

Chapter 2

Heterotrophic-Autotrophic Denitrification

Abstract A novel heterotrophic-autotrophic denitrification (HAD) approach supported by granulated spongy iron, pine bark and mixed bacteria was proposed for remediation of nitrate contaminated groundwater in an aerobic environment. The HAD involves biological deoxygenation, chemical reduction (CR) of nitrate and dissolved oxygen (DO), heterotrophic denitrification (HD) and autotrophic denitrification (AD). The experimental results showed 0.121 d, 0.142 d and 1.905 d were needed to completely remove DO by HAD, spongy iron and mixed bacteria respectively. Spongy iron played a dominant role in deoxygenation in the HAD. After 16 days, NO₃-N removal was approximately 100, 6.2, 83.1, 4.5 % by HAD, CR, HD, AD, respectively. CR, HD and AD all contributed to the overall removal of NO₃-N, but HD was the most important denitrification mechanism. There existed symbiotic, synergistic and promotive effects of CR, HD and AD within the HAD. The different environmental parameters (e.g. water temperature) showed different effects on HAD. HAD was capable of providing steady denitrification rate (1.233–1.397 mg/L/d) for 3.5 months. Pine bark could provide sufficient organic carbon, spongy iron could steadily remove DO, and microbial activity maintained relatively constant. HAD denitrification was zero order with a reaction rate constant (K) of 1.3220 mg/L/d.

Keywords Groundwater remediation • Aerobic environment • Heterotrophic-autotrophic denitrification (HAD) • Chemical reduction (CR) • Heterotrophic denitrification (HD) • Autotrophic denitrification (AD) • Granulated spongy iron • Pine bark

Abbreviations

ACS	American Chemical Society
AD	Autotrophic denitrification
BD	Biological denitrification
BET	Brunauer-Emmett-Teller
CR	Chemical reduction
DO	Dissolved oxygen

DNRA	Dissimilatory nitrate reduction to ammonium
HAD	Heterotrophic-autotrophic denitrification
HD	Heterotrophic denitrification
HRT	Hydraulic retention time
MDR	Maximum denitrification rate
MNPR	Maximum NO ₃ -N percent removal
PRB	Permeable reactive barrier
RO	Reverse osmosis
TOC	Total organic carbon
ZVI	Zero-valent iron

2.1 Introduction

Nitrate contamination of groundwater has become an environmental and health issue in developed and developing countries (Della Rocca et al. 2007a). Nitrate contaminated groundwater often contains dissolved oxygen (DO) (≤ 7 mg/L) to form an aerobic environment (Gómez et al. 2002; Schnobrich et al. 2007).

Zero-valent iron (ZVI) in the forms of steel wool, nanoscale iron, granulated iron, cast iron, iron chips, iron powder, etc. has been worldwide reported to chemically or biotically remove nitrate from water (Cheng et al. 1997; Till et al. 1998; Huang et al. 1998; Westerhoff and James 2003; Choe et al. 2004; Yang and Lee 2005; Ahn et al. 2008; Rodríguez-Maroto et al. 2009). However, ZVI in the form of granulated spongy iron has been neglected.

Actually, the most economical, environmentally sound, promising and versatile approach being studied for nitrate denitrification is biological denitrification (BD). The majority of BD processes relies on heterotrophic denitrifiers requiring an organic carbon source, however, groundwater has a low carbon content. As a result, an external organic carbon has to be supplied for bacterial growth. Recently, various studies have been conducted to evaluate the potential use of cellulose-rich solid organic carbon sources in heterotrophic denitrification (HD) processes, such as cotton, wood chips, wheat straw, newspaper, sawdust (Volkita et al. 1996a, b; Soares and Abeliovich 1998; Saliling et al. 2007; Kim et al. 2003; Robertson et al. 2008). Some of these carbon sources have been successfully contained in permeable reactive barrier (PRB) for in situ remediation or in reactors for *ex situ* remediation. However, valuable information on pine bark as a solid carbon substrate is lacking.

More recently, a heterotrophic-autotrophic denitrification (HAD) approach supported by steel wool and cotton was put forward by Della Rocca et al. (2006, 2007b). This HAD process involves chemical reduction (CR), HD and autotrophic denitrification (AD). Cotton could act as a source of organic carbon for HD; while carbon dioxide generated by HD could be employed as a source of inorganic

carbon by autotrophic denitrifiers. Concomitantly, steel wool could reduce DO in water and produce cathodic hydrogen, which enhances both HD and AD. Soares et al. (2000) reported 1,200 kg of cotton was strongly compressed and water flowed through channels of lower resistance at a relatively high flow rate. As a consequence, the denitrification rate declined considerably. Therefore it's necessary to seek a "harder" matrix as a solid organic carbon source to both support biofilm development and optimize the hydrodynamic environment which favors HAD processes. Continuous column experiments were carried out to investigate the denitrification capacity, the effects of flow-rate, ZVI amount, inlet nitrate and phosphate on the HAD performance and the formation of by-products (such as iron and bacterial biomass) (Della Rocca et al. 2006, 2007b). Although high nitrate-nitrogen ($\text{NO}_3\text{-N}$) removal efficiencies were encouraging, questions arise regarding the deoxygenation capacities and pathways, the respective contributions of AD, HD, ZVI-based CR to the overall $\text{NO}_3\text{-N}$ removal, the kinetics of $\text{NO}_3\text{-N}$ denitrification, and the effects of environmental parameters (e.g. water temperature) on denitrification behavior in HAD processes.

In the above context, a new HAD approach supported by mixing granulated spongy iron, pine bark and mixed bacteria was proposed to remediate nitrate contaminated groundwater in an aerobic environment and further develop HAD processes. The objectives of this study were to: (1) explore the feasibility and efficiency of $\text{NO}_3\text{-N}$ removal using the HAD process; (2) investigate the deoxygenation capacities and pathways of HAD; (3) determine the contributions of AD, HD, granulated spongy iron-based CR to the overall $\text{NO}_3\text{-N}$ removal; (4) confirm the kinetics of $\text{NO}_3\text{-N}$ denitrification; (5) evaluate the effects of mass of pine bark, water temperature, high-concentration nitrate, nitrite, ammonium and coexistent inorganic anions on the denitrification behavior in the HAD; and (6) provide information for future column studies and field in situ applications.

2.2 Materials and Methods

2.2.1 Materials and Chemicals

Granulated spongy iron (60.60 % of Fe^0 ; 0.425–1.000 mm in diameter) was obtained from Kaibiyuan Co., Beijing, China. Pine bark (0.15–20.00 mm in diameter) was obtained from a local nursery store in Adelaide, South Australia. Moist sub-surface soil (0.3 m depth from surface) was taken from a pristine and humic-acid-rich area on the campus of the Flinders University of South Australia. Milli-Q (Millipore) water was used to prepare reagent solutions. Reverse osmosis (RO) water was spiked with mineral salts media acting as synthetic groundwater. In addition to $\text{NO}_3\text{-N}$ (10–100 mg/L), the mineral salts media contained the following (final concentration in mg/L): NaHCO_3 (482), K_2HPO_4 (17.4), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.53), Na_2EDTA (7.4), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (40.6), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (49), $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$

(21.9), NaCl (58.5), and trace elements which was composed of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.504), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.08), $\text{ZnSO}_4 \cdot 10\text{H}_2\text{O}$ (0.088), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.72) (Huang et al. 2012). Unless otherwise indicated, all chemicals used were analytical or American Chemical Society (ACS) reagent grade as received.

2.2.2 Enrichment Culture Protocol to Establish a Denitrifying Bacterial Population

The soil was passed through 0.425 mm and 0.075 mm sieves and then stored in a cold room (3.2 ± 1.0 °C) before use (Huang et al. 2012). Initial enrichment culture was undertaken using a 1 L Schott bottle to which was added: (1) 5.00 g of soil; (2) 800 ml of RO water which was enriched with 22.6 mg $\text{NO}_3\text{-N/L}$ and 3.0 mg $\text{K}_2\text{HPO}_4\text{-P/L}$; (3) 5.00 g of pine bark; (4) 5.00 g of granulated spongy iron. The N:P weight ratio was 22.6:3 and initial DO was 3.95 mg/L. Following initial enrichment the denitrifying population was maintained by successively subculturing. Upon depletion of $\text{NO}_3\text{-N}$ a bacterial suspension was transferred into fresh mineral salts media containing spongy iron and pine bark without soil at a bacterial suspension: mineral salts media volume ratio of 1:9. The enrichment culture was conducted in water bath (15 °C).

2.2.3 Batch Experiments

Batch incubations were conducted in sterile, screw capped, 2 L Schott bottles, to which was added mineral salts media. Spongy iron was sterilized (160 °C for 4 h). All the glassware, pipette tips, storage containers and mineral salts media were steam autoclaved (121 °C for 15–17 min) prior to use (Huang et al. 2012). When BD, biological deoxygenation and environmental parameters were being assessed an inoculum with mixed bacteria from an enriched denitrifying bacterial subculture was used, and the inoculum accounted for 10 % of the total mineral salts media volume (Huang et al. 2012). The granulated spongy iron: mineral salts solution ratio of 5:800 (mg:ml) was maintained. The Schott bottles were equipped with membrane screw caps and covered with aluminium foil and statically incubated in the dark (Huang et al. 2012). Water samples were drawn with a syringe and filtered through a 0.45 μm glass fibre filter (Whatman GF/C, 47 mm in diameter). The filtrate was placed in a screw capped plastic tube and stored at 3.2 ± 1.0 °C until analysis for $\text{NO}_3\text{-N}$, nitrite-nitrogen ($\text{NO}_2\text{-N}$), ammonium-nitrogen ($\text{NH}_4\text{-N}$), pH and T. DO was monitored online in the incubation bottles. The experimental conditions of batch incubations are shown in Table 2.1.

Table 2.1 The design of batch incubations used in this study

Incubations	Granulated spongy iron (g)	Pine bark (g)	Inoculum (ml)	Initial NO ₃ - N (mg/L)	Initial DO (mg/L)	Mineral salts media (g)	Water temperature (°C)	Initial pH
Deoxygenation by HAD	12.50	12.50	200	0	4.2	2000	15	8.04
Deoxygenation by CR	12.50	0	0	0	4.7	2000	15	7.87
Deoxygenation by mixed bacteria	0	12.50	200	0	4.1	2000	15	8.04
NO ₃ -N removal by HAD	12.50	12.50	200	20.35	4.3	2000	15	7.83
NO ₃ -N removal by HAD (control)	0	12.50	0	21.96	4.3	2000	15	7.83
NO ₃ -N removal by CR	12.50	0	0	22.57	0	2000	15	8.32
NO ₃ -N removal by HD	0	12.50	200	21.64	0	2000	15	7.72
NO ₃ -N removal by AD + CR	12.50	0	200	23.46	4.3	2000	15	8.32
Effect of the mass of pine bark on HAD	12.50	2.50 7.50	200	21.09 21.53	4.3	2000	15	8.16
Effect of water temperature on HAD	12.50	12.50	200	22.00 21.32 21.47	4.5	2000	15 27.5 33	8.16
Effect of high-concentration nitrate on HAD	12.50	12.50	200	22.06 97.58 20.35	4.5	2000	15	8.16
Effect of nitrite on HAD	12.50	12.50	200	20.48	4.5	2000	15	8.16
Effect of ammonium on HAD	12.50	12.50	200	21.46	4.5	2000	15	8.16
Effect of coexistent inorganic cations on HAD	12.50	12.50	200	21.99 - 22.56	4.5	2000	15	8.16

(continued)

Table 2.1 (continued)

Incubations	Granulated spongy iron (g)	Pine bark (g)	Inoculum (ml)	Initial NO ₃ - N (mg/L)	Initial DO (mg/L)	Mineral salts media (g)	Water temperature (°C)	Initial pH
Longevity of pine bark	12.50	12.50	200	20.77	4.5	2000	15	8.16
Kinetics of NO ₃ -N denitrification by HAD				-				
				23.62				
	12.50	12.50	200	44.20	4.1	2000	15	8.08
Kinetics of NO ₃ -N denitrification by HAD (control)				20.35				
				11.15				
	0	0	0	20.35	4.1	2000	15	8.08

2.2.4 Analytical Methods

Inorganic nitrogen ($\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$ and $\text{NH}_4\text{-N}$) was analysed using a FOSS-Tecator FIAStar 5,000 flow injection analyser (Sweden) equipped with a FOSS 5027 Auto-Sampler, a nitrite/nitrate method cassette, a ammonium method cassette (including gas diffusion cell and gas diffusion membrane), a 40 μL sample loop, a 400 μL sample loop, a cadmium reduction column and interference filters ($M = 590\text{ nm}$ and $R = 720\text{ nm}$; $M = 540\text{ nm}$ and $R = 720\text{ nm}$) employing American Public Health Association Standard Methods (APHA et al. 1992). The lower detection limits for $\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$ and $\text{NH}_4\text{-N}$ were 0.005, 0.005 and 0.010 mg/L respectively. Temperature, pH, and DO were measured using a pH meter (Hanna, Model 8417, Italy) and a digital DO meter (Hanna, Model 9143, Italy).

2.2.5 Data Processing Methods

Variation of $\text{NO}_3\text{-N}$ (or $\text{NO}_2\text{-N}$ or $\text{NH}_4\text{-N}$ or DO) (Eq. 2.1)

$$\Delta C = C_t - C_0 \quad (2.1)$$

where, ΔC is variation of $\text{NO}_3\text{-N}$ (or $\text{NO}_2\text{-N}$ or $\text{NH}_4\text{-N}$ or DO) concentration (mg/L); t is reaction time (d); C_t is $\text{NO}_3\text{-N}$ (or $\text{NO}_2\text{-N}$ or $\text{NH}_4\text{-N}$ or DO) concentration at time t (mg/L); C_0 is initial $\text{NO}_3\text{-N}$ (or $\text{NO}_2\text{-N}$ or $\text{NH}_4\text{-N}$ or DO) concentration at time 0 (mg/L) $\text{NO}_3\text{-N}$ (or $\text{NO}_2\text{-N}$ or $\text{NH}_4\text{-N}$ or DO) percent removal (Eq. 2.2)

$$\sigma = (C_t - C_0)/C_t \times 100\% \quad (2.2)$$

where, σ is $\text{NO}_3\text{-N}$ (or $\text{NO}_2\text{-N}$ or $\text{NH}_4\text{-N}$ or DO) percent removal (%) Variation of pH (Eq. 2.3)

$$\Delta pH = pH_t - pH_0 \quad (2.3)$$

where, ΔpH is variation of pH; pH_t is pH at time t ; pH_0 is initial pH at time 0 $\text{NO}_3\text{-N}$ (or $\text{NO}_2\text{-N}$) denitrification rate (Eq. 2.4)

$$\Phi = (C_t - C_0)/t \quad (2.4)$$

where, φ is $\text{NO}_3\text{-N}$ (or $\text{NO}_2\text{-N}$) denitrification rate (mg/L/d).

2.3 Results and Discussion

2.3.1 Deoxygenation Capacity of the Heterotrophic-Autotrophic Denitrification Process

To apply HAD processes to nitrate contaminated groundwater, the initial DO in raw water must be eliminated to create an anaerobic environment for chemical nitrate reduction and bionitrification. Dissolved oxygen is thermodynamically more reducible than dissolved nitrate by ZVI due to a higher standard cell-reaction potential of oxygen reduction than that of nitrate reduction (Su and Puls 2007). On the other hand, BD produces less energy yield than oxygen respiration, therefore, a bacterial cell growing in aerobic environments will choose to use oxygen as a terminal electron acceptor (Gómez et al. 2002). In addition to this competitive effect, oxygen negatively controls BD at two levels: reversible inhibition of the activities of denitrification enzymes and regulation of gene expression (Gómez et al. 2002).

Figure 2.1 shows the changes in DO percent removal in mineral salts media by HAD, granulated spongy iron only, and mixed bacteria only. Linear regression analysis was used to describe DO removal over reaction time and estimate the time when complete reduction of DO was achieved. Strong positive linear correlations between percent removal and reaction time in the HAD, granulated spongy iron only, and mixed bacteria only incubations were determined (Eqs. 2.5, 2.6, and 2.7; Fig. 2.1).

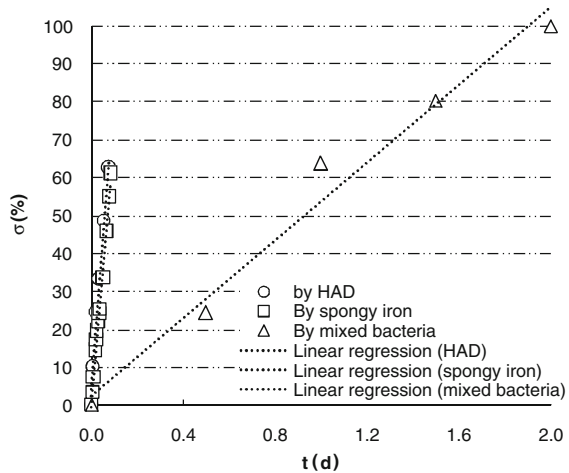
For HAD:

$$\sigma(\%) = 793.0137t(d) + 4.2059(R^2 = 0.9854, P < 0.001) \quad (2.5)$$

For granulated spongy iron:

$$\sigma(\%) = 702.3783t - 0.4572(R^2 = 0.9951, P < 0.001) \quad (2.6)$$

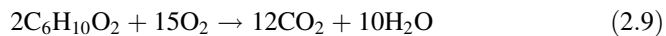
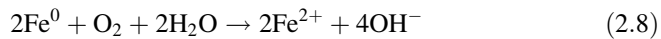
Fig. 2.1 Removal of dissolved oxygen (DO) by heterotrophic-autotrophic denitrification (HAD), granulated spongy iron and mixed bacteria at the initial DO of 4.2, 4.7 and 4.1 mg/L, respectively



For mixed bacteria:

$$\sigma(\%) = 51.1922t + 2.4818 (R^2 = 0.9781, P < 0.001) \quad (2.7)$$

Linear least square analysis demonstrated that 0.121 d, 0.142 d and 1.905 d were needed to completely remove DO by HAD, granulated spongy iron and mixed bacteria respectively. Clearly, HAD, granulated spongy iron, and mixed bacteria all had deoxygenation capacity, and the mixed bacteria contained aerobic heterotrophs. Therefore, granulated spongy iron and aerobic heterotrophs both contributed to DO removal in the HAD incubation. Granulated spongy iron consumes DO via CR (Eq. 2.8) (Della Rocca et al. 2005a). Aerobic heterotrophs employed total organic carbon (TOC) released by pine bark to deoxygenate via aerobic respiration (Eq. 2.9). Undoubtedly, HAD depended on granulated spongy iron via CR and aerobic heterotrophs via aerobic respiration to remove DO. Any incubation that had granulated spongy iron included reduced DO more rapidly than the mixed bacteria alone (Fig. 2.1). Consequently, granulated spongy iron played a dominant role in deoxygenation in the HAD (Huang et al. 2012).



Oxygen reduction is considered necessary prior to the onset of nitrate removal. The HAD process has shown the rapid deoxygenation capacity (Fig. 2.1). Therefore, this process facilitated an anaerobic environment which avoids the competitive and inhibitory effects of DO. This is a desirable attribute because most nitrate laden groundwater also contains oxygen (Huang et al. 2012).

2.3.2 Contributions of Chemical Reduction, Heterotrophic Denitrification and Autotrophic Denitrification to the Performance of the Heterotrophic-Autotrophic Denitrification Process

2.3.2.1 Performance of Heterotrophic-Autotrophic Denitrification

Figure 2.2 shows the changes in $\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$, $\text{NH}_4\text{-N}$ and pH in the HAD incubation.

As shown in Fig. 2.2a, $\text{NO}_3\text{-N}$ decreased with increasing reaction time. 19.92 mg/L $\text{NO}_3\text{-N}$ was removed during the 16 day period (Fig. 2.2a), suggesting that the HAD approach not only was effective at removing $\text{NO}_3\text{-N}$ but also is worthy of further study. 0.27 mg/L $\text{NO}_3\text{-N}$ was lost from the control incubation for $\text{NO}_3\text{-N}$ in 16 days (Fig. 2.2a). This indicated that the effect of $\text{NO}_3\text{-N}$ physical adsorption onto pine bark was negligible, i.e., $\text{NO}_3\text{-N}$ was not removed by adsorption in the HAD incubation. $\text{NO}_2\text{-N}$ tended to reach its maximum (0.15 mg/L) at day 10, and

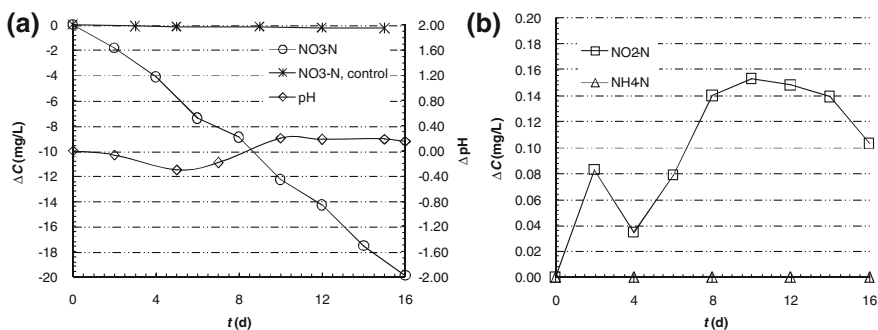


Fig. 2.2 Performance of heterotrophic-autotrophic denitrification (HAD) in the HAD incubation: **a** variations of $\text{NO}_3\text{-N}$ and pH; **b** variations of $\text{NO}_2\text{-N}$ and $\text{NH}_4\text{-N}$. The control incubation contained $\text{NO}_3\text{-N}$ of 21.96 mg/L and pine bark of 12.50 g (sterilized, 160 °C for 4 h) without granulated spongy iron and mixed bacteria

subsequently decreased to 0.10 mg/L at day 16 (Fig. 2.2b). $\text{NO}_2\text{-N}$ accounted for $\leq 0.77\%$ of the $\text{NO}_3\text{-N}$ removed. No $\text{NH}_4\text{-N}$ variations were found (Fig. 2.2b). Actually, $\text{NH}_4\text{-N}$ concentrations were below the detection limit at all times. As a result, the HAD approach transformed nitrate into gaseous nitrous compounds (such as NO , N_2O) or nitrogen gas (N_2). The small variations in pH ranging from -0.30 to 0.20 were noted at the initial pH of 7.83 between days 0 and 16 (Fig. 2.2a). It is likely that the bicarbonate in the mineral salts media acting as a pH buffer controls the pH change, but no measurements were made to confirm this. As is well known, pH increase is probably detrimental to microbial metabolism and may limit nitrate and particularly nitrite BD. In combination with the nitrate and nitrite changes, it can be concluded that the solution pH never inhibited the HAD performance. For the case of groundwater remediation, it's possible that a denitrification PRB filled with spongy iron and pine bark could be installed in aquifers. Obviously, the HAD approach was potentially a feasible and effective approach for in situ groundwater remediation.

2.3.2.2 Performance of Chemical Reduction Denitrification

Figure 2.3 shows the changes in $\text{NO}_3\text{-N}$ and pH in the CR incubation. Only 1.34 mg/L $\text{NO}_3\text{-N}$ was removed during the 16 day period at the initial pH of 8.32 (Fig. 2.3), demonstrating that the capacity of nitrate CR by granulated spongy iron was limited at weakly alkaline pHs, which was consistent with the literature describing CR (Westerhoff and James 2003). The reason for the low reduction capacity was a limited supply of electrons which come from granulated spongy iron either directly or indirectly via the acidic corrosion products of Fe such as hydrogen. Part of Fe^0 on the surface of granulated spongy iron was oxidized in the deoxygenation process, and then granulated spongy iron was covered with a passive oxide film consisting of an inner layer of Fe_3O_4 and an outer passive layer

Fig. 2.3 Performance of chemical reduction (CR) denitrification in the CR incubation at the initial pH of 8.32: variations of $\text{NO}_3\text{-N}$ and pH

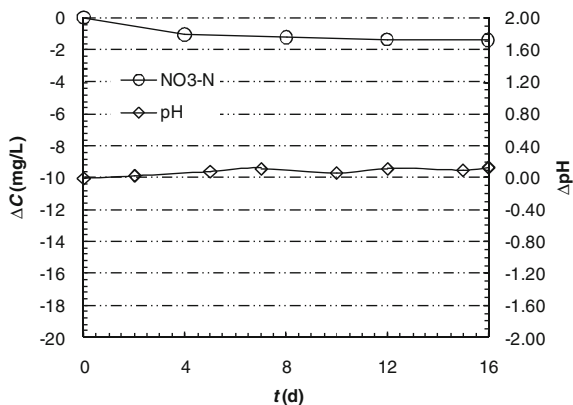
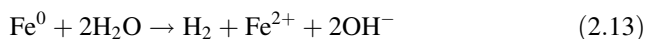
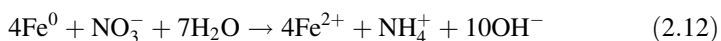
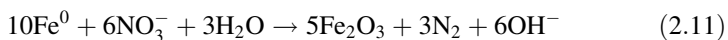
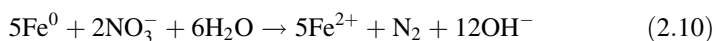


Table 2.2 Performance of chemical reduction (CR) denitrification in the CR incubation at the initial pH of 8.32: variations of $\text{NO}_2\text{-N}$ and $\text{NH}_4\text{-N}$

	0 d	4 d	8 d	12 d	16 d
$\Delta C(\text{NO}_2\text{-N})$ (mg/L)	0.00	0.00	0.01	0.01	0.00
$\Delta C(\text{NH}_4\text{-N})$ (mg/L)	0.00	0.00	0.01	0.02	0.00

of Fe_2O_3 (Tsai et al. 2009). This passive layer prevented the onset of electron transfer (Tsai et al. 2009). In weakly alkaline solutions, possible pathways of chemical $\text{NO}_3\text{-N}$ reduction by Fe^0 are proposed in Eqs. 2.10, 2.11, and 2.12 (Siantar et al. 1996; Kielemoes et al. 2000). Table 2.2 illustrates the changes in $\text{NO}_2\text{-N}$ and $\text{NH}_4\text{-N}$ in the CR incubation. Almost no $\text{NO}_2\text{-N}$ and $\text{NH}_4\text{-N}$ variations were observed (Table 2.2). This implied that the $\text{NO}_3\text{-N}$ removed was converted to gaseous nitrogen. It has been well demonstrated and generally accepted that regardless of Fe^0 types, rapid and complete nitrate CR mainly occurs at acidic $\text{pH} \leq 4$ or 5, and neutral or alkaline conditions are not normally favorable for nitrate reduction (Huang et al. 1998; Huang and Zhang 2004; Yang and Lee 2005; Rodríguez-Maroto et al. 2009), but the main disadvantage of low pHs is the release of ammonium as a major undesirable nitrogen product.

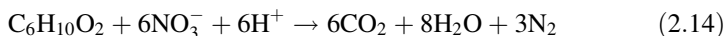


Theoretically, the rise in pH can be achieved from Fe^0 -based chemical nitrate reduction as well as anaerobic Fe^0 corrosion (Eqs. 2.10, 2.11, 2.12, and 2.13).

Accordingly, the minor rise in pH ($\Delta\text{pH} = 0 \sim 0.13$) (Fig. 2.3) could also indicate that the CR process did not progress significantly via single granulated spongy iron at weakly alkaline pHs.

2.3.2.3 Performance of Heterotrophic Denitrification

Figure 2.4 shows the changes in $\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$, $\text{NH}_4\text{-N}$ and pH in the HD incubation under an anaerobic condition. 17.97 mg/L $\text{NO}_3\text{-N}$ was depleted during the 16 day period (Fig. 2.4a), demonstrating that the mixed bacteria contained heterotrophic denitrifiers. Heterotrophic denitrifiers could utilize TOC released by pine bark to reduce $\text{NO}_3\text{-N}$, for synthesis of new bacterial cells and maintenance of the existing cell mass (Eq. 2.14) (Ovez et al. 2006; Della Rocca et al. 2005b). $\text{NO}_2\text{-N}$ concentration was less than 0.08 mg/L (Fig. 2.4b). Hence, no nitrite accumulation occurred. Nitrite accumulation during HD has previously been reported. $\text{NO}_2\text{-N}$ concentration did not drop below 13 mg/L in a batch experimental study where pine shavings served as a solid carbon source. In other studies, nitrite accumulated when sucrose was used as a liquid carbon source (Piñar and Ramos 1998; Gómez et al. 2000). However, ethanol and methanol as liquid carbon sources did not cause the occurrence of nitrite accumulation (Gómez et al. 2000). It can be deduced that nitrite accumulation seems to depend on carbon source types. No $\text{NH}_4\text{-N}$ variations were observed (Fig. 2.4b).



According to Eq. 2.14, pH should increase in HD as a result of the consumption of hydrogen ions ($\text{H}^+ \downarrow$). But the results suggested just the opposite at the initial pH of 7.72 (Fig. 2.4a). The solution pH was between 7 and 8, indirectly indicating the heterotrophic denitrifying bacteria functioned effectively. It is widely believed that heterotrophic denitrifying microbial activity is at an optimal around neutrality (pH 6.0–8.0) (Beaubien et al. 1995; Elefsiniotis and Li 2006).

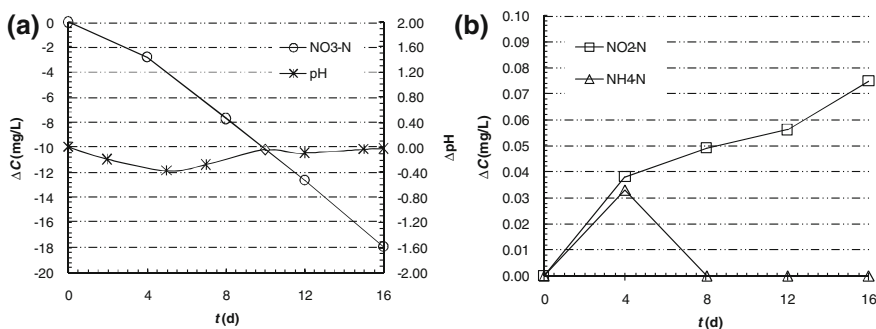


Fig. 2.4 Performance of heterotrophic denitrification (HD) in the HD incubation under an anaerobic condition: **a** variations of $\text{NO}_3\text{-N}$ and pH; **b** $\text{NO}_2\text{-N}$ and $\text{NH}_4\text{-N}$. The mineral salts media used was sparged with N_2 gas for 5 min at 100 kPa after sampling

2.3.2.4 Performance of Autotrophic Denitrification

Considering the explosion and safety concerns associated with hydrogen gas, H_2 was not employed to investigate the performance of AD. AD was identified by investigating the difference in performance between of AD coupled with CR (AD + CR) and CR only. Likewise, pH caused by AD at time t ($pH_{t,AD}$) was obtained by determination of the difference between AD + CR ($pH_{t,AD+CR}$) and CR ($pH_{t,CR}$). To preclude the probable interference of TOC and suspended solid from the mixed inoculum, the procedures were introduced as follows: first, filter the inoculum suspension through a $0.45\ \mu m$ fiber filter membrane; second, filter the filtrate through a $0.2\ \mu m$ filter membrane; third, add the $0.2\ \mu m$ membrane into a AD + CR incubation without pine bark. Figure 2.5 shows the changes in NO_3^- -N, NO_2^- -N, NH_4^+ -N and pH caused by AD and also shows their changes in the AD + CR incubation. $0.87\ mg/L$ NO_3^- -N was removed during the 16 day period (Fig. 2.5a), indicating that AD occurred but also the mixed bacteria contained autotrophic denitrifiers.

Under anaerobic environments, the corrosion of spongy iron by water formed cathodic hydrogen and ferrous iron (Eq. 2.13) (Choe et al. 2000; Su and Puls 2004). Hydrogenotrophic denitrifying bacteria utilized hydrogen and inorganic carbon (CO_2 , HCO_3^- , etc.) to reduce NO_3^- -N (Haugen et al. 2002). The *beta-Proteobacteria*, including *Rhodocyclus*, *Hydrogenophaga*, and *beta-Proteobacteria* HTCC 379, probably played an important role in hydrogenotrophic denitrification (Zhang et al. 2009). Also, a group of lithoautotrophic bacteria might couple an AD process to the oxidation of Fe^{2+} with an inorganic carbon source (Straub and Buchholz-Cleven 1998; Weber et al. 2006). Additionally, the spongy iron used served as a solid-carrier for biofilm formation. Autotrophic denitrifying growth on Fe^0 has been previously demonstrated (Till et al. 1998). Little NO_2^- -N ($\leq 0.04\ mg/L$) and NH_4^+ -N ($\leq 0.06\ mg/L$) were generated via AD (Fig. 2.5b).

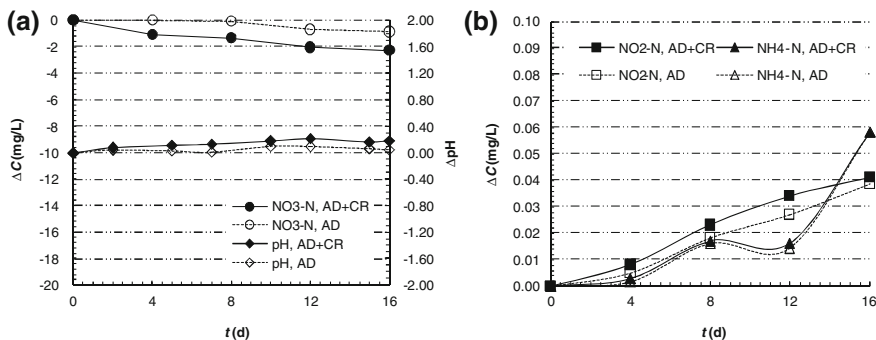
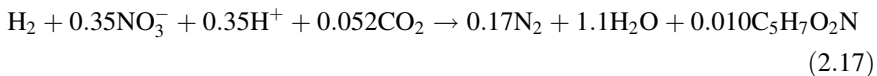
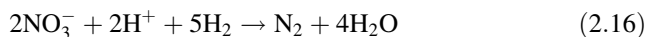
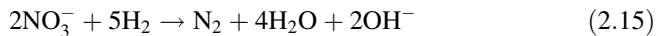


Fig. 2.5 Performance of autotrophic denitrification (AD) and autotrophic denitrification coupled with chemical reduction (AD + CR): **a** variations of NO_3^- -N and pH; **b** NO_2^- -N and NH_4^+ -N. For NO_3^- -N (or NO_2^- -N or NH_4^+ -N), $C_{t,AD}$ (mg/L) = $C_{t,AD+CR}$ (mg/L) - $C_{t,CR}$ (mg/L) at time t ; For pH, $pH_{t,AD} = pH_{t,AD+CR} - pH_{t,CR}$ at time t

While pH values ranging from 7.0 to 8.0 are optimal for HD, previous researchers have observed that hydrogenotrophic denitrification did not appear to be inhibited by pH values up to approximately 8.6. Both abiotic and biotic $\text{NO}_3\text{-N}$ reduction result in the elevation of pH in water due to either the production of hydroxide ions ($\text{OH}^- \uparrow$) or the consumption of hydrogen ions ($\text{H}^+ \downarrow$) in Fe^0 -supported hydrogenotrophic denitrification (Eqs. 2.13, 2.15, 2.16, and 2.17).



The minor rise in pH ($\Delta\text{pH } 0 \sim 0.21$ for AD + CR; $\Delta\text{pH } 0 \sim 0.13$ for CR) (Fig. 2.5a) at the initial pH of 8.32 therefore appeared to demonstrate that the AD + CR and CR only processes did not progress significantly at weakly alkaline pHs. It's well known that Fe^0 corrosion is slow at $\text{pH} \geq 7$ and autotrophic denitrifiers grow slowly, causing the low capacity of the AD.

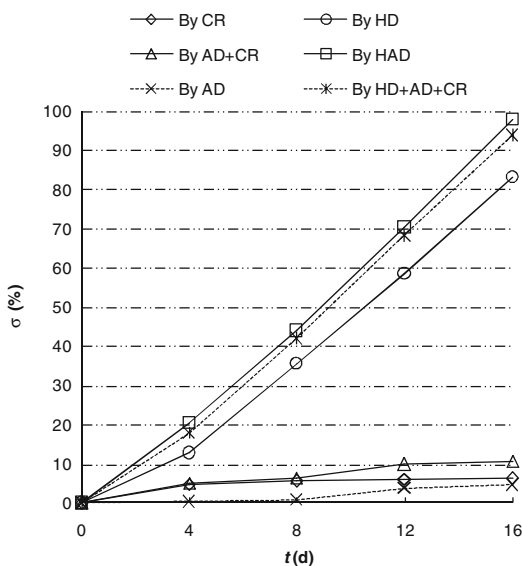
2.3.2.5 Contributions of Chemical Reduction, Heterotrophic Denitrification and Autotrophic Denitrification

Figure 2.6 shows the changes in $\text{NO}_3\text{-N}$ percent removal in mineral salts media by granulated spongy iron-based CR only, HD only, AD + CR, HAD, AD and HD + AD + CR. Percent removal of $\text{NO}_3\text{-N}$ for AD was defined by percent removal for AD + CR minus percent removal for HD. Percent removal of $\text{NO}_3\text{-N}$ for HD + AD + CR was defined by percent removal for AD + CR plus percent removal for HD.

The percent removal of $\text{NO}_3\text{-N}$ escalated with increasing reaction time in the CR, HD, AD + CR and HAD incubations (Fig. 2.6). After 16 days little $\text{NO}_3\text{-N}$ was lost from the CR incubation (6.2 % removed) at the initial pH 8.32. After 16 days there was large loss of $\text{NO}_3\text{-N}$ in the HD incubation (83.1 % removed). This value was greater than 6.2 % in the single CR incubation, indicating AD not only occurred but also reduced 4.5 % of the initial $\text{NO}_3\text{-N}$. After 16 days $\text{NO}_3\text{-N}$ removal was approximately 100 % in the HAD incubation.

There is no doubt that CR, HD and AD all contributed to the overall removal of $\text{NO}_3\text{-N}$ in the HAD incubation in 16 days, demonstrating their symbiotic, synergistic and promotive effects within the HAD. The $\text{NO}_3\text{-N}$ losses were greater for HD than for CR + AD, confirming that HD was the most important $\text{NO}_3\text{-N}$ denitrification mechanism in the HAD (Huang et al. 2012). It is interesting to note that the combined percent removal for AD + CR + HD was slightly less than that for HAD at any point in time. The largest difference (4.2 %) between them was attained at day 16. An explanation is that in the HAD incubation, carbon dioxide

Fig. 2.6 The percent removal of $\text{NO}_3\text{-N}$ by chemical reduction (CR), heterotrophic denitrification (HD), autotrophic denitrification coupled with chemical reduction (AD + CR), heterotrophic-autotrophic denitrification (HAD) and the calculated percent removal of $\text{NO}_3\text{-N}$ by autotrophic denitrification (AD) and HD + AD + CR



generated by aerobic heterotrophs and heterotrophic denitrifiers was consumed by autotrophic denitrifiers as an additional inorganic carbon source to enhance the capacity of AD (Eqs. 2.9, and 2.14) (Della Rocca et al. 2006; Ovez et al. 2006). Concomitantly, the consumption of molecular hydrogen overcame the decrease of available reactive surface area of Fe^0 (Choe et al. 2004).

2.3.3 Effects of Environmental Parameters on the Heterotrophic-Autotrophic Denitrification Process

2.3.3.1 Effect of the Mass of Pine Bark

As mentioned in Sect. 2.3.2.5, the majority of $\text{NO}_3\text{-N}$ removal by the HAD relied on HD that necessarily requires an organic carbon source, so the mass of pine bark might affect the HAD performance. Three different amounts of pine bark (2.5, 7.5 and 12.5 g) were incubated in a constant mineral salts media (2.0 L). A comparison was made of denitrification rates among the three amounts in Fig. 2.7a. The changes in $\text{NO}_2\text{-N}$ and $\text{NH}_4\text{-N}$ over reaction time at the three weights of pine bark are shown in Fig. 2.7b.

In the first 4 days of incubation, the denitrification rates for the three amounts were almost the same. There was a minor effect of pine bark amount on the HAD denitrification during this period of time (Fig. 2.7a). This is attributed to the acclimation of the denitrifying bacteria to this substrate (Elefsiniotis and Li 2006).

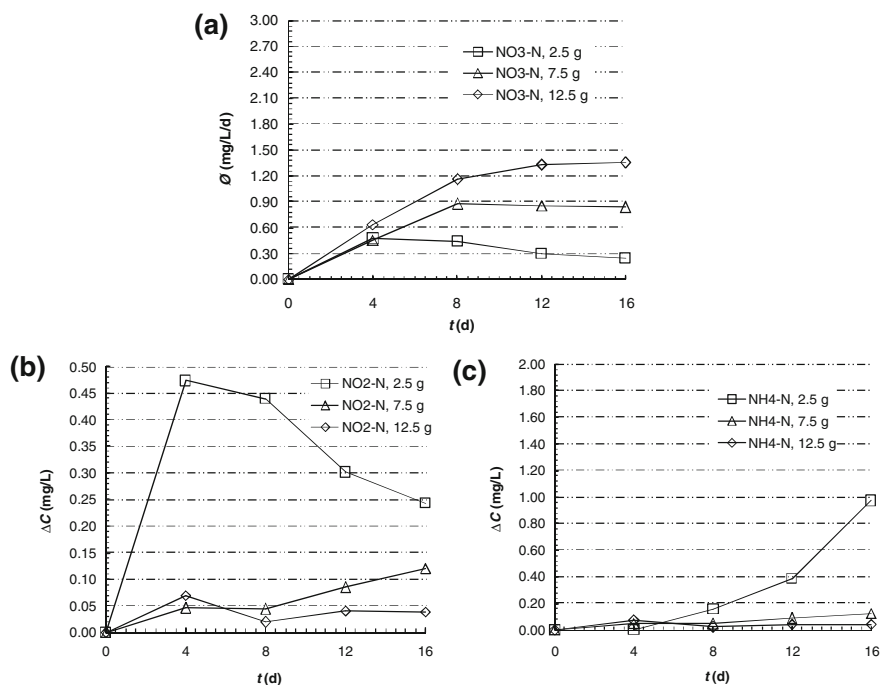


Fig. 2.7 Effect of pine bark amount on the performance of heterotrophic-autotrophic denitrification (HAD) over reaction time at 2.5, 7.5 and 12.5 g of pine bark in 2000 mL of mineral salts media: **a** denitrification rates; **b** variations of NO₂-N; **c** variations of NH₄-N

In the last 4 days of incubation, the denitrification rate increased in the order of pine bark weight: 12.5 g > 7.5 g > 2.5 g (Fig. 2.7a). There was a noticeable effect of pine bark mass on the HAD performance from days 4 to 16. Larger amounts of pine bark could provide more organic carbon and electron donors for heterotrophic bacterial respiration and growth. Besides, increasing the pine bark mass could increase the surface area of pine bark available to the denitrifying population (Ovez et al. 2006). Overall, denitrification rates were closely and positively related to the mass of pine bark.

The most significant NO₂-N accumulation at day 4 at the pine bark of 2.5 g (Fig. 2.7b) resulted from a lack of pine bark supply. Limitation of organic carbon causes the competition between nitrite and nitrate reductase for electron donors, and induces delayed syntheses and inhibited activities of nitrite reductase relative to nitrate reductase (Hunter 2003). These leads to denitrification rates of NO₃-N being lower than those of NO₂-N (Betlach and Tiedje 1981), which gives rise to the incomplete reduction of NO₃-N.

Low dissolved NH₄-N concentrations of ≤0.12 mg/L were found at the pine bark of 7.5 and 12.5 g, but high concentrations of 0.38–0.97 mg/L, which accounted for 10.89–24.95 % of the NO₃-N transformed, were observed during the

last 4 days of incubation at the pine bark of 2.5 g (Fig. 2.7c). Dissimilatory nitrate reduction to ammonium (DNRA) by microorganisms might be a mechanism of $\text{NH}_4\text{-N}$ production (Kim et al. 2003). However, DNRA requires a carbon rich and high organic carbon to nitrate environment (Yin et al. 2002; Robertson et al. 2008). Therefore it is unlikely that DNRA was the major cause of $\text{NH}_4\text{-N}$ production in the incubation with the smallest weight of pine bark; notwithstanding it only accounted for <1 % of the $\text{NO}_3\text{-N}$ transformed in HD systems (Greenan et al. 2006). Elucidation of $\text{NH}_4\text{-N}$ production in the HAD is not a primary focus of this study and the exact cause remains unknown.

2.3.3.2 Effect of Water Temperature

Water temperature has different effects on different BD processes. Volokita et al. (1996a) reported that denitrification rates at 14 °C were approximately half of the rates observed at 30 °C when cotton was used as a carbon source for HD. Volokita et al. (1996b) reported denitrification rates at 14 °C were approximately one third of the rates at 32 °C when newspaper was used as another carbon source. Robertson et al. (2000) reported that denitrification rates at 2–5 °C were 5 mg N/L/d, whereas the corresponding rates at 10–20 °C were 15–30 mg N/L/d, when coarse wood mulch acted as a carbon source (Robertson et al. 2000). Huang et al. (2012) reported denitrification rate at 27.5 °C was 1.36 times higher than at 15.0 °C for a HAD denitrification with methanol and spongy iron. It's not clear how water temperature influences the HAD process with pine bark and spongy iron from literature. The optimum temperature for BD is between 25 and 35 °C, and meanwhile denitrification can take place in the range 2–50 °C due to the bacteria capacity to survive in extreme environmental conditions (Karanasios et al. 2010). In the study reported here, a cold temperature (15 °C) was chosen based on the average ambient groundwater temperature in Shenyang, China. A medium (27.5 °C) and a warm (33.0 °C) temperatures were chosen to allow the growth and good performance of denitrifiers based on the optimum temperature (25–35 °C) (Karanasios et al. 2010). A comparison was made of denitrification rates among different water temperatures in Fig. 2.8a. The changes in $\text{NO}_2\text{-N}$ and $\text{NH}_4\text{-N}$ over reaction time at different water temperatures are shown in Fig. 2.8b, c.

Denitrification capacity increased as temperature increased (Fig. 2.8a). The denitrification rate at 33 °C was 3.19 times higher than at 15.0 °C and 1.59 times higher than at 27.5 °C, and the denitrification rate at 27.5 °C was 2.02 times higher than at 15.0 °C, when complete $\text{NO}_3\text{-N}$ removal was attained (Fig. 2.8a). These results demonstrated that the HAD process was temperature dependent and sensitive to change in temperature. These two conclusions were consistent with the literature describing cellulose-based HD (Volokita et al. 1996b; Robertson et al. 2000) in that HD was the most important $\text{NO}_3\text{-N}$ denitrification mechanism in the HAD process (see Sect. 2.3.2.5). High temperatures positively affect bacterial enzyme kinetics, specific growth rates and reaction rate constants. On the other hand, the higher water temperature, the easier the breakdown of the carbon chain

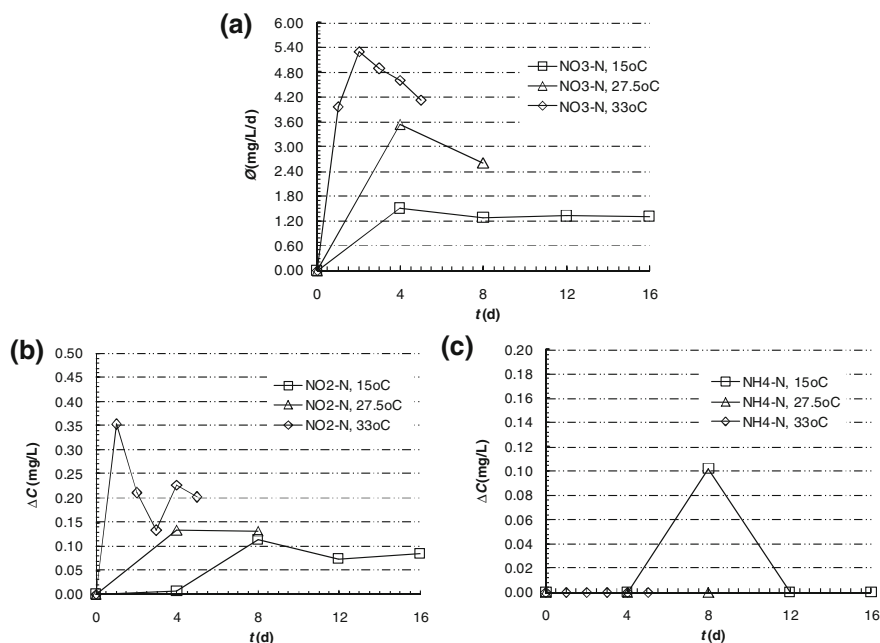


Fig. 2.8 Effect of water temperature on the performance of heterotrophic-autotrophic denitrification (HAD) over reaction time at 15, 27.5 and 33 °C: **a** denitrification rates; **b** variations of $\text{NO}_2\text{-N}$; **c** variations of $\text{NH}_4\text{-N}$

of pine bark by denitrifying microorganisms, and thus the greater available soluble fraction of organic carbon. Zhao et al. (2009) found the elevation of temperature in the range of 10–30 °C resulted in a comparable positive influence on HD.

The maximum concentration of $\text{NO}_2\text{-N}$ (0.35 mg/L $\text{NO}_2\text{-N}$) was recorded at 30 °C compared with 0.13 mg/L $\text{NO}_2\text{-N}$ at 27.5 °C and 0.11 mg/L $\text{NO}_2\text{-N}$ at 15 °C (Fig. 2.8b). Apparently, the activities of nitrate reductase at the warm temperature were slightly higher than those at the other two temperatures. No variations in $\text{NH}_4\text{-N}$ were found at 15, 27.5 and 30 °C respectively, except after 8 days at 15 °C (0.10 mg/L) (Fig. 2.8c). These results indicated that water temperature did not alter the HAD denitrification pathways.

2.3.3.3 Effect of High-Concentration Nitrate

As discussed in Sect. 2.3.2.5, at the normal initial $\text{NO}_3\text{-N}$ concentration of approximately 22.6 mg/L (around the acceptable China, India and Australia values for public health), the HAD was able to completely remove $\text{NO}_3\text{-N}$ within 16 days. However, from the literature it's not known how high-concentration nitrate affects the HAD performance. Based on a literature survey, a high $\text{NO}_3\text{-N}$ concentration of around 100 mg/L was set. A comparison was made of denitrification rates between

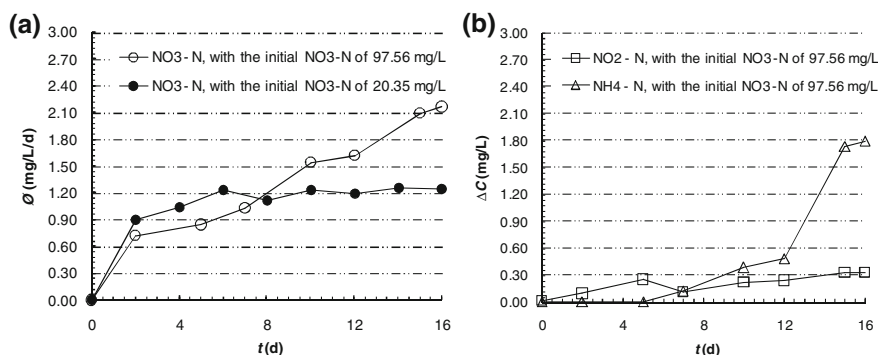


Fig. 2.9 Effect of high-concentration nitrate on the performance of heterotrophic-autotrophic denitrification (HAD) over reaction time: **a** denitrification rates at the initial $\text{NO}_3\text{-N}$ of 97.58 and 20.35 mg/L; **b** variations of $\text{NO}_2\text{-N}$; **c** variations of $\text{NH}_4\text{-N}$

the two $\text{NO}_3\text{-N}$ concentrations (20.35 and 97.58 mg/L) in Fig. 2.9a. The changes in $\text{NO}_2\text{-N}$ and $\text{NH}_4\text{-N}$ over reaction time at the high concentration (97.58 mg/L) are shown in Fig. 2.9b.

While the percent removal of $\text{NO}_3\text{-N}$ decreased to 35.53 % at day 16 at the high $\text{NO}_3\text{-N}$ of 97.58 mg/L (data not shown), the corresponding denitrification rate of 2.166 mg/L/d was much greater than that of 1.245 mg/L/d at the $\text{NO}_3\text{-N}$ of 20.35 mg/L (Fig. 2.9a). These results suggested the high $\text{NO}_3\text{-N}$ level was not seriously detrimental to the denitrifying biofilm and the $\text{NO}_3\text{-N}$ substrate inhibition did not occur. It is thus clear that the denitrifiers had strong shock resistance ability. In an earlier study, Della Rocca et al. (2006) concluded that denitrifiers in the iron powder/cotton system could resist $\text{NO}_3\text{-N}$ of 49.78 mg/L at hydraulic retention time (HRT) of approximate 4 days.

The variation in $\text{NO}_2\text{-N}$ reached to 0.34 mg/L at day 16 at the high concentration of $\text{NO}_3\text{-N}$ (Fig. 2.9b). To further increase percent removal as high as possible and decrease $\text{NO}_2\text{-N}$ as low as possible at the high $\text{NO}_3\text{-N}$ concentration incubation, increasing reaction time or decreasing mineral salts media volume might be required. It can be seen from Fig. 2.9b, starting from day 5, increasing amounts of $\text{NH}_4\text{-N}$ were generated at the high $\text{NO}_3\text{-N}$ concentration, with time. Since $\text{NH}_4\text{-N}$ was not produced at the normal initial $\text{NO}_3\text{-N}$ concentration (Fig. 2.2b), $\text{NH}_4\text{-N}$ production at the high concentration implied that the release rate of carbon was lower than its consumption rate, and then the HAD denitrification was limited without sufficient supply of soluble organic carbon. Other researchers have also reached a similar conclusion (Su and Puls 2007).

2.3.3.4 Effect of Nitrite

Nitrite, an intermediate product in BD, is a highly toxic compound, whose toxicity may inhibit microbial enzyme activities. Nitrite accumulation was often observed

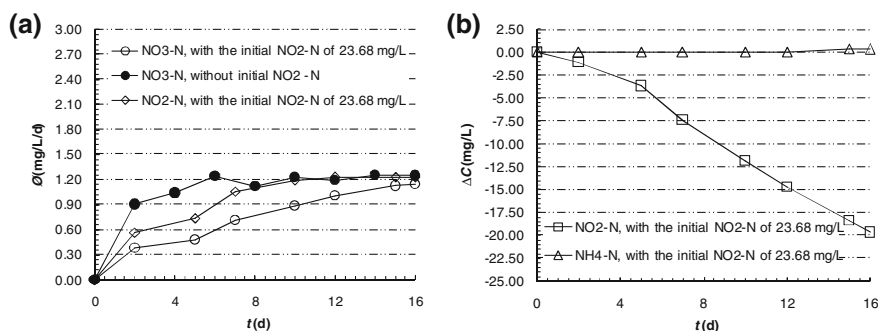


Fig. 2.10 Effect of nitrite on the performance of heterotrophic-autotrophic denitrification (HAD) over reaction time: **a** denitrification rates with and without the initial $\text{NO}_2\text{-N}$ of 23.68 mg/L; **b** variations of $\text{NO}_2\text{-N}$; **c** variations of $\text{NH}_4\text{-N}$

in laboratory experiments and field applications by numerous researchers in literature (Piñar and Ramos 1998; Chang et al. 1999; Gómez et al. 2000; Smith et al. 2001; Lee and Rittmann 2002; Della Rocca et al. 2006). Nitrite reductase is rather sensitive to environmental conditions which include carbon source types, temperature, H_2 pressures, DO, phosphate, toxic compounds (e.g. heavy metals, pesticides or their derivatives), electrical conductivity, and so forth. In addition, nitrite accumulation is strongly influenced by microbial species present. As discussed in Sect. 2.3.2.1, only $\leq 0.77\%$ of the $\text{NO}_3\text{-N}$ was converted to $\text{NO}_2\text{-N}$ at the initial $\text{NO}_3\text{-N}$ of 20.35 mg/L. Even so, it is desirable to investigate the effect of nitrite on the HAD performance due to common occurrence of nitrite accumulation and complexity of a groundwater environment. 23.68 mg/L $\text{NO}_2\text{-N}$ was added into a 2 L Schott bottle containing 20.48 mg/L $\text{NO}_3\text{-N}$. A comparison was made of $\text{NO}_3\text{-N}$ denitrification rates between incubations containing 20.48 mg/L $\text{NO}_3\text{-N}$ with and without initial $\text{NO}_2\text{-N}$ (Fig. 2.10a), and the change in $\text{NO}_2\text{-N}$ denitrification rates is shown in Fig. 2.10a. The changes in $\text{NO}_2\text{-N}$ and $\text{NH}_4\text{-N}$ over reaction time at the initial $\text{NO}_2\text{-N}$ of 23.68 mg/L are shown in Fig. 2.10b.

The $\text{NO}_3\text{-N}$ denitrification rates without initial $\text{NO}_2\text{-N}$ were constantly greater than with it, but the difference between them became smaller and smaller (Fig. 2.10a). These indicated that: (1) nitrate reductase was temporarily, but significantly, inhibited by the toxicity of nitrite at the beginning of incubation; (2) nitrate reductase gradually adapted itself to the highly toxic environment with the added $\text{NO}_2\text{-N}$ decreasing and reaction time increasing; (3) nitrate reductase refreshed its activities and syntheses at the end of incubation; and (4) there was a remarkable negative impact of nitrite on the HAD performance.

Unexpectedly, the $\text{NO}_2\text{-N}$ denitrification rates were higher than the $\text{NO}_3\text{-N}$ ones (Fig. 2.10a) and most of the added $\text{NO}_2\text{-N}$ (19.68 mg/L) was reduced during the 16 day period at the initial $\text{NO}_2\text{-N}$ of 23.68 mg/L (Fig. 2.10b). These suggested that: (1) the HAD was capable of simultaneously removing $\text{NO}_3\text{-N}$ and $\text{NO}_2\text{-N}$; (2) the activities and syntheses of nitrite reductase was enhanced due to adequate

nitrite substrate; (3) the activities of nitrite reductase were higher than those of nitrate reductase; (4) nitrite as an electron acceptor appeared to be superior to nitrate; and (5) soluble organic carbon released by pine bark met the needs of HD of nitrate and nitrite. Variations of $\text{NH}_4\text{-N}$ were not found before day 15, and ascended to levels between 0.32 and 0.33 mg/L afterwards at the initial $\text{NO}_2\text{-N}$ of 23.68 mg/L (Fig. 2.10b). Likewise, nitrite did not alter the HAD denitrification passways.

2.3.3.5 Effect of Ammonium

Although nitrate is most commonly associated with groundwater nitrogen contamination, combined contamination of nitrate and ammonium is also found, primarily due to the discharge of wastewater from sources such as septic systems and wastewater infiltration beds. It is possible that partial nitrate is reduced to ammonium by the HAD when denitrification conditions such as solution pH are changed. Ammonium is potentially toxic to aquatic organisms, particularly microorganisms, at high concentrations (Su and Puls 2007). Della Rocca et al. (2006) point out denitrifiers were inhibited by ammonium (14.62 mg/L as $\text{NH}_4\text{-N}$). For these reasons, the effect of ammonium on the HAD performance was investigated. 21.22 mg/L $\text{NH}_4\text{-N}$ was added into a 2L Schott bottle containing 21.46 mg/L $\text{NO}_3\text{-N}$. A comparison was made of denitrification rates between incubations containing 21.46 mg/L $\text{NO}_3\text{-N}$ with 21.22 mg $\text{NH}_4\text{-N/L}$ and without initial $\text{NH}_4\text{-N}$ (Fig. 2.11a). The changes in $\text{NO}_2\text{-N}$ and $\text{NH}_4\text{-N}$ over reaction time at the initial $\text{NH}_4\text{-N}$ of 21.22 mg/L are shown in Fig. 2.10b.

The denitrification rates of incubations without initial $\text{NH}_4\text{-N}$ were higher, in the first 7 days of incubation, than those containing $\text{NH}_4\text{-N}$ (Fig. 2.11a), indicating nitrate and nitrite reductase was significantly inhibited by ammonium at the beginning of incubation. In contrast the rates for incubations without $\text{NH}_4\text{-N}$ were

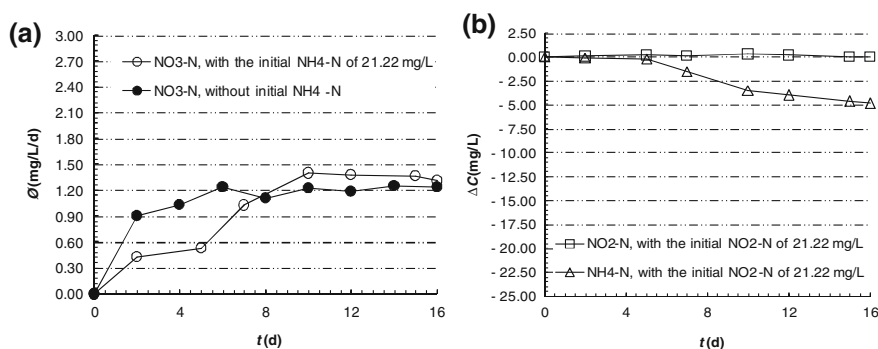
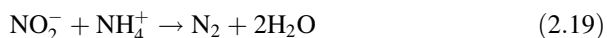
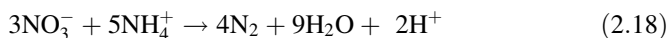


Fig. 2.11 Effect of ammonium on the performance of heterotrophic-autotrophic denitrification (HAD) over reaction time: **a** denitrification rates with and without the initial $\text{NH}_4\text{-N}$ of 21.22 mg/L; **b** variations of $\text{NO}_2\text{-N}$; **c** variations of $\text{NH}_4\text{-N}$

lower than those containing $\text{NH}_4\text{-N}$ after day 8 (Fig. 2.11a), indicating both reductase had adapted themselves to the high ammonium (21.22 mg/L as $\text{NH}_4\text{-N}$).

Variations of $\text{NO}_2\text{-N}$ were observed to vary between 0 and 0.32 mg/L (Fig. 2.11b), suggesting that complete denitrification was attained during the 16 day period in the presence of 21.22 mg/L $\text{NH}_4\text{-N}$. 4.85 mg/L $\text{NH}_4\text{-N}$ was lost from the Schott bottle with initial $\text{NH}_4\text{-N}$ in 16 days (Fig. 2.11b). Ammonia volatilization could not explain the $\text{NH}_4\text{-N}$ loss as the pH values (≤ 8.45) (data not shown) were not above 9. The mixed bacteria used possibly contained anaerobic ammonium-oxidizing bacteria. Under anaerobic conditions, anaerobic ammonium-oxidizing bacteria could directly oxidize ammonium to nitrogen gas with nitrate and nitrite as the electron acceptors in the presence of both nitrate and ammonium (Eqs. 2.18, and 2.19) (Su and Puls 2004).



Little added $\text{NH}_4\text{-N}$ (≤ 0.20 mg/L) was lost before day 5 (Fig. 2.11b). This behavior indicated adsorption onto pine bark and spongy iron was not a cause of the $\text{NH}_4\text{-N}$ loss (4.85 mg/L).

2.3.3.6 Effects of Coexistent Inorganic Anions

Successful implementation of an in situ groundwater remediation process requires a thorough understanding of the effect of geochemical composition on its behavior. It is expected that some coexistent inorganic anions (such as SO_4^{2-} , SO_3^{2-} and BO_3^{3-}) will affect the behavior of the HAD process. However, their effects have not been well understood. A comparison was made of denitrification rates in incubations with and without coexistent inorganic anions in Fig. 2.12a. The changes in $\text{NO}_2\text{-N}$ and $\text{NH}_4\text{-N}$ over reaction time in the presence of different coexistent anions are shown in Fig. 2.12b, c.

Sulfate, sulfite and borate all had strongly similar inhibitory effects on the HAD, from days 2 to 16 (Fig. 2.12a). It's possible that the toxicity of sulfate, sulfite (more toxic) and borate (125, 104 and 513 mg/L respectively) inhibited the microbial activity of heterotrophic and autotrophic denitrifiers. The blockage of reactive sites on the surface of Fe^0 and its corrosion products by specific adsorption of the inner-sphere complex forming ligands (sulfate, sulfite and borate) (Su and Puls 2004) might be responsible for the decreased nitrate CR by spongy iron in the HAD. The denitrification rates with sulfate, sulfite and borate at day 16 were lower than without them (Fig. 2.12a), suggesting $\text{NO}_3\text{-N}$ was not completely depleted due to the existence of sulfate, sulfite and borate. Robertson et al. (2007) reported sulfate and sulfite reduction consuming organic carbon (Eqs. 2.20, 2.21) did not occur until after $\text{NO}_3\text{-N}$ depletion was complete. It can be deduced that sulfate and sulfite reduction did not take place in the 16 days.

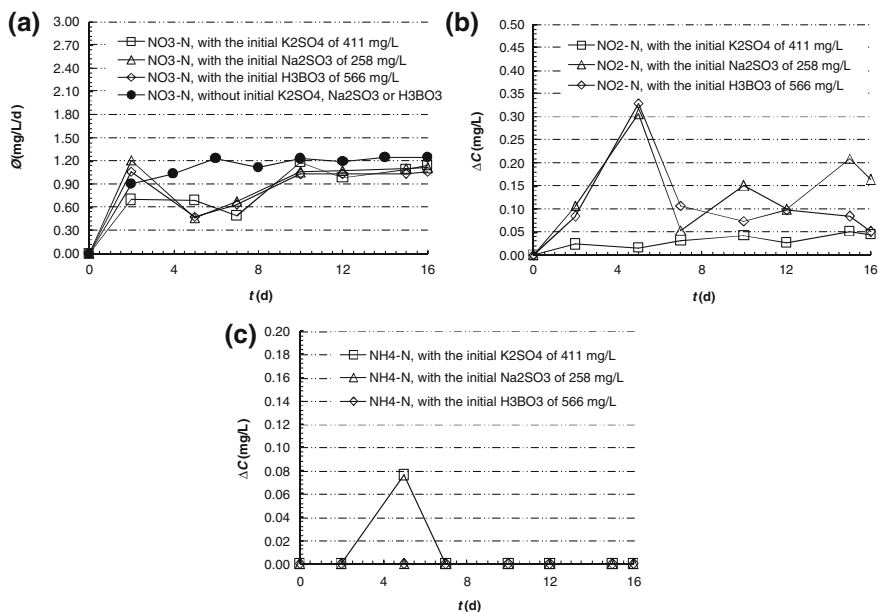
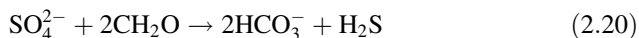


Fig. 2.12 Effects of coexistent inorganic anions on the performance of heterotrophic-autotrophic denitrification (HAD) over reaction time: **a** denitrification rates with the initial K₂SO₄ of 411 mg/L, the initial Na₂SO₃ of 258 mg/L, the initial H₃BO₃ of 566 mg/L, and without any of them; **b** variations of NO₂-N; **c** variations of NH₄-N



From trace to small amounts of NO₂-N (up to 0.33 mg/L) were detected in the presence of SO₄²⁻, SO₃²⁻ and BO₃³⁻ (Fig. 2.12b), which was consistent with our studies in the above sections. NH₄-N was lower than 0.1 mg/L (Fig. 2.12c), so it was not a predominant denitrification product in the presence of the three coexistent inorganic anions.

The presence of SO₄²⁻, SO₃²⁻ and BO₃³⁻ in groundwater will retard NO₃-N denitrification by the HAD process. Their negative effects should thus be taken into consideration when a PRB is designed for field application. Consequently, a greater mass of spongy iron and/or pine bark may be needed for sites where SO₄²⁻, SO₃²⁻ and BO₃³⁻ are significant co-contaminants with nitrate in groundwater.

2.3.4 Long-term Performance of the Heterotrophic-Autotrophic Denitrification Process

To investigate the long-term performance of HAD, a HAD bottle was kept in incubation continuously for 105 days. When both $\text{NO}_3\text{-N}$ and $\text{NO}_2\text{-N}$ concentrations were no longer detected, $\text{NO}_3\text{-N}$ was added to return the solutions to final concentrations of between 20.77–23.62 mg/L, however, neither pine bark nor spongy iron was supplemented.

The experimental results for the changes in $\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$, $\text{NH}_4\text{-N}$ and denitrification rate are illustrated in Fig. 2.13a–c. It was observed that complete $\text{NO}_3\text{-N}$ removal was achieved rapidly in approximately 16 days after each $\text{NO}_3\text{-N}$ addition (Fig. 2.13a), and almost equal denitrification rates (1.233–1.397 mg/L/d) were obtained when $\text{NO}_3\text{-N}$ was completely removed at days 15, 34, 53, 70, 87, 105 (Fig. 2.13c). All the evidence from the present study indicated pine bark could provide sufficient organic carbon, spongy iron could steadily remove DO, and microbial activity maintained relatively constant during 105 days. Pine bark, rich in cellulose fibers, released organic carbon by means of cellulose-degrading bacteria, and simultaneously heterotrophic denitrifiers consumed organic carbon to finish their metabolism. Apparently, the net result of these two different processes was sufficient supply of organic carbon. Ovez et al. (2006) showed that the time

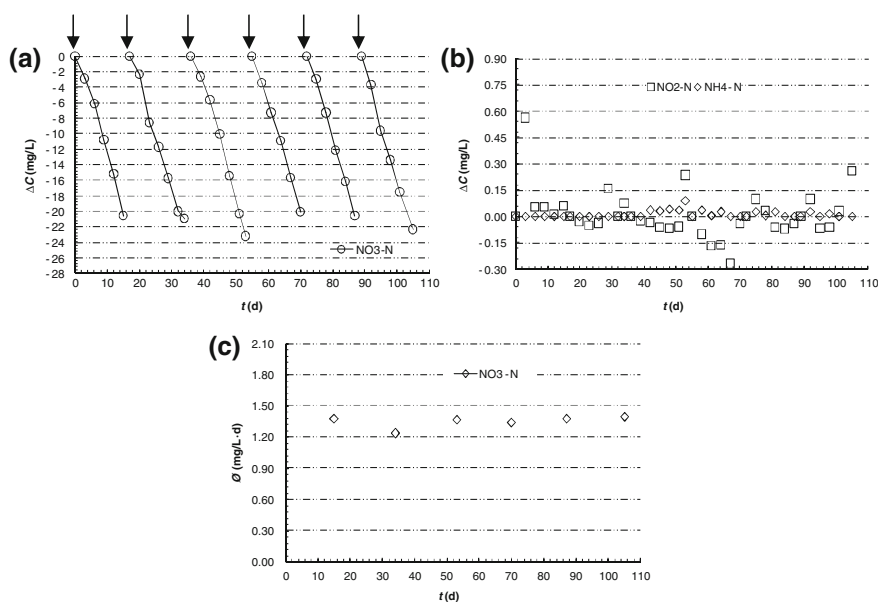


Fig. 2.13 Long-term performance of heterotrophic-autotrophic denitrification (HAD) over reaction time at the initial $\text{NO}_3\text{-N}$ of 20.77–23.62 mg/L: **a** variations of $\text{NO}_3\text{-N}$; **b** variations of $\text{NO}_2\text{-N}$ and $\text{NH}_4\text{-N}$; **c** variations of denitrification rates. Arrows indicate the addition of $\text{NO}_3\text{-N}$ to the Schott bottle

(<10 days) for 100 % $\text{NO}_3\text{-N}$ removals was always shorter in liquorice and giant reed reactors at the beginning of incubation and both of the reactors provided higher denitrification rates for a long-time (70 days), when compared to the HAD bottle in this study. Nevertheless, denitrification rates for liquorice and giant reed declined as the process continued because of the reduction in the usable organic carbon content of the substances. Volokita et al. (1996b) found that a time-dependent decay in denitrification rate was noticeable after several months of operation when newspaper served as a sole carbon source. Of the four cellulose-based organic substances, pine bark was capable of providing the most steady denitrification rates for 3.5 months. Pine bark was superior to liquorice, giant reed and newspaper. Only at day 3 was a significant, high and transient $\text{NO}_2\text{-N}$ concentration (0.56 mg/L) generated (Fig. 2.13b). $\text{NO}_2\text{-N}$ concentrations of > 0.3 mg/L generated represented 2.63 % of the 38 water samples collected. These phenomena suggested that no $\text{NO}_2\text{-N}$ accumulated during the long-term incubation (105 days). A maximum of $\text{NH}_4\text{-N}$ generated (0.09 mg/L) was observed at day 53 (Fig. 2.13b), which only accounted for 0.4 % of $\text{NO}_3\text{-N}$ removed. This indicated that $\text{NO}_3\text{-N}$ was not converted into $\text{NH}_4\text{-N}$ during the long-term incubation.

2.3.5 Kinetics of $\text{NO}_3\text{-N}$ Denitrification by the Heterotrophic-Autotrophic Denitrification Process

To study the kinetics of $\text{NO}_3\text{-N}$ denitrification by the HAD process, three different initial $\text{NO}_3\text{-N}$ concentrations (44.20, 20.35 and 11.15 mg/L) were incubated and the changes in $\text{NO}_3\text{-N}$ over reaction time were monitored and compared with an incubation containing 20.35 mg/L $\text{NO}_3\text{-N}$ at a DO concentration of 4.1 mg/L in the absence of pine bark, spongy iron and mixed bacteria (Fig. 2.14a-c).

From Fig. 2.14a-c, about 32, 16 and 8 days were needed to completely remove nitrate at the initial $\text{NO}_3\text{-N}$ concentrations of 44.20, 20.35 and 11.15 mg/L respectively. The higher the initial $\text{NO}_3\text{-N}$ concentration was, increased the time for complete nitrate removal. It can also be seen from Fig. 2.14a-c that $\text{NO}_3\text{-N}$ decreased with increasing reaction time and the change in trends of $\text{NO}_3\text{-N}$ concentration for all the three concentrations were similar. The results for a blank solution containing $\text{NO}_3\text{-N}$ (20.35 mg/L) but no spongy iron, pine bark and mixed bacteria are included in Fig. 2.14b and show losses of $\text{NO}_3\text{-N}$ to be insignificant in the absence of pine bark, spongy iron and mixed bacteria.

The above observation implied that $\text{NO}_3\text{-N}$ denitrification under these conditions would comply with zero order reactions with respect to $\text{NO}_3\text{-N}$ concentration. A zero order reaction with respect to $\text{NO}_3\text{-N}$ concentration can be described by a differential equation as given in Eq. 2.22.

$$\frac{-d[\text{NO}_3^-]}{dt} = K \quad (2.22)$$

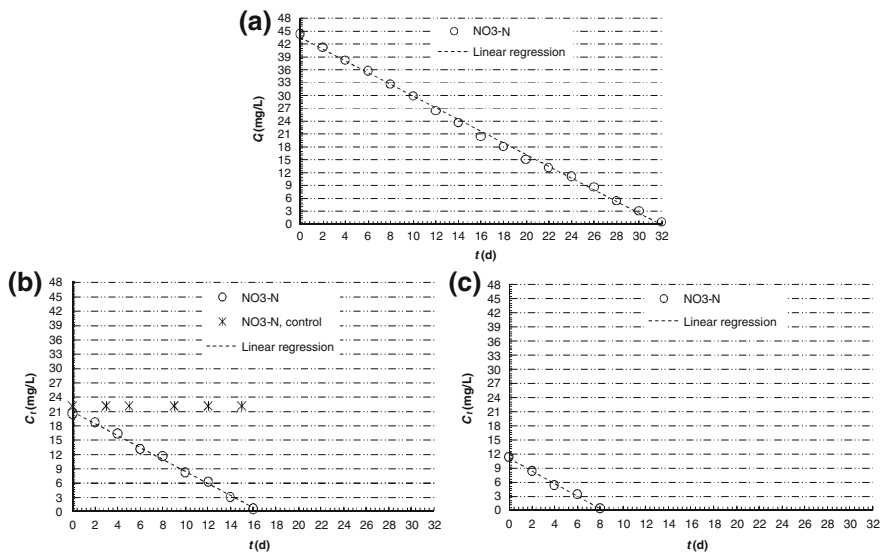


Fig. 2.14 Kinetics of $\text{NO}_3\text{-N}$ denitrification by heterotrophic-autotrophic denitrification (HAD): **a** the initial $\text{NO}_3\text{-N}$ of 44.20 mg/L; **b** the initial $\text{NO}_3\text{-N}$ of 20.35 mg/L; **c** the initial $\text{NO}_3\text{-N}$ of 11.15 mg/L. The control solution contained $\text{NO}_3\text{-N}$ of 20.35 mg/L and DO of 4.1 mg/L in the absence of granulated spongy iron, pine bark and mixed bacteria

If Eq. 2.22 is integrated it gives an equation often called the integrated zero order rate law as given in Eq. 2.23.

$$[\text{NO}_3^-] = -Kt + [\text{NO}_3^-]_0 \quad (2.23)$$

where K is the zero order reaction rate constant, whose negative is the observed slope of the regression line from plotting $\text{NO}_3\text{-N}$ concentration against reaction time.

A linear regression of the zero order kinetic Eq. 2.22 was performed for each data set (Fig. 2.14a–c). The linear regression model was statistically significant ($R^2 > 0.9949$) in all cases (Eqs. 2.24, 2.25, and 2.26) providing strong evidence that the HAD denitrification was zero order with respect to $\text{NO}_3\text{-N}$ concentration, which suggested that there were sufficient denitrifiers and (organic) carbon sources in the HAD process.

At the initial $\text{NO}_3\text{-N}$ of ~ 45.2 mg/L:

$$C(\text{mg/L}) = -1.3654t(\text{d}) + 43.3647 (R^2 = 0.9978) \quad (2.24)$$

At the initial $\text{NO}_3\text{-N}$ of ~ 22.6 mg/L:

$$C(\text{mg/L}) = -1.2676t(\text{d}) + 20.8864 (R^2 = 0.9965) \quad (2.25)$$

At the initial $\text{NO}_3\text{-N}$ of ~ 11.3 mg/L:

$$C(\text{mg/L}) = -1.3333t(\text{d}) + 10.9350 (R^2 = 0.9949) \quad (2.26)$$

The values of K ranged from 1.2676 to 1.3654 mg/L/d (Eqs. 2.24, 2.25, and 2.26) with a mean of 1.3220 mg/L/d. Little variation in K among the three different initial $\text{NO}_3\text{-N}$ concentrations indicated that altering the concentration of initial $\text{NO}_3\text{-N}$ did not alter the rate of reaction, i.e., the amount of $\text{NO}_3\text{-N}$ removed was positively proportional to the reaction time. It is therefore concluded that under the conditions of the experiment, the HAD denitrification was zero order with respect to $\text{NO}_3\text{-N}$ concentration and the reaction rate constant (K) was independent of the initial $\text{NO}_3\text{-N}$ concentrations within the applied concentration range.

The K , intimately related to environmental conditions (water temperature, pH, DO, etc.) and experimental parameters (denitrifier concentration, masses of spongy iron and pine bark, etc.), had great potential application prospects in designing the HAD process. For example, if influent and effluent $\text{NO}_3\text{-N}$ concentrations are given, the hydraulic retention time will be determined most easily with the K .

2.3.6 Mechanisms for Dissolved Oxygen Removal and $\text{NO}_3\text{-N}$ Denitrification in the Heterotrophic-Autotrophic Denitrification Process

The main mechanisms for DO removal and $\text{NO}_3\text{-N}$ denitrification in the HAD process are illustrated in Fig. 2.15. Cellulose fiber-rich pine bark released organic carbon into mineral salts media by cellulose-degrading bacteria to support biological deoxygenation and HD. Spongy iron and aerobic heterotrophs rapidly removed DO respectively (Eqs. 2.8, 2.9), creating anaerobic environments to favor BD, chemical nitrate reduction and anaerobic iron corrosion. Simultaneously, biological deoxygenation produced carbon dioxide, favoring AD. 0.142 d and 1.905 d were needed to completely remove DO by granulated spongy iron-based CR and aerobic heterotrophs respectively. Granulated spongy iron-based CR played a dominant role in DO removal. Under anaerobic conditions, the corrosion of spongy iron was coupled with the reduction of water-derived protons, forming cathodic hydrogen (Eq. 2.13) to favor AD. At the same time, bacteria maybe took advantage of hydrogenase enzymes to accelerate the anodic dissolution of iron and hydrogen production to some extent when H^+ or H_2S was generated (Eqs. 2.18, 2.20, and 2.21).

Cathodic hydrogen and inorganic carbon (CO_2 and HCO_3^-) allowed autotrophic denitrifiers to reduce nitrate to innocuous nitrogen gas (Eq. 2.17). 4.5–8.7 % of the initial $\text{NO}_3\text{-N}$ was denitrified by AD in 16 days. At neutral or weak alkaline pH values, spongy iron chemically reduced nitrate. 6.2 % of the initial $\text{NO}_3\text{-N}$ was reduced by granulated spongy iron-based CR in 16 days. Possible pathways of chemical nitrate reduction are shown in Eqs. 2.10, 2.11, and 2.12. Organic carbon released by pine bark acted both as an electron donor and as carbon and energy source. Heterotrophic denitrifiers utilized the organic carbon to reduce

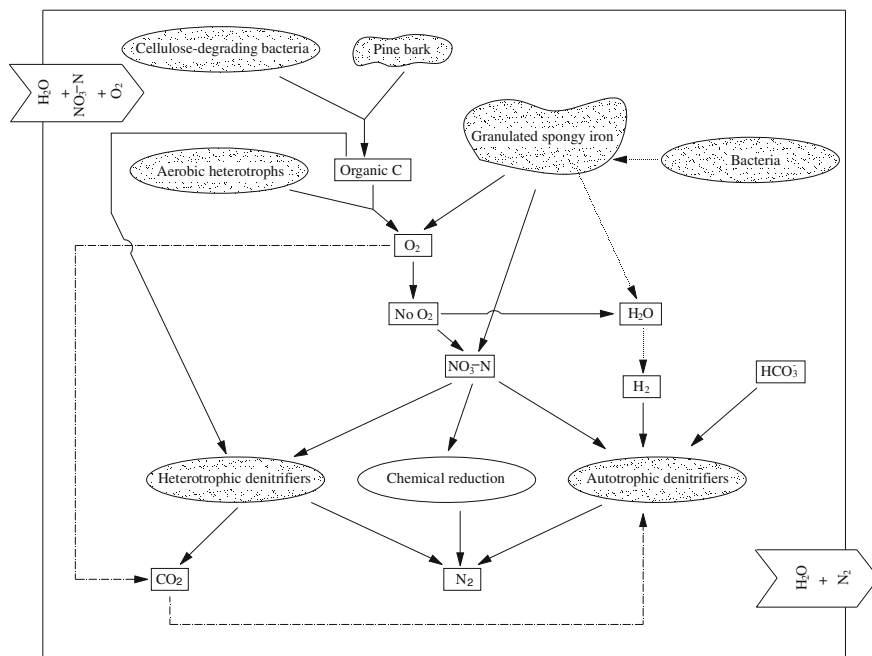


Fig. 2.15 The mechanisms for dissolved oxygen (DO) removal and $NO_3^- - N$ denitrification occurring in the heterotrophic-autotrophic denitrification (HAD) process

nitrate and meanwhile generated carbon dioxide, which further favored AD (Eq. 2.14). 83.1 % of the initial $NO_3^- - N$ was removed by HD in 16 days. HD was the most important mechanism for $NO_3^- - N$ denitrification in the HAD. Pine bark providing a large amount of surface area served as a solid-carrier for biofilm formation. Generally, spongy iron has a porous internal structure and large surface area. The spongy iron used having a Brunauer-Emmett-Teller (BET) specific surface of $0.49 \text{ m}^2/\text{g}$ served as another solid-carrier.

In short, the combination of cellulose-degrading bacteria, aerobic heterotrophs, granulated spongy iron-based CR, anaerobic iron corrosion, heterotrophic and autotrophic denitrifiers contributed to DO removal and $NO_3^- - N$ denitrification in the HAD process.

2.3.7 Comparison of Denitrification Capacity Among Different Denitrification Approaches

Based on a literature survey, the comparison of maximum $NO_3^- - N$ percent removal (MNPR) (%) and maximum denitrification rate (MDR) (mg/L/d) among different denitrification approaches is presented in Table 2.3. In spite of the similar MNPRs (≥ 96.3 %) for all the processes, the differences in denitrification rate among these

Table 2.3 Comparison of denitrification capacity among different denitrification processes

Material	System description	Maximum NO ₃ -N percent removal (%)	Maximum denitrification rate (mg/L/d)	Water temperature (°C)	Denitrification mechanism	Reference
Fe powder (10 µm)	Batch study	100	780.48 ^a	No ^b	CR	Liao et al. (2003)
Fe powder (100–200 mesh)	Batch study	~100	1290.86 ^a	22	CR	Siantar et al. (1996)
Nanoscale Cu/Fe (5.0 %, w/w)	Batch study	100	280 ^a	25	CR	Liou et al. (2005)
Iron grains (~0.5 mm)	Batch study	~100	1509.6 ^a	No ^b	CR	Huang and Zhang (2004)
Acetate	Batch study	~100	0.55 ^a	No ^b	HD	Devlin et al. (2000)
Cotton burl compost	Batch study	~100	6.67 ^a	23 ± 1	HD	Su and Puls. (2007)
Cornstalks	Batch study	No ^b	13.54 ± 1.74 ^a	20 ± 2	HD	Greenan et al. (2006)
Cardboard fibers	Batch study	No ^b	5.18 ± 0.11 ^a	20 ± 2	HD	Greenan et al. (2006)
Wood chips	Batch study	No ^b	2.19 ± 0.20 ^a	20 ± 2	HD	Greenan et al. (2006)
Wood chips amended with soybean oil	Batch study	No ^b	3.75 ± 0.09 ^a	20 ± 2	HD	Greenan et al. (2006)
Pure H ₂	Batch study	96.3 ^a	7.30 ^a	30	AD	Smith et al. (1994)
Pure H ₂ + CO ₂	Batch study	~100	75.98	30 ± 1	AD	Vasiliadou et al. (2006)
Cathodic hydrogen + HCO ₃ ⁻ +nanoscale Fe	Batch study	~100	46.67 ^a	25	AD	Shin and Cha (2008)
Elemental sulfur + limestone	Batch study	100	No ^b	22	AD	Zhang and Lampe (1999)
Methanol + spongy Fe (0.075–0.425 mm)	Batch study	~100	4.2	27.5	HD + AD + CR	Huang et al. (2012)
Pine bark + spongy Fe (0.075–0.425 mm)	Batch study	~100	1.25	15	HD + AD + CR	This study

^a Estimated or calculated based on the data given in the article; ^b No data

processes were very evident (Table 2.3). This is the consequence of the differences in denitrification mechanism and experimental condition. Even though the MDRs for the single CR processes were very high (Table 2.3), but the main problems of Fe^0 -based CR were the release of ammonium and ferrous ions, and a requirement for a low acidic pH (or a pH buffer or a bimetallic catalyst), which limit their applicability. Although the MDRs for the single hydrogenotrophic denitrification processes were ideal (Table 2.3) and hydrogenotrophic denitrification could remove nitrate more cleanly with the production of fewer residual organics compared to HD, the explosion and safety concerns and the low solubility associated with pure H_2 have prevented widespread acceptance of hydrogenotrophic denitrification as a remediation technology. The agglomeration or clumping of nanoscale iron particles to each other or to the substrate surface has impeded field applications of nanoscale iron-based CR or hydrogenotrophic denitrification. The sulfur-autotrophic denitrification process has showed some drawbacks: the low solubility of sulfur compounds; the production of sulfate and nitrite; the use of limestone for pH adjustment (Zhang and Lampe 1999; Karanasios et al. 2010). These drawbacks limit its applicability. The MDR for the HAD process reported in this study was lower than the values given in Table 2.3 for the HD processes (except the acetate-supported HD process). Nevertheless, the HD processes had low or even no capacities of deoxygenation. When encountering DO, these processes would weaken or even lose their denitrification performances. In contrast, the HAD process was able to effectively remove DO (Fig. 2.1), which could maintain its stable denitrification rate. The MDR for the HAD process supported by methanol and spongy iron was acceptable, but maintaining contact between methanol and spongy iron would require a large amount of methanol in field applications in that methanol is water soluble and mobile, and spongy iron is neither. More important, methanol is considerably more toxic than nitrate, which makes its use restricted to the treatment of wastewater. Both disadvantages have hindered field applications of methanol-supported HD and HAD for in situ groundwater remediation.

2.4 Conclusions

Based on the research findings the main conclusions were as follows.

1. The HAD process's deoxygenation capacity depended on granulated spongy iron via CR and aerobic heterotrophs via aerobic respiration to remove DO, but granulated spongy iron played a dominant role in deoxygenation. 0.121 d, 0.142 d and 1.905 d were needed to completely remove DO by HAD, granulated spongy iron and mixed bacteria respectively.
2. The HAD process was potentially a feasible and effective approach for groundwater remediation. 19.92 mg/L $\text{NO}_3\text{-N}$ was removed during the 16 day period. No $\text{NO}_2\text{-N}$ accumulation occurred. No $\text{NH}_4\text{-N}$ variations were found.
3. There existed symbiotic, synergistic and promotive effects of CR, HD and AD within the HAD. CR, HD and AD all contributed to the overall removal of

NO₃-N, but HD was the most important NO₃-N denitrification mechanism. After 16 days, NO₃-N removal was approximately 100, 6.2, 83.1, 4.5 % by HAD, CR, HD, AD, respectively.

4. The HAD denitrification was zero order with respect to NO₃-N concentration, and the reaction rate constant (*K*) of 1.3220 mg/L/d was independent of the initial NO₃-N concentrations within the applied concentration range (11.15–44.20 mg/L).
5. There was a minor effect of pine bark amount on the HAD denitrification from days 0 to 4, whereas there was a noticeable effect from days 4 to 16.
6. Denitrification capacity increased as temperature increased. The denitrification rate at 33 °C was 3.19 times higher than at 15.0 °C and 1.59 times higher than at 27.5 °C.
7. The high nitrate level (97.58 mg/L as NO₃-N) was not seriously detrimental to the denitrifying biofilm. The denitrifiers had strong shock resistance ability. The denitrification rate of 2.17 mg/L/d at the high NO₃-N concentration was much greater than that of 1.25 mg/L/d at the normal NO₃-N concentration (20.35 mg/L).
8. Nitrite (23.68 mg/L as NO₃-N) had a remarkable negative impact on the HAD performance. Nitrate reductase was temporarily, but significantly, inhibited by the toxicity of nitrite at the beginning of incubation, and then it refreshed its activities and syntheses at the end.
9. High ammonium (21.22 mg/L NH₄-N) significantly inhibited nitrate and nitrite reductase at the beginning of incubation. Afterwards both reductase adapted themselves to the high ammonium.
10. Coexistent inorganic anions (125 mg/L sulfate, 104 mg/L sulfite and 513 mg/L borate) all had strongly similar inhibitory effects on the HAD performance, from days 2 to 16. NO₃-N was not completely depleted at day 16 due to their existence.
11. The HAD approach was capable of providing steady denitrification rate (1.233–1.397 mg/L/d for 3.5 months. Pine bark could provide sufficient organic carbon, spongy iron could steadily remove DO, and microbial activity maintained relatively constant. Almost no NO₂-N accumulated and no NH₄-N was generated during the long-term incubation.

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