Nitrate removal by nitrate-dependent Fe(II) oxidation in an upflow denitrifying biofilm reactor

Jun Zhou, Hongyu Wang, Kai Yang, Yuchong Sun and Jun Tian

ABSTRACT

A continuous upflow biofilm reactor packed with ceramsite was constructed for nitrate removal under an anaerobic atmosphere without an organic carbon source. Denitrifying bacteria, *Pseudomonas* sp. W1, *Pseudomonas* sp. W2 and *Microbacterium* sp. W5, were added to the bioreactor as inocula. Nitrate concentration, nitrite accumulation and nitrogen removal efficiency in the effluent were investigated under various conditions set by several parameters including pH, hydraulic retention time (HRT), ratios of carbon to nitrogen (C/N) and temperature. The results illustrated that the maximum removal efficiency of nitrogen was 85.39%, under optimum reaction parameters, approximately pH 6.5–7, HRT = 48 hours and C/N = 13.1:1 at temperature of 30 °C, which were determined by experiment.

Key words | ceramsite, continuous upflow biofilm reactor, nitrate-dependent Fe(II) oxidation, nitrate removal

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INTRODUCTION

Nitrate is a prevalent contaminant in many wastewaters and groundwaters and needs to be removed in order to prevent oxygen consumption and eutrophication of surface waters. Substandard industrial wastewater and domestic sewage discharge, landfill leachate and the extensive use of fertilizers contribute to nitrate-polluted water, and nitrate could also originate from the nitrification process (Chen *et al.* 2014). Increased nitrate concentration in drinking water is a potential risk, which may threaten human health by leading to methemoglobinemia in infants (Bhatnagar & Sillanpaa 2011). Moreover, nitrite formed by nitrate reduction can also react with some amines to produce carcinogenic nitrosamines (Devlin *et al.* 2000). The common methods of nitrate removal are ion exchange, reverse osmosis, adsorption, electrodialysis, and denitrification; biological denitrification is identified as a cost-effective technology for its relatively low cost, no by-products generated and no requirement for post-treatment (Liu *et al.* 2012).

Biological denitrification is normally known as an anaerobic process with the reduction of nitrate to dinitrogen ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) (Adav *et al.* 2010). Heterotrophic denitrificans have a rapid growth rate with sufficient organic carbon provided by adding organics or by pre-denitrification (Lotter *et al.* 1986). These may lead to increased cost and the sacrifice of nitrogen removal efficiency. In contrast, autotrophic denitrification has promise for application in the treatment of oligotrophic waters (such as groundwater) and low C/N ratio wastewater (Morgenroth & Wilderer 1998). Widdel *et al.* (1993) reported O_2-independent oxidation of ferrous iron with light as the energy source using purple non-sulphur photosynthetic bacteria.

Microbial nitrate-dependent Fe(II) oxidation (NDFO) was first discovered in 1996, using nitrate-reducing bacteria with ferrous iron as the sole electron donor (Straub *et al.* 1996). This anaerobic process utilizes nitrate-reducing bacteria for the reduction of nitrate to nitrogen gas with Fe(II) as an electron donor. NDFO bacteria also contribute to the biogeochemical cycles of iron and nitrogen (Zhang *et al.* 2014). A number of NDFO microorganisms have been isolated from diverse environments. Nevertheless, most of these microorganisms are heterotrophic (Chakraborty *et al.* 2011), which indicates that they need an organic co-substrate for the growth and oxidation of different forms of Fe(II), such as chelated Fe(II) (Dong *et al.* 2013), soluble Fe(II) (Vereda *et al.* 2013) and solid-phase Fe(II) (Weber *et al.* 2001). Autotrophic pure and enrichment cultures have also been reported (Kumaraswamy *et al.* 2006). Heterotrophic denitrification carries the risk of secondary pollution (Chung *et al.* 2014). Most researchers focus on

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microbial physiology, while very few reports are about NDFO bacteria in a biological reactor.

In this study, a bioreactor was constructed with pure cultures of *Pseudomonas* sp. W1, *Pseudomonas* sp. W2 and *Microbacterium* sp. W5 capable of nitrate reduction with anaerobic Fe(II) oxidation. The major objective of this study was to remove nitrate utilizing nitrate-dependent Fe(II) oxidation in a continuous upflow bioreactor packed with ceramsite. Some environmental conditions have been investigated to operate the reactor effectively.

MATERIALS AND METHODS

Reactor operation

The experiment was carried out in a plexiglass column. A schematic diagram of the reactor used in this study is shown in Figure 1. The working volume of the reactor was 4.71 L with a height of 750 mm and an internal diameter of 100 mm; below this was a filter plate, 80 mm above the bottom of the reactor, with dozens of holes, each with a diameter of 2 mm. The bioreactor was filled with a height of 600 mm of ceramsite filter media (3–5 mm diameter, 1,730 kg/m^3 density, 650 m^2/m^3 specific surface). A cover plate with a gas vent was used to ensure anaerobic conditions. The effluent was discharged from the outlet 150 mm above the top of the ceramsite by gravity.

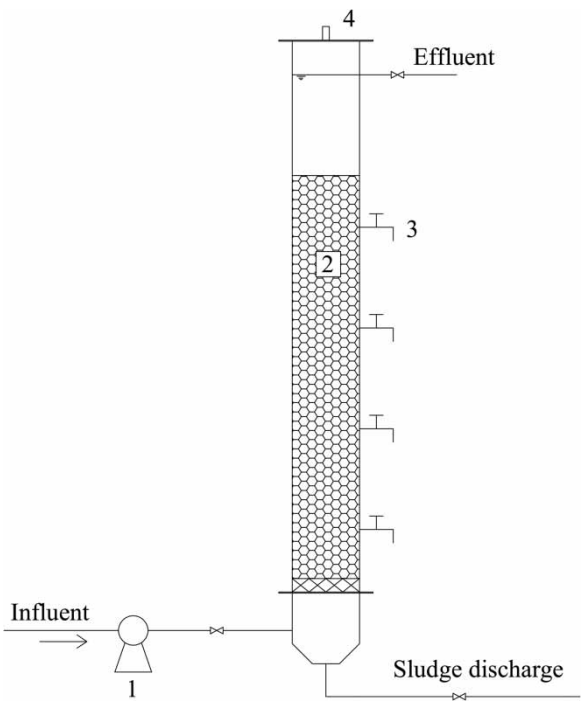

Figure 1 | Schematic diagram of anaerobic continuous upflow biofilm reactor: (1) peristaltic pump; (2) ceramsite; (3) sampling port; (4) gas vent.

The influent was supplied continuously from the bottom of the reactor by a peristaltic pump. To form the biofilm, the precultured *Pseudomonas* sp. W1, *Pseudomonas* sp. W2 and *Microbacterium* sp. W5 isolates (around 6×10^6 cells per mL) were inoculated into the reactor and the oxygen was expelled. In the initial 7 days of the start-up period, the culture medium containing mixed bacteria solution flowed into the reactor from a jar (5 L volume) with a cork, through the ceramsite and was recycled into the jar from the outlet with a flow rate of 3.27 mL/min; the hydraulic retention time (HRT) was 24 hours. The culture medium characteristics were as follows (per liter): KNO_3, 100 mg; $MgSO_4$, 200 mg; $CaCl_2$, 200 mg; K_2HPO_4, 300 mg; KH_2PO_4, 500 mg; NaCl, 1,000 mg; $NaHCO_3$, 2,000mg; $FeSO_4$, 800 mg; and pH, 7.0. $NaHCO_3$ was used as the carbon source. When the concentration of KNO_3 increased to 300 mg/L, culture medium was discharged without recycling when the nitrogen removal rate reached 30–40%. Black biofilm adhering to the surface of the ceramsite was obtained with a nitrogen removal rate achieving approximately 40–50%, which demonstrated that the start-up of the bioreactor was basically complete. During the experiment, the reactor was operated in continuous mode without recycling of synthetic water.

Synthetic wastewater with the following composition was supplied continuously in the experiments (per liter): nitrate-N, 30–40 mg; nitrite-N, 2–10 mg; total phosphorus, 1.5–2 mg; Fe^{2+} (form of $FeSO_4$), 800 mg; HCO_3^- (form of $NaHCO_3$), 1,000–3,000 mg. If not specified, the optimal value of the conditions will be used in the following experiments to replace the original value, and the conditions uninvestigated remain unchanged. Based on other reports (Li *et al.* 2014; Wang *et al.* 2014), the molar ratio of Fe/N = 2.27–5.0, and the stoichiometric value of Fe and N, Fe^{2+} was set at the value of 800 mg/L with an Fe/N molar ratio value of 5.0. The pH, HRT and temperature were 6–8 hours, 12–60 hours and 15 °C –35 °C, respectively. When operating conditions were changed, the effluent of four continuous cycles was evaluated once the reactor had obtained a stable response, and average variations of nitrate and nitrite of three samples were measured. The pH was regulated using 0.5 M NaOH and 0.5 M HCl.

Analytical methods

Nitrate nitrogen and nitrite nitrogen were measured according to *Standard Methods* (American Public Health

Association (APHA) 2005). The pH of the water was measured by an 828 Orion pH meter (Waltham, MA, USA). The temperature of the solution was measured with a 52 YSI dissolved oxygen (DO) meter (Yellow Springs, OH, USA). X-ray diffraction (XRD) spectra from 5 to 75° of the biofilm attached to the ceramsite were obtained using a Bruker D8 Advance X-ray diffractometer (Karlsruhe, Germany).

RESULTS AND DISCUSSION

Nitrogen removal under different pH

Strains W1, W2 and W5 were able to grow with pH ranging from 5.0 to 8.0. The optimal pH of 6.8 ± 0.3 indicated that *Pseudomonas* sp. W1, *Pseudomonas* sp. W2 and *Microbacterium* sp. W5 were neutrophilic bacteria. As shown in Figure 2(a), within a pH range of 6–8, there is a small variation in nitrogen removal efficiency, which slightly drops following an increase with pH 6.5–7 as optimal. Some other researchers achieved similar results. An NDFO bacterium strain, BDN-1, was reported as having a growth pH from 5.0 to 8.5, with 7.0 as optimal pH (Kumaraswamy *et al.* 2006). Other researchers found that the nitrate- and Fe(III)-reducing bacterium *Paracoccus versutus* LYM performs Fe(II)EDTA–NO reduction coupled with Fe(II) EDTA oxidation at approximately pH 7.2 (Dong *et al.* 2013). The results were basically in agreement with the conclusion that anaerobic NDFO occurs under circumneutral pH conditions (Straub *et al.* 1996).

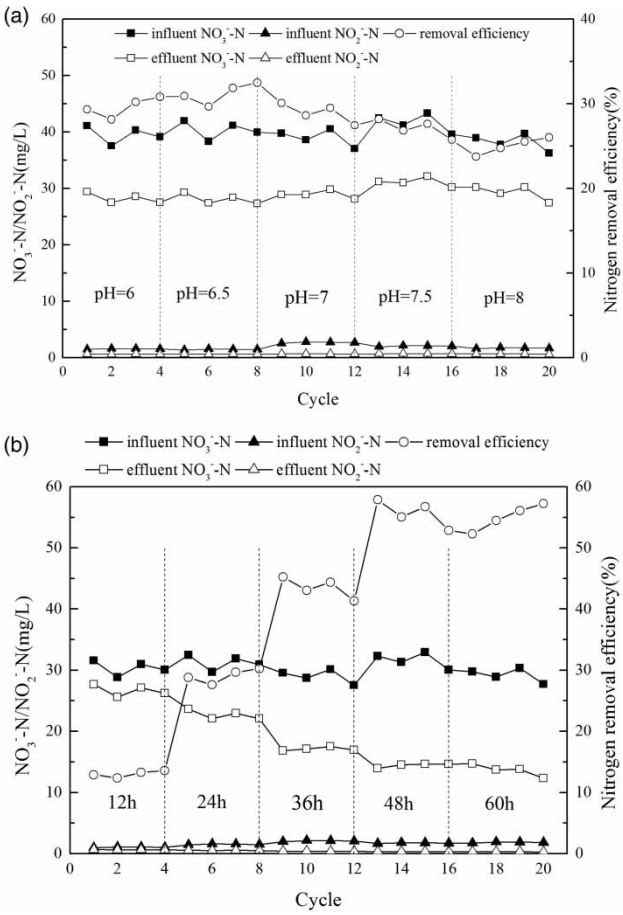

Figure 2 | Variation of NO_3^--N, NO_2^--N and nitrogen removal efficiency with (a) different pH and (b) different HRT.

As pH is a significant environmental factor for bacteria, the optimal pH of the environment is a key parameter for the NDFO process. Acidic and alkaline environments were not fit for NDFO. The main reason for this might have been that pH influenced the enzyme activity of bacteria (Xu & Long 2000). The higher nitrate removal efficiency might be attributed to the high activity of nitrate and nitrite reductases, which are partly dependent on the pH of the environment. The activity of nitrate reductases or nitrite reductases might have been inhibited when the pH was too high or under acidic conditions. In addition, the amount of Fe(II) utilized as an electron donor by nitrate-reducing bacteria was significant for the NDFO process. Nevertheless, under circumneutral conditions with the presence of bicarbonate, the redox potential of Fe^{3+}/Fe^{2+} was favorable for the NDFO (Straub *et al.* 1996; Kumaraswamy *et al.* 2006). Under acidulous conditions, more soluble Fe (II) (e.g., $FeSO_4$) could exist as an electron donor, but not ferrous hydroxide precipitates (Kumaraswamy *et al.* 2006), which were not accessible to cells for further denitrification. In addition, the precipitates adhered to the surface of cells, ceasing the mass transfer and hindering the NDFO process under alkaline conditions, which led to a decrease in nitrate removal efficiency. Furthermore, the stoichiometric nitrate-dependent Fe^{2+} oxidation reaction is $10Fe^{2+} + 2NO_3^- + 12H^+ \rightarrow 10Fe^{3+} + N_2 + 6H_2O$, while the NDFO was not a complete chemical process (Weber *et al.* 2006). This indicated that the $[H^+]$ might promote the oxidation of Fe^{2+} and nitrate reduction. Moreover, formation of a low-pH micro-environment in the immediate vicinity of the cells might be a possible means of avoiding the cellular encrustations by Fe(II) oxidizers (Hegler *et al.* 2010), contributing to the nitrate removal. Nevertheless, another report found that the nitrate conversion rate increased when pH increased from 7.0 to 8.0, and pH 8.0 was shown to be the optimal value (Zhang *et al.* 2014).

The stable variations of the nitrate removal efficiencies revealed that this bioreactor was easy to control for pH. A small quantity of nitrite nitrogen was observed during the experiments, and no ammonia nitrogen was detectable. Taken together, these results illustrated that nitrate might be removed by transforming to gaseous nitrogen under circumneutral and acidulous conditions.

Comparison of nitrogen removal under different HRT

Figure 2(b) shows how the concentrations of NO_3^--N and NO_2^--N in the influent and effluent of the NDFO bio-ceramsite reactor changed with HRT ranging from 12 to 60 hours, acquired by controlling the flow rates.

It can be observed from Figure 2(b) that the amount of nitrate in the effluent was almost as much as that in the influent, and the concentration of nitrate-N in the effluent decreased as the HRT increased. Meanwhile, the concentration of NO_2^--N also declined when the HRT was longer. The concentration of NO_3^--N in the effluent was always below 15.00 mg/L when the HRT was longer than 48 hours. The nitrogen removal efficiency reached a peak value of 57.85% at an HRT of 48 hours. Moreover, the effluent NO_2^--N always fluctuated at a low level below 0.65 mg/L during this phase.

It is very important to determine the appropriate HRT for the reactor because HRT markedly affected the performance of the denitrification process. The concentration of NO_3^--N in the effluent would be too high if the HRT was too short. This is attributed to the fact that a short HRT led to slow growth rate and slow reaction rate of the denitrifying bacteria for the denitrification occurring. Moreover, a short HRT led to a high flow rate, which might cause the biofilm adhering to the surface of the ceramsite to be washed out and denitrifying bacteria to not propagate well. Hence, the nitrogen removal efficiency would decline. The result revealed that the denitrification effect was better for constant influent if the processing time was longer. Nonetheless, the nitrogen removal efficiency increased more slowly when the HRT was longer than 48 hours, while the growth of removal efficiency was faster when the HRT was prolonged from 12 to 48 hours.

The nitrogen removal efficiency was investigated when the HRT was extended to 72, 84, and 96 hours (data were detected once at each HRT). The result (data not shown) indicated that the nitrogen removal efficiency tended to decline as the HRT was extended from 72 to 96 hours. The possible reason for this might be that the low flow rate resulted in insufficient substrates for the microorganisms to remove nitrate at the terminal of the reactor, and microorganisms might have died without a carbon source. Beyond that, since the nitrate removal in the reactor was partly performed by adsorption of the biofilm, desorption occurred as the HRT was too long. In consideration of cost and removal efficiency, the most appropriate HRT in this study was 48 hours.

Nitrogen removal under different C/N ratios

Figure 3(a) presents the variation of concentrations of NO_3^--N and NO_2^--N in the influent and effluent under different C/N ratios. The applied C/N ranged from 6.6:1 to 19.7:1. It is apparent that the concentration of NO_3^--N in the effluent decreased dramatically from 20.02 ± 0.65 to 9.92 ± 0.45 mg/L when the C/N ratio increased from 6.6:1 to 13.1:1. However, an increase of the concentration of NO_3^--N in the effluent was observed as the C/N ratio rose

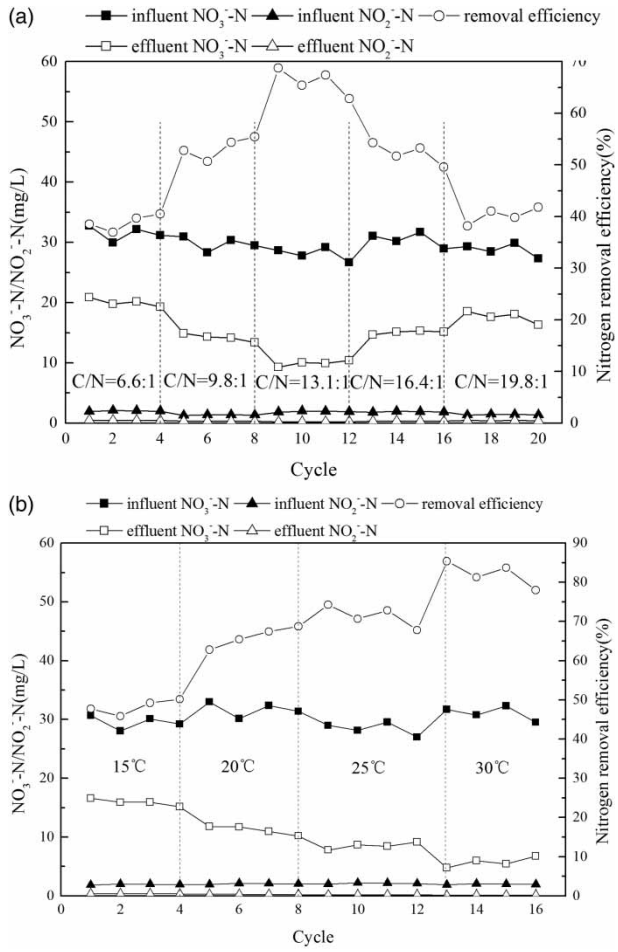

Figure 3 | Variation of NO_3^--N, NO_2^--N and nitrogen removal efficiency with (a) different C/N ratios and (b) different temperatures.

to 17.63 ± 0.95 mg/L. A peak value of average nitrogen removal efficiency of 66.10% was achieved at a C/N ratio of 13.1:1, with the lowest concentration of NO_3^--N of 9.30 mg/L in the effluent. Meanwhile, a good level of nitrite removal was also achieved and accumulation of ammonia nitrogen was not detected. The nitrite-N in the effluent fluctuated between 0.22 and 0.49 mg/L, which are low levels, during the experimental phase.

As a carbon source is necessary for the denitrification process and microbial growth, it is essential to determine the optimal C/N ratio. The optimal C/N ratios are different, depending on the forms of carbon source, and must be determined experimentally. In this study, the C/N of 6.6:1 was not sufficient for microorganisms to grow as the concentration of the nitrate was constant. The bacteria had enough carbon sources for growth and the denitrification process by increasing the C/N ratio with a constant concentration of nitrate. Therefore, nitrogen removal efficiency increased. However, precipitates of iron carbonate were formed using $FeSO_4$ in bicarbonate medium (Benz *et al.* 1998). It has also been reported that in the presence of CO_2 and HCO_3^- as a naturally important buffer system, the prevailing forms of ferric and ferrous iron were Fe_2O_3 hydrates and $FeCO_3$, respectively, which constitute a redox pair favorable for nitrate-reducing and ferrous iron oxidation (Straub *et al.* 1996). Hence, the precipitates could be utilized. Nevertheless, as the C/N ratio increased, cell encrustation was observed. It has been reported by numerous studies that microbial NDFO might result in cell encrustation (Dippon *et al.* 2012; Dong *et al.* 2013). The formation of Fe-oxide cell encrustations might limit the nutrient and substrate uptake (Hallberg & Ferris 2004) or constrain cell mobility (Kappler & Straub 2005), hence inhibiting the denitrification process exhibiting as more nitrate in the effluent when the C/N ratio increased from 13.1:1 to 19.7:1. In this study, an equilibrium was found between the promotion of nitrate removal attributed to increasing the C/N ratio and the limitation by cell encrustation, at a C/N ratio of 13.1:1.

The impact of temperature on reactor performance

Figure 3(b) shows the variation in the concentration of NO_3^--N and NO_2^--N in the influent and effluent at different temperatures, which ranged from 15 to 30 °C. It can be seen from Figure 3(b) that the concentration of NO_3^--N and NO_2^--N in the effluent decreased with the increase of temperature. The increase of nitrogen removal efficiency from about 48.26% to approximately 82.10% indicated that temperature has a significant effect on the NDFO process.

The microbial cell membrane is in gel state and nutrients transport through it; microbes, which cannot utilize nutrients for growth and metabolism due to the disturbance of substrates, transfer into cells at a lower temperature. When the temperature is appropriate, intracellular enzyme activity increases, coupling with acceleration of the intracellular biochemical reaction rate and microbial growth, which lead to a higher denitrification rate. The results revealed that strains W1, W2, W5 and other dominant bacteria in the reactor were mainly mesophilic bacteria. This result was similar to a report by other researchers, which demonstrated the mesophilic proteobacteria *Paracoccus ferrooxidans* strain BDN-1 (Kumaraswamy *et al.* 2006) grew autotrophically using ferrous iron as the only electron donor and nitrate as the electron acceptor. Conversely, the nitrate conversion rate increasing as the temperature increased from 20 to 31 °C has also been reported (Zhang *et al.* 2014). Li *et al.* (2014) demonstrated that nitrate concentration declined dramatically from 9.5 mM to about 6.6 mM after 72 hours at 30 °C through NDFO by an autotrophic bacterium, *Citrobacter freundii* Strain PXL1. At the temperature of 30 °C, both nitrate and nitrite were reduced rapidly and nitrate reductase and nitrite reductase had high activity. The appropriate temperature for this reactor could be chosen as 30 °C.

The characteristics of the biofilm were analyzed by the XRD technique, and chemical composition and crystallinity were also studied by the XRD technique. The XRD spectrum of the biofilm is shown in Figure 4. Distinct diffractive acutiapices were found in the XRD spectrum, which implied crystalline Fe(III) oxides identified as mitridatite ($Ca_3Fe_4(PO_4)_4(OH)_6{\cdot}3H_2O$). On the other hand, iron

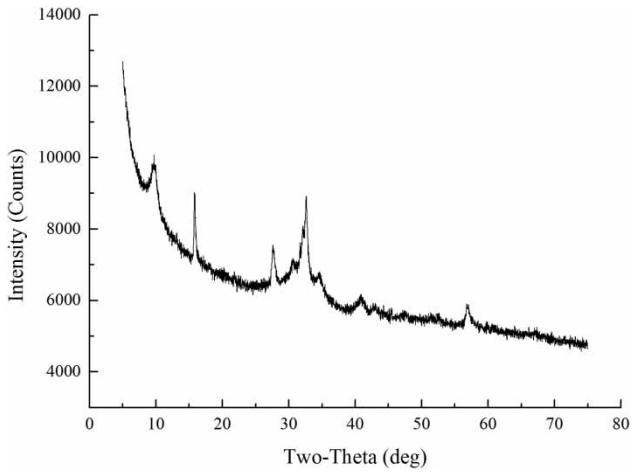

Figure 4 | XRD spectrum of biofilm.

compounds were thought to be a potential precipitant for the control of phosphorus pollution (Nguyen *et al.* 2014).

CONCLUSION

The continuous upflow biofilm reactor packed with ceramsite inoculated with three novel isolated nitrate-reducing and Fe(II) oxidizing autotrophic bacteria, *Pseudomonas* sp. W1, *Pseudomonas* sp. W2 and *Microbacterium* sp. W5, was able to effectively remove NO_3^--N as well as NO_2^--N under an anaerobic atmosphere. The appropriate pH, HRT, C/N ratio and temperature were 6.5 hours–7.0 hours, 48 hours, 13.1:1 and 30 °C, respectively. It was demonstrated that this biofilm reactor is effective for treatment of groundwater polluted by nitrate. The results of the present study can be used as a reference for nitrate removal through the nitrate-dependent Fe(II) oxidation process.

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