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Simultaneous nitrate and phosphate removal from wastewater lacking organic matter through microbial oxidation of pyrrhotite coupled to nitrate reduction



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ABSTRACT

This study investigated the efficiency of a pyrrhotite autotrophic denitrification biofilter (PADB) technology for simultaneous N and P removal from wastewater lacking organic matter. A PADB was constructed with natural pyrrhotite as the biofilter medium and inoculated with autotrophic denitrifies enriched from anaerobic sludge. Over an operating period of 247 days, PADB efficiently removed NO₃ and PO₄²⁻ simultaneously from wastewater that lacked organic matter. The hydraulic retention time (HRT), and influent NO₃ and PO₄²⁻ concentrations affected the removal of NO₃ and PO₄²⁻. A longer HRT led to lower concentrations of NO₃ and PO₄²⁻ in the effluent. The PO₄²⁻ removal was influenced by NO₃ removed; the more NO₃³⁻ removed, the more PO₄²⁻ nemoved. As the synthetic wastewater containing NO₃ –N of 28 mg L⁻¹ and PO₄²⁻ –P of 6 mg L⁻¹ in the absence of organic matter was treated by PADB at HRT of 24 h, total oxidized nitrogen (TON; NO₂ – N + NO₃ – N) and PO₄²⁻ –P of c.62 mg L⁻¹, respectively. When treatment of municipal wastewater treatment plant (WWTP) secondary effluent with TON of 21.11 mg L⁻¹ and PO₄²⁻ –P of 2.62 mg L⁻¹ at HRT of 24 h, the effluent TON was 1.89 mg L⁻¹ and PO₄²⁻ –P was 0.34 mg L⁻¹. PO₄²⁻ was removed through the formation of secondary minerals with Fe and Ca. These secondary minerals contained elevated phosphorus, which presents a potential for P recovery from wastewater.

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1. Introduction

The discharge of nitrogen (N) and phosphorus (P) from wastewater to water bodies is the main causes of eutrophication (Kim et al., 2012) so prior to wastewater discharge, removals of N and P are critical in order to reduce eutrophication (Conley et al., 2009). However, the costs associated with the removals of N and P are substantial (Schindler and Hecky, 2009). The primary concern for wastewater treatment is the removal of nitrate (NO₃) and phosphate (PO₄³⁻) (Zhu and Jyo, 2005). Therefore it is necessary to develop efficient and cost-effective technologies that successfully remove N and P from wastewater. Heterotrophic denitrification is the most efficient and costeffective nitrogen removal process and has been widely used (Park and Yoo, 2009). However, the efficiency of heterotrophic denitrification is dependent on the availability of easily biodegradable organic matter in the wastewater. When wastewater does not contain sufficient readily biodegradable organic matter, the addition of external carbon sources (e.g. methanol) is required, thus increasing operational costs.

 NO_3^- can also be removed from wastewater via sulfur autotrophic denitrification, in which reduced sulfur compounds, such as $S_2O_3^{2-}$, elemental sulfur (S⁰), H₂S, etc., are used as electron donors (Batchelor and Lawrence, 1978). Recently sulfur autotrophic denitrification has attracted increasing interest for NO_3^- removal from wastewater in the absence of biodegradable organic matter due to its three major advantages over heterotrophic denitrification: no requirement for external carbon sources; lower sludge production; and lower operational costs (Sierra-Alvarez et al., 2007). S⁰ is the







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most extensively studied reduced sulfur compound in autotrophic denitrification because of its low cost and ease of use. Using S⁰ as the electron donor and limestone as the pH buffer and inorganic carbon source, the sulfur-limestone autotrophic denitrification process (SLAD) has been shown to be effective in the removal of NO_3^- from wastewater (Zhang, 2002), groundwater (Soares, 2002), and landfill leachate (Koenig and Liu, 1996) in accordance with the following stoichiometric equations:

$$5S + 6NO_3^- + 2H_2O \rightarrow 5SO_4^{2-} + 3N_2 + 4H^+$$
(1)

$$CaCO_3 + H^+ \rightarrow Ca^{2+} + HCO_3^-$$
⁽²⁾

Iron sulfide (FeS) can function as an alternative reduced sulfur compound for autotrophic denitrification in the aquatic environment (Schippers and Jorgensen, 2002), and is even more efficient than S⁰ (Trouve et al., 1998). The addition of synthetic FeS to sediments quantitatively enhanced denitrification (Golterman, 1989; Haaijer et al., 2007), and for a given concentration of NO₃ the denitrification rate increased with the FeS concentration in sediments until saturation was reached (Garciagil and Golterman, 1993). Autotrophic denitrification using FeS as an electron donor is an important NO_3 removal process and as much as one third of nitrate removal in a range of freshwater and marine environments is due to FeS autotrophic denitrification (Vaclavkova et al., 2014). FeS-based autotrophic denitrification can proceed in accordance with the following stoichiometric equations:

$$5\text{FeS} + 8\text{NO}_3^- + 4\text{H}_2\text{O} \rightarrow 4\text{N}_2 + 5\text{SO}_4^{2-} + 5\text{Fe}^{2+} + 8\text{OH}^- \tag{3}$$

$$NO_{3}^{-} + 5Fe^{2+} + 6H^{+} \rightarrow \frac{1}{2}N_{2} + 5Fe^{3+} + 3H_{2}O$$
(4)

The overall reaction is expressed as follows:

$$10\text{FeS} + 18\text{NO}_3^- + 16\text{H}_2\text{O} \rightarrow 9\text{N}_2 + 10\text{Fe}(\text{OH})_3 + 10\text{SO}_4^{2-} + 2\text{H}^+$$
(5)

One of the main methods for removing PO_4^{-} from wastewater in wastewater treatment plants is chemical precipitation with compounds containing Fe²⁺, Fe³⁺, Al³⁺ and Ca²⁺ (De-Bashan and Bashan, 2004). PO₄³⁻ precipitation with Fe³⁺ proceeds in accordance with the stoichiometric equation as follows:

$$\mathrm{Fe}^{3+} + \mathrm{PO}_4^{3-} \to \mathrm{FePO}_4 \downarrow \tag{6}$$

In the SLAD process, Ca^{2+} is released and PO_4^{3-} is primarily removed by the formation of calcium phosphate precipitates. However, the pH of the SLAD effluent is usually around 6.5, far lower than 12 which is the optimal pH for the formation of calcium phosphate precipitates (Wang and Nancollas, 2008), so PO_4^{3-} removal is limited in the SLAD process (Li et al., 2014).

According to Eqs. (3)–(5), FeS autotrophic denitrification produces Fe^{2+} , Fe^{3+} and $Fe(OH)_3$, which are capable of removing PO_4^{3-} from wastewater with a broad range of pH values (Parsons and Smith, 2008; Ruihua et al., 2011; Wei et al., 2008). Therefore, autotrophic denitrification with synthetic FeS as the sulfur source can efficiently remove N and P simultaneously from wastewater despite the absence of easily biodegradable organic matter, as previously reported in batch experiments (Li et al., 2013b).

Pyrrhotite, one of the most abundant reduced sulfur mineral sources in nature, has a non-stoichiometric composition of Fe_{1-x}S, where x varies from 0 (FeS) to 0.125 (Fe₇S₈) (Belzile et al., 2004). Pyrrhotite is much less expensive than S⁰. It is reasonable to hypothesize that pyrrhotite autotrophic denitrification could be advantageous over S⁰ autotrophic denitrification, for example, less

production of H⁺ and SO₄²⁻, and efficient PO_4^{3-} removal. However, compared with a number of studies on S⁰ and synthetic FeS autotrophic denitrification for wastewater treatment, very few studies have focused on pyrrhotite autotrophic denitrification for the removals of N and P.

From an application prospective, it is probable that the natural pyrrhotite mineral can be used as the filling media of the biofilters which can simultaneously remove NO_3^- and PO_4^{3-} from wastewater lacking easily biodegradable organic matter through pyrrhotite autotrophic denitrification. In the present study, a pyrrhotite autotrophic denitrification biofilter (PADB) was constructed with the natural pyrrhotite mineral as the filling media. The main objectives of this research were to establish a PADB with autotrophic denitrifiers enriched from anaerobic sludge, and investigate the simultaneous removal of NO₃ and PO₄³⁻ from wastewater lacking organic matter and the mechanisms of NO₃ and PO₄⁻ removals. A multi-disciplinary approach, based on environmental engineering, mineralogy, microscopy, and spectroscopy techniques, was used to investigate the efficiency of PADB. These techniques included X-ray diffraction (XRD), X-ray fluorescence (XRF), scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS), and cloning and sequencing analysis.

2. Materials and methods

2.1. Pyrrhotite and limestone

Pyrrhotite (obtained from Tongling City, Anhui Province, China) was fragmented in a jaw crusher and sieved into particles of 2.36–5.12 mm in size. Pyrrhotite particles were placed in a 10% (v/ v) HCl solution for 2 h for the removal of oxides formed on the surface, and rinsed with deionized water until the pH of the rinse reached 7 \pm 0.1. The clean pyrrhotite particles were dried at 20 °C in a vacuum drying oven for 24 h and subsequently stored in airtight bags, which were flushed with nitrogen gas to prevent oxidation.

The pyrrhotite sample for XRF analysis was ground into a powder and subsequently analyzed in accordance with the manufacturer's instructions (ARL-9800XP, ARL, Switzerland). The XRF analysis identified the chemical composition of the pyrrhotite as follows: Fe 56.69%, S 38.46%, Si 1.22%, Cu 0.74%, Ca 0.34%, Mg 0.24%, Al 0.21%, Cl 0.10%, W 0.05%, K 0.03%, Zn 0.02%, Mn 0.02%, Cr 0.01%, and the residue was O. The combined total content of Fe and S was 92.43%, with a molar ratio of Fe to S of 0.948. X-ray diffraction (X'TRA, ARL, Switzerland) analysis indicates that the pyrrhotite mineral was composed of pyrrhotite mineral phase (Supporting Information Fig. S2). Pulverized limestone was obtained from Galway, Ireland and was sieved into particles of 2.36–5.12 mm. All reagents used were analytical grade (Trace Metal Grade, Fisher, UK).

2.2. Biofilters

Three identical biofilters were constructed and each was an anoxic upflow fixed-bed reactor comprising a stainless column with an internal diameter of 10 cm, a height of 27 cm, and a working volume of 1800 mL. The three biofilters were packed with: limestone (biofilter B1); pyrrhotite (biofilter B2); and a combination of both pyrrhotite and limestone (volume ratio of 1:1) (biofilter B3). The porosity of every biofilter was approximately 45% with a capacity of 810 mL for wastewater in each column. The hydraulic retention time (HRT) was calculated as follows:

$$HRT = \frac{24Vn}{Q} \tag{7}$$

where, HRT: hydraulic retention time, h; V: Volume of the filling

medium in the column, mL; *n*: porosity of the filling medium; and *Q*: water volume feeding into the column in 24 h, mL.

Three outlets were located on each biofilter at heights of 5 cm (A), 15 cm (B) and 25 cm (C) from the bottom of the column, which allowed water sampling along the columns. The treated wastewater flowed out of the biofilters from Outlet C.

2.3. Enrichment of autotrophic denitrifiers and culturing of biofilm

At first, sulfur-based autotrophic denitrifiers were enriched from anaerobic sludge. 50 mL of anaerobic sludge taken from a local WWTP was inoculated in 1000 mL conical flasks with the culturing medium made from deionized water containing 5 g L^{-1} Na₂S₂O₃·5H₂O, 2 g L^{-1} KH₂PO₄, 2 g L^{-1} KNO₃, 1 g L^{-1} NaHCO₃, $0.5 \text{ g } \text{L}^{-1} \text{ NH}_4 \text{Cl}, 0.5 \text{ g } \text{L}^{-1} \text{ MgCl}_2 \cdot 6\text{H}_2 \text{O} \text{ and } 0.01 \text{ g } \text{L}^{-1} \text{ FeSO}_4 \cdot 7\text{H}_2 \text{O}$ (Baalsrud and Baalsrud, 1954). The conical flask was sealed with a rubber stopper with two glass tubes, A and B. Tube A was for flushing pure N₂, and Tube B for collecting N₂ gas produced. The conical flask was flushed with pure N₂ for 10 min to exclude air, sealed and kept at an ambient temperature $(20 \pm 3 \circ C)$ for 4 days. Successful enrichment of autotrophic denitrifiers was evaluated by the volume of the gas generated, which was measured using the water displacement method. After five enrichments the volume of the gas generated was stable at approximately 240 mL for each culturing, indicating successful enrichment (the theoretical volume of N₂ generated was equal to 240.4 mL according to the equation: $5S_2O_3^{2-} + 8NO_3^{-} + H_2O \rightarrow 10SO_4^{2-} + 4N_2 + 2H^+).$

In order to ensure sufficient biofilm growth, the three biofilters were fed with a solution generated from the culturing medium (95%) and the inoculum solution (5%) obtained from the preceding enrichment experiment. The solution was flushed with N_2 for 15 min and then pumped into the three biofilters at a flow rate of 15 mL/min continuously. The solution was circulated in the biofilters for 4 days, and then replaced with fresh one. The duration of the biofilm enrichment lasted for 10 cycles (40 d).

2.4. Biofilter operation

Following formation of the biofilm, synthetic or real wastewater was fed into the biofilters. The synthetic wastewater was prepared with tap water, KH_2PO_4 and KNO_3 with various concentrations of NO_3^- and PO_4^{3-} but without NO_2^- (Table 1), and the real wastewater was the secondary effluent of a local WWTP with TON of 21.1 \pm 6.5 mg L⁻¹ (NO_2^--N <0.3 mg L⁻¹), and $PO_4^{3-}-P$ of 3.4 \pm 2.2 mg L⁻¹.

Wastewater was pumped into the biofilters from the bottom four times a day, with control of a timer, and was collected from Outlet C (top outlet). The three biofilters were continuously operated at various conditions (Table 1) for 247 days. During the operation phase, the effects of HRT, influent TON and PO_4^{3-} –P levels on the N and P removal efficiencies were studied. Phase 1 was

| Table 1 | |
|-------------|------------|
| Operational | conditions |

considered to be the start-up phase of the PADB. Effects of HRT (Phases 2–5), influent PO_4^{3-} –P concentrations (Phases 3, 6 and 7) and influent TON concentrations (Phases 7 and 8) on the performance of PADB were investigated. During Phase 9 (Day 218 to Day 247) real secondary effluent of a local WWTP was treated. The local WWTP uses a conventional A/O activated sludge process for nitrogen removal while phosphorus is removed by ferric sulphate tertiary treatment. Efficient nitrification is achieved in the WWTP with a yearly mean NH¹/₄-N concentration of 0.56 mg L⁻¹ in secondary treatment effluent.

2.5. Sampling and analysis

The wastewater was sampled according to HRT. In general, the effluent from the biofilters between two sampling events (the interval between two sampling events was usually 2 days or 1 HRT), was collected with a vessel for each biofilter, tested for pH (WTW 340, Germany) and subsequently used for further analysis. Wastewater samples were filtered (0.45 μ m) and measured for TON, NO₂⁻-N, PO₄³⁻-P, NH₄⁺-N, alkalinity, and SO₄²⁻ with a nutrient analyzer (Konelab 20, Thermo Clinical Labsystems, Vantaa, Finland). Settled solids in the effluent of the biofilters were collected and analyzed with XRF.

Filtered wastewater samples were acidified to 1% using nitric acid (HNO₃) (Trace Metal Grade 67–69%, Fisher, UK) and metal concentrations (Cd, Pb, As, Al, Ca, Mg, Fe, etc) were determined using Inductively Coupled Plasma Mass Spectrometry (ICP-MS (ELAN DRCe, Perkin Elmer, Waltham, USA) in a class 1000 (ISO class 6) cleanroom (Staunton et al., 2014).

Mineral samples taken from Outlets A, B, and C of each biofilter were treated following Torrento (Torrento et al., 2012), and gold coated (Emitech K550) for scanning electron microscopy (SEM; Hitachi S-4700, Hitachinaka, Japan). Energy Dispersive Spectroscopy (EDS) point analysis (spectra generated from the analysis of spot locations) was performed to determine the presence of a range of elements (e.g. K, Fe, S) (INCA[®]; Oxford Instruments; High Wycombe, UK). The analysis was performed at an acceleration voltage of 20 kV, an emission current (I_e) of 10 µA and a working distance of 12 mm (Morrison et al., 2009).

Samples were also collected for genomic DNA extraction; the bacterial 16 rRNA gene was amplified, followed by cloning, sequencing and sequence analysis (protocols are included in Supporting Information).

3. Results and discussion

3.1. Start-up of PADB (phase 1)

Fig. 1 shows the variation of TON, NO_2^--N , $PO_4^{3-}-P$ and SO_4^{2-} in the effluent of the three biofilters over the entire operational period lasting 247 days. B1 was the control biofilter packed with

| Phase | Days | HRT (h) | Influent TON (mg L ⁻¹) | Influent $PO_4^{3-}-P (mg L^{-1})$ | Influent SO_4^{2-} (mg L^{-1}) |
|----------------|---------|---------|------------------------------------|------------------------------------|-------------------------------------|
| 1 | 0-58 | 48 | 28.1 ± 0.5 | 12.6 ± 0.3 | 48.6 ± 5.9 |
| 2 | 58-88 | 48 | 27.5 ± 0.4 | 5.9 ± 0.2 | 41.6 ± 4.8 |
| 3 | 88-118 | 24 | 27.2 ± 0.6 | 6.1 ± 0.2 | 34.6 ± 2.9 |
| 4 | 118-147 | 12 | 26.7 ± 0.4 | 6.1 ± 0.1 | 36.6 ± 1.1 |
| 5 | 147-179 | 72 | 27.2 ± 0.6 | 5.9 ± 0.1 | 34.6 ± 1.3 |
| 6 | 179-187 | 24 | 26.5 ± 0.3 | 12.0 ± 0.1 | 35.5 ± 0.3 |
| 7 | 187-195 | 24 | 26.6 ± 0.2 | 24.3 ± 0.3 | 34.1 ± 1.6 |
| 8 | 195-211 | 24 | 12.7 ± 0.3 | 24.4 ± 0.4 | 27.0 ± 1.4 |
| 9 ^a | 211-247 | 24 | 21.1 ± 6.5 | 3.4 ± 2.2 | 59.1 ± 18.5 |

^a During Phase 9, the influent was the secondary effluent taken from a local WWTP.



Fig. 1. Variation of TON, NO₃⁻-N, PO₄³-P and SO₄²⁻ during biofilter operation.

limestone, so theoretically TON would not be removed in B1 from the influent wastewater and SO_4^{2-} would not be produced. It was observed that during the first 10 days of Phase 1, the SO_4^{2-} concentration of B1 effluent decreased steeply from 960 to 128 mg L^{-1} , and decreased slowly from 128 mg L^{-1} to similar values of the influent in the remaining days of Phase 1. The TON concentrations of B1 effluent increased continuously from 0.6 mg L^{-1} to nearly the same as that of the influent during Phase 1. The culturing medium used during the biofilm enrichment stage contained very high concentrations of SO_4^{2-} , and very low concentrations of TON. As the wastewater was pumped into the biofilters, the culturing solution was gradually washed out of the biofilter, causing SO_4^{2-} concentrations in B1 effluent to decrease steeply in the first 10 days of Phase 1. It was observed in B1 that SO_4^{2-} production coupled to TON removal continued throughout the entirety of Phase 1, indicating that sulfur autotrophic denitrification still occurred during Phase 1. When sulfide is used as an electron donor, whether it is partially oxidized to S^0 or completely oxidized to SO_4^{2-} depends on the $NO_3^{-}/$ S^{2-} ratios (Cardoso et al., 2006). During the biofilter enrichment stage of the present study, the $NO_3^{-}/S_2O_3^{2-}$ ratio in the culturing medium was approximately 1:1, which is lower than the stoichiometric ratio of 1.6 (Mora et al., 2014) required for complete oxidation of $S_2O_3^{2-}$ to SO_4^{2-} . Therefore, some $S_2O_3^{2-}$ was partially oxidized to S^0 , which remained in the biofilters. Using the remaining S^0 as the electron donor to reduce TON, B1 became a SLAD system to remove NO₃ during Phase 1. By the end of Phase 1 the remained S⁰ was exhausted, so the levels of TON and SO_4^{2-} in the effluent were equal to B1 influent, indicating that B1 did not reduce TON. In the reported SLAD system there was accumulation of NO_2^- during denitrification (Wei et al., 2008). NO₂ accumulation was also observed in B1 in Phase 1 and NO_2^- peaked at 5.92 mg L⁻¹ on Day 10. With the operating time increasing, NO_2^- decreased gradually to almost zero at the end of Phase 1.

 $PO_4^{3-}-P$ concentrations in the B1 effluent decreased quickly from 74 to 17 mg L⁻¹ in the first 10 days of Phase 1, which was also due to the washing out of the culturing medium. However, at the end of Phase 1, $PO_4^{3-}-P$ levels in B1 effluent fluctuated between 16 and 23 mg L⁻¹, which were still higher than that of the influent wastewater. The PO_4^{3-} level in the culturing medium was 455.9 mg L⁻¹ and limestone was a good adsorbent for PO_4^{3-} (Karageorgiou et al., 2007), therefore PO_4^{3-} was adsorbed on the limestone during the biofilm enrichment stage. As the wastewater, which had a much lower $PO_4^{3-}-P$ concentration ($12.6 \pm 0.3 \text{ mg L}^{-1}$), was pumped into B1, the adsorbed PO_4^{3-} on the limestone was released into the wastewater, causing effluent $PO_4^{3-}-P$ levels to exceed those of the influent.

In the control biofilter (B1), TON removal and SO_4^{-} production did not occur during Phases 2 to 8. During Phase 2 to Phase 6, the $PO_4^{3-}-P$ concentrations of B1 effluent were even higher than that of the influent. $PO_4^{3-}-P$ of B1 effluent was a little lower than that of the influent during Phase 7 and nearly the same as that of the influent during Phase 8. Only when the $PO_4^{3-}-P$ concentration of the influent was rather high, such as 24 mg L⁻¹ during Phase 7, the limestone recovered somewhat the adsorption capacity for PO_4^{3-} , but it lost that capacity during Phase 8. This was assumed to be due to adsorption saturation.

During Phase 1 with the operating time increasing, TON levels in effluent from B2 and B3 increased, peaked at 2.5 and 2.7 mg L⁻¹ respectively around Day 26, subsequently decreased and finally remained stable at 0.4 and 1.5 mg L⁻¹, respectively. During Phase 1, both the remaining S⁰ and pyrrhotite were used as electron donors, therefore effluent TON levels from B2 and B3 were much lower than in B1 effluent (Fig. 1). Around Day 26 the remained S⁰ was almost used up, which was deduced from the very small difference in SO₄^{2–} levels between the effluent and influent of B1 after 26 days of operation. The autotrophic denitrifiers in B2 and B3 possibly

were not completely acclimated to pyrrhotite, therefore the TON concentration peaked on Day 26. By the end of Phase 1 autotrophic denitrifiers in B2 and B3 were gradually acclimated to pyrrhotite as the only sulfur source, and TON levels of B2 and B3 effluent were reduced to 0.4 and 1.5 mg L⁻¹, respectively. Since there was more pyrrhotite in B2 than in B3, TON levels in effluent were lower in B2 than B3. NO₂⁻-N levels of B2 and B3 effluent peaked at 2 and 1.5 mg L⁻¹, respectively, around Day 26, and finally stabilized at zero by the end of Phase 1. The peaks of TON and NO₂⁻-N in B2 and B3 effluent occurred at approximately the same time, likely related to the exhaustion of the produced S⁰.

SO₄²⁻ concentrations in B2 and B3 effluent decreased steeply from over 960 mg L⁻¹ to 220 and 140 mg L⁻¹, respectively, around Day 12 due to the gradual washing out of the culturing medium from the biofilters. From Day 12 to Day 58 SO_4^{2-} concentrations in B2 and B3 effluent fluctuated between 220 and 279 mg L^{-1} , 140 and 174 mg L^{-1} , respectively. By the end of Phase 1 the removed TON in B2 and B3 was about 27.1 and 26 mg L^{-1} , respectively, and the levels of SO²⁻ produced were approximately 198 and 97 mg L⁻¹, respectively. Because NO₂⁻ concentration was very low during Phases 2 to 8, TON was nearly the same as NO₃. Namely, TON removed could be considered as $NO_{\overline{3}}$ removed. Thus the molar ratios of TON removed/SO₄²⁻ produced (Δ TON/ Δ SO₄²⁻) were 0.96 and 1.84 for B2 and B3, respectively. $\Delta TON/\Delta SO_4^{2-}$ of B3 was very close to the stoichiometric value of Eq (5), which proves pyrrhotite autotrophic denitrification described by Eq. (5). However, $\Delta TON/\Delta SO_4^{2-}$ of B2 was lower than the stoichiometric value of Eq. (5).

Reduced sulfur can be oxidized with O₂ by autotrophic denitrifiers (Annachhatre and Suktrakoolvait, 2001), therefore, pyrrhotite can be aerobically oxidized by autotrophic denitrifiers using dissolved oxygen in the influent wastewater, expressed by Eq. (8):

$$4\text{FeS} + 90_2 + 10\text{H}_2\text{O} \rightarrow 4\text{Fe}(\text{OH})_3 + 4\text{SO}_4^{2-} + 8\text{H}^+$$
(8)

According to Eq. (8), SO_4^{2-} and H^+ were produced without NO_3^- reduction. It can be inferred that aerobic oxidation of pyrrhotite would have taken place in B2, leading to $\Delta TON/\Delta SO_4^{2-}$ of B2 lower than the stoichiometric value of Eq (5) in B2.

Due to PO_4^{-} released from the surface of limestone and pyrrhotite, which is also an efficient phosphate adsorbent (Li et al., 2013a), PO_4^{-} -P levels in B2 and B3 effluent decreased continuously until stabilizing in Day 38. After Day 38 the removals of PO_4^{-} by B2 and B3 were 63.6% and 45.8%, respectively. Therefore, Phase 1 was regarded as the start-up stage of PADB.

3.2. Simultaneous NO_3^- and PO_4^{3-} removals of PADB biofilters in phases $2{-}9$

Experiments were conducted in Phases 2–8 of the PADB biofilters, in order to investigate effects of HRT and influent TON and $PO_4^{3-}-P$ concentrations on simultaneous NO_3^{-} and PO_4^{3-} removals and SO_4^{2-} release. Table 2 shows average effluent TON and $PO_4^{3-}-P$ concentrations, average TON and PO_4^{3-} removals, SO_4^{2-} produced, and molar ratios of NO_3^{-} removed and SO_4^{2-} produced of B2 and B3 during these phases. During each phase, the data in the second half period was more stable than that in the first half period because of the transit of the operational conditions from the preceding phase. Therefore all average data and standard deviations presented in Table 2 are calculated from stable testing results obtained in the second half period of each phase.

According to Fig. 1 NO_2^--N was less than 0.2 mg L⁻¹ during Phases 2 to 9, showing that nitrite accumulation was insignificant in the PADB biofilter under the conditions tested.

During Phases 2, 3, 4, and 5, the TON and PO_4^{3-} levels of the influent were similar, and the HRT was 48, 24, 12, and 72 h,

respectively. With increasing HRT, effluent TON and $PO_4^{3-}-P$ concentrations decreased, and the removals of TON and $PO_4^{3-}-P$ increased. As HRT was 48 h, the average effluent TON concentration and removal for B2 were 0.28 \pm 0.03 mg L⁻¹ and 98.97 \pm 0.14%, respectively, and further increasing HRT did not enhance the performance of B2. The shorter the HRT, the higher the rate of denitrification achieved in B2. The average SO₄²⁻ concentration in effluent increased with HRT, and was 196.60 \pm 12.12 mg L⁻¹ at HRT 72 h for B2. For the influent with 27.3 mg L⁻¹ TON and 6.1 mg L⁻¹ PO₄³⁻-P, the average TON and PO₄³⁻-P concentrations of B2 effluent were 6.61 \pm 0.33 and 0.32 \pm 0.04 mgL⁻¹, respectively, at HRT of 12 h. In conventional biological nutrient removal activated sludge systems (Brown et al., 2011; Xu et al., 2014), the optimal HRT is usually around 12 h, so the PADB technology is very practical and is a potentially applicable nutrient removal technology.

PO³₄−−P levels in B2 effluent decreased during the first half of Phase 2 (Fig. 1) indicating that PO³₄[−] was continued to be released from the surface of the pyrrhotite. Influenced by PO³₄[−] release from the surface of limestone and pyrrhotite in B3, the PO³₄[−]−P removal of B3 was low. As the HRT was 12 h, the effluent PO³₄[−]−P concentrations and the PO³₄[−] removal of B2 reached 0.32 ± 0.04 mg L^{−1} and 94.66 ± 0.74%, respectively, and further increasing HRT to 72 h only increased the PO³₄[−] removal to 96.33 ± 0.86%. When using the SLAD process to treat wastewater containing 15 mg L^{−1} PO³₄[−]−P, at the HRT of 24 h, the PO³₄[−] removal was below 60% (Li et al., 2014). Therefore, the PADB technology is much more efficient than the SLAD in PO³₄[−] removal.

During Phases 3, 6 and 7, $PO_4^{3-}-P$ concentrations of the influent were 6, 12 and 24 mg L^{-1} , respectively. With increasing $PO_4^{3-}-P$ levels of the influent $(6-24 \text{ mg L}^{-1})$ the TON removal and the produced SO_4^{2-} of B2 slightly decreased from 95.84 \pm 0.48 to 90.19 \pm 1.25%, and from 117.38 \pm 6.73 to 109.73 \pm 1.70 mg L⁻¹, respectively. As the influent $PO_4^{3-}-P$ concentrations were 6 and 12 mg L⁻¹ the PO₄³⁻ removal of B2 was similar, 95.84 \pm 0.48% and 96.18 \pm 0.40%, respectively, and decreased to 84.85 \pm 2.67% quickly as the influent PO_4^{3-} – P concentration was increased to 24 mg L⁻¹, and the PO_4^{3-} -P removed was 20.36 mg L⁻¹. According to Eqs. (3), (5) and (6), the stoichiometric ratio of NO_3^--N removed and PO_4^{3-} – P removed is 1.23. Therefore, as more than 90% of 28 mg L⁻¹ NO_3^- –N was removed, the PO_4^{3-} –P removed should have been more than 31 mg·L-1, which is much higher than the highest of 20.36 mg L^{-1} observed in the experiment. Because effluent pH values of B2 and B3 were about 7 and 7.5, respectively, it may be that a portion of the produced Fe^{3+} may not precipitate with PO_4^{3-} but form iron hydroxides (Eq. (9)), which reduced Fe³⁺ availability for PO₄³⁻ precipitation:

$$Fe^{3+} + 3OH^{-} \rightarrow Fe(OH)_{3} \tag{9}$$

During Phases 7 and 8, influent NO_3^--N concentrations were 28 and 14 mg L⁻¹ respectively. Though the TON of effluent during Phase 8 was lower than that during Phase 7, the removed TON during Phase 8 was much lower than that during Phase 7, and accordingly the produced SO_4^{2-} and removed PO_4^{3-} were lower during Phase 8 than those during Phase 7.

According to Table 2, $\Delta TON/\Delta SO_4^-$ in B3 was in the range of 1.74–1.92 during Phases 2 to 4, which were close to 1.80 of the stoichiometric value of Eq. (5), indicating pyrrhotite autotrophic denitrification described by Eq. (5) also occurred. However, during Phases 5 to 8, $\Delta TON/\Delta SO_4^{2-}$ in B3 ranged from 1.99 to 2.29, a little higher than that of the stoichiometric value. Heterotrophic bacteria were found in SLAD, using dead and lysed bacterial cells as their energy source to reduce NO₃, which did not produce SO₄²⁻ (Koenig et al., 2005). As a similar biological process, it is possible that heterotrophic denitrification also occurred in PADB. Therefore, during

| Table 2 | |
|--|---------------|
| Average TON and $PO_4^{-}-P$ concentrations in the effluent, average TON and PO_4^{-} removal, SO_4^{-} generation, and $\Delta TON/\Delta SO_4^{-}$ | of B2 and B3. |

| Phase | HRT(h) | Biofilter | Average effluer | (mg L ⁻¹) Average re | | al (%) | Average produced SO_4^{2-} (mg L ⁻¹) | $\Delta TON/\Delta SO_4^{2-}$ |
|-------|--------|-----------|------------------|----------------------------------|------------------|----------------------------------|--|-------------------------------|
| | | | TON | PO ₄ ³⁻ -P | TON | PO ₄ ³⁻ -P | | |
| 2 | 48 | B2 | 0.28 ± 0.04 | 0.21 ± 0.05 | 98.97 ± 0.14 | 96.33 ± 0.86 | 155.74 ± 25.23 | 1.20 |
| | | B3 | 4.56 ± 0.37 | 6.26 ± 0.39 | 83.32 ± 1.22 | -7.47 ± 8.36 | 87.64 ± 14.93 | 1.80 |
| 3 | 24 | B2 | 1.13 ± 0.13 | 0.28 ± 0.11 | 95.84 ± 0.48 | 95.49 ± 1.68 | 117.38 ± 6.73 | 1.52 |
| | | B3 | 13.47 ± 0.49 | 3.63 ± 0.59 | 50.45 ± 1.85 | 40.13 ± 8.63 | 49.06 ± 2.01 | 1.92 |
| 4 | 12 | B2 | 6.61 ± 0.33 | 0.32 ± 0.04 | 75.06 ± 1.26 | 94.66 ± 0.74 | 102.31 ± 2.38 | 1.35 |
| | | B3 | 21.06 ± 0.26 | 3.49 ± 0.10 | 20.70 ± 1.74 | 42.40 ± 1.91 | 22.24 ± 1.53 | 1.74 |
| 5 | 72 | B2 | 0.24 ± 0.07 | 0.19 ± 0.07 | 99.11 ± 0.24 | 96.80 ± 1.20 | 196.60 ± 12.12 | 0.94 |
| | | B3 | 5.88 ± 0.64 | 1.05 ± 0.05 | 78.55 ± 2.68 | 82.16 ± 0.88 | 73.60 ± 12.08 | 1.99 |
| 6 | 24 | B2 | 1.97 ± 0.17 | 0.46 ± 0.05 | 92.97 ± 0.71 | 96.18 ± 0.40 | 114.06 ± 1.58 | 1.48 |
| | | B3 | 17.07 ± 0.29 | 5.49 ± 0.39 | 35.83 ± 1.60 | 54.15 ± 3.16 | 30.48 ± 0.35 | 2.12 |
| 7 | 24 | B2 | 2.61 ± 0.33 | 3.71 ± 0.64 | 90.19 ± 1.25 | 84.85 ± 2.67 | 109.73 ± 1.70 | 1.50 |
| | | B3 | 18.08 ± 0.34 | 15.30 ± 0.25 | 32.13 ± 1.32 | 37.51 ± 1.19 | 25.56 ± 0.91 | 2.29 |
| 8 | 24 | B2 | 0.71 ± 0.39 | 9.51 ± 0.30 | 94.42 ± 3.07 | 61.26 ± 1.15 | 55.99 ± 2.26 | 1.48 |
| | | B3 | 5.55 ± 1.09 | 15.14 ± 0.55 | 56.41 ± 8.48 | 37.25 ± 2.45 | 23.99 ± 0.95 | 2.04 |

 $^{*}\Delta TON/\Delta SO_{4}^{2-}$: Molar ratio of NO₃ removed to SO₄²⁻ produced.

Phases 5 to 8 in B3 a slightly greater $\Delta NO_3^-/\Delta SO_4^{2-}$ was possibly due to heterotrophic denitrification. During Phases 2 to 8 $\Delta NO_3^-/\Delta SO_4^{2-}$ of B2 was less than that of the stoichiometric value of Eq. (5), and the longer the HRT, the smaller the $\Delta TON/\Delta SO_4^{2-}$. These should be as a result of aerobic pyrrhotite oxidation, like during Phase 1.

During Phase 9 the secondary effluent taken from a local municipal WWTP was treated. As the average concentrations of NO₃⁻-N and PO₄³⁻-P in the influent were 21.11 \pm 6.53 and 3.4 \pm 2.2 mg L⁻¹, respectively, the average effluent concentrations of NO₃⁻-N and PO₄³⁻-P were 18.72 \pm 4.69 and 9.53 \pm 1.61 mg L⁻¹ for B1, 1.89 \pm 1.39 and 0.34 \pm 0.23 mg L⁻¹ for B2, and 10.89 \pm 4.63 and 1.88 \pm 1.18 mg L⁻¹ for B3, respectively. The results clearly prove that the PADB was able to simultaneously remove N and P effectively from WWTP secondary effluent. In the limestone biofilter (B1), only 3 mg L⁻¹ of NO₃⁻-N was removed, which may have resulted from heterotrophic denitrification, because there was a small amount of organic matter present in the secondary effluent. The effluent PO₄³⁻-P concentration was much higher than that in the influent which should be due to continuous PO₄³⁻ release from the limestone.

The B1, B2, and B3 effluents on Days 84 (Phase 2) and 172 (Phase 5) were analyzed with ICP_MS for metal content (Supporting Information Table S1). Concentrations of all metals (Fe, Cu, Cd, Pb, etc) measured in the effluent were much lower than the discharge standards of pollutants for municipal WWTPs (GB 18918-2002, China). Therefore, there will be no secondary risk of metal pollution after polishing the secondary effluent with the PADB process.

Limestone and pyrrhotite particle samples were taken out of B1 and B2 from outlet A, respectively, on Day 68, and analyzed with SEM. The SEM images show that biofilm was grown on the limestone even though there had been no sulfur sources supplied for 68 days. Biofilm was well established on the pyrrhotite particle surface (Fig. 2).

TRFLP analysis was carried out and the results are shown in Table 3. Autotrophic denitrifiers, such as *Sulfurimonas denitrificans* (Pu et al., 2014) and *Thiobacillus denitrificans* (Torrento et al., 2012), became the dominant species present in the biofilters B1 and B2, and *Sulfurimonas denitrificans* were overall the most abundant in the biofilters. This indicates that enrichment of autotrophic denitrification was the most significant process in the PADB technology. Though there were no sulfur sources supplied for 68 days in B1, the biofilm mainly comprising autotrophic denitrifiers still existed. Because pyrrhotite was used as the sulfur source for 68 days, the microbial community of B2 had some differences from that of B1 (Table 3).

3.3. P removal mechanisms in the PADB biofilter

Pyrrhotite particles were collected from B2 on Day 68 from Outlets A, B and C, and analyzed with SEM. The SEM images in Fig. 2 shows that the biofilm was formed on the surface of pyrrhotite and the order of biomass abundance was A > B > C. With increasing height from the bottom to the top, NO_3^- levels in the solutions decreased and consequently less biomass occurred at the top of the biofilter.

SEM observation shows secondary minerals in a ball-shape aggregate of platy crystals and plate-shape secondary minerals covering the surface of pyrrhotite particles, and the former was more abundant than the latter (Fig. 3). According to Fig. 1 TON levels in the effluent increased slightly with the operation time, increasing in each testing phase, which was possibly caused by



Fig. 2. SEM micrographs of particles from the bottom outlet of the limestone biofilter (B1) and pyrrhotite biofilter (B2) on Day 68. Scale bars: (a) 5 µm, (b) 5 µm.

| Table 3 | | | |
|----------------------|-----------------------|-----------------|------------|
| Identification and r | elative abundances of | f the TRFLPs in | B1 and B2. |

| Identification of the TRFLPs | Relative abundances (%) | | |
|--|-------------------------|-------|--|
| | B1 | B2 | |
| Sulfurimonas denitrificans | 75.11 | 68.05 | |
| Thiobacillus denitrificans/Thiobacillus thioparus/Rhodocyclaceae bacterium | 18.08 | 19.05 | |
| Melioribacter roseus/Paludibacter propionicigenes | 0.34 | 2.03 | |
| Simpliscispira | 0.46 | 1.54 | |
| Sphingobacterium sp. | 5.22 | 4.36 | |
| Rhizobium sp. | 0.79 | 4.98 | |



Fig. 3. SEM micrographs of pyrrhotite particles in biofilter B2 from outlets A (a, b), B (c, d), C (e, f) and original pyrrhotite (g, h). Scale bars: (a) 10 μ m, (b) 50 μ m, (c) 10 μ m, (d) 50 μ m, (e-h) 5 μ m.

covering of pyrrhotite particles by these secondary minerals. The covering of secondary minerals would prevent autotrophic denitrifiers from using pyrrhotite as an electron donor to reduce TON. Figs. 3 and 4 show SEM-EDS analysis of the secondary minerals. The elemental composition of the ball-shape aggregate of platy crystals was mainly made of O, C, Fe and P, and the plate-shape



Fig. 4. Scanning electron microscopy of secondary minerals (with ball-shape aggregate of platy crystal (X), and plate-shape (Y) crystal) (a, b). EDS point analysis (c, d) of mineral fragments marked X and Y, indicating the presence of phosphorous (P), potassium (K), iron (Fe), calcium (Ca), sodium (Na) and carbon (C). Scale bars: (a–b) 7 μm.

secondary mineral was mainly made of O, C, Ca and P (Supporting Information Table S2 and S3). Thus, the secondary minerals with ball-shape aggregates and plate-shape ones can be interpreted as FePO₄ and CaHPO₄, respectively. The P content of the secondary minerals were up to 20%.

From Phase 7, the turbidity of B2 effluent increased. The settled

solids in the effluent were collected after settling and analyzed with XRF and the content of P_2O_5 was 37.5% (Table 4). In China contents of P_2O_5 in the phosphate ore for producing phosphate fertilizer is usually 36%. Therefore, collecting the secondary minerals is a potential measure for recovering P resources from wastewater as a fertilizer.

| Table 4 | |
|---|-------|
| Chemical composition of the settled solids in the efflu | ient. |

| Constituent | P ₂ O ₅ | Fe ₂ O ₃ | CaO | SiO ₂ | SO ₃ | K ₂ O | CuO | Na ₂ O | MgO | Cl |
|-------------|-------------------------------|--------------------------------|------|------------------|-----------------|------------------|------|-------------------|------|------|
| Content | 37.50 | 47.99 | 7.31 | 3.83 | 1.05 | 0.89 | 0.43 | 0.41 | 0.28 | 0.31 |

As the WWTP secondary effluent was treated by PADB at HRT of 24 h, the effluent TON and $PO_4^{3-}-P$ concentrations were below 2 and 0.4 mg L⁻¹, respectively, and based on the pyrrhotite price of \$100/ton, the cost of pyrrhotite consumed in the PADB is estimated as 0.01 $/m^3$. However, for conventional heterotrophic denitrification, in order to efficiently remove NO_3^- from wastewater by adding external carbon sources, the cost is about $0.2/m^3$ (Sun et al., 2010). Therefore, PDAD is a simple, efficient and cost-effective method for simultaneous N and P removal from wastewater lacking organic matter, attaining low N and P levels in order to meet strict wastewater discharge regulations.

P resource is limited and will be exhausted in 100–400 years and is becoming a strategic material (Cisse and Mrabet, 2004; Cordell et al., 2009; Sengupta, 2013). P recovery from wastewater may help solve the dwindling supply of phosphorus resources (Gilbert, 2009). In PDAD process, PO_4^{3-} was removed through the formation of secondary minerals with Fe and Ca. These secondary minerals contained elevated P contents and can be potentially used to manufacture P fertilizer. Therefore, PADB could even potentially recover phosphate resources from wastewater. As a novel way of simultaneous N and P removal, and P recovery from wastewater, PADB would make the double gains.

4. Conclusion

This study has shown that PADB biofilters efficiently removed NO_3^- and PO_4^{3-} simultaneously from wastewater lacking organic matter. HRT, and influent NO $_3$ and PO $_4^{3-}$ concentrations affected the removals of NO₃ and PO₄³⁻. A longer HRT was beneficial to a low NO_3^- concentration in the effluent while the removal of PO_4^{3-} was influenced by the amount of TON removed (the more TON removed, the more PO_4^{3-} removed). As synthetic wastewater with NO_3^--N of 28 mg L⁻¹ and $PO_4^{3-}-P$ of 6 mg L⁻¹ at HRT of 12 h, the effluent TON and PO_4^{3-} –P concentrations were as low as 6.61 and 0.32 mg L^{-1} , respectively. When treating the secondary effluent with NO₃-N of 21.11 mg L⁻¹ and PO₄³⁻-P 2.62 mg L⁻¹ at HRT of 24 h, the effluent had NO_3^--N of 1.89 mg L^{-1} and $PO_4^{3-}-P$ of 0.34 mg L^{-1} . This clearly indicates that the PADB process can be used as a valid technology for treating secondary effluent, attaining low N and P levels in order to meet strict discharge regulations for wastewater. In addition, the PADB can recover phosphorus from wastewater.

Notes

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2016.03.034.

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