Hydrogenotrophic denitrification for treating nitrate contaminated without/with reactive black 5 dye

Tippawan Singhopon, Kenta Shinoda, Suphatchai Rujakom and Futaba Kazama

ABSTRACT

NO_3-N and dye colors discharged from textile wastewater pose environmental problems in Thailand. This study aimed to observe the nitrogen removal rate (NRR) with and without RB-5 color contamination via hydrogenotrophic denitrification (HD) processing, which uses H_2 gas as electron donor to reduce NO_3-N and NO_2-N; comparing with bioreactors treatment to evaluate systems that can simultaneously remove NO_3-N and dye color. Five reactors under different operation and gas supply conditions were set-up under HRT of 24 h, including an aerobic reactor using air, two anaerobic reactors using argon and H_2, and a combined process using intermittent air/argon and air/H_2. NRR without dye varied between 45 and 90% for H_2 and air/H_2 by HD processing, while it was completely removed when adding color. H_2 and air/H_2 reactors experienced partial decolorization of approximately 20–30%, whereas the other three reactors remained unchanged. Effluent of NO_3-N were close to wastewater standards, but the color was still easy to detect, which indicated that the treatment time needs to be sufficient. In conclusion, HD and intermittent air/H_2 processing can completely remove NO_3-N and NO_2-N when contaminated with RB-5 color. Furthermore, RB-5 did not affect the NRR, whereas some particles of dye color can also reduce in these processes.

Key words | biodegradation, dye color removal, hydrogenotrophic denitrification, nitrate removal rate, Reactive Black 5 dye color

HIGHLIGHTS

- NRR with and without RB-5 dye contamination was evaluated in HD reactors and compared with bioreactors under various conditions using air, argon and H_2 gas supplies.
- Simultaneous NO_3-N, NO_2-N and RB-5 dye removal occurred in H_2 and combined air/H_2 reactors.
- HD processing accelerated when contaminated with RB-5 dye.
- RB-5 dye does not affect the NRR removal, while some partial removal can occur with HD processing.
- NO_3-N, NO_2-N was removed faster than RB-5 dye color.

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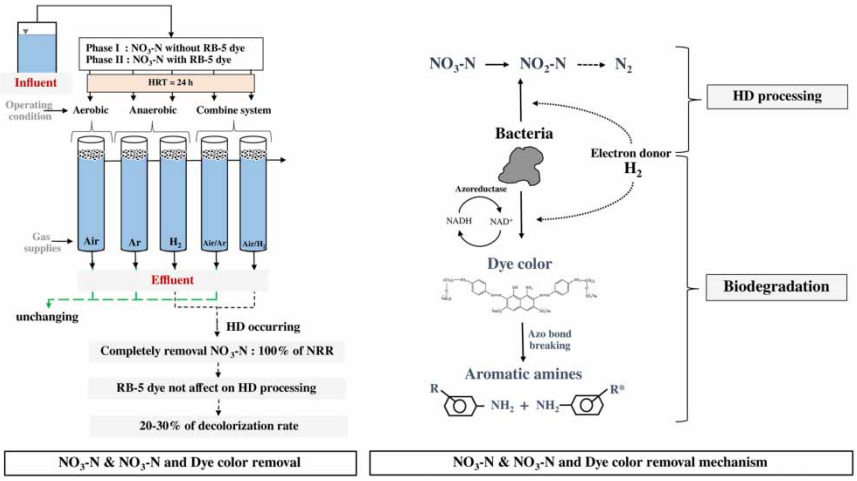
GRAPHICAL ABSTRACT



INTRODUCTION

Thailand is a newly industrialized and developing country, and textiles are one of its most important export industries, with 14,400 tons/yr of leather and textile fibers being produced and exported. Various processing stages such as bleaching, dyeing, printing, finishing, sizing and stiffening use chemical reagents and large volumes of water, thereby generating huge quantities of effluent wastewater that require discharging. Presently, the textile industry in Thailand generates approximately 2.55×10^6 m^3/yr of wastewater contaminated with various toxic pollutants, in particular, nitrate (NO_3-N) (Sahinkaya *et al.* 2017; Sairiam *et al.* 2019). NO_3-N is a common pollutant that can be converted into nitrite (NO_2-N), which is another toxic pollutant generally found in groundwater, surface water, and wastewater. Normally, NO_3-N from textile wastewater is produced from salts such as sodium nitrate used in dye baths to improve textile fibers. The concentration of NO_3-N can be in the range of 40–100 g/L in textile effluents (Cirik *et al.* 2013). These levels are problematic and have a harmful impact on groundwater and human health, for example, they cause methemoglobin in children (Rahman *et al.* 2020). The World Health Organization (WHO) recommends NO_3-N and NO_2-N concentration to be less than 11.3 and 0.91 mg/L in drinking water, respectively (WHO 2011). Many techniques have been developed to treat NO_3-N pollution, including chemical–physical processes such as ion exchanger and ultra-filtration reverse osmosis, and biological denitrification. The biological denitrification system involves both heterotrophic and autotrophic processes and is commonly applied for treating NO_3-N and NO_2-N. The system is based on the respiration of facultative bacteria as heterotrophic denitrification bacteria (HDB) and autotrophic denitrification bacteria (ADB), with NO_3-N used as an electron acceptor under anaerobic and anoxic conditions.

Hydrogenotrophic denitrification (HD) or hydrogen-based autotrophic denitrification is a type of treatment processing that is used to reduce inorganic compounds such as NO_3-N and NO_2-N using H_2 as electron donors, and bicarbonate or carbon dioxide for biosynthesis. Owing to its low biomass, low residual organic compounds, low system costs and high nitrogen removal efficiency compared with heterotrophic denitrification, HD is most commonly used to treat groundwater (Eamrat 2017). Theoretical equations for heterotrophic (Equation (1)) and hydrogenotrophic (Equation (2)) denitrification are given below.

Heterotrophic denitrification using methanol as a carbon source (Rezvani *et al.* 2017):

$$NO_3^- + 1.08\,CH_3OH + H^+ \rightarrow 0.065\,C_5H_7NO_2 + 0.47\,N_2 + 0.76\,CO_2 + 2.44\,H_2O \quad (1)$$

HD using bicarbonate as an inorganic carbon source and H_2 as electron donors (Rujakom *et al.* 2019):

$$NO_3^- + 2.892\,H_2 + 0.171\,HCO_3^- \rightarrow 0.483\,N_2 + 2.268\,H_2O + 0.17\,OH^- + 0.034\,C_5H_7NO_2 \quad (2)$$

However, various HD systems have been developed for municipal wastewater treatment, while only a few studies have reported on its application in industrial and textile wastewater treatment. As textile wastewater has a high pollutant contamination, mainly due to the variety of dyes used in the textile industry, the concentration of pollutants in this type of wastewater often does not adhere to the current wastewater standards in Thailand. The characteristics of textile wastewater in Thailand are presented in Table 1.

Dye colors that are mostly made from organic acids and salts, inorganic acid and salts, a bleaching agent, and trace metals, are usually discharged into an effluent as 50% of the influent feed. Even a small portion of dye color is highly visible and can influence the visual appearance of streams and other water resources. Generally, dye colors can be removed under anaerobic and combined systems through anoxic processes because the reductive cleavage of the azo bond that occurs as an electron acceptor can transfer to aromatic amines under anaerobic conditions. Consequently, aromatic amines are generated and then reduced under aerobic conditions (Sheng *et al.* 2018). Therefore, modern wastewater treatment processes such as adsorption, electrochemical oxidation, ozone, ion exchange, membrane and biological processing are used to treat textile wastewater. However, effective treatments of NO_3-N and dye color are often low in anaerobic treatment processes due to bacterial activities and the deficiency of electron donors. Electron donation is a key step in the removal of NO_3-N, NO_2-N, and color (Li *et al.* 2014). A previous study that focused on the effect of NO_3-N removal on anaerobic azo dye reduction with various electron donors, i.e. acetate, methyl-group, and CO_2/H_2, found no observable dye color in the effluent, whereas CO_2/H_2 was determined to be the best electron donor (Saroyan *et al.* 2019). The study also found that H_2 gas and formate are better electron-donors for decolorization than acetate and methanol.

Hence, the primary aim of this study was to apply HD treatment processing to remove high concentrations of NO_3-N from wastewater when it is contaminated with dye color. This study also attempts to evaluate the systems that can simultaneously reduce NO_3-N contaminated with dye color in a single reactor. For this purpose, five reactors were set up under the following process conditions, i.e. aerobic, anaerobic, and combined system, to investigate the treatment performance in the simultaneous removal of NO_3-N and NO_2-N, without and with dye contamination. In the aerobic system, air was constantly supplied to a reactor to observe the effect of all parameters when mixed with oxygen. Two anaerobic reactors were operated using various types of biological denitrification: the first was heterotrophic and operated using argon supplies, while the other used HD processing and was supplied with H_2 gas, which acted as the

Table 1 | Characteristics of wastewater from the textile industry in Thailand

Parameters	Effluent industrial wastewater standard in Thailand	Influent[a]	Effluent[b]	Effluent[c]
pH	5.5–9	7.0	7.77	–
BOD (mg/L)	< 60	800–1200	1050	90–250
COD (mg/L)	≤ 400	6000–8000	3056	800
TN (mg/L)	≤ 100	100–700	154	50
TSS (mg/L)	≤ 150	1500–3000	975	–
TDS (mg/L)	$\leq 3{,}000$	8725	4000–9000	–
Cr^{3+}(mg/L)	≤ 0.5	–	13	0.2
Color (ADMI)	Not objectionable	800–1200	–	450
Color (mg/L)[d]		40–500	–	2–112

[a,c]Influent and effluent qualities from a textile factory in Thailand).
[b]Effluent textile wastewater from the central wastewater treatment plant in Thailand.
[d]Influent and effluent color concentrations from anaerobic and combined anaerobic/aerobic treatment processing in Thailand (2015).

electron donor. The combined system (anaerobic/aerobic), which is widely used in the removal of dye color and organic matter, was supplied by an intermittent feeding of air/argon and an air/H_2 supply. Reactive Black 5 (RB-5), which is a type of azo dye color and is most commonly used in dyeing processes, was selected as the model dye color combined with NO_3-N contamination.

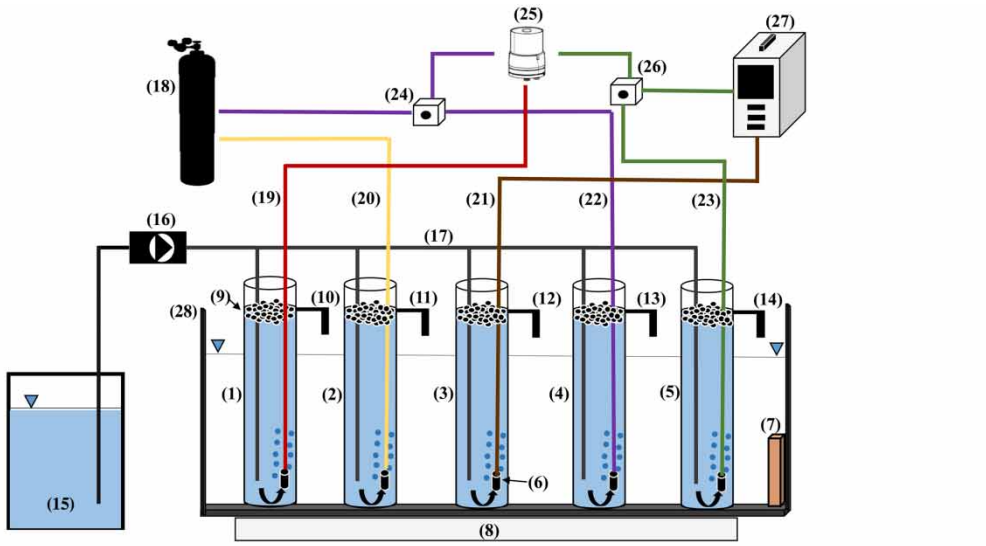
MATERIALS AND METHODS

Experimental configuration

Five reactors under various operating conditions were set up including an aerobic (with air), two anaerobic (one with argon and another with H_2), and a combined system reactor (one with air/argon and another with air/H_2); these reactors were used to observe the treatment performance for the removal of NO_3-N, NO_2-N, and dye color. All the reactors that were made from plastic were fabricated in a cylindrical form; 3.5 cm in diameter and 28 cm in height. The working volume of each of the reactors was approximately 2 L. All the reactors were fixed inside a water tub to control their temperatures in the range of 30–33 °C. Furthermore, the reactors were connected with an influent synthetic wastewater tank, and for gas feeding, the pipe lines were fixed on the operating conditions of the reactors. The schematic of the experimental configuration of the reactors is depicted in Figure 1.

Preparation of synthetic textile wastewater

Synthetic wastewater was prepared using tap water based on the actual concentrations and the chemical reagents (g/L)



(1)-(5).	Reactors under various gas supplied (air, argon, H_2, air/argon and air/ H_2)	(6).	Air stone dif$_{fuser}$
(7).	A heating rod in a water tub	(8).	A magnetic stirrer
(9).	Plastic beads	(10)-(14).	Effluent pipes from each reactor
(15).	Synthetic wastewater feeding tank	(16).	A peristatic pump
(17).	Influent pipe	(18).	An Argon cylinder tank
(19)-(23)	Gas distribution pipes line (air, argon, H_2, air/argon and air/H_2)	(24).	A timer for controlling air/argon gas
(25).	An Air pump	(26).	A timer for controlling Air/H_2 gas
(27).	A hydrogen generator	(28).	A water tub

Figure 1 | Schematic of the experimental setup.

adapted from a previous study (Panswad & Luangdilok 2000). The composition of wastewater is presented in Table 2. The influent NO_3-N concentrations considered in this study ranged from 40, 50 and reached 80 mg-N/L at the end of the experiment. In the second phase, RB-5 dye color was added into these reactors. The dye concentration for all the reactors was 80 mg/L in the primary phase, reduced to 20 mg/L toward the end of the experiment. RB-5 was obtained from the Fujifilm Wako Pure Chemical Corporation. Co., Ltd (Sigma-Aldrich, USA). More information about the dye is provided in Table 3 (Bouraie & Din 2016).

Influent loading rate and gas supply conditions

To examine the influent loading rates, experiments were conducted in two phases: NO_3-N without RB-5 contamination, and NO_3-N with RB-5 contamination. The treatment performance of each reactor was tested for 24 h, in a continuous feeding mode. At the beginning, the enhanced sludge from the HD reactor, which was operating for more than 400 d, was added to each reactor. This reactor was operated at an HRT of 8 h at a controlled temperature of 30 °C and fed with influent water at approximately 8.7 mL/min, with an H_2 supply of 30 mL/min. The nitrogen loading rate (NLR) was 313 g-N/m^3/d. The HD reactor showed the best performance, with the removal rates of NO_3-N and NO_2-N close to 85–95%. The enriched sludge was added between 0.2 and 0.4 g of volatile suspended solids (VSS)/L to each reactor. In the first phase in days 1–7, the air, argon, air/argon, and air/H_2 gas reactors were set up to maintain an NLR of 40 g-N/m^3/d. On day 8, the H_2 reactor was constructed, and an NLR of 50 g-N/m^3/d was maintained in all the reactors. On day 18, an NLR of 80 g-N/m^3/d was maintained in the air, argon, air/argon, and air/H_2 reactors; this change was also done on day 11 for the H_2 reactor. These changes were made to observe the effect on the nitrogen removal rate (NRR) when the influent loading is increased. In the second phase, NO_3-N with RB-5 was introduced on day 33 for all the reactors except for the H_2 reactor, where the pollutant was introduced on day 27, at 80 mg/L of RB-5 (amount equivalent to NLR). On day 48, for the four reactors, and on day 42, for the H_2 reactor, the RB-5 dose was reduced to 20 mg/L to isolate the effect of NO_3-N removal on the dye.

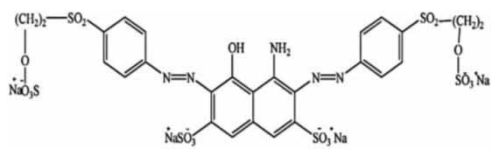
For gas supply feeding, the pipelines were fixed depending on the operating conditions of each reactor. One of the aerobic reactors was continuously supplied with air to grow aerobic bacteria, while the two anaerobic reactors were continuously supplied with argon and H_2 gas for bacterial growth and for the comparison of treatment performances under the presence of heterotrophic and hydrogenotrophic denitrifying bacteria, respectively. Of the other two reactors, one was intermittently supplied with air/argon and the other with air/H_2, and controlled under the combined anaerobic/aerobic system. Air was released from an air pump to each

Table 2 | Composition of synthetic wastewater

Chemical	Concentration (mg/L
$NaNO_3$	0.486 g/L as 80 mg NO_3-N/L
KH_2PO_4	0.110 g/L as 12.5 mg P
$NaHCO_3$	1.0 g/L as an inorganic carbon source
$CaCl_2$	0.021 g/L as 7.5 mg Ca
$MgSO_4.7H_2O$	0.038 g/L as 3.75 mg Mg
Trace elements (I)[a]	1 ml/L
Trace elements (II)[b]	1 ml/L
Reactive Black 5 dye	0.08 g/L as 80 mg/L color

[a]Trace element I contained: 5 g/L EDTA and 5 g/L $FeSO_4$.
[b]Trace element II contained: 15 g/L EDTA, 0.43 g/L $ZnSO_4.7H_2O$, 0.24 g/L $CoCl_2.6H_2O$, 1 g/L $MnCl_2.4H_2O$, 0.25 g/L $CuSO_4.6H_2O$, 0.22 g/L $MnCl_2.5H_2O$, 0.22 g/L $NaMoO_4.5H_2O$, 0.19 g/L $NiCl_2.6H_2O$, 0.21 g/L $NaSeO_4.10H_2O$, and 0.014 g/L H_3BO_4.

Table 3 | Commercial information of Reactive Black 5 dye color

Color index	Reactive Black 5	Dye structure
Type	Reactive	
Synonyms	Remazol Black B	
λ_{max}	597 nm	
Molecular formula	$C_{26}H_{21}N_5Na_4O_{19}S_6$	
Molecular weight	991.82 g/mol	

reactor, while argon was discharged from a cylinder tank and H_2 was supplied from an H_2 generator, via an air stone diffuser. Initially, the volume of the continuous supply of air and argon was controlled at 300 mL/min, and H_2 at 100 mL/min. After a supply of 18 days of air and argon supply, and 12 days of H_2, it was reduced to 50 mL/min. For the intermittently fed reactors of air/argon and air/H_2, all the gases including air, argon, and H_2 were supplied at 50 mL/min under an aerobic to non-aerobic time ratio of 30:60 min and controlled using a timer. Plastic beads were selected to cover the top of each reactor to maintain the anaerobic conditions, whereas a magnetic stirrer was used to mix the liquid and sludge. The experimental conditions are detailed in Table 4.

Analytical methods

The influent and effluent from all the reactors were centrifuged (Hitachi CF 16RXII, Japan) at 10,000 rpm for 10 min, and stored in a freezer (–18 °C) until the analyses. pH, dissolved oxygen (DO), and dissolved hydrogen (DH) were directly measured inside the reactors using a pH meter (Horiba-B712, Japan) and DO probe (YSI 58 Dissolved Oxygen Meter, Japan), respectively. The temperature was checked with a digital thermometer (WT-6, China). NO_3-N and NO_2-N concentrations were then measured using the exhausting ultraviolet spectrophotometric screening method for NO_3-N and a colorimetric method for NO_2-N (UV-1800, Shimadzu-Spectrophotometer, Japan) (APHA 2012). Moreover, the rate of decolorization was calculated on the basis of the Lambert–Beer law as various concentrations of RB-5 were prepared, and their absorbance values were measured at a maximum wavelength of RB-5 at 597 nm in a spectrophotometer. The abscissa presented dye concentrations and the absorbance values were the ordinate, a standard curve draw of the correlation coefficient corrected was $R^2 = 0.9980$ and linear equation was $y = 0.0214x + 0.0201$ (Assadi *et al.* 2018). However, the nitrogen removal and decolorization efficiency were calculated using Equations (3)–(6):

$$\text{Nitrogen loading rate (NLR)}[\text{g} - \text{N/m}^3/\text{d}] = \frac{\text{Influent nitrate [g} - \text{N/L]} \times \text{Flow rate [L/d]}}{\text{Reactor volume [m}^3\text{]}} \quad (3)$$

$$\text{Nitrogen removal rate (NRR)}[\text{g} - \text{N/m}^3/\text{d}] = \frac{(\text{Nitrate removed} + \text{Nitrite removed})\,[\text{g} - \text{N/L}] \times \text{Flow rate [L/d]}}{\text{Reactor volume [m}^3\text{]}} \quad (4)$$

$$\text{Nitrogen removal efficiency (\%)} = \frac{\text{Nitrogen removal rate (NRR) [g} - \text{N/m}^3/\text{d]}}{\text{Nitrogen loading rate (NLR) [g} - \text{N/m}^3/\text{d]}} \quad (5)$$

$$\text{Decolorization (\%)} = \left(\frac{\text{Initial absorbance} - \text{Absorbance after treatment}}{\text{Initial absorbance}}\right) \times 100 \quad (6)$$

Statistical analysis

To test the differences between the treatments performance of decolorization, all the reactors during the second phase were analyzed by the analysis of variance (ANOVA) and least significant difference (LSD) using the SPSS statistical software tool version 25. Statistical significance was kept at $p < 0.05$.

Table 4 | Summary of the operating conditions used in this study

Operating condition			Phase I (without RB-5 dye)			Phase II (with RB-5 dye)	
Time (day)	Air, Argon, Air/argon and Air/H_2 reactors		1–7	8–17	18–32	33–47	48–66
	H_2 reactor		–	0–10	11–26	27–41	42–60
Influent feeding	NO_3-N (mg/L)		40	50	80–85	80–85	80
	RB-5 dye color (mg-N/L)		–	–	–	80	20
Gas supply conditions	Continuous feeding (mL/min)	Air	300	300	50	50	50
		Argon	300	300	50	50	50
		H_2	100	100	50	50	50
	Intermittent feeding (mL/min)	Air/Argon Air/H_2	Cycle times	30 min of aerobic and 60 min of non-aerobic			
			Gas flow	50 mL/min in each type			

RESULTS AND DISCUSSION

Effluent NO_3-N and NO_2-N concentrations

In this study, the reactors were continuously fed with synthetic wastewater at an HRT of 24 h to observe the nitrogen removal rate (NRR). The experiments were conducted in two phases: the first NO_3-N did not contain the RB-5 dye color contamination, while the second did. Influent concentrations of NO_3-N feeding varied between 40 and 85 g-N/m^3/d. The actual effluent NO_3-N and NO_2-N concentrations and nitrogen removal efficiency from all the reactors by operating times are provided in Figure 2(a)–2(j).

Figure 2(a) and 2(b) shows the results from the reactor that was continuously fed with air: the effluent concentration of NO_3-N was stable at the same amount of influent feeding, while NO_2-N concentration and NRR were close to zero. Under anaerobic conditions, the reactors were separated into two reactors to compare the treatment performance by various types of denitrifying bacteria under continuous feeding with argon to make heterotrophic conditions as shown in Figure 2(c) and 2(d), and the other with H_2 as hydrogenotrophic conditions (HD) as shown in Figure 2(e) and 2(f). The results of NO_3-N and NO_2-N concentrations from the argon reactor had still accumulated with the same amount of influent feeding; this was similar to the results obtained from the air reactor; however, NO_2-N concentration and NRR were also close to zero. However, the conditions in these reactors were almost entirely anaerobic; the DO concentrations were found to be ranging from 0.15 to 4.70 mg/L. On the contrary, the results showed that the NO_3-N and NO_2-N removal rates were acceptable when H_2 was supplied as shown in Figure 2(e) (without RB-5 dye) and Figure 2(f) (with RB-5 dye). During the addition of 50 g-N/m^3/d from day 1 to 10 into this reactor, with a 100 mL/min H_2 gas supply, the NO_3-N and NO_2-N effluent concentrations reached only around 10 mg-N/L on day 1 due to the start-up process and slow growth of denitrifying bacteria. On day 11, after the influent loading rate increased to 80 g-N/m^3/d and the H_2 supply dropped to 50 mL/min, there was a minimal change in the NO_3-N and NO_2-N concentrations owing to the lack of electron donors (H_2). We found that H_2 and a high concentration of influent load can affect the nitrogen accumulation rate. Therefore, the nitrogen removal efficiency of this phase decreased but remained stable in the range of 80–100%. After day 27, 80 mg/L of RB-5 was added. This amount was decreased to 20 mg/L on day 42. Concentrations of NO_3-N and NO_2-N in the effluent at this stage were nearly zero, whereas the nitrogen removal efficiency was stable and close to 100%. Hence, the nitrogen removal efficiency improved in the second phase compared to that in the first phase where RB-5 was not added.

In the combined system under intermittent gas supplies including air/argon and air/H_2, as shown in Figure 2(g)–2(j), respectively, the NO_3-N and NO_2-N concentration decreased noticeably in the air/H_2 reactor. In the first phase, as shown in Figure 2(i), the results from air/H_2 highlighted that NO_3-N and NO_2-N concentrations in this reactor were still high due to the HD process being incomplete. The treatment performance of NRR was reduced when the influent loading rate increased. However, after the addition of dye color (day 33), as shown in Figure 2(j), NO_3-N effluent concentrations decreased; and the increase in NO_2-N concentrations was significantly higher than that without dye color feeding. Moreover, after day 48, influent dye concentration decreased from 80 to 20 mg/L. The results showed that NO_3-N and NO_2-N effluent concentrations were higher than the previous concentration while NRR also dropped. Therefore, the concentration of dye color may be effective for the NO_3-N and NO_2-N removal. However, the nitrogen removal efficiency in this reactor fluctuated more as compared to the H_2 reactor and was dependent on the amount of NO_3-N and NO_2-N accumulations in each operating time, in the range of 45–90% and 80–100%, with and without RB-5 dye, respectively. Hence, the air/H_2 reactor showed a reduction to the levels lower than in the H_2 reactor because the air or oxygen usually slowed down the HD process. The DO concentrations confirmed the effect of oxygen on NRR in this reactor. The DO concentrations in the air/H_2 reactor ranged from 0.04 to 5.90 mg/L. The recommended value of DO concentration for improving NRR varies between 0.5 and 0.8 mg/L. However, DO concentrations higher than 4.5 mg/L in the anaerobic and combined system can affect nitrogen removal. Previous studies found that when oxygen ranges from 0.36 to 3.36% under anaerobic conditions, it can inhibit nitrate removal while denitrifying bacteria in the high oxygen environment does not

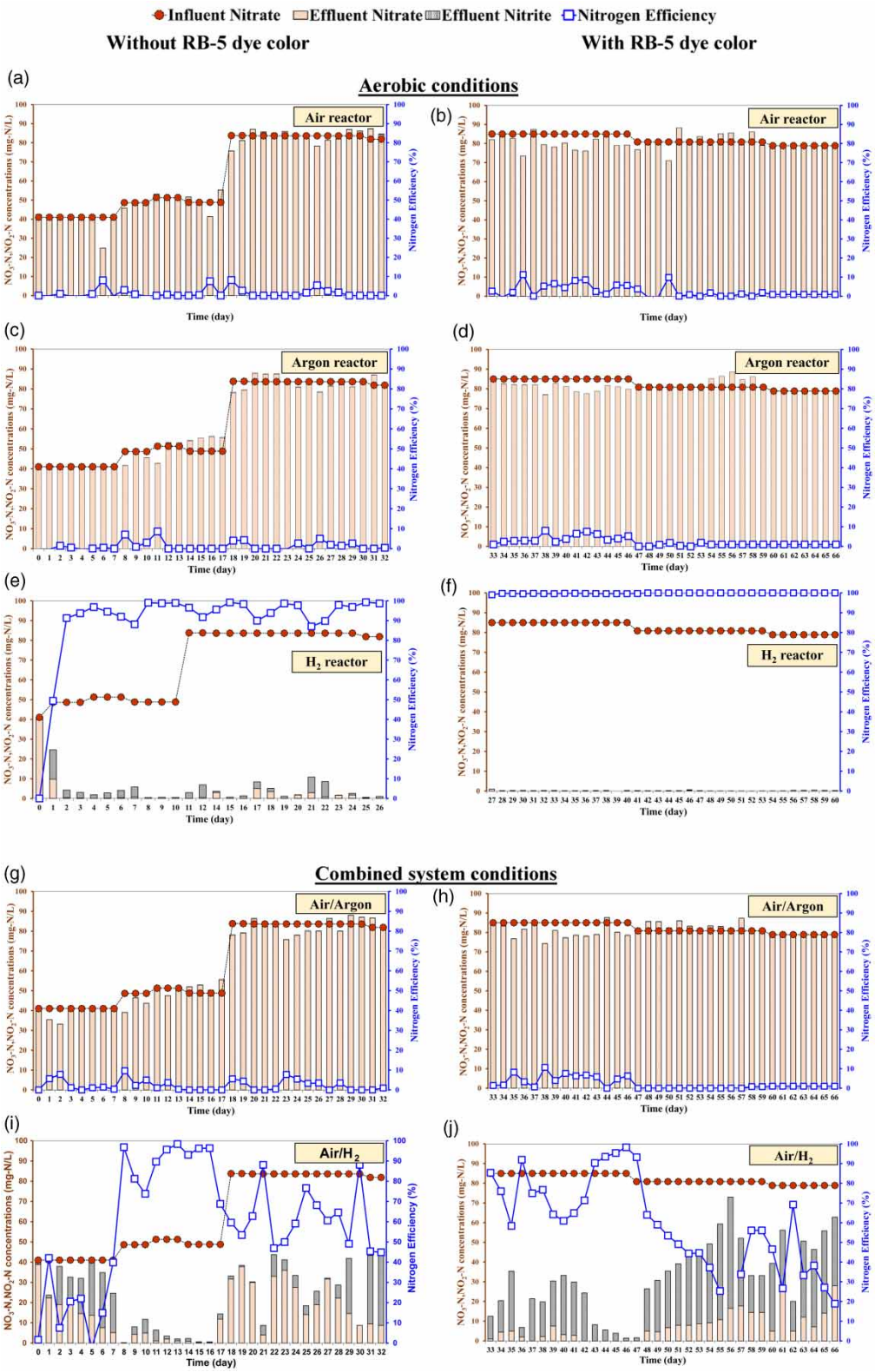


Figure 2 | NO_3-N and NO_2-N concentrations and nitrogen efficiency during two phases of operation (a) and (b) from Air reactor, (c) and (d) from Argon reactor, (e) and (f) from H_2 reactor, (g) and (h) from Air/argon reactor and (i) and (j) from Air/H_2 reactor.

synthesize the nitrogen-reducing enzyme (Griessmeier *et al.* 2019). As the results for the air reactor are the same; NRR is close to zero because high value oxygen mixing may have affected the bacterial growth rate in this reactor.

In this experiment, NO_3-N and NO_2-N of the H_2 and air/H_2 reactors were completely removed by the HD process that occurred in both reactors. Denitrifying bacteria, which are autotrophic organisms, were used as the electron donor from H_2 with HCO_3^- as an inorganic carbon source to completely reduce NO_3-N and NO_2-N under hydrogenotrophic processing. Notably, the pH increased in both reactors. The pH of effluent wastewater varied between 9.47 and 11.17 in the H_2 reactor and between 9.21 and 11.05 in the air/H_2 reactor; the original pH values were in the range of 8.02–8.50 during influent feeding. As the HD process occurred in both reactors, one acid equivalent (H^+) was consumed based on a stoichiometric equation for NO_3-N reduced to N_2 gas, which converts to an alkalinity rate of 3.579 g $CaCO_3^-$ per 1 g of NO_3-N reduced. This concentration can lead to an increase in the pH values in these reactors. In contrast, the pH concentrations from the air, argon and air/argon reactors were the same as those in influent feeding, thereby proving that no reaction occurred in these reactors. According to the previous reports, the optimum pH conditions for the HD process ranged from 7.6 to 8.6 (Eamrat 2017). Similarly, pH levels from 9.13 to 9.93 can lead to high denitrification rates (Li *et al.* 2013). Conversely, owing to the lack of carbon sources in air, argon and air/argon can lead to a slower NO_3-N reduction and bacterial growth compared to the heterotrophic process using organic matter.

In conclusion, HD processing can completely reduce NO_3-N and NO_2-N when contaminated with RB-5 dye; in contrast, this dye color did not affect the combined NO_3-N and NO_2-N removal. The dye color also did not significantly affect NO_3-N removal because NO_3-N had a faster oxidation-reduction reaction than the dye color (Panswad & Luangdilok 2000; Cirik *et al.* 2013). Furthermore, dye color can lead to an increase in the reaction rates of NO_3-N and NO_2-N, thus reducing the time required for treatment.

Effluent dye color concentrations

The biological degradation of a dye mostly occurs in anaerobic processes by the reductive cleavage of dye bonds and through an oxidation-reduction reaction that removes aromatic amines in the aerobic process. Conversely, the dye is an electron acceptor with limited reduction when it exists alongside more effective electron acceptors (Assadi *et al.* 2018; Chen *et al.* 2018). In this experiment, the treatment efficiencies of RB-5 dye when contaminated with NO_3-N under different operating conditions were investigated using various dye concentrations. In the first stage, 80 mg/L of RB-5 dye color was fed into the air, argon, air/argon, and air/H_2 reactors on days 33–47, which was then decreased to 20 mg/L on days 48–66. Similarly, 80 mg/L of RB-5 dye coloring was added to the H_2 reactor on day 27, which was then reduced to 20 mg/L on day 42. The effluent RB-5 dye concentrations and decolorization rate of all reactors are shown in Figure 3(a)–3(e).

The statistical analysis results showed that different operating conditions and gas feeding had significant interaction effects on the effluent concentrations of the dye color. The dye color concentration was slightly reduced in both the H_2 and air/H_2 reactors as shown Figure 3(c)–3(e). The dye color amounts in these reactors decreased at a slightly faster rate than in the other reactors. The dye concentrations in the air, argon and air/argon reactors accumulated at a constant influent feeding rate, as presented in Figure 3(a), 3(b) and 3(d). A lack of bacterial growth incompletely influenced the biodegradation processing. The results show that the decolorization rate was approximately 20–35% in H_2 and 10–20% in air/H_2 reactors, respectively. The average effluent concentrations in the H_2 and air/H_2 reactors were approximately 58 and 67 mg/L at a feeding rate of 80 mg/L of RB-5, and 16 and 18 at a feeding rate of 20 mg/L of RB-5, respectively. However, the decolorization rates remained unchanged when the dye concentration was reduced from 80 to 20 mg/L. Hence, the RB-5 dye color can cause a slight reduction together with NO_3-N and NO_2-N during HD processing.

Dissolved organic carbon (DOC) was measured for the influent and effluent to check the bacterial activities in both reactors. The results of DOC increased by 23.4 and 13.7 mg/L for the H_2 and air/H_2 reactors, respectively, while the influent feeding DOC result was 8.5 mg/L. The DOC value may have increased from ADB activity that produced extracellular polymeric substances (ESP), which are

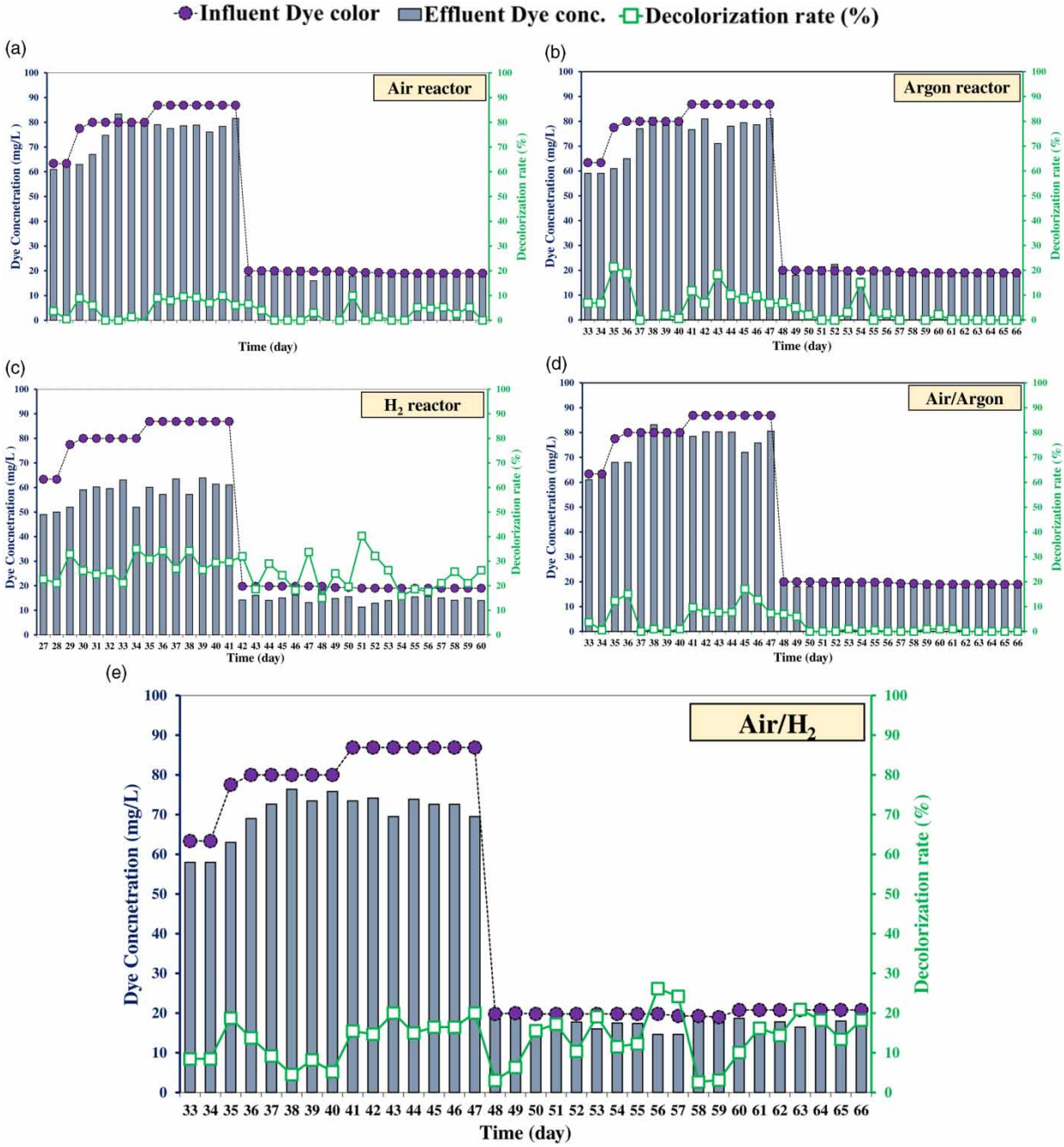


Figure 3 | RB-5 dye concentrations and decolorization rate. (a) from Air reactor; (b) Argon reactor; (c) H_2 reactor; (d) Air/argon reactor; (e) Air/H_2 reactor.

biopolymers used to protect cells from external environmental or strength conditions. Bacterial activities in these reactors can help in the removal of NO_3-N along with the dye color by the process of denitrification. Hence, the metabolism of ADB under HD processing can also generate NADH, which is another factor that facilitates dye color removal using azo-reductases to break azo bonds in the dye molecule, which then act as electron acceptors to

produce colorless aromatic amines. One of the advantages of using H_2 gas for decolorization is that its electron-donating substrates can reduce and charge form of azo dye to aromatic amines (Saroyan *et al.* 2019). However, the reduction in dye concentrations was very minimal in the H_2 reactor and, to a slightly larger extent, in the air/H_2 reactor in the presence of NO_3-N. The dye color was removed faster in the presence of lower NO_3-N concentration (Lourenço *et al.* 2000). Conversely, oxygen and NO_3-N are more active electron acceptors than a dye, and lead to incomplete decolorization (Lee *et al.* 2010).

Table 1 shows that the actual influent and effluent concentrations of dye color under anaerobic and combined anaerobic/aerobic treatment processing in Thailand were found in the range of 40–500 mg/L reducing to 2–112 mg/L due to effective treatment processing and long-term operation. Hence, HRT is a crucial factor that can improve the treatment efficiency of decolorization using HD and the combined system in this study.

Relationship between nitrogen removal and decolorization rate

Table 5 presents the relationship between NRR compared with the decolorization rate from all the reactors under different influent feeding regimes, including actual NO_3-N and NO_3-N contaminated with RB-5 dye. NRR and dye were mostly removed in the anaerobic and combined systems when H_2 gas was used during HD processing, whereas the aerobic, anaerobic, and combined systems using air and argon gas remained unchanged.

The average NRR results without and with RB-5 dye were reduced in the H_2 and air/H_2 reactors, at close to 95% in the H_2 reactor without RB-5 and close to 100% with RB-5 dye, although the latter dropped slightly to 90–100% after the azo dye concentration was reduced from 80 to 20 mg/L. Similarly, there were decreases in the nitrogen removal efficiency of the air/H_2 reactor after the RB-5 dye concentration was reduced from 80 to 20 mg/L, at 80 and 42%, respectively.

Table 5 | Summary of the average nitrogen efficiency compared to the decolorization rate

		Nitrogen efficiency (%)			Decolorization rate (%)		
Reactor	**HRT (h)**	**Without RB-5**	**With 80 mg/L of RB-5**	**With 20 mg/L of RB-5**	**With 80 mg/L of RB-5**	**With 20 mg/L of RB-5**	**Reference**
Air		1	4	1	5	3	This study
Argon	24	1	4	1	8	2	
H_2		95	100	100	28	24	
Air/Argon		1	2	0	7	1	
Air/H_2		61	80	42	13	14	
Previous studies							
1. Anaerobic-biofilm anoxic–aerobic membrane bioreactor	24	–	100		60–80		Spagni *et al.* (2010)
2. Sequencing batch reactors	6	–	100		63		Cirik *et al.* (2013)
3. Membrane bioreactor	72	–	–		80–100		Friha *et al.* (2015)
4. Anaerobic reactor	120–240	–	–		95–99		Thailand (2015)
5. Up-flow anaerobic filter	48	–	–		> 95		
6. Combine anaerobic–aerobic reactor	48	–	–		58		
7. Membrane bioreactor	70	–	100		20–60		Luong *et al.* (2016)
8. Anaerobic–aerobic–anoxic sequencing batch reactors	8	–	40–75		60–85		Chen *et al.* (2018)
9. Hydrolysis/acidification and multiple anoxic/aerobic process	6	–	100		63		Gu *et al.* (2018)

Hence, the HD process was accelerated when contaminated with dye color while it was delayed when the dye concentrations decreased at this stage. Previous studies suggested that NO_3-N and NO_2-N is an electron acceptor similar to dye color but it is highly effective; therefore these substances can compete for electrons with dye color leading to an increase in oxidation-reaction that grew NRR contaminated with high amount of dye color and thus limit decolorization (Lee *et al.* 2010; Chen *et al.* 2018).

For the decolorization rate, both the reactors had a steady and low percentage of color removal, although there was a reduction in the influent concentrations of dye color owing to the lack of electron donors. In the previous studies that focused on the treatment schemes to remove color contaminated with NO_3-N, the denitrification process began at the first stage and dye color reduced only after the denitrification had been completed (Chen *et al.* 2018). However, in our study the RB-5 removal rate from HD processing with a high dye concentration was 28%, which subsequently dropped to 24% when the dye color concentration was reduced. The rate of decolorization was still low when compared with the previous studies due to the small size of the reactor, high amount of NO_3-N contamination and short operating time.

According to previous studies that evaluated advanced treatment processing to simultaneously remove nitrogen and dye color in a single reactor, the treatment efficiency of both the parameters was varied based on the operation time (i.e. HRT) and the treatment process, such as anaerobic-biofilm anoxic–aerobic membrane bioreactor, sequencing batch reactor, anaerobic reactor, up-flow anaerobic filter, and hydrolysis/acidification, or multiple anoxic/aerobic process. The results obtained in most of these studies showed the complete removal of nitrogen, while the decolorization rate was found to be in the range of 20–100%, except in the case of the membrane bioreactor and up-flow anaerobic filter, which exhibited high decolorization rates. However, the combined system is generally used to improve the quality of textile wastewater.

CONCLUSIONS

HD processing was performed under different operating and gas supply conditions; the reactors included an aerobic, two anaerobic (one supplied with argon, and the other with H_2) and two combined systems (one with intermittent air/argon, and the other with air/H_2). The results of NRR and decolorization rate showed high treatment efficiencies of the anaerobic processes and combined systems that used H_2 and air/H_2 as electron donors. The concentrations of NO_3-N and dye color were significantly and simultaneously reduced when HD processing was performed in a single reactor. However, in the air, argon and air/argon reactors, no oxidation-reduction reactions occurred owing to a lack of bacterial growth. Furthermore, H_2 reactors showed higher NRR than the air/H_2 reactors because oxygen negatively affected the HD process. The NRR and decolorization rates were high in H_2 reactors when both NO_3-N and RB-5 dye color were presented, thereby providing optimal conditions for H_2 to donate electrons. The performance of the H_2 reactors in terms of nitrogen removal was 90–100% and the decolorization ranged from 20 to 35%, whereas the air/H_2 reactor showed a 40–100% NRR and a 10–20% decolorization rate after an HRT of 24 h. This is because NO_3-N is a better electron acceptor compared to the azo dye, and therefore, the decolorization rate was low. However, there was a reduction in the nitrogen removal efficiency after the concentration of RB-5 in the influent was reduced. The dye color may compete with NO_3-N and NO_2-N to improve the oxidation-reduction reaction rates of nitrogen. Hence, HD processing can still completely remove NO_3-N and NO_2-N when contaminated with RB-5 dye color, and RB-5 did not affect the NO_3-N and NO_2-N removal rates. However, the concentration of NO_3-N after processing met the effluent wastewater standard in Thailand, and the concentration of color was easy to detect even in contaminated wastewater. In the future, HD and combined systems should use appropriate H_2 levels and sufficient treatment time to help facilitate the reduction of the concentration of dye color in a single reactor. Furthermore, these systems should be applied to textile wastewater to observe their performance in a real situation. This method provides an opportunity to improve and develop advanced technologies that can lower treatment costs, ensure sustainability and improve wastewater quality before being discharged, as well as reduce freshwater consumption in developing countries like Thailand.

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DATA AVAILABILITY STATEMENT

Data cannot be made publicly available; readers should contact the corresponding author for details.

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