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Riboflavin Enhanced Denitrification of Artificial Wastewater under Low C/N Condition in Cold Season

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Nitrogen removal under low C/N ratio in cold season is an arduous task. In this study, riboflavin was used as an eco-friendly electron mediator to improve the denitrification process in an SBR reactor under conditions of low temperature (10 °C to 15 °C) and limited carbon source (C/N ratio = 4.0). The results indicated that riboflavin created a suitable pH in the system for denitrification. Under water temperature of 10 °C to 15 °C, riboflavin (10 mg/L) stimulated the NO_3^--N and TN removal rate by 16.5%, and 51%, respectively. Riboflavin promoted the utilization efficiency of limited carbon source, driving the denitrification process with low residual acetate as electron donor. The rising cost of riboflavin supplement (10 mg/L) was 0.025 USD per m^3 of wastewater. To satisfy the efficient nitrogen removal from municipal wastewater, the optimum C/N ratio and the selection of solid/immobilized redox mediators should be developed in future work.

INTRODUCTION

The combined process of nitrification-denitrification is a traditional but very effective method for nitrogen removal from municipal wastewater. Denitrification is affected by available carbon source, temperature, pH, and dissolved oxygen (Elefsiniotis and Li 2006). In China, municipal wastewater is characterized by low C/N ratio (< 6.0), meaning that low energy metabolism drives the biological redox-reaction of NO_3^--N to NO_2^--N, and finally to N_2 (Liang *et al.* 2014). In addition, water temperature below 15 °C reduces or even ceases the microbial/enzymatic activity, decelerating the denitrification process (Ge *et al.* 2012). These problems urge the development of a cost-effective method to achieve high and quick nitrogen removal from wastewater with low C/N ratio in cold seasons.

To facilitate nitrogen removal, the external addition of carbon sources such as methanol, sodium acetate, glucose, and other bio-available organic wastes are applied to drive the denitrification process (Zhang *et al.* 2019; Mielcarek *et al.* 2020). Reducing reagents such as zero valent iron (Fe^0), sulfide (S^{2-}), and hydrogen (H_2) are also inorganic electron donors (Oh *et al.* 2016; Zhang *et al.* 2019). However, this process consumes industrial resources and raises operating fees. Electron mediators can be applied to lower the activation energy (E_a), which enhances the denitrification rate and the utilization of limited carbon sources under low C/N ratio (Guo *et al.* 2015). The standard redox potential (E_0') of a possible electron mediator should be between the two half reactions of donor-

oxidation and denitrification, improving the extracellular electron transport (Van der Zee and Cervantes 2009). Artificial quinoid redox mediators, including anthraquinone (AQ), anthraquinone-2,6-disulphonate (AQDS), 2-hydroxy-1,4-naphthoquinone (LAW), and 1,2-naphthoquinone-4-sulphonate (NQS) can be reversibly transferred between their hydroquinone forms, shuttling electrons from the donor to nitrate and nitrite (Yuan *et al.* 2017). However, these quinone-associated artificial electron mediators are quite expensive, and they pose potential toxicity to the functional microbe and environment. Cadena Ramírez *et al.* (2019) reported an inhibitory effect of quinoid redox mediators on denitrification. In recent years, natural or eco-friendly redox mediators, *e.g.*, chlorophyll and biochar, have been used for promoting denitrification (Wu *et al.* 2019; Lu *et al.* 2020).

Riboflavin is a biogenic product with electron mediating ability. Although many microorganisms could generate crude riboflavin, its production is very small. Thus, commercial riboflavin was served by chemical synthesis for a long time during the last century. Currently, crude riboflavin could be efficiently fermented by *Bacillus subtilis* and *Ashbya gossypii* strains with ordinary carbon sources such as vegetable oils (Revuelta *et al.* 2017). In comparison to chemical synthesis, biogenic production has its advantage in reducing the cost and the secondary pollution. Riboflavin is a relatively economical and available choice as an external electron mediator. It could eco-friendly improve the electron transport ability and shift the abundance of denitrifying bacteria (Guo *et al.* 2019). However, other studies reported that riboflavin could not benefit the isolation of denitrifier due to the high C/N ratio (20) at 37 °C (Heylen *et al.* 2006). Under conditions of low C/N ratio in cold seasons, the effect of riboflavin on denitrification process has not received enough research attention. This study aims to improve the nitrogen removal rate under low temperature and to maximize the utilization of limited carbon sources. The feasibility and future works of using riboflavin to upgrade the denitrification with low C/N in cold seasons are considered.

EXPERIMENTAL

Influent, Bioreactors, and Inoculum

Artificial wastewater in this study was prepared with analytical grade potassium nitrate (KNO_3) and sodium acetate (CH_3COONa). Riboflavin was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). It was dissolved in alkaline conditions to a stock concentration of 18.8 g/L. Influent C/N ratio was fixed at 4.0, with NO_3^--N of 75 mg/L, and sodium acetate of 384 mg/L because its COD equivalent was 0.78. $NaHCO_3$ buffer in the influent was supplied at 500 mg/L. Ca, Mg, Fe, and trace metals (Mn, Zn, Ni, Co) were supplemented as described in a previous study (Huang *et al.* 2020). A plexiglass-made sequential batch reactor (SBR, 5L) was operated for two months in winter to examine the effect of riboflavin on nitrogen removal in the cold season. The inoculum was the returned activated sludge from a secondary clarifier of Qige Municipal Wastewater Treatment Plant (MWTP) in Hangzhou, China. The sludge in the SBR was 3 g VSS/L.

SBR Operation

The SBR was continuously operated in winter season for 61 days from Nov. 2017 to Jan. 2018, with two batches per day. The working procedure in each batch was as follows, 30 min feeding with a peristaltic pump, 10 h stirring (100 rpm) with a mechanical

stirrer, 1 h gravity settling, and 30 min draining. No air was aerated during any operation. Despite the lack of aeration, dissolved oxygen (DO) in the initial 1 h was up to 1.8 mg/L, due to the residual DO in the artificial wastewater. After that, DO in the SBR decreased to anoxic condition (< 0.5 mg/L).

The total hydraulic retention time (HRT) was designed at 10.71 h, and sludge retention time (SRT) was at 40 days. The entire operation was defined for three ongoing stages, *i.e.*, Stage I (Denitrification acclimatization, Days 1 to 22), Stage II (Operating stabilization, Days 23 to 48), and Stage III (Riboflavin responding, Days 49 to 61). During the stages, different operational conditions were conducted to elucidate the effect of riboflavin on denitrification. Stages I and II were conducted with riboflavin-free influent. And the water temperature during Stage I was usually above 15 °C. After that, the water temperature decreased below 15 °C. In Stage III, riboflavin (10 mg/L) was added to the influent to assess the advantage of riboflavin on nitrogen removal under low temperature. During the 61-day operation period, the effluent samples were collected every one or two days, for the analysis of various nitrogen species and chemical oxygen demand (COD).

Chemical Analysis

The pH and temperature were detected *in situ* using a portable pH meter (Mettler Toledo, F2 Standard, Greifensee, Switzerland). DO was detected by a portable DO meter (SANXIN, SX716, Shanghai, China). The collected samples were centrifuged at $4000 \times g$ and then filtered through disposable syringe filters (0.45 μm, Anpel Co., Shanghai, China). The filtrate was analyzed for COD and nitrogen speciation, *i.e.*, nitrate (NO_3^--N), nitrite (NO_2^--N), and total nitrogen (TN). The COD was analyzed in accordance with standard methods (APHA/AWWA/WEF 2005). NO_3^--N was detected by the ultraviolet spectrophotometric method (PhotoLab 6600). NO_2^--N was measured using the N-(1-naphthalene)-diaminoethane spectrophotometry method; TN was analyzed by the alkaline potassium persulfate ultraviolet spectrophotometry method.

RESULTS AND DISCUSSION

SBR Operating Conditions

Temperature and pH are significant factors affecting bio-chemical process during wastewater treatment. The variations of water temperature and pH in the SBR system are shown in Fig.1. Stage I was considered as the acclimation for the denitrification process using returned sludge from MWTP. During this period, the water temperature ranged between 15 °C to 20 °C, and the pH was 8.0 to 8.7. In the following Stage II and Stage III, the water temperature declined successively, and it mainly ranged between 10 °C to 15 °C. Stages II and III were considered as the stages for operating-stabilization and riboflavin-responding in the cold season.

Low temperature below 15 °C inhibits the denitrification activity and functional gene expression, rapidly lowering the nitrogen removal efficiency (Racys *et al.* 2018). A common engineering resolution of this issue in cold seasons (such as winter) is to enhance the hydraulic/sludge retention time, or to decrease the nitrogen load rate (Song *et al.* 2020). Elsewhere, new bioreactors such as the moving bed biofilm reactor (MBBR) and biochar-packed SBR were developed to solve this problem (He *et al.* 2018; Kowalski *et al.* 2019). However, these modifications required larger reconstruction cost for scale-up applications.

After the stabilization, riboflavin (10 mg/L) was added to the influent at Stage III to assess its promotion on denitrification resisting cold temperature. Notably, pH slightly decreased after the supplement of riboflavin in Stage III, ranging from 7.5 to 8.0. This decrease would be attributed to the potential sludge fermentation triggered by riboflavin (Huang *et al*. 2019). Moreover, denitrification was an alkalinity-producing process, which would be repressed when ambient pH was higher than 8.0, or lower than 6.0 (Lin *et al*. 2009). Thus, riboflavin played a positive role for denitrification by creating a suitable pH condition, accelerating the start-up of the SBR operation in cold seasons.
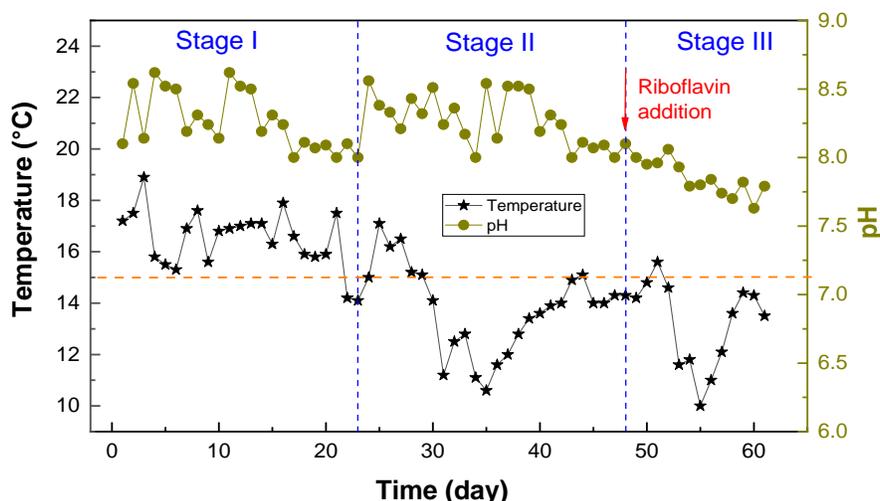
Fig. 1. Variation of water temperature and pH in different operating stages

Variation of Nitrogen Species in the Effluent

The concentrations of various nitrogen species, including NO_3^--N, NO_2^--N and TN, are shown in Fig. 2. The results indicated a remarkable removal of NO_3^--N after acclimating for six days. In the following Stage II and Stage III, the effluent NO_3^--N declined to the minimum of 4.5 mg/L (94% removal). NO_2^--N showed an increasing trend during Stage I, a slight fluctuation during Stage II, and a tiny decrease during Stage III.

Although NO_3^--N removal was high, TN removal was relative lower with C/N ratio of 4.0 under low temperature (< 15 °C). The results indicated that NO_2^--N accumulation was gradually increased since the end of Stage I, resulting in a higher effluent TN at ~40 mg/L with removal efficiency below 50%. This occurrence was mainly attributed to the lack of bioavailable electron donor, inducing a partial denitrification of nitrate (NO_3^--N) to nitrite (NO_2^--N) rather than complete denitrification to nitrogen gas (N_2). Higher pH promotes the partial denitrification process during the long-term operation (Qian *et al*. 2019). In this study, higher pH (8.0 to 8.7) in Stage II was observed (Fig.1), which in turn resulted in a larger NO_2^--N accumulation. During Stage III, effluent TN was strikingly decreased after the addition of riboflavin (10 mg/L). In this condition, nitrite concentration was comparable as that in Stage II, while TN removal achieved by up to 70%, much higher than that in Stage II (< 50%). Notably, the removed TN was not balanced by the produced nitrate and nitrite. This could be attributed to the potential abiotic oxidation of nitrite to nitrate in the presence of riboflavin after the analysis of nitrite (Doane 2017), resulting in a doubled contribution to TN. The exact mechanism needs a further investigation.

Furthermore, the denitrification rate at the end of Stage II (Day 47) was 1.88 mg $NO_3^--N/(L \cdot h)$, which was increased to 2.19 mg $NO_3^--N/(L \cdot h)$ at the end of Stage III (Day

61). The TN removal rate was increased from 1.02 mg TN/(L·h) to 1.54 mg TN/(L·h). Riboflavin increased NO_3^--N and TN removal rate by 16.5% and 51%, respectively. This result was consistent with those of a previous study, which indicated that artificial electron mediators of AQDS, LAW and NQS could improve the denitrification efficiency by 11% to 18.7% under low water temperature condition (10 °C to 15 °C) (Yuan *et al.* 2017).

As a biogenic and eco-friendly electron mediator, riboflavin could stimulate the denitrification rate and TN removal under low water temperature (10 °C to 15 °C) in cold season. However, NO_3^--N and the generated NO_2^--N were unable to be completely reduced because of the insufficient energy pool (low C/N ratio) in this study. High and efficient electron sink to denitrification with limited carbon source is recommended for future studies.
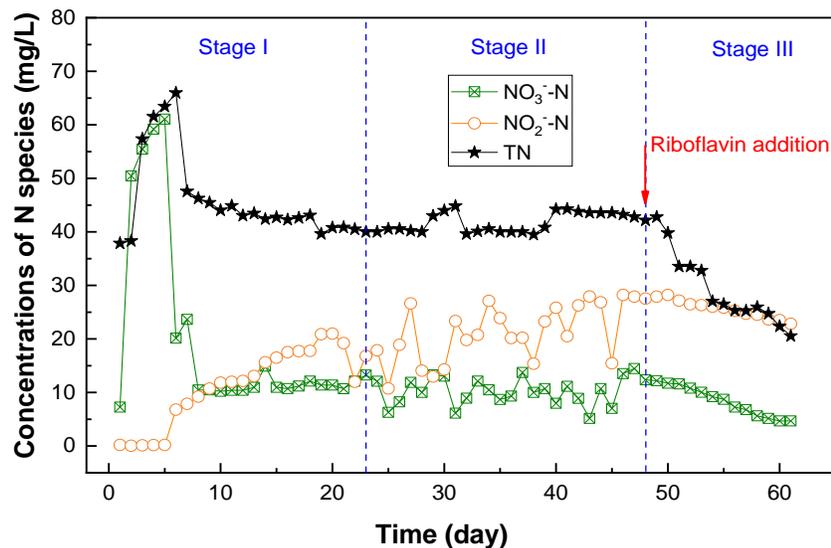
Fig. 2. Variation of NO_3^--N, NO_2^--N and TN in the effluent at different operating stages

Variation of COD in the Effluent

Effluent COD concentrations was detected to investigate whether riboflavin could improve the utilization efficiency of limited carbon source, *i.e.*, sodium acetate (Fig. 3).
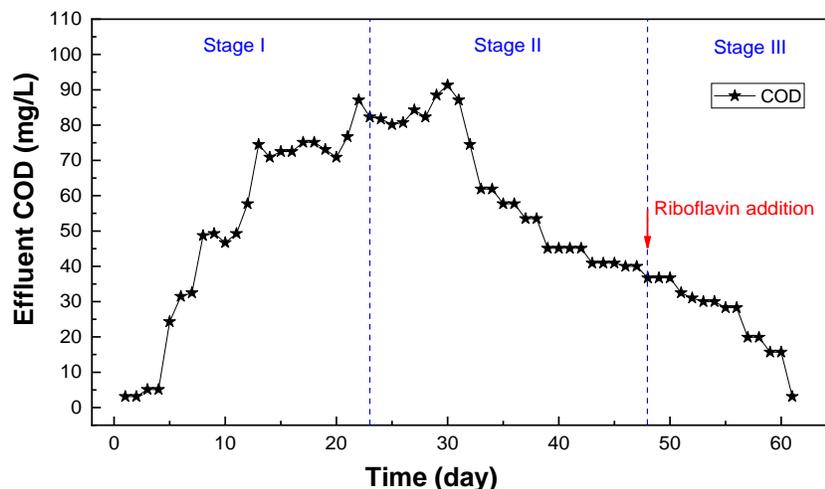
Fig. 3. Variation of COD in the effluent at different operating stages

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During Stage I, the effluent COD was gradually increased to ~80 mg/L, indicating a declined consumption of carbon source in acclimation period. The authors attributed this occurrence to the high pH and decreased water temperature along with the operation time (Fig. 1), which is not ready for the denitrification gene expression yet (Saleh-Lakha *et al.* 2009). Furthermore, riboflavin might promote the sludge fermentation to generate soluble organics, which also contributed to the upward trend of effluent COD (Huang *et al*. 2019).

During Stage II, effluent COD dropped (Fig. 3), indicating a stronger denitrification activity, which in turn consumed more carbon source. At the end of Stage II, effluent COD was stabilized at ~40 mg/L. This low carbon source concentration could hardly drive the denitrification process further, due to the kinetic disadvantage. Notably, after the addition of riboflavin in Stage III, effluent COD first slightly but then sharply declined to undetectable level at the end of Stage III (Day 61). These findings implied that riboflavin could efficiently lower the E_a, promoting the effectiveness of residual acetate and potential fermentative by-products as electron donors for denitrification. However, the low C/N ratio in this study lacked sufficient energy to drive complete denitrification. Thus, TN could not be further removed to satisfy the Class 1A discharge standard according to Chinese national standard (GB18918-2002).

Operational Cost and Future work

Efficient nitrogen removal under low C/N condition in cold season is an arduous task and an ongoing topic. In this study, the cost of riboflavin addition (10 mg/L) was calculated at 0.025 USD per m^3 of wastewater, based on the industrial riboflavin price of 2.5 USD/kg (Shi *et al.* 2020). This would raise the operating cost by ~20% for municipal wastewater treatment (Qi *et al.* 2020). In the future, research should be focused on the following: (1) the optimum dosage of added riboflavin should be optimized to reduce the increase in costs; (2) the selection of cheap solid materials with electron mediating ability are highly appreciated to replace soluble redox mediators; (3) the immobilization of soluble redox mediators on special carriers are expected to be developed.

CONCLUSIONS

1. Riboflavin decreased the pH to the range 7.5 to 8.0 in the SBR system, which was suitable for the denitrification process, accelerating the start-up of the SBR operation in cold season.

2. Under low water temperature (10 °C to 15 °C), riboflavin stimulated NO_3^--N and TN removal by 16.5% and 51%, respectively; TN removal was lower than NO_3^--N due to the partial denitrification and larger accumulation of NO_2^--N.

3. Under low C/N ratio (4.0), riboflavin promoted the utilization of limited carbon source, driving the denitrification process with low residual acetate as electron donor.

4. Riboflavin was judged to be a relative cheap electron mediator for denitrification, and the rising cost of its addition (10 mg/L) was calculated as 0.025 USD per m^3 of wastewater.

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