Simultaneous removal of Fe^{3+} and nitrate in the autotrophic denitrification immobilized systems

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ABSTRACT

In this study, strain CC76, identified as *Enterobacter* sp., was tested for the reduction of Fe^{3+} and denitrification using immobilized pellets with strain CC76 as experimental group (IP) and immobilized pellets with strain CC76 and magnetite powder as experimental group (IPM) in the autotrophic denitrification immobilized systems (ADIS). Compared with IP, a higher nitrate removal rate was obtained with IPM by using three levels of influent Fe^{3+} (0, 5, and 10 mg/L), four levels of pH (5.0, 6.0, 7.0, and 8.0), and three levels of hydraulic retention time (HRT) (12, 14, and 16 h), respectively. Furthermore, response surface methodology (RSM) analysis demonstrated that the optimum removal ratios of nitrate of 87.21% (IP) and 96.27% (IPM) were observed under the following conditions: HRT of 12 h, pH of 7.0 and influent Fe^{3+} concentration of 5 mg/L (IP) and 1 mg/L (IPM).

Key words | autotrophic denitrification, Fe^{3+} reduction, immobilized pellets, magnetite powder, nitrate removal, RSM

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INTRODUCTION

Groundwater is an important source of municipal water supply for domestic and industrial use (Showers *et al.* 2008). Nitrate is simply transported to groundwater through uncontrolled discharge of nitrate-containing sources, such as chemical fertilizers, industrial or domestic wastes, and landfill leachate (Ghafari *et al.* 2009). High nitrate concentration in drinking water may bring us various health effects. For instance, infants under six months fed with nitrate contaminated water could have blue baby syndrome, and if untreated, may die (Ghafari *et al.* 2008; Mousavi *et al.* 2012). The recommended nitrate concentration limit in the drinking water by WHO and the Chinese Ministry of Health is 10 mg/L (NO_3^--N) (Fu *et al.* 2014). Therefore, several nitrate removal technologies such as electro-dialysis, reverse osmosis, adsorption and chemical and biological methods have been used in water treatment (Bhatnagar & Sillanpää 2011; Loganathan *et al.* 2013). However, biological denitrification is considered to be the most appropriate technology compared to other techniques for treatment of nitrate-contaminated steams. Several bioreactors have been developed for the biological denitrification of wastewater.

Nitrification and denitrification processes have proved individually successful in biofilm reactors, and there are already many different biofilm systems in use, such as trickling filters, rotating biological contactors (RBCs), fixed media reactors, biofilters, fluidized bed reactors, etc. (Makkulath & Thampi 2012; Biswas *et al.* 2014). The sequencing batch biofilm reactor (SBBR) system, one type of biofilm technology, has attracted much attention because of its ability to take advantage of both a biofilm reactor and a sequencing batch reactor (Ding *et al.* 2011). In addition, biofilm reactors are also characterized by a lower biomass growth and better sedimentation properties compared to activated sludge flocs (Helness & Qdegaard 2001).

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Immobilization of bacteria as biofilm on the surface of the carrier can reduce the risk of biomass wash-out (Magri et al. 2012). These immobilization techniques include self-immobilization as granular biomass (Dapena-Mora et al. 2004; López et al. 2008), attachment on the surface of a carrier forming biofilm (Tsushima et al. 2007; Ni et al. 2010), and entrapment of the microbial biomass into gel pellets (Isaka et al. 2007; Furukawa et al. 2009).

Meanwhile, scholars have found that some microorganisms can utilize ferrous iron as an electron donor to convert nitrate into nitrogen gas and these microorganisms have been found in various habitats, such as swine waste lagoons, lake sediments, even freshwater (Chaudhuri et al. 2001; Straub et al. 2004; Muehe et al. 2009).

Iron-reducing bacteria (IRBs) commonly occur in anaerobic systems and play an essential role in iron cycling (Kim et al. 2014). In the present study, we aim to investigate the adaptability of iron-reducing bacterium strain CC76 under different conditions in autotrophic denitrification immobilized systems (ADIS). In this study, the recycling of iron is shown in the ADIS. Furthermore, an ADIS with magnetically immobilized CC76 cells has been designed and operated to enhance the ability for denitrification in groundwater. Herein, we discuss the abilities for nitrate removal and iron reduction through the addition of magnetite, and without it, in the immobilized pellets under different conditions.

MATERIALS AND METHODS

Bacterial strain and culture conditions

Iron reducing bacteria CC76, which has the ability to reduce nitrate and Fe^{3+} simultaneously, was obtained from Tang Yu oligotrophic reservoir (Shaanxi Province, China) (Su et al. 2016a).

The basal medium was comprised of the following reagents per litre: $NaHCO_3$, 0.5 g; $NaNO_3$, 0.05 g; KH_2PO_4, 0.05 g; $MgSO_4{\cdot}7H_2O$, 0.05 g; $Fe_2(SO_4)_3$, 0.1 g; $CaCl_2$, 0.05 g. A trace element solution (2 mL) was added, and the final pH of the medium was adjusted to 7.0 with 1 mol/L NaOH or HCl solution. Ultra-pure water was used in this study. The trace element solution components were as follows: 0.5 mg/L $FeSO_4{\cdot}7H_2O$; 0.5 mg/L $CuSO_4{\cdot}5H_2O$; 0.5 mg/L $MgSO_4{\cdot}7H_2O$; 1.0 mg/L EDTA; 0.2 mg/L $ZnSO_4$; 0.1 mg/L $MnCl_2{\cdot}4H_2O$ and 0.2 mg/L $CoCl_2{\cdot}6H_2O$ (Su et al. 2016b). The medium was heated with a high pressure steam cooker to 121 °C under an anoxic atmosphere, which put it into a sterile stage. The strain was grown in 1 L bottles containing 0.9 L medium.

Experimental setup

In this study, three reactors were set up: (1) the immobilized pellets without the addition of extra bacteria as the control group; (2) the immobilized pellets with the corresponding bacteria strain CC76 as experimental group (IP); (3) the immobilized pellets with strain CC76 and magnetite powder as experimental group (IPM). Herein, 2% (m/v) sodium alginate (SA) mixed with strain CC76, strain CC76 and magnetite powder, and without either, was slowly injected into 2% (m/v) $CaCl_2$ with a syringe needle (with a diameter of 2 mm) to form homogeneous pellets. And 0.2 g magnetite was added in 100 mL solution of SA mixed with strain CC76. Meanwhile, the reactors were maintained at 30 °C under anaerobic conditions and the water level was kept at the same point. The pH was monitored at regular time intervals through the pH monitoring system during the operation. According to the experimental requirement, the operational conditions were under different hydraulic retention times (HRT) (Phase 1); pH (Phase 2); and influent Fe^{3+} concentrations (Phase 3). The results of the three phases of the experiments are clearly listed in Table 1. The running of the entire experiment lasted for 1,260 h.

Analytical methods

Water sample collection was performed every day during the 1,260 h operational running time for the control group, IP and IPM. These samples were used for testing Fe^{2+}, nitrite (NO_2^--N) and nitrate (NO_3^--N). Meanwhile, pH was measured with a pH meter (HQ11d, HACH, USA). Nitrate-N concentration was measured by calculating the difference between OD_{220} and $2 \times OD_{275}$ of an UV spectrophotometric screening method. Nitrite-N concentration was determined by colorimetry using the N-(1-naphthyl)-1, 2-diaminoethane dihydrochloride method at wavelengths of

Table 1 | Summary of the performance in the ADIS

Phase		HRT during the test [h]	pH during the test	Initial NO_3^--N [mg/L]	Initial Fe (III) [mg/L]	Cycle times
Phase 1	Phase 1.1	12	7	30	20	10
	Phase 1.2	14	7	30	20	10
	Phase 1.3	16	7	30	20	10
Phase 2	Phase 2.1	14	5	30	20	10
	Phase 2.2	14	6	30	20	10
	Phase 2.3	14	8	30	20	10
Phase 3	Phase 3.1	12	7	10	10	10
	Phase 3.2	12	7	10	5	10
	Phase 3.3	12	7	10	0	10
Phase 4	Phase 4.1	12	6	10	3	7
	Phase 4.2	12	7	10	1	7
	Phase 4.3	12	7	10	5	7
	Phase 4.4	12	8	10	3	7
Phase 5	Phase 5.1	10	6	10	1	7
	Phase 5.2	10	6	10	5	7
	Phase 5.3	10	8	10	1	7
	Phase 5.4	10	8	10	5	7
	Phase 5.5	10	7	10	3	7
Phase 6	Phase 6.1	8	6	10	3	7
	Phase 6.2	8	7	10	1	7
	Phase 6.3	8	7	10	5	7
	Phase 6.4	8	8	10	3	7

540 nm. Fe^{2+}concentration was measured spectrophotometrically with phenanthroline at 510 nm. The reduction rates of NO_3^--N and Fe^{3+} were calculated using the formula (C_0-C_n)/h. C_0 was the initial concentration of NO_3^--N. C_n was the final concentration of NO_3^--N at n hours, and h was the time of strain CC76 treatment. All experiments were performed at least in duplicate.

RESULTS AND DISCUSSION

Effect of HRT on denitrification performance

Figure 1(a) presents the average nitrate concentrations of effluent, which were maintained at 10.37 mg/L (IP) and 7.51 mg/L (IPM), 7.24 mg/L (IP) and 5.97 mg/L (IPM), 1.84 mg/L (IP) and 0.67 mg/L (IPM) at an HRT of 12 h, 14 h and 16 h, respectively. The maximum efficiency of 93.84% (IP) and 97.76% (IPM) was observed at an HRT of 16 h, which was higher than 63.47% (IP) and 73.57% (IPM) at an HRT of 12 h, 75.88% (IP) and 80.13% (IPM) at an HRT of 14 h. The removal rate of nitrate decreased when the HRT was changed from 16 h to 12 h (Figure 1(b)). As a result, a longer HRT could facilitate nitrate removal by such denitrifying bacteria, in accordance with Zhou *et al.* (2011). Moreover, the nitrate concentration of the effluent in the control group was also accompanied by a small drop compared to the influent (the removal efficiency less than 20%), this might be due to the growth of other bacteria in the reactor, which required nitrogen sources, the phenomenon which appeared in the control group also appeared in the next phase of the operation.

From Figure 1(c), it could be seen that the average concentration of Fe^{2+} in IP (3.40 mg/L) was lower than in IPM (5.23 mg/L). Meanwhile, the reduction rate of Fe^{3+} in IP and IPM had been in a state of fluctuation, especially when the HRT was 12 h (Figure 1(d)). Since Fe^{2+} was the electron donor for nitrate removal, and the nitrate removal rate was obviously in fluctuation, it is concluded that a higher nitrate removal rate was obtained with a higher concentration of Fe^{2+}. The reason is that the immobilized pellets with strain CC76 can use existing Fe^{2+} as an electron donor for denitrification (Su *et al.* 2016b). In addition, Figure 1(e) shows that the concentration of nitrite in IP and IPM had remained at a low level, which was no more than 0.77 mg/L and 0.50 mg/L, and the nitrite of the control group was also maintained in the normal range (less than 0.04 mg/L). It can be concluded that pellets with magnetite were beneficial to improving the efficiency of nitrate removal, and accelerating the degradation of nitrate. Meanwhile, the nitrate removal efficiency of the effluent increased with the increase in HRT.

Effect of pH on denitrification performance

From Figure 1(a), it is shown that the nitrate was removed basically in the reactor when the HRT was 16 h. However, the extension of the HRT did not significantly improve the removal rate of nitrate. Therefore, the HRT was adjusted to 14 h in order to describe the effect of pH on the nitrate removal more accurately, and the pH was set to 5.0, 6.0, 8.0 just as Table 1 shows, pH of 7.0 had been studied at an HRT of 14 h. The highest average nitrate removal efficiency was obtained in the neutral (pH of 7.0) condition

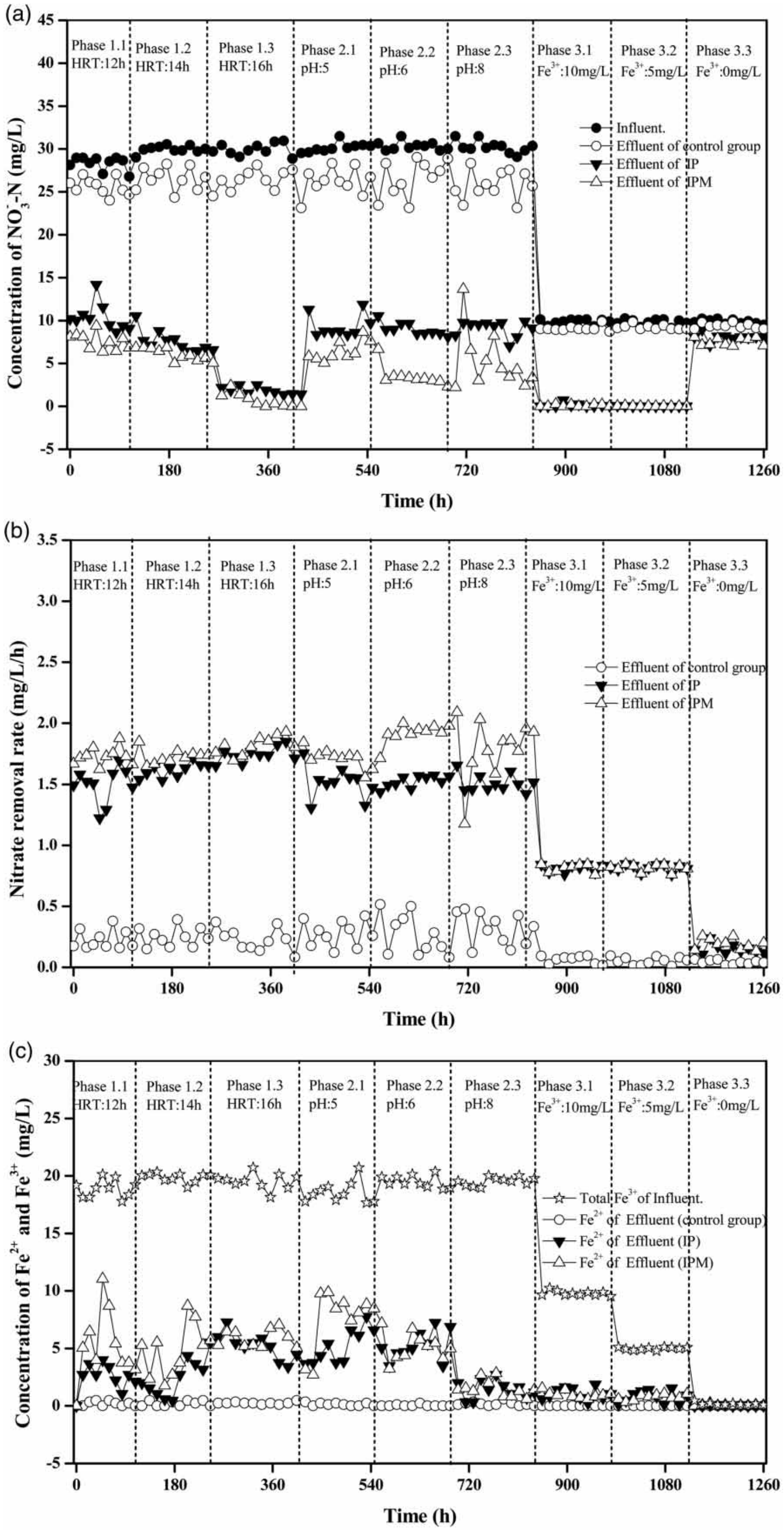
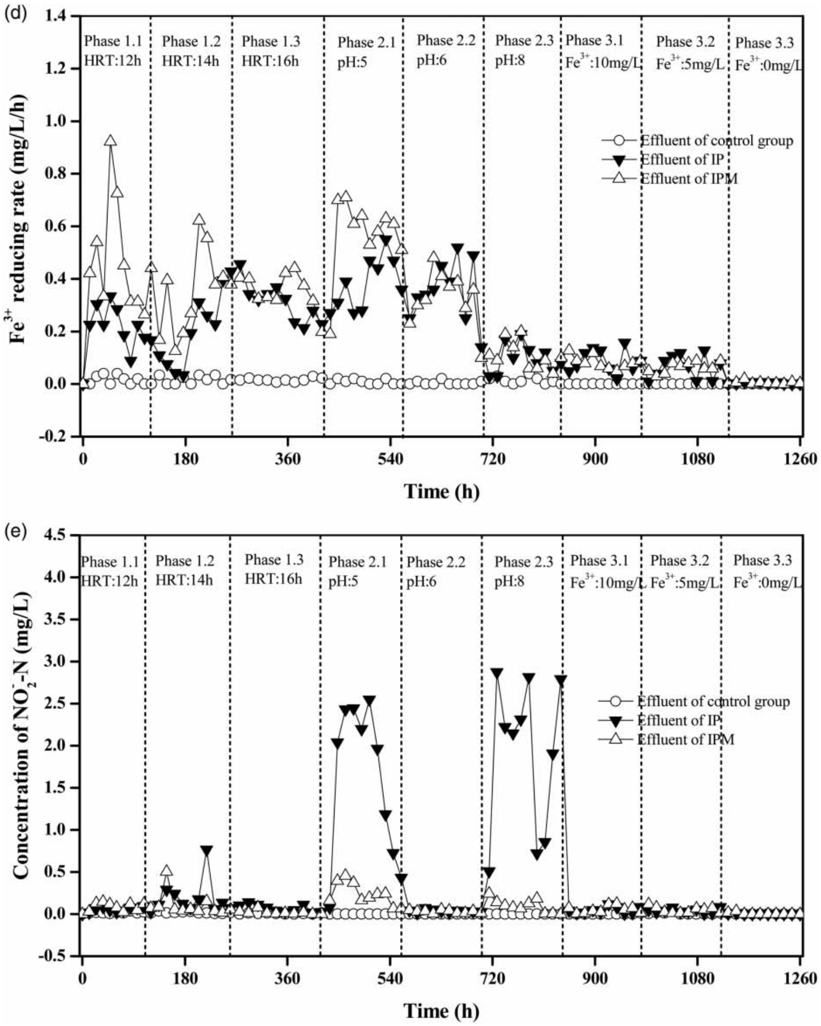


Figure 1 | Operation performance of the ADIS: (a) changes of NO_3^--N concentration; (b) changes of NO_3^--N removal rate; (c) changes of Fe^{2+} and Fe^{3+} concentration; (d) changes of Fe^{3+} reducing rate; (e) changes of NO_2^--N concentration, IP: the immobilized pellets with corresponding bacteria strain CC76 as experimental group, IPM: the immobilized pellets with strain CC76 and magnetite powder as experimental group. (*Continued*.)



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and weak acid (pH of 6.0) condition compared to other treatments, and the corresponding average removal efficiencies of 75.88% and 90.05% were obtained. It was observed from Figure 1(b) that the average nitrate removal rate of IP rose from 1.48 mg/L/h to 1.63 mg/L/h when the pH increased from 5.0 to 7.0, but then dropped to 1.50 mg/L/h at a pH of 8.0. Meanwhile, the average nitrate removal rate of IPM was higher than IP under the same pH conditions. The maximum average nitrate removal rate (1.97 mg/L/h) of IPM was observed at a pH of 6.0. Genarally, the results showed neutral or weakly acidic conditions were beneficial for the removal of nitrate, which was consistent with a previous study (Su *et al.* 2015).

During the pH experiment, the concentration of effluent Fe^{2+} was also in a state of fluctuation; this was probably caused by the iron cycle (Su *et al.* 2016b). This process was described as follows: strain CC76 can convert Fe^{3+} to Fe^{2+}. Fe^{2+} which has been converted could become an electron donor for denitrification to convert Fe^{2+} to Fe^{3+} at the same time. It can be seen from Figure 1(c) that the concentration of Fe^{2+} decreased with the increase of pH; especially when the pH was 8.0, the average concentration of Fe^{2+} was only 1.35 mg/L (IP) and 1.53 mg/L (IPM), and the other phases were: 5.22 mg/L and 7.98 mg/L (pH of 5.0); 4.91 mg/L and 4.56 mg/L (pH of 6.0). Figure 1(e) shows that the concentration of effluent NO_2^- was 1.60 mg/L (IP) and 0.23 mg/L (IPM) when the pH was 5.0, and 1.92 mg/L (IP) and 0.095 mg/L (IPM) when the pH was 8.0. Different levels of accumulation in the effluent nitrite were obtained in acidic (pH of 5.0) and alkalescent conditions (pH of 8.0), which might be due to the nitrite reductase being inhibited in the partial acid and alkaline conditions (Sorokin *et al.* 2011).

Effect of the influent Fe^{3+} concentration on denitrification performance

Figure 1(a) shows that the concentration of Fe^{3+} was set to 10 mg/L (Phase 3.1), 5 mg/L (Phase 3.2), 0 mg/L (Phase 3.3) under the condition of a low concentration of nitrate (10 mg/L). As the HRT of a low concentration nitrate would be

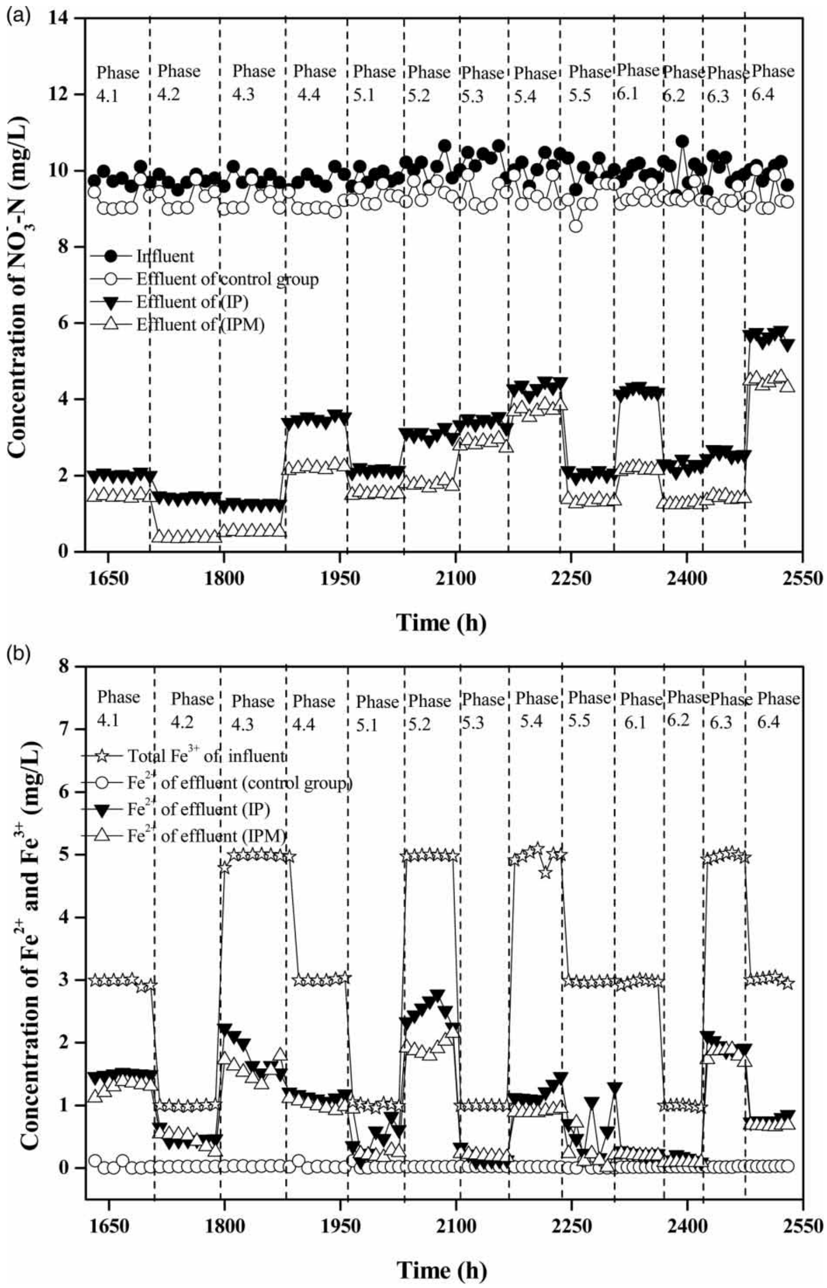

Figure 2 | Operation performance of the reactors: changes of NO_3^--N concentration (a); changes of Fe^{2+} and Fe^{3+} concentration (b); changes of NO_2^--N concentration (c) at 1,632–2,530 h. (*Continued*.)

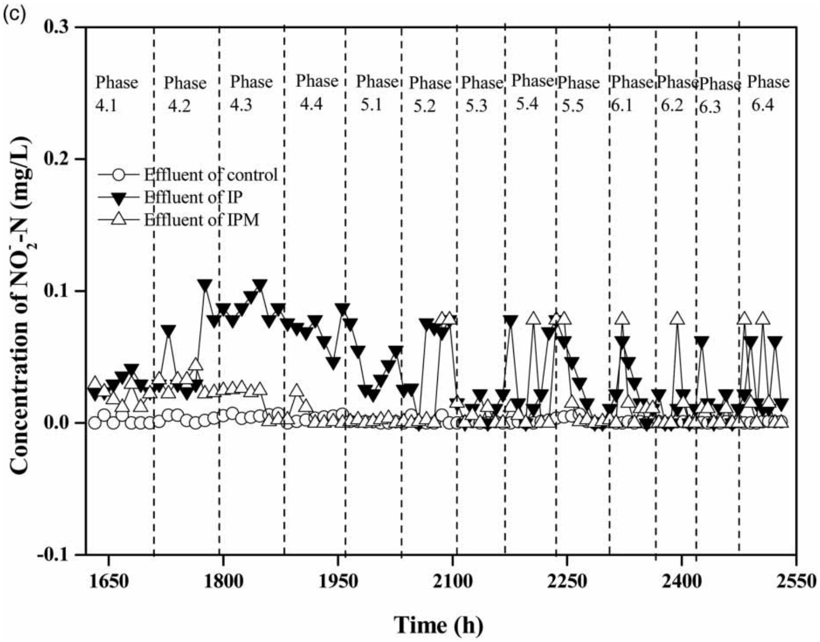

Figure 2 | Continued.

shortened, the HRT of this stage was set to 12 h. The results showed that the average nitrate removal efficiency and removal rate of IP were 98.72% and 0.81 mg/L/h (Phase 3.1), 99.79% and 0.81 mg/L/h (Phase 3.2), 18.22% and 0.15 mg/L/h (Phase 3.3); The corresponding average nitrate removal efficiency and removal rate of IPM were 99.08% and 0.81 mg/L/h, 99.75% and 0.81 mg/L/h, 23.49% and 0.19 mg/L/h, respectively. As shown in Figure 1(c), when the concentration of the influent Fe^{3+} decreased from 10 mg/L to 5 mg/L, the effluent Fe^{2+} did not vary obviously. It could be explained by sufficient concentration of Fe^{2+} being provided for denitrification by using Fe^{2+} as an electron donor when the concentration of the influent Fe^{3+} was 5 mg/L. When the concentration of the influent Fe^{3+} increased to 10 mg/L, the concentration of the effluent Fe^{2+} did not increase, probably due to chemical oxidation and sedimentation. It was found that the nitrate removal efficiency was increased slightly in the experimental groups (IP and IPM). The nitrate could be removed effectively in the presence of a small amount of Fe^{3+}, but more Fe^{3+} did not mean the removal efficiency and rate would be higher. As in the autotrophic environment, strain CC76 can convert the oxidized Fe (III) to Fe (II), and the iron cycle was formed in this process (Su *et al.* 2016b).

In this study, there was no discovery of a large amount of nitrite accumulation (Figure 1(e)), which would provide a safe theoretical basis for the low concentration of nitrate in groundwater treatment.

Analysis of ADIS in the low concentration of effluent nitrate by RSM

The Box-Behnken design was used to analyze the interactive effects of important variables that significantly affect the removal of nitrate by strain CC76 at a low concentration, including HRT, pH, and the influent Fe^{3+} concentration as shown in Table 1. (Phase 4.1–6.4). Statistical analysis was performed using the Design-Expert (8.0.6.1) program with the SAS software package. Effluent concentration of nitrate is shown in Figure 2(a). In the IP, response surface methodology (RSM) analysis demonstrated that the maximum removal ratio (87.21%) and rate (0.70 mg/L/h) of nitrate occurred under the conditions of an HRT of 12 h, pH of 7.0, and influent Fe^{3+}concentration of 5 mg/L; in the IPM, RSM analysis demonstrated that the maximum removal ratio (96.27%) and rate (0.79 mg/L/h) of nitrate occurred under the conditions of an HRT of 12 h, pH of 7.0, and influent Fe^{3+} concentration of 1 mg/L. This difference of optimal conditions could due to magnetite powder being added in the IPM. During the whole experiment, the concentration of nitrite is always maintained at a low level (Figure 2(c)). Moreover, a higher removal ratio of nitrate was obtained in the IPM, since a high concentration zone was formed by the adsorption of magnetite to increase the nitrate removal. It has been reported that magnetic nanomaterial could be used for adsorption removal of heavy metals (Pb (II), Cr (III)) with its large surface area and high adsorption capacity (Lingamdinne *et al.* 2017). Similarly, with added magnetite powder in the immobilized pellets, its adsorption ability could promote nitrate removal.

The response surfaces, as shown in Figure 3(a) and 3(b), show that the nitrate removal ratio increased with increasing HRT as well as pH ranging from 6.0 to 6.8. The reason is that the bacteria are able to make better use of Fe^{2+} as an electron donor in the acidic condition (Yang *et al.* 2006). In addition, it could also be concluded that the nitrate removal ratio enhanced significantly as the HRT increased. This might be due to the increasing residence times in the

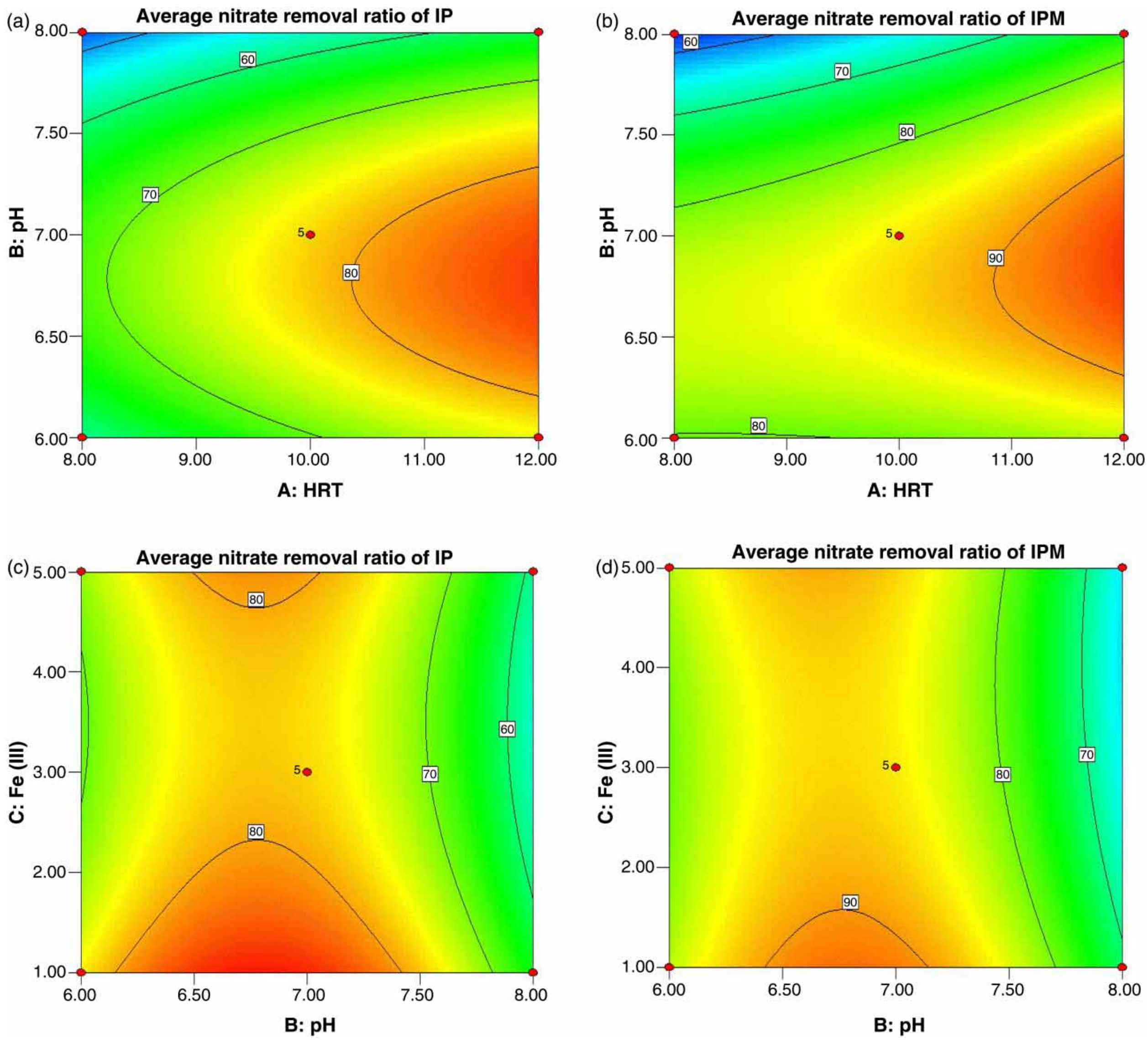

Figure 3 | Design-Expert plots: Average nitrate removal ratio as a function of HRT and pH in IP (a) and IPM (b); average nitrate removal ratio as a function of pH and influent Fe^{3+} concentration in IP (c) and IPM (d).

reactors, which allowed the bacteria to adapt to the new environment and the nitrate contaminant had enough time to degrade successfully. Zhou *et al.* (2011) suggested that good nitrate removal efficiency was obtained at a long HRT in a lab scale up flow biofilter. Figure 3(c) and 3(d) illustrated the effects of the interaction of initial pH and influent Fe^{3+}concentration in the response process. As shown in Figure 3(c) and 3(d), the nitrate removal ratio increased with increasing pH and Fe^{3+} to attain optimum conditions, and then decreased with a further increase.

CONCLUSIONS

In this study, the iron reducing bacteria CC76, with the ability of removal nitrate and Fe^{3+}, was obtained from Tang Yu oligotrophic reservoir. Immobilized pellets with corresponding bacteria strain CC76 as experimental group (IP) were compared with immobilized pellets with strain CC76 and magnetite powder as experimental group (IPM) to reflect the denitrification performance. During the whole experiment, higher nitrate removal efficiency was obtained in the IPM. It is concluded that the removal rate was increased with a longer HRT and acid conditions. RSM analysis demonstrated that the optimum nitrate removal efficiency and removal rate of 87.21% and 0.70 mg/L/h (IP), 96.27% and 0.79 mg/L/h (IPM) were observed under the conditions of HRT of 12, initial pH of 7.0 and influent Fe^{3+} concentration of 5 mg/L (IP) and 1 mg/L (IPM) in the low concentration of nitrate of 10 mg/L. Owing to the ability to simultaneously undertake Fe^{3+} reduction and nitrate removal, CC76 is a promising candidate in the extensive application of effective removal of nitrate by the iron cycle.

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