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Evaluation of Performance and Microbial Community of NH_4-N and NO_3-N Bioreactors

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Abstract

Nitrogen contamination of groundwater has become an increasingly serious issue affecting the quality of drinking water. An energy efficient and low cost drinking water treatment method involving two attached growth bioreactors were developed for both NH_4-N removal and NO_3-N removal. Continuous flow of the groundwater through the NH_4-N bioreactor resulted in the removal of NH_4-N by nitrification without any aeration. The efficiency of NH_4-N removal was determined to be 70% in the laboratory and 95% in on-site trials. The higher efficiency of the on-site bioreactor resulted from the presence of various groups of local microorganisms (8 groups and 3 classes) which were cultivated from the on-site groundwater. The NO_3-N bioreactor was capable of removing NO_3-N from the groundwater efficiently by hydrogenotrophic denitrification at low H_2 supply rates. A high NO_3-N removal efficiency of 98% was found in the bioreactors that used both local microorganisms and other microorganisms that were cultivated from a drinking water system. Although the microbial community present in both NO_3-N bioreactors were different, the dominant bacterial taxonomic groups were found to be similar, i.e., *Betaproteobacteria* and *Gammaproteobacteria*. The NH_4-N and NO_3-N bioreactors are alternative methods with high efficiency and various microbial groups for nitrogen-contaminated groundwater treatment.

Keywords: Nitrogen contaminated groundwater; Nitrification; Hydrogenotrophic denitrification; Microbial community

Introduction

Nitrogen is one of the most significant contaminants commonly present in groundwater. Nitrogen can be present in different forms in contaminated water and these include ammonium-nitrogen (NH_4-N), nitrite-nitrogen (NO_2–N) and nitrate-nitrogen (NO_3–N). Groundwater is commonly polluted by anthropogenic activities such as disposal of sewage, and industrial effluents and fertilizer uses [1,2] and produced naturally by mineralization of organic matter *in situ* and by sorption of metal oxide [3]. Groundwater is a major drinking water source and there are severe health risks that arise from consumption of nitrogen-contaminated water. The World Health Organization (WHO) has set up guidelines for safe drinking water, whereby the specified concentrations of NH_4-N, NO_2-N and NO_3-N must be lower than 1.5, 0.9 and 11.3 mg/L, respectively [4].

Several technologies have been developed for removing nitrogen from the groundwater to provide safe drinking water. These technologies can be broadly categorised as in-situ technology (applying to aquifer) [5,6] and *ex-situ* technology (applying to pumped groundwater) [7,8]. The *ex-situ* technology is more preferable compared to the former because of the ease in operation and maintenance. Two well-known *ex-situ* technologies for nitrogen removal are nitrification and hydrogenotrophic denitrification. The nitrification process has been proposed for treating water containing NH_4-N contaminants; the basic operating concept involves NH_4-N oxidation to NO_3-N under a supply of oxygen (air). The hydrogenotrophic denitrification process is used for removing NO_2-N and NO_3-N under hydrogen supply and involves the reduction of both NO_2-N and NO_3-N to nitrogen gas (N_2). One of the major issues with the bioreactors for nitrification and hydrogenotrophic denitrification developed in previous studies are the high costs which make them unsuitable for use in remote areas. These high costs arise from the costs of infrastructure and maintenance, the high levels of energy consumption and the technical difficulties in operation.

The objective of this research work is to develop attached growth bioreactors that are simple to operate, energy-efficient and economical for removing NH_4-N and NO_3-N from groundwater. The performance of both bioreactors containing various initial microorganisms is discussed, while tests were done to determine the major groups present in the microbial communities.

Materials and Methods

Reactor set-up and operation

Bioreactor for NH_4-N removal: The NH_4-N bioreactor consisted of a 2 cmϕ×100 cm long acrylic column that contained 250 cm^2 polyester fibre carriers (supported by NET Co. Ltd., Japan). The fibre carriers were kept along the column for the purpose of microorganisms' attachment and water pathway. The synthetic NH_4-N groundwater (influent) was allowed to flow to the top of the fibre carriers at a flow rate of 2.9 L/day; then the influent penetrated through the fibre carriers until the end of column (effluent). The effluent was collected frequently

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for further analysis. A schematic diagram of the operating NH_4-N bioreactor is presented in Figure 1a. Before starting the bioreactor, 200 mL of concentrated activated sludge from the drinking water system in Kofu city (in Yamanashi, Japan) was fed to the bioreactor for providing the initial microorganisms on the fibre carriers.

Another NH_4-N bioreactor was scaled up and established at Chyasal area (Kathmandu Valley, Nepal), which was the location of this research program. The on-site NH_4-N bioreactor was composed of a 25 cmϕ×160 cm long acrylic column and contained approximately 1m^2 of polyester fibre carriers. The fibre carriers covered three stainless steel holders (2 cmϕ×150 cm, 8 cmϕ×150 cm and 12 cmϕ×150 cm), which were concentrically arranged in the bioreactor (Figure 1b). Droplets of groundwater were generated via 20 small droppers provided around the top of the fibre carriers and the overall flow rate was 200-250 L/day. During the experiment (with no activated sludge addition), the local microorganisms present in the groundwater were cultivated and attached to the fibre carriers [9].

Bioreactor for NO_3-N removal: The NO_3-N bioreactor consisted of an 11.5×16×16 cm acrylic container (working volume 3L) that contained 660 cm^2 polyester fibre carriers (supported by NET Co. Ltd., Japan). The fibre carriers covered a stainless steel holder and were provided for microorganism attachment (Figure 2). The synthetic NO_3-N groundwater (influent) was fed continuously to the bioreactor at a flow rate of 9.6 L/day. H_2 gas was supplied via a H_2 generator (HG260, GL Science, Japan) to the reactor at a flow rate of 70 mL/min. The liquid inside the reactor was completely mixed at 150 rpm using a stirrer. A schematic diagram of the set up is illustrated in Figure 2. Before starting the experiment, 200 mL of concentrated activated sludge (from the drinking water system in Kofu city) was fed to the bioreactor to provide initial microorganisms for attachment on the fibre carriers.

Another laboratory NO_3-N bioreactor (11.5×16×16 cm; working volume of 3 L) was set up, and this was comprised of the fibre carriers taken from the on-site NH_4-N bioreactor. The local microorganisms were used as the initial microorganisms for this bioreactor. In this experiment, the bioreactor was operated under the same conditions as the previous NO_3-N bioreactor. The operating conditions used for all experiments are summarised in Table 1.

Synthetic groundwater preparation

In this research, the groundwater at Chyasal was standardised in order to prepare the synthetic groundwater. The amount (mg/L) of different ions in the groundwater at Chyasal was determined to be:

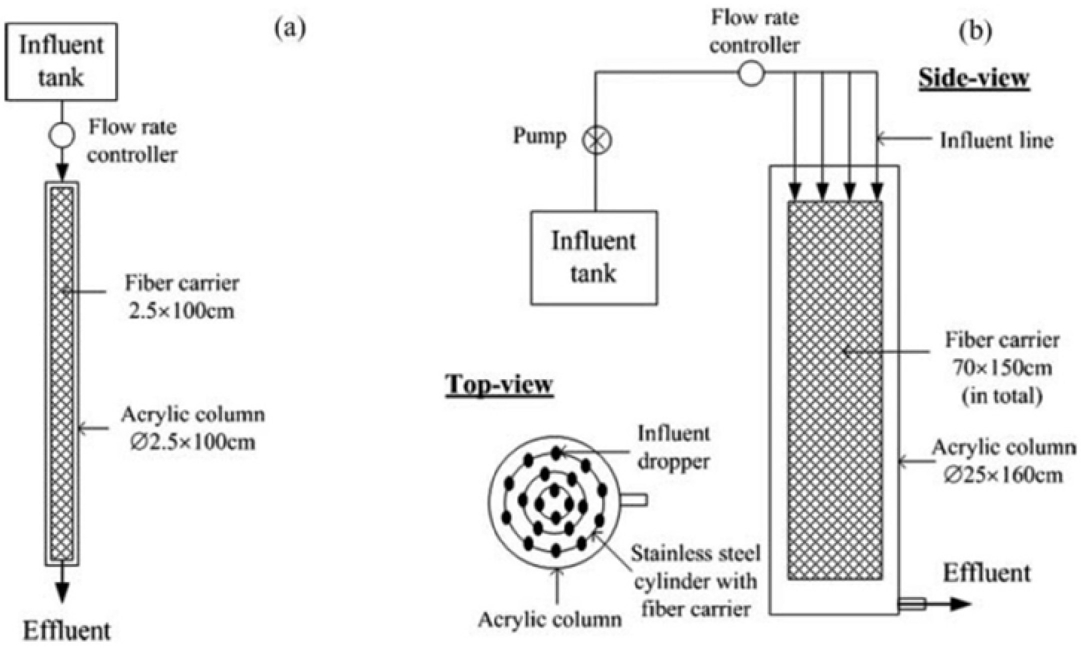

Figure 1: (a) Schematic diagram of laboratory NH_4-N bioreactor and **(b)** schematic diagram of on-site NH_4-N bioreactor.

Bioreactor	Experiment	Initial Microorganisms Source	Operating Conditions				Period (days)
			NH_4-N (mg/L)	NO_3-N (mg/L)	Air Supply (mL/min)	H_2 Supply (mL/min)	
NH_4-N bioreactor	I	Activated sludge from drinking water system	30	-	-	-	60
	II	On-site groundwater	30	-	-	-	300
NO_3-N bioreactor	III	Activated sludge from drinking water system	-	30	-	70	30
	IV	On-site groundwater	-	30	-	70	30

Table 1: Summary of the operating conditions used in the experimental studies.

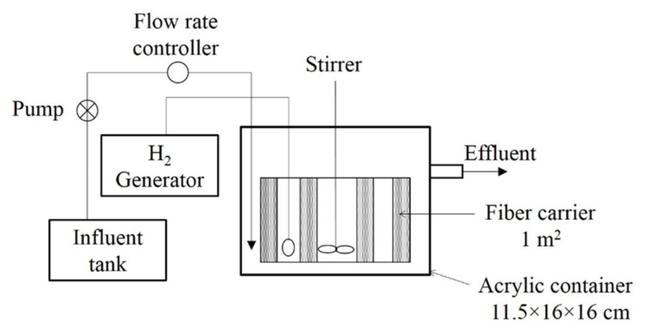

Figure 2: Schematic diagram of the NO_3-N bioreactor.

NH_4-N 15; Ca^{2+} 34; Mg^{2+} 10; K^+ 20; Na^+30; SO_4^{2-}30; and Cl^- 42 [10]. The NH_4-N containing synthetic groundwater was prepared by adding the following chemicals (g/L):$(NH_4)_2SO_4$ 0.14; $NaHCO_3$ 0.48; KCl 0.05; $CaCl_2 \cdot 2H_2O$ 0.11; $MgSO_4 \cdot 7H_2O$ 0.10; and $Na_2HPO_4 \cdot 12H_2O$ 0.02. The synthetic NO_3-N containing groundwater was prepared by adding the following chemicals (g/L): $NaNO_3$ 0.18; $NaHCO_3$ 0.48; KCl 0.05; $CaCl_2 \cdot 2H_2O$ 0.11; $MgSO_4 \cdot 7H_2O$ 0.10; and $Na_2HPO_4 \cdot 12H_2O$ 0.02.

Analytical methods

Water quality: The concentrations of NH_4-N, NO_2-N and NO_3-N in both the influent and effluent were measured using phenate, colorimetric and ultraviolet spectrophotometric screening methods, respectively in accordance with the standard methods used for the examination of water and wastewater [11]. The NH_4-N and NO_3-N removal efficiency of the NH_4-N and NO_3-N bioreactors were calculated using Equations 1 and 2, respectively.

$$NH_4-N\ \text{removal efficieincy} = (1-\frac{[NH_4-N]_{eff}}{[NH_4-N]_{inf}})\times 100 \qquad (1)$$

$$NO_3-N\ \text{removal efficieincy} = (1-\frac{[NO_3-N]_{eff}+[NO_2-N]_{eff}}{[NO_3-N]_{inf}})\times 100 \qquad (2)$$

where, $[NH_4\text{-}N]_{inf}$ = NH_4-N concentration (mg/L) in the influent

$[NH_4\text{-}N]_{eff}$ = NH_4-N concentration (mg/L) in the effluent

$[NO_3\text{-}N]_{inf}$ = NO_3-N concentration (mg/L) in the influent

$[NO_3\text{-}N]_{eff}$ = NO_3-N concentration (mg/L) in the effluent

$[NO_2\text{-}N]_{eff}$ = NO_2-N concentration (mg/L) in the effluent

Microbial analysis

The microbial communities present on the fibre carriers were identified by using a culture-independent method based on 16S rRNA gene sequencing. The total nucleic acids extracted from the fibre carriers were used as the template for amplifying 16S rRNA genes by polymerase chain reaction (PCR). The amplified DNA fragments were cloned into the *E. coli* strain DH5α [12-14]. The clonal DNAs obtained from the 16S rRNA gene libraries were subjected to restriction fragment length polymorphism (RFLP) analysis by separate digestion with HhaI and HaeIII (Takara, Shiga, Japan). The nucleotide sequence data from the representative clones of each of the RFLP groups were compared with those in the database of Ribosomal Database project by using the CLASSIFIER program developed by Michigan State University [15].

Results and Discussion

Performance of NH_4-N bioreactor

The NH_4-N bioreactor (containing initial microorganisms from the drinking water system) was operated by feeding the synthetic NH_4-N groundwater through it. The experimental results showed that the NH_4-N removal efficiency was 28% on the 1st day and it increased significantly to 68% on the 4th day. This indicates that microorganisms are present which are responsible for NH_4-N removal (e.g. nitrifiers), and moreover, the concentrations of these microorganisms were increasing rapidly. The presence of high amounts of these microorganisms is indicated by the stable value (70%) of the NH_4-N removal efficiency for 50 days. Previous studies [16,17] have identified that the major biological process for removing NH_4-N from contaminated water is nitrification. In the nitrification process, NH_4-N is oxidised to NO_3-N via the formation of intermediate NO_2-N, and high amounts of oxygen are required for complete nitrification (Equation3 [18]).

$$NH_4^+ + 1.86\ O_2 + 0.10\ CO_2 \rightarrow 0.02\ C_5H_7NO_2 + 0.98\ NO_3^- + 0.09\ H_2O + 1.98\ H^+ \qquad (3)$$

From Figure 3a, the NH_4-N concentration was seen to decrease from 40 mg/L in the influent to 10 mg/L in the effluent, while the NO_3-N concentration increased from zero in the influent to 20 mg/L in the effluent. These results clearly support the occurrence of nitrification in this bioreactor. It should be noted that although the NH_4-N bioreactor had no air and/or oxygen supply entering it, oxygen from the air could have diffused into the reaction, and this appears to have been utilized for nitrification by the microorganisms. However, the oxygen levels appear to be insufficient for complete NH_4-N removal and thus the maximal removal efficiency was ~70% in this experiment. From the results, it

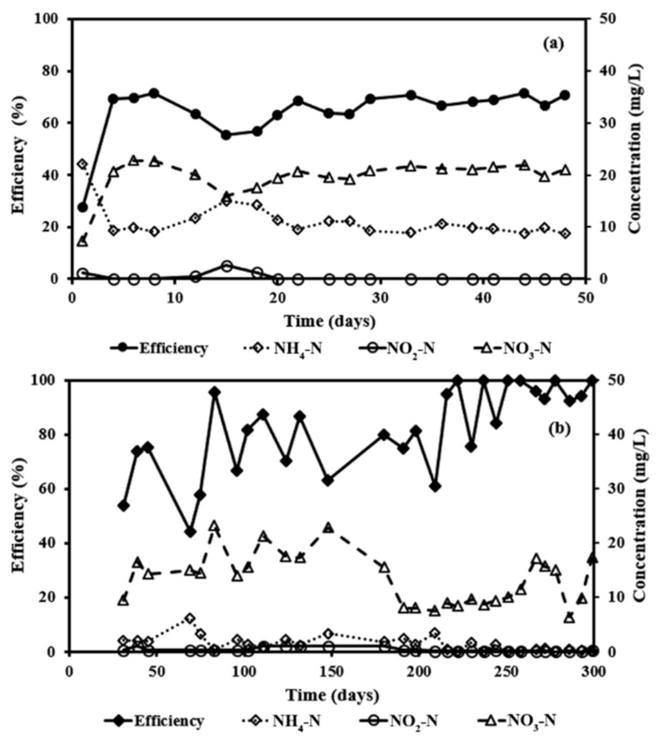

Figure 3: (a) Performance of NH_4-N bioreactor containing microorganisms from drinking water system, and **(b)** performance of NH_4-N bioreactor containing microorganisms from on-site ground water.

is seen that the NH_4-N bioreactor developed in this research can be used as an alternative method for biological groundwater treatment. The advantages of this bioreactor are lower energy consumption from aeration and pumping systems comparing to the reactors used in previous studies [19,20].

The NH_4-N bioreactor was scaled-up and operated at the site (Chyasal) and for this purpose; the microorganisms attached on the fibre carriers were cultivated from the local microorganisms present in the groundwater at Chyasal. From the experimental results, it is seen that the on-site bioreactor required a longer period to achieve the NH_4-N removal efficiency of 70%; however the efficiency of NH_4-N removal was seen to gradually increase to ~95% in 220 days. The NO_2-N in the effluent was very low (<3 mg/L) as the previous NH_4-N bioreactor. The higher efficiency of the on-site NH_4-N bioreactor is believed to result from the differences in the microbial community present in these two bioreactors, and this is discussed in the following section.

Microbial community in NH_4-N bioreactor

At the conclusion of the previous experiments, the microorganisms attached to the fibre carriers of the two NH_4-N bioreactors were identified. As seen in Figures 4a and 4b, the bioreactor that used microorganisms from the drinking water system contained 5 groups and 3 classes of bacteria, of which *Alphaproteobacteria* (25%), *Betaproteobacteria* (24%) and *Nitrospirae* (20%) were the most abundant phylogenetic groups. In contrast, bacteria in the on-site NH_4-N bioreactor consisted of 8 groups and 4 classes of which *Firmicutes* (34%) and *Alphaproteobacteria* (26%) were the dominant groups. Therefore, the greater variety of bacteria and the rich of *Firmicutes* were reasons for enhancing the nitrification process of the NH_4-N bioreactor. Another significant reason for enhancement of the bioreactor performance was the increase in total microorganisms in accordance with increasing fibre carriers area. *Firmicutes* contains the 3 classes of *Bacilli*, *Clostridia* and *Mollicutes* and are found in food- and beverage-related industries. Moreover, the abundance of *Firmicutes* in laboratory-scale nitrification bioreactor and wastewater treatment plant was also reported in literatures [21,22].

Performance of NO_3-N bioreactor

From the previous sections, the effect of the microbial community on the performance of bioreactor and dominant microbial community was observed to be different in different initial microorganisms (i.e., from the drinking water system and on-site groundwater). Two NO_3-N bioreactors were set up: one using the initial microorganisms from the drinking water system and another using the local microorganisms which were taken from the on-site NH_4-N bioreactor. The results for 30 days of experimental testing are shown in Figures 5a and 5b; both bioreactors were able to achieve high NO_3-N removal efficiencies >90%. The efficiency of bioreactor that used initial microorganisms from the drinking water system reached 95% within two days, with both the NO_2-N and NO_3-N concentrations in the effluent being <5 mg/L. On the other hand, the bioreactor that used local microorganisms required a longer period of 20 days to achieve a similar efficiency of 95%. This longer duration is attributed to the following: the microorganisms responsible for nitrification were present in greater concentrations in the fibre carriers, and thus the microorganisms responsible for denitrification (i.e., hydrogen-oxidising denitrifiers) were cultivated at a slower rate. The presence of NO_2-N in the effluent indicates the cultivation of small numbers of hydrogen-oxidising denitrifiers. The decrease in the NO_2-N concentration to almost zero in 25 days reflects the rich presence of hydrogen-oxidising denitrifiers in the bioreactor. To confirm the occurrence of hydrogenotrophic denitrification in the NO_3-N bioreactor, the supply of H_2 to the bioreactors was stopped after finishing the experiments. However, this resulted in a cessation of the NO_3-N removal (data not shown). Therefore, NO_3-N was removed by hydrogenotrophic denitrification, as presented in Equation 4 [23]). From the results, it can be concluded that the NO_3-N bioreactor can remove NO_3-N from groundwater at a very high efficiency, and moreover, this system has advantages of being simple, easy to operate and requiring less H_2comparing to the reactors used in previous studies [24,25].

$$H_2 + 0.35NO_3^- + 0.35H^+ + 0.05CO_2 \rightarrow 0.01C_5H_7NO_2 + 0.17N_2 + 1.10H_2O \quad (4)$$

Microbial community of NO_3-N bioreactor

At the end of the experimental work, the microbial community in the fibre carriers in both NO_3-N bioreactors were identified. The results reveal that the microbial community in the NO_3-N bioreactor that used initial microorganisms from the drinking water system consisted of 7 bacterial taxonomic groups and 3 classes, with the *Betaproteobacteria*

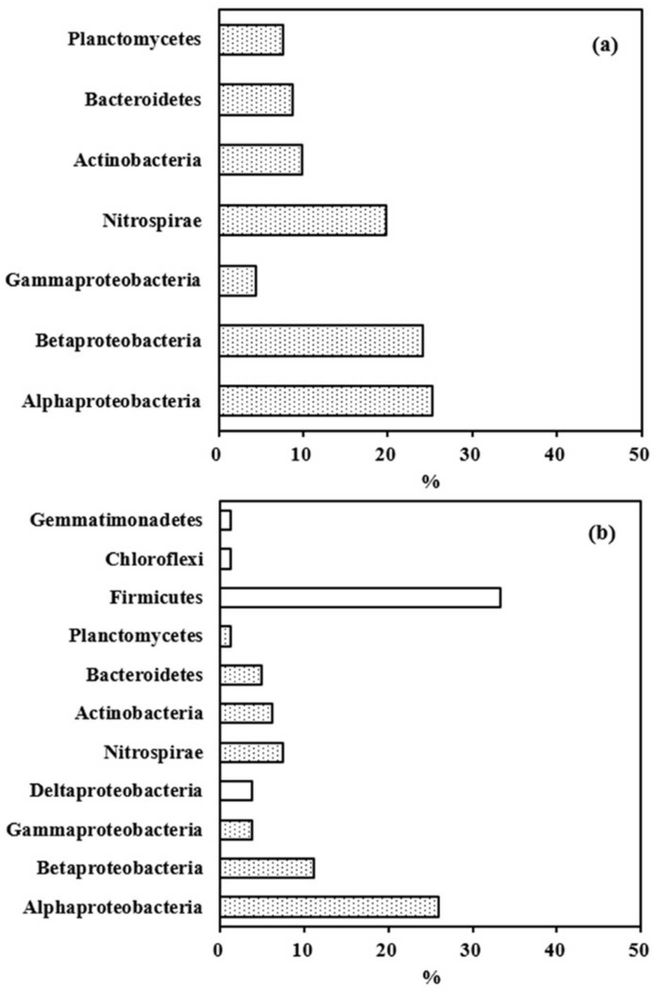

Figure 4: Details of the microbial community present in the NH_4-N bioreactor based on the use of initial microorganisms from (a) the drinking water system and (b) the on-site groundwater.

being the most abundant phylogenetic group (47%). On the other hand, in the NO_3-N bioreactor that used local microorganisms, the microorganism community consisted of 5 groups and 3 classes, with *Gammaproteobacteria* and *Beta proteobacteria* being the dominant types at 50% and 30%, respectively. Regarding literatures [24,26], *Proteobacteria* is the most microorganisms reported as hydrogen-oxidising denitrifiers and especially of *Betaproteobacteria*. *Thauera* is example of *Beta proteobacteria* responsible for hydrogenotrophic denitrification, its denitrification rate was 0.1-0.2 mg N/mg VSS·d [27]. In addition, *Gammaproteobacteria* including *Escherichia, Acinetobacter* and *Methylobacter* was detected significantly in the groundwater in the Kathmandu Valley [9].

The microbial community in the latter had lower numbers of bacterial groups compared to both the former NO_3-N reactor and also compared to the on-site NH_4-N bioreactor. This is because in the second NO_3-N bioreactor, the microorganisms responsible for hydrogenotrophic denitrification were cultivated from the local microbial community which is rich in nitrifiers. Therefore the groups of hydrogen-oxidising denitrifiers were limited in the microbial groups in the on-site bioreactor alone, as indicated by the similarity in the microbial groups in Figures 5b and 6b.

Based on the experimental results, the groundwater is kept in the bioreactor for 1-2 hours (NH_4-N bioreactor) and 4-6 hours (NO_3-N bioreactor). The effect of the presence of the microorganisms (e.g. *Firmicutes*, *Betaproteobacteria*, etc.) on the drinking water quality is currently unknown or very limited, and thus further studies are required to investigate these effects.

Conclusions

Simplistic NH_4-N and NO_3-N bioreactors were developed for removing nitrogen-containing species (NH_4-N and NO_3-N) from the groundwater. In the NH_4-N bioreactor, nitrification occurred and its efficiency was in the range of 70-95%. The high amounts of *Firmicutes* phylogenetic group, along with a diverse variety of other microbes resulted in the greater NH_4-N removal efficiency of the on-site NH_4-N bioreactor that used local microorganisms. A very high NO_3-N removal efficiency of 98% was achieved in the NO_3-N bioreactors using local microorganisms and microorganisms from the drinking water system. This is because *Proteobacteria* is the most abundant microorganisms in both NO_3-N bioreactors. However, the NO_3-N bioreactor using local microorganisms required a longer duration for cultivation. Furthermore, the microorganisms remaining in the treated groundwater will be further analysed before implying the bioreactors to the drinking water system in remote areas.

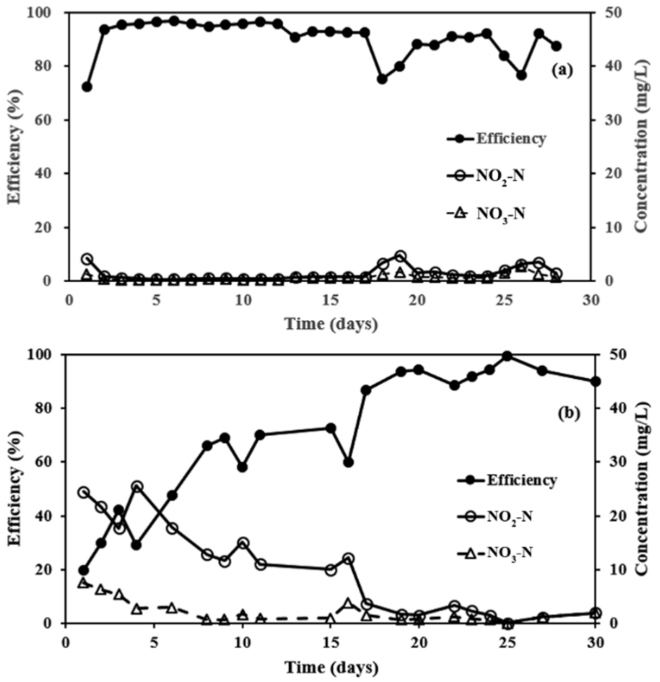

Figure 5: (a) Performance of NO_3-N bioreactor containing microorganisms from drinking water system, and (b) performance of NO_3-N bioreactor

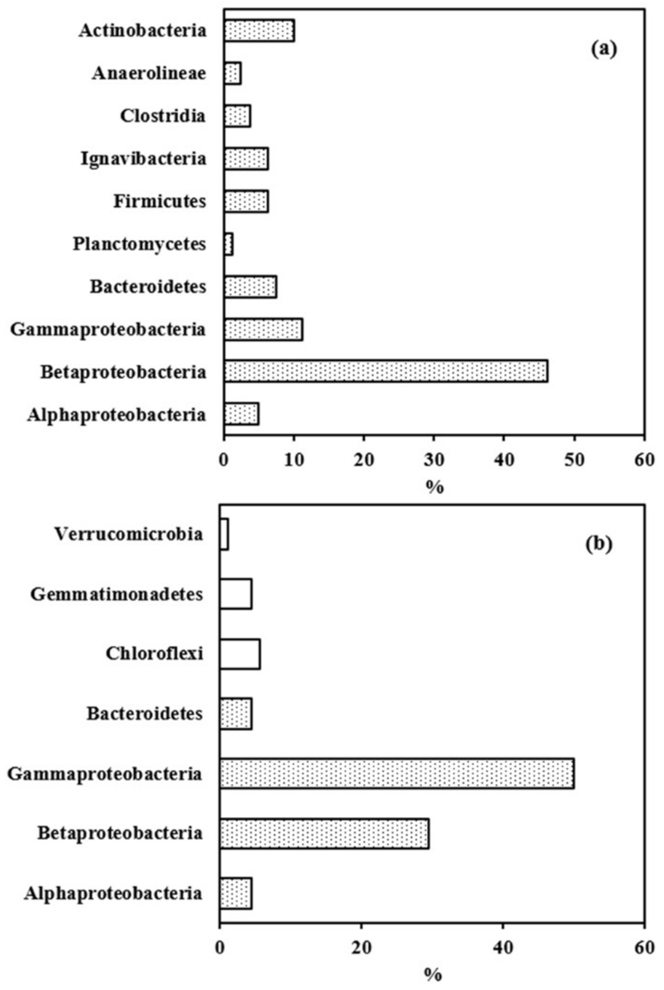

Figure 6: Details of the microbial community present in NO_3-N bioreactor using different initial microorganisms from (a) the drinking water system and (b) the on-site groundwater.

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