Effect of Bicarbonate on the Performance of Hydrogen-based Denitrification at Different Hydraulic Retention Times

SUPHATCHAI RUJAKOM*, KENTA SHINODA1, TIPPAWAN SINGHOPON1, MAI NAKANO1, TATSURU KAMEI2, and FUTABA KAZAMA3

1 Integrated Graduate School of Medicine, Engineering and Agricultural Sciences, University of Yamanashi/4-4-37 Takeda, Kofu, Yamanashi 400-8510, Japan
2 Department of Life and Environmental Sciences, University of Yamanashi /4-4-37 Takeda, Kofu, Yamanashi 400-8510, Japan
3 Interdisciplinary Research Centre for River Basin Environment, University of Yamanashi /4-4-37 Takeda, Kofu, Yamanashi 400-8510, Japan

Abstract

Bicarbonate (HCO$_3^-$) can be used as an inorganic carbon source for hydrogen-based denitrification (HD). Since HCO$_3^-$ is considered to accelerate the NO$_2^-$ reduction rate, this study is attempted to minimize the hydraulic retention time (HRT) of HD systems using varied amounts of HCO$_3^-$: deficient, moderate, and abundant amounts. The results implied that a low NO$_2^-$ amount was removed at unsuitably short HRTs, resulting in poor HD efficiency despite being supplemented with abundant HCO$_3^-$ amounts. HCO$_3^-$ assisted in rapidly acclimatizing the bacteria having nirS gene, causing higher NO$_2^-$ reduction rate and aided in changing the bacterial communities. Thauera spp. were the most dominant bacteria in abundant HCO$_3^-$ conditions, achieving high HD efficiency at 8 to 24 h HRT whereas satisfactory efficiency was achieved in the deficient and moderate HCO$_3^-$ amount-systems through the collaboration of Rhodocyclaceae, Alcaligenaceae, and Xanthomonadaceae as predominant bacteria in the community. A strong correlation between the abundance of nirS gene and Thauera spp. was also found. The findings in this study revealed the importance of using HCO$_3^-$ for the enrichment of H$_2$-oxidizing denitrifiers containing nirS gene in order to reduce NO$_2^-$ accumulation to enhance the HD efficiency.

Key words: Hydrogen-based denitrification; Hydraulic retention time; Bicarbonate; Functional gene; Bacterial community

INTRODUCTION

Nitrate-contaminated groundwater has been found in both developed and developing countries such as Serbia$^1$, Spain$^2$, Afghanistan$^3$, India$^4$, Vietnam$^5$, and Myanmar$^6$. The high levels of nitrate (NO$_3^-$) and nitrite (NO$_2^-$) contamination in drinking water sources pose a serious risk to human health, and can cause serious disorders such as methemoglobinemia, cancer, and thyroid diseases$^7$, as observed in some of the aforementioned countries, where people use groundwater in their daily lives. Biological denitrification has been widely practiced as a remediation process for NO$_3^-$-contaminated groundwater. The reduction of NO$_3^-$ to N$_2$ gas is catalyzed by several denitrification-functional genes present in the bacteria$^{8-13}$ through four reduction steps as described in Eq. (1)$^{14}$.

\[ \text{NO}_3^- \xrightarrow{\text{narG}} \text{NO}_2^- \xrightarrow{\text{nirK}} \text{NO} \xrightarrow{\text{norB}} \text{N}_2O \xrightarrow{\text{nosZ}} \text{N}_2 \] (1)
Biological denitrification is classified into two types, based on the group of bacteria functioning in the process: heterotrophic and autotrophic denitrification. Although a high nitrogen removal rate can be achieved through heterotrophic denitrification, a post-treatment of the effluent is required to deal with the excess of organic carbon, which was supplied as electron donor and energy source\(^1\). For autotrophic denitrification, an electron donor is required in the form of sulfur compounds, ferrous iron, arsenite, manganese, or H\(_2\) gas\(^2\), together with inorganic carbon. CO\(_2\) gas and HCO\(_3^-\) have often been used as an inorganic carbon source for the bacteria in autotrophic denitrification system\(^3, 4, 5\). Ghafari et al.\(^4\) found that faster acclimatization of autotrophic denitrification system was achieved when HCO\(_3^-\) was used as inorganic carbon compared with CO\(_2\) gas. Hydrogen-based autotrophic denitrification using H\(_2\) as an electron donor is called hydrogenotrophic denitrification (HD). HD has several advantages over the use of other donors, for the following reasons: the by-products are harmless, H\(_2\) gas is a non-toxic electron donor, and no post-treatment is required to deal with an excess of organic carbon\(^6\). Moreover, the denitrification performance was found to be efficient as indicated in previous studies\(^7-10\).

\[
\begin{align*}
NO_3^- + 2.892H_2 + 0.171HCO_3^- &\rightarrow 0.483N_2 + 0.034C_5H_7O_2N + 2.268H_2O + 1.171OH^- \\
\end{align*}
\]

Eq. (2) expresses the HD reaction, showing the stoichiometric amount of HCO\(_3^-\). Furthermore, intermediate NO\(_2^-\) is produced during the denitrification process. As the accumulation of NO\(_2^-\) in the denitrification system is undesirable, it is crucial to increase the NO\(_2^-\) removal rate to be equal to or to exceed the NO\(_3^-\) reduction rate. This helps in achieving greater denitrification efficiency. By using HCO\(_3^-\) as an inorganic carbon source under a controlled pH of 7.0 to 8.2, the growth rate of the denitrifying bacteria is increased\(^1\), which in turn increases the removal rates of NO\(_3^-\) and NO\(_2^-\). This results not only in better control of NO\(_3^-\) accumulation, which is a common phenomenon in denitrification processes but also in the shortening the operation time of the process. The purpose of this study was to investigate the effect of HCO\(_3^-\) on the efficiency of HD system operated at several hydraulic retention times (HRTs), in order to gain a better insight into the role of varied HCO\(_3^-\) amounts on H\(_2\)-oxidizing denitrifiers in HD system as no study on the HD bacterial communities affected by HCO\(_3^-\) amount has been reported. The findings of this study can be used as a strategy on acclimatizing the HD bacterial communities to enhance the system efficiency while minimizing HRT.

**MATERIALS AND METHODS**

**Experimental method** In this study, three suites of 1 L volume working bottles were used as sequencing batch reactors (SBRs): S1, S2, and S3 (Fig. 1). Each reactor was tightly closed with a rubber stopper equipped with straight tube connectors for discarding effluent, filling influent, and sparging H\(_2\) gas. As an inoculum, mixed liquor sludge of 1.4 ± 0.1 g VSS was used. This sludge was enriched over a year under identical conditions as mentioned in the previous study\(^2\). The temperature was controlled by placing the reactors in a water tub, heated by a heating stick. To avoid the limitation of H\(_2\) for the HD system, the reactors were also continuously sparged with H\(_2\) gas throughout the operation using an HG260 generator (GL Sciences Inc., Japan) at 20 mL/min as 5 mL/min H\(_2\) sparging rate was found to be optimal\(^1\). Table 1 summarizes the operating conditions of each reactor used in the experiment, where the reactors S1, S2, and S3 represent the conditions for the deficient, moderate, and abundant HCO\(_3^-\) amounts, respectively. The operating approach for the reactors was adapted from Ghafari et al.\(^2\), following four operating steps: reaction, settlement, discarding, and refilling. These steps were performed onto each SBR and were completed within HRTs. Fifteen additional minutes were needed for the biological sludge to settle completely, which is a requirement to retain the sludge inside the reactors for as long as possible. As the study started with 24 h HRT and then changed to 12, 8, and 4 h HRTs,
two peristaltic pumps and three timers were used to conduct the four operation steps mentioned above. Two timers were used to prompt the discarding pump to start working prior to the filling pump, in order to control the varied HRTs; and the third timer was used to stop the magnetic stirrer used to control sludge settlement, before discarding the supernatant as an effluent for each operating cycle. The mixed liquor sludge was inoculated into each reactor, following which synthetic water was added to fill up to 1 L. Prior to the inoculation, the sludge was thoroughly washed with tap water. All the reactors in this study were carried out in the freed pH conditions. The effluent, i.e., the treated water, was sampled each day from each reactor and was preserved at a temperature of −4 °C for the NO₃, NO₂, and HCO₃ analyses. The pH of the treated samples was measured in the moment of collecting, using a pH meter (Horiba Scientific, Japan). On the last operating day of each HRT, the sludge samples were collected from each reactor for DNA extraction to be used in bacterial community analysis through next-generation sequencing (NGS) analysis and quantification of denitrification-functional genes based on quantitative polymerase chain reaction (qPCR).

**Synthetic feeding water preparation**

Synthetic feeding water was synthesized for adding to the mixed cultures of denitrifiers that were supplied with different amounts of HCO₃ as inorganic carbon source. This synthetic feeding water was prepared using
tap water mixed with NaNO₃ to obtain an initial NO₃⁻ concentration of 40 mgN/L. Based on the results obtained from our previous study²⁵, using 200 mg HCO₃⁻/L (moderate amount) could be sufficient to remove 40 mg NO₃⁻-N/L as the required stoichiometric HCO₃⁻ amount is 30 mg approximately, estimated based on Eq. (2) and the abundant HCO₃⁻ amount (5000 mg/L) was able to accelerate NO₂⁻ reduction rate whereas nitrogen removal was also observed when no additional HCO₃⁻ was supplemented. In the present study, selected HCO₃⁻ concentrations of 0, 200, and 5000 mg/L, using NaHCO₃, were then added to represent the conditions of deficient, moderate, and abundant HCO₃⁻ amounts, respectively. The chemical compositions added for synthesizing the feeding water are described in Table 1.

**Water sample analysis** NO₃⁻, NO₂⁻, and HCO₃⁻ concentrations were determined following the standard methods for the analysis of water and wastewater²⁷. The concentrations of NO₃⁻ and NO₂⁻ were analyzed colorimetrically using a UV-1800 spectrophotometer (Shimadzu, Japan), following the methods described in sections 4500-NO₂⁻ B and 4500-NO₃⁻ B²⁷. As this study is aimed to investigate the effect of varied HCO₃⁻ amounts on denitrification behavior, HCO₃⁻ was determined through titration for alkalinity analysis in 2320-Alkalinity B²⁷.

**Bacterial community analysis** An approximate 100 mg sludge sample taken from different operating conditions was used to extract total DNA using a FastDNA® Spin Kit for Soil (MP Biomedicals, USA) according to the provided protocol. Subsequently, DNA concentration was determined by Nanodrop using Quantum™ Fluorometer (Promega Corp., USA) prior to the commercial NGS analysis service (FASMAC Co., Ltd., Japan). The NGS analysis was carried out based on the analysis of partial region of bacterial 16S rRNA amplified by Univ-515F and Univ-806R primers (Table 2). The MiSeq platform was used to obtain the amplified metagenomic sequences. The raw sequence data was then taxonomically classified using QIIME software version 1.9.0 in order to acquire the operational taxonomic units (OTUs), clustered at 97% similarity, and the bacterial relative abundance²⁸. A heatmap presenting the abundance in bacterial community among the different operating conditions was subsequently created through R software version 3.6.0 using Heatplus (version 2.30.0)²⁹, vegan (version 2.5.5)³⁰ packages following the instructions mentioned by Wongkiew et al.³¹.

**Denitrification-functional genes quantification** Extracted DNA samples from the sludge samples were also used to quantify the number of each functional gene (narG, nirK, nirS, norB, and nosZ) present in the sludge obtained from different operating conditions to observe whether the denitrifying processes occurred in the system as a result of HCO₃⁻ amounts and HRTs. Furthermore, 16S rRNA gene was determined using Univ-

| Functional genes | Primers | Sequence (5’ - 3’) |
|------------------|---------|-------------------|
| 16S rRNA         | Univ-515F⁸ | GTG YCA GCM GCC GCG GTA A |
|                  | Univ-806R⁸ | GGA CTA CNV GGG TWT CTA AT |
| narG             | narG-1960m2f⁹ | TAY GTS GGG CAG GAR AAA CTG |
|                  | narG-2050m2r⁹ | CGT AGA AGA AGC TGG TGC TGG T |
| nirK             | nirK583F¹³ | TCA TGG TGC TGC CGC GKG ACG G |
|                  | nirK909R¹³ | GAA CTT GCC GGT KGC CCA GAC |
| nirS             | nirScd3aF¹⁰ | GTS AAC GTS AAG GAR ACS GG |
|                  | nirSR3cd¹⁰ | GAS TTC GGR TGS GTC TGG A |
| norB             | cnorB2F¹¹ | GAC AAG NNN TAC TGG TGG T |
|                  | cnorB6R¹¹ | GAA NCC CCA NAC NCC NGC |
| nosZ             | nosZ1527F¹⁰,¹² | CGC TGT TCH TCG ACA GYC A |
|                  | nosZ1773R¹⁰,¹² | ATR TCG ATC ARC TGB TCG TT |

---

Table 2 Primers used for quantitative polymerase chain reaction.
curves were prepared from the serial diluted sample; 0.2 µM forward and reverse primer (Eurofins Genomics Inc., Japan); 12.5 µL SYBR® Premix EX Taq™ II (Takara Bio Inc., Japan); and 10.3 µL sterile DNAse-free water. The experiments were carried out in triplicate. The thermal cycling conditions of qPCR for 16S rRNA were 95 °C for 30 s and 35 cycles at 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 20 s. The qPCR conditions for narG amplification were 300 s at 95 °C and 40 cycles of 15 s at 95 °C, 15 s at 58 °C, and 60 s at 72 °C. Amplification of nirK included 30 s at 95 °C and 40 cycles at 98 °C for 5 s, 63 °C for 50 s, and 72 °C for 60 s. The conditions for nirS qPCR were 30 s at 95 °C; afterward, 40 cycles were added as follows: 98 °C for 5 s, 57 °C for 50 s, and 72 °C for 60 s. Amplification of norB included 300 s at 95 °C; and 40 cycles at 95 °C for 30 s, 54 °C for 45 s, and 72 °C for 45 s. The conditions for nosZ qPCR were 30 s at 95 °C, and 40 cycles of 5 s at 98 °C, 50 s at 58 °C, and 60 s at 72 °C. A synthetic plasmid consisting of the sequences of each targeting gene was used to prepare calibration curves. The calibration curves were prepared from the serial diluted standard plasmid of particular genes (10³ to 10⁷ gene copies/µL).

**Calculation**

For this study, the amounts of NO₃⁻ and NO₂⁻ [mgN/d] were calculated as initial N loading converted NO₃⁻ and removed NO₂⁻ amounts, which will reveal more information than calculating concentrations [mgN/L] of NO₃⁻ and NO₂⁻ alone. The calculations were performed following Eq. (3) - (6) where \( C_{0, NO_3} \), \( C_{t, NO_3} \), \( C_{0, NO_2} \), and \( C_{t, NO_2} \) are the concentrations [mgN/L] of initial NO₃⁻, effluent NO₃⁻, initial NO₂⁻, and effluent NO₂⁻, respectively. \( V \) [L] is the reactor volume and \( t \) [h] is the HRT operated to the reactors.

\[
NO_3_{\text{initial}} = \frac{C_{0, NO_3} \times V \times 24}{t} \quad (3)
\]

\[
NO_3_{\text{converted}} = \frac{[(C_{t, NO_3}) - (C_{0, NO_3})] \times V \times 24}{t} \quad (4)
\]

\[
NO_2_{\text{accumulated}} = \frac{[(C_{t, NO_2}) - (C_{0, NO_2})] \times V \times 24}{t} \quad (5)
\]

\[
NO_2_{\text{removed}} = (NO_2_{\text{converted}}) - (NO_2_{\text{accumulated}}) \quad (6)
\]

Moreover, denitrification efficiency for varying operating conditions was calculated as a percentage, with 100 % representing complete and efficient denitrification, and the lower percentages representing incomplete to poor denitrification. The efficiency was calculated using Eq. (7).

\[
\text{Denitrification efficiency} = \frac{\left( C_{0, NO_3} - \left( C_{t, NO_3} + C_{t, NO_2} \right) \right)}{C_{0, NO_3}} \times 100 \quad (7)
\]

**Statistical analysis**

Since the data on denitrification efficiency obtained in this study was not found to be normally distributed (data not shown), transformation of the data was done based on Templeton⁵, prior to performing a one-way ANOVA, multiple comparisons through Least Significant Difference (LSD) tests, and Pearson correlation using SPSS software version 20. Probability values \((p)\) related to the tests are provided for some results.

**RESULTS AND DISCUSSION**

**Effect of HCO₃⁻ on HD efficiency at different operating HRTs**

Figure. 2 illustrates the trends of HD efficiency, converted NO₃⁻, removed NO₂⁻, and initial N amounts, observed during 85 days of operation in the reactors achieved with different HCO₃⁻ amounts (S1: deficient; S2: moderate; and S3: abundant), and at varying HRTs (24, 12, 8, and 4 h). As shown in Fig. 2, at HRT of 24 h, the converted amounts of NO₃⁻ between the three reactors were not different \((p > 0.05)\), which indicated a complete NO₃⁻ removal of the HD system. Whereas, the removed NO₂⁻ amount in reactor S1 was found to be lower than reactor S2 and S3 \((p < 0.0001)\). Hence, extremely high efficiency was achieved in reactors S2 (98.2 ± 4.9 %) and S3 (99.6 ± 0.3 %). This showed that the efficiency of reactor S1 (73.5 ± 17.3 %) was fluctuating and was lower than that of reactors S2 and S3 due to the imbalance between the amounts of...
converted NO\textsubscript{3} and removed NO\textsubscript{2} as shown in Fig. 2a, which caused NO\textsubscript{2} accumulation. HRT was subsequently changed to 12 h on the 37th day. High efficiency of denitrification was still observed at 12 h HRT in reactors S2 (95.2 ± 6.1 %) and S3 (99.5 ± 0.6 %). However, the efficiency in reactor S1 became gradually lower, 44.9 ± 11.6 %, reaching values below those observed for 24 h HRT. For HRT of 8 h, the ability of NO\textsubscript{3} conversion in reactor S3 was found to be greater than that in reactors S1 and S2 (p < 0.0001), which again resulted in high denitrification efficiency of reactor S3 (96.0 ± 6.5 %) whereas a decline in the efficiency was found in both reactors S1 (39.6 ± 20.9 %) and S2 (68.9 ± 14.6 %). Interestingly, although the removed NO\textsubscript{2} amount in reactor S2 was less than that in reactor S3, it preserved the balance between NO\textsubscript{3} and NO\textsubscript{2} removal ability (Fig. 2b) at HRT of 8 h, which caused no nitrite accumulation, achieving a good HD efficiency. When the HRT was further reduced to 4 h, which was the shortest operating HRT in this study, all reactors eventually showed extremely poor HD efficiency (Fig. 2). The above observations highlight the variation in denitrification efficiency in all reactors for each operating HRT (p < 0.0001). Reactor S1, that did not receive supplementary HCO\textsubscript{3}, had the lowest denitrification efficiency with all the operating HRTs, compared to reactor S2 and S3, except for the 4 h HRT where low denitrification efficiency was found in all the reactors (p < 0.05). However, both NO\textsubscript{3} and NO\textsubscript{2} were completely removed in reactor S3 at 8 h HRT as the shortest operation time, but not in the other two reactors, which were supplied with a lower amount of HCO\textsubscript{3}. This is in agreement with the results obtained in our previous study, showing that the NO\textsubscript{2} removal rate increased when a higher HCO\textsubscript{3} amount was supplied to the HD system which resulted in the preservation of the balance between the removals of NO\textsubscript{3} and NO\textsubscript{2} in order to eliminate NO\textsubscript{2} accumulation\textsuperscript{30} even when decreased HRT. This suggests that using a higher amount of bicarbonate could lead the HD system to achieve better denitrification efficiency at shorter HRTs. All reactors nonetheless explicitly showed a decrease in denitrification efficiency through the operation of 8 h HRT, in the order S3 > S2 > S1 (p < 0.05). Consequently, H\textsubscript{2}-oxidizing denitrifiers in this study need a given minimum amount of inorganic carbon, 200 mg HCO\textsubscript{3}/L, in order to have a suitable effect on nitrogen reduction within the denitrification process while given minimum operating HRTs are also simultaneously required, 12 h and 8 h for the system supplemented with 200 and 5000 mg HCO\textsubscript{3}/L, respectively, to enhance the denitrification efficiency.

**Depletion of HCO\textsubscript{3} during HD process**

Since HCO\textsubscript{3} was supplied as the inorganic carbon source for the H\textsubscript{2}-oxidizing denitrifiers, registering the amount of HCO\textsubscript{3} consumed by the bacteria is crucial to better understand its effects on the HD process. This would allow to observe the amount of HCO\textsubscript{3} which is sufficient for completing the HD process. Furthermore, the activity of the H\textsubscript{2}-oxidizing denitrifiers under unsuitable operating HRTs...
can be analyzed for given HCO₃⁻ amounts. In this study, the amounts of HCO₃⁻ in the influents of reactors S1, S2, and S3 were found to be 22 ± 5, 156 ± 45, and 3386 ± 745 mg, respectively. This suggests that a certain amount of HCO₃⁻ present in the tap water was used for the synthesis of the feeding water, as no additional HCO₃⁻ was supplied to reactor S1. The HD efficiency of 73.5 ± 17.3 % observed in reactor S1 during a 24 h HRT (Fig. 2a) suggested that the low amount of HCO₃⁻ present in the tap water was sufficient to achieve the removal of N compounds in the reactor, for a longer HRT. However, lower efficiencies were observed in reactor S1 when the HRT was shortened to 12, 8, and 4 h (Fig. 2a). Additionally, Fig. 3a shows that the entire amount of HCO₃⁻ was consumed in reactor S1 for all the operating HRTs, as the initial HCO₃⁻ amount in this reactor was lower than the required amount to remove 40 mg NO₃⁻ N/L. Similar results were found in reactor S2, except for the HRT of 4 h (Fig. 3a), after which some residual HCO₃⁻ was still found in the effluent (89 ± 14 mg). This suggests that a 4 h HRT is relatively short for the H⁺-oxidizing denitrifiers to perform a complete HD process. The remaining amount of HCO₃⁻ indicated that denitrifiers did not consume HCO₃⁻ properly within such a short HRT, therefore showing an adverse impact on HD efficiency as illustrated in Fig. 2b. The results obtained from reactor S3 imply that smaller amounts of HCO₃⁻ were consumed when the operating HRT was shortened (Fig. 3a); however, high denitrification efficiency was achieved for all the operating HRTs, except for that of 4 h (Fig. 2c), as the complete HD was not achieved under an extremely short operating HRT. Additionally, the remaining HCO₃⁻ amount at the HRT below the requirement was found in larger than when the longer HRTs were applied to the systems. The decrease in the reduction ability of NO₃⁻ and NO₂⁻ might be the result of the malfunction of the bacteria in undesirably short HRTs as the trace of HCO₃⁻ consumption reduced by reducing the operating HRT. From this study, an HRT of 8 h for an HD system supplied with an abundant amount of HCO₃⁻ was found to be the shortest achievable operating time for remarkably high denitrification efficiency. Additionally, when operating under unsuitably short HRTs, the denitrification efficiency decreased due to the accumulation of NO₂⁻ in the HD system. Higher NO₃⁻ accumulation continued to occur for the same short HRT, even when abundant HCO₃⁻ was supplied. Therefore, it is crucial to allow a suitable, longer operation time when performing HD, as sufficient HRT reduces accumulated NO₂⁻ and improves the overall HD efficiency. Besides, considering the consumed HCO₃⁻ between the actual amount and the stoichiometric amount in the present study reveals that the amount of HCO₃⁻ consumed during the denitrification process is consistent with stoichiometry (Eq. (2)) as satisfactory denitrification efficiency was even observed in the system supplemented with no additional HCO₃⁻, containing 22 ± 5 mg HCO₃⁻. Nonetheless, providing the only stoichiometric amount of HCO₃⁻ might limit the denitrification

Fig. 3 Profiles of (a) HCO₃⁻ consumption percentage; (b) produced OH⁻ amount; and (c) pH of the effluent in hydrogen-based denitrification system at various hydraulic retention times in different reactors. The plots show the averages of the data observed in each operating hydraulic retention time. Error bars indicate the standard deviations.
activity and would require longer operating HRT to achieve greater efficiency.

**Effect of HRTs and HCO$_3^-$ on pH** As shown in Eq. (2), the denitrification process produces OH$^-$ as a by-product that increases the pH of the solution. Fig. 3b shows the produced OH$^-$ amount determined in this study. Amount of OH$^-$ was found higher when long HRTs were operated to the systems and it decreased as the HRT was shorted as well. This implies that the activity of H$_2$-oxidizing denitrifying bacteria was inferior when the shorter HRTs were operated as the effluent pH in all reactors was lower when operating under shorter HRTs (Fig. 3c), and it is associated with the consumed amounts of HCO$_3^-$ (Fig. 3a). Besides, OH$^-$ was produced nearly zero in the reactor S3 even the desirable denitrification efficiency was achieved in this reactor suggesting a powerful ability to keep the pH more stable than the other reactors as a result of the abundant amount of HCO$_3^-$ (Fig. 3a). Whereas, the produced OH$^-$ amount was found higher in reactors S1 and S2, which were supplemented with deficient and moderate HCO$_3^-$ amounts since there was no additional buffer in these systems. Fig. 3b also implies that the denitrification activity in reactor S2 was greater than in reactor S1 as the OH$^-$ amount was produced higher in the reactor S2.

**Quantification of denitrification-functional genes** Sludge samples collected from the different operating conditions were analyzed for qPCR to obtain better insights of specific denitrification-functional genes. The quantity of 16S rRNA gene ranged between $10^{10}$ and $10^{12}$ copies/g sludge, varied by different operating conditions. The quantity of narG, nirK, nirS, norB and nosZ genes detected in the bacterial sludge obtained from the experiments were about $10^8$ to $10^9$, $10^9$ to $10^{10}$, $10^8$ to $10^{10}$, and $10^9$ to $10^{10}$ copies/g sludge, respectively. The quantity of narG, nirK, nirS, norB, and nosZ genes are presented by the relative abundance of genes to the 16S rRNA gene as shown in Fig. 4. The abundance of nirK, nirS, and norB genes significantly increased when the higher HCO$_3^-$ amount was supplemented ($p < 0.01$) whereas narG and nosZ genes were found insignificantly correlated to HCO$_3^-$ amount ($p > 0.05$). Additionally, the relative abundance of nirS gene to the 16S rRNA gene in the sludge taken from HD reactor supplemented with abundant HCO$_3^-$ amount (reactor S3) was found to be high at all the operating HRTs compared to the other reactors, as presented in Fig. 4. However, the abundance of nirS gene in reactor S3 decreased as operating HRT was reduced. Since the nirS gene reveals the activities of NO$_2^-$ reductase in the HD process, a high amount of HCO$_3^-$ can increase the bacteria having nirS gene on NO$_2^-$ reduction. This is in accordance with the observed high efficiency of the HD system when an abundant amount of HCO$_3^-$ was supplied as presented in Fig. 2c and the efficiency decreased when HRT was reduced. This implies that HCO$_3^-$ played an important role in this process.
Effect of Bicarbonate on the Performance of Hydrogen-based Denitrification at Different Hydraulic Retention Times

Role in increasing NO$_2$-reductase activity. There was no significant variation in the abundance of denitrifying genes, observed in reactors S1 and S2; however, the cooperation among the bacteria in the community could lead the systems to achieve the desirable HD efficiency as observed at HRT of 24 h for reactor S1 (Fig. 2a) and at HRTs of 24, 12, and 8 h for reactor S2 (Fig. 2b). Additionally, Fig. 5 shows the adaptation of the bacteria-containing $nirS$ gene in the bacterial sludge obtained on days 0, 7, 14 and 36 during the 24 h HRT operation. The bacteria-containing $nirS$ gene was rapidly adapted by seven days from about $10^{10}$ to $10^{11}$ copies/g sludge when the abundant HCO$_3^-$ amount was given, compared to the conditions of deficient and moderate HCO$_3^-$ amounts. This suggests that an abundant HCO$_3^-$ amount could be used to acclimatize the H$_2$-oxidizing denitrifiers having $nirS$ gene quickly.

Bacterial community under different operating conditions The bacterial community structure (Fig. 6) in the reactor with abundant HCO$_3^-$ amount (S3) was significantly different from the HCO$_3^-$ deficit reactor (S1) and reactor with moderate HCO$_3^-$ amount (S2). *Thauera* spp. (NO$_3^-$-oxidizing bacteria$^{24}$) and *Xanthomonadaceae* (autotrophic denitrifiers that used pyrite as an electron donor found in water and sediment$^{29}$) were considered as the dominant bacteria, when

![Color Key](image)

**Fig. 5** Relative abundance of *nirS* genes over 16S rRNA gene quantity in different conditions of HCO$_3^-$ amounts operated at the hydraulic retention time of 24 h on days 0, 7, 14 and 36.

**Fig. 6** Heatmap presenting dissimilarities of bacterial community structures (relative abundance percentage) among the sludge samples collected from different operating conditions of HCO$_3^-$ amount and operating hydraulic retention time (HRT). g, f, and o define genus, family, and order levels, respectively.
the abundant HCO₃⁻ amount was supplemented since the relative abundances of *Thauera* spp. and *Xanthomonadaceae* were found significantly high, 30.5-77.2 and 2.7-32.0 %, respectively. *Thauera* spp. were observed to be highly abundant in the community, and its relative abundance decreased as the HRTs were reduced. As the *nirS* gene was detected in *Thauera* spp., in this present study, the *nirS* gene abundance among 16S rRNA gene (Fig. 4) and the relative abundance of *Thauera* spp. in the bacterial community (Fig. 6) were found strongly correlated (p < 0.0001) as shown in Fig. 7. This implies that *Thauera* spp. were greatly acclimatized in the system supplemented with abundant HCO₃⁻ amount. However, the relative abundance of *Xanthomonadaceae* was augmented where the abundance of *Thauera* spp. decreased. This probably indicated that there was a collaboration of these bacteria to achieve the desirable HD efficiency. Conversely, *Rhodocyclaceae* (28.9-67.4 %), bacteria found in heterotrophic denitrification, *Alcaligenaceae* (8.4-25.5 %), the family found in the bacterial population of denitrification system, and *Xanthomonadaceae* (9.4-31.7 %) were found to be the dominant bacteria in the reactors S1 and S2, which were supplemented with deficient and moderate HCO₃⁻ amounts, respectively. It was reported that although the optimum range of pH is between 7.5 and 9.5 for denitrifying bacteria, the change in pH can also be affected by the denitrifiers as well since OH⁻ was produced during the denitrification process which increases the pH in the denitrification system. In the present study, in the freed pH condition systems, a negative correlation was found between effluent pH and *Thauera* spp. abundance (p < 0.01), suggesting the reduction in *Thauera* spp. abundance when the system reached the alkaline zone. Whereas, positive correlations were found between pH and the abundance of *Rhodocyclaceae* (p < 0.0001) and *Alcaligenaceae* (p < 0.01), which implied the increase in *Rhodocyclaceae* and *Alcaligenaceae* abundances in the alkaline conditions. Hence, further studies on the effects of pH parallely with varied HCO₃⁻ amounts by controlling the pH are needed to firmly discuss whether the HCO₃⁻ amount or pH was an important factor leading to the changes in HD bacterial community.

**CONCLUSIONS**

The effect of HCO₃⁻ on the efficiency of HD system at several operating HRTs was investigated in order to enhance the system efficiency while minimizing the HRT. The shortest HRTs achieving a desirable HD efficiency were 12 and 8 h, for the system supplemented with moderate and abundant HCO₃⁻ amounts, respectively. HCO₃⁻ played a significant role in increasing the NO₂⁻ reduction and enhancing HD efficiency, as profusely bacteria containing *nirS* gene found in abundant HCO₃⁻ conditions; and it was also found crucial that the abundant HCO₃⁻ amount could rapidly acclimatize the bacteria having *nirS* gene. Besides, the evidence of HCO₃⁻ consumption indicated the denitrifying activities as the consumption of HCO₃⁻ decreased when the HRT was reduced. The lower production of OH⁻, having an impact on no pH increase inside the systems, was also observed when HRT was reduced in which it reveals the inferior activity of H₂-oxidizing denitrifiers. As it is, however, well-known that HCO₃⁻ can be used as a buffer for the solution, using abundant HCO₃⁻ amount can also protect the system from pH increasing. This implies that a sufficient HRT was also a factor required for the reduction of accumulated NO₂⁻ for enhancing the overall HD efficiency. Moreover, an important role of varied HCO₃⁻ amounts in the bacterial community shift in the HD system was found.
in this study. With abundant HCO₃⁻, Thauera spp. were dominant among the community and resulted in high HD efficiency. A satisfactory HD efficiency, however, was achieved in low HCO₃⁻ amount system through the collaboration of Rhodocyclaceae, Alcaligenaceae, and Xanthomonadaceae bacterial communities. Besides, the strong correlation between the relative abundance of nirS gene and Thauera spp. was found in this study, suggesting that Thauera spp. were acclimatized quickly when abundant HCO₃⁻ was supplemented to the HD system. In brief, an adequate HD efficiency was achieved over the different structure of bacterial communities, which was affected by HCO₃⁻. However, to firmly reveal the causes of shifting in the bacterial community structure, the effect of varied pH values and HCO₃⁻ amounts on the behavior of H₂-based denitrification deserves further studies. The study on the effect of HCO₃⁻ on the pure cultures isolated from mixed HD cultures is also needed as the identified bacteria sensitive to HCO₃⁻ can support a strategy on using HCO₃⁻ to well enrich the bacteria in HD system for enhancing the HD efficiency.

ACKNOWLEDGMENTS

This work was partially supported by the Science and Technology Research Partnership for Sustainable Development (SATREPS) program of the Japan Science and Technology Agency (JST) and the Japan International Cooperation Agency (JICA). English Language editing and review services were supplied by Editage (www.editage.com).

REFERENCES

1) Devic, G., Djordjevic, D., Sakan, S.: Natural and anthropogenic factors affecting the groundwater quality in Serbia. Sci. Total Environ., 468–469, 933–942 (2014)
2) Otero, N., Torrentó, C., Soler, A., Menció, A., Mas-Pla, J.: Monitoring groundwater nitrate attenuation in a regional system coupling hydrogeology with multi-isotopic methods: The case of Plana de Vic (Osona, Spain). Agric. Ecosyst. Environ., 133(1–2), 103–113 (2009)
3) Houben, G., Tünnermeier, T., Eqrar, N., Himmelsbach, T.: Hydrogeology of the Kabul Basin (Afghanistan), part II: Groundwater geochemistry. Hydrogeol. J., 17(4), 935–948 (2009)
4) Suthar, S., Bishnoi, P., Singh, S., Mutiyar, P. K., Nema, A. K., Patil, N. S.: Nitrate contamination in groundwater of some rural areas of Rajasthan, India. J. Hazard. Mater., 171(1–3), 189–199 (2009)
5) Cam, P. D., Lan, N. T. P, Smith, G. D., Verma, N.: Nitrate and bacterial contamination in well waters in Vinh Phuc province, Vietnam. J. Water Health., 6(2), 275–279 (2008)
6) Grzybowski, M., Lenczewski, M. E., Oo, Y. Y.: Water quality and physical hydrogeology of the Amarpura township, Mandalay, Myanmar. Hydrogeol. J., 27(4), 1497–1513 (2019)
7) Ward, M. H., Jones, R. R., Brender, J. D., de Kok, T. M., Weyer, P. J., Nolan, B. T., Villanueva, C. M., van Breda, S. G.: Drinking water nitrate and human health: An updated review. Int. J. Environ. Res. Public Health., 15(7), 1–31 (2018)
8) Pichler, M., Coskun, Ö. K., Ortega-Arbulú, A. S., Conci, N., Wörheide, G., Vargas, S., Orsi, W. D.: A 16S rRNA gene sequencing and analysis protocol for the Illumina MiniSeq platform. MicrobiologyOpen., 7(6), 1–9 (2018)
9) López-Gutiérrez, J. C., Henry, S., Hallet, S., Martin-Laurent, F., Catroux, G., Philippot, L.: Quantification of a novel group of nitrate-reducing bacteria in the environment by real-time PCR. J. Microbiol. Meth., 57(3), 399–407 (2004)
10) Throßbäck, I. N., Enwall, K., Jarvis, Å., Hallin, S.: Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiol. Ecol., 49(3), 401–417 (2004)
11) Braker, G., Tiedje, J. M.: Nitric oxide reductase (norB) genes from pure cultures and environmental samples. Appl. Environ. Microbiol., 69(6), 3476–3483 (2003)
12) Scala, D. J., Kerkhof, L. J.: Nitrous oxide reductase (nosZ) gene-specific PCR primers for detection of denitrifiers and three nosZ genes from marine sediments. FEMS Microbiol. Lett., 162(1), 61–68
13) Yan, T., Fields, M. W., Wu, L., Zu, Y., Tiedje, J. M., Zhou, J.: Molecular diversity and characterization of nitrite reductase gene fragments (nirK and nirS) from nitrate- and uranium-contaminated groundwater. Environ. Microbiol., 5(1), 13–24 (2003)

14) Ghafari, S., Hasan, M., Aroua, M. K.: Effect of carbon dioxide and bicarbonate as inorganic carbon sources on growth and adaptation of autohydrogenotrophic denitrifying bacteria. J. Hazard. Mater., 162(2–3), 1507–1513 (2009)

15) Karanasios, K. A., Vasiliadou, I. A., Pavlou, S., Vayenas, D. V.: Hydrogenotrophic denitrification of potable water: A review. J. Hazard. Mater., 180(1–3), 20–37 (2010)

16) Capua, F. D., Pirozzi, F., Lens, P. N. L, Esposito, G.: Electron donors for autotrophic denitrification. Chem. Eng. J., 362(3), 922–937 (2019)

17) Koenig, A., Liu, L. H.: Kinetic model of autotrophic denitrification in sulphur packed-bed reactors. Water Res., 35(8), 1969–1978 (2001)

18) Xing, W., Li, J., Cong, Y., Gao, W., Jia, Z., Li, D.: Identification of the autotrophic denitrifying community in nitrate removal reactors by DNA-stable isotope probing. Bioreour. Technol., 229, 134–142 (2017)

19) Eamrat, R., Tsutsumi, Y., Kamei, T., Khanichaidecha, W., Tanaka, Y., Kazama, F.: Optimization of Hydrogenotrophic Denitrification Behavior Using Continuous and Intermittent Hydrogen Gas Supply. J. Water Environ. Technol., 15(2), 65–75 (2017)

20) Li, P., Xing, W., Zuo, J., Tang, L., Wang, Y., Lin, J.: Hydrogenotrophic denitrification for tertiary nitrogen removal from municipal wastewater using membrane diffusion packed-bed bioreactor. Bioreour. Technol., 144, 452–459 (2013)

21) Rezania, B., Oleszkiewicz J. A., Cicke, N., Mo, H.: Hydrogen-dependent denitrification in an alternating anoxic-aerobic SBR membrane bioreactor. Water Sci. Technol., 51(6–7), 403–409 (2005)

22) Visvanathan, C., Hung, N. Q., Jegatheesan, V.: Hydrogenotrophic denitrification of synthetic aquaculture wastewater using membrane bioreactor. Process Biochem., 43(6), 673–682 (2008)

23) Chang, C. C., Tseng, S. K., Huang, H. K.: Hydrogenotrophic denitrification with immobilized Alcaligenes eutrophus for drinking water treatment. Bioreour. Technol., 69, 53–58 (1999)

24) Mao, Y., Xia, Y., Zhang, T.: Characterization of Thauera-dominated hydrogen-oxidizing autotrophic denitrifying microbial communities by using high-throughput sequencing. Bioreour. Technol., 128, 703–710 (2013)

25) Rujakom, S., Shinoda, K., Kamei, T., Kazama, F.: Investigation of hydrogen-based denitrification performance on nitrate accumulation under various bicarbonate doses. EnvironmentAsia., 12 (Special issue), 54–63 (2019)

26) Ghafari, S., Hasan, M., Aroua, M. K.: Improvement of autohydrogenotrophic nitrite reduction rate through optimization of pH and sodium bicarbonate dose in batch experiments. J. Biosci. Bioeng., 107(3), 275–280 (2009)

27) APHA: American Public Health Association. Standard methods for the examination of water and wastewater. 22nd ed. Washington, D. C., USA: American Public Health Association (APHA), American Works Association (AWWA) and Water Environment Federation (WEF) (2012)

28) Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Knight, R.: QIIME allows analysis of high-throughput community sequencing data. Nat. Methods, 7(5), 335–336 (2010)

29) Ploner, A. Heatplus: Heatmaps with row and/or column covariates and colored clusters. R package version 2.30.0. https://github.com/alexploner/Heatplus (2019)

30) Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O’Harra, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E., Wagner, H.: vegan: Community Ecology Package. R package version 2.5–5. https://CRAN.R-project.org/package=vegan (2019)

31) Wongkiew, S., Park, M. R., Chandran, K., Khanal, S. K.: Aquaponic Systems for Sustainable Resource Recovery: Linking Nitrogen Transformations to Microbial Communities. Environ. Sci. Technol., 52(21), 12728–12739 (2018)
32) Angnes, G., Nicoloso, R. S., da Silva, M. L. B., de Oliveira, P. A. V., Higarashi, M. M., Mezzari, M. P., Miller, P. R. M.: Correlating denitrifying catabolic genes with N₂O and N₂ emissions from swine slurry composting. Bioresour. Technol., 140, 368–375 (2013)

33) Templeton, G. F.: A Two-Step Approach for Transforming Continuous Variables to Normal: Implications and Recommendations for IS Research. Comm. Assoc. Inform. Syst., 28(4), 41–58 (2011)

34) Torrentó, C., Urmeneta, J., Otero, N., Soler, A., Viñas, M., Cama, J.: Enhanced denitrification in groundwater and sediments from a nitrate-contaminated aquifer after addition of pyrite. Chem. Geol., 287(1–2), 90–101 (2011)

35) Liu, B., Mao, Y., Bergaust, L., Bakken, L. R., Frostegård, Å.: Strains in the genus Thauera exhibit remarkably different denitrification regulatory phenotypes. Environ. Microbiol., 15(10), 2816–2828 (2013)

36) Xu, G., Peng, J., Feng, C., Fang, F., Chen, S., Xu, Y., Wang, X.: Evaluation of simultaneous autotrophic and heterotrophic denitrification processes and bacterial community structure analysis. Appl. Microbiol. Biotechnol., 99(15), 6527–6536 (2015)

37) Yamada, T., Tsuji, H., Daimon, H.: Nitrate removal performance and diversity of active denitrifying bacteria in denitrification reactors using poly (L-lactic acid) with enhanced chemical hydrolyzability. Environ. Sci. Pollut. Res., 26(36), 36236–36247 (2019)

38) Dong, H., Jiang, X., Sun, S., Fang, L., Wang, W., Cui, K., Yao, T., Wang, H., Zhang, Z., Zhang, Y., Zhang, Z., Fu, P.: A cascade of a denitrification bioreactor and an aerobic biofilm reactor for heavy oil refinery wastewater treatment. RSC Adv., 9(13), 7495–7504 (2019)

39) Yasuda, T., Waki, M., Fukumoto, Y., Hanajima, D., Kuroda, K., Suzuki, K.: Characterization of the denitrifying bacterial community in a full-scale rockwool biofilter for compost waste-gas treatment. Appl. Microbiol. Biotechnol., 101(17), 6779–6792 (2017)

40) Albina, P., Durban, N., Bertron, A., Albrecht, A., Robinet, J. C., Erable, B.: Influence of hydrogen electron donor, alkaline pH, and high nitrate concentrations on microbial denitrification: A review. Int. J. Mol. Sci., 20(20), 5163 (2019) (Submitted 2020. 4. 7) (Accepted 2020. 5. 19)
