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The key factor in High Pressure Hydrogenotrophic Denitrification: Hydrogen partial pressure

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ABSTRACT

In the envisaged hydrogen economy, H_2 could be an interesting alternative electron donor for the denitrification of drinking water or wastewater. The main obstacle to engineering the hydrogenotrophic denitrification process is the low solubility of H_2 in water under atmospheric pressure, which limits denitrification rate and nitrogen removal efficiency. In this paper, we demonstrated a novel configuration of hydrogenotrophic denitrification, namely High Pressure Hydrogenotrophic Denitrification (HPHD). Elevated H_2 partial pressure (pH_2) was employed to increase dissolved H_2 concentration and concomitantly enhance denitrification rate. Our results showed that the specific denitrification rate increased from 9.6 mg N/(gVSS·h) at 0.5 bars to 51.0 mg N/(gVSS·h) at 9 bars in HPHD. The denitrification effect could be retained with elevated pH_2 at a low temperature. The specific denitrification rate at 3 bars and 15 °C was 20.5 mg N/(gVSS·h), approximately 1.5 times that at 1 bar and 30 °C, which was quite beneficial for hydrogenotrophic denitrification under cold conditions. Different from nitrite reduction, less impact was observed on nitrate reduction by low temperature, which explained high nitrite accumulation in HPHD at 15 °C. Overall, our investigations shed light on the role of pH_2 in the promising solution for nitrogen removal in HPHD.

1. Introduction

Increasing urban density, with incomplete nitrogen removal from urban sewage streams and excess agricultural run-off, has been raising nitrogen concentrations in the environment beyond safe planetary boundaries. In particular, the increasing concentration of nitrate in groundwater aquifers as a critical indicator of potable water quality has raised the concerns of researchers due to potential health risks associated with long-term direct or indirect exposure to contaminated water resources [1]. The observed nitrate concentration values in urban aquifers in many countries and regions are considerably above the safe limit of 11.3 mg NO_3^--N/L in potable water as suggested by the World Health Organization and the Council of European Communities [2].

Nitrification/denitrification (i.e., the A_N/O process) is the most commonly used biological nitrogen removal (BNR) technology in municipal wastewater treatment systems. However, it is also the most energy-intensive [3], and not capable of complying with the increasingly stringent wastewater discharge standards, especially at low temperatures [4]. In addition, the common characteristic of municipal wastewater in many areas is low C/N ratio [5,6]. Under such circumstances, an incessant supply of external organic electron donors for the traditional BNR process is required to ensure smooth denitrification [6]. This undoubtedly increases operation cost while triggering the risk of substandard COD in the final effluent. Therefore, efficient, clean, and economical alternative technologies are urgently required to address these challenges. Hydrogenotrophic denitrification is an excellent option that has attracted considerable attention recently, because of the clean nature of H_2 and its relative cost effectiveness [1,7]. Moreover, it can be produced relatively easily from water electrolysis, and thus, is more readily accessible in remote locations. In addition, utilizing peak power production, which increasingly exceeds local maximum grid capacity, in decentralized water treatment schemes can be an interesting option [8].

H_2 is one of the most thermodynamically favorable and universally bioavailable electron donor for denitrification [7,9]. The feasibility of hydrogenotrophic denitrification to efficiently remove nitrate has been verified through principle description and a variety of reactor configurations [1,10–12]. Previous studies were mostly carried out towards

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attached growth systems, such as H_2-based membrane biofilm reactor (MBfR), which mainly performed under atmospheric pressure and focused on potable water or the small-scale treatment of oligotrophic surface water and groundwater [1,13,14]. Nevertheless, the low solubility of H_2 and potential safety concerns form barriers to further translating the promising "lab-scale" results of this technology into full-scale practical applications [9,15,16].

Notably, liquid-phase H_2 concentration increases with increasing pressure in accordance with Henry's law. Unfortunately, current studies on hydrogenotrophic denitrification in a pressurized reactor remain limited. Epsztein et al. proposed a micro-pressure hydrogenotrophic reactor, i.e., maximum pH_2 was only 1.3 bars, which was mostly for removing low nitrate in groundwater [15]. Evidently, enhancing gas-liquid mass transfer in a pressurized manner does provide new ideas for improving reactor design. However, conclusive evidence for the effective treatment of high-nitrate wastewater using pressurized hydrogenotrophic denitrification is not yet available in the literature.

To date, most denitrification processes are operated within an optimal temperature range of 25 °C–35 °C [17]. The ambient temperature of groundwater and the seasonal temperature of municipal wastewater in high-latitude regions are lower. For the conventional A_N/O process, the activity of denitrifying microorganisms declines rapidly at psychrophilic temperature (<20 °C), resulting in a sharp decrease in denitrification rate [18,19]. When temperatures are considerably below the optimal value, stable nitrification-denitrification could be technically achieved by increasing sludge concentration and prolonging hydraulic and sludge retention time. With H_2 solubility increasing at a lower temperature, hydrogenotrophic denitrification at higher pH_2 may potentially offset operating at psychrophilic temperature, providing a worthy alternative to conventional approaches.

Given the aforementioned considerations, the specific objective of this study was to investigate the performance of high pressure hydrogenotrophic denitrification (HPHD) system in the treatment of high-nitrate contaminated wastewater. First, the influences of pH_2 on denitrification rate were studied. Then, the competition between nitrate and nitrite reduction with non-H_2-limiting was explored by analyzing nitrate consumption and nitrite accumulation at various pH_2. Finally, the response of hydrogenotrophic denitrification to psychrophilic conditions was evaluated, and the direct effects of low temperature on nitrate and nitrite reduction rates were investigated. To the best of our knowledge, this is the first report of low temperature hydrogenotrophic denitrification in a pressurized bioreactor.

2. Methods and materials

2.1. Reactor set up and operation

The reactor depicted in Fig. 1 was designed for operation up to 1 MPa. The working volume was 650 mL, and the gas phase of the headspace was manually controlled at 150 mL. To seal the reactor, eight high-strength hexagonal bolts were tightened using a torque wrench [20]. A pressure gauge was connected to the top of the reactor through X-tec joint fittings (X-tec, SS-RV11F-K6, China). Online monitoring was performed to obtain accurate real-time temperature and pH data (METTLER TOLEDO, InPro4550VP pH Probe, Switzerland) during the operation. The temperature in the reactor was controlled at the designed value via the water bath (APTIO, XOYS-4006 N, China). Magnetic stirring was conducted with two bladed impellers mounted on the central stirring shaft and operated at 200 rpm for all the experiments.

The reactor was flushed by H_2 to remove air in the headspace before each run. In this study, H_2 pressure referred to relative pressure (gauge pressure). Total absolute pressure was maintained constant throughout the experiments by keeping the H_2 cylinder connected to the reactor through a pressure regulator [15]. The N_2 partial pressure produced by the exhaustion of nitrate in the reactor was small and negligible (p_{N_2} < 0.31 bars). Therefore, the pH_2 in the reactor was deemed to be relatively constant.

2.2. Cultivation of microorganisms

The mixed culture inoculated in the experiments was anoxic sludge enriched in the A_N/O process of a wastewater treatment plant located in Shanghai, China. Synthetic wastewater, which was composed of ultrapure water, nitrate (0.722 g/L KNO_3), bicarbonate (2 g/L $NaHCO_3$), phosphate (1.60 g/L KH_2PO_4 and 1.44 g/L K_2HPO_4), and other trace elements required for the growth of microorganisms, was used as feed (Text S1, Supplementary Materials). Hydrogenotrophic denitrifiers are sensitive to pH, with an optimum value in the range of 7.6–8.6, but it may slightly exceed this range due to the different inoculated cultures [21]. A pH range of 7.2–9.0 was controlled by phosphate buffer.

Prior to the start of the experiments, the acclimation and cultivation of hydrogenotrophic denitrifying bacteria present in the inoculum were realized by incubating the microorganisms in a fed-batch reactor under 1 bar of H_2 pressure at 30 °C. Each batch was flushed with H_2 for 3 min to ensure anoxic conditions for denitrification. Then, a 12 h running cycle was completed, in which the specific nitrogen loading rate was 80 mgNO_3^--N/(gVSS·d). The steady state of the enrichment culture was confirmed when the denitrification rate remained constant with the removal of total nitrogen exceeding 99.0 % [15]. The incubations lasted

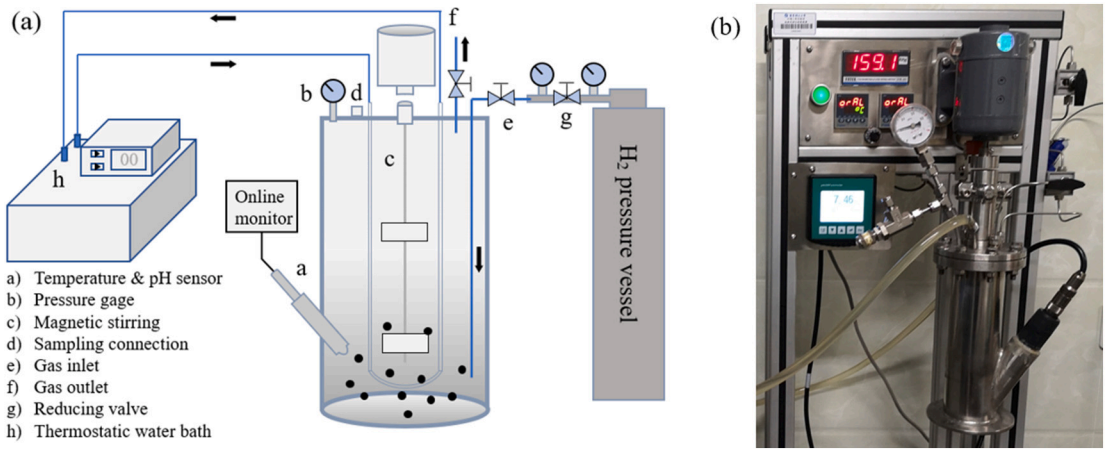

Fig. 1. Schematic view of the pressurized reactor (a), and photo of the used reactor (b).

at least 3 months.

2.3. Description of experiments

2.3.1. Experiment 1: effects of pH_2 on denitrification rates at 30 °C

The adapted stock culture was added to synthetic wastewater to conduct a series of draw-fill operations in a pressurized reactor. Each fed-batch test contained 500 mL of mixed solution (initial NO_3^--N 100 mg/L). The H_2 pressure applied was 0.5, 1, 3, 5, 7, and 9 bars at a temperature of 30 °C, respectively. For each tested pressure, the system was first operated to reach a steady state with low effluent total nitrogen (<10 mg/L). After nitrate degradation was completed, the supernatant was decanted after properly settling the suspended biomass. Then, the reactor was reloaded with synthetic wastewater to the original concentration. This process completed one operating cycle. After reaching a stable nitrogen conversion rate, additional 15 feeding cycles were performed in this fed-batch experiment to ensure reproducibility. Biomass concentration could be regarded as constant due to the slow growth of autotrophic denitrifying bacteria and the minor loss of microorganisms in decanting supernatants. Effluent samples were collected in each cycle for nitrate and nitrite analyses. The MLVSS concentration was measured in triplicate at the end of each pressure batch test.

2.3.2. Experiment 2: competition between nitrate and nitrite reduction under pressurized conditions

Experiment 2 was conducted following each pressure batch test in Experiment 1 to determine the specific reduction rates of nitrate and nitrite. All batch tests in this part consisted of three parallel replicates. Nitrate and nitrite were used as the sole electron acceptors respectively. The initial NO_3^--N and NO_2^--N concentrations were controlled at approximately 100 mg/L using KNO_3 and KNO_2, respectively. The pH_2 and temperature were consistent with those in Experiment 1. Each test lasted for 3 h, and mixed liquor samples were taken every 30 min for nitrate and nitrite analyses.

2.3.3. Experiments 3: response of HPHD to low temperature

For Experiments 3, the temperature in the reactor was controlled at 15 °C to evaluate the performance of the HPHD system at low temperature. The stock culture was acclimated at a pH_2 of 1 bar until the denitrification rate was constant. Then, Experiments 3–1 and 3–2 were performed in a manner similar to those in Experiments 1 and 2, respectively. The specific denitrification rates in HPHD at 15 °C were demonstrated in Experiment 3–1. Meanwhile nitrate reduction and nitrite accumulation were evaluated in Experiment 3–2. The H_2 pressure applied in the batch tests was 1, 3, and 7 bars. The sampling analyses of nitrate, nitrite, and MLVSS were identical to the aforementioned methods.

2.4. Sampling and analytical methods

The supernatant was collected using disposable syringes after properly precipitating the suspended biomass and immediately filtering through a disposable Millipore filter unit (0.22 μm pore size). NO_3^--N, NO_2^--N were measured by an UV-VIS spectrophotometer (Shimadzu UV1900, Japan). The pH and temperature were monitored online through the VarioPin connector equipped with an electrode sensor (InPro4550VP pH Probe, Switzerland). NO_3^--N, NO_2^--N and MLVSS were determined in accordance with standard methods [22].

3. Results

3.1. Effects of pH_2 on specific denitrification rate at 30 °C

The effects of pH_2 on specific denitrification rate were studied in Experiment 1. Fig. 2 presents the specific denitrification rates under different pH_2 at a steady state. In the applied range of 0.5–5 bars, the effects of pH_2 on the specific denitrification rate could be observed, with its average gradually increasing five-fold from 9.6 mg N/(gVSS·h) at 0.5 bars to 48.7 mg N/(gVSS·h) at 5 bars. This result was attributed to the dissolved H_2 concentration increasing with elevated pH_2. Then, pH_2 in the reactor was increased to the range of 5–9 bars, while the specific denitrification rate remained basically steady. The maximum specific denitrification rate obtained in this study was 51.0 mg N/(gVSS·h), which was much higher than those in previous studies (9.1–29.6 mg N/(gVSS·h)) [10,18,23].

In contrast, the reaction time for the effluent TN concentration to below 10 mg/L in the batch experiment shortened as pH_2 increased. The reaction time stepwise decreased five-fold from 15 h to 3 h as pH $_2$ reached 5 bars. Compared with 15 h at 0.5 bars, a reaction time of 3 h is more feasible in full-scale applications. The operating parameters and corresponding specific denitrification rates of each stage in Experiment 1 are summarized in Table 1.

3.2. Competition between nitrate and nitrite reduction in HPHD

The specific reduction rates of NO_3^--N, NO_2^--N were evaluated using

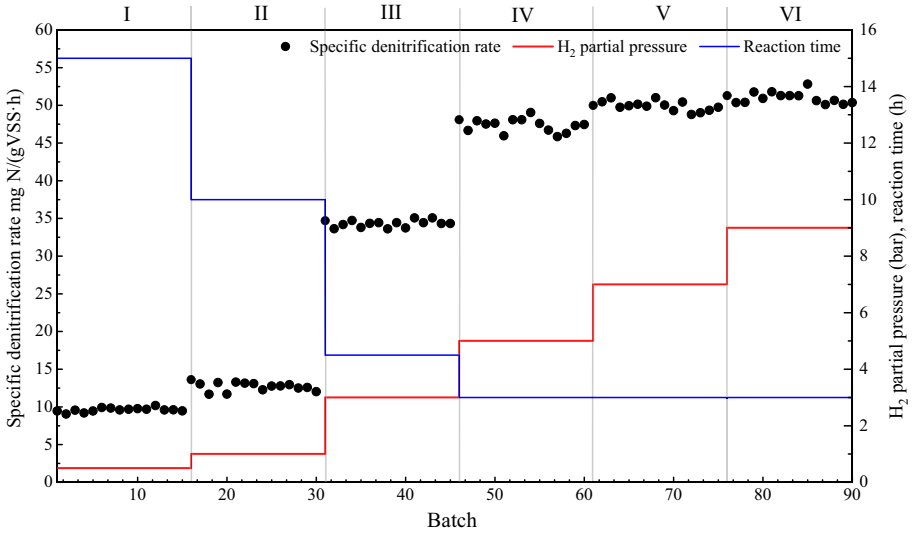

Fig. 2. Specific denitrification rates and reaction time in HPHD under different pH_2 at 30 °C.

Table 1
Overview of operating parameters and results of specific denitrification tests under different pressures at 30 °C.

Stage	pH_2 (bar)	Feed NO_3^--N (mg/L)	Effluent NO_3^--N[a] (mg/L)	Effluent NO_2^--N[a] (mg/L)	Reaction time (h)	MLVSS[b] (g/L)	Specific denitrification rate[a] (mg N/(gVSS·h))
I	0.5	100	8.1	0.1	15	0.63	9.6
II	1	100	3.5	2.3	10	0.68	14.0
III	3	100	NA	0.4	4.5	0.64	34.3
IV	5	100	NA	0.5	3	0.68	48.7
V	7	100	NA	0.7	3	0.66	49.9
VI	9	100	NA	0.1	3	0.65	51.0

NA: below detection limits.
[a] Average of 15 batches.
[b] Average in triplicate.

nitrate and nitrite as the sole electron acceptor, respectively. The specific reduction rates of NO_3^--N, NO_2^--N were defined as the first-step specific denitrification rate (FSDR) and the second-step specific denitrification rate (SSDR), respectively. The details of the NO_3^--N, NO_2^--N profiles under different pH_2 in Experiment 2 are available in the Supplementary Materials (Fig. S1). Here, the results of 5 bars were presented as an example (Fig. 3). Given that excess H_2 was supplied neither NO_3^--N nor NO_2^--N reduction was limited by electron donors. Thus, the maximum FSDR and SSDR under each pH_2 could be determined through the linear regression distribution of NO_3^--N and NO_2^--N, along with the MLVSS concentration (average of triplicate), respectively [24]. The results are summarized in Fig. 4.

As shown in Fig. 3, the concentrations of NO_3^--N, NO_2^--N linearly declined in the two electron acceptor addition schemes. On the other hand, the maximum FSDR, SSDR showed a similar trend, both showing an increase with elevated pH_2 (Fig. 4). The maximum SSDR was almost equal or very close to FSDR at the same pH_2, implying no remarkable difference in nitrate or nitrite reduction capacity at 30 °C in HPHD. This is for example evidenced, when pH_2 was 5 bars, the maximum SSDR was slightly higher than the maximum FSDR (63.6 mg N/(gVSS·h) and 61.3 mg N/(gVSS·h), respectively). Similarly, Rezania et al. observed that the maximum SSDR consistently exceeded maximum FSDR when nitrate and nitrite were used separately as the sole electron acceptor, regardless of temperature and pH. Nitrite appeared to be the more favorable electron acceptor [19]. Therefore, it is reasonable to predict that nitrite would not be detected or appreciable accumulation would not be observed. However, when nitrate was the sole electron acceptor, unexpected nitrite accumulations were observed in this study (Fig. 3a).

In comparison with the FSDR, the SSDR was much lower in the scheme where nitrate was the sole electron acceptor. The FSDR was

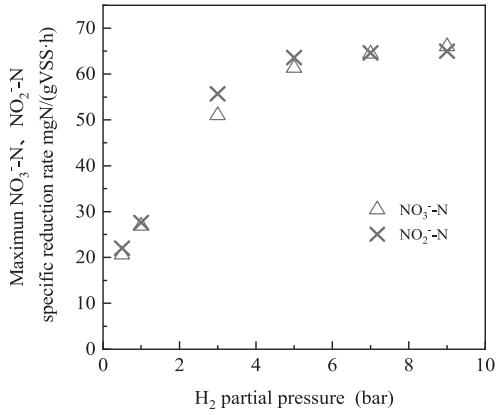

Fig. 4. Effects of pH_2 on the maximum NO_3^--N, NO_2^--N specific reduction rates at 30 °C.

61.3 mg N/(gVSS·h) at a pH_2 of 5 bars, while SSDR was only 47.3 mg N/(gVSS·h). Therefore, nitrite started to accumulate with the concomitant reduction of nitrate. Accumulation continued until nitrate was exhausted. The maximum nitrite accumulation was 24.8 mg N/L, accounting for 29.5 % of that produced by nitrate reduction (Fig. 3a). Previous studies on the kinetics of hydrogenotrophic denitrification under varying temperatures displayed that the concentration of accumulated nitrite remained constant over time until residual nitrate was exhausted [19,23]. Although not fully consistent with the results obtained in this study, the difference could be owed to the different microbial cultures used and the varying operational conditions.

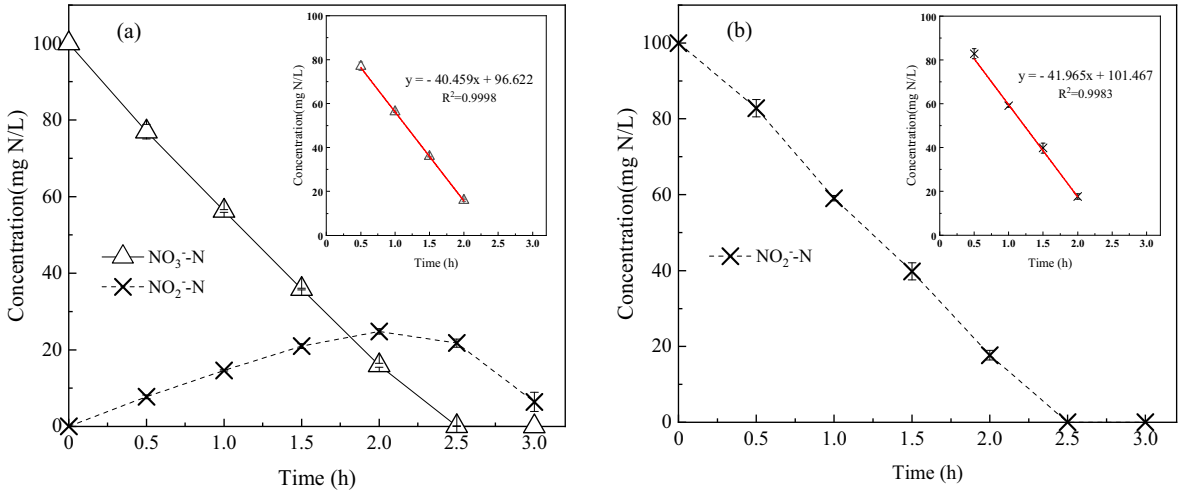

Fig. 3. NO_3^--N, NO_2^--N profiles at a pH_2 of 5 bars, where NO_3^--N(a) and NO_2^--N (b) were used as the sole electron acceptor. The red lines indicate the linear regression of NO_3^--N and NO_2^--N data with $R^2 > 0.99$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Response of specific denitrification rate to low temperature with elevated pH_2

Low efficiency or even failure has been a problem for denitrification processes operating under low temperatures. In order to investigate the effect of low temperature on HPHD, the operating temperature was set to 15 °C in Experiments 3. The effects of elevated pH_2 on specific denitrification rate in Experiment 3–1 are shown in Fig. 5. The results resembled those at 30 °C, with higher pH_2 leading to a higher specific denitrification rate. However, the specific denitrification rates in HPHD at 15 °C, as expected, were much less than those at 30 °C under the same pH_2. When pH_2 was 7 bars, for example, it was only 25.9 mg N/(gVSS·h) a decrease of 48.1 % compared to that of 30 °C.

However, the hydrogenotrophic denitrifying microorganisms cultivated in previous study showed they could adapt to low temperatures. [25]. After a closer inspection of these results, it was noticed that the specific denitrification rate at 1 bar pH $_2$ and 15 °C was 9.2 mg N/(gVSS·h), which was approximately equal to 9.6 mg N/(gVSS·h) that of 0.5 bars at 30 °C. Similarly, the specific denitrification rate of 3 bars at 15 °C was 20.5 mg N/(gVSS·h), which was about 1.5 times that of 1 bar at 30 °C. These results suggested that effective nitrogen removal could be achieved under low temperatures by off-setting the rate losses induced by low ambient temperature with elevated pH_2.

3.4. Effects of temperature on nitrate and nitrite reduction

The high-level of nitrite accumulations were observed at all tested pressures in Experiment 3–2. The NO_3^--N, NO_2^--N profiles at a pH_2 of 3 bars are shown in Fig. 6. Nitrate concentration decreased sharply from 100 mg N/L to 1.6 mg N/L within 2.5 h, with a corresponding increase of nitrite accumulation. The maximum nitrite accumulation reached 79.4 mg N/L, accounting for 81.5 % of nitrate reduced. Notably, the percentage of nitrite accumulation was constant at approximately 85.9 % within the first 2 h, and it gradually decreased as nitrate was depleted. The inhibition of nitrate reduction due to the biotoxicity of nitrite was not observed with increasing nitrite accumulation.

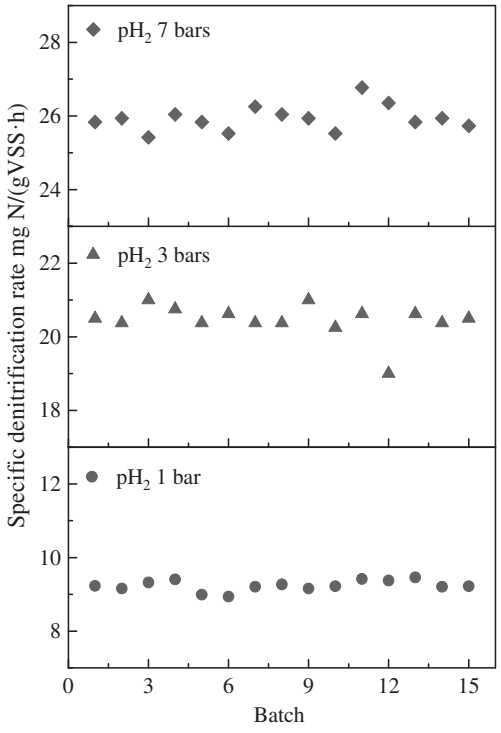

Fig. 5. Specific denitrification rates in HPHD under different pH_2 at 15 °C.

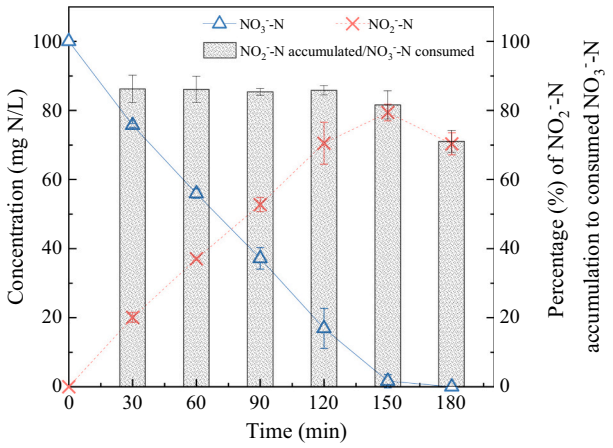

Fig. 6. NO_3^--N, NO_2^--N profiles and accumulation percentage of NO_2^--N under a pH_2 of 3 bars at 15 °C.

The temperature dependence of nitrate and nitrite reduction in HPHD was investigated, and the FSDR, SSDR at 15 °C and 30 °C were compared in Fig. 7. The FSDR decreased slightly when temperature was 15 °C, and the maximum percentage of decrease was only 7.0 % (pH $_2$ 3 bars). In contrast, a sharp decrease in the SSDR was observed in these experiments at 15 °C. It was reduced by 67.0 % at a pH $_2$ of 3 bars, i.e., from 28.5 mg N/(gVSS·h) to 9.4 mg N/(gVSS·h), and the ratio of SSDR/FSDR was only 18.5 %, further explaining the phenomenon of high nitrite accumulation at 15 °C.

Fig. 7 also shows that the FSDR increased while SSDR remained almost constant when pH_2 was elevated from 3 bars to 7 bars at 15 °C. The difference between FSDR and SSDR in the trend relative to pressure changes under low temperature (i.e., SSDR/FSDR ratio decreased) would lead to a considerably higher nitrite accumulation as pH_2 increased. The results indicated that nitrite reduction was the rate-limiting step in HPHD, especially at low temperatures.

4. Discussion

4.1. High pressure driven hydrogenotrophic denitrification

Although H_2 is considered the cleanest electron donor for denitrification, the application of hydrogenotrophic denitrification in full-scale plants is limited by its low solubility under atmospheric pressure [9,21]. In this study, the dissolved H_2 concentration was increased by pressurization in order to increase denitrification rates. The hypothesis was confirmed with a positive correlation between pH_2 and denitrification rate till a pH_2 of 5 bars, as shown in Fig. 2. Furthermore, denitrification rate increased moderately with a slight increase in pH_2, but reaction time was significantly shortened. When pH_2 rose from 0.5 bars to 1 bar, the specific denitrification rate increased from 9.6 mg N/(gVSS·h) to 14.0 mg N/(gVSS·h), and the corresponding reaction time was reduced by 5 h. These results suggest that pH_2 could be critical to improving the performance of nitrogen removal in HPHD.

For a specific biomass, specific denitrification rate was almost at a constant level when pH_2 reached a threshold (Fig. 2). That is, H_2 was no longer the limiting substrate. Generally, the kinetics model of hydrogenotrophic denitrification can be described by zero-order kinetics when substrate concentration is much higher than the half-saturation constant (i.e., $S_{NO_3^-} \geq K_{NO_3^-}$ and $S_{H_2} \geq K_{H_2}$) [19,23,26]. The kinetics of denitrification at concentrations of >1 mg NO_3^--N/L are independent of nitrate, suggesting that the supply of electron donors controls the rate [17]. Our results showed that the hydrogenotrophic denitrification process was a zero-order reaction when $pH_2 \geq 5$ bars (absolute pressure, 6 bars). According to Henry's law, the dissolved H_2 concentration was about 8.98 mg/L in aqueous solutions at 30 °C (Henry's law constant,

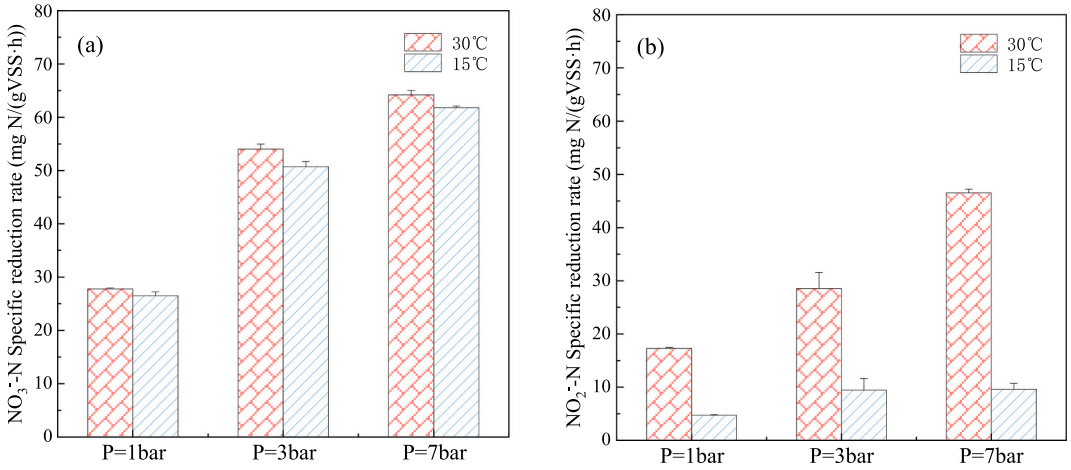

Fig. 7. Effects of temperature on the specific reduction rate of NO_3^--N and NO_2^--N in HPHD at different pressures and temperatures.

0.749 mmol/(L·bar)). This finding is significant for the kinetics and the modelling of hydrogenotrophic denitrification. In addition, a pH_2 of 5 bars is relatively realistic for reactor design and safety considerations in practical applications. The concerns of H_2 loss in the effluent stream and the associated security risks should be addressed, but appear manageable given the low H_2 solubility under atmospheric pressure. Moreover, the combination of pressurized hydrogenotrophic denitrification and the subsequent polishing unit resulted in high H_2 conversion rates and could achieve almost 100 % utilization of H_2 [27].

H_2-limitation has been widely reported as one of the main causes of nitrite accumulation [1,28,29]. The low critical limit of dissolved H_2 concentration was reported to be 0.2 mg/L [28], when nitrite reductase would be inhibited. However, Li et al. [18] reported that nitrate could be effectively removed without observing the accumulation of nitrite, even if the dissolved H_2 concentration was as low as 0.02 mg/L. Nitrite accumulation was also evaluated by applying different pH_2 in this study. Nitrite accumulation occurred under all pH_2 applied conditions in HPHD, and the accumulation level depended on the difference between FSDR and SSDR. These results indicated that dissolved H_2 concentration is not directly responsible for the accumulation of hydrogenotrophic denitrification intermediates.

Although the reduction of nitrate and nitrite, which are catalyzed by different enzymes, proceed in parallel at the end of the respiratory chain [30], our results indicate that these denitrification steps in HPHD are linked and nitrite reduction is affected by nitrate. Nitrate reductase (NAR) and nitrite reductase (NIR) acquire electrons from a common electron source (ubiquinol pool) in which electrons are produced by the oxidation of H_2. Pan et al. [24] confirmed that the competition of reducing equivalents could happen between denitrification steps despite the oversupply of electron donors, with the consequence of denitrification intermediates accumulation. In this study, nitrate was always preferentially reduced regardless of pH_2, which might be due to the nitrate competitive inhibition of nitrite reduction [31] or the higher electrophilic capability of nitrate for specific denitrifying culture [32]. In addition, the unique microbial community structure may cause an imbalance of nitrogen species reduction reactions. Nitrate respiring bacteria were reported to reduce nitrate using H_2 with nitrite as the final reduction product [33].

4.2. Possible inhibitory mechanism of low temperature and prevention strategies

The consensus was achieved that the performance of denitrification systems under low temperatures would be negatively affected [21]. A significant decrease in the denitrification rates of the HPHD system was observed when temperature decreased from 30 °C to 15 °C. Especially, nitrite reduction rate was more sensitive to temperature drops, which was supported by considerable reports [18,19]. This was also confirmed by denitrifying enzymes activity. Compared with those at 30 °C, NAR and NIR activities at 15 °C decreased by 27.7 % and 39.4 %, respectively (Table S1). The decrease in temperature enlarged the gap in the relative rates of nitrate and nitrite reduction, which was consistent with more nitrite accumulation at 15 °C. However, nitrate reduction rate was seemingly unaffected by the decrease in NAR activity. As shown in Fig. 7a, nitrate reduction rates at 15 °C were very close to those at 30 °C. These specific denitrification characteristics in HPHD may be attributed to the enrichment of facultative anaerobes capable of reducing nitrate only to nitrite [34].

Microbial community structures were identified by high-throughput sequencing, and the genera *Paracoccus* and *Hydrogenophaga* were the dominate members in HPHD (accounting for >90 % of the microbial population). The results also showed that temperature variation altered microbial community composition. At the genera level, the relative abundance of *Paracoccus* declined sharply while that of *Hydrogenophaga* increased greatly in response to a lower temperature, and *Hydrogenophaga* dominated the microbial community (Fig. S4). The study on genotypic and phenotypic characterization of hydrogenotrophic denitrifiers showed that high nitrite accumulation was associated with denitrification regulatory phenotypes, with *Hydrogenophaga taeniospiralis* exhibiting a typical progressive denitrification process that electrons from H_2 oxidation initially flow to NAR until all nitrate had been reduced to nitrite and then to NIR [35]. Furthermore, the abundance and expression of denitrifying functional genes regulated by low temperature stress may make nitrite reduction a rate-limiting step in denitrification [36–39].

The effectiveness of HPHD for nitrate remediation in wastewater at a relatively low temperature was also verified in this study. The higher pH_2 resulted in higher nitrite accumulation, however it sharply decreased after reaching the plateau (Fig. 6), when the competitive inhibition for nitrite was relieved with the exhaustion of nitrate. The removal of nitrate and nitrite could be achieved in a much shorter time for higher pH_2. These results further support the idea that increasing the pH_2 of reaction system to attain the desired denitrification rate at a low temperature is a simple and effective method. For hydrogenotrophic denitrification, the availability of liquid-phase H_2 when compared to other environmental conditions, such as temperature, is a crucial factor that controls denitrification rates. As to influent fluctuations, the HPHD has the advantage to ensure denitrification by easily increasing the electron donor, which is more difficult for heterotrophic denitrification and sulfur autotrophic denitrification. In addition, preconditioning mixed culture with nitrite and the synergistic optimization of pH and bicarbonate dose could be employed to successfully circumvent nitrite

accumulation [40]. Combining biological denitrification with iron-based abiotic nitrite reduction (the addition of Fe(II)-containing minerals) was also suggested to counteract nitrite accumulation and foster complete hydrogenotrophic denitrification [41].

5. Conclusions

High Pressure Hydrogenotrophic Denitrification was first reported for high efficient nitrate removal in wastewater. The denitrification rates were strongly dependent on pH_2 in HPHD. The specific denitrification rate at 30 °C increased from 9.6 mg N/(gVSS·h) at 0.5 bars to 51.0 mg N/(gVSS·h) at 9 bars. Nitrite accumulation occurred even with excessive H_2, indicating that dissolved H_2 concentration could not be directly responsible for nitrite accumulation. Moreover, the HPHD could well cope with psychrophilic temperatures by off-setting the denitrification rate loss with elevated pH_2. The specific denitrification rate at 3 bars and 15 °C was 20.5 mg N/(gVSS·h), approximately 1.5times that at 1 bar and 30 °C. The SSDR declined dramatically at 15 °C whereas the FSDR remained almost unaffected, resulting in substantial and steady nitrite accumulation in HPHD.

CRediT authorship contribution statement

Jianmin Zhou: Conceptualization; Investigation; Data curation; Formal analysis; Visualization; Validation; Writing - original draft. **Lei Ding:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing. **Mu Shi:** Investigation, Methodology, Data curation. **Ralph E. F. Lindeboom:** Writing - review & editing. **Changzheng Cui:** Writing - review & editing.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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