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$_2$-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$_2$-N bioreactor was capable of removing NO$_2$-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$_2$-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$_2$-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 oxides [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 (applied to aquifer) [5,6] and ex-situ technology (applied 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$_2$-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$_2$-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 cm$^2$×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...
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 cmØ×160 cm long acrylic column and contained approximately 1 m$^2$ of polyester fibre carriers. The fibre carriers covered three stainless steel holders (2 cmØ×150 cm, 8 cmØ×150 cm and 12 cmØ×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 3 L) 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.](image-url)
NH₄-N 15; Ca²⁺ 34; Mg²⁺ 10; K⁺ 20; Na⁺ 30; SO₄²⁻ 30; and Cl⁻ 42 [10]. The NH₄-N containing synthetic groundwater was prepared by adding the following chemicals (g/L): (NH₄)₂SO₄ 0.14; NaHCO₃ 0.48; KCl 0.05; CaCl₂·2H₂O 0.11; MgSO₄·7H₂O 0.10; and Na₂HPO₄·12H₂O 0.02. The synthetic NO₃-N containing groundwater was prepared by adding the following chemicals (g/L): NaNO₃ 0.18; NaHCO₃ 0.48; KCl 0.05; CaCl₂·2H₂O 0.11; MgSO₄·7H₂O 0.10; and Na₂HPO₄·12H₂O 0.02.

**Analytical methods**

**Water quality:** The concentrations of NH₄-N, NO₂-N and NO₃-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 NH₄-N and NO₃-N removal efficiency of the NH₄-N and NO₃-N bioreactors were calculated using Equations 1 and 2, respectively.

\[
\text{NH₄-N removal efficiency (1)} = \left(1 - \frac{[\text{NH₄-N}]_{\text{inf}}}{[\text{NH₄-N}]_{\text{eff}}} \right) \times 100
\]

\[
\text{NO₃-N removal efficiency (2)} = \left(1 - \frac{[\text{NO₃-N}]_{\text{inf}} + [\text{NO₂-N}]_{\text{eff}}}{[\text{NO₃-N}]_{\text{eff}}} \right) \times 100
\]

where, \([\text{NH₄-N}]_{\text{inf}} = \text{NH₄-N concentration (mg/L)}\) in the influent

\([\text{NH₄-N}]_{\text{eff}} = \text{NH₄-N concentration (mg/L)}\) in the effluent

\([\text{NO₃-N}]_{\text{inf}} = \text{NO₃-N concentration (mg/L)}\) in the influent

\([\text{NO₃-N}]_{\text{eff}} = \text{NO₃-N concentration (mg/L)}\) in the effluent

\([\text{NO₂-N}]_{\text{eff}} = \text{NO₂-N concentration (mg/L)}\) 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 NH₄-N bioreactor**

The NH₄-N bioreactor (containing initial microorganisms from the drinking water system) was operated by feeding the synthetic NH₄-N groundwater through it. The experimental results showed that the NH₄-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 NH₄-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 NH₄-N removal efficiency for 50 days. Previous studies [16,17] have identified that the major biological process for removing NH₄-N from contaminated water is nitrification. In the nitrification process, NH₄-N is oxidised to NO₃-N via the formation of intermediate NO₂-N, and high amounts of oxygen are required for complete nitrification (Equation 3 [18]).

\[
\text{NH₄}^+ + 1.86 \text{O}_2 + 0.10 \text{CO}_2 \rightarrow 0.02 \text{C}_5\text{H}_7\text{NO}_2 + 0.98 \text{NO}_3^- + 0.09 \text{H}_2\text{O} + 1.98 \text{H}^+
\]

From Figure 3a, the NH₄-N concentration was seen to decrease from 40 mg/L in the influent to 10 mg/L in the effluent, while the NO₃-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 NH₄-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 NH₄-N removal and thus the maximal removal efficiency was ~70% in this experiment. From the results, it
is seen that the NH₄-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 compared to the reactors used in previous studies [19,20].

The NH₄-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 NH₄-N removal efficiency of 70%; however the efficiency of NH₄-N removal was seen to gradually increase to ~95% in 220 days. The NO₂-N in the effluent was very low (<3 mg/L) as the previous NH₄-N bioreactor. The higher efficiency of the on-site NH₄-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 NH₄-N bioreactor**

At the conclusion of the previous experiments, the microorganisms attached to the fibre carriers of the two NH₄-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 NH₄-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 NH₄-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 NO₃-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 NO₃-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 NH₄-N bioreactor. The results for 30 days of experimental testing are shown in Figures 5a and 5b; both bioreactors were able to achieve high NO₃-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 NO₃-N and NO₂-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 NO₂-N in the effluent indicates the cultivation of small numbers of hydrogen-oxidising denitrifiers. The decrease in the NO₂-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 NO₃-N bioreactor, the supply of H₂ to the bioreactors was stopped after finishing the experiments. However, this resulted in a cessation of the NO₃-N removal (data not shown). Therefore, NO₃-N was removed by hydrogenotrophic denitrification, as presented in Equation 4 [23].

\[
\text{H}_2 + 0.35\text{NO}_3^- + 0.35\text{H}^+ + 0.05\text{CO}_2 \rightarrow 0.01\text{C}_5\text{H}_7\text{NO}_2 + 0.17\text{N}_2 + 1.10\text{H}_2\text{O}
\]

(4)

**Microbial community of NO₃-N bioreactor**

At the end of the experimental work, the microbial community in the fibre carriers in both NO₃-N bioreactors were identified. The results reveal that the microbial community in the NO₃-N bioreactor that used initial microorganisms from the drinking water system consisted of 7 bacterial taxonomic groups and 3 classes, with the Betaproteobacteria and Firmicutes being the most abundant groups. In contrast, the on-site NO₃-N bioreactor had 10 groups and 3 classes of bacteria, of which Alphaproteobacteria (34%), Betaproteobacteria (24%) and Nitrospirae (20%) were the most abundant phylogenetic groups. Therefore, the greater variety of bacteria and the rich of Firmicutes were reasons for enhancing the nitrification process of the NO₃-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].

**Figure 4:** Details of the microbial community present in the NH₄-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\textsubscript{3}-N bioreactor that used local microorganisms, the microbial 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. \textit{Thauera} is example of Betaproteobacteria responsible for hydrogenotrophic denitrification, its denitrification rate was 0.1–0.2 mg N/mg VSS\textsubscript{d} [27]. In addition, Gammaproteobacteria including Escherichia, Acinetobacter and Methyllobacter 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\textsubscript{3}-N reactor and also compared to the on-site NH\textsubscript{4}-N bioreactor. This is because in the second NO\textsubscript{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\textsubscript{4}-N bioreactor) and 4–6 hours (NO\textsubscript{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\textsubscript{4}-N and NO\textsubscript{3}-N bioreactors were developed for removing nitrogen-containing species (NH\textsubscript{4}-N and NO\textsubscript{3}-N) from the groundwater. In the NH\textsubscript{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\textsubscript{4}-N removal efficiency of the on-site NH\textsubscript{4}-N bioreactor that used local microorganisms. A very high NO\textsubscript{3}-N removal efficiency of 98% was achieved in the NO\textsubscript{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\textsubscript{3}-N bioreactors. However, the NO\textsubscript{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.

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References


