

## Investigation of Hydrogen-Based Denitrification Performance on Nitrite Accumulation Under Various Bicarbonate Doses

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### Abstract

Nitrate-contamination in groundwater sources has been globally taken as a crucial issue due to the effects of nitrate on human health, e.g., methaemoglobinaemia. Various processes have been applied for the treatment of contaminated groundwater with nitrate. Hydrogen-based autotrophic denitrification (hydrogenotrophic denitrification) is an alternative process used for abatement of the nitrate-contamination. Although high efficiency on nitrate removal was achieved by former research, it is unclear whether the growth of *true denitrifiers* is affected by inorganic carbon in which this is essential to comprehend the principal cause of nitrite accumulation. This study found that inorganic carbon had no impact on the nitrate reduction rate whereas the nitrite reduction rate was improved when a higher amount of inorganic carbon was supplied. Also, using bicarbonate as an inorganic carbon source had a positive effect on the growth and adaptation of *true denitrifiers* that can reduce the nitrite accumulation in hydrogen-based denitrification system.

**Keywords:** Hydrogenotrophic, Denitrification, Hydrogen, Bicarbonate, Nitrite

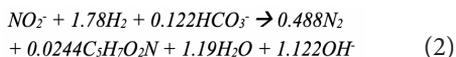
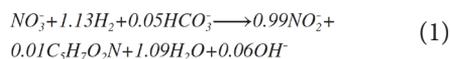
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### 1. Introduction

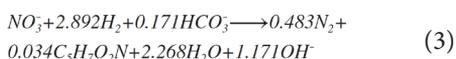
Water is essential for human activities in which groundwater is also one of the crucial water resources. There is approximately 21.1 percent of the groundwater used for domestic purposes globally as stated by Marget and Gun (2013). Remarkably, a huge amount of deaths in developing countries have been annually reported due to water-borne diseases e.g. methaemoglobinaemia which is an effect of nitrate and nitrite existing in drinking water. The critical guideline value of nitrate and nitrite are 11 and 0.9 mg/L as nitrogen, respectively, according to the guidelines released by World

Health Organization (2011); it has been observed that nitrite is more harmful than nitrate in bottle-fed infants. To get rid of these nitrogen contents from contaminated water, several processes such as distillation, reverse osmosis, electro dialysis, chemical precipitation, ion-exchange, and biological denitrification; have been introduced to abate the contamination (Dahab, 1991). To date, there have been much research on biological denitrification process that the heterotrophic and the autotrophic denitrification are known as two types of biological denitrification. Autotrophic denitrification is an alternative process for nitrate removal by using nitrate

and either sulfur compounds or hydrogen gas as an electron acceptor and an electron donor, respectively. Hydrogen-based autotrophic denitrification has been introduced as hydrogenotrophic denitrification in which the stoichiometric reaction equations are shown as follows (Mao *et al.*, 2013):



Overall equation;



Besides, hydrogenotrophic bacteria can provide several advantages: generating lower cell than heterotrophic denitrification which reduces the cost on post-treatment for sludge treatment; and no organic substances is required; for example, ethanol or methanol is required for heterotrophic denitrification as a carbon source for heterotrophic denitrifying bacteria (Karanasios *et al.*, 2010) whereas an inorganic carbon source is vital for hydrogenotrophic denitrification process such as bicarbonate as described in Eq. (3). In addition, nitrite accumulation was found by many researchers under several limited conditions, e.g., unsuitable pH, operated temperature, inadequate intensity of buffers and flow of hydrogen supply in which denitrification process was halted by accumulated nitrite (Cao *et al.*, 2013; Saleh-Lakha *et al.*, 2009; Rezanian *et al.*, 2005; Lee and Rittmann, 2003; Nguyen *et al.*, 2018). Also, Mousavi *et al.* (2012) suggested that the importance of applying an appropriate type of carbon can neglect the malfunction of the denitrification process affected by the unsuitable carbon source. The selection of buffer is also crucial to minimize nitrite accumulation as Visvanathan *et al.* (2008) concluded that nitrite accumulated, observed when carbon dioxide gas was used as a buffer, was lower than when phosphate buffers were used. Seeing that (1) bicarbonate is an inorganic carbon source for autotrophic bacteria and also has the ability

on stabilizing pH of the system as reported by Ghafari *et al.*, (2009) and (2) bicarbonate of 0.171 moles is required to remove 1 mole of nitrate, various doses of bicarbonate was supplied for the denitrification process in this study in order to investigate the behavior of the denitrification system in the absence of pH control.

## 2. Materials and Methods

### 2.1 Enrichment of biological sludge

As a continuous stirred-tank reactor (CSTR) can be used to accomplish the complete mixing between fluid, sludge and the other contents entering into the reactor instantaneously (Metcalf and Eddy, 2003), a 2-L working volume CSTR (Fig. 1) was used as an enrichment reactor for the sludge obtained from the previous study. The freshly prepared feeding water (compositions shown in Table 1) was constantly filled up in the reactor. The conditions applied to the system were 24 h, 31±1°C and 150 rpm for hydraulic retention time (HRT), temperature and stirring speed, respectively. Note that a heating rod and a magnetic stirrer were used as a temperature controller and a mixer, respectively, in this stage. Hydrogen gas was used to continuously sparge into the reactor at 10 mL/min. The concentration of nitrate and nitrite was measured daily as well as the pH in both of the inlet feeding water and the treated water. As soon as the system became acclimatized, the enriched sludge was used for the following studies.

### 2.2 Effects of bicarbonate on hydrogenotrophic denitrification performance

As the investigation of hydrogenotrophic denitrification performance depending on bicarbonate dose was expected, the experiment was divided into five batches at different C/N ratios: 0, 1, 5, 25 and 125. Bicarbonate dose was accounted as the inorganic carbon source for hydrogenotrophic bacteria; hence, different amounts of bicarbonate (presented in Table 2) were used in order to vary the dose of bicarbonate at 40 mg NO<sub>3</sub><sup>-</sup>-N/L based. It is crucial to note that the C/N ratio of zero

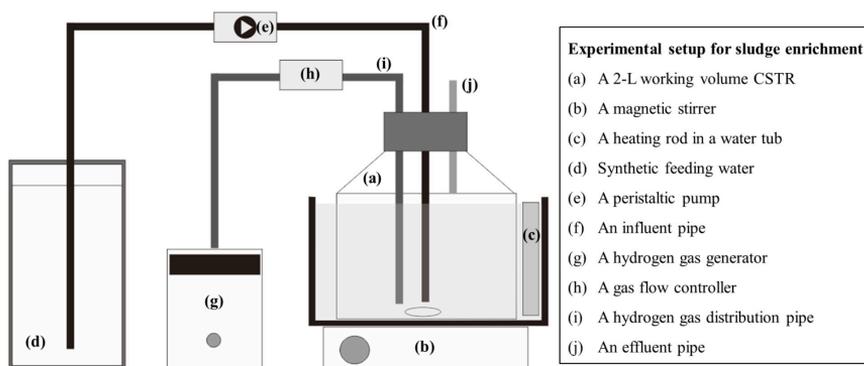


Figure 1. A schematic diagram of a CSTR for sludge enrichment

Table 1. Compositions of feeding water for the biological sludge enrichment (Eamrat et al., 2017)

Chemicals	Amount
NaNO <sub>3</sub>	0.2425 g/L (40 mg NO <sub>3</sub> <sup>-</sup> -N/L)
NaHCO <sub>3</sub>	0.5000 g/L
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.3000 g/L
KH <sub>2</sub> PO <sub>4</sub>	0.0270 g/L
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.1800 g/L
Trace elements I <sup>a</sup>	1 mL/L
Trace elements II <sup>b</sup>	1 mL/L

<sup>a</sup> Consisting of EDTA and FeSO<sub>4</sub>

<sup>b</sup> Consisting of EDTA, ZnSO<sub>4</sub>·7H<sub>2</sub>O, CoCl<sub>2</sub>·6H<sub>2</sub>O, MnCl<sub>2</sub>·4H<sub>2</sub>O, CuSO<sub>4</sub>·5H<sub>2</sub>O, NaMoO<sub>4</sub>·2H<sub>2</sub>O, NiCl<sub>2</sub>·6H<sub>2</sub>O, NaSeO<sub>4</sub>·10H<sub>2</sub>O, and H<sub>3</sub>BO<sub>4</sub>

Table 2. Bicarbonate doses used in the experiment

Desired HCO <sub>3</sub> <sup>-</sup> concentration (mg/L)	NaHCO <sub>3</sub> required (g/L)	C/N ratios
0	0	0
40	0.056	1
200	0.275	5
1000	1.337	25
5000	6.886	125

which contained no bicarbonate was applied in this study since this also can be used to investigate the occurrence of heterotrophic denitrification process in inorganic carbon deficit condition. As the doses of bicarbonate used were estimated from the stoichiometric equation of hydrogenotrophic denitrification (Eq. (3)), 30 mg HCO<sub>3</sub><sup>-</sup>/L is a stoichiometric amount which is equivalent to 40 mg NO<sub>3</sub><sup>-</sup>

-N/L synthesized in the feeding water. The synthetic water was consequently prepared following the compositions in Table 1 excluding NaHCO<sub>3</sub> that the amounts of NaHCO<sub>3</sub> added in this experiment were presented in Table 2. Besides, twenty-one glass vials were used in the experiment as closed-system SBRs per batch. The acclimatized sludge taken from the enrichment was washed down by the synthetic

water as shown in Table 1 in which trace elements I, II and  $\text{NaHCO}_3$  were not supplemented. The prepared sludge was thoroughly mixed with the freshly prepared synthetic water as a mixed liquor sludge separately for each batch. Then, a 14 mL-synthetic water was filled into each glass vial and 1 mL of the mixed liquor sludge was inoculated afterward. All the vials were sealed tightly with a rubber cap and an aluminum cover before getting them sparged with  $\text{H}_2$  gas for 5 min. A shaking water bath was used to control the temperature of those vials at  $31 \pm 1^\circ\text{C}$  and to agitate the vials throughout the experiments. The samples were taken every 2 h for 12 h in each batch. At each sampling time, three vials were taken as triplication. Subsequently, the supernatant of the samples was used to measure the pH immediately. The supernatant of samples was preserved at  $-4^\circ\text{C}$  for further analysis of nitrate and nitrite.

### 2.3 Effects of bicarbonate on nitrite reduction

Besides the batch tests mentioned above, this experiment was conducted to observe the difference between nitrate and nitrite reduction behavior in the different doses of applied bicarbonate subsequently for verifying the results obtained formerly. Since the C/N ratios of 1 and 125 were selected from the results obtained from the batch studies, four sets of Duran bottle with 0.5 L working volume were used as experimental sequencing batch reactors (SBR) for the investigation. A rubber cap was also used to tightly close the reactors to maintain anoxic condition. The synthetic water used in this study was prepared according to the batch experiments. The experiments were initiated with  $40 \text{ mg NO}_2^- \text{-N/L}$  and  $40 \text{ mg NO}_3^- \text{-N/L}$  for each dose of the bicarbonate by using  $\text{NaNO}_2$  and  $\text{NaNO}_3$ , respectively. The approach of SBRs operation was performed according to the study of Ghafari *et al.* (2009) that there were four steps of operation: reaction, settlement, sampling, and refill. The reaction was 23 h and the other three steps had to be finished within 1 h to make 24 h for each cycle. The enriched sludge of 5 mL ( $3180 \text{ mg MLVSS/L}$ ) was used to inoculate into each reactor. At the refilling step, the reactors were sparged with hydrogen gas for

5 min. An incubator and a magnetic stirrer were used to maintain the operating temperature at  $35^\circ\text{C}$  and to agitate the liquid in reactors. The measurements of pH, nitrate, and nitrite were carried out immediately after sampling.

### 2.4 Analytical methods

A pH meter (HORIBA Scientific, Japan) was used to measure pH. The standard methods for the examination of water and wastewater (APHA, 2012) were followed to determine nitrite and nitrate concentrations through the colorimetric method. Nitrite was examined by using color reagent, prepared from diazotized sulfonamide with N-(1-naphthyl)-ethylene-diamine dihydrochloride, to form a reddish purple azo dye and measured the absorbance at 543 nm whereas 1 N hydrochloric acid was used to determine nitrate concentration, that the absorbance at 220 nm and 275 nm were read and subtracted to obtain the absorbance due to nitrate presenting in the samples. Note that nitrite and nitrate concentrations were determined against the standard curves prepared by using standard solutions of nitrite and nitrate. Besides, Eq. (4) and (5) were used to calculate nitrate reduction rate (NRR) and specific denitrification rate (SDR), respectively; in which  $\Delta\text{C}_{\text{NO}_3}$  and  $\Delta\text{C}_{\text{TN}}$  are subtraction of final from initial concentration of nitrate and total nitrogen [ $\text{mg N/L}$ ], respectively; S is the concentration of inoculated sludge [ $\text{g MLVSS/L}$ ] and t is the operation time [h].

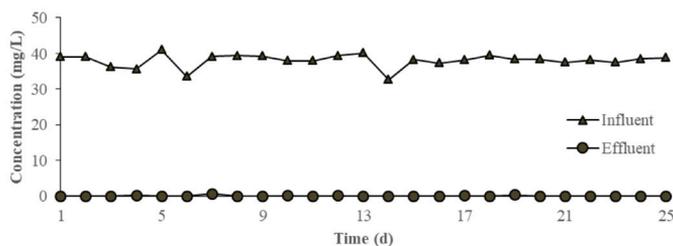
$$\text{NRR} [\text{mg NO}_3^- \text{-N/L-h}] = \Delta\text{C}_{\text{NO}_3} / t \quad (4)$$

$$\text{SDR} [\text{mg N/g MLVSS-h}] = \Delta\text{C}_{\text{TN}} / (S \times t) \quad (5)$$

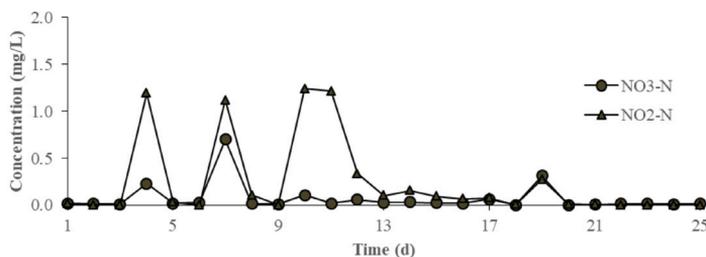
## 3. Results and Discussion

### 3.1 Sludge acclimatization

Fig. 2 illustrates that although nitrate was completely reduced to nearly zero after 24 h of operation, the enrichment was continuously operated. As revealed in Fig. 3; however, nitrite accumulation was observed from the 4<sup>th</sup> day onwards that it reached to about 1.2  $\text{mg NO}_2^- \text{-N/L}$ . This obviously shows that the



**Figure 2.** Nitrate profiles of influent and effluent when 40 mg  $\text{NO}_3^-$ -N/L was applied for the biological sludge acclimatization



**Figure 3.** Nitrate and nitrite profiles during the acclimatization of the biological sludge

complete denitrification was not achieved. Afterward, nitrate and nitrite were not found on the 20<sup>th</sup> day; thus, the enrichment of the sludge was operated till the 25<sup>th</sup> day to ensure that the sludge acclimatization with complete denitrification was achieved. As the seed sludge of this enrichment was obtained from hydrogenotrophic denitrification reactor of the former study, high denitrification efficiency was observed during the sludge acclimatization.

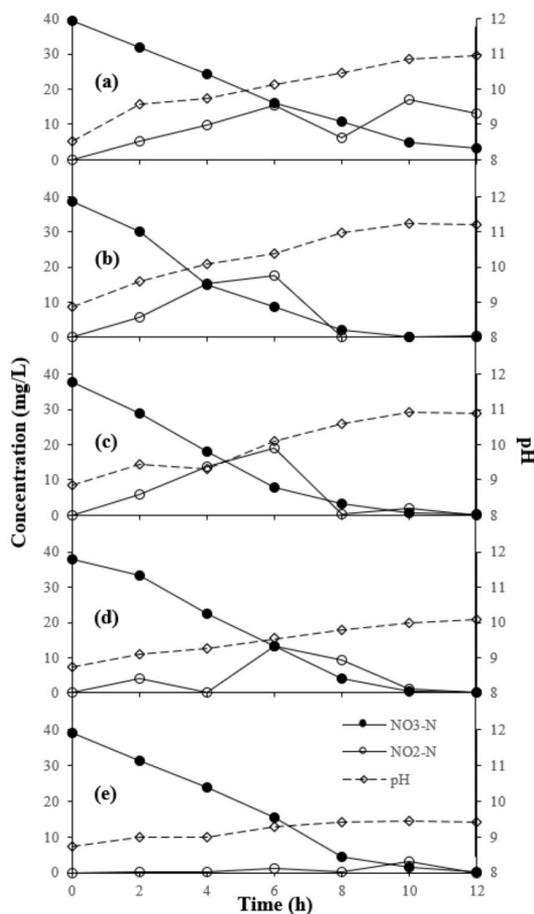
### 3.2 Effect of bicarbonate dose

Five batches of the investigation were parallelly conducted after the acclimatized sludge was achieved. The acclimatized sludge of  $14 \pm 3$  mg MLVSS was inoculated into each vial as a seed of denitrifying bacteria. All the batches were initiated with a nitrate concentration of 40 mg  $\text{NO}_3^-$ -N/L. Since Fig. 3 can reveal that nitrate removal was completely achieved by 10 h in all batches of the study except the batch lacking bicarbonate (Fig. 4a), the nitrate reduction rates at the 10<sup>th</sup> hour were in the range of 3.4 to 3.8 mg N/L-h which is a narrow range as presented in Table 3. Subsequently, the nitrate reduction rate can affirm the apparently same trends of nitrate reduction obtained from all the applied doses of bicarbonate as illustrated in Fig. 4.

Thus, the significant effect of bicarbonate on the nitrate reduction rate was not found in this study. It is explicit that pH increased during the nitrite reduction process due to producing  $\text{OH}^-$  at higher amount than the nitrate reduction process since there is a production of only 0.06 moles  $\text{OH}^-$  to convert 1 mole  $\text{NO}_3^-$  into  $\text{NO}_2^-$  whereas 1.122 moles of  $\text{OH}^-$  is produced when converting 1 mole  $\text{NO}_2^-$  into gaseous nitrogen according to the stoichiometric equations of autohydrogenotrophic denitrification expressed in Eq. (1) and (2). Hence, it can be deduced that the pH value kept increasing when there was the presence of nitrite inside the system and reached stable when the complete reduction of nitrite content was nearly attained. This is therefore consistent with the results shown in Fig. 4 that the peak of nitrite accumulated in the system was found where nitrate removal was almost to be accomplished and the accumulated nitrite was rapidly reduced afterward. This was also seen by Cao *et al.* (2012). Additionally, the initial pH values of the synthetic water were different at the different bicarbonate dose used. The initial pH values were 8.52, 8.86, 8.84, 8.72 and 8.72 at the C/N ratio of 0, 1, 5, 25 and 125, respectively. The pH values observed during 12 h experimental operation are presented in Fig. 4. The trends of

pH change reveal that the pH sharply increased until the accumulated  $\text{NO}_2^-$  was reduced to almost zero. When the differences in pH were considered as presented in Table 3, it obviously shows that higher bicarbonate doses were able to rescue the system from raising the pH into the alkaline zone in which the additional buffer was not applied to the systems; for instance, the difference between the initial and final pH as a result of operation at C/N ratio of 125 was 0.69 where 2.43, 2.34, 2.04 and 1.35 were observed at C/N ratio of 0, 1, 5 and 25, respectively. Ghafari *et al.* (2009) also summarized that bicarbonate provided a buffer for pH stabilization to assist the system on controlling pH especially when  $\text{NaHCO}_3$  higher than 1000 mg/L was applied. Interestingly, the complete denitrification process was observed at the C/N ratio of 1,

which provided bicarbonate almost equal to the stoichiometric amount as presented in Eq. (3). However, the high accumulation of nitrite and the steep shift in pH value were seen during the investigation. This noted that an adequate amount of buffer was required to stabilize the pH value as the high pH in the system is unpleasant for hydrogenotrophic denitrification and it can result in precipitation problems (Tang *et al.*, 2011). Theoretically, autotrophic denitrification requires inorganic carbon for the process. While, there are several possible approaches to enhance the denitrification process: (1) the ability of heterotrophs on using the decomposed bacterial cells as an organic carbon to complete the denitrification process, (2) the use of extracellular polymeric substances (EPS) as an alternative source for



**Figure 4.** Nitrate, nitrite and pH profiles at different C/N ratios: 0 (a), 1 (b), 5 (c), 25 (d) and 125 (e) during 12 h of investigation

**Table 3.** Comparison of pH and reaction rates at different C/N ratios

C/N ratio	0	1	5	25	125
<b>NR<sup>R</sup><sup>a</sup></b>	3.4	3.8	3.7	3.8	3.8
<b>SDR<sup>b</sup></b>	1.9	3.2	3.3	4.0	3.0
<b>Initial pH</b>	8.52	8.86	8.84	8.72	8.73
<b>Final pH</b>	10.95	11.20	10.88	10.07	9.42

<sup>a</sup> Nitrate reduction rate [mg NO<sub>3</sub><sup>-</sup>-N/L-h]

<sup>b</sup> Specific denitrification rate [mg N/g MLVSS-h]

heterotrophs when lacking both of inorganic and organic carbon (Nguyen *et al.*, 2017) and (3) the existence of the inorganic carbon from tap water used to synthesize the feeding water which can be utilized by autotrophic bacteria. In regard to Fig. 4a, the denitrification process was also observed in the system with lack of bicarbonate as mentioned above. Since the mixed cultures sludge was used in this study, there were coexistences between heterotrophic and autotrophic bacteria in the system. This indicated that the heterotrophs had the ability to reduce nitrate greater than autotrophic bacteria. However, the denitrification was not able to complete within 12 h in the batch without bicarbonate supplied.

### 3.3 Effects of bicarbonate on nitrite reduction

According to the results obtained previously, the C/N ratio of 1 and 125 were chosen to apply in this study. The experiments were initiated with 40 mg NO<sub>3</sub><sup>-</sup>-N/L and 40 mg NO<sub>2</sub><sup>-</sup>-N/L for both of the selected C/N ratios. Fig. 5 reveals that nitrate and nitrite were completely removed by 6 days of operation when C/N ratio was 125 whereas the complete removal of nitrate and nitrite were not achieved even operated to 10 days at C/N ratio of 1. The trends of pH change were almost the same in both of the experiment initiated with nitrate and nitrite when separately considered the C/N ratios. In the case of the C/N ratio of 1, nitrite reached a peak when nitrate was removed to less than 10 mg NO<sub>3</sub><sup>-</sup>-N/L. A peak of pH was simultaneously observed when nitrite accumulation reached a peak. The pH slightly

increased until the 10<sup>th</sup> day which resembled the trend of pH change when initiated the experiment with nitrite as illustrated in Fig. 5a. Besides, nitrite accumulation was not found at high concentration in the system applied with the C/N ratio of 125 and initiated with nitrate. Nitrite was reduced to zero within 6 days when spiked nitrite as initial contamination to the system at the C/N ratio of 125 (Fig. 5b). As the findings of Eamrat *et al.* (2017), there were various main players in the mixed culture sludge: e.g., family *Rhodocyclaceae* and *Alcaligenaceae*, and genus *Hydrogenophaga*, *Thauera*, and *Methyloversatilis*; which were found during the operation of the hydrogenotrophic denitrification system using different approaches to supply hydrogen gas. This is the evidence indicating that the mixed culture sludge consists of both heterotrophs and autotrophs since the bacteria can grow in the different trophic conditions. Additionally, there are two types of bacteria in the mixed cultures sludge functioning on the denitrification process: *nitrate respiring bacteria* and *true denitrifying bacteria*. The nitrate respiring bacteria can convert nitrate to nitrite while conversion of nitrite to gaseous nitrogen is performed by the *true denitrifiers* (Ghafari *et al.*, 2009). Fig. 5a reveals that the growth of the *true denitrifiers* seemed to be slower than the *nitrate respiring bacteria* under low bicarbonate dose applied. As nitrite reduction rates were also found to be 0.14 and 0.29 mg NO<sub>2</sub><sup>-</sup>-N/L-h for C/N ratios of 1 and 125, respectively. This caused a high accumulation of nitrite. However, there was a seemingly high growth rate of the *true*

denitrifiers that the balance between the *nitrate respiring bacteria* and the *true denitrifying bacteria* resulted in an elimination of nitrite accumulation problem in the denitrification.

### 3.4 Balance between the conversion of nitrate and nitrite causing nitrite accumulation

As discussed previously, the balance between  $(NO_3^-)_{converted}$  representing the amount of nitrate converted to nitrite and  $(NO_2^-)_{removed}$  representing the amount of nitrite removed from the system according to the activities of the *nitrate respiring bacteria* and the *true denitrifiers* was estimated using the results from the investigation of bicarbonate dose effect on denitrification as shown in Fig. 4 to comprehend the possible cause of nitrite accumulation using Eq. (6), (7) and (8). Note that  $(NO_3^-)_i$  is nitrate concentration at time (i) [mg/L];  $(NO_2^-)_i$  is nitrite concentration at time (i) [mg/L]; and V is the working volume of reactor [L]. Besides, (j) is the sampling interval which was 2 h in this study since the samples were taken every other hour.

$$(NO_3^-)_{converted} [mg] = [(NO_3^-)_{i,j} - (NO_3^-)_i] \times V \quad (6)$$

$$\Delta NO_2^- [mg] = [(NO_2^-)_i - (NO_2^-)_{i,j}] \times V \quad (7)$$

$$(NO_2^-)_{removed} [mg] = (NO_3^-)_{converted} - \Delta NO_2^- \quad (8)$$

The relationships among the ratio of  $(NO_2^-)_{removed}$  over  $(NO_3^-)_{converted}$ , and the concentration of nitrate and nitrite during the experiment when applied several bicarbonate doses are illustrated in Fig. 6. Since  $(NO_3^-)_{converted}$  and  $(NO_2^-)_{removed}$  can represent the amount of nitrite, which is converted from nitrate; and the actual amount of nitrite, which is removed from the system, respectively; nitrite accumulation will not occur when  $(NO_3^-)_{converted}$  is equal to  $(NO_2^-)_{removed}$ . Thus, the ratio of  $(NO_2^-)_{removed}$  over  $(NO_3^-)_{converted}$  was calculated as the bars shown in Fig. 6 that the ratio equal to or higher than 1 should result in the absence of nitrite accumulation. The results show that the ratios reached 1 or even higher at 10 h when C/N ratios were 0, 1 and 5; and at 4 and 2 h when C/N ratios were 25 and 125, respectively. This can be concluded that the higher bicarbonate dose, the faster growth and adaptation rate of the true denitrifiers on nitrite removal.

## 4. Conclusions

Investigation of hydrogen-based denitrification performance carried out under various bicarbonate doses shows that the denitrification occurred even in the inorganic carbon deficit system. Denitrification, however, was not completed in 12 h under the absence

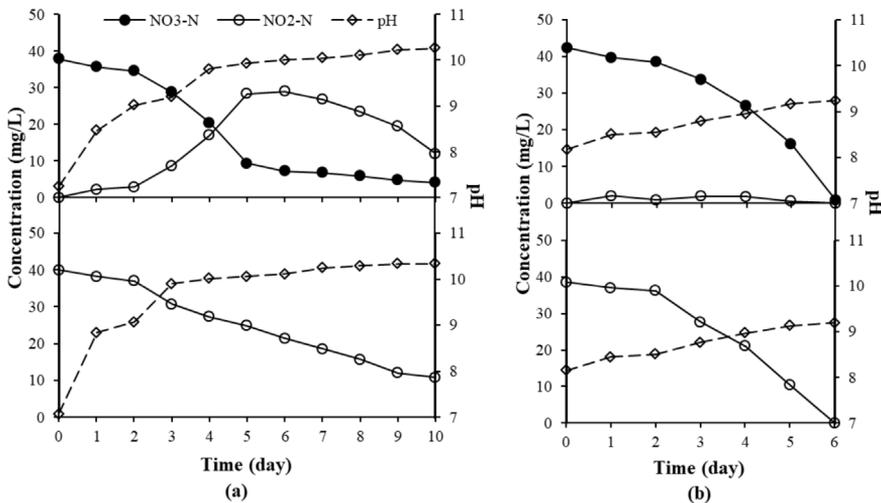
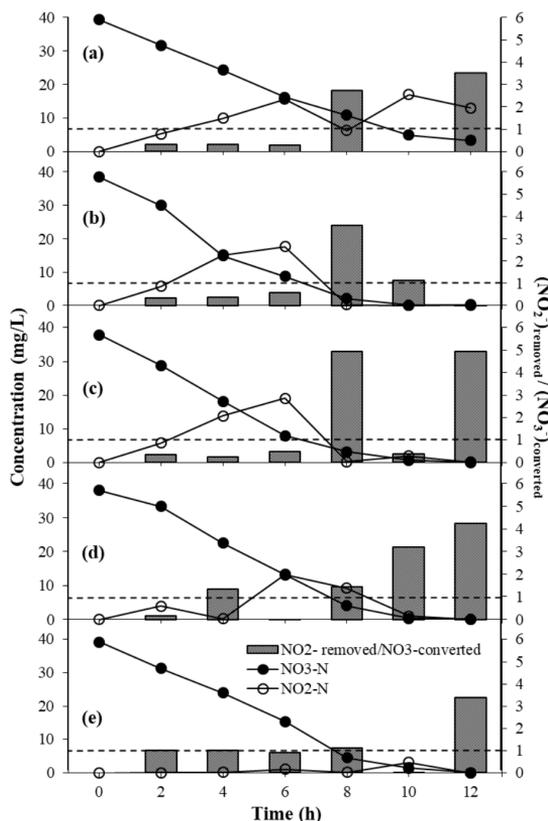


Figure 5. Nitrate, nitrite and pH profiles at C/N ratio of 1 (a) and 125 (b)



**Figure 6.** Relationship among the ratio of  $(NO_2^-)_{removed}$  over  $(NO_3^-)_{converted}$  and the nitrate and nitrite profiles at different C/N ratios: 0 (a), 1 (b), 5 (c), 25 (d) and 125 (e)

of bicarbonate. It was also found that nitrate reduction was independent of bicarbonate doses but an improvement in nitrite reduction rate was found to be dependent on increasing bicarbonate amount, the inorganic carbon. Likewise, it explicitly reveals that the shift of pH value to the alkaline zone, which was a result of the conversion of nitrite, can be rescued by supplying higher bicarbonate dose since bicarbonate can be used as buffer solutions for the system. To avoid the accumulation of nitrite in the denitrification process, preserving the balance between the *nitrate respiring bacteria* and the *true denitrifying bacteria* can result in the constancy between nitrate removal and nitrite removal. The results obtained in this study can confirm that bicarbonate dose had a positive effect on the growth and adaptation of *true denitrifiers*, reducing the nitrite accumulation in the hydrogen-

based denitrification process. The cooperative working between heterotrophs and autotrophs, resulting in the conversion of nitrate to nitrite, was found in this study. Besides, the autotrophs were mostly responsible for the conversion of nitrite to gaseous nitrogen as the increase in bicarbonate ultimately accelerates the nitrite reduction rate. Still, optimization of the suitable pH range deserves further investigation to comprehend whether pH control is necessary when use bicarbonate as an inorganic carbon source for hydrogenotrophic denitrification.

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