Desalination and Water Treatment
www.deswater.com
doi: 10.1080/19443994.2013.831788

52 (2014) 7937–7950
December

Taylor & Francis
Taylor & Francis Group

Simultaneous removal of nitrate and aniline from groundwater by cooperating heterotrophic denitrification with anaerobic ammonium oxidation

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Received 22 March 2013; Accepted 17 July 2013

ABSTRACT

To investigate the performance of cooperating heterotrophic denitrification (HD) with anaerobic ammonium oxidation (ANAMMOX) in removing nitrate and aniline simultaneously from organic limitation combined contamination groundwater, batch cultures, and flow column experiments were constructed. The two experimental results showed that it was feasible to remove nitrogen and carbon simultaneously by indigenous HD and ANAMMOX bacteria existing ubiquitously in groundwater aquifer medium, and the cooperation effect was significant. Batch cultures results demonstrated that high nitrate, aniline, and COD removal efficiency (over 79.0, 96.6, and 69.6%) were achieved at selected C/N of 0.3, 0.46, and 0.91 conditions, and the maximum activities of HD and ANAMMOX reached to 25.22 mg/(d·l), 5.54 mg/(d·l), and 7.06 mg/(d·l) after 150 days inoculation. As such, nitrate, aniline, and COD removal efficiency were equal to 99.8, 99.9, and 89.5% in continuous flow column with nitrate 1,000 mg L^{-1} and aniline 50 mg L^{-1}. The variations of NO_2^--N, NH_4^+-N, pH, and *EC* could indicate HD and ANAMMOX coupling characteristics accurately. Moreover, carbon source utilization efficiency was dramatically improved by the synergetic effect of HD and ANAMMOX, as ANAMMOX could use CO_2 and other inorganic carbon produced by HD process, which confirmed its great potential and feasibility to develop energy-saving and high efficiency *in situ* technology in the remediation of complex polluted groundwater.

Keywords: Nitrate and aniline; HD; ANAMMOX; Synergistic effect; Groundwater

1. Introduction

Throughout the world, groundwater resource is used extensively as a source of drinking water [1,2]. In China, about 70% of the populations rely on groundwater for their drinking water and therefore groundwater plays a leading role in assuring drinking water safety [3]. However, groundwater is faced with an unprecedented risk of combined pollution due to the increased release of various organic and inorganic substances from industrial, agricultural, and domestic activities [4–7]. In particular, in some areas where cities and industries are concentrated, complex pollution

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has become a very prominent problem of groundwater contamination [8–11]. The Weihe River is the mother river of far-flung Guanzhong region in Shaanxi Province west-central China, which covers an area of 135,000 km^2 and plays a very important role in the development of industry and agriculture of Shaanxi. Recent survey on the groundwater along the river, it was not only the average nitrate (NO_3^--N) concentration exceeded the national standard of 20 $mg\,L^{-1}$ and even to 146.77 $mg\,L^{-1}$ in some regions [12,13], but also aromatic amine compounds aniline was detected in some monitoring points [14]. Because both nitrate and aniline are carcinogenic, teratogenic and mutagenic contaminants, and when the two contaminants are coexist, the damage to health and safety of coastal residents taking groundwater as their drinking water will be strengthen. Therefore, cost-effective and appropriate remediation technologies must be explored and implemented to treat nitrate and aniline compound polluted groundwater.

Various physical, chemical, and biological methods either *ex-situ* or *in situ* for remediation of contaminated groundwater environments have been proposed [15,16]. Compared with other processes, *in situ* bioremediation mostly relies on indigenous microbial metabolism with the advantaged superiority of no groundwater transfer, benign products and low energy consumption. Thus, using aquifer indigenous microbial self-purification and enhancing natural attenuation of underground aquifer to remove nitrate and aniline is generally considered the most promising cleanup option to ensure groundwater quality safety. But the content of dissolved oxygen (DO) in groundwater circumstance is only 1-7 $mg\,L^{-1}$[17], so that the underground aquifers contaminated by organics are usually in anaerobic or anoxic state due to aerobic degradation of organics by indigenous microorganisms. Accordingly, *in situ* anaerobic or anoxic biological degradation has already become a major concern method to remove nitrate and aniline from complex contaminated groundwater.

In the anaerobic/anoxic conditions, nitrate removal in groundwater mainly depended on denitrification. Denitrification generally proceeds through some combination of the following intermediate forms:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO + N_2O \rightarrow N_2(g) \quad (1)$$

The complete denitrification process can be expressed as reaction (2):

$$2NO_3^- + 10e^- + 12H^+ \rightarrow N_2 + 6H_2O, \quad \Delta G^0 = -333\text{kJ/mol} \quad (2)$$

Denitrification can be divided into heterotrophic denitrification (HD) and autotrophic denitrification (AD) according to carbon source utilization way. HD requires organics to provide electron donor and energy source, while AD needs the participation of inorganic carbon. Site investigation and laboratory experiments by several researchers confirmed that refractory organics aniline could be sole carbon, nitrogen, and energy sources of heterotrophic denitrifiers [18–23]. The reaction of aniline biodegradation when nitrate nitrogen (NO_3^--N) as an electron acceptor without taking into account the growth of bacterial cells is as follows:

$$C_6H_5NH_2 + 6NO_3^- + 2H_2O = 6HCO_3^- + 2N_2 + NH_4^+ + OH^- + N_2O \quad (3)$$

Thus, it is theoretical possible and experimental feasible for simultaneous removal of nitrate and aniline from groundwater via *in situ* HD.

Yet, further research indicated that nitrate enhanced aniline degradation, and aniline removal efficiency was closely related to the availability of electron acceptor nitrate [14,24,25]. As a result, nitrate was often required overdose in order to obtain a high aniline removing rate. In other words, aniline was completely removed from mixed polluted groundwater by HD, but nitrate was still residual, it is difficult to treat the high nitrate and organic carbon limited groundwater only through HD [26,27]; moreover, the nitrite (NO_3^--N) and ammonium (NH_4^+-N) accumulation in HD process is a problem not to be ignored; furthermore, the CO_2 and HCO_3^- produced during HD were wasted in all of the studies mentioned above [28]. And these problems not only limit the effect of simultaneous removal nitrogen and carbon from compound polluted groundwater, but also increase the difficulty and cost of subsequent treatment.

Anaerobic ammonium oxidation (ANAMMOX) is conversion of ammonium and nitrite/nitrate to dinitrogen gas (N_2) [29]. The reactions are as follows:

$$5NH_4^+ + 3NO_3^- \rightarrow 4N_2 + 9H_2O + 2H^+, \quad \Delta G^0 = -297\text{kJ/M(ANAMMOX)} \quad (4)$$

$$NH_4^+ + NO_2^- \rightarrow N_2 + H_2O, \quad \Delta G^0 = -357\,\text{kJ/M(ANAMMOX)} \quad (5)$$

ANAMMOX is autotrophic biological reaction and hence no organics are needed. It can occur naturally in the presence of inorganic carbon source (CO_2,

HCO_3^-, and CO_3^{2-}, etc.) under anaerobic or anoxic conditions ($Eh = +350$ to $+100$ mV), where nitrogen is used as an electron acceptor in place of oxygen [30]. ANAMMOX has the advantage of low microbes and activated sludge output, and minor secondary pollution. Therefore, if the autotrophic ANAMMOX can be used to remove the excess nitrate of low-dissolved organic carbon groundwater, it not only can save energy consumption, but also can alleviate nitrite and ammonium accumulation in the HD process. Fortunately, it is notable that, there produces NH_4^+-N in the process of aniline degradation, while denitrification produces NO_2^--N, and there is a cumulative process [19]. In addition, denitrification degradation process produces inorganic carbon HCO_3^-, CO_2, and so on. These substances will provide substrates and carbon source for ANAMMOX to stimulate and ensure its occurrence.

In fact, the ANAMMOX micro-organisms in groundwater environmental media are prevalence, especially in heavily nitrogen polluted sites [31,32]. A few filamentous and short rod bacteria were presence in groundwater environment reported by several researchers [33–35]; and in anaerobic membrane bioreactor, ANAMMOX bacteria with other microbial populations like aerobic ammonia-oxidizing bacteria, nitrite oxidizing bacteria, and denitrifiers were harmonious coexistence; reactor experiments also showed that when NH_4^+-N, NO_2^--N, and NO_3^--N were complicated, denitrification and ANAMMOX were coexist in the same reactor [36–38], Sabumon [39] confirmed ANAMMOX microbes and heterotrophic denitrifiers coexisted in cow dung biomass, and there was synergetic effect to remove organics and carbon simultaneously. These researches revealed that it was feasible to co-culture denitrification and ANAMMOX bacteria [31–39]. That is to say in anoxic ecosystems, when organic matter is available microbes may prefer reactions (1), (2), and (3), and when organic carbon is limiting, reaction (4) or (5) may be preferable [39–41].

Based on the analysis of previous studies, there are strong indications that heterotrophic denitrifiers coexist with ANAMMOX microbes in groundwater environment. It is theoretical and experimental feasible to remove nitrate and refractory organics aniline simultaneously combining HD and ANAMMOX in groundwater, and there may have a synergistic effect between the two biological reactions, which may greatly increase simultaneous removal efficiency and improve carbon source utilization efficiency to save costs. Thus, it is meaningful to develop high efficiency and low cost *in situ* bioremediation technology of groundwater. Utilization of such coupling systems, however, requires a thorough understanding of characteristics of HD and ANAMMOX cooperation which currently does not exist. The objective of the present work was, therefore, simulation natural conditions of groundwater using batch and continuous bioreactors, to study the feasibility and performance of simultaneous removing nitrate and aniline by cooperating HD with ANAMMOX.

2. Materials and methods

2.1. Materials

Aquifer sediments were collected from the Weihe riverbed about 500 m downstream from Xianyang Bridge (34°24′31.32″ N, 108°54′42.58″ E) on the Weihe River, which is close to the populous city of Xi'an located in ShaanXi province of China. The groundwater used for testing in this study was taken from a 30 m well at Wangjiazhuang Village (34°19′41.01″ N, 108°44′31.60″ E) located in 600 m downstream of Weihe Bridge also in ShaanXi Xi'an. The characteristics of groundwater were as follows: DO 2.0 mg L^{-1}; aniline 12.49 mg L^{-1}; nitrate (NO_3^--N) 45.0 mg L^{-1}; nitrite (NO_2^--N) 0.04 mg L^{-1}; ammonium (NH_4^+-N) 0.05 mg L^{-1}; Cl^- 100.0 mg L^{-1}; chemical oxygen demand (COD_{Mn}) 47.0 mg L^{-1}; pH 7.58; Ca^{2+} 3.82 mg L^{-1}; Mg^{2+} 2.25 mg L^{-1}; temperature 23.5°C.

Anaerobic techniques were used in samples collection. Both groundwater and sediments were collected anaerobically from sampling points in sealed glass bottles with no headspace and transported back to the laboratory on ice for prompt processing. The sediments and groundwater samples were processed for baseline physical and chemical characterizations. Sediments were naturally air-dried and then sieved through an 80-mesh screen for using to keep the same medium in the study.

Synthetic groundwater was prepared for using in batch tests and column study by analytical grade chemicals and collected groundwater. Under room temperature (about 25°C), the analytical pure aniline (C_6H_5NH) and potassium nitrate (KNO_3) were dissolved in groundwater to make simulating complex wastewater for testing, and the concentration was adjusted based on design of the experiments.

A series of chemicals (C_6H_5NH, KNO_3, etc.) were purchased from Xi'an chemical reagents company, Xi'an city, Shaanxi province, P.R. China. All of them are analytical grade.

2.2. Experimental process

This work was carried out as an initial step towards development of simultaneous removing

carbon and nitrogen for *in situ* biological treatment of polluted groundwater plume containing nitrate and recalcitrant compounds. So the HD and ANAMMOX process in nitrate and aniline degradation were characterized by static batch tests and dynamic flow column experiments, respectively.

2.2.1. Batch experiment

Static batch experiments were carried out by sacrificial batch cultures. The batches were setup by following methods described by Harrison et al. [42], each batch (250-mL amber glass bottle) contained sediments (5 g) and groundwater (150 mL). Nitrate and aniline were amended in corresponding treatments with the initial concentrations as aniline 30, 50, 100 mg L^{-1} when nitrate 1,000 mg L^{-1} and nitrate 500, 1,000, 1,500 mg L^{-1} when aniline 50 mg L^{-1}. After vigorous mixing for 10 min under room temperature (~25˚C), these bottles were purged with nitrogen gas (N_2) for 30 min to maintain anoxic conditions (oxidation–reduction potential was lower than 200 mv), and sealed with Teflon-coated stoppers immediately. The batches were setup 90 repeats as one batch under each concentration condition, and were incubated in an incubator with temperature at 24 ± 1˚C. Three samples were taken every time (no-reused) and analyzed regularly for aniline, NO_3^--N, COD, NO_2^--N, and NH_4^+-N concentrations as well as electrical conductivity (*EC*) and pH values. To minimize error, only the mean values were considered.

2.2.2. Dynamic column experiment

Soil column constituted by aquifers medium is one effective way to simulate the actual hydro-geologic conditions of the groundwater basin [26]. The dynamic experimental reactor with its instrumentation and control system is schematized in Fig. 1. The experimental apparatus consists of water supply unit (A: groundwater, B_1: nitrate, B_2: aniline, B_3: mixture water) and a cylindrical organic glass biological reactor region. Soil columns (R_1) are 15 cm inner diameter and 40 cm height, and sand columns (R_2 and R_3) are 10 cm diameter and 50 cm height. R_1 were filled with aquifer sediments; R_2 and R_3 were filled with sand soils with an effective diameter of 0.5 mm and uniformity coefficient of 1.23; bottom of R_1, R_2, and R_3 was filled with 2–3 cm quartz sand for anti-clogging. The sieves, which consist of stainless steel, were placed at the inlet and the exit of the reactor to prevent washout of soil fragments.

The flow rate was adjusted using peristaltic pump, and the column was operated in upflow mode to keep the aquifer media saturated and avoid the appearance of preferential flow. The inoculation lasted 20 days in batch mode by circulation of water with daily replenishment of nitrate. Samples were collected daily from sampling points S_1 (inlet), S_2 and S_3 (outlet), and the collection samples were routinely assayed for aniline, NO_3^--N, NO_2^--N, NH_4^+-N, and COD concentrations as well as *EC* and pH values. The experimental study was carried out at temperature 24 ± 1˚C.

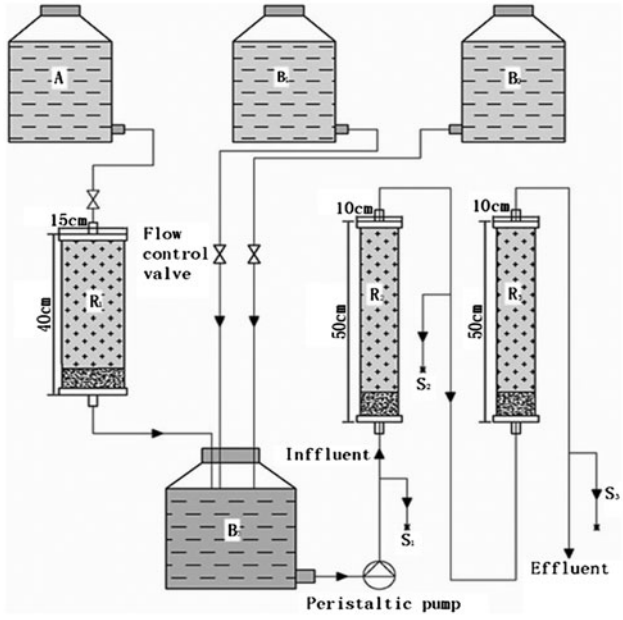

Fig. 1. Schematic view of the experimental apparatus.

2.3. Analytical methods

Water quality indexes were determined according to the Water and Wastewater Monitoring Analysis Methods published by State Environmental Protection Administration of China (State Environmental Protection Administration, 2002) [43]. Aniline concentration in aqueous solution was monitored by high-performance liquid chromatography (HPLC, Agilent 1100 Series, Agilent, USA) by comparison with the retention times of standard compounds. Aliquots of 10 μl were injected into the HPLC to determine the concentration of aniline, running with a mobile phase of 0.05 mol/L ammonium acetate-acetic acid buffer solution/methanol (65/35 v/v). The separation was performed on a Zorbax ODS column (250 mm × 4.6 mm (i.d.)) at a flow rate of 0.8 mL/min and column temperature of 30˚C. A UV detector was used with the wavelength set at 285 nm.

NO_3^--N and NO_2^--N were determined by ion chromatography (IC) (792Basic IC, Metrohm, Switzerland) with a separating column (A Supp 5(6.1006.530),

250 mm × 4.0 mm) and *EC* detector, with $NaHCO_3$ (1.0 mmol/l)/Na_2CO_3 (3.2 mmol/l)/methanol (3%) eluent and a total flow rate of 0.6 mL/min. The samples (20 μl) were injected manually through an injection port. The concentration of NH_4^+-N released during aniline degradation was determined according to the Nessler's reagent spectrophotometric method (GB7479-87, China). COD was measured using a Hach COD analyzer (Loveland, Co., USA) following method described by Jirka and Carter [44].

EC values were measured by Conductivity Meter DDB-303A type and pH was recorded by pHS-3C analyzer (Shanghai Precision Scientific Instrument Co., Ltd, China). All samples were immediately analyzed to avoid further reaction.

3. Results and discussion

3.1. Batch tests

3.1.1. Nitrate and aniline removal

HD and ANAMMOX bacteria are ubiquitous in nature [31,37], and HD occurs naturally when certain bacteria use nitrate as terminal electron acceptor in their respiratory process in the absence of oxygen [15]. Thus, five group tests to examine the potential and efficiency of removing nitrate and aniline simultaneous from groundwater through batch cultures according to aforementioned concentrations were conducted, and the results are given in Fig. 2.

From Fig. 2, we can see that both nitrate and aniline concentration decreased with increasing incubation time and the final removal rate of nitrate, aniline, and COD exceeded 79.03, 96.6, and 69.7%, respectively, was achieved after operation of 150 days. But the removal rate evidently changed in different incubation stage as the simulation system was at different microbial metabolism periods. The whole removing process can be divided into three phases, in addition to the group with nitrate and aniline concentration 1,500 and 50 mg L^{-1}, the other groups were no lag period, it is mainly because the collected sediment are undisturbed aquifer medium polluted by nitrate and some refractory organics, indigenous micro-organisms had been domestication and adapted to such special organic carbon limited circumstance [14,30], and these microbes playing an overwhelming role in the degradation could utilize aniline as carbon and energy, however, some acclimatization period was needed for the nitrate concentration high to 1,500 mg L^{-1}.

During the first period of 30 days, nitrate, aniline, and COD were rapid decline, suggesting that HD began to occur, and relatively high removal rate was achieved in this stage mainly relying on HD. Subsequently, from days 30 to 105, nitrate, aniline, and COD concentration continued to decrease although the removal rate declined slightly. Nitrate decreased over than 215.09 mg L^{-1}, which was significantly higher than the stoichiometric consumption of 160 mg L^{-1} only HD existing according to Eq. (3), but in this stage, the ORP values were below zero (data were not shown), so nitrate assimilated was unlikely to occur [15,37], therefore, we infer that autotrophic ANAMMOX may occur as explanation in reaction Eqs. (4) and (5). Biodegradation process of batch transited into the coexistent phase of HD and ANAMMOX, and degradation rate declined as a result of autotrophic efficiency lowers than heterotrophic generally. Then, in the last 30 days from 120 to 150, the performances of the system maintained at a stable stage that nitrate concentration continued to reduce and aniline was almost completely removed. However, as the interspecific competition for COD of HD and ANAMMOX bacteria reached a new balance, biodegradation turned into autotrophic ANAMMOX phase when bacteria could could use NH_4^+-N to remove the remainder nitrate efficiently and stably, and the reactions (4) and (5) provided a possible explanation for the observation [35,39].

It was apparent that single-stage autotrophic nitrogen removal was achieved by the close cooperation between HD and ANAMMOX bacteria. However, both HD and ANAMMOX effect including the start-up time and duration time were conspicuously influenced by initial concentrations of nitrate and aniline. Under experimental conditions, HD was mainly affected by the initial aniline concentration, and the duration time increased with aniline increased (Fig. 2(b)). When aniline concentration increased from 30 mg L^{-1} to 100 mg L^{-1}, the duration time of HD prolonged from about 25 days to 90 days, and the start-up time of ANAMMOX lagged correspondingly (Fig. 2(b)). Accordingly, the autotrophic ANAMMOX reaction time increased with the nitrate concentration increase (Fig. 2(a)). When nitrate concentration increased from 500 mg L^{-1} to 1,500 mg L^{-1}, the autotrophic ANAMMOX duration time increased from about 60 days to more than 60 days removing to the same level (Fig. 2(a)). This showed that the combined degradation features of HD and ANAMMOX were closely related to the initial concentrations of target pollutants, which were confirmed by other researchers [41,45]. Heterotrophs are micro-organisms that require organic substrates as carbon and energy source for growth and development. In contrast, ANAMMOX bacteria are autotrophic micro-organisms that can use inorganic carbon substances to proliferation [37,39]. Hence, when the substrate concentration changed, HD

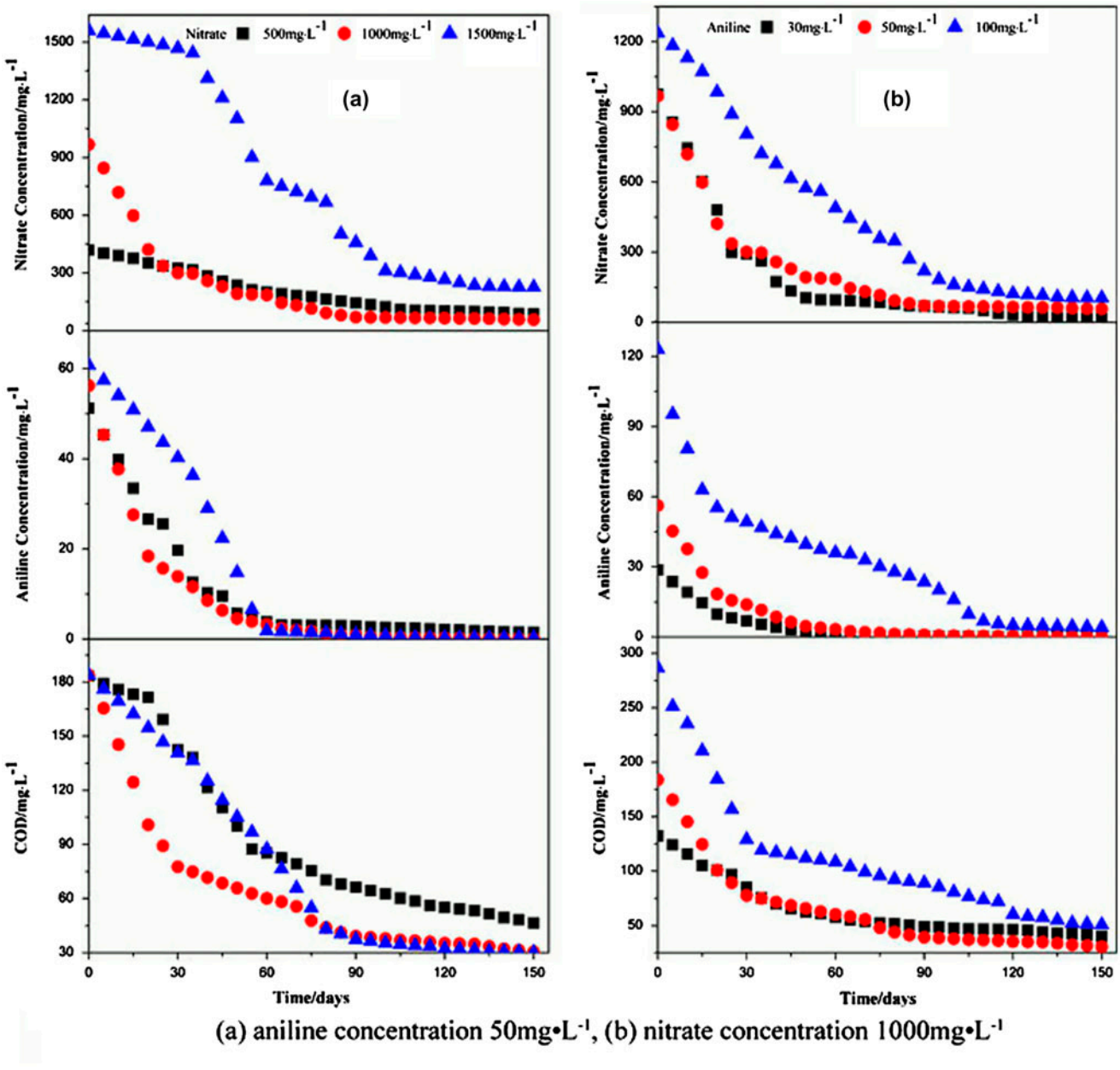

Fig. 2. NO_3^--N, aniline and COD removal in batch tests (a) aniline concentration 50 mg L^{-1} and (b) nitrate concentration 1,000 mg L^{-1}.

and ANAMMOX biological activities altered correspondingly result in the removal rate to nitrate and aniline change.

However, the results of COD demonstrated that the combination to remove organics was stable and the COD removal efficient was less affected by C/N (Fig. 2). COD was consumed gradually by various indigenous micro-organisms in the batch tests, and the final removal was over 77.7% at the selected C/N concentrations. It is worthwhile to note that the variation of COD was similar to the change of aniline, illustrating that there was a relationship between aniline degradation and COD consumption. This phenomenon is attributable to the medium used in this study containing little dissolved organic carbon, and thus a great part of COD was caused by aniline. Moreover, about 100 days later, the COD concentration decreased slightly despite degradation was on the ANAMMOX stage, this is because although ANAMMOX can occur without organic carbon, the growth or action of microorganisms may consume some organic carbon, which also can explain the occurrence of ANAMMOX.

Generally, it was not only nitrate and refractory organics could be effectively removed simultaneously by cooperating HD with ANAMMOX in the simulated groundwater reactor, but also the synergistic characteristics of removal process was obvious. In the first stage, HD hold a dominant position, and nitrate and aniline degradation mainly depended on HD, but in later stage, nitrate residue removal mainly through ANAMMOX. These strongly implied that ANAMMOX had a potential advantage in dealing with high nitrogen and organic carbon limited groundwater.

3.1.2. Ammonia and nitrite

Biological nitrogen removing is a complex microbial metabolic process including a series of successive reactions as shown in Eqs. (1)–(3). In the process, a number of nitrogen intermediates were formed, for example, NO_2^--N and NH_4^+-N are important intermediates produced by HD in aniline and nitrate degradation; in addition, NH_4^+-N is also the important substrate of ANAMMOX. The research findings presented by Matsumoto et al. [46] and Grigatti et al. [47] suggested that NO_2^--N and NH_4^+-N concentration change had a close relation to biological degradation characteristics and removing efficiency of nitrogen and organics. Li et al. [48] also found that NO_2^--N and NH_4^+-N could be indicators of nitrification and denitrification in a sequencing batch reactor. Therefore, NO_2^--N and NH_4^+-N concentrations were measured to evaluate the cooperating removal characteristics and the results are presented in Fig. 3.

Apparently, NO_2^--N and NH_4^+-N concentrations change can be approximately divided into three phases. Both of them rapidly increased in the first stage and followed by increasing slowly to the maximum, and then slightly declined with incubation time prolonged. This phenomenon is mainly attributed to that in first stage HD was the main way to remove nitrate and aniline, so NO_2^--N and NH_4^+-N concentrations increased rapidly as to serious accumulation. Biological activity of ANAMMOX bacteria was excited with the substrate of NO_2^--N and NH_4^+-N concentrations increase, and thus ANAMMOX began to occur. Meanwhile, the degradation process got into HD and ANAMMOX coexistence phase, and the accumulation of NO_2^--N and NH_4^+-N was weakened.

However, from Fig. 3 and it was obvious that conversion time of each stage and the maximum of NO_2^--N and NH_4^+-N as well as the reached time were closely related to the initial nitrate and aniline concentration. With initial nitrate and aniline concentrations increase, both the time of NO_2^--N and NH_4^+-N concentration reached the maximum and the coexist time of HD with ANAMMOX increased. Moreover, the time for NO_2^--N and NH_4^+-N reduced to the same level owing to ANAMMOX also increased. When nitrate concentrations increased from 500 to 1,500 mg L^{-1}, the maximum of NO_2^--N increased from 4.87 mg L^{-1} (on days 35) to 43.68 mg L^{-1} (on days 89) while time reached to the maximum increased from 35 to 89 days. Comparably, NH_4^+-N accumulation and existing time were mainly affected by the initial aniline concentration, when the aniline concentration increased from 30 to 100 mg L^{-1}, the maximum of NH_4^+-N increased from 5.48 mg L^{-1} (on days 47) to 15.25 mg L^{-1} (on days 99), and time of reached to the maximum increased from 47 to 99 days.

So, NH_4^+-N and NO_2^--N could indicate the important features of HD and ANAMMOX cooperation process to remove nitrogen and organic carbon simultaneously, and the indication was closely related to nitrate and aniline initial concentrations. For example, when nitrate and aniline concentration were (1,000, 30 mg L^{-1}), (1,000, 50 mg L^{-1}), and (1,000, 100 mg L^{-1}) (theoretical C/N were 0.27, 0.46 and 0.91), ANAMMOX began to show its effect at 30, 50, and 90 days approximately (Fig. 3(b)), and the duration time also increased from 60 to 120 days (Fig. 3(a)). NH_4^+-N and NO_2^--N concentration change not only confirmed the occurrence of autotrophic ANAMMOX, but also could determine the stage of cooperating biodegradation HD with ANAMMOX. Hence, it is possible to be efficient and convenient chemical indicators for the combination of HD and ANAMMOX.

3.1.3. pH and EC

HD and ANAMMOX consist of a series of enzymatic reactions involving formation various nitrogenous and no-nitrogenous intermediates [37]. So water quality parameters, such as pH and *EC*, will be changed with the intermediates produced in HD and ANAMMOX; otherwise, enzyme activity is easily influenced by water quality parameters. That is to say that biological growth and environmental parameters are interactive influenced process, and the removing efficiency will be influenced indirectly. Therefore, water quality parameters *EC* and pH in the simulation batch tests were monitored and shown in Fig. 4.

As can be seen from Fig. 4, pH and *EC* values change also were divided into three phases. In first 30 days, *EC* and pH values decreased, then gradually increased to each maximum value and decreased to the minimum again at tested C/N concentrations (Fig. 4). The change time of each phase was about 35, 45, and 95 days, which were consistent with the

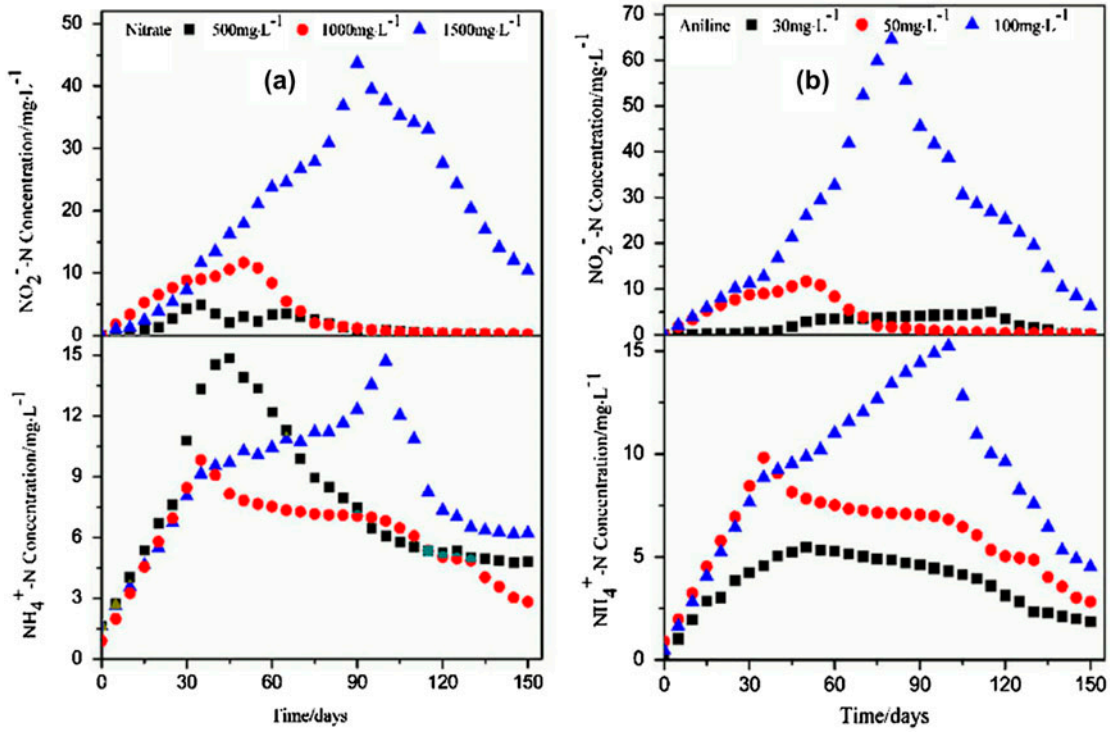

Fig. 3. Nitrite and ammonium concentration changes in the batch tests (a) aniline concentration 50 mg L^{-1} and (b) nitrate concentration 1,000 mg L^{-1}.

change time of HD and ANAMMOX discussed above. Thereby, pH and *EC* can also indicate HD and ANAMMOX process.

Due to initial degradation stage, there were some carbon dioxide (CO_2) produced by indigenous microbial respiration (Eq. (6)), and the CO_2 dissolved in

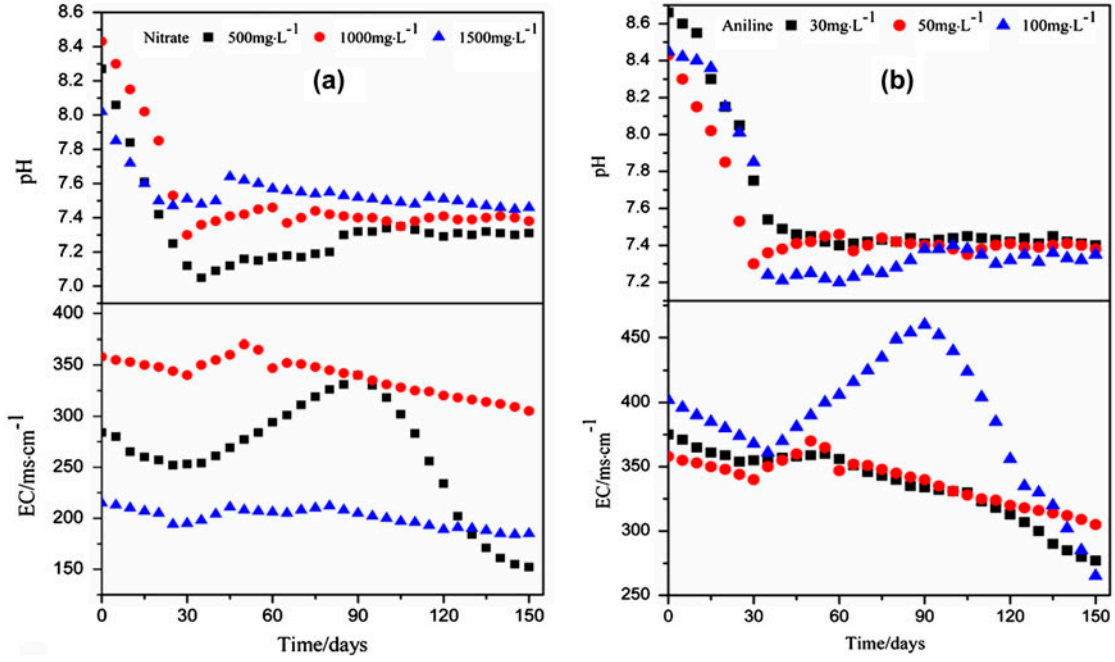

Fig. 4. *EC* and pH changes in the batch tests (a) aniline concentration 50 mg L^{-1} and (b) nitrate concentration 1,000 mg L^{-1}.

water to produce hydrogen ion (the proton, H^+) to cause pH values declined directly (Eq. (7)). Furthermore, the generated bicarbonate (HCO_3^-) consumed hydroxide (OH^-) in water solution making pH declined indirectly (Eqs. (8)–(10)). The similar conclusion that CO_2 produced in biodegradation dissolved in water reduced of pH values was found by researchers like Dong et al. [49] and Wan et al. [50].

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 12H_2O + \text{energy} \quad (6)$$

$$CO_2 + H_2O \rightarrow HCO_3^- + H^+ \quad (7)$$

$$HCO_3^- + H^+ \rightarrow H_2CO_3 \quad (8)$$

$$H_2CO_3 + OH^- \rightarrow HCO_3^- + H_2O \quad (9)$$

$$HCO_3^- + OH^- \rightarrow CO_3^{2-} + H_2O \quad (10)$$

Next, from days 30 to 105, pH values increased as HD occurred, which induced the environment alkalinity increase. From formula (2), there are 17 g OH^- generated theoretically for every 14 g N reduced to N_2 by HD without considering biological growth. This results were consistent with finding that HD brought system pH values rising [51,52] and it also well illustrated that the degradation was at HD stage. And then, from 105 to 150 days, pH declined slightly owing to some H^+ released from organics and intermediates of ANAMMOX and eventually stabilized at about 7.7. The final pH was in the optimum range of autotrophic ANAMMOX bacteria growth 7.8–8.6 reported by Lee et al. [53], and when pH exceeded this range, TN removal efficiency would be greatly reduced. On the basis of evidence, we deduced that autotrophic ANAMMOX had occurred and consumed acidity produced by HD to weaken alkalinity rise, so that the system had a good pH buffering capacity in the synergistic of HD and ANAMMOX.

Similar to pH values, *EC* values of samples were measured too. *EC* is a measurement that correlates with water properties affected directly by charged ions dissolved in water. Therefore, *EC* can estimate the total amount of charged ions dissolved in water although it still does not provide any information about the ion composition in the water.

As culture time went on, *EC* values decreased slowly on the initial time, followed by rapid increased and then decreased until gradually stabilized, whereas in different NO_3^--N and aniline initial concentrations the change time and values were different distinctly. The reasons for this trend were that the kind and concentration of conductive substances changed with the activities of indigenous micro-organisms (synthesis, decomposition, *etc.*). In the beginning, indigenous bacteria were stimulated to proliferation through respiration or fermentation to produce energy, in this process, large amounts of nutrients were consumed, and free charged ions decreased, result in *EC* dropped. However, in this process, CO_2 was generated by microbial respiration and NH_4^+-N was produced by HD. Because CO_2 could rapidly dissolve in water to form carbonic acid (H_2CO_3), bicarbonate ions (HCO_3^-), and carbonate ions (CO_3^{2-}), which increased conductive ions concentration, and therefore *EC* increased on days 20–45. In addition, *EC* declined with NO_3^--N concentration increased, and the final *EC* values were smaller than the initial. This may relative to N_2 generated by ANAMMOX eventually overflowed outside the simulated system, which is a further illustration of ANAMMOX existence in the last stage of batch tests.

From the analysis of pH and *EC*, we can summarize that *EC* and pH values increased in HD stage and decreased in ANAMMOX stage, and the overall changes could indicate the degradation process, which is feasible to be utilized as fuzzy control parameters to remove organics and nitrate. Consequently, chemical parameters as accurate and effective indicators for removal process were proved. Furthermore, the ANAMMOX process in batch tests showed a high ANAMMOX activity and shock resistance for high-strength ammonium wastewater, which provided good foundation for the following experiments.

3.2. Dynamic soil column experiment

The basic principle of *in situ* bioremediation contaminated groundwater is using metabolism of indigenous micro-organisms to break down pollutants to innocuous CO_2, H_2O, or other substances necessary for life. Studies presented by Smith et al. [54] shown that the key to obtain good degradation efficiency was enriching special indigenous micro-organisms having efficient purification capabilities. Normally, under natural conditions, special indigenous micro-organisms were isolated from nature and cultivated by adding targeted contaminants. Accordingly, on the basis of static batch cultures results, a group of nitrate and aniline with concentration of 1,000 and 50 mg L^{-1} (theoretical C/N ratio 0.46) was selected in flow column experiment and added once the column flow and reduction circumstance had been stable after domestication about 20 days. The performance and effect to remove nitrogen and organics in the continuous soil column along the hydraulic direction are shown in Fig. 5.

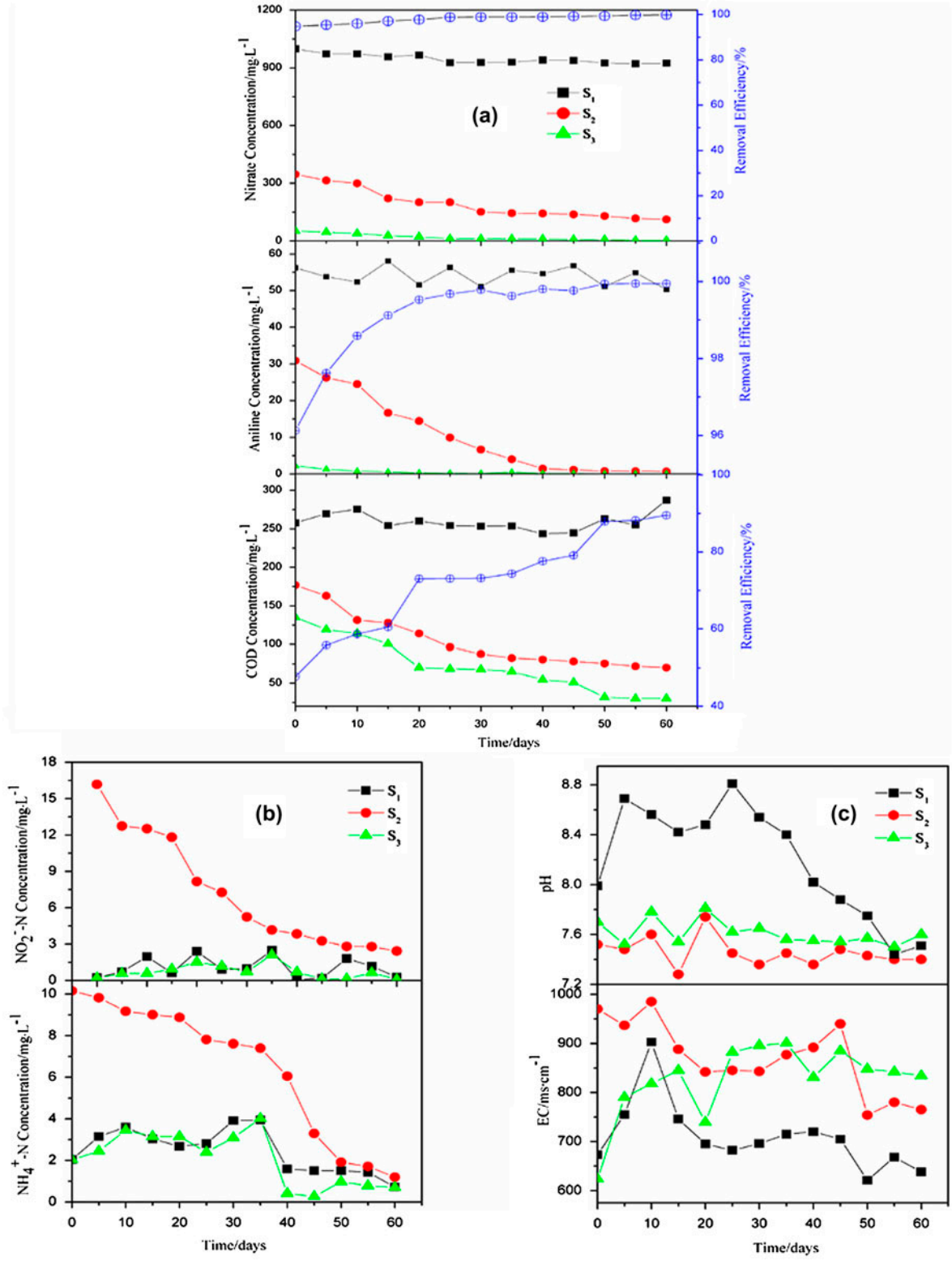

Fig. 5. (a) Nitrate, aniline, and COD concentration, (b) Nitrite and ammonium, and (c) *EC* and pH change in the column tests.

3.2.1. Nitrate and aniline removal

Fig. 5(a) presents the change in aniline, nitrate, and COD concentration in continuous column experiment. From Fig. 5(a), high removal efficiency of aniline, nitrate, and COD was obtained in all phases although a slight increase with operating time increasing, and there was no lag phase of degradation. The strategy of the experiment was that no organics were added in acclimation stage and micro-organisms had consumed most of the organic matters in sediments. When aniline was added and NO_3^--N concentration increased from 300 mg L^{-1} to 1,000 mg L^{-1}, sufficient electron donor (aniline) and acceptor (NO_3^--N) as well as nutrition were available for the "starvation" period bacteria, so pollutants were strongly depredated by HD microbe; additionally, some NO_3^--N was used as nitrogen source of microbial metabolism, so the removal efficiency of nitrate, aniline and COD increased rapidly and reached to 98.83, 93.67, and 73.02% on days 25, respectively. Next 25–45 days, NO_3^--N, aniline, and COD removal efficiency increased slowly, and increase rate decreased. After 45 days, the removing was stable and removal efficiency high to 99.8, 99.9, and 85.5% on 60 days, respectively. This reason is that, after "starvation" period, denitrifying bacteria participating in metabolism must undergo adaptive period to the new substrates generated in former degradation to improve activities [55]. The following 15 days, with the consumption of nutritional and intermediate products, denitrification circumstance and biological activities were stable. Consequently, the removal rate changed slowly from 25 to 45 days and gradually stabled in days 45–60.

In addition, as Fig. 5(a) presents, it was significant that although NO_3^--N and aniline of 10.81 mg L^{-1} and 0.18 mg L^{-1} were monitored in effluent (S_3) on days 25 and COD was still higher than 65 mg L^{-1}, the removal rate increased sharply; on 40 days, aniline concentration was near to detection limit in S_2 while NO_3^--N concentration was over than 200 mg L^{-1} and COD was lower than 100 mg L^{-1} after 45 days. From these phenomenon, we can infer that there was almost HD effect in the whole soil column before 25 days, and from days 25 to 45 were period of HD and ANAMMOX coexistence, but ANAMMOX played a leading role on days 45 to 60 in column Ⅱ.

3.2.2. NO_2^--N and NH_4^+-N

From Fig. 5(b), there were a few NO_2^--N and NH_4^+-N in influent (S_1), and concentrations increased first (S_2) and then decreased (S_3) along the water flow direction. The main cause for this phenomenon is that, there had been abundant indigenous micro-organisms for 30 days culture in column Ⅰ, and microbial metabolism utilized nutrient of sediment to produce a few NO_2^--N and NH_4^+-N which were detected in S_1, with aniline and nitrate added, microbial activities changed result in the concentration of NO_2^--N and NH_4^+-N increased first and then decreased.

There were dramatic accumulation of NO_2^--N and NH_4^+-N in S_2, especially 25 days ago, for an average reached to 10.56 and 8.92 mg L^{-1}, but with time increasing accumulation decreased. This can be interpreted as HD microbes rapidly proliferated with organic matter added, and consumed aniline to produce NO_2^--N and NH_4^+-N; but meanwhile, HD products were not enough to excite ANAMMOX bacteria activities to the maximum, and ANAMMOX was still weak leading to NO_2^--N and NH_4^+-N accumulation. With the HD process going on, ANAMMOX microbial activities were stimulated, and we inferred that under the substrate induction, after about 40 days enhancement, there were a large amount of ANAMMOX bacteria that could utilize the products of HD, such as CO_2, CO_3^{2-}, as carbon source for autotrophic ANAMMOX, therefore, NO_2^--N and NH_4^+-N accumulation were weakened.

Moreover, from 20 to 50 days, NO_2^--N and NH_4^+-N concentrations in S_2 decreased simultaneously (NO_2^--N decreased from 16.17 mg L^{-1} to 3.27 mg L^{-1}, and NH_4^+-N decreased from 10.16 mg L^{-1} to 3.30 mg L^{-1}), and the reduction ratio was close to the theoretical values of ANAMMOX calculated from equation (5), and at this stage NO_3^--N in effluent (S_3) was almost completely removed, implying that ANAMMOX reaction began to occur and played essential functions, especially in column Ⅱ. After 50 days, not only NO_2^--N and NH_4^+-N concentrations in S_2 and S_3 dropped to low level (similar to influent), but also NO_3^--N and aniline were almost completely removed, suggesting that HD and ANAMMOX had good synergistic effect to simultaneous removal of nitrogen and carbon.

3.2.3. pH and EC

Meanwhile, pH and *EC* characteristics are shown in Fig. 5(c). The pH was decreased from over 8.0 in S_1 (influent) to lower than 7.5 in S_2 and then increased to 7.7 in S_3 (effluent), and the change was significant before 45 days. The variation characteristics could well explain the biological metabolism process, as reactions (2)–(4) illustrated that HD generated alkalinity while ANAMMOX consumed alkalinity. And more important, the final pH value (days 45 to 60) was stabilized at around 7.5 within the optimal pH scope

of biological anaerobic denitrification, which demonstrated that the combination had very good buffer role on the synergistic effect of HD with ANAMMOX.

However, the changes of *EC* were more complex compared with NO_2^--N, NH_4^+-N, and pH (Fig. 5). Nevertheless, the overall features were S_1 (influent) *EC* values smaller than S_2 and S_3 (effluent), and S_3 *EC* increased in all experiments; S_2 *EC* reached the maximum in HD period (on 25 days), and the change trend were $S_1 < S_2 < S_3$ during ANAMMOX period (about days 45–60). This is largely attributable to indigenous micro-organisms consumed limited DO of water in reactor Ⅰ, and free O_2 was transformed into O^{2-} making the inlet water conductive ions increased, so S_1 *EC* increased in despite of lower than S_2 before 20 days. From days 20 to 45, S_2 *EC* increased which indicated the occurrence of HD, since NO_3^--N and aniline were transformed into NO_2^--N and NH_4^+-N, and microbial metabolism activities (synthesis, decomposition, etc.) changed in HD degradation process, so that conductive substances quantity increased. However, after 45 days, all *EC* values from S_1 to S_3 were lowered; it was not only relative to nutrient consumption in soil column I, II, and III, but also to N_2 overflow generated by synergistic effect of HD and ANAMMOX. Moreover, the results also indirectly indicated that indigenous microbes were active in soil column and synchronization removing NO_3^--N and aniline was a complicated biological degradation process.

Overall, the constructed experimental system could rapidly establish denitrifying environments, which was suitable for making full use of collaboration of HD and ANAMMOX. The results confirmed that removing nitrate and organics simultaneous by the cooperation HD with ANAMMOX of indigenous micro-organisms ubiquitous in underground aquifer was feasible; and synergistic effect of HD and ANAMMOX such as good pH buffer capacity was remarkable; furthermore, pH and *EC* could be used as indicators of HD and ANAMMOX process.

4. Conclusions

In this study, both batch tests and flow column results showed that HD and ANAMMOX were coexistent in groundwater conditions, it is feasible, therefore, to remove nitrate and refractory organics aniline simultaneous coupling HD with ANAMMOX, and moreover, high and stable removing efficiency was achieved. In batch tests, the average removal rate of nitrate, aniline, and COD at selected C/N ratios (about 0.30, 0.46, and 0.91) were 89.1, 98.4, and 77.7%, respectively; and in soil column experiment, the final removal rate reached to 99.8, 99.9, and 89.5% after an operation of 60 days. The combination HD and ANAMMOX demonstrated good synergistic effect that no NO_2^--N and NH_4^+-N accumulation and having a pH buffer effect in removing nitrate and aniline simultaneously, and the cooperation among these bacteria was considered to be responsible for simultaneous nitrogen and COD removing. The results also showed that chemical parameters could be effective indicators of the cooperating process. When NO_2^--N and NH_4^+-N increased simultaneously, indicated HD occurrence, while decreased indicated ANAMMOX; when pH values decline as well as *EC* values increase, HD occurred and took effect, while reverse were ANAMMOX. Overall, the combination process will offer a great future potential for saving of energy and resource for efficient nitrogen and carbon removing from groundwater *in situ* application. Given the low-cost of our system, it is feasible to use synergistic effect of HD with ANAMMOX of indigenous microbes existence in underground aquifer to remove nitrogen and carbon from complex pollution groundwater, and although the findings are obtained directly from the experimental data, their further practical application in field is valuable to be explored in future study.

Acknowledgments

We would like to thank the support given by evaluation and investigation of groundwater pollution in the main cities of Northwest China (No. 1212011220982) from China Geological Survey, NPU Foundation for Fundamental Research (NPU-FFR-JCR20130145) and Graduate Starting Seed Fund of NPU (Z2013157; Z2013158).

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