for 10.0 min made its dissolved oxygen (DO) less than 0.200 mg L<sup>-1</sup> and blowing carbon dioxide into the wastewater for 2.00 min provide some carbon sources for AnAOB.

ABR<sub>U</sub> was exposed to the ultrasonic irradiation with the optimal ultrasonic irradiation intensity and exposure duration gained by the batch tests while ABR<sub>C</sub> was taken as the contrast system without ultrasonic treatment. Other operation parameters of ABR<sub>U</sub> and ABR<sub>C</sub> were identical. The influent was continuously provided by a peristaltic pump and the effluent was discharged through the overflow port. For each reactor, the temperature was controlled at the normal temperature condition (25.0 °C), the pH was kept at 7.80 ± 0.200, and the hydraulic retention time (HRT) was 24 h.



**Fig. 2.** The effects of ultrasound on the Anammox activities under different ultrasound intensities and exposure time.

2.4. Chemical analysis

The influent and effluent were measured every day according to the standard method to monitor the concentration of  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_2^-\text{-N}$ , and  $\text{NO}_3^-\text{-N}$  [16]. The concentration of DO was measured with a digital portable DO meter and the pH was measured with a digital portable pH meter.

2.5. Microbial community and morphology analysis

Real-time quantitative PCR (RTQ-PCR) was used to determine the 16S rRNA gene copies of AnAOB (in triplicate), and quantify the enrichment level of AnAOB in the seed sludge and mature sludge after cultivation. DNA extraction and purification procedure were as described previously [17]. DNA was extracted in an OMEGA kit (E.Z.N. A™ Mag-Bind Soil DNA Kit, USA). Two pairs of primers targeting for

AnAOB (AMX809F-AMX1066R) and eubacteria (Eub341F-Eub534R) were designed by Software Premier 5.0 and their information was listed in Table 1. The 16S rRNA gene was amplified by the polymerase chain reaction (PCR) of AMX809F-AMX1066R and Eub341F-Eub 534R, and each sample was subjected to triplicate PCR amplification treatment. The fluorescence quantitative PCR system (ABI StepOne plus, USA) was used for absolute quantification of AnAOB and eubacteria.

On day 101, both  $\text{ABR}_U$  and  $\text{ABR}_C$  achieved a relatively high nitrogen loading and stable nitrogen removal performance, sludge samples were taken from the two reactors for morphological observation. The macroscopic morphology and microstructure of the sludge samples were observed. The microstructure of the samples was observed by a scanning electron microscope (SEM). The sludge samples were fixed with 2.50% glutaraldehyde, washed with 0.100 M phosphate buffered saline (PBS) and dehydrated with a series of ethanol solutions with gradient volume percentages (30.0, 50.0, 70.0, 90.0, 95.0 and 100%) [18]. The

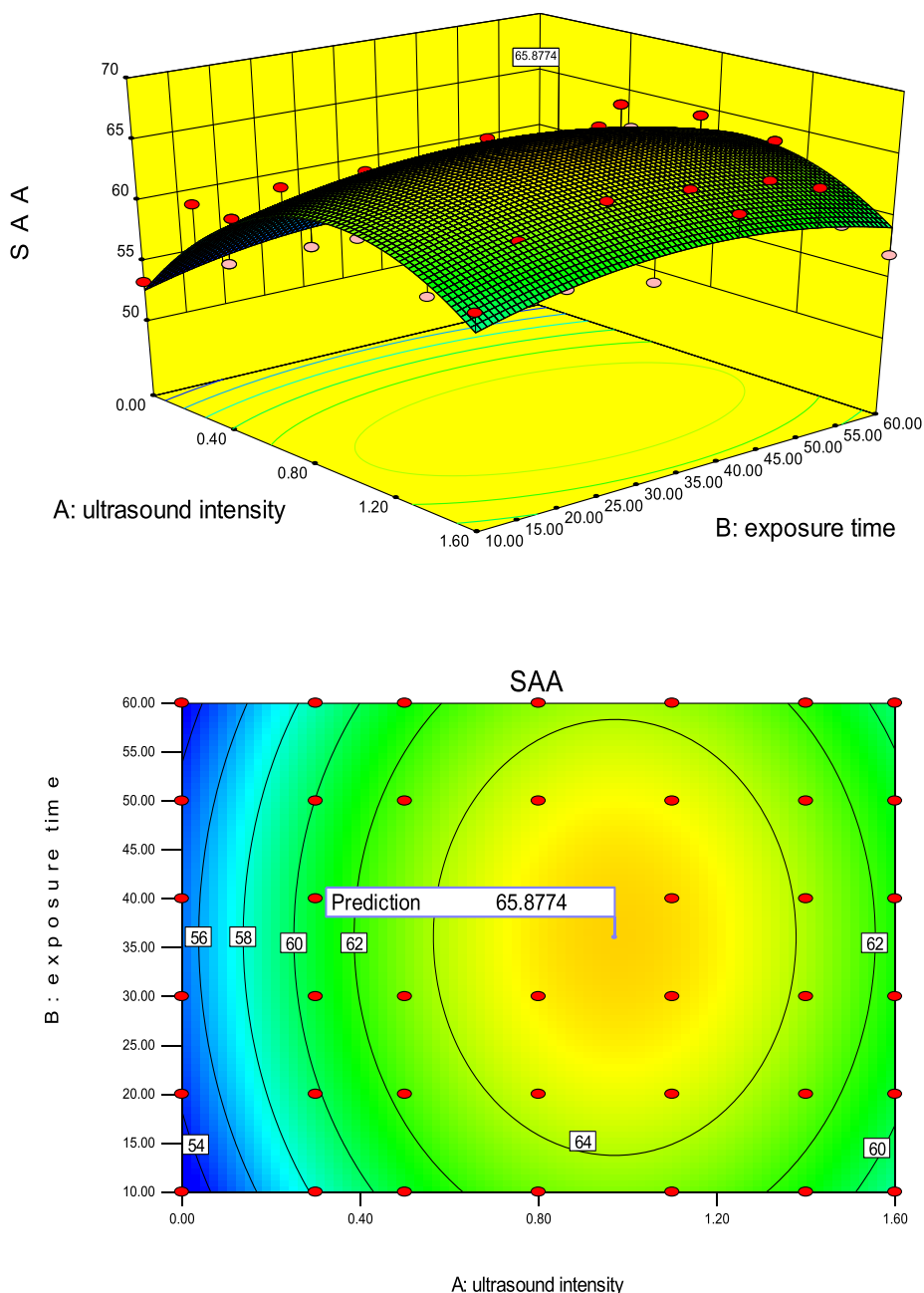


Fig. 3. The response surface of Anammox activity to exposure time and ultrasound intensity by RSM.

dehydrated samples were then rinsed with *tert*-butanol, and after desiccated and spaying coated with gold, the samples were observed by an SEM (Nova Nano SEM 450, FEI, USA).

### 3. Results and discussion

#### 3.1. Optimal ultrasound parameters selection

To investigate the impact of ultrasound intensity and exposure time on SAA, batch tests coupled with the RSM were used. The ultrasound frequency was set at 25.0 kHz. As shown in Fig. 2, under the irradiation of different ultrasonic intensity and exposure time, the AnAOB showed different degrees of activity. A series of different ultrasonic intensities (0.300, 0.500, 0.800, 1.10, 1.40, 1.60  $\text{W cm}^{-2}$ ) was adopted in the batch tests and all played a positive role in the improvement of SAA to a certain extent. The ultrasonic intensity of 1.10  $\text{W cm}^{-2}$  exhibited a better effect on improvement of Anammox activity than any other intensity. When the ultrasonic intensity was fixed at 1.10  $\text{W cm}^{-2}$ , the ultrasonic irradiation with 10.0, 20.0, 30.0, 40.0, 50.0, 60.0 s increased SAA by 18.0, 18.7, 22.7, 29.8, 25.9 and 19.7%, respectively. Under the same ultrasonic intensity, SAA was first increased and then decreased with the exposure time prolonging. This change rule of SAA under ultrasonic irradiation was similar to that in the research reported by Duan et al. (2011) [12]. This indicated that appropriate ultrasonic irradiation could improve the activity of AnAOB but excessive ultrasonic irradiation (excessive intensity or exposure time) would destroy the cell structure of AnAOB and thereby reduce their activity [19].

Based on the results of batch tests, an RSM was established to optimize the ultrasound parameters. The effects of two factors (the ultrasonic intensity and exposure time) were shown in Fig. 3, and the equation obtained by the reduced quadratic equation model fitting experiment results was as followed:

$$\text{SAA} = 65.54 + 3.10 \times A + 0.20 \times B - 7.26 \times A^2 - 2.36 \times B^2 \quad (1)$$

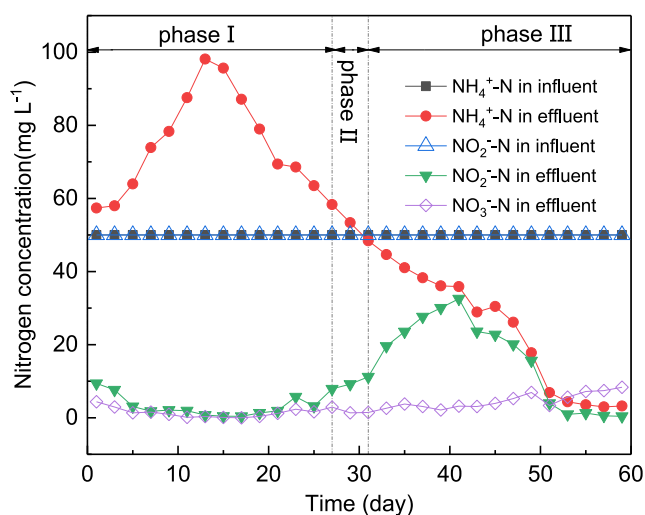
where A is the value of ultrasound intensity ( $\text{W cm}^{-2}$ ) and B is the value of exposure time (second, s).

The Model F-value of 44.1 implied the model was significant. The P value of the model was less than 0.100%, which means there was only a 0.0100% chance that a "Model F-Value" this large could occur due to noise. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. This model ratio of 22.1 indicated an adequate signal. This model can be used to navigate the design space.

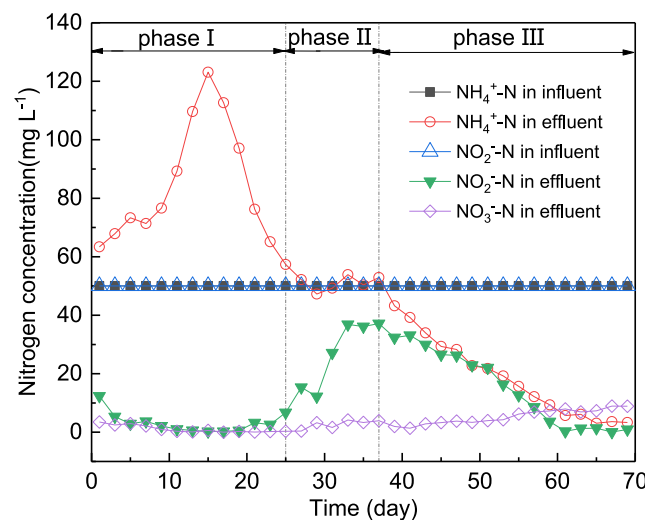
As calculated by numeric optimization of the reduced quadratic equation model and the convenience of actual operation, the optimum ultrasound intensity was 1.00  $\text{W cm}^{-2}$  and the optimum exposure time was 36.0 s. The ultrasound irradiation with the optimum intensity and exposure time resulted in a peak SAA of 65.9  $\text{mg N (g VSS)}^{-1} \text{d}^{-1}$ , which was significantly higher than SAA of the contrast group (53.3  $\text{mg N (g VSS)}^{-1} \text{d}^{-1}$ ). The optimum ultrasound intensity and exposure time combined with the ultrasound frequency was taken as the optimal ultrasound parameters for enhancement of Anammox performance in ABR<sub>U</sub>.

#### 3.2. Comparison of Anammox performance in ABR<sub>U</sub> and ABR<sub>C</sub>

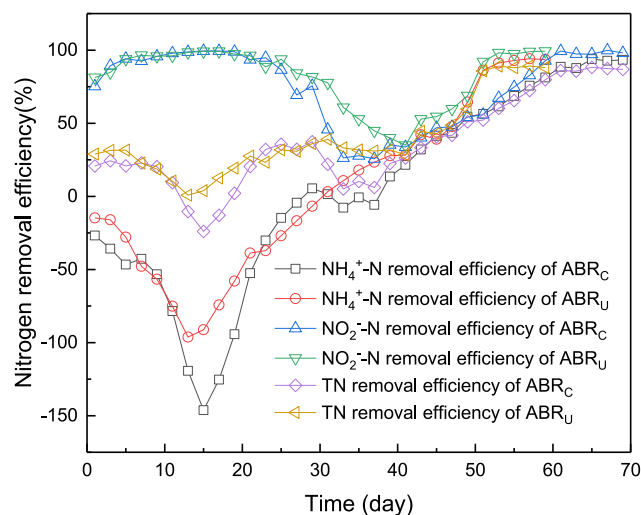
To investigate the effects of ultrasound irradiation on Anammox process performance in both start-up period and load-enhancing period, two systems defined as ABR<sub>C</sub> and ABR<sub>U</sub>, respectively, were operated in the identical parameters in parallel to formed a set of contrasts. But one was with ultrasonic treatment and the other was without ultrasonic treatment. According to batch tests and RSM calculation results, as well as referring to convenient, the determination of ultrasound irradiation were intensity of 1.00  $\text{W cm}^{-2}$  and exposure time of 36.0 s for actual implement in ABR<sub>U</sub> continuous experiment.



(a)



(b)



(c)

Fig. 4. Nitrogen variations during the start-up period of Anammox process: (a) Variations of nitrogen concentration with time during the start-up period in ABR<sub>U</sub>; (b) Variations of nitrogen concentration with time during the start-up period in ABR<sub>C</sub>; (c) Nitrogen removal efficiency in ABR<sub>C</sub> and ABR<sub>U</sub>.

**Table 2**

The summary of different ANAMMOX start-up processes.

T (°C)	Reactor	Inoculum	Day	Reference
30 ± 1	Amended UASB <sup>a</sup>	Mixed activated sludge	85	[19]
37	UFBR <sup>b</sup>	Sludge obtained from Anaerobic digester	107	[20]
33–34	SBR	Excess sludge from an upflow anaerobic sludge blanket	120	[21]
Room temperature	UBF <sup>c</sup>	The mixture of nitrification sludge (60%), anaerobic activated sludge (20%) and 20% anaerobic granular sludge	98	[22]
25 ± 1	ABR <sub>C</sub>	Aerobic activated sludge	69	This study
25 ± 1	ABR <sub>U</sub>	Aerobic activated sludge	59	This study

<sup>a</sup> UASB with amendment of bamboo charcoal.<sup>b</sup> Up-flow fixed-bed biofilm column reactor.<sup>c</sup> Up-flow blanket filter reactor run with the operational strategy of high nitrogen loading rate.

### 3.2.1. Start-up period of Anammox process

To start up the Anammox process, the influent concentration of  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_2^-\text{-N}$  in both reactors were  $50.0 \text{ mg L}^{-1} \text{ d}^{-1}$  in constant, respectively. Anammox processes in ABR<sub>C</sub> and ABR<sub>U</sub> were successfully started after day 69 and day 59, and the total nitrogen (TN) removal efficiency exceeded 70.0% and 80.0%, respectively. In terms of the variations of nitrogen concentration in effluent (showed in Fig. 4), the start-up period of Anammox process both in ABR<sub>C</sub> and ABR<sub>U</sub> was divided into three phases: sludge conversion phase, lagging phase and activity appearance phase.

In phase I, the sludge conversion phase, the  $\text{NH}_4^+\text{-N}$  concentration in the effluent was higher than that in the influent in both reactors, conversely, the  $\text{NO}_2^-\text{-N}$  concentration in the effluent was sharply decreased below approximately  $10.0 \text{ mg L}^{-1}$  at the very beginning and even closed to  $0 \text{ mg L}^{-1}$  during this course. It was due to the cell autolysis caused by the adaption of microorganism to the new environment and the inhibition of microbe survival in the anaerobic conditions thence leading to  $\text{NH}_4^+\text{-N}$  release from the inside of the lysed cell. The concentration of  $\text{NH}_4^+\text{-N}$  in the effluent increased in the first half phase and then decreased, while that change trend of  $\text{NO}_2^-\text{-N}$  and  $\text{NO}_3^-\text{-N}$  was on the contrary. In addition, the  $\text{NO}_2^-\text{-N}$  removal efficiency of ABR<sub>C</sub> and ABR<sub>U</sub> were both above 90.0%, and the maximum removal efficiency of  $\text{NO}_2^-\text{-N}$  was as high as 99.5% and 99.2%, respectively. In this duration, the main reaction leading to  $\text{NO}_2^-\text{-N}$  consumption in the reactors was endogenous denitrification, which was advanced by the organism released from the autolyzed cells. The decrease of the  $\text{NH}_4^+\text{-N}$  concentration and the increase of the  $\text{NO}_2^-\text{-N}$  concentration in the effluent was caused by the weakened autolysis in the last half phase as microorganisms gradually adapted to the new environment. The duration of the phase I of the two reaction systems was roughly the same, day 25 in ABR<sub>C</sub> and day 27 in ABR<sub>U</sub>. However, it was worth noting that the maximum concentration of  $\text{NH}_4^+\text{-N}$  in the ABR<sub>C</sub> effluent ( $110 \sim 123 \text{ mg L}^{-1}$ ) was more than twice the influent, while that in the effluent of ABR<sub>U</sub> was much lower ( $98.1 \text{ mg L}^{-1}$ ). It might be owing to that the selected ultrasound irradiation resulted in a better adaptation of microbe to the anaerobic condition in ABR, although at normal temperature.

In phase II, the lagging phase, the  $\text{NH}_4^+\text{-N}$  concentration of the effluent in two reactors was close to that of the influent. We regarded this as the characteristic of this phase, that was, the difference in  $\text{NH}_4^+\text{-N}$  concentration between influent and effluent was within  $5.00 \text{ mg L}^{-1}$ . Although there was still no sign of  $\text{NH}_4^+\text{-N}$  being removed in this phase, its concentration of the effluent had already gradually decreased. Simultaneously, the concentration of the  $\text{NO}_2^-\text{-N}$  in the effluent increased, which indicating that the endogenous denitrification turned to be weaker. The above two phenomena were related to the weakening of cell autolysis. As the process ran, microorganism in the reactors adapted the operational condition step by step and the selective retention of functional bacterial was gradually completed, wherefore the biomass of autolytic lysis was getting less. It not only reduced the  $\text{NH}_4^+\text{-N}$  flowing out with the lysed intracellular material in the effluent, but also inhibited the endogenous denitrification. Since the organic matter produced by cell autolysis was gradually washed out, which made endogenous denitrification lack of organic carbon source and was inhibited, so

the removal efficiency of  $\text{NO}_2^-\text{-N}$  in the ABR<sub>C</sub> and ABR<sub>U</sub> was reduced to 25.7% and 77.6%, respectively. What's more, the duration of lagging phase in ABR<sub>U</sub> was much shorter than that in ABR<sub>C</sub>, one was 4 days while the other was 12 days, which demonstrated the ability to shorten the start-up time of the Anammox process by the specific ultrasound irradiation. Moreover, the  $\text{NO}_2^-\text{-N}$  removal efficiency of ABR<sub>U</sub>, under the condition of ultrasonic intervention, could reach to 77.6%~81.7% even during the lagging phase, which was almost two or three times that of the control group in ABR<sub>C</sub>. It exhibited a better nitrogen removal performance in ABR<sub>U</sub> than in ABR<sub>C</sub>, although there was no Anammox activity appearance in this phase.

In phase III, the activity appearance phase, the  $\text{NH}_4^+\text{-N}$  concentration of the effluent in two reactors both decreased continuously. The removal of  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_2^-\text{-N}$  of the influent began to be synchronized a continued since day 39 in ABR<sub>C</sub> and day 33 in ABR<sub>U</sub>, which showed that the Anammox activity appeared. In this phase, the average removal efficiency of  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_2^-\text{-N}$  reached about 90.0% in ABR<sub>C</sub> and 97.0% and ABR<sub>U</sub> at steady state while the mean removal efficiency of TN was 86.0% and 88.0%, correspondingly. This marked the successful and stable start-up of Anammox process at the normal temperature in both ABRs with or without specific ultrasound irradiation. It could not be ignored that the time of Anammox activity in ABR<sub>U</sub> appeared 6 days earlier than in ABR<sub>C</sub>. Also, the start-up period in ABR<sub>U</sub> was 10 days shorter than in ABR<sub>C</sub>, since one was 59 days and the other was 69 days. This explained that the stimulation of specific ultrasound irradiation could accelerate the start-up period of Anammox process and achieve an efficient nitrogen removal performance even at normal temperature.

According to two reactors Anammox process start-up period time-spending and nitrogen removal performance comparison, the specific ultrasound irradiation determined based on the results of RSM not only accelerated the start-up course but also promoted the Anammox activity at normal temperature. The main reason of ultrasound advantageous stimulation of the Anammox process might be that the low intensity ultrasound cause cavitation and shearing stress could produce beneficial effect on the biological reaction. On the one hand, owing to the ultrasonic exposure, the stable cavitation caused by the relatively low intensity ultrasound irradiation could be favorable to enhance the mass transfer and fluid mixing, which leads to positive effect on the rate of Anammox bacterium reaction in the ultrasound irradiation system. On the other hand, the shearing stress caused by the low intensity ultrasound irradiation could thin the wall and the membrane of the cell so as to increase its permeability and strengthen the rate of mass transfer.

As mentioned above, the Anammox process was successfully started up after 69 days in ABR<sub>C</sub> and 59 days in ABR<sub>U</sub>, which was signed by the phenomenon that most of the  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_2^-\text{-N}$  in the influent were removed and the maximum of TN removal efficiency reached about 85.0% in both reactors.  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_2^-\text{-N}$  are both not only the matrix and the self-inhibitor of the Anammox reaction. It was reported that the Anammox reaction is more sensitive to  $\text{NO}_2^-\text{-N}$  inhibition [15]. Therefore, the phenomenon that the concentration of  $\text{NO}_2^-\text{-N}$  in effluent is lower than  $1.00 \text{ mg L}^{-1}$  for two consecutive days is also regarded as a decisive factor for the successful start of the Anammox process. Compared with other studies on Anammox start-up before, the time-

spending on Anammox start-up period in this study was much shorter (showed in Table.2 [20-23]), even though at normal temperature, which was a relatively negative living condition of AnAOB. Except the specific ultrasound irradiation bio-stimulation, the superiority of ABR on the cultivation of bacteria with low growth rate and long doubling time like AnAOB also contributed to the rapid start-up of the Anammox process. Moreover, the check valve was used to replace the exhaust of the water seal device, which avoided the matter of dissolved oxygen being brought in by the water seal device reverse suction, and better ensured the anaerobic environment and the stability of the reactor operation. In addition, the inoculated sludge collected from the sewage treatment plant had AnAOB pre-enrichment, so its indigenous AnAOB content was higher. Due to the factors discussed above, under anaerobic and normal temperature conditions, non-functional aerobic microorganisms in the Anammox system in two reactors were gradually inactivated and washed out in a relatively fast process, while AnAOB were selectively retained, making the Anammox reaction in the two reactors start quickly.

### 3.2.2. Nitrogen load-enhancing period of Anammox process

To evaluate the long-term effect of specific ultrasonic radiation on the performance of the Anammox process and its nitrogen removal performance under the condition of enhancing the nitrogen loading rate (NLR), the nitrogen removal effects in ABR<sub>C</sub> and ABR<sub>U</sub> were observed. And the nitrogen load enhancement was achieved by the increasing of NH<sub>4</sub><sup>+</sup>-N and NO<sub>2</sub><sup>-</sup>-N concentration in the influent. During the nitrogen load-enhancing period, the concentration variation of the three nitrogen forms (NH<sub>4</sub><sup>+</sup>-N, NO<sub>2</sub><sup>-</sup>-N and NO<sub>3</sub><sup>-</sup>-N), nitrogen removal rate (NRR) and nitrogen removal efficiency (NRE) of ABR<sub>C</sub> and ABR<sub>U</sub> (without or with the application of ultrasound field) were recorded and illustrated in Fig. 5. Under the same operational strategy parameters as the start-up period, like pH and temperature, the two reactors showed differentiated nitrogen removal performance. For ABR<sub>C</sub>, without the implication of ultrasound, from day 73 to day 87, the influent NH<sub>4</sub><sup>+</sup>-N and NO<sub>2</sub><sup>-</sup>-N concentrations were raised stepwise from 50.0 mg L<sup>-1</sup> to 200 mg L<sup>-1</sup>, respectively and synchronously. And correspondingly, the NRR increased stepwise from 0.0900 kg N m<sup>-3</sup> d<sup>-1</sup> to 0.320 kg N m<sup>-3</sup> d<sup>-1</sup>. At the end of the experiment (from day 71 onwards), the maximal NRR in ABR<sub>C</sub> was 0.333 kg N m<sup>-3</sup> d<sup>-1</sup>, achieved on day 93 with the influent NH<sub>4</sub><sup>+</sup>-N and NO<sub>2</sub><sup>-</sup>-N concentrations of 200 mg L<sup>-1</sup>. The steady treatment performance was considered to be arrived from day 71 to day 101, with an average TN, the NH<sub>4</sub><sup>+</sup>-N and the NO<sub>2</sub><sup>-</sup>-N removal efficiency of 81.9, 81.5 and 97.0%, respectively. For ABR<sub>U</sub>, under the exposure to the specific ultrasound, the NLR gradually increased with a stable and a high NRR. And the maximal NRR reached up to as high as 0.430 kg N m<sup>-3</sup> d<sup>-1</sup>, achieved on day 97 with the influent NH<sub>4</sub><sup>+</sup>-N and NO<sub>2</sub><sup>-</sup>-N concentrations of 250 mg L<sup>-1</sup>. The remarkable Anammox process treatment performance can be explained that the AnAOB became more active under the positive influence of specific ultrasound irradiation and exhibited strong activity. The period from day 61 to day 101 was regarded as the steady operation phase, in which the mean TN, the NH<sub>4</sub><sup>+</sup>-N and the NO<sub>2</sub><sup>-</sup>-N removal efficiency was 85.1, 84.8 and 98.9%, respectively.

There is a significant observation that there was a more superior and more quickly increase of treatment performance of Anammox reactor with specific ultrasound field application during nitrogen load-enhancing period. Thanks to the positive effects of ultrasonic irradiation, the max NRR (0.430 kg N m<sup>-3</sup> d<sup>-1</sup>) and NLR (0.500 kg N m<sup>-3</sup> d<sup>-1</sup>) in ABR<sub>U</sub> was obviously better than the max NRR (0.333 kg N m<sup>-3</sup> d<sup>-1</sup>) and NLR ((0.400 kg N m<sup>-3</sup> d<sup>-1</sup>) in ABR<sub>C</sub> (Fig. 5). Besides, under the ultrasonic irradiation, the NRR and NLR increased by 25.0% and 30.3% respectively, which was a considerable performance improvement. AnAOB were the functional bacteria responsible for Anammox process. The positive effect of the ultrasound on biological processes could be mainly attributed to that the low frequency and intensity ultrasound could stimulate enzyme activity, improve cell membrane permeability,

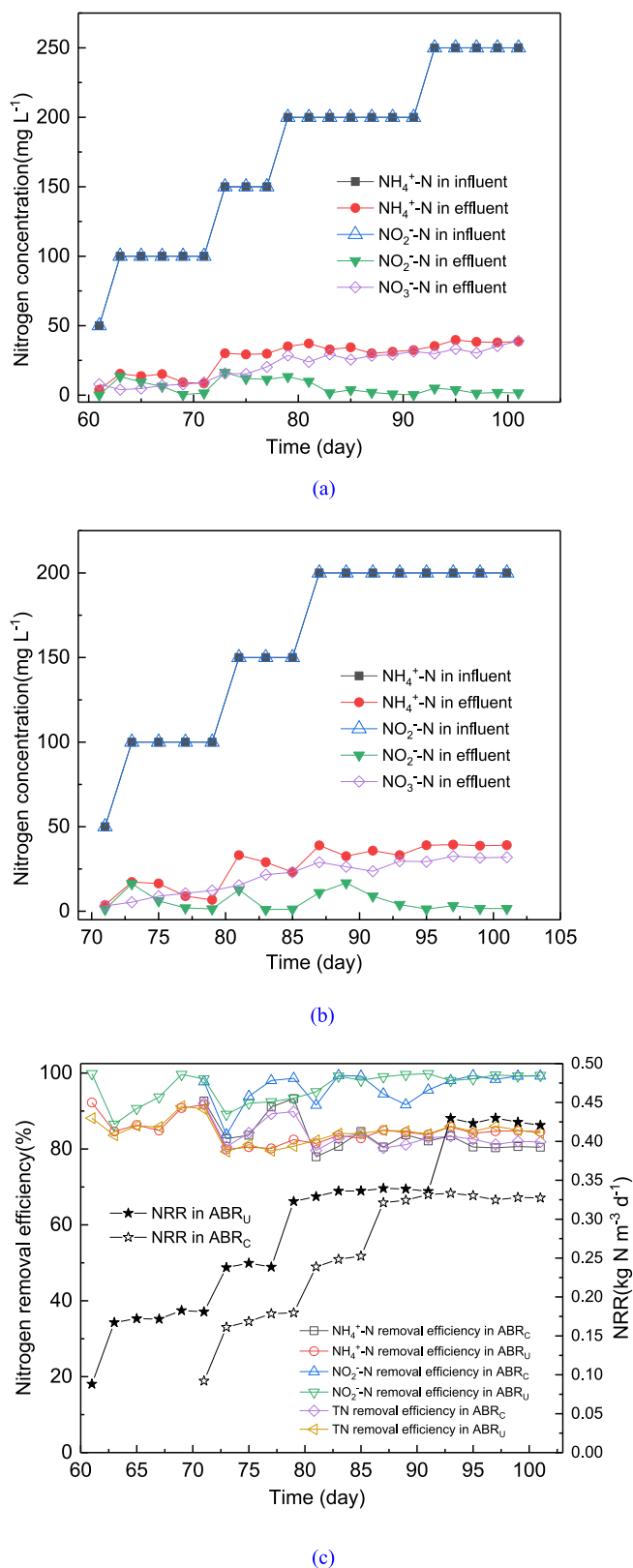


Fig. 5. Nitrogen variations during the nitrogen load-enhancing period of Anammox process: (a) Variations of nitrogen concentration with time in the Anammox nitrogen load enhancement period in ABR<sub>U</sub>; (b) Variations of nitrogen concentration with time in the Anammox nitrogen load enhancement period in ABR<sub>C</sub>; (c) Nitrogen removal efficiency and NRR in ABR<sub>C</sub> and ABR<sub>U</sub>.

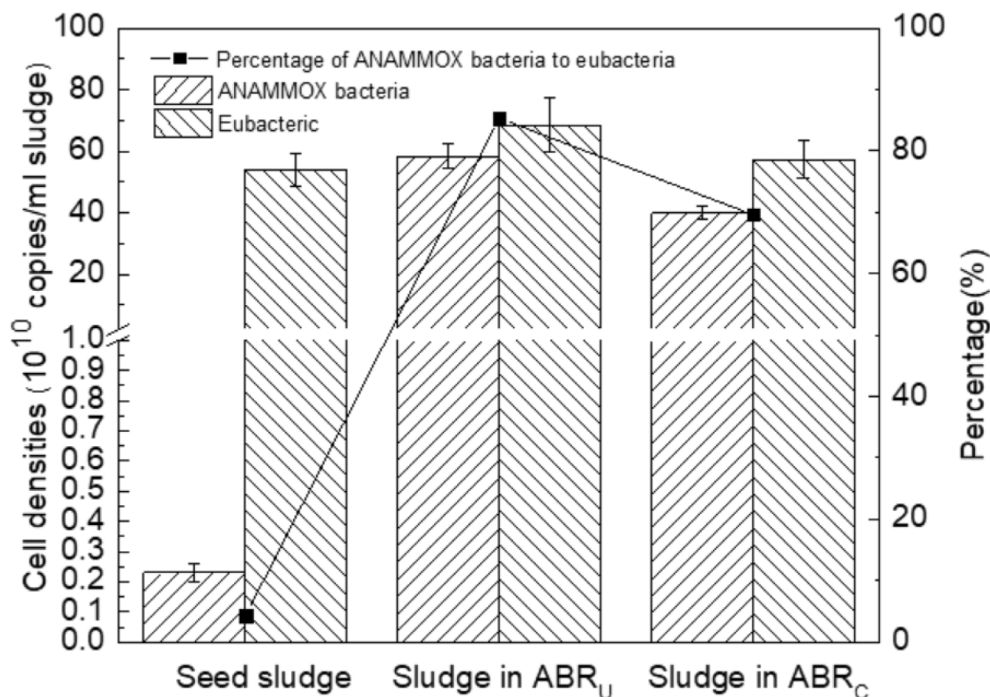


Fig. 6. The enrichment levels of AnAOB in the mature sludge in ABR<sub>C</sub> and ABR<sub>U</sub>.

promote cell growth and biosynthesis of the functional bacteria [24]. With the similar biological mechanism, Anammox process was enhanced by ultrasound irradiation with the low frequency and intensity. The ABR<sub>U</sub> displayed an excellent Anammox performance at a normal temperature (25.0 °C) might on account of the treatment of specific ultrasound field. Furthermore, the local turbulences and liquid micro-circulations (acoustic streaming) formed by the ultrasound might be utilized to strengthen the mass transfer processes [25]. Meanwhile, the shearing stress generated by ultrasound is conducive to sludge granulation, which can accelerate the enrichment of AnAOB and the washed-out of other non-functional bacteria, thereby improving the competitive advantage of AnAOB in the system, and making the reactor exhibit intentional Anammox nitrogen removal performance. Additionally, the outstanding resistance of ABR to load enhancement and microbial retention capacity also make a non-negligible contribution to the Anammox performance. In conclusion, it became clear that the application of specific ultrasound irradiation is an effective method to promote the treatment performance of Anammox process and quicken the start-up time of it.

### 3.3. Microbial community analysis and morphology observation

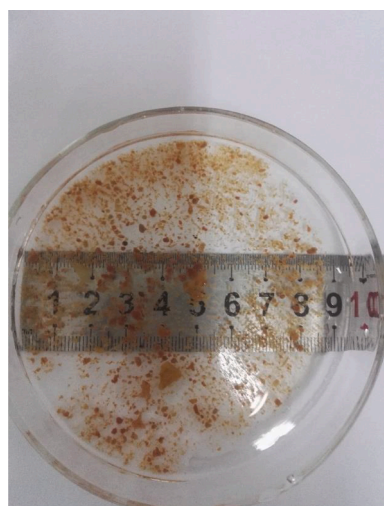
In order to further explore the mechanism of specific ultrasonic radiation at normal temperature to shorten the start-up time of the Anammox process and improve its nitrogen removal performance, RTQ-PCR was used to evaluate the enrichment levels of AnAOB in seed sludge and mature sludge (day 101) after cultivation. As shown in the Fig. 6, after 101 days of continuous operation of the Anammox process, the densities of AnAOB cell and eubacterial cell in the sludge of the ABR<sub>U</sub> and the ABR<sub>C</sub> were quite different, and there were significant changes compared with the densities of bacterial communities in the seed sludge. The percentage of AnAOB to eubacteria in the seed sludge, the mature sludge in ABR<sub>U</sub> and the mature sludge in ABR<sub>C</sub> were 0.430, 85.3 and 69.6%, respectively. The results indicated that after a period of operation, the AnAOB in the two reactors were both effectively enriched. The multi-stage retention capacity of the three compartments of the ABR enabled the reactors to hold high biomass.

However, the density of AnAOB in ABR<sub>U</sub> irradiated by specific

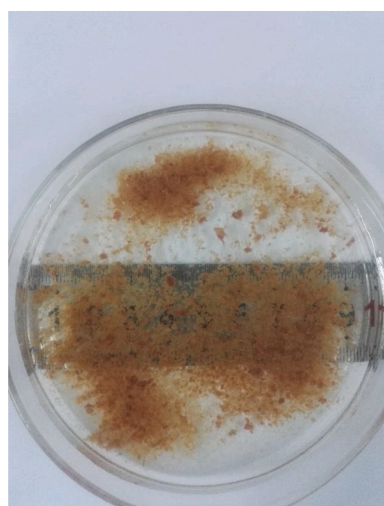
ultrasonic waves and their proportion in eubacteria were significantly higher than that of ABR<sub>C</sub> without ultrasonic treatment. In this experiment, since the seed sludge inoculated was aerobic activated sludge, the accumulation of AnAOB and the elimination of other bacteria were important, which links for the rapid start-up and high efficiency and stability of the Anammox process. Specific ultrasonic irradiation enhanced the succession process of other bacteria in the inoculated sludge in ABR<sub>U</sub> to AnAOB. On the one hand, ultrasonic treatment has different effects on the structure of microorganisms, so different microorganisms have different tolerance and sensitivity to ultrasonic stimulation [26]. Taking advantage of this difference, the specific ultrasonic irradiation in this experiment formed a specific growth environment pressure on the microbial colonies in the sludge, which was uniquely favorable to AnAOB, thereby promoting the growth and accumulation of AnAOB while eliminating non-target bacteria. Moreover, the shear stress generated by the vibration of the ultrasonic stream also promoted the washing of inactivated bacteria from the inside of the reactor [27]. On the other hand, transient ultrasound irradiation can accelerate the exchange of substances and strengthen biological activity [28]. The shear stress and steady cavitation caused by low-intensity ultrasound irradiation can change the cell morphology, make the cell wall and cell membrane thinner, enhance the permeability of the cell membrane, promote the mass transfer efficiency of substances inside and outside the cell, accelerate cell proliferation and produce more metabolites, and improve the activity expression AnAOB [12,29]. In addition, the application of ultrasound can reduce the sensitivity of Anammox process to the temperature during operation [14]. Even if the temperature condition in this study was lower than the optimum temperature for AnAOB, they could still accumulate at a faster rate under the action of specific ultrasonic irradiation.

The mature sludge samples were taken from ABR<sub>U</sub> (sludge A) and ABR<sub>C</sub> (sludge B) for morphological observation on day 101. Previous studies have shown that the color of sludge and the content of heme-C can be used as two indicators of Anammox activity [30]. Heme-C is an important part of enzymes in AnAOB and is related to Anammox activity. Both sludge A and sludge B were reddish brown (shown in Fig. 7), which reflected that the dominant flora in the mature sludge at that time was AnAOB. However, sludge A was formed into granulated sludge,





(a)

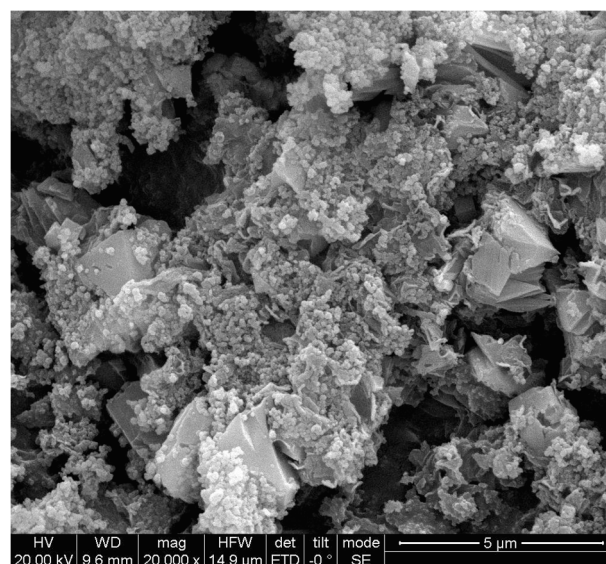


(b)

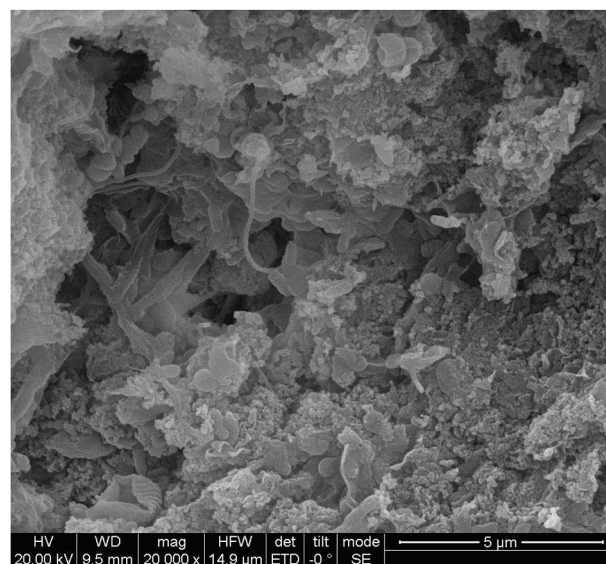
**Fig. 7.** Image of mature sludge (sludge A and sludge B) in ABR<sub>C</sub> and ABR<sub>U</sub>. (a) Image of sludge A at the end of the nitrogen load-enhancing period in ABR<sub>U</sub>. (b) Image of sludge B at the end of the nitrogen load-enhancing period in ABR<sub>C</sub>.

while sludge B was mostly flocculent sludge. Compared with flocculent sludge, granular sludge has stronger settling performance, and is easier to separate from the treated wastewater, which is beneficial to maintain the higher effluent water quality. The vibration of the ultrasonic sound stream creates holes in the sludge and promotes the granulation of the sludge. SEM was used to observe the microstructure of sludge A and sludge B more deeply. The microscopic morphology of the two sludges is significantly different (shown in Fig. 8). For sludge A, the cultivated granular sludge had a single strain, mainly spherical bacteria, coated with fragmented colloidal substance. The fragmented colloidal substance is EPS, which is conducive to the formation of Anammox granular sludge, and at the same time can improve the ability to resist load impact and maintain the stability of the Anammox process [2,31]. For sludge B, the structure of the flora is mainly coccus, but also a small number of filamentous bacteria and brevis-bacterium. The results showed that a more compacted microbial structure was formed by the stimulation of the specific ultrasound irradiation, and the microbial morphology changed from multiple to single. The compacted microbial structure of Anammox sludge in the ABR<sub>U</sub> made the Anammox process stable.

Microbial analysis demonstrated that ultrasonic irradiation with the specific parameters play key roles on Anammox process. On one hand,



(a)



(b)

**Fig. 8.** SEM of mature sludge (sludge A and sludge B) at the end of the nitrogen load-enhancing period in ABR<sub>U</sub> and ABR<sub>C</sub> respectively. (a) SEM of sludge A at the end of the nitrogen load-enhancing period in ABR<sub>U</sub>. (b) SEM of sludge B at the end of the nitrogen load-enhancing period in ABR<sub>C</sub>.

the ultrasound improved the growth and reproduction ability of AnAOB and its competitive advantage over other bacteria, so that AnAOB reached a higher cell densities and enrichment levels in ABR<sub>U</sub>. On the other hand, the ultrasound promote granulation of Anammox sludge so as to enhance the biomass retention of ABR<sub>U</sub>. Therefore, the rapid start-up of Anammox process and its efficient and stable nitrogen removal performance was achieved in ABR<sub>U</sub>.

#### 4. Conclusions

The low-intensity ultrasonic treatment enhanced the Anammox start-up and operational performances in the ABR at normal temperature. Due to the positive stimulation of AnAOB by the specific ultrasonic irradiation, the start-up time of Anammox process in ABR<sub>U</sub> (59 d) was evidently shorter than in ABR<sub>C</sub> (69 d). At the end of the nitrogen load-enhancing period, NLR ( $0.500 \text{ kg N m}^{-3} \text{ d}^{-1}$ ) and NRR ( $0.430 \text{ kg N m}^{-3} \text{ d}^{-1}$ ) in

ABR<sub>U</sub> were both higher than NLR (0.400 kg N m<sup>-3</sup> d<sup>-1</sup>) and NRR (0.333 kg N m<sup>-3</sup> d<sup>-1</sup>) in ABR<sub>C</sub>. What's more, the low-intensity ultrasound treatment enhanced the stability of the Anammox process in the ABR. The results of RTQ-PCR and SEM demonstrated that the specific low-intensity ultrasound treatment improved the enrichment levels of AnAOB and strengthened the granulation of cultivated sludge so as to realize the stable and efficient operation of Anammox process at normal temperature.

#### CRedit authorship contribution statement

**Luzi Yuan:** Methodology, Investigation, Data curation, Writing - original draft. **Tao Wang:** Funding acquisition, Project administration, Supervision. **Fanghua Xing:** Investigation, Resources. **Xian Wang:** . **Hongying Yun:** .

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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