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Nitrogen removal from ammonium-contaminated groundwater using dropping nitrification–cotton-based denitrification reactor

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ABSTRACT

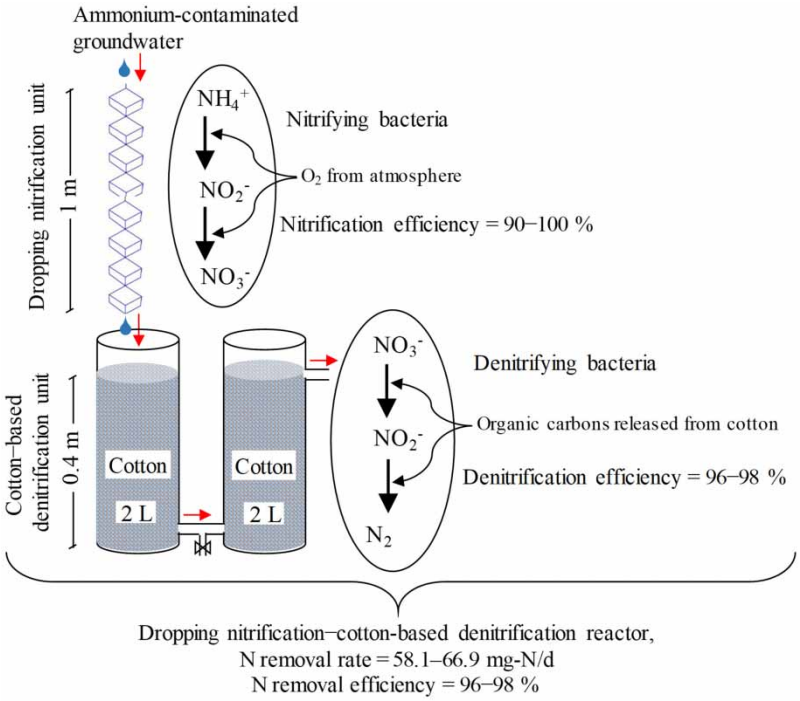
A novel dropping nitrification–cotton-based denitrification reactor was developed for total nitrogen (N) removal from ammonium (NH_4^+)-contaminated groundwater. The nitrogen removal ability of the reactor was evaluated for 91 days. A 1 m-long dropping nitrification unit was fed with synthetic groundwater containing 30 mg-NH_4^+-N/L at a flow rate of 2.16 L/d. The outlet of the dropping nitrification unit was connected to the cotton-based denitrification unit. The NH_4^+ present in the groundwater was completely oxidized (>90% nitrification efficiency) by nitrifying bacteria to nitrite (NO_2^-) and nitrate (NO_3^-) in the dropping nitrification unit. Subsequently, the generated NO_2^- and NO_3^- were denitrified (96%–98% denitrification efficiency) by denitrifying bacteria in the cotton-based denitrification unit under anoxic conditions. Organic carbons released from the cotton presumably acted as electron donors for heterotrophic denitrification. Nitrifying and denitrifying bacteria were colonized in higher abundance in the dropping nitrification and cotton-based denitrification units, respectively. The total N removal rate and efficiency of the dropping nitrification–cotton-based denitrification reactor for 91 days were 58.1–66.9 mg-N/d and 96%–98%, respectively. Therefore, the dropping nitrification–cotton-based denitrification reactor will be an efficient, sustainable, and promising option for total N removal from NH_4^+-contaminated groundwater.

Key words: biological process, cotton-based denitrification, dropping nitrification, NH_4^+-contaminated groundwater, nitrogen removal

HIGHLIGHTS

- A dropping nitrification–cotton-based denitrification reactor was proposed for N removal.
- NH_4^+ in the groundwater was nitrified to NO_3^- in dropping nitrification unit.
- The generated NO_3^- was denitrified to N_2 in cotton-based denitrification unit.
- Nitrifying and denitrifying bacteria were colonized in nitrification and denitrification units, respectively.
- The reactor achieved 96%–98% total N removal from groundwater.

GRAPHICAL ABSTRACT



INTRODUCTION

Drinking water is an essential resource for life. United Nations Sustainable Development Goal 6 is targeted to ensure the availability of water for all by 2030. Groundwater is preferred to surface water for domestic water supplies because of its quality and consistency in quality and quantity throughout the year. Approximately 50% of the drinking water supply worldwide is obtained from groundwater (Smith *et al.* 2016). Groundwater is also used for agricultural (El-Din *et al.* 2021; Zhao *et al.* 2021) and industrial purposes (Amiri *et al.* 2021; Rao *et al.* 2021). However, groundwater is contaminated with ammonium-nitrogen (NH_4^+-N) in many countries (Patterson *et al.* 2002; Lindenbaum 2012; Huang *et al.* 2015; Choudhary *et al.* 2016; Shakya *et al.* 2019) at levels higher than the limit set by the World Health Organization for drinking water (WHO 2011). Also, NH_4^+-N remains for a longer time in groundwater due to hydro-geochemical conditions (Böhlke *et al.* 2006; Atta & Yaacob 2015). Excess NH_4^+ in groundwater makes it undrinkable because of bad taste and odor, reduces the effectiveness of chlorine disinfection, and increases the possibility of pathogenic contamination during water distribution. The NH_4^+ can also be converted to nitrite (NO_2^-) and nitrate (NO_3^-) through oxidation during water treatment and distribution processes. Excess NO_2^- and NO_3^- cause methemoglobinemia and can also produce *N*-nitroso compounds (Soares 2000). Therefore, total N removal (and not only NH_4^+-N removal) from NH_4^+-contaminated groundwater before the conventional water treatment process is essential.

Several methods are used for removing NH_4^+ from groundwater and environmental water. Examples include physicochemical methods such as reverse osmosis (Kubal *et al.* 2011), ion exchange (Wang *et al.* 2007), and adsorption (Šiljeg *et al.* 2010), and biological methods such as biofilters (Štembal *et al.* 2004; Štembal *et al.* 2005), trickling sand filters (de Vet *et al.* 2009), and permeable reactive barriers (PRB) (Gibert *et al.* 2008; Robertson *et al.* 2008). Biological methods are considered eco-friendly and highly economical (Soares 2000; Huang *et al.* 2018). Biofiltration (Štembal *et al.* 2004; Štembal *et al.* 2005) is more efficient for NH_4^+ removal than trickling sand filters (de Vet *et al.* 2009). However, both systems were evaluated for lower concentrations of NH_4^+ (2.6 and 4.4 mg-N/L, respectively) and required continuous aeration and external energy. Although PRB is cost-effective in the long term, it requires a large-scale construction and incurs a high initial cost (Obiri-Nyarko *et al.* 2014). In a previous study (Maharjan *et al.* 2020), dropping nitrification was proposed as a new effective and sustainable method of NH_4^+ removal from contaminated groundwater with low costs and reduced energy consumption.

The dropping nitrification could efficiently oxidize NH_4^+ to NO_3^- and NO_3^- accumulated in the treated water. Therefore, NO_3^- removal, that is, denitrification, is essential for total N removal through nitrification.

In this study, we developed a novel biological N removal reactor by combining dropping nitrification and cotton-based denitrification units (Figure S1, Supplementary Information) and investigated its total N removal ability from NH_4^+-contaminated groundwater. Cotton, a low-cost agricultural product and a suitable and effective carbon source/electron donor for heterotrophic denitrifying bacteria (Rocca *et al.* 2006; Park & Yoo 2009; Fowdar *et al.* 2015), was selected as the attaching media and organic carbon source for the denitrification unit. We hypothesized that the NH_4^+ in groundwater will be oxidized to NO_2^- and NO_3^- in the dropping nitrification unit and the generated NO_2^- and NO_3^- will be reduced to N_2 gas in the cotton-based denitrification unit. However, to the best of our knowledge, the combination of dropping nitrification and cotton-based denitrification has not been studied yet. This is the first report proposing the novel dropping nitrification–cotton-based denitrification reactor. The objectives of this study were to demonstrate efficient and sustainable total N removal from contaminated groundwater using a dropping nitrification–cotton-based denitrification reactor and investigate the N removal mechanisms of the reactor based on nitrifying (ammonia-oxidizing bacteria [AOB] and nitrite-oxidizing bacteria [NOB]) and denitrifying bacterial abundance values.

MATERIALS AND METHODS

Synthetic NH_4^+-contaminated groundwater

Synthetic groundwater containing 30 mg-NH_4^+-N/L was prepared and used in this study. The synthetic groundwater constituents included $Na_2HPO_4{\cdot}12H_2O$ (104.5 mg/L), KH_2PO_4 (17 mg/L), NaCl (37.5 mg/L), KCl (17.5 mg/L), $CaCl_2{\cdot}2H_2O$ (23 mg/L), $MgSO_4{\cdot}7H_2O$ (25.6 mg/L), $NaHCO_3$ (353 mg/L), and $(NH_4)_2SO_4$ (141.6 mg/L); the pH was 7.84 $\pm$ 0.12. The constituents and their concentrations in the synthetic groundwater were determined based on the chemical composition of NH_4^+-contaminated groundwater sampled from the Kathmandu Valley, Nepal (Khanitchaidecha *et al.* 2012). In addition, an NO_3^--contaminated groundwater containing 30 mg-NO_3^--N/L (182.12 mg of $NaNO_3$ per L instead of $(NH_4)_2SO_4$ in the above NH_4^+-contaminated groundwater) was also synthesized and used for the start-up of denitrification units.

Experimental setup and conditions

Laboratory-scale N removal reactors, composed of a dropping nitrification unit and a cotton-based heterotrophic denitrification unit, were prepared (Figure S1). The dropping nitrification unit was made of polyolefin sponges (82 in number; 10 mm $\times$ 10 mm $\times$ 10 mm; Sekisui Aqua Systems Company, Osaka, Japan) and diagonally connected to a nylon thread. The effective length and dry weight of the nitrification unit were 1 m and 2.9 g, respectively. The dropping nitrification units (in four replicates) were incubated with 5 L synthetic NH_4^+-contaminated water mixed with activated sludge (20:1, v/v) through aeration for seven days to colonize the nitrifying bacterial community in the sponge materials. The activated sludge was collected from a municipal wastewater treatment plant in Kofu, Yamanashi, Japan. The cotton-based heterotrophic denitrification unit was made of two Pyrex columns (50 cm height and 8 cm internal diameter) for down-flow and up-flow (Figure S1). Each column was filled with approximately 104 g of cotton balls (2–3 cm diameter; 95% unbleached cotton and 5% polyester; Yoshiyuki Sawada Co. Ltd). The cotton materials were fixed in the column using a polypropylene mesh. The cotton balls were incubated in 4 L synthetic NO_3^--contaminated water mixed with activated sludge (20:1, v/v) for seven days to colonize the denitrifying bacterial community before filling in the columns. A cotton-based denitrification unit was placed immediately below the dropping nitrification unit (Figure S1). For the control experiment of the cotton-based denitrification unit, a polyolefin sponge-based denitrification unit was prepared and connected to the dropping nitrification unit. The two types of N removal reactors were prepared in two replicates and set in a greenhouse without artificial lights and temperature controllers at the University of Yamanashi, Kofu, Yamanashi, Japan. The four replicates of the dropping nitrification units were separated for two replicates of two types of denitrification units. Thus, nitrification and denitrification experiments were conducted in four and two replicates, respectively. The reactors were covered with a sun-shielding sheet from all sides to prevent microalgal growth.

Synthetic NH_4^+-contaminated groundwater was fed to the top of each dropping nitrification unit at a flow rate of 2.16 L/d (1.5 mL/min) and flowed through the nitrification and denitrification units to the final outlet. The duration of the experimental tests was 91 days. During the experimental period, water samples were collected from the top (1; inlet), middle (2), and bottom (3) of the dropping nitrification unit: the middle of the first (4), junction of the two (5), middle of the second (6), and final outlet (7; outlet) of the denitrification units (Figure S1).

Analysis of samples

The pH, redox potential (ORP), and dissolved oxygen (DO) of the inlet and outlet samples were measured on-site using a pH/temp meter (AS600, As One Corporation, Osaka, Japan), waterproof ORP meter (ORP-6041, Custom Corp., Chiyoda-ku, Japan), and DO meter (PDO-520, Fuso Inc., Chuo-ku, Japan), respectively. The water samples were filtered through a membrane filter (polypropylene, pore size = 0.45 μm; Membrane Solutions Co. Ltd, Minato-ku, Japan) and subjected to the following N and organic carbon concentration analyses. NH_4^+-N, NO_2^--N, and NO_3^--N concentrations were determined with a spectrophotometer (UVmini-1280; Shimadzu Co. Ltd, Japan) using the indophenol method, *N*-(1-naphthyl) ethylenediamine method, and ultraviolet spectrophotometric screening method, respectively, according to the *Standard Methods for the Examination of Water and Wastewater* (APHA/AWWA/WEF 1998). The dissolved organic carbon (DOC) concentration in the filtered sample was measured using a total organic carbon analyzer (ASI-L, Auto Sampler Shimadzu and TOC-L, Shimadzu TOC Analyzer, Kyoto, Japan).

The nitrification and denitrification efficiency in the dropping nitrification and denitrification units, respectively, and the total N removal rate and efficiency of dropping nitrification–denitrification reactors were calculated using Equations (1)–(4);

$$\text{Nitrification efficiency (\%)} = \frac{(NO_2^-\text{-N} + NO_3^-\text{-N})_{\text{outlet}} - (NO_2^-\text{-N} + NO_3^-\text{-N})_{\text{inlet}}}{(NH_4^+\text{-N})_{\text{inlet}} - (NH_4^+\text{-N})_{\text{outlet}}} \times 100 \quad (1)$$

$$\text{Denitrification efficiency (\%)} = \frac{(NO_2^-\text{-N} + NO_3^-\text{-N})_{\text{inlet}} - (NO_2^-\text{-N} + NO_3^-\text{-N})_{\text{outlet}}}{(NO_2^-\text{-N} + NO_3^-\text{-N})_{\text{inlet}}} \times 100 \quad (2)$$

$$\text{Total N removal rate } \left(\frac{\text{mg}}{\text{d}}\right) = (\text{Inlet N} - \text{Outlet N}) \times \text{flow rate} \quad (3)$$

$$\text{Total N removal efficiency (\%)} = \frac{(\text{Inlet N} - \text{Outlet N})}{(\text{Inlet N})} \times 100 \quad (4)$$

where inlet N and outlet N are the sums of NH_4^+-N, NO_2^--N, and NO_3^--N in the inlet and outlet samples, respectively.

Microbial community analyses

The sponge materials (approximately 100 mg wet weight) in the dropping nitrification units and cotton or sponge materials (approximately 100 mg wet weight) in the denitrification units were collected for the microbial community analysis on the final day, that is, the 91st day of operation. Microbial DNA was extracted from the sponge and cotton materials using NucleoSpin Tissue (Macherey-Nagel GmbH, Duren, Germany) according to the manufacturer's protocol. Bacterial 16S rRNA and the ammonia monooxygenase (*amoA*) gene of AOB, nitrite oxidoreductase (*nxrA*) gene of NOB, and nitrate reductase (*narG*), nitrite reductase (*nirK* and *nirS*), and nitrous oxide reductase (*nosZ*) genes of denitrifying bacteria were quantified through real-time quantitative polymerase-chain-reaction (qPCR) in a thermal cycler dice real-time system II (Takara Bio Inc., Shiga, Japan). Each 25 μL reaction mixture contained 12.5 μL of SYBR Premix Ex Taq (TaKaRa Bio), 0.5 μM of each forward and reverse primer (Table S1), 2 μL of template DNA, and 9.5 μL of deionized H_2O. The qPCR reaction conditions were as follows: initial denaturation at 95 °C for 30 s, 40 cycles at 98 °C for 5 s, annealing at the specified temperatures (which varied with the primer type; Table S1) for 50 s, and an extension at 72 °C for 1 min, followed by a dissociation stage (95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s). A standard curve for each gene based on a synthetic plasmid carrying the target sequence was plotted. All qPCRs were conducted in duplicate, and the average gene abundance in the cotton or sponge media (copies/mg of media) was calculated.

Statistical analysis

The mean and standard deviation (SD) of the physicochemical parameters, N concentrations, and DOC concentrations were calculated. The gene abundance values (± SD) in the media were also calculated. A paired *t*-test was used to examine significant differences ($P < 0.05$). The data were processed using SPSS version 20 (IBM Corp., Armonk, NY, USA).

RESULTS AND DISCUSSION

Long-term performance of dropping nitrification unit

Changes in the pH and DO in the inlet (1) and outlet samples (3) of the dropping nitrification unit for 91 days are shown in Figure 1. The pH of the outlet sample (7.22–7.95) was significantly lower ($P < 0.05$) than that of the inlet sample (7.62–7.99). The pH decrease indicated microbial nitrification (Richardson & Watmough 1999; Schmidt *et al.* 2003).

The DO contained in the inlet (1) and outlet samples (3) of the dropping nitrification unit were 1.0–2.9 and 2.9–4.1 mg/L, respectively. The DO in the outlet samples was significantly higher ($P < 0.05$) than that in the inlet samples. The synthetic groundwater trickled from the top of the nitrification unit to the bottom. During the dropping, oxygen from the atmosphere dissolved into the groundwater and increased the DO. Higher DO levels created an aerobic environment and favored microbial nitrification (Machdar *et al.* 2018).

Changes in NH_4^+-N, NO_2^--N, and NO_3^--N concentrations in the inlet (1) and outlet samples (3) and the nitrification efficiency of the dropping nitrification unit for 91 days are shown in Figure 2. The NH_4^+-N concentration in the inlet groundwater samples was 27.6–31.9 mg/L. The NH_4^+-N concentration in the outlet samples of the dropping nitrification unit was lower than 1.6 mg/L after five days. The concentrations of NO_2^--N and NO_3^--N in the outlet samples of the dropping nitrification unit were 0.0–3.0 mg/L and 22.4–29.1 mg/L, respectively. The results indicated that NH_4^+ in the groundwater sample was efficiently and sustainably converted to NO_2^- and NO_3^- through biological nitrification during the dropping process. The nitrification efficiency of the dropping nitrification unit for 91 days was 90%–100%.

Long-term performance of cotton-based denitrification unit

Variations in the pH, ORP, and DO in the inlet (average value of the outlet of the dropping nitrification units, for both denitrification units), and outlet samples of the cotton-based and sponge-based denitrification units for 91 days are shown in Figure 3. The ranges of the pH values for the inlet and outlet samples of the cotton-based and sponge-based denitrification units were 7.22–7.95, 5.08–6.53, and 6.91–7.57, respectively. The pH of the outlet sample of the cotton-based denitrification unit was significantly lower ($P < 0.05$) than that of the inlet and outlet samples of the sponge-based denitrification unit. The decrease in the pH could be attributed to proton generation during cotton-supported heterotrophic denitrification (Soares *et al.* 2000; Rocca *et al.* 2006). The ranges of the ORP values for the inlet and outlet samples of the cotton-based and sponge-based denitrification units were 8–64, (−191)–(−138), and 106–157 mV, respectively. The DO concentrations of the inlet and outlet samples of the cotton-based and sponge-based denitrification units were 2.9–4.1, 1.6–2.6, and 2.7–4.1 mg/L, respectively. The ORP and DO

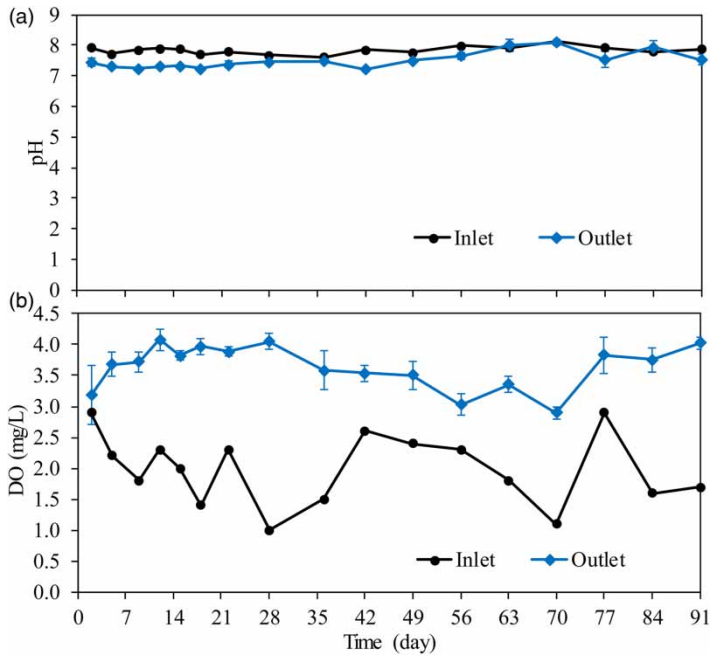


Figure 1 | Temporal variations in (a) pH and (b) DO in inlet and outlet samples for 91 days of dropping nitrification. Plots present mean values and error bars correspond to SD ($n = 4$).

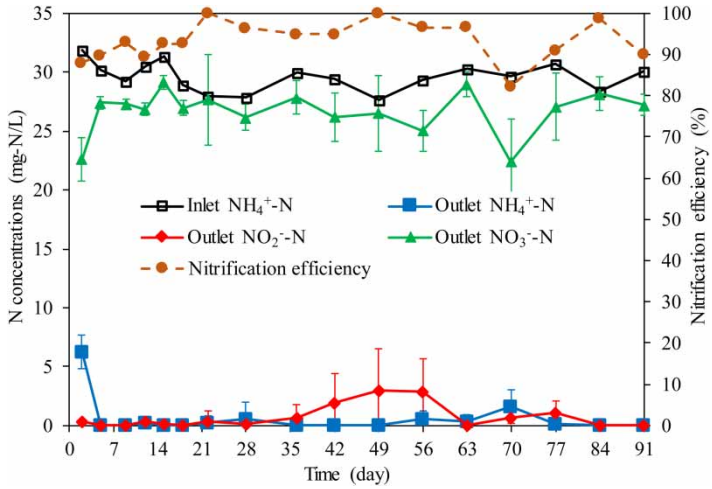


Figure 2 | Temporal variations in NH_4^+-N, NO_2^--N, and NO_3^--N concentrations in inlet and outlet samples and nitrification efficiency for 91 days of dropping nitrification. Plots present mean values and error bars correspond to SD ($n = 4$).

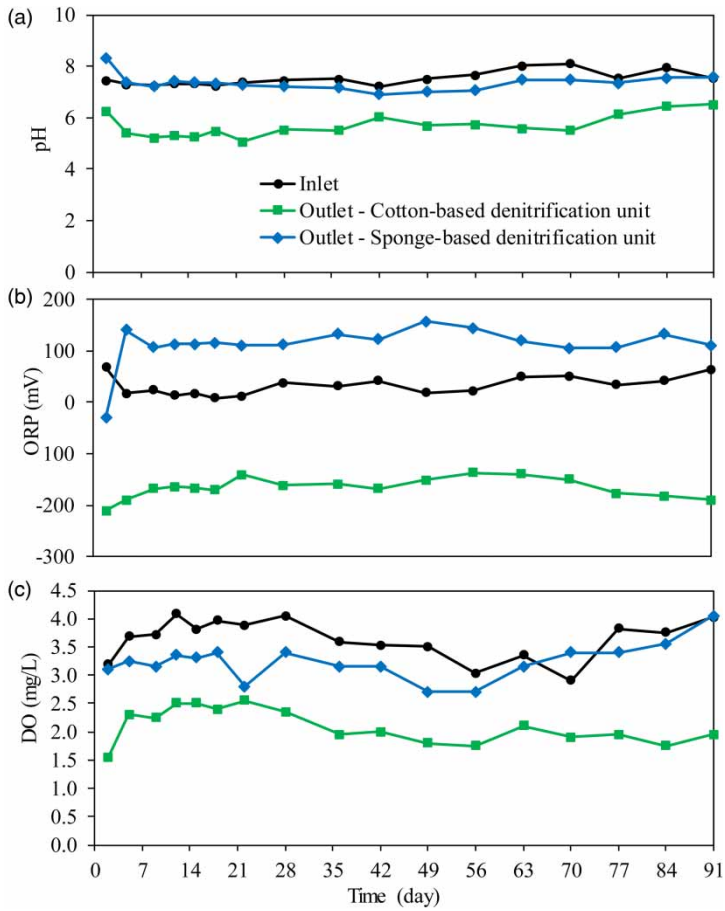


Figure 3 | Temporal variations in (a) pH, (b) ORP, and (c) DO in inlet and outlet samples of cotton-based and sponge-based denitrification units for 91 days. Plots present mean values ($n = 4$ for inlet and $n = 2$ for outlet samples).

values of the outlet samples of the cotton-based denitrification units were significantly lower ($P < 0.05$) than those of the inlet and outlet samples of the sponge-based denitrification units. The lower ORP and DO values of samples in the cotton-based denitrification units enhanced the growth and activity of denitrifying bacteria.

Changes in NH_4^+-N, NO_2^--N, and NO_3^--N concentrations in the inlet and outlet samples and denitrification efficiencies of the cotton-based and sponge-based denitrification units for 91 days are depicted in Figure 4. The concentrations of NH_4^+-N, NO_2^--N, and NO_3^--N in the outlet of the cotton-based denitrification units for 91 days were 0.0–0.4, less than the detectable limit, and 0.6–1.0, respectively. The corresponding concentrations in the outlet of the sponge-based denitrification units between 5 and 91 days were 0.0–0.6, 0.0–0.1, and 26.8–30.7, respectively. The denitrification efficiencies of the cotton-based and sponge-based denitrification units were 96%–98% and 0%–3%, respectively. These results confirmed that efficient and sustainable NO_3^- removal occurred in the cotton-based denitrification units.

The DOC concentration in the outlet sample of the cotton-based denitrification units was significantly higher ($P < 0.05$) than those in the inlet and outlet samples of the sponge-based denitrification units (Figure 5). The DOC in the cotton-based denitrification unit must have been released from the cotton. Cotton is composed of biodegradable cellulose fibers (Kim & Triplett 2001; Tokumoto *et al.* 2002) that can be gradually hydrolyzed by bacteria to release organic carbon (Aloni & Brenner 2017). The organic carbons presumably acted as electron donors for heterotrophic-denitrifying bacteria and facilitated high denitrification efficacy in the cotton-based denitrification units. Cotton is a sustainable carbon source/electron donor for denitrification in several groundwater and wastewater treatment reactors (Volokita *et al.* 1996; Fowdar *et al.* 2015; Aloni & Brenner 2017). However, the higher DOC in the outlet samples of the cotton-based denitrification units makes them unsuitable for drinking. Therefore, further studies should be conducted to reduce the release of DOC in the treated water, by optimizing the quantity of cotton and N loading rate in cotton-based denitrification units. Moreover, for practical applications of cotton-based denitrification, an organic carbon removal process using post-treatment methods, such as activated carbons and sand filters, is required.

Total N removal ability of dropping nitrification–cotton-based denitrification reactor

The dropping nitrification unit and the cotton-based denitrification unit were combined to form a dropping nitrification–cotton-based denitrification reactor. The total N removal rate and efficiency of the dropping nitrification–cotton-based

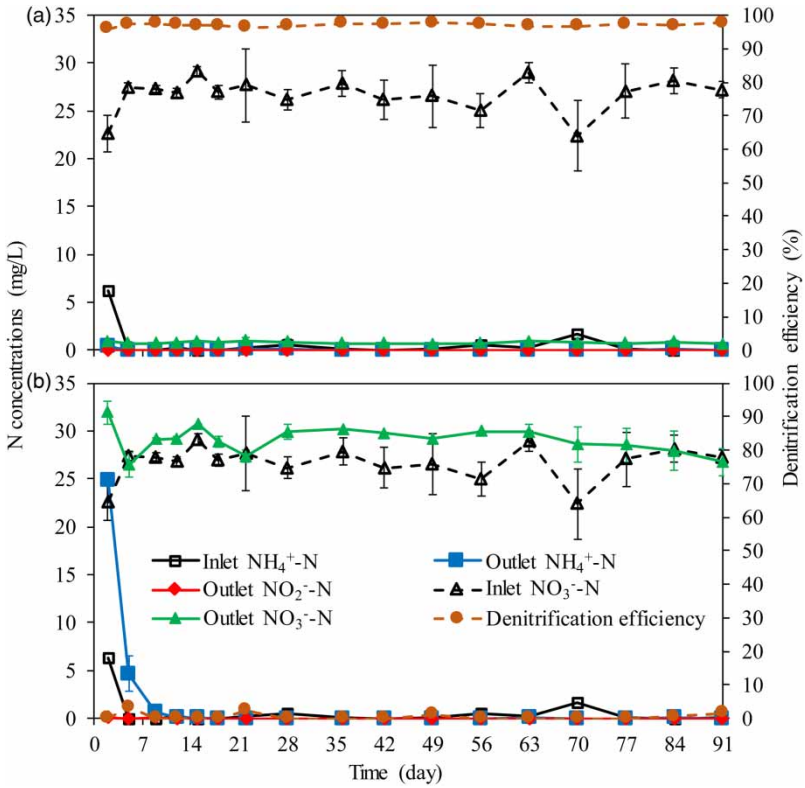


Figure 4 | Temporal variations in NH_4^+-N, NO_2^--N, and NO_3^--N concentrations in inlet and outlet samples and denitrification efficiencies of (a) cotton-based and (b) sponge-based denitrification units for 91 days. Plots present mean values and error bars correspond to SD ($n = 4$ for inlet samples and $n = 2$ for outlet samples).

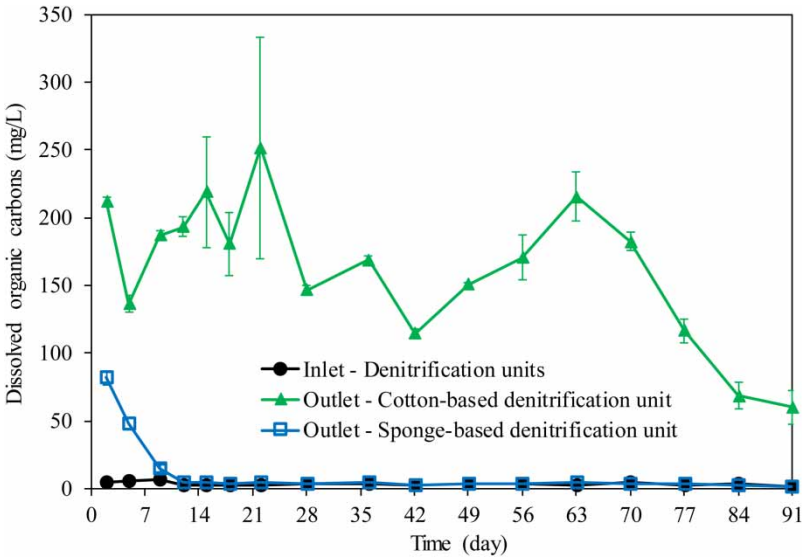


Figure 5 | Concentration of DOC in inlet and outlet samples of cotton-based and sponge-based denitrification units for 91 days. Plots present mean values and error bars correspond to SD ($n = 2$).

denitrification reactor and dropping nitrification–sponge-based denitrification reactor (control) are shown in Figure 6. The N removal rate and efficiency of the dropping nitrification–cotton-based denitrification reactor were 58.1–66.9 mg-N/d and 96%–98%, respectively, whereas those of the control reactor were limited to 0–7.6 mg-N/d and 0%–12%, respectively. A biofilter and trickling sand filters removed up to 96% and 39% of NH_4^+-N from groundwater, respectively, in an aeration facility (Štembal *et al.* 2005; de Vet *et al.* 2009). However, the reactors were investigated for low-level NH_4^+-contamination (2.6 and 4.4 mg-N/L, respectively) and the biofilter oxidized NH_4^+ only to NO_3^-; hence, the N removal efficiency was less than 12%, and that of trickling sand filters was not analyzed.

N transformation in dropping nitrification–cotton-based denitrification reactor

The variations in NH_4^+-N, NO_2^--N, and NO_3^--N concentrations in the dropping nitrification–cotton-based denitrification reactor (i.e., from sampling points 1 to 7; Figure S1) are shown in Figure 7. The NH_4^+-N concentration efficiently decreased and was converted to NO_2^--N and NO_3^--N at the dropping nitrification unit (sampling points 1 to 3). The NH_4^+-N, NO_2^--N, and

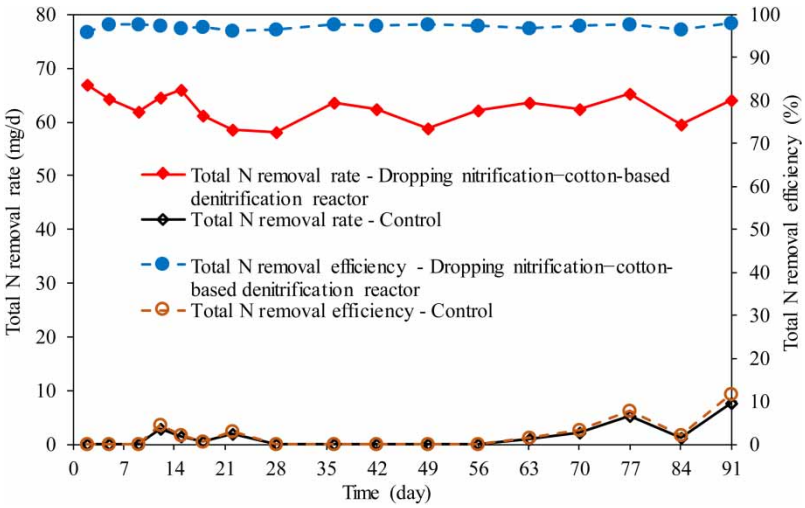


Figure 6 | Total N removal rate and efficiency of dropping nitrification–cotton-based denitrification reactor and dropping nitrification–sponge-based denitrification reactor (control) for 91 days ($n = 2$).

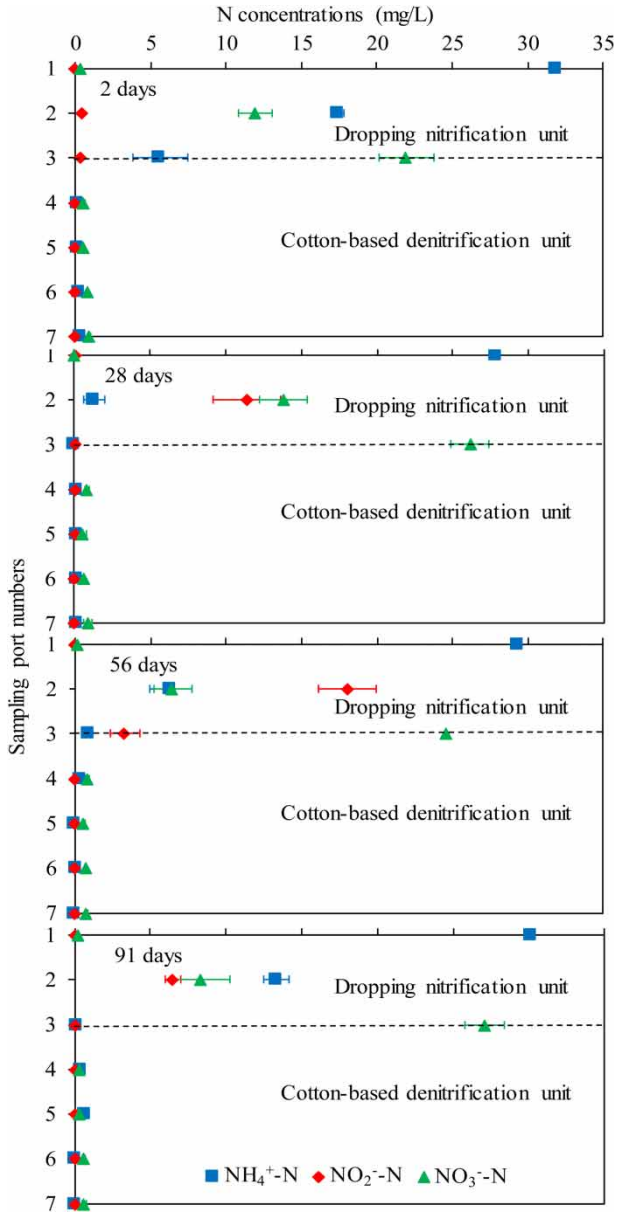


Figure 7 | Monthly transitions in NH_4^+-N, NO_2^--N, and NO_3^--N concentrations along the dropping nitrification–cotton-based denitrification reactor (i.e., in inlet (1), intermediate (2, 3, 4, 5, and 6), and outlet (7) samples). Plots present mean values and error bars correspond to SD ($n = 2$).

NO_3^--N concentrations in the cotton-based denitrification unit (sampling points 4 to 7) were minimal. The results suggested that the NH_4^+ in the synthetic groundwater was converted to NO_2^- and NO_3^- through nitrification in the dropping nitrification unit, and the generated NO_2^- and NO_3^- were converted to nitrogen gas (N_2) and entirely removed through denitrification in the cotton-based denitrification unit.

Distribution of nitrifying and denitrifying bacteria in dropping nitrification–cotton-based denitrification reactor

The abundance of bacterial 16S rRNA and AOB-*amoA*, *nxrA*, *narG*, *nirK*, *nirS*, and *nosZ* genes in the dropping nitrification–cotton-based denitrification reactor on the final day of operation (91st day) is shown in Figure 8. The bacterial 16S rRNA gene abundance ranged from 1.82×10^8 to 9.32×10^8 copies/mg-sponge in the dropping nitrification unit and 2.22×10^8 to 1.13×10^9 copies/mg-cotton in the denitrification unit. The abundance of the AOB-*amoA* gene was 9.10×10^3 copies/mg-sponge at the top; this value increased to 2.80×10^5 copies/mg-sponge in the middle of the dropping nitrification unit and then

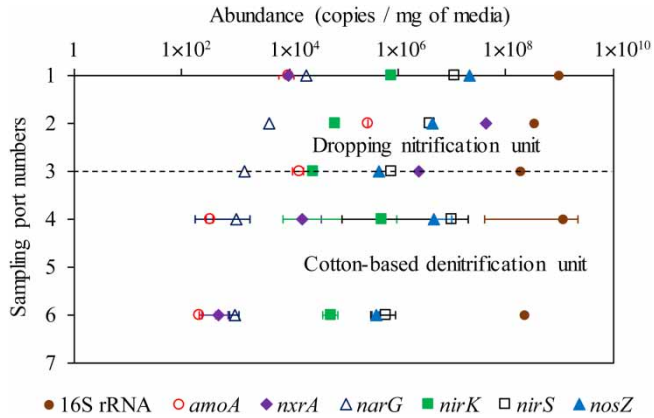


Figure 8 | Abundance of bacterial 16S rRNA and AOB-*amoA*, *nxrA*, *narG*, *nirK*, *nirS*, and *nosZ* genes in dropping nitrification–cotton-based denitrification reactor on final day (91st day) of operation. Plots present mean values and error bars correspond to SD of duplicate experiments ($n = 2$).

decreased to 2.09×10^2–3.28×10^2 copies/mg-cotton in the denitrification unit. Similarly, the abundance of the *nxrA* gene was 9.56×10^3 copies/mg-sponge at the top, which increased to 2.43×10^6–4.32×10^7 copies/mg-sponge in the dropping nitrification unit and then decreased to 4.63×10^2–1.69×10^4 copies/mg-cotton in the denitrification unit. Both AOB and NOB were detected in aerated biofilters and trickling sand filters (Štembal *et al.* 2005; de Vet *et al.* 2009).

The abundance values of *narG*, *nirK*, *nirS*, and *nosZ* were 1.44×10^3–2.01×10^4, 2.73×10^4–7.54×10^5, 7.34×10^5–1.09×10^7, and 4.51×10^5–2.08×10^7 copies/mg-sponge, respectively, in the dropping nitrification unit and 6.31×10^2–2.00×10^4, 5.78×10^4–4.92×10^5, 6.13×10^5–9.95×10^6, and 1.74×10^6–1.18×10^7 copies/mg-cotton in the denitrification unit. The abundance values of *narG*, *nirK*, *nirS*, and *nosZ* genes decreased from top to bottom of the nitrification unit, which was similar to the results of a previous dropping nitrification study (Maharjan *et al.* 2020), subsequently increased, and maintained their higher abundances in the denitrification unit. The results indicated that all types of N-transforming bacteria were present in the reactor from the top of the dropping nitrification unit to the bottom of the cotton-based denitrification unit. Nitrifying bacteria (AOB and NOB) were colonized at relatively higher abundance in the dropping nitrification unit, and denitrifying bacteria were colonized at relatively higher abundance in the cotton-based denitrification unit. The colonized nitrifying and denitrifying bacterial distribution satisfied the aerobic and anaerobic conditions of the dropping nitrification and denitrification units (Figures 1 and 3) and enabled highly efficient nitrification, that is, complete NH_4^+ oxidation to NO_3^-, and denitrification, that is, the generated NO_3^- were converted to N_2 in each respective unit (Figures 2, 4 and 7) for the total N removal in the dropping nitrification–cotton-based denitrification reactor.

Applicability and future study

The results clearly demonstrated that the laboratory-scale dropping nitrification–cotton-based denitrification reactor can efficiently and sustainably remove total N (not only NH_4^+-N, but also NO_2^--N and NO_3^--N) from NH_4^+-contaminated groundwater. The reactor may also be applicable for the treatment of NH_4^+-contaminated surface waters. We plan to investigate the N removal ability of the dropping nitrification–cotton-based denitrification reactor for different types of contaminated waters and wastewaters. Moreover, it is necessary to investigate the N removal performance of the dropping nitrification–cotton-based denitrification reactor at pilot scale, and analyze the initial and running costs of the reactor for its practical use.

CONCLUSIONS

In this study, a novel dropping nitrification–cotton-based denitrification reactor was proposed and investigated for total N removal (not only NH_4^+-N, but also NO_2^--N and NO_3^--N) from NH_4^+-contaminated groundwater. Synthetic NH_4^+-contaminated groundwater (30 mg-N/L) was treated at a flow rate of 2.16 L/d for 91 days. The NH_4^+ present in the groundwater was completely oxidized to NO_3^- in the dropping nitrification unit (>90% nitrification efficiency). Subsequently, the generated NO_3^- were almost entirely denitrified by the denitrifying bacteria in the cotton-based denitrification unit under anoxic conditions (96%–98% denitrification efficiency). The organic carbon released by the cotton presumably acted as an electron donor for heterotrophic denitrification. Nitrifying bacteria (AOB and NOB) and denitrifying bacteria were found to be colonized at a

relatively higher abundance in the dropping nitrification and cotton-based denitrification units, respectively. The total N removal rate and efficiency of the dropping nitrification–cotton-based denitrification reactor were 58.1–66.9 mg-N/d and 96%–98%, respectively, for 91 days. Hence, the dropping nitrification–cotton-based denitrification reactor will be an efficient, sustainable, and promising option for total N removal from NH_4^+-contaminated groundwater.

AUTHOR CONTRIBUTIONS

A.K.M. and T.T. conceptualized the study design. A.K.M. performed the experiments, interpreted the results, and prepared a draft of the manuscript. T.T. supervised the experiments, checked and interpreted the results, and corrected the draft of the manuscript. K.M. and K.N. discussed the results and critically reviewed the manuscript. All the authors have read and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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