Research Article

Received: 12 January 2021 Revised: 22 April 2021 Accepted article published: 6 May 2021 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jctb.6788

Enhancement of nitrate removal under limited organic carbon with hydrogen-driven autotrophic denitrification in low-cost electrode bio-electrochemical reactors

Prarunchaya Peungtim,[a,b] Orapan Meesungnoen,[c] Palisa Mahachai,[c] Pakpoom Subsoontorn,[c] Thu Nga Do,[d] Auppatham Nakaruk[b,e] and Wilawan Khanitchaidecha[a,b*]

Abstract

BACKGROUND: Nitrate-contaminated water is a concerning environmental and health problem in agricultural areas. Due to its low organic carbon content, such water cannot be purified using a conventional bioreactor with heterotrophic denitrification. Low-cost bio-electrochemical reactors were established with the aim of enhancing denitrification performance by cooperation of heterotrophic and hydrogenotrophic denitrification.

RESULTS: At the lowest C/N ratio of 1.0, the bioreactor reached only 67.4% for NO_3-N removal and 47.5% for total N removal (total N removal rate of *ca* 1.2 mg L^{-1} h^{-1}), whereas the Cu reactor (bio-electrochemical reactor using copper wire as cathode) achieved the best efficiencies of 73.7% and 53.8% for NO_3-N and total N removal (total N removal rate of *ca* 1.3 mg L^{-1} h^{-1}), and followed by the SS reactor (bio-electrochemical reactor using stainless steel wire as cathode). On the other hand, the greatest total organic carbon (TOC) removal efficiency was observed for the bioreactor and followed by the SS reactor and the Cu reactor. The low TOC consumption of 1.2–1.4 mg-TOC (mg-N)$^{-1}$ in the Cu reactor and the SS reactor indicated the enhanced denitrification from coexisting hydrogenotrophic denitrification. In addition, the performance of hydrogenotrophic denitrification was improved by increasing the applied current from 10 to 30 mA.

CONCLUSIONS: The abundance of autotrophic denitrifying bacteria (identified as hydrogenotrophs) was higher than that of heterotrophic denitrifying bacteria in both the Cu reactor and the SS reactor. *Archromobacter* and *Flavobacterium* were the most dominant genera in the biofilm of the Cu reactor cathode and in the suspended sludge, respectively. *Hydrogenophaga* were detected in both biomass samples, but were absent from the biomass samples of the conventional bioreactor.

Keywords: bio-electrochemical reactor; hydrogenotrophic denitrification; low-cost electrodes; water contamination and treatment

INTRODUCTION

Nitrate contamination in groundwater is one of the most important environmental issues in many countries such as Thailand, India, China and Vietnam.[1-4] This nitrate contamination results from an extreme use of chemical fertilizer in agricultural areas, an excessive application of nitrogen in agro-livestock farming and a daily manure discharge.[5,6] The consumption of nitrate-contaminated water leads to health hazards such as blue baby syndrome in infants and blue-gray discoloration of the skin in adults. The World Health Organization has recommended that nitrate (NO_3-N) and nitrite (NO_2-N) in drinking water should not exceed 11.3 and 1.0 mg L^{-1}, respectively. However, a high nitrate level of approximately 21.9 ± 18.3 mg L^{-1} was detected in rural groundwater in Yantai, China[7] and a wide nitrate range from 0.1 to 325.1 mg L^{-1} was observed in groundwater of agro-livestock farming in Korea.[5]

Pump-and-treat technology becomes a key process to improve the groundwater quality to meet drinking water standards.

* *Correspondence to: W Khanitchaidecha, Department of Civil Engineering, Faculty of Engineering, Naresuan University, Phitsanulok 65000, Thailand. E-mail: wilawank1@gmail.com*

a *Department of Civil Engineering, Faculty of Engineering, Naresuan University, Phitsanulok, Thailand*

b *Centre of Excellence for Innovation and Technology for Water Treatment, Faculty of Engineering, Naresuan University, Phitsanulok, Thailand*

c *Department of Biochemistry, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand*

d *Faculty of Energy Technology, Electric Power University, Hanoi, Vietnam*

e *Department of Industrial Engineering, Faculty of Engineering, Naresuan University, Phitsanulok, Thailand*

Hydrogenotrophic denitrification has been recommended for groundwater treatment in recent years. This process can remove nitrate efficiently under limited organic carbon such as groundwater resource; hydrogen gas (H_2) functions as electron donor while assimilating inorganic carbon (i.e. CO_2) provides an energy source for microbial activity. In addition, the metabolism in this process generates less excess biomass than conventional heterotrophic denitrification. In a typical hydrogenotrophic denitrification system, gaseous hydrogen is generated through a high-pressure hydrogen tank[8] and hydrogen generator.[9] Hydrogen gas is nontoxic but its low water solubility, ranging only from 0.5 to 1.6 mg L^{-1},[10] is the main concern for researchers and engineers in developing an efficient hydrogen-driven denitrification system to improve groundwater quality.

In the literature, a biofilm system is one type of economic nitrogen and organic carbon removal process[11-14]; nitrogen is efficiently removed together with electricity production.[15,16] Similarly, a bio-electrochemical system or biofilm–electrode system, a combination of biological and electrochemical technologies, has been developed for small water treatment systems. Inert substances including platinum, graphite, gold and stainless steel are used as electrodes. Gaseous hydrogen is generated through water hydrolysis reaction (Eqn (1)) at the cathode, whereas anode oxidation reaction takes place at graphite (Eqn (2)). Then the produced H_2 and CO_2 are consumed through hydrogenotrophic denitrification. Various microbial genera from the Proteobacteria phylum have been reported as hydrogenotrophs such as *Thauera*, *Hydrogenophaga* and *Rhodocyclaceae*.[9] A high abundance of *Pseudomonas* capable of autotrophic and heterotrophic denitrification was detected in a bio-electrochemically assisted nitrate removal system.[17] The synergy between heterotrophic denitrification, widely used in nitrate removal processes and requiring high organic content, and hydrogenotrophic (autotrophic) denitrification is believed to be the key to a high and stable nitrate removal performance for limited-organic groundwater.

$$2H_2O + 2e^- \rightarrow H_2 + 2OH^- \quad (1)$$

$$C + 2H_2O \rightarrow CO_2 + 4H^+ + e^- \quad (2)$$

In the study reported here, a simple, functional and economical bio-electrochemical reactor was established for treating nitrate-contaminated water. The performances of two reactors using low-cost copper wire and stainless steel wire as a cathode were compared. Graphite plate was used as an anode for both reactors. Nitrate-contaminated groundwater commonly contains low organic carbon; therefore the reactors were started to operate at a sufficient organic condition (C/N ratio of 2) and then the organic content was decreased to groundwater levels of C/N ratios of 1.5 and 1. Meanwhile, the applied current was increased stepwise from 10 to 30 mA to contribute the enhancing hydrogenotrophic denitrification. The microbial community was also clarified. The results would be valuable for further development of small-scale groundwater treatment in nitrate-contaminated areas.

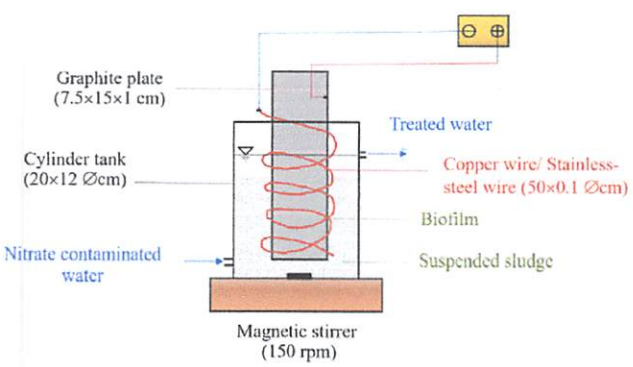

Figure 1. Schematic of bio-electrochemical reactor.

METHODOLOGY

Experimental apparatus

Two bio-electrochemical reactors with a working volume of 1.4 L and low-cost electrodes were established: one reactor used a copper wire as cathode (named Cu reactor) and the other reactor used a stainless steel wire as cathode (named SS reactor). The wires (30 cm × 0.1 cm diameter submerged area) were spirally covered on a graphite plate (7.5 cm × 8 cm × 1 cm submerged area) as anode. The water surface was covered by small plastic beads to avoid oxygen transfer across the air–water interface. A conventional bioreactor without electrodes and applied current (named bioreactor) was set up as a control reactor which allowed only heterotrophic denitrification to occur.

Microorganism preparation

Active sludge was collected from an activated sludge treatment plant at Wang Tong Hospital (Phitsanulok province, Thailand). The sludge was of a dark brown color and had good settleability. The sludge was acclimatized in a feed-batch mode under anaerobic condition until achieving >90% nitrate removal. The nitrate-contaminated water of 20 ± 2 mg L^{-1} was prepared by mixing 0.121 ± 0.003 g L^{-1} $NaNO_3$, 0.227 ± 0.005 g L^{-1} $CH_3COONa{\cdot}3H_2O$ and 0.018 ± 0.001 g L^{-1} KH_2PO_4. During acclimatization, the *in situ* dissolved oxygen (DO) was low (<0.5 mg L^{-1}).

Operation method

An amount of 100 mL of acclimatized sludge was added into each of the Cu reactor, SS reactor and bioreactor. The reactors were operated under continuous mode; the contaminated water influent was continuously fed to the reactors, while the effluent simultaneously overflowed from the reactors (hydraulic retention time was *ca* 8 h). In phase 1, the C/N ratio was decreased stepwise from 2.0 to 1.5 to 1.0 to investigate the effect of C/N ratio on nitrate removal and activity of denitrifying bacteria. The effect of applied current on denitrification performance and microbial activity was evaluated in phase 2 by increasing the applied current from 10 to 20 to 30 mA (the current densities were approximately 0.08, 0.16 and 0.24 mA cm^{-2}, respectively). The *in situ* DO was measured and kept low (<0.5 mg L^{-1}). A schematic of the bio-electrochemical reactors and the bioreactor is shown in Fig. 1. The experimental conditions and chemicals used in contaminated water are summarized in Table 1.

Morphology and element characterization

The copper wire and stainless steel wire cathodes were analyzed in terms of morphology and elemental composition at the end of the experiment through scanning electron microscopy and energy dispersive X-ray spectrometry (SEM-EDS; Leo1455VP) at an accelerating voltage of 20 kV.

Microbial characterization

The biomass including biofilm on cathode and suspended sludge was collected at the end of the experiment. The population density of autotrophic denitrification bacteria and heterotrophic denitrification bacteria was determined using serial dilution plating assay on selective agar media. Later, genomic DNA was extracted from

Table 1. Experimental conditions

Phase	Reactor	Cathode	Anode	Applied current (mA)	C/N ratio
1	Bio-electrochemical reactor (Cu reactor)	Copper wire	Graphite plate	10.0 ± 0.1	2.0[a]
					1.5[b]
					1.0[c]
	Bio-electrochemical reactor (SS reactor)	Stainless steel wire	Graphite plate	10.0 ± 0.1	2.0[a]
					1.5[b]
					1.0[c]
	Control reactor (bioreactor)	—	—	—	2.0[a]
					1.5[b]
					1.0[c]
2	Bio-electrochemical reactor (Cu reactor)	Copper wire	Graphite plate	10.0 ± 0.1	1.0[c]
				20.0 ± 0.1	
				30.0 ± 0.1	
	Bio-electrochemical reactor (SS reactor)	Stainless steel wire	Graphite plate	10.0 ± 0.1	1.0
				20.0 ± 0.1	
				30.0 ± 0.1	

[a] Chemicals used (g L^{-1}): 0.121 ± 0.003 $NaNO_3$, 0.227 ± 0.005 $CH_3COONa{\cdot}3H_2O$ and 0.018 ± 0.001 KH_2PO_4.
[b] Chemicals used (g L^{-1}): 0.121 ± 0.003 $NaNO_3$, 0.170 ± 0.004 $CH_3COONa{\cdot}H_2O$ and 0.018 ± 0.001 of KH_2PO_4.
[c] Chemicals used (g L^{-1}): 0.121 ± 0.003 $NaNO_3$, 0.113 ± 0.003 $CH_3COONa{\cdot}3H_2O$ and 0.018 ± 0.001 KH_2PO_4.

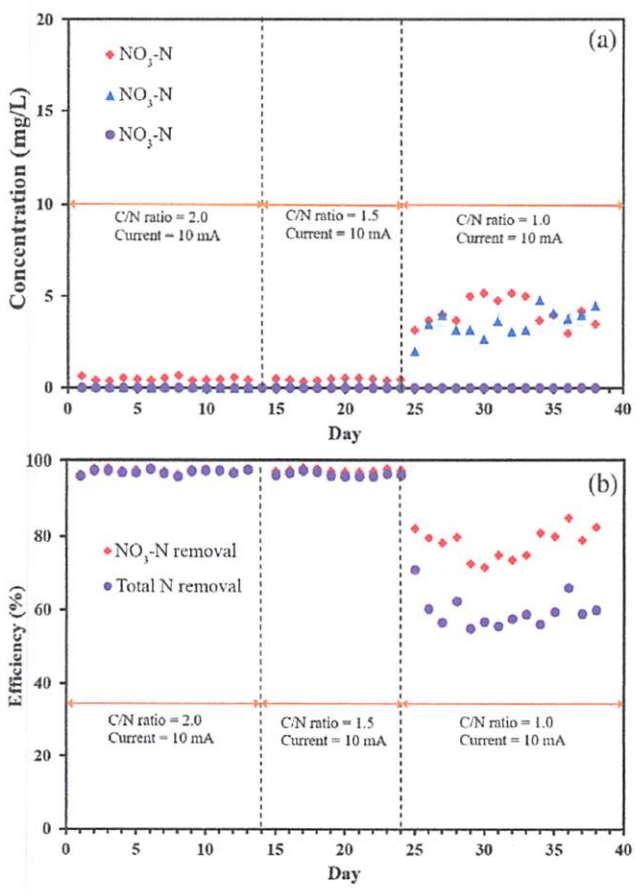

Figure 2. (a) Effluent concentration and (b) removal efficiency of Cu reactor under different C/N ratios.

0.2 g of biomass using Nucleospin® DNA tool (Macherey-nagel, Germany). DNA quantification was carried out using nanodrop. Sequencing was performed by Macrogen Inc. (South Korea) with an Illumina MiSeq system. Amplification of V3 and V4 region of 16S ribosomal DNA gene was performed by PCR using standard IUPAC nucleotide nomenclature. The protocol targeting of this region was PCR forward primer: 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; and PCR reverse primer: 5′ GTCTCGTGGGCTCGGAGATGTG TATAAGAGACAGGACTACHVGGGTATCTAATCC.

Analysis method

Water samples were collected daily and analyzed for pH, DO, nitrate (NO_3-N), nitrite (NO_2-N), ammonium (NH_4-N) and total organic carbon (TOC). The pH and DO were determined using a pH meter (Eutech Instruments) and a DO meter (CyberScan DO 110 model). In addition, ion interactions with chemicals were dependent on the prevailing environmental conditions such as pH.[18] The TOC was measured with a TOC analyzer (Analytikjena, multi N/C 2100s). NH_4-N, NO_2-N and NO_3-N were determined in accordance with the standard methods for the examination of water and wastewater.[19] The denitrification performance was measured as NO_3-N removal and total nitrogen (total N) removal as in Eqns (3) and (4):

$$\text{NO}_3\text{–N removal efficiency}\,(\%) = \left(1 - \frac{[\text{NO}_3^- - \text{N}]_{\text{effluent}}}{[\text{NO}_3^- - \text{N}]_{\text{influent}}}\right) \times 100 \quad (3)$$

$$\text{Total N removal efficiency}\,(\%) = \left(1 - \frac{[\text{NO}_3^- - \text{N}]_{\text{effluent}} + [\text{NO}_2^-]_{\text{effluent}} + [\text{NH}_4^+]_{\text{effluent}}}{[\text{NO}_3^- - \text{N}]_{\text{influent}}}\right) \times 100 \quad (4)$$

$$\text{TOC removal efficiency}\,(\%) = \left(1 - \frac{[\text{TOC}]_{\text{effluent}}}{[\text{TOC}]_{\text{influent}}}\right) \times 100 \quad (5)$$

Table 2. Summary of NO_3-N, total N and TOC removal efficiency at various operating conditions

Reactor	Applied current (mA)	C/N 2.0				C/N 1.5				C/N 1.0			
		TOC consumption (mg-TOC/mg-N)	TOC removal (%)	NO_3-N removal (%)	Total N removal (%)	TOC consumption (mg-TOC/mg-N)	TOC removal (%)	NO_3-N removal (%)	Total N removal (%)	TOC consumption (mg-TOC/mg-N)	TOC removal (%)	NO_3-N removal (%)	Total N removal (%)
Cu reactor	10	1.7	80.0 ± 0.4	97.4 ± 0.5	97.2 ± 0.6	1.1	73.3 ± 0.4	97.5 ± 0.4	96.6 ± 0.5	1.2	65.0 ± 0.3	73.7 ± 8.7	53.8 ± 11.5
	20	—	—	—	—	—	—	—	—	0.8	50.0 ± 1.2	80.5 ± 3.7	59.7 ± 4.8
	30	—	—	—	—	—	—	—	—	0.7	40.0 ± 1.3	80.9 ± 3.9	60.1 ± 4.5
SS reactor	10	1.7	85.0 ± 0.1	97.9 ± 1.1	97.6 ± 1.5	1.2	80.0 ± 0.7	97.6 ± 0.9	96.8 ± 1.4	1.4	75.0 ± 0.6	67.2 ± 12.5	52.6 ± 16.1
	20	—	—	—	—	—	—	—	—	1.2	70.0 ± 0.5	76.4 ± 3.1	58.0 ± 3.9
	30	—	—	—	—	—	—	—	—	0.9	50.0 ± 0.5	76.7 ± 3.2	57.4 ± 3.8
Bioreactor	10	1.8	92.5 ± 0.6	99.8 ± 0.3	99.8 ± 0.3	1.7	93.0 ± 0.3	93.1 ± 1.9	80.7 ± 3.7	1.7	85.0 ± 0.8	67.4 ± 10.7	47.5 ± 10.7

RESULTS AND DISCUSSION

Comparison of denitrification performance under various C/N ratios

The three reactors were operated to treat nitrate-contaminated water containing different C/N ratios to investigate the effect of the C/N ratio on nitrate removal and activity of denitrifying bacteria. The Cu reactor and SS reactor were operated at a constant 10 mA to contribute to water hydrolysis and graphite oxidation at the electrodes. The reactions mentioned above induced hydrogenotrophic denitrification to remove the contaminating NO_3-N. From the results in Fig. 2 and Table 2, the Cu reactor achieved high NO_3-N and total N removal efficiencies of approximately 97% at C/N ratios of 2.0 and 1.5. The low effluent NO_3-N and NO_2-N of *ca* 0.5 and *ca* 0.1 mg L^{-1} revealed that the contaminating NO_3-N was biologically removed through denitrification, possibly by heterotrophic and hydrogenotrophic processes. Similarly, a efficient denitrification performance of 97–98% was observed for the SS reactor (Fig. 3 and Table 2). At the lowest C/N ratio of 1.0, the best denitrification performance was found for the Cu reactor, followed by the SS reactor and the bioreactor, respectively (Figs 2–4; results summarized in Table 2). Without the applied current in the bioreactor, the contaminating NO_3-N was removed through only heterotrophic denitrification; the conventional mechanism is presented in Eqn (6).[20] Therefore, the bioreactor results revealed that heterotrophic denitrification alone could not efficiently remove NO_3-N at a low organic content, especially at C/N ratios of 1.5 and 1.0. In comparison, the combination of heterotrophic and hydrogenotrophic denitrification led to better NO_3-N and total N removal efficiencies for both Cu reactor and SS reactor. The results show that the activity of hydrogenotrophic denitrification was able to be enhanced at low organic content. The mechanism of hydrogenotrophic denitrification is explained in Eqn (7).[21] Furthermore, the total N removal rate reached a maximum of *ca* 2.4 mg L^{-1} h^{-1} at a C/N ratio of 2.0, and later reduced to *ca* 1.3 mg L^{-1} h^{-1} at a C/N ratio of 1.0 in the Cu reactor and SS reactor (*ca* 1.2 mg L^{-1} h^{-1} in the bioreactor).

$$NO_3^- + 1.08CH_3OH + H^+ \rightarrow 0.46N_2 + 0.76CO_2 + 2.44H_2O + 0.065C_5H_7O_2N \quad (6)$$

$$H_2 + 0.35NO_3^- + 0.35H^+ + 0.05CO_2 \rightarrow 0.17N_2 + 1.10H_2O + 0.01C_5H_7O_2N \quad (7)$$

From Fig. 2(a), at a C/N ratio of 1.0, similar NO_3-N and NO_2-N concentrations, ranging from 4 to 5 mg L^{-1}, remained in the Cu reactor effluent. However, a higher NO_3-N of *ca* 6.3 mg L^{-1} was significantly observed in the SS reactor effluent than NO_2-N of *ca* 2.8 mg L^{-1} (Fig. 3(a)). Therefore, the different electrode materials had significant impacts on electrochemical reactions, H_2 generation and consequently denitrification performance. In this study, using copper wire and graphite plate as electrodes in the bio-electrochemical reactor led to a higher hydrogenotrophic denitrification performance than using stainless steel wire and graphite plate as electrodes. However, the denitrification performance of the Cu reactor and the SS reactor sharply dropped during days 25–30, especially that of the SS reactor. We hypothesized that the dissolved H_2 was insufficient to promote the effective hydrogenotrophic denitrification. Therefore, increasing currents were applied to the Cu reactor and SS reactor in the next experiments.

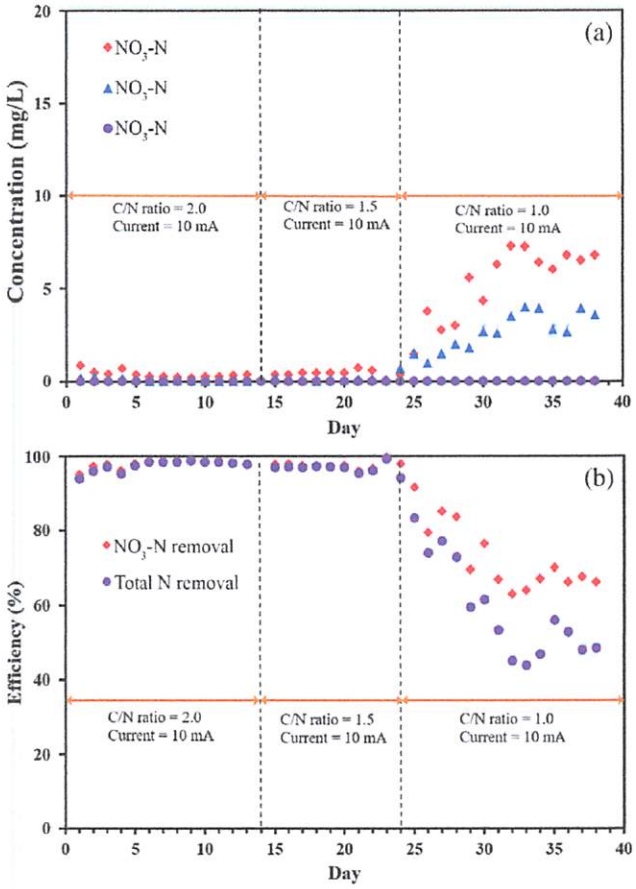

Figure 3. (a) Effluent concentration and (b) removal efficiency of SS reactor under different C/N ratios.

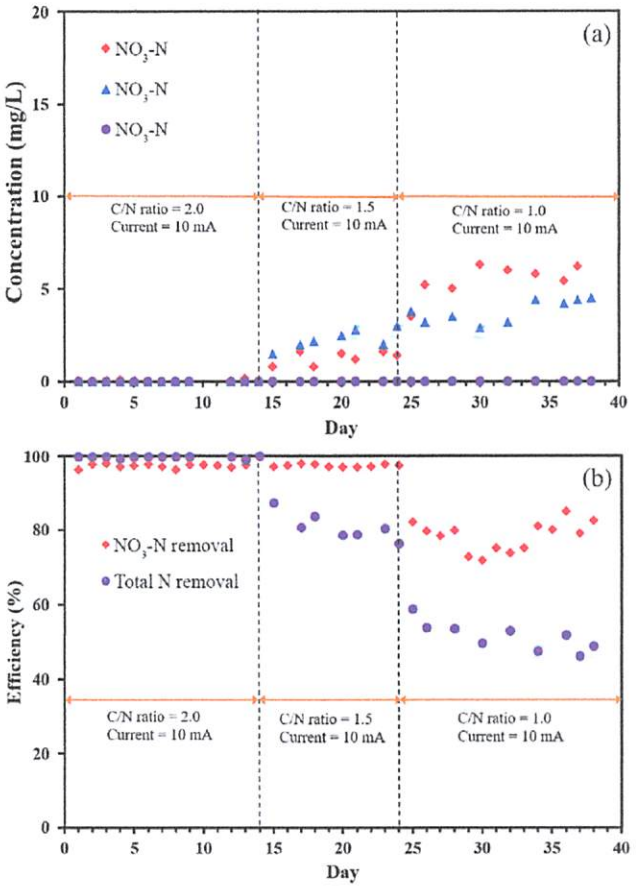

Figure 4. (a) Effluent concentration and (b) removal efficiency of bioreactor under different C/N ratios.

Comparison of denitrification performance under various applied currents

The Cu and SS reactors were continuously operated under increasing applied currents to improve the denitrification performance by enhancing hydrogenotrophic denitrification (Figs 5 and 6). In the Cu reactor, the NO_3-N and total N removal was slightly raised from 73.7% and 53.8% at 10 mA to *ca* 81% and *ca* 60% at higher currents of 20 and 30 mA. Partial denitrification still occurred; the effluent ratio of NO_2-N/NO_3-N was approximately 1.1. Similar results were observed for the SS reactor; a maximal removal of *ca* 76% for NO_3-N and *ca* 58% for total N was reached at both 20 and 30 mA, and the effluent ratio of NO_2-N/NO_3-N was nearly 1.0. In terms of the total N removal rate, it was 1.4–1.5 mg L^{-1} h^{-1} at an applied current of 30 mA for the Cu reactor and SS reactor. In comparison, the total N removal rate was similar to that of a moving bed biofilm reactor of 1.4 mg L^{-1} h^{-1},[16] and lower than a traditional biofilm reactor of 4.3 mg L^{-1} h^{-1}.[14] In addition, no NH_4-N was observed during the experiments, indicating that no NH_4-N reduction occurred at the cathode. As reported previously,[14] excessive free ammonia (>10 mg L^{-1}) caused the inhibition of a biofilm reactor for pilot-scale nitrogen removal.

In the two bio-electrochemical reactors, the microorganisms (i.e. hydrogenotrophs) formed a biofilm on the cathode, generating H_2 from water hydrolysis reaction. Surface fracture and chemical composition of the copper wire with biofilm were analyzed, as presented in Fig. 7. According to the average of three points, its elements consisted of C, 28; Mg, 27; P, 9; K, 0.5; Ca, 28 wt%. The abundant C element implied active microorganisms. The small amounts of P and K elements were from the chemicals used during contaminated water preparation. In addition, Mg and Ca were from water hardness. Since tap water containing approximately 100 mg L^{-1} as $CaCO_3$ of hardness was used for synthesizing the contaminated water, the hardness including Mg and Ca was chemically precipitated on the copper wire. The CO_2 produced from anode was dissolved in the water and generated CO_3^{2-} according to Eqns (8) and (9). The chemical precipitation from water hardness is represented in Eqns (10)–(12).[22] Therefore, the water hardness is another concerning factor for a bio-electrochemical reactor when used for actual contaminated water.

$$CO_2 + H_2O \rightarrow HCO_3^- + H^+ \quad (8)$$

$$HCO_3^- + OH^- \rightarrow CO_3^{2} + H_2O \quad (9)$$

$$Ca^{2+} + CO_3^{2-} \rightarrow CaCO_{3(s)} \quad (10)$$

$$Mg^{2+} + CO_3^{2-} \rightarrow MgCO_{3(s)} \quad (11)$$

$$Ca^{2+}\left(Mg^{2+}\right) + CO_3^{2-} \rightarrow Ca(Mg)CO_{3(s)} \quad (12)$$

Although the bio-electrochemical reactors developed in this study could not effectively remove the NO_3-N and NO_2-N, the reactor performance can be enhanced by improving reactor designs, electrode material and arrangement as well as regular electrode replacement to avoid the negative effects from

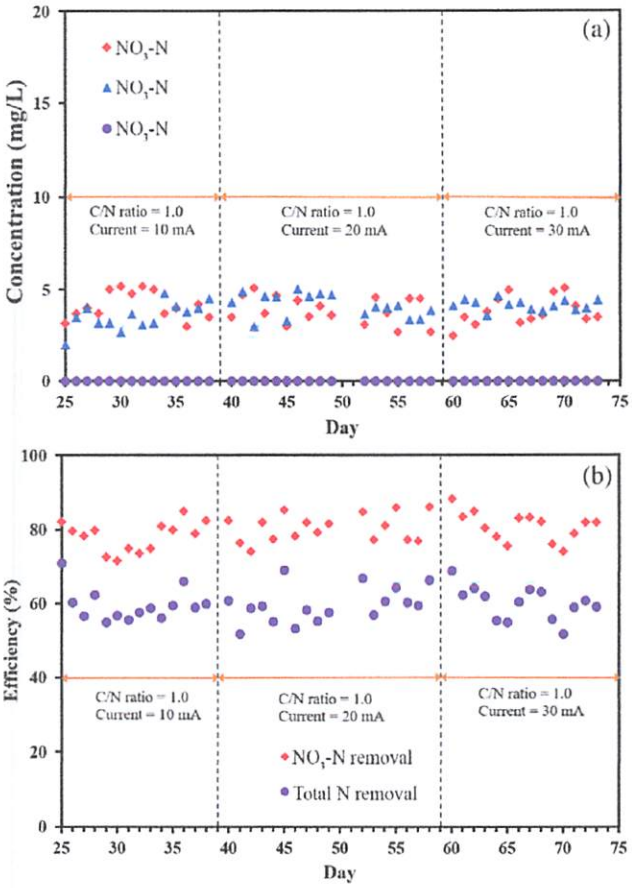

Figure 5. (a) Effluent concentration and (b) removal efficiency of Cu reactor under different applied currents.

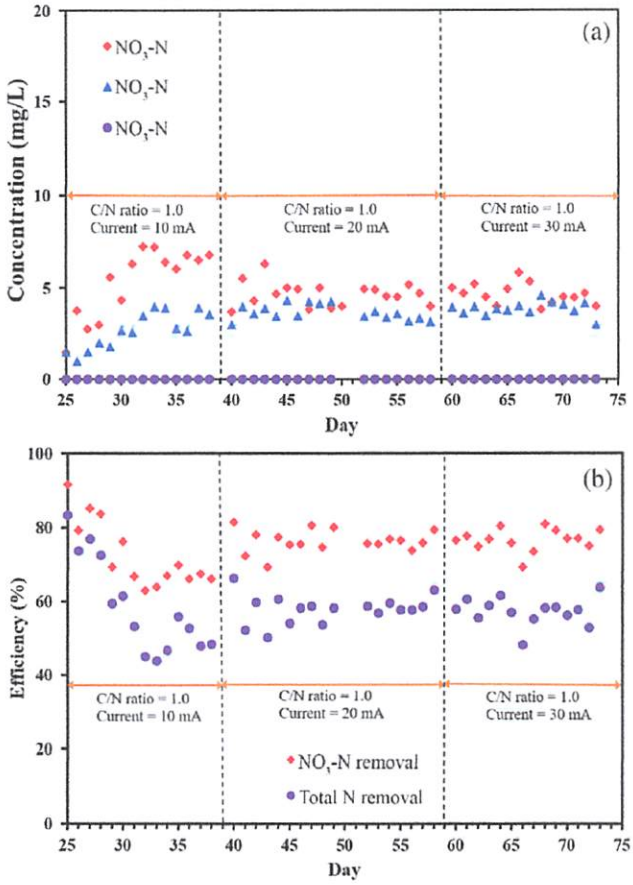

Figure 6. (a) Effluent concentration and (b) removal efficiency of SS reactor under different applied currents.

chemical deposits on the cathode. Moreover, the combination of anammox and denitrification is another solution to enhance the nitrogen removal efficiency.[23]

Organic carbon consumption for denitrification

In a comparison of various C/N ratios, the reactors achieved the maximal TOC removal efficiency at a C/N ratio of 2.0 (Fig. 8(a)). This was because heterotrophic denitrification occurred mostly at a sufficient organic carbon condition. Meanwhile, the TOC removal was decreased at the lower organic contents from inhibited heterotrophic denitrification and together with enhanced hydrogenotrophic denitrification. Among the three reactors, the highest TOC removal efficiency was in the bioreactor, followed by the SS reactor and the Cu reactor, respectively. It should be noted that the low TOC removal efficiency in the Cu reactor (i.e. 65% at a C/N ratio of 1.0) did not mean it had a poor performance. On the other hand, the Cu reactor exhibited the best cooperation of heterotrophic denitrification and hydrogenotrophic denitrification. In addition, the hydrogenotrophic denitrification in the Cu reactor could be improved by increasing the applied current, as indicated by the lowest TOC removal of 40% at an applied current of 30 mA (Fig. 8(b)).

The TOC consumption was calculated and the results are presented in Table 2. The TOC consumption was approximately 1.7–1.8 in the bioreactor and 1.1–1.7 in the Cu reactor and SS reactor. The low TOC consumption in the Cu reactor and SS reactor resulted from cooperating hydrogenotrophs and heterotrophs for denitrification. However, performance was inhibited at the lowest C/N ratio used in this study, because chemical deposit (i.e. $CaCO_3$, $MgCO_3$ and $Ca(Mg)CO_3$) accumulated on the cathode surface and prevented H_2 release into solution. When the applied current was increased in the Cu reactor and SS reactor, the TOC consumption value was gradually decreased to 0.7–0.9 due to the enhancement of hydrogenotrophic denitrification. It should be noted that nitrate removal from contaminated water containing low organic content is priority rather than organic carbon removal.

Enumeration of denitrifying bacteria

Denitrifying bacteria are common observed in a limited oxygen environment and reported as extreme-conditions-tolerating bacteria.[24,25] The number of denitrifying bacteria including autotrophic (i.e. hydrogenotrophic) and heterotrophic denitrifying bacteria were measured after phase 2 of various applied currents. Autotrophic denitrifying bacteria and heterotrophic denitrifying bacteria in biofilm from the Cu reactor cathode and SS reactor cathode and in suspended sludge appeared during reactor operation. Table 3 presents the CFU values, with autotrophic denitrifying bacteria in the biofilm being calculated as 6.7×10^6 CFU mL^{-1} in the Cu reactor and 7.7×10^5 CFU mL^{-1} in the SS reactor. Autotrophic denitrifying bacteria were more abundant than heterotrophic denitrifying bacteria in both reactors, implying that hydrogenotrophs dominated denitrification process in the biofilm. In comparison, the amount of autotrophic denitrifying bacteria in the biofilm was larger than that in the suspended sludge. The explanation was that the H_2 generated at the cathode was

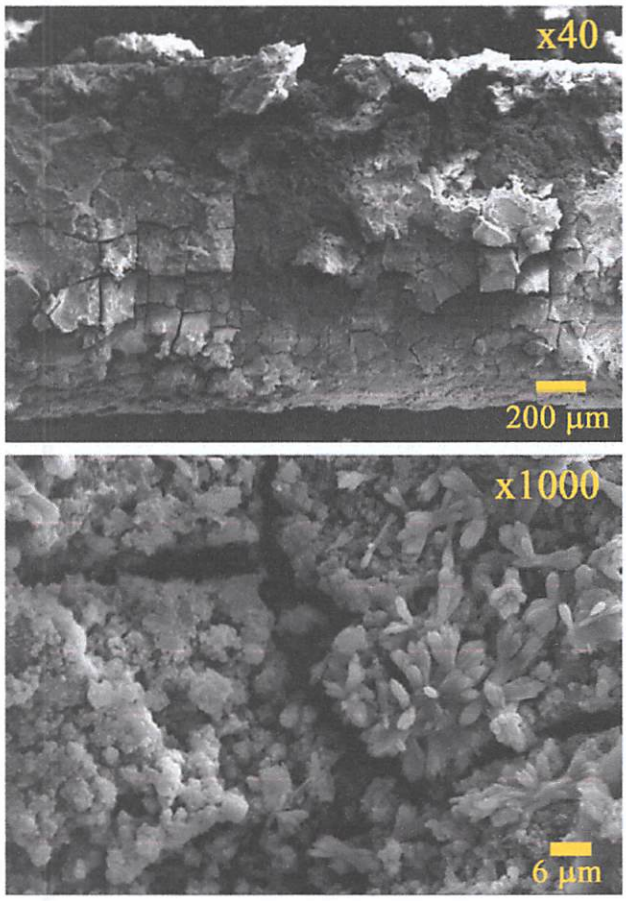

Figure 7. SEM-EDS images of copper wire.

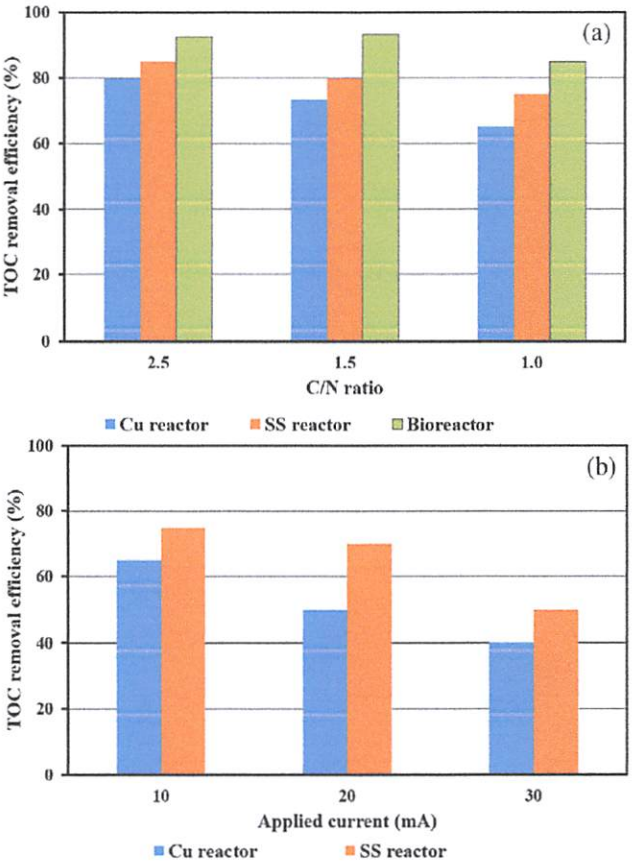

Figure 8. (a) Average TOC removal efficiency (influent TOC; C/N 2.5 = 40 ± 2 mg L^{-1}, C/N 1.5 = 30 ± 1.5 mg L^{-1} and C/N 1.0 = 20 ± 1 mg L^{-1}). (b) TOC removal efficiency as a function of applied current.

significantly consumed by autotrophic denitrifying bacteria at the biofilm. The small volume of H_2 was released into the solution and later consumed by autotrophic denitrifying bacteria in the suspended sludge. In addition, the chemical deposit at the cathode possibly reduced gaseous H_2 transfer. The greater numbers of autotrophs and heterotrophs in the Cu reactor could explain why this reactor had a better denitrification performance than the SS reactor under low C/N ratio.

The microbial community at the biofilm and in the suspended sludge of the Cu reactor was identified at phylum and genus levels using 16S rDNA gene amplicon sequencing (Fig. 9). Proteobacteria was the dominant phylum in the biofilm, accounting for over 90% of the population; the rest was classified as Actinobacteria. The bacterial community in the suspended sludge mainly consisted of Proteobacteria and Bacteriodetes, each accounting for about 40% of the population. The rest of this community was Actinobacteria and Verrucomicrobia. In comparison, the active sludge from the conventional bioreactor had the most diverse bacterial community. Similar to the suspended sludge, this community was dominated by Proteobacteria and Bacteriodetes, together with an observable level of Actinobacteria. Unlike the suspended sludge, Verrucomicrobia was absent but Ignavibacteriae, Chloroflexi and Firmiticutes were present. Over 20% sequence reads of this community cannot be assigned to any known bacterial phylum. Numerous bacterial phyla have been identified as heterotrophs and play a dominant role in nitrogen removal under aerobic and anaerobic conditions, such as Proteobacteria, Firmicutes, Bacteroidetes, Chloroflexi, Acidobacteria, Actinobacteria and Gemmatimonadetes.[26] Significantly, heterotrophic denitrifying bacteria are tolerant and commonly found in various environments including soil, river, sediment and active sludge in treatment plants[27] which was a seed sludge source in this study.

The relative abundance of the bacterial community at the genus level for the Cu reactor is shown in Fig. 9(b). *Hydrogenophaga*, *Thermomonas*, *Gammobacter* and *Nocardia* were found in both biofilm and suspended sludge. Relative abundance of *Hydrogenophaga* was higher in the biofilm (6.7%) than in the suspended sludge (3.5%). This observation is in agreement with previous reports that *Hygrogenophaga* is always found in denitrification systems and carries out hydrogenotrophic denitrification.[9,28,29] On the contrary, *Thermomonas* was less abundant in the biofilm (1.9%) than in the suspended sludge (11.9%). This genus is related to heterotrophic denitrification and tends to become more abundant when more organic carbon is available.[29] Genus *Nocadia* is defined as hydrogen-oxidizing bacteria, having the ability to utilize gaseous hydrogen as electron donor with oxygen as electron acceptor and to fix carbon dioxide.[30]

Archromobacter was absent from both suspended sludge and active sludge but accounted for nearly half (44.7%) of the biofilm bacterial community. Previous work identified functional genes such as nitrite reductase (nirS), nitric oxide reductase (qnorB) and nitrous oxide reductase (nosZ) in *Archomobacter*, implying its oxygen-tolerant denitrification potential.[31] *Flavobacterium* made up 33% and 17.4% of the bacterial community in the suspended sludge and active sludge, respectively. This genus

Table 3. Most probable number count for autotrophic and heterotrophic denitrifying bacteria

	Autotrophic denitrifying bacteria			Heterotrophic denitrifying bacteria		
Sample	Dilution	Average number of colony (CFU)	CFU mL^{-1}	Dilution	Average number of colony (CFU)	CFU mL^{-1}
Cu reactor: biofilm	10^{-4}	66	6.7×10^6	10^{-4}	52	5.2×10^6
Cu reactor: suspended sludge	10^{-3}	138	1.4×10^6	10^{-3}	88	8.8×10^5
SS reactor: biofilm	10^{-3}	77	7.7×10^5	10^{-3}	67	6.7×10^5
SS reactor: suspended sludge	10^{-2}	157	1.6×10^5	10^{-2}	211	2.1×10^5

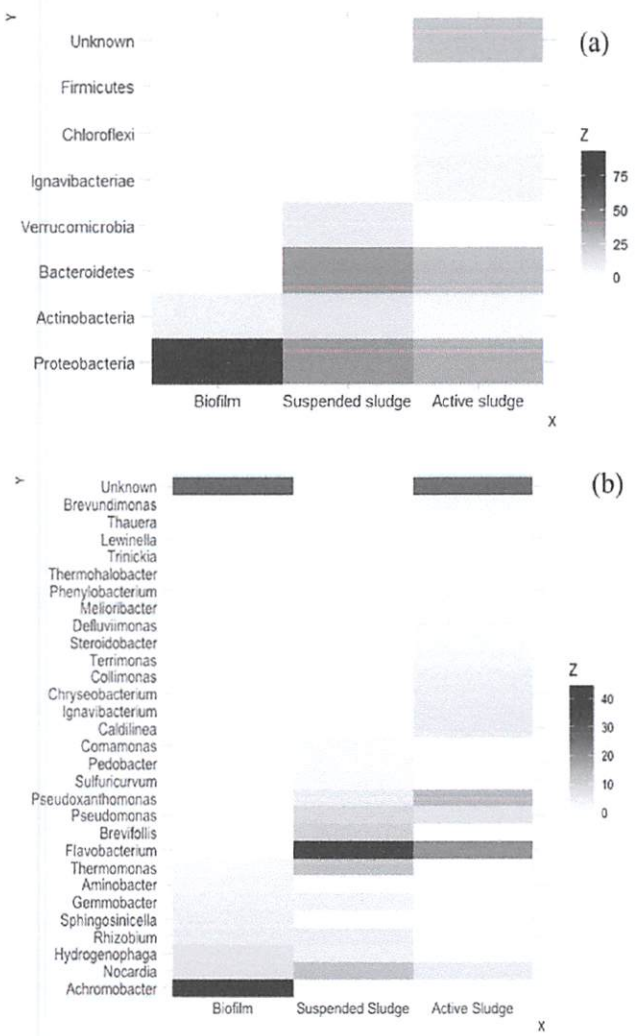

Figure 9. Abundance of bacterial community at (a) phylum and (b) genus levels; biofilm of Cu reactor, suspended sludge of Cu reactor and active sludge of bioreactor.

contributes to sludge granulation and nitrite production in a partial denitrification system.[32] The hydrogenotrophic and heterotrophic denitrification bacteria coexisted in either biofilm or suspended sludge of the Cu reactor. It is likely that the synergistic interaction between hydrogenotrophs and heterotrophs in the bio-electrochemical reactor caused the enhancement of denitrification performance. Further studies of actual contaminated water and wastewater treatment as well as microbial analysis (i.e. coexisting anammox bacteria[15,33]) are necessary to understand the reactor mechanisms and limitations.

CONCLUSIONS

The bio-electrochemical reactors (i.e. Cu reactor and SS reactor) could improve the NO_3-N and total N removal efficiency compared with the conventional bioreactor for limited-organic-carbon water. Hydrogenotrophic denitrification was an important mechanism in the Cu reactor and SS reactor at a low C/N ratio. This led to high removal efficiencies of NO_3-N and total N being observed, whereas the TOC removal efficiency was decreased: 73.7% for NO_3-N, 53.8% for total N and 65.0% for TOC in the Cu reactor operating at a C/N ratio of 1.0 and an applied current of 10 mA. Meanwhile, hydrogenotrophic denitrification was enhanced on increasing the applied current: 80.9% for NO_3-N, 60.1% for total N and 40.0% for TOC in the Cu reactor operating at a C/N ratio of 1.0 and an applied current of 30 mA. However, water hardness affected chemical deposition at the cathode, reducing gaseous H_2 generation for hydrogenotrophic denitrification. Comparing the electrode materials, the Cu reactor using copper wire as cathode provided better denitrification performance and greater bacterial number than the SS reactor using stainless steel wire as cathode. For microbial analysis, autotrophic denitrifying bacteria (i.e. hydrogenotrophs) were more abundant in the biofilm covering on the cathode than in the suspended sludge. Further studies are needed to improve the denitrification performance of the Cu reactor and SS reactor as well as application to actual water and wastewater containing high nitrogen levels, such as contaminated groundwater and agro-livestock wastewater in rural areas of Vietnam.

ACKNOWLEDGEMENTS

The study was supported by the Asia-Pacific Network for Global Change Research [grant number CBA2019-05SY-Do] and Faculty of Engineering, Naresuan University [R2564E011].

REFERENCES

1 Sajil Kumar PJ, Geostatistical modeling of fluoride enrichment and nitrate contamination in the groundwater of lower Bhavani Basin in Tamil Nadu India. *Model Earth Syst Environ* **3**:1 (2016). https://doi.org/10.1007/s40808-016-0260-1.

2 Chen J, Wu H and Qian H, Groundwater nitrate contamination and associated health risk for the rural communities in an agricultural area of Ningxia Northwest China. *Exposure Health* **8**:349–359 (2016). https://doi.org/10.1007/s12403-016-0208-8.

3 Strady E, Dang VBH, Némery J, Guédron S, Dinh QT, Denis H *et al.*, Baseline seasonal investigation of nutrients and trace metals in surface waters and sediments along the Saigon River basin impacted by the megacity of Ho Chi Minh (Vietnam). *Environ Sci Pollut Res* **24**: 3226–3243 (2017). https://doi.org/10.1007/s11356-016-7660-7.

4 Wongsanit J, Teartisup P, Kerdsueb P, Tharnpoophasiam P and Worakhunpiset S, Contamination of nitrate in groundwater and its potential human health: a case study of lower Mae Klong river basin

Thailand. *Environ Sci Pollut Res* **22**:11504–11512 (2015). https://doi.org/10.1007/s11356-015-4347-4.
5 Kim H-R, Yu S, Oh J, Kim KH, Oh YY, Kim HK *et al.*, Assessment of nitrogen application limits in agro-livestock farming areas using quantile regression between nitrogen loadings and groundwater nitrate levels. *Agric Ecosyst Environ* **286**:106660 (2019). https://doi.org/10.1016/j.agee.2019.106660.
6 Zhang H, Xu Y, Cheng S, Li Q and Yu H, Application of the dual-isotope approach and Bayesian isotope mixing model to identify nitrate in groundwater of a multiple land-use area in Chengdu plain, China. *Sci Total Environ* **717**:137134 (2020). https://doi.org/10.1016/j.scitotenv.2020.137134.
7 Yu G, Wang J, Liu L, Li Y, Zhang Y and Wang S, The analysis of groundwater nitrate pollution and health risk assessment in rural areas of Yantai, China. *BMC Public Health* **20**:437 (2020). https://doi.org/10.1186/s12889-020-08583-y.
8 Epsztein R, Beliavski M, Tarre S and Green M, Pressurized hydrogenotrophic denitrification reactor for small water systems. *J Environ Manage* **216**:315–319 (2018). https://doi.org/10.1016/j.jenvman.2017.03.010.
9 Eamrat R, Tsutsumi Y, Kamei T, Khanichaidecha W, Ito T and Kazama F, Microbubble application to enhance hydrogenotrophic denitrification for groundwater treatment. *Environ Nat Resour J* **18**:156–165 (2020). https://doi.org/10.32526/ennrj.18.2.2020.15.
10 Liang D, He W, Li C, Wang F, Crittenden JC and Feng Y, Remediation of nitrate contamination by membrane hydrogenotrophic denitrifying biofilm integrated in microbial electrolysis cell. *Water Res* **188**:116498 (2021). https://doi.org/10.1016/j.watres.2020.116498.
11 Daija L, Selberg A, Rikmann E, Zekker I, Tenno T and Tenno T, The influence of lower temperature, influent fluctuations and long retention time on the performance of an upflow mode laboratory-scale septic tank. *Desalin Water Treat* **57**:18679–18687 (2016). https://doi.org/10.1080/19443994.2015.1094421.
12 Zekker I, Artemchuk O, Rikmann E, Ohimai K, Dhar Bhowmick G, Madhao Ghangrekar M *et al.*, Start-up of Anammox SBR from non-specific inoculum and process acceleration methods by hydrazine. *Water* **13**:350 (2021). https://doi.org/10.3390/w13030350.
13 Zekker I, Bhowmick GD, Priks H, Nath D, Rikmann E, Jaagura M *et al.*, ANAMMOX-denitrification biomass in microbial fuel cell to enhance the electricity generation and nitrogen removal efficiency. *Biodegradation* **31**:249–264 (2020). https://doi.org/10.1007/s10532-020-09907-w.
14 Rikmann E, Zekker I, Tenno T, Saluste A and Tenno T, Inoculum-free start-up of biofilm- and sludge-based deammonification systems in pilot scale. *Int J Environ Sci Technol* **15**:133–148 (2018). https://doi.org/10.1007/s13762-017-1374-3.
15 Zekker I, Kivirüüt A, Rikmann E, Mandel A, Jaagura M, Tenno T *et al.*, Enhanced efficiency of nitritating-anammox SBR achieved at low decrease rates of oxidation-reduction potential. *Environ Eng Sci* **36**:350–360 (2019). https://doi.org/10.1089/ees.2018.0225.
16 Zekker I, Raudkivi M, Artemchuk O, Rikmann E, Priks H, Jaagura M *et al.*, Mainstream-sidestream wastewater switching promotes anammox nitrogen removal rate in organic-rich, low-temperature streams. *Environ Technol* **41**:1–10 (2020). https://doi.org/10.1080/09593330.2020.1721566.
17 Xu D, Xiao E, Xu P, Zhou Y, He F, Zhou Q *et al.*, Performance and microbial communities of completely autotrophic denitrification in a bioelectrochemically-assisted constructed wetland system for nitrate removal. *Bioresour Technol* **228**:39–46 (2017). https://doi.org/10.1016/j.biortech.2016.12.065.
18 Tenno T, Uiga K, Mashirin A, Zekker I and Rikmann E, Modeling closed equilibrium systems of H_2O–dissolved CO_2–solid $CaCO_3$. *Chem Eur J* **121**:3094–3100 (2017). https://doi.org/10.1021/acs.jpca.7b00237.
19 Rice EW, Baird RB and Eaton AD, *Standard Methods for the Examination of Water and Wastewater*, 23rd edn. American Public Health Association, American Water Works Association, Water Environment Federation, USA (2017).
20 Ingole DN, Biological denitrification: a review. *Int J Res Environ Sci Technol* **3**:78–85 (2014).
21 Khanitchaidecha W, Koshy P, Kamei T, Shakya M and Kazama F, Investigation of the effects of hydrogenotrophic denitrification and anammox on the improvement of the quality of the drinking water supply system. *J Environ Sci Health A* **48**:1533–1542 (2013). https://doi.org/10.1080/10934529.2013.797249.
22 Zhao Y, Feng C, Wang Q, Yang Y, Zhang Z and Sugiura N, Nitrate removal from groundwater by cooperating heterotrophic with autotrophic denitrification in a biofilm–electrode reactor. *J Hazard Mater* **192**:1033–1039 (2011). https://doi.org/10.1016/j.jhazmat.2011.06.008.
23 Kamei T, Naitoh D, Khanitchaidecha W and Kazama F, Simultaneous removal of ammonium and nitrate by a combination of ANAMMOX and hydrogenotrophic denitrification. *J Water Environ Technol* **13**:167–178 (2015). https://doi.org/10.2965/jwet.2015.167.
24 Mandel A, Zekker I, Jaagura M and Tenno T, Enhancement of anoxic phosphorus uptake of denitrifying phosphorus removal process by biomass adaption. *Int J Environ Sci Technol* **16**:5965–5978 (2019). https://doi.org/10.1007/s13762-018-02194-2.
25 Rubin S, Marín I, Gómez MJ, Morales EA, Zekker I, San Martín-Uriz P *et al.*, Prokaryotic diversity and community composition in the Salar de Uyuni, a large scale, chaotropic salt flat. *Environ Microbiol* **19**:3745–3754 (2017). https://doi.org/10.1111/1462-2920.13876.
26 Gao L, Han F, Zhang X, Liu B, Fan D, Sun X *et al.*, Simultaneous nitrate and dissolved organic matter removal from wastewater treatment plant effluent in a solid-phase denitrification biofilm reactor. *Bioresour Technol* **314**:123714 (2020). https://doi.org/10.1016/j.biortech.2020.123714.
27 Rajta A, Bhatia R, Setia H and Pathania P, Role of heterotrophic-aerobic denitrifying bacteria in nitrate removal from wastewater. *J Appl Microbiol* **128**:1261–1278 (2019). https://doi.org/10.1111/jam.14476.
28 Liu X, Huang M, Bao S, Tang W and Fang T, Nitrate removal from low carbon-to-nitrogen ratio wastewater by combining iron-based chemical reduction and autotrophic denitrification. *Bioresour Technol* **301**:122731 (2020). https://doi.org/10.1016/j.biortech.2019.122731.
29 Xing W, Li J, Li P, Wang C, Cao Y, Li D *et al.*, Effects of residual organics in municipal wastewater on hydrogenotrophic denitrifying microbial communities. *J Environ Sci* **65**:262–270 (2018). https://doi.org/10.1016/j.jes.2017.03.001.
30 Aragno M and Schlegel HG, in *The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria*, ed. by Starr MP *et al.* Springer, Berlin, pp. 865–893 (1981).
31 Kathiravan V and Krishnani K, *Pseudomonas aeruginosa* and *Achromobacter* sp.: nitrifying aerobic denitrifiers have a plasmid encoding for denitrifying functional genes. *World J Microbiol Biotechnol* **30**:1187–1198 (2014). https://doi.org/10.1007/s11274-013-1543-6.
32 Cao S, Du R, Zhang H and Peng Y, Understanding the granulation of partial denitrification sludge for nitrite production. *Chemosphere* **236**:124389 (2019). https://doi.org/10.1016/j.chemosphere.2019.124389.
33 Zekker I, Rikmann E, Tenno T, Vabamäe P, Tomingas M, Menert A *et al.*, Anammox bacteria enrichment and phylogenic analysis in moving bed biofilm reactors. *Environ Eng Sci* **29**:946–950 (2012). https://doi.org/10.1089/ees.2011.0146.

