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Research Article

Feasibility of low-intensity ultrasound treatment with hydroxylamine to accelerate the initiation of partial nitrification and allow operation under intermittent aeration

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ABSTRACT

Partial nitrification is a key aspect of efficient nitrogen removal, although practically it suffers from long start-up cycles and unstable long-term operational performance. To address these drawbacks, this study investigated the effect of low intensity ultrasound treatment combined with hydroxylamine (NH_2OH) on the performance of partial nitrification. Results show that compared with the control group, low-intensity ultrasound treatment (0.10 W/mL, 15 min) combined with NH_2OH (5 mg/L) reduced the time required for partial nitrification initiation by 6 days, increasing the nitrite accumulation rate (NAR) and ammonia nitrogen removal rate (NRR) by 20.4% and 6.7%, respectively, achieving 96.48% NRR. Mechanistic analysis showed that NH_2OH enhanced ammonia oxidation, inhibited nitrite-oxidizing bacteria (NOB) activity and shortened the time required for partial nitrification initiation. Furthermore, ultrasonication combined with NH_2OH dosing stimulated EPS (extracellular polymeric substances) secretion, increased carbonyl, hydroxyl and amine functional group abundances and enhanced mass transfer. In addition, 16S rRNA gene sequencing results showed that ultrasonication-sensitive *Nitrospira* disappeared from the ultrasound + NH_2OH system, while *Nitrosomonas* gradually became the dominant group. Collectively, the results

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of this study provide valuable insight into the enhancement of partial nitrification start-up during the process of wastewater nitrogen removal.

Introduction

Partial nitrification is a part of the ammonia oxidation process ($NH_4^+ \rightarrow NO_2^-$) and is currently one of the more commonly applied process steps in the field of biological nitrogen removal from wastewater, often being used in combination with denitrification or anaerobic ammonia oxidation processes. Compared with complete nitrification and denitrification, partial nitrification-denitrification can theoretically reduce the system requirements for aeration by 25% and for organic carbon by 40%, while partial nitrification coupled with anaerobic ammonia oxidation can avoid the need for organic carbon supplementation and reduce residual sludge production by 80% (Li et al., 2019; Wang et al., 2018). Therefore, partial nitrification can save the energy requirements of wastewater treatment processes and reduce organic carbon consumption, resulting in it becoming an efficient and indispensable part of many novel nitrogen removal processes. The key to achieving partial nitrification is to control the ratio of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB), leading to the accumulation of NO_2^--N and the elimination of nitrate nitrogen (NO_3^--N) (Duan et al., 2019). Currently, many strategies have been used to regulate, selectively inhibit or eliminate NOB, mainly by combining the different physiological characteristics of AOB and NOB, including intermittent aeration, the control of sludge retention time (SRT), pH, free ammonia (FA), dissolved oxygen (DO), free nitrous acid (FNA) and sulfide concentrations (Katsogiannis et al., 2003; Sekine et al., 2020; Wang et al., 2018; Yang et al., 2018). All these strategies have been shown to effectively suppress NOB to varying extents, although they suffer from disadvantages such as complex operational constraints, long partial nitrification initiation cycles and unstable long-term process performance. In recent years, some novel partial nitrification process start-up methods have received much research attention, including hydroxylamine (NH_2OH) dosing and low-intensity ultrasound treatment, both of which are rapid and effective NOB inhibition strategies with a low impact on AOB (Katsogiannis et al., 2003; Tian et al., 2021).

During the nitrification process, ammonia is first oxidized to NH_2OH by ammonia monooxygenase (AMO) and NH_2OH is then oxidized to NO_2^--N by hydroxylamine oxidoreductase (HAO). NH_2OH is an intermediate in the nitrification process, which can promote the rate of ammonia oxidation and increase AOB activity (Harper et al., 2009; Tian et al., 2021). Furthermore, NH_2OH can inhibit NOB activity through NO and N_2O produced during ammonia-nitrogen conversion, leading to nitrite accumulation (Zhao et al., 2021). Therefore, the use of NH_2OH as an inhibitor rapidly triggers the accumulation of nitrite, which is an effective strategy for achieving partial nitrification. Zhao et al. (2021) showed that after the addition of NH_2OH, the nitrite accumulation rate (NAR) reached 95.06% and the ammonia nitrogen removal rate (NRR) reached 94.78%. However, the short-term addition of NH_2OH during nitrification could not maintain stable partial nitrification long-term, as shown by the decrease in NAR from 95.08% to 0.19% within 21 days after the cessation of NH_2OH dosing (Zhao et al., 2021). This phenomenon indicates that NOB gradually adapt to NH_2OH inhibition and the abundance of NOB gradually increases. However, low-intensity ultrasound is able to continuously inhibit NOB activity, overcoming the limitations associated with NH_2OH dosing.

Ultrasound treatment has been widely investigated and applied in the field of enhanced aerobic sludge. High-intensity ultrasound (>100 W/cm^2) can be used for sludge disintegration to achieve a reduction in sludge volume, while low-intensity ultrasound (<10 W/cm^2) also has multiple beneficial effects on the target sludge, including mechanical, cavitation and thermal effects (Arefi-Oskoui et al., 2019). It has previously been demonstrated that suitable ultrasound treatment can promote cell wall penetration and enhance transfer efficiency, thus facilitating cellular anabolic processes (Lin and Wu, 2002). Furthermore, it has been shown that low-intensity ultrasound increased the relative abundance of *nitrosomonas*, while reducing the number of nitrifying bacteria, achieving up to 99% nitrite accumulation (Zhang et al., 2011; Zheng et al., 2019). The different tolerance of AOB and NOB to ultrasonic stimulation (Zheng et al., 2016), allows low-intensity ultrasound treatment to improve partial nitrification by affecting the activity of both AOB and NOB, increasing the activity of key enzymes required for partial nitrification. However, in order to prevent excessive microbial destruction by low-intensity ultrasound treatment, the duration of ultrasonic treatment during each operation cycle must be kept relatively short, providing AOB with sufficient recovery time and extending partial nitrification periods to up to 24 days (Tian et al., 2021). The addition of NH_2OH accelerates microbial metabolism and induces the secretion of more extracellular polymeric substances (EPS) to enhance cell resistance, thus accelerating the partial nitrification start-up process. Eventually, the limitations of ultrasound treatment alone could be overcome.

Although previous studies have reported that both ultrasonication and NH_2OH have positive effects on partial nitrification, they also have certain drawbacks. Previous studies have investigated the effects of different means of promotion on partial nitrification, although they have generally assessed promotion mechanisms in isolation. Therefore, the present study investigated the effects of two means of promotion in combination on partial nitrification, with the aim of further improving partial nitrification systems. This study investigated whether a combination of NH_2OH dosing and low-intensity ultrasound treatment under intermittent aeration conditions, can synergistically affect the operational stability of partial nitrification and overcome the limitations of long start-up periods using ultrasonication and unstable performance during long-term operation using NH_2OH dosing. The NAR and NRR were measured under different operational conditions and the characteristic changes to sludge morphology, microbial community structure and microbial-mediated EPS were determined during reactor operation, to investigate the mechanism of the effect of ultrasound treatment coupled with NH_2OH dosing on partial nitrification.

1. Materials and methods

1.1. *Inoculated sludge and experimental water*

Inoculated sludge was obtained from nitrifying sludge that had been domesticated in the laboratory. After inoculation of the reactor, the mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) concentrations were approximately 1960 and 1445 mg/L, respectively. The shrimp farming wastewater was collected from an aquaculture base in Huadu, Guangdong Province (China). The wastewater contained a high ammonia nitrogen concentration and low COD, with the water quality meeting the requirements of the partial nitrification process. Synthetic wastewater was used in this study to simulate real wastewater while maintaining a consistent wastewater composition without experimental interference. The NH_4^+-N concentration and pH of synthetic wastewater were supplemented by the addition of $(NH_4)_2SO_4$ and $NaHCO_3$, respectively, while sodium acetate was added as a readily biodegradable COD substrate. Detailed information on other chemicals can be found in Appendix A Text S1.

1.2. *Experimental setup and operational procedure*

1.2.1. *Single-factor experiments*

In order to carry out sequencing batch reactor (SBR) operation experiments using the control reactor (CR), ultrasound reactor (UR), hydroxylamine reactor (HR) and coupled ultrasound-hydroxylamine reactor (UHR), it was necessary to initially determine the optimal operating parameters for each group of reactors. Therefore, single-factor experiments were conducted. The ideal optimal working parameters were determined from preliminary experiments (Appendix A Text S7) and the available literature (Miao et al., 2020; Tora et al., 2011; Ye et al., 2019). The initial parameters were as follows: $T = 26 \pm 2$ °C, pH = 8.3, solid-liquid ratio = 1:3, single aeration time = 15 min, DO = 0.8–1.2 mg/L, NH_4^+-N concentration = 125 mg/L, COD concentration = 10–20 mg/L and HRT = 13.33 hr. The NH_4^+-N concentration of the synthetic wastewater was set at 125 mg/L, as the NH_4^+-N concentration of the actual wastewater is too low to facilitate the partial nitrification reaction (Ye et al., 2019). In addition, the partial nitrification process is intended to pretreat shrimp aquaculture wastewater with NH_4^+-N concentrations ranging between 100 and 150 mg/L, so 125 mg/L NH_4^+-N was considered a relevant concentration. The single-factor experiments were conducted in borosilicate glass bottles with a working volume of 400 mL, and the experimental time was determined based on the time required for the complete removal of ammonia nitrogen. The NAR and ammonia nitrogen complete removal time were selected as the dependent variables for all single factor experiments, reflecting the partial nitrification performance of the reactor.

The effects of various factors were determined on the NAR and ammonia nitrogen complete removal time, including varying ratios of anaerobic time to aeration time, aeration flow rate, ultrasonication intensity, ultrasonication duration and NH_2OH concentration. The optimal operating schemes for CR, UR and HR can be derived from the results of the single-factor experiments. Finally, the UHR operating scheme was derived by combining the optimal operating schemes for the UR and HR systems as the operational parameters for the UHR. The design of single-factor experimental parameters is shown in Table 1.

1.2.2. *SBR design and experimental procedure*

A Plexiglass SBR (400 mL working volume) was used for the partial nitrification experiments, equipped with an oxygenation pump for aeration and a magnetic stirring device for continual mixing during the reaction phase. Four reactor systems were prepared: CR, UR, HR and UHR reactors (Fig. 1). The reactor was operated in sequential batch mode for 15 days, completing 2 cycles per day for a total of 30 cycles. Each cycle lasted for a 12 hr period, which was divided into feeding (stage I), ultrasonication (stage II), intermittent aeration (stage III) and drainage (stage IV). In the feeding stage (I) (30 min duration), 300 mL of synthetic wastewater was pumped into the SBR systems via a peristaltic pump, with each SBR having been loaded with 100 mL of partial nitrification sludge. The CR and UR systems only had synthetic wastewater fed into the system, while HR and UHR also had a NH_2OH solution added to the synthetic wastewater, controlling the NH_2OH concentration at 5 mg/L. The wastewater exchange rate was 75%. In the ultrasonication stage (II) (30 min duration), ultrasonication was performed at the end of the feeding period, with one 15 min ultrasonic treatment applied to the UR and UHR systems each cycle at a frequency of 25 kHz and power of 900 W, while the other reactor groups remained in an idle state. Ultrasonication was performed in intermittent mode with 2 sec of operation and 5 sec of rest, ensuring that the temperature of the solution did not increase during ultrasonication. Schlafer et al. (2000) showed that intermittent and repeated sonication was more beneficial than continuous and prolonged sonication for the activation of microbial activity. In the intermittent aeration phase (III) (10 hr duration), after the sonication period, a 45 min anoxic phase was followed by alternate aerobic (15 min) and anoxic (45 min) phases until the end of the intermittent aeration period. In drainage stage (IV) (1 hr duration), the unit was stopped at the end of the reaction and drained (15 min) after a 30 min settling period. Finally, the reactor remained idle (15 min) prior to the next cycle. The reactor was agitated continually during the intermittent aeration phase by magnetic stirring and the temperature was maintained at 26 ± 2°C. The pH was maintained between 7.8 and 8.2. The HRT remained constant at 13.33 hr. The changes in nitrogenous pollutant concentration, NAR, NRR, sludge particle size, sludge morphology, ORP (oxidation–reduction potential), EPS and microbial community were investigated during the

Table 1 – Parameters of single factor experiment.

Parameters	CR	UR	HR
Aeration flow rate (mL/min)	200, 400, 600, 800	400	400
Anaerobic to aerobic period ratios	1:1, 3:1, 5:1, 7:1	3:1	3:1
Ultrasonication intensity (W/mL)	/	0.025, 0.05, 0.1, 0.2	/
Ultrasonication duration (min)	/	1, 5, 10, 15	/
NH_2OH concentration (mg/L)	/	/	1, 5, 10, 15

CR: control reactor; UR: ultrasound reactor; HR: hydroxylamine reactor.

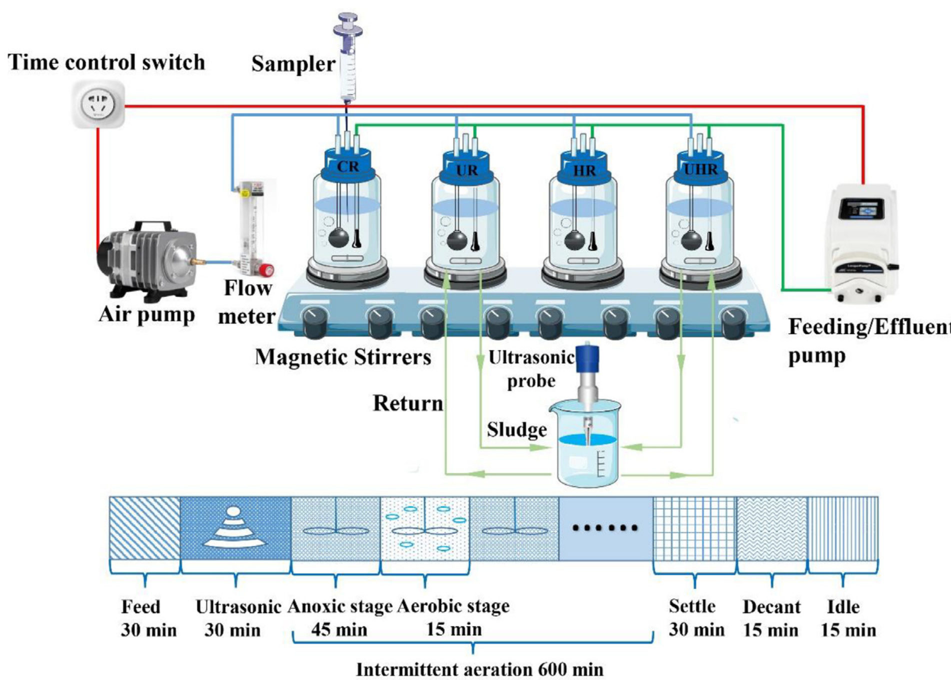

Fig. 1 – Schematic diagram of sequencing batch reactor.

partial nitrification process under continuous SBR operation conditions.

1.3. Analytical methods

MLSS, MLVSS, SV_{30}, VSS, NH_4^+-N, NO_2^--N and NO_3^--N were measured according to standard methods (Gilcreas, 1966). Protein (PN) and polysaccharide (PS) concentrations were determined using the Lowry and anthrone colorimetric methods, respectively (Tian et al., 2021). EPS was extracted by a modified heating method and functional group analysis was performed using a Fourier Transform infrared spectroscopy (FTIR) (Nicolet 6700, Thermo-Fisher, USA) (Tian et al., 2021). Simultaneously, fluorescence spectrometry (Hitachi F-4600, Hitachi, Japan) was used to determine the changes in EPS fluorescent organic substances. The Illumina Hisep PE250 sequencing platform was used for 16S rRNA gene sequencing to reveal changes in the microbial community composition. The specific ammonia oxidation rate of AOB (SAOR), specific nitrate accumulation rate of NOB (SNaPR), NAR and NRR were measured according to the methods of Zhang et al. (2019a). Temperature, pH and ORP were measured using a HACH portable monitor (STARTER 2100/3C pro-B, USA). Ultrasonication treatment was performed using a probe type ultrasonic generator (SM-900A, Shunmatech, China) with a 10 mm probe and a frequency of 25 kHz. A laser diffraction analyzer (Mastersizer 3000, Malven, UK) was used to determine the sludge particle size and scanning electron microscopy (SEM) (MIRA, Tescan, Czech) was used to observe the microscopic morphology of sludge particle surfaces. A detailed description of the methods used is provided in Appendix A Text S2.

2. Results and discussion

2.1. Determination of optimal working parameters for sbr operation

Real-time aeration control has been proven a viable strategy for the initiation and maintenance of partial nitrification in wastewater (Tian et al., 2021; Zhao et al., 2021). Therefore, the

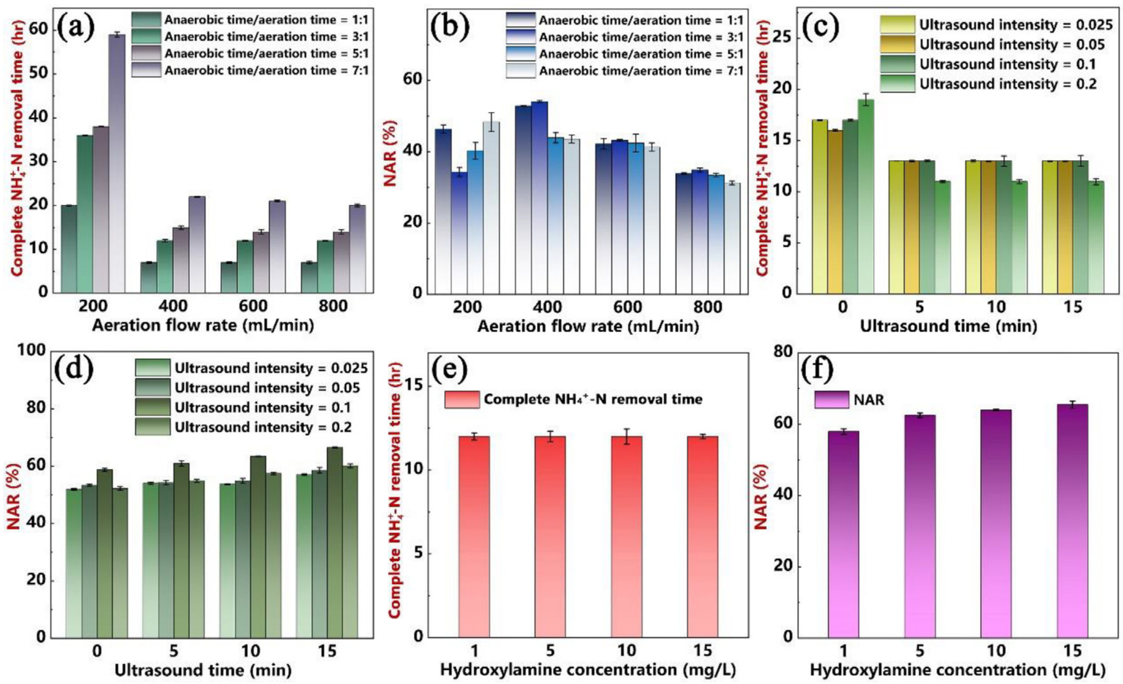

Fig. 2 – Variation in complete removal time for ammonia nitrogen and the nitrite accumulation rate (NAR) under different operational conditions: (a) and (b) ratio of anaerobic time to aeration time and aeration flow rate; (c) and (d) ultrasonication intensity and duration; (e) and (f) hydroxylamine concentration.

effects of varying ratios of anaerobic to aerobic periods and aeration flow rates were investigated (Fig. 2a-b). At an aeration flow rate of 200 mL/min, a longer time was required for the complete removal of ammonia nitrogen from the reactor (>20 hr) and the NAR varied significantly under different anaerobic to aerobic period ratios. This variation was due to the difference in partial nitrification performance caused by low aeration rates, causing microorganisms to compete for oxygen (Pollice et al., 2002). When the aeration flow rate was increased from 200 to 400 mL/min, the time required for the complete removal of ammonia nitrogen ranged from 7 to 20 hr under different anaerobic to aerobic period ratios. When the aeration flow rate was increased further from 400 to 800 mL/min, the time required for the complete removal of ammonia nitrogen generally remained unchanged, indicating that the microbial requirements for oxygen had reached saturation when the aeration flow rate was 400 mL/min. When the anaerobic to aerobic period ratio was increased from 1:1 to 3:1 at an aeration flow rate of 400 mL/min, the NAR increased from 52.83% to 54.05%. This is because intermittent aeration induces alternating anoxic and aerobic conditions, allowing NOB to be suppressed, while the AOB recovery time is not affected by DO concentrations (Gilbert et al., 2014). With a further increase in the anaerobic to aerobic period ratio from 3:1 to 5:1, there was a tendency for the NAR to decrease, reducing the partial nitrification performance of the system due to insufficient availability of DO within the reactor. Therefore, the optimal anaerobic to aerobic period ratio and aeration flow rate for the partial nitrification sludge reactor were 3:1 and 400 mL/min, respectively, with aeration periods lasting for 15 min per hr. The effects of ultrasonic treatment intensity and duration on the NAR and the time required for complete removal of ammonia nitrogen, were investigated at an anaerobic to aerobic period ratio of 3:1 and an aeration flow rate of 400 mL/min (Fig. 2c-d). At an ultrasonic treatment intensity of 0.025–0.2 W/mL, the average time required for the complete removal of ammonia nitrogen reduced from 16 to 19 hr to 11–13 hr as the duration of ultrasonication treatment increased from 0 to >5 min. When the ultrasonication treatment duration was increased further, the time required for complete removal of ammonia nitrogen remained stable. In contrast, the NAR exhibited an increasing trend, reaching a maximum of 66.49% after 15 min of ultrasonication at an intensity of 0.1 W/mL. Therefore, the optimal ultrasonication intensity was determined to be 0.1 W/mL for a duration of 15 min, which in combination with intermittent aeration can effectively improve the partial nitrification performance of SBR systems (Tian et al., 2021).

The effect of NH_2OH concentration on the NAR and time required for the complete removal of ammonia nitrogen, was investigated at an aeration flow rate of 400 mL/min and an anaerobic to aerobic period ratio of 3:1 (Fig. 2e-f). After the addition of NH_2OH, the time required for the complete removal of ammonia nitrogen was about 12 hr in all four reactor groups at different NH_2OH concentrations, with the lowest NAR of 54.94% achieved using 1 mg/L NH_2OH. When the NH_2OH concentration was increased from 5 to 15 mg/L, the NAR increased marginally from 62.49% to 65.48%. Therefore, 5 mg/L NH_2OH was selected as the optimal NH_2OH concentration in terms of operational costs and performance.

In conclusion, the optimal UHR working parameters were determined to include an anaerobic to aerobic period ratio of

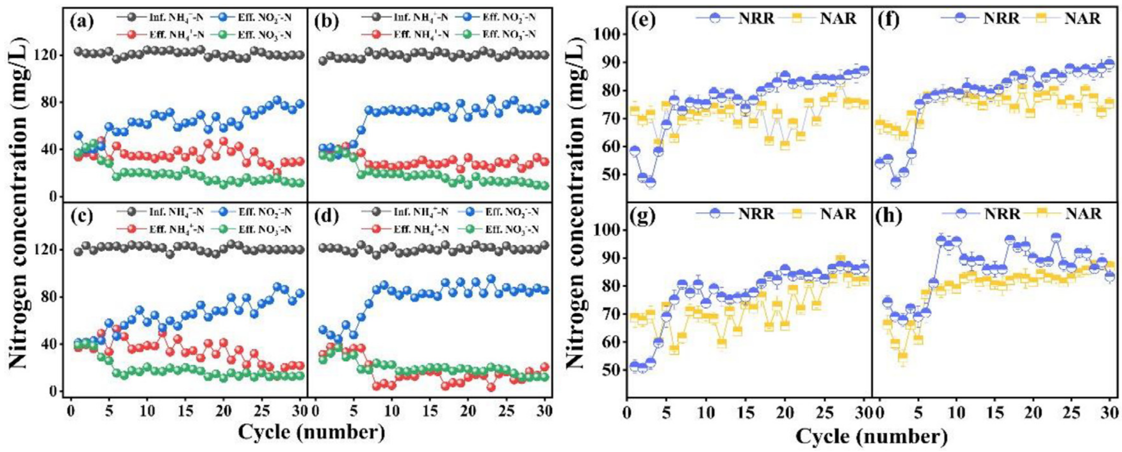

Fig. 3 – Variations in nitrogenous pollutant concentrations during the operation of the sequencing batch reactor (SBR) systems: (a) control reactor (CR), (b) ultrasound reactor (UR), (c) hydroxylamine reactor (HR) and (d) coupled ultrasound-hydroxylamine reactor (UHR); Ammonia nitrogen removal rate (NRR) and nitrite accumulation rate (NAR) during the operation of the SBR systems: (e) CR, (f) UR, (g) HR and (h) UHR.

3:1, aeration flow rate of 400 mL/min, aeration period duration of 15 min per hr, ultrasonication treatment intensity of 0.1 W/mL, ultrasonication duration of 15 min and NH_2OH concentration of 5 mg/L.

2.2. *Continuous sbr operation in partial nitrification mode*

Fig. 3 shows the partial nitrification performance of all four groups of reactors over 30 cycles of continuous SBR operation. In the CR, the NO_2^--N concentration increased gradually during continuous SBR operation from an initial level of 52.02 to 78.71 mg/L after 30 cycles, with an effluent NH_4^+-N concentration of 29.93 mg/L, NAR of 87.13% and NRR of 75.06% (Fig. 3e). These results indicate that real-time aeration period control can effectively mitigate the conversion of nitrite to nitrate, achieving high levels of nitrite accumulation (Guo et al., 2009).

The partial nitrification performance of the UR is shown in Fig. 3b and f. Effluent NO_2^--N concentrations started to stabilize in the 7th cycle of continuous SBR operation and by the 30th cycle, the effluent concentrations of NH_4^+-N and NO_2^--N were 29.53 mg/L and 78.64 mg/L, respectively. The NAR and NRR reached 89.38% and 75.39%, respectively (Fig. 3f). The partial nitrification performance of the UR system (NAR = 89.38%, NRR = 75.39%) was better than that of the CR system (NAR = 87.13%, NRR = 75.06%). This difference was attributed to low-intensity ultrasonication treatment effectively promoting AOB bioactivity, which can improve mass transfer, increase enzyme activity and accelerate cell metabolism, resulting in stable partial nitrification performance (Zheng et al., 2013). In summary, moderate ultrasonication treatment can effectively enhance the partial nitrification performance of SBRs.

In the HR system, the changes in nitrogenous pollutant concentrations, NAR and NRR at a NH_2OH concentration of 5 mg/L, are shown in Fig. 3c and g. At the initial point of NH_2OH dosing, the partial nitrification performance of the reactor was not significantly enhanced and the effluent NAR and NRR remained relatively low. However, with ongoing continuous cycles of operation, effluent NO_2^--N concentrations exhibited a gradually increasing trend, NH_4^+-N and NO_3^--N concentrations gradually decreased, while the NAR and NRR increased simultaneously. After 30 cycles of operation, the NAR and NRR had reached 86.32% and 81.85%, respectively. Overall, these results indicate that the good partial nitrification performance of the HR system was due to NH_2OH dosing, accelerating the conversion of NH_4^+-N to NO_2^--N and increasing the NRR (Chandran and Smets, 2008). In addition, maximum NOB inhibition was achieved at a NH_2OH concentration of 5 mg/L (Sui et al., 2020; Wang et al., 2020). As a result, the addition of NH_2OH caused the NAR and NRR to increase steadily and helps to maintain the stable performance of partial nitrification.

The variations in nitrogenous pollutant concentrations in the UHR are shown in Fig. 3d. In the 8th cycle of continuous SBR operation, the UHR effluent NO_2^--N concentration (85.99 mg/L) was higher than the CR (63.41 mg/L), UR (71.21 mg/L) and HR (60.74 mg/L), indicating that the highest level of NO_2^--N accumulation occurred in the UHR. In addition, the UHR effluent NH_4^+-N and NO_3^--N concentrations were lower than in all other reactor groups. Notably, the NRR exceeded 85% after 8 cycles (Fig. 3h), while all other reactor groups required 20 cycles to reach NRRs of >85% (Fig. 3e, g), indicating that the UHR system achieved a faster start-up of the partial nitrification process, with higher levels of NO_2^--N accumulation and lower NH_4^+-N effluent concentrations. This phenomenon may be attributed to the synergistic effect of low-intensity ultrasonication and NH_2OH dosing, on the promotion of AOB growth and the inhibition of NOB activity (Zhang et al., 2019b; Zheng et al., 2019). The variation in AOB and NOB activities was expressed in terms of the SAOR and SNAPR, as shown in Appendix A Fig. S1. The use of low-intensity ultrasonication achieved continuous inhibition

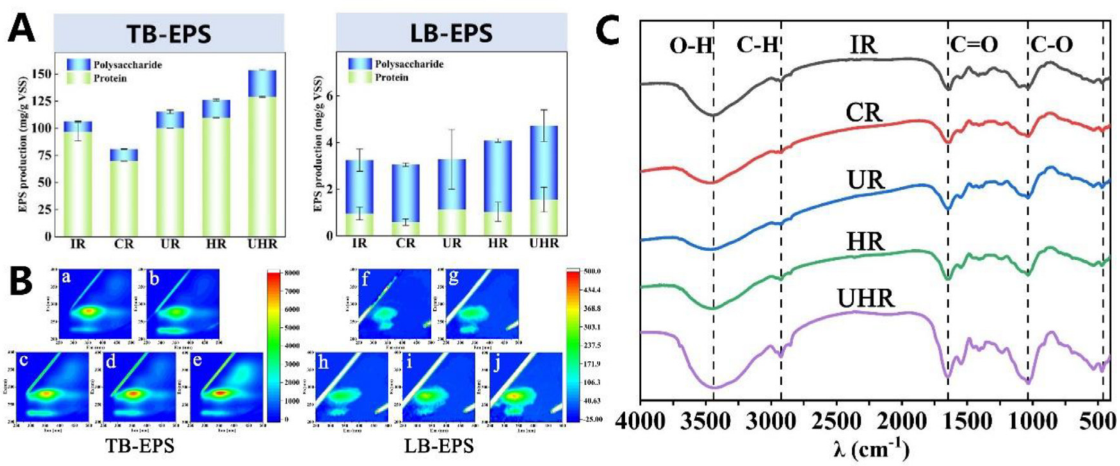

Fig. 4 – (A) Extracellular polymeric substances (EPS) composition and concentrations, (B) Fluorescence spectral characteristics of EPS fractions in sludge from different reactor groups: (a) and (f) IR (initial reactor); (b) and (g) CR; (c) and (h) UR; (d) and (i) HR; (e) and (j) UHR, (C) Fourier Transform infrared spectroscopy analysis of total EPS.

of NOB growth and activity, resulting in NOB being unable to recover and therefore, overcoming the limitation of unstable performance during long-term operation with NH_2OH dosing.

In summary, the combination of ultrasonication treatment and NH_2OH dosing was able to accelerate the start-up period of the partial nitrification process, achieving a NRR and NAR of >85% after only 8 cycles of continuous SBR operation (4 days), improving NO_2^--N accumulation and the removal of NH_4^+-N.

2.3. *Variations in eps*

2.3.1. *EPS composition and concentrations*

EPS consists of complex polymeric polymers secreted extracellularly during microbial metabolism, which can act as ion transport channels between biofilms and liquids, improving the substrate mass transfer efficiency. In addition, EPS provides protection for microbial cells against environmental stress such as heavy metals, low temperatures and high salinity (Hou et al., 2015). Therefore, EPS characteristics such as composition and concentration, can adapt according to the matrix mass transfer effect and provide resistance to external environmental changes in partial nitrification sludge (Ma et al., 2019). In order to determine the effect of ultrasonication treatment in conjunction with NH_2OH dosing on the EPS properties in partial nitrification sludge, the initial sludge was analyzed along with sludge samples collected after 30 cycles of continuous SBR operation, as shown in Fig. 4A. Results showed that EPS in the partial nitrification sludge consisted mainly of TB-EPS (>90%), with LB-EPS accounting for only a small fraction (<10%). The TB-EPS fraction consisted mainly of PN and PS, with PN being the most abundant component. However, after 30 cycles of continuous SBR operation, the PN content of EPS decreased from 96.80 to 70.04 mg/g VSS, while the PS did not change significantly. The decrease in PN content may be due to changes in influent water quality, operational method or reaction stage, leading to the inactivation of some NOB or other heterotrophic bacteria in the nitrifying sludge and PN decomposition (Huang et al., 2022). The PN and PS contents of EPS were significantly higher in the UR and HR systems compared to the CR, which was attributed to the use of either ultrasonication or NH_2OH dosing alone, increasing AOB activity and promoting bacterial EPS secretion (Tian et al., 2021; Zhao et al., 2021). In addition, the PN and PS contents of the TB-EPS fraction in the UHR were 128.99 and 24.91 mg/g VSS, respectively, which were higher than all other reactor groups, indicating that ultrasonication treatment in combination with NH_2OH dosing could promote EPS secretion in partial nitrification sludge. An increased EPS content could improve the substrate mass transfer performance of the reactor system and accelerate the entry of NH_4^+ into AOB cells, thus promoting the ammonia oxidation rate and increasing the accumulation of nitrite nitrogen, while enhancing the structural stability of the partial nitrification sludge (Li et al., 2020; Liao et al., 2001; Ma et al., 2019). Ultimately, the NRR of partial nitrification sludge was improved and the process start-up time was reduced.

2.3.2. *3D-EMM analysis of eps*

Fig. 4B shows the differences in EPS 3D-fluorescence spectra between the initial reactor (IR) sludge and the sludge from all four reactor groups after 30 cycles of continuous SBR operation. Results show that the fluorescence spectra of the TB-EPS fraction contained four characteristic peaks corresponding to tyrosine/tryptophan-like amino acids (peak I), tyrosine/tryptophan-like proteins (peak II), aromatic-like humic acids (peak III) and polycarboxylic acid-like humic acids (peak IV) (Wang and Zhang, 2010). The results of 3D-EEM analysis of EPS are showed in Table 2. In the IR system, the intensity of II peak was significantly higher than the other three peaks, indicating that TB-EPS is dominantly composed of tyrosine/tryptophan-like proteins (Fig. 4A). After 30 cycles of intermittent aeration (Fig. 4b), the intensity of peak I was enhanced while the intensity of peak II reduced slightly. This phenomenon may be due to the change in the sludge en-

Table 2 – Results of the 3D-EEM analysis of extracellular polymeric substances.

EPS	Reactor	Peak A		Peak B		Peak C		Peak D	
		Ex/Em (nm)	Intensity	Ex/Em (nm)	Intensity	Ex/Em (nm)	Intensity	Ex/Em (nm)	Intensity
TB-EPS	IR	225/340	1954.07	280/350	6721.14	275/435	1591.17	360/445	988.22
	CR	225/345	3656.16	280/350	5082.14	275/450	1352.24	355/440	620.44
	UR	225/345	3041.16	285/350	6626.69	275/440	1700.10	350/440	1318.44
	HR	225/350	2869.66	285/355	7220.63	275/445	1769.74	355/440	1138.74
	UHR	225/340	2632.07	285/355	7917.63	275/435	1846.17	340/430	2367.49
LB-EPS	IR	235/345	170.76	275/335	183.37	–	–	–	–
	CR	225/325	202.52	270/335	230.06	–	–	–	–
	UR	230/335	204.03	275/325	239.55	–	–	–	–
	HR	230/325	150.65	275/330	347.36	–	–	–	–
	UHR	230/335	242.23	275/330	413.26	–	–	–	–

vironment, causing small amounts of tyrosine/tryptophan-like proteins to breakdown into amino acids. Compared to the CR, the fluorescence intensity of peak II was significantly enhanced in the UR system (Fig. 4c), which may be due to the mechanical and steady-state cavitation effects of low-intensity ultrasonication, inducing mild repairable damage to microbes, triggering metabolic compensation mechanisms in cells and increasing the synthesis of PN for the repair of organisms (Huang et al., 2020). In addition, the intensity of TB-EPS peak II was also significantly enhanced when NH_2OH was added to the reactor (Fig. 4d). This was attributed to the combined effects of NH_2OH dosing and optimized reactor operation, increasing the stability of partial nitrification, promoting the ammonia conversion process of AOB and accelerating metabolism, resulting in the secretion of more EPS and increased bacterial activity (Tian et al., 2021; Xu et al., 2012). Notably, the fluorescence intensity of peak II in the UHR was significantly higher than all other groups and the intensity of peak IV was also significantly increased. These results suggest that ultrasonication coupled with NH_2OH dosing can synergistically promote bacterial EPS secretion. Furthermore, the trend observed for characteristic peaks in LB-EPS was consistent with the trend in TB-EPS. Previous studies have shown that humic acid exhibits strong hydrophobicity, facilitating the mutual attraction between sludge aggregates and improving settling performance (Shi et al., 2017). Meanwhile, the adhesion of PN on cells not only contributes to the improved settling performance, but also enhances ion exchange between cells and the solution (Huang et al., 2020). Therefore, ultrasonication treatment coupled with NH_2OH dosing can stimulate sludge EPS secretion, improving the settling properties of sludge, enhancing mass transfer, increasing AOB activity and the partial nitrification performance of the system.

2.3.3. *Fourier infrared spectroscopy scanning*

To further illustrate the differences in the functional groups of partial nitrification sludge in each group of reactors, FTIR was used to analyze the total EPS of sludge (Fig. 4C). The peaks at 3433 – 3478 cm^{-1} were attributed to the hydroxyl stretching vibrations of carboxylic acids, sugars and other substances. Among these, the hydrogen bonding interactions within and between carboxylic acid molecules, generally form broad peaks and due to intermolecular hydrogen bonding O–H stretching vibrations. The peaks at 2850 – 2960 cm^{-1} corresponded to the stretching vibrations of C–H in alkanes and PS. The peak at 1650 – 1690 cm^{-1} was assigned to the stretching vibration of C = O and the bending vibration of amide NH_2 groups in the secondary structure of EPS PN. The peak present in the range 975 – 1078 cm^{-1} corresponded to the C–O stretching vibration in PS. The peaks in the range of 496 – 530 cm^{-1} were attributed to unsaturated bonds (Guibaud et al., 2003; Omoike and Chorover, 2006). These results verify that the EPS contained both PS and PN components. The higher intensity of the O–H peak in the IR system was attributed to the initial growth of microbes within the sludge in the original partial nitrification reactor, which is rich in EPS. As the sludge entered the CR system for incubation, the intensity of the O–H peak decreased, while the intensity of the C = O peak associated with PN also decreased simultaneously. This was due to the environmental changes caused by the intermittent aeration mode, leading to a decrease in EPS content (Fig. 4A). When ultrasonication treatment was applied, the intensity of the UR system O–H peak was further reduced, owing to the degradation of PS biomolecules that function as binders. Ultrasonication treatment also stimulated the production of EPS, leading to an increase in the intensity of the C = O peak, corresponding to an increase in PN content. The addition of NH_2OH enhanced microbial metabolism and stimulated EPS secretion by the sludge. Therefore, the intensity of both the O–H and C = O peaks increased in the HR system. However, the variation in peak intensities was more pronounced in the UHR system, with O–H, C–O and C = O peak intensities being much higher than in all other reactor groups. This was due to the synergistic effects of NH_2OH dosing and ultrasonication on partial nitrification properties, resulting in a large increase in EPS, corresponding to an increase in both PN and PS (Fig. 4A) (Badireddy et al., 2008). The results of FTIR assay showed that low-intensity ultrasonication combined with NH_2OH dosing increased the sludge content of carbonyl, hydroxyl and amino functional groups, improved the microbial mass transfer efficiency and increased NH_4^+-N removal, thus shortening the time required for the initiation of partial nitrification (Tian et al., 2021). Therefore, low-intensity ultrasonication treatment coupled with NH_2OH dosing can increase the abundance of various func-

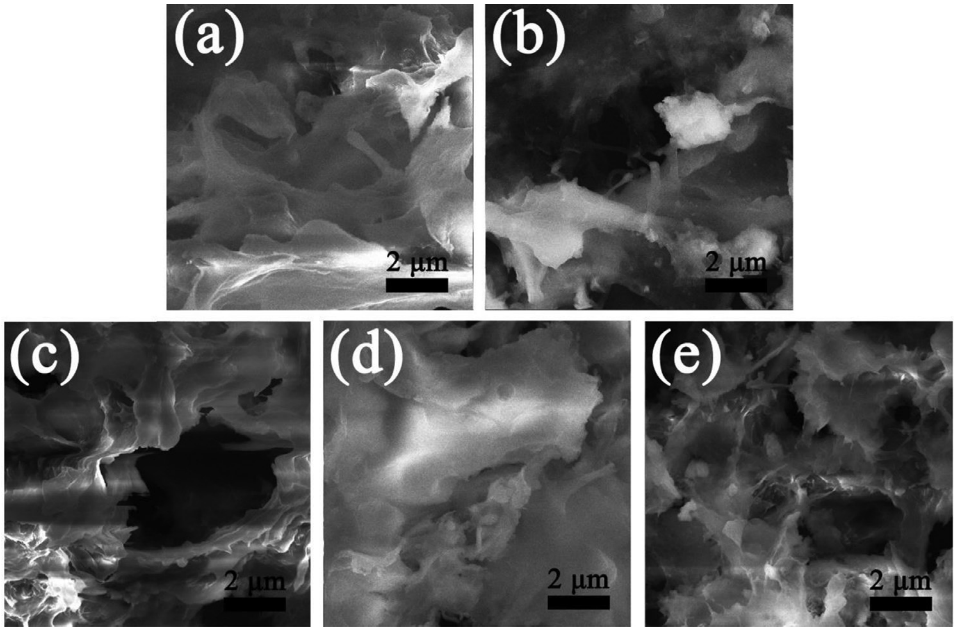

Fig. 5 – Scanning electron microscopy images of the sludge in the different reactors: (a) IR; (b) CR; (c) UR; (d) HR; (e) UHR.

tional groups and improve the activity of partial nitrification sludge.

2.4. *Changes in sludge morphological characteristics*

To further explore the effect of ultrasonication treatment in combination with NH_2OH dosing on the micromorphology of sludge, SEM observations were performed on the initial sludge and sludge from all four reactor groups after the 30th cycle of continuous SBR operation (Fig. 5a-e). The initial sludge appeared uneven overall, although its surface morphology was relatively smooth and no bacterial contours could be observed, as the initial sludge was suspended within the SBR and mainly in a flocculated form. After 30 cycles of operation under intermittent aeration conditions (CR) (Fig. 5b), the sludge gradually solidified, its smooth surface disappeared and the outline of bacteria were clearly observable, showing them to be clusters of rod-shaped bacteria. This indicates that the flocculated sludge gradually solidified and that bacteria grew well under controlled aeration conditions (Guo et al., 2009). As shown in Fig. 5c, the UR sludge surface was relatively rough and uneven, with loose floc structures and a large number of wavy flocs, increasing the specific surface area of sludge. This indicates that low-intensity ultrasonication leads to floc dispersion and increases the porosity and solid-liquid contact area of sludge, which plays a key role in enhancing mass transfer and improving the biological treatment efficiency of wastewater (Zhang et al., 2008). When NH_2OH was added to the system, the sludge surface exhibited a smooth floc-like morphology with a fuller and denser profile than the initial sludge (Fig. 5c). This may be due to the addition of NH_2OH stimulating cells to secrete EPS and attach to the sludge surface (Xu et al., 2012), while the formation of EPS on bacterial cell surfaces allows cells to be protected from damage caused by the harsh environment (Miao et al., 2018). When the sludge was treated with a combination of low-intensity ultrasonication and NH_2OH dosing (Fig. 5e), the sludge surface became relatively rough and loosely structured, with sludge particles adhered closely to each other. The looseness of sludge in the UHR reactor was less than in the UR, but greater than in the HR, indicating that ultrasonication improves the porosity of sludge and enhances mass transfer, while the addition of NH_2OH causes AOB to secrete more EPS as a form of protection. Both low-intensity ultrasonication and NH_2OH dosing have a synergistic effect on the stability of partial nitrification performance, improving the $NH_4{}^+$-N removal rate and reducing the time required for the start-up of partial nitrification. Determination of sludge particle size also indicated that combined treatment with low-intensity ultrasonication and NH_2OH dosing can strengthen the mass transfer ability between sludge and the surrounding solution, maintain sludge structural stability (Table 3).

Table 3 – Particle size analysis of sludge from different partial nitrification reactors.

Reactor group	IR	CR	UR	HR	UHR
dv50 (μm)	48.6	29.5	12.4	30.1	13.3

dv50: medium particle size; IR: initial reactor; HR: hydroxylamine reactor; UR: ultrasound reactor; UHR: coupled ultrasound-hydroxylamine reactor.

The variations in SV_{30}, SVI (Sludge Volume Index), MLSS and MLVSS of sludge during each treatment, are shown in Table 4. SVI can be used to characterize the settling performance of sludge, with lower SVI values indicating a better settling performance (Li and Yang, 2007). As shown in Table 4, the CR system had the lowest SVI (139.42), while the UHR system

Table 4 – Change in sludge properties.

Sludge index	IR	CR	UR	HR	UHR
SV_{30} (%)	28	29	36	34	36
SVI	142.85	139.42	146.15	148.60	154.84
MLSS (mg/L)	1960 ± 120	2080 ± 110	2258 ± 120	2288 ± 110	2325 ± 110
MLVSS (mg/L)	1445 ± 70	1510 ± 80	1636 ± 80	1674 ± 70	1678 ± 70

SVI: sludge Volume Index; MLSS: mixed liquor suspended solids, MLVSS: mixed liquor volatile suspended solids.

had the highest (154.84). Therefore, the settling performance of the CR system was the best and the settling performance of UHR system was the worst. This is because the settling performance of the system is closely related to the LB-TBS content of EPS, with a higher LB-EPS content associated with poorer settling performance (Liu et al., 2022). As shown in Fig. 4A, the variation in SVI in each reactor group was linearly correlated with the variation in LB-EPS content (Li and Yang, 2007).

2.5. 16S rRNA gene sequencing

To further determine the effect of low-intensity ultrasonication in combination with NH_2OH treatment on microorganisms, the composition and relative abundance of the sludge microbial community was analyzed at the phylum and genus levels. The 10 most dominant bacterial phyla (in terms of relative abundance) are shown in Fig. 6a, consisting of Proteobacteria, Bacteroidetes, Ignavibacteriae, Chloroflexi, Nitrospirae and others. Among them, Proteobacteria and Nitrospirae were identified as the main nitrifying functional phyla associated with nitrogen removal, performing ammonia oxidation and nitrite oxidation in nitrification reactions (Flemming and Wingender, 2010). After 30 cycles of intermittent aeration, the abundance of Proteobacteria increased from 27.04% (CR) to 37.26%, while the abundance of Nitrospirae decreased from 5.82% (CR) to 2.50%. Therefore, the intermittent aeration operation mode was favorable for NOB growth and AOB inhibition, which was beneficial to the NRR. After ultrasonication treatment was applied with intermittent aeration (UR), the abundance of Proteobacteria and Nitrospirae decreased by 0.57% and 0.32%, respectively. This was attributed to the disruption of cell structures by low-intensity ultrasonication, reducing the abundance of the microbial community (Li et al., 2016). However, the AOB community were consistently dominant, causing the NRR and NAR to be higher in the UR than in the CR. Furthermore, the abundance of the phylum Bacteroidetes was higher in the UR than in the CR, as Bacteroidetes can adapt to extreme environments and are beneficial to the partial nitrification performance of the system (Zhang et al., 2019c). The phylum Chloroflexi provides a supporting and skeletal function in sludge and the relative abundance of Chloroflexi decreased due to the disruption to sludge structure by ultrasonication (Chen et al., 2013). Compared to the CR, the abundance of Proteobacteria increased in the HR to 37.91%, while the abundance of Nitrospirae decreased by 2.01%. This is due to NH_2OH dosing increasing AOB bioactivity and inhibiting NOB growth (Huang et al., 2020). The abundance of the phylum Proteobacteria was 47.24% after 30 cycles of continual operation in the UHR, becoming the dominant phylum in sludge and accounting for a major portion of AOB. However, the abundance of Nitrospirae, the phylum corresponding to NOB, was the lowest (1.85%) in the UHR, indicating that ultrasonication with NH_2OH dosing effectively increased the abundance of AOB and strongly inhibited NOB activity. Therefore, low-intensity ultrasonication coupled with NH_2OH dosing improved the NRR and shortened the partial nitrification start-up time by altering microbial community abundances.

Genus-level abundance analysis was performed on partial nitrification sludge and a genus-level taxonomic phylogenetic tree was established (Fig. 6b-c). Sequencing results showed that seven main genus were present in the partial nitrification sludge of all reactor groups. Among them, *Nitrosomonas* and *Nitrospira* are the main genus involved in the nitrification reaction. The abundance of *Nitrosomonas* was 16.89%, which as the main AOB genus and the dominant genus of Proteobacteria in sludge, played a main role in ammonia oxidation (Yang et al., 2017). After 30 cycles of continuous SBR operation, the abundance of *Nitrosomonas* in the CR, UR, HR and UHR systems was 27.79%, 25.70%, 28.91% and 35.38%, respectively. This indicates that in the UHR sludge AOB had the highest abundance and the highest activity, while also verifying that the AOB-dominated microbial community plays a major role in partial nitrification (Cui et al., 2019). However, the NOB dominant genus *Nitrospira*, which belongs to the phylum Nitrospirae, had a lower relative abundance (Zhou et al., 2018). This is because ultrasonication combined with NH_2OH treatment changes the microbial community structure of the sludge, with ultrasonication-sensitive *Nitrospira* disappearing from the system while *Nitrosomonas* gradually became the dominant group (Huang et al., 2020; Zhang et al., 2019b; Zheng et al., 2013). Thus, under the influence of both ultrasonication and NH_2OH dosing, AOB became the dominant genus while the abundance of NOB decreased and the partial nitrification performance of the reactor was enhanced.

2.6. Principle analysis of ultrasonication coupled with NH_2OH dosing for the promotion of partial nitrification

Low-intensity ultrasonication and the addition of NH_2OH are considered the effective means to promote partial nitrification. However, most previous studies have focused on single promotion methods in isolation, with relatively little known about the synergistic promotion principles and effects of these two methods. In this study, the principles of synergy between ultrasonication and NH_2OH dosing for the promotion

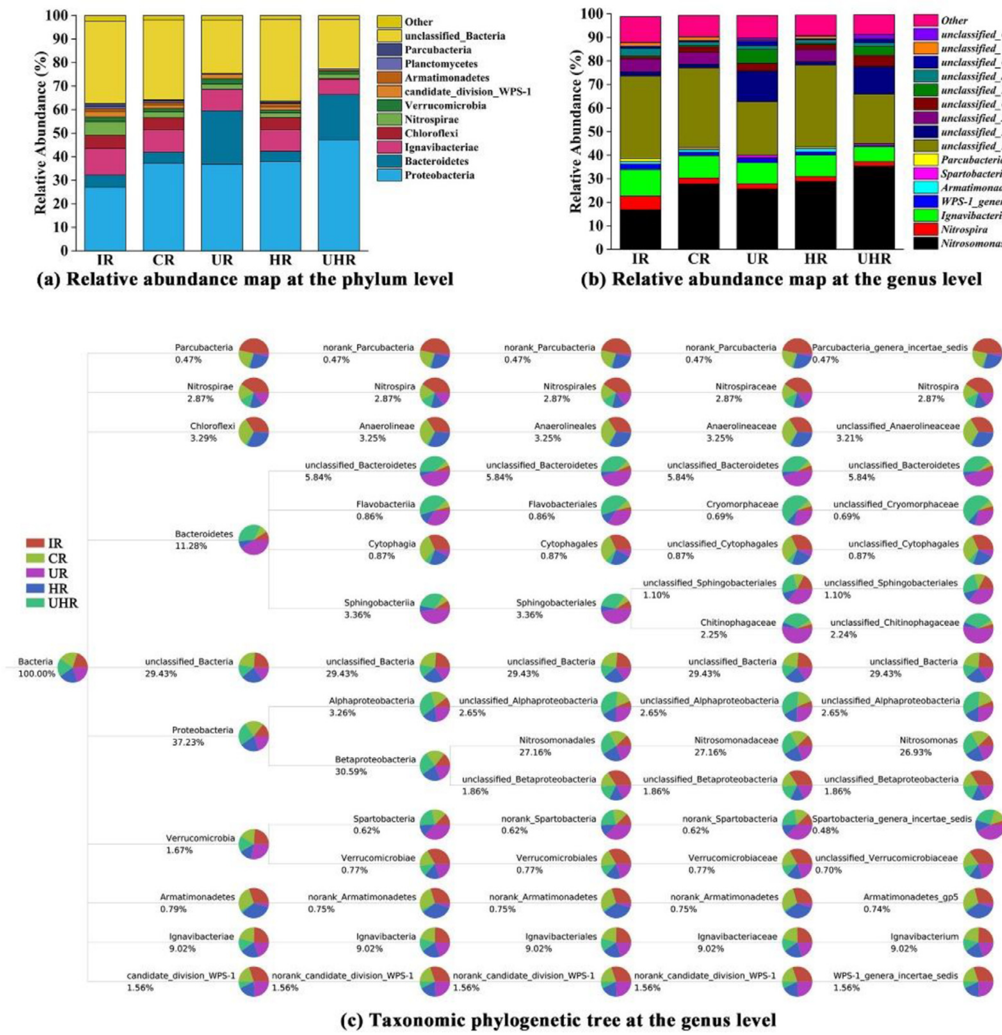

Fig. 6 – High-throughput sequencing of 16Sr RNA: (a) Relative abundance map at the phylum level; (b) Relative abundance map at the genus level; (c) Taxonomic phylogenetic tree at the genus level.

of partial nitrification performance were speculated, proposing the synergistic mechanism of effect for the improvement of partial nitrification system performance.

The possible mechanistic principle of ultrasonication in combination with NH_2OH dosing on the promotion of partial nitrification, is shown in Fig. 7. During the partial nitrification reaction, NH_4^+ is first converted to NH_2OH by ammonia monooxygenase (AMO) and subsequently, NH_2OH is oxidized to NOH by hydrolymine oxidase (HAO). A portion of NOH continues to be converted to NO_2^- by HAO and to NO by nitrite reductase (NIR), while another portion of NOH is rapidly catabolized to produce NO. Meanwhile, a small amount of NO will be reduced to N_2O by nitric oxide reductase (NOR) (Poughon et al., 2001). NO and N_2O inhibit nitrate oxidoreductase (NXR), which converts NO_2^- to NO_3^-, resulting in the accumulation of NO_2^--N. The addition of NH_2OH promotes the metabolism of AOB, accelerating the rate of ammonia conversion and improving the rate of NH_4^+-N removal, producing more NO_2^-, NO and N_2O, while also secreting more EPS to enhance the mass transfer effect and provide resistance to environmental changes. ORP represents the redox potential and its dynamics reflect the change in NH_2OH concentration in solution, indicating the extent of NH_2OH utilization by the partial nitrifying sludge. In addition, the changes in ORP can also indirectly reflect the production of NO and N_2O (Appendix A Text S4 and Fig. S2) (Zhao et al., 2021). Low-intensity ultrasonication changed the structure of the sludge, increasing the porosity of the sludge surface and improving cell permeability (Tian et al., 2021; Xie et al., 2008). As a result, NO and N_2O were able to enter NOB more easily, inhibiting the activity of the key enzyme NXR, increasing the NAR and reducing the effluent NO_3^--N concentration. Due to differences in bacterial tolerance, low-intensity ultrasonication can also directly reduce or inhibit NOB activity, increasing the abundance of AOB phyla and reducing the abundance of NOB. Therefore, the coupled use of low-intensity ultrasonication and NH_2OH dosing has a synergistic effect on partial nitrification, achieving rapid start-up of partial nitrification and improving the NRR and NAR of the reactor, providing a convenient start-up method for the rapid realization of partial nitrification.

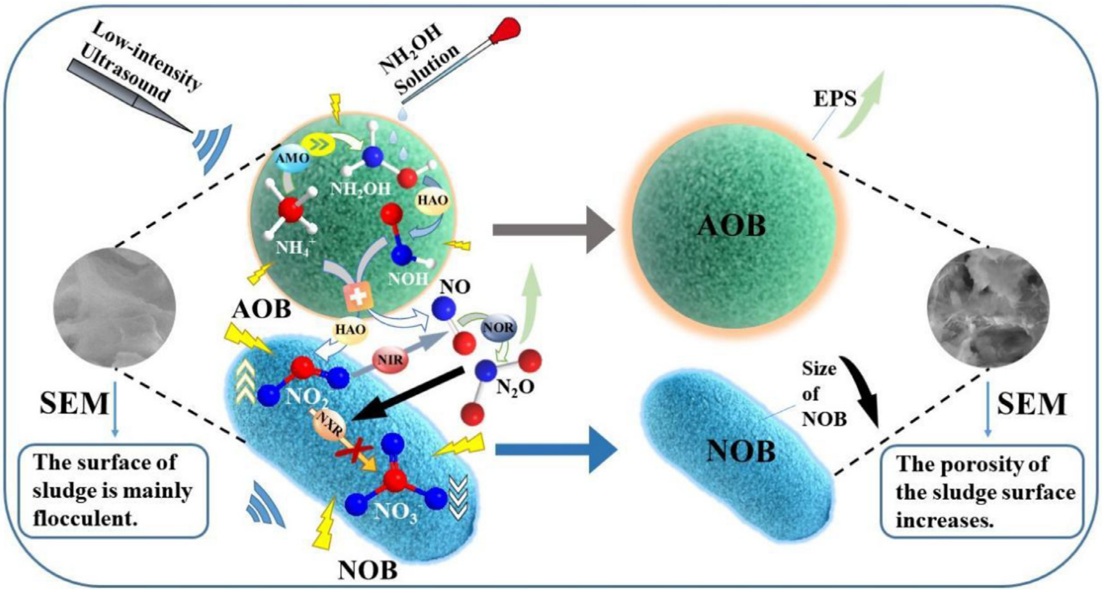

Fig. 7 – Possible mechanism of promotion of partial nitrification by synergistic ultrasonication treatment and hydroxylamine dosing.

2.7. *Potential applications and environmental impact*

Achieving rapid start-up of the partial nitrification process has been a major research focus in the field of wastewater nitrogen removal. However, some key problems remain, such as slow start-up times and inconsistent long-term operational performance. Tian et al. (2021) achieved more than 90% NAR by adding NH_2OH, but the cessation of NH_2OH dosing resulted in deterioration of the partial nitrification performance. NOB also gradually adapted to the inhibition of NH_2OH during long-term operation of partial nitrification processes. Therefore, NH_2OH dosing has the problem of unstable performance during long-term operation. In addition, Harper et al. (2009) achieved 85% NAR in 11 days by promoting partial nitrification with low-intensity ultrasonication. The start-up time for the partial nitrification process was long.

In this study, a strategy of simultaneous low-intensity ultrasonication and NH_2OH dosing was proposed to promote the rapid start-up of partial nitrification processes. Combined low-intensity ultrasonication and NH_2OH dosing achieved a NAR and NRR of >85% after 4 days of SBR operation in intermittent aeration mode (0.1 W/mL ultrasonication intensity for 15 min and NH_2OH dosing concentration of 5 mg/L). The addition of NH_2OH accelerated the rate of ammonia conversion and increased the NRR. Low-intensity ultrasonication increased the porosity of sludge surfaces, improved cell permeability and inhibited the activity of NXR, reducing the effluent NO_3^--N concentration and increasing the NAR. Therefore, the use of low-intensity ultrasonication with NH_2OH dosing, shortened the partial nitrification start-up time. In addition, the results of continuous SBR operation in the partial nitrification scale-up test (Appendix A Fig. S3 and Text S6), show that the long-term partial nitrification performance remained stable after treatment with low-intensity ultrasonication and co-hydroxylamine.

As a result, the use of ultrasonication in cooperation with NH_2OH can overcome the shortcomings of these methods alone, supporting the improvement of partial nitrification system performance. This approach may be an effective, convenient and economical method capable of further coupling with other nitrogen removal processes. However, the capacity for this system to initiate the partial nitrification process requires further investigation to explore the feasibility and stability of the process in pilot or large-scale applications, as well as to determine its potential for combination with anaerobic ammonia oxidation or denitrification nitrogen removal processes.

3. Conclusions

In this study, the effect of ultrasound combined with NH_2OH on the initiation and stable operation of the partial nitrification process was investigated, with an in depth analysis of their collaborative mechanism of effect. The following conclusions were obtained.

(1) Compared to the control reactor, >85% NAR and NRR were achieved within 4 days using low-intensity ultrasonication combined with NH_2OH dosing, shortening the partial nitrification start-up time by 6 days, with the maximum NAR reaching 96.48%.
(2) Ultrasonication with NH_2OH dosing stimulated EPS secretion, increased carbonyl, hydroxyl and amine functional group abundances and enhanced mass transfer.
(3) Ultrasonication combined with NH_2OH also promoted the growth of AOB and inhibited NOB activity, avoiding the occurrence of complete nitrification when NH_2OH was added alone and improving the systems stability.

Overall, this study shows that low-intensity ultrasonication with NH_2OH dosing can enhance partial nitrification performance, exhibiting high potential for practical application in nitrogen-containing wastewater treatment.

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 article.

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Appendix A Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2023.06.003.

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