

Article

Removal of Nitrate Nitrogen by *Rhodotorula graminis* Immobilized in Alginate Gel for Groundwater Treatment

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Abstract: Groundwater is the source of all tap water in Kumamoto City, Japan. However, the concentration of nitrate nitrogen (NO_3^- -N) tends to increase every year due to the influences of over-fertilization, field disposal of livestock manure, and inflow of domestic wastewater. A heterotrophic nitrification–aerobic denitrification (HN-AD) system is an attractive approach for nitrate-nitrogen removal. In this study, *Rhodotorula graminis* NBRC0190, a naturally occurring red yeast that shows high nitrogen removal performance in glucose, was immobilized on calcium alginate hydrogel beads. NO_3^- -N removal efficiency exceeded 98% in the region of NO_3^- -N concentration below 10 mg/L in the model groundwater. Even after the same treatment was repeated five times, the denitrification performance of the *R. gra* immobilized alginate hydrogel beads was maintained. Finally, when this treatment method was applied to actual groundwater in Kumamoto City, it was possible to make the water of even higher quality.

Keywords: *Rhodotorula graminis*; nitrate nitrogen; groundwater; heterotrophic nitrification; aerobic denitrification; nitrogen removal



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1. Introduction

Kumamoto City, with a population of 0.7 million, uses groundwater as its water source for all its tap water. The water quality of groundwater sources in the city is comparable to commercial mineral water, except for nitrate nitrogen [1]. The concentration of nitrate nitrogen in the groundwater tends to increase every year due to the influences of overfertilization, field disposal of livestock manure, and inflow of domestic wastewater. The removal of nitrate nitrogen from groundwater is an important issue to be solved from the perspective of maintaining sustainability. Excess organic nitrogen entering the soil decomposes into ammonium, nitrite, and nitrate ions in the soil. These ions return to nitrogen molecules and organic nitrogen through denitrification and assimilation, respectively. In this way, the nitrogen cycle proceeds, but excess nitrate nitrogen pollutes groundwater. Various treatments have been applied to remove nitrate nitrogen from water sources; i.e., biological removal [2–5], ion exchange [6,7], reverse osmosis [8], and chemical reduction [9–11].

Biological nitrification and denitrification are widely applied because they do not pollute the environment and have low maintenance costs. Traditionally, nitrification reactions under aerobic conditions and denitrification reactions under anaerobic conditions have been treated in independent processes. Robertson and Kuenen [12] reported that *Thiosphaera pantotropha*, a heterotrophic bacterium, acts on the denitrification reaction even under aerobic conditions. The heterotrophic–aerobic denitrification system using such bacteria has attracted much attention because of their advantages such as high tolerance in acidic condition, high growth rate, and simultaneous nitrification and denitrification [13]. Bacteria with the ability to remove nitrogen by HN-AD include *Agrobacterium* sp. LAD9 [14], *Acinetobacter junii* YB [15], *Pseudomonas stutzeri* T1 [16], *Pseudomonas tolaasii* Y-11 [17], and

Sporidiobolus pararoseus Y1 [4]. These studies have focused on wastewater treatment, and there have been no reports of denitrification reactions in the dilute concentration range of nitrate nitrogen below 10 mg/L.

Rhodotorula species have been found in soil, air, and plant-associated organisms in a variety of environments ranging from the deep sea to deserts [18,19]. *Rhodotorula graminis* has properties suitable for the production of L-Phenylalanine ammonia-lyase [20] and lipids [21]. However, water treatment using *Rhodotorula graminis* has not yet been studied.

In the previous research [22], we found that the environmental-friendly and naturally occurring yeast *Rhodotorula graminis* NBRC0190 (hereinafter referred to as *R. gra*) has a high nitrate assimilation performance. Evaluated by *R. gra* flask culture experiments, it was found that the system could remove 97% and 99% of nitrate nitrogen from dairy well water (nitrate-nitrogen concentration = 23 mg/L) and treated water of a water purification center (nitrate-nitrogen concentration = 45 mg/L), respectively. For practical application, the immobilization of *R. gra* on porous substrates is necessary. The advantages of the yeast immobilization system include increased activity due to high accumulation, reduced risk of coexisting bacterial contamination, and recycling of yeast. Calcium alginate hydrogel beads are commonly used carriers for the immobilization of biocatalysts [23] due to their advantages of low cost, high porosity, and simplicity of preparation.

In this study, *R. gra* was immobilized in calcium alginate hydrogel beads. A leakage test of *R. gra* from *R. gra*-immobilized alginate beads was carried out. The immobilized *R. gra* beads were added to model water containing 10 mg/L nitrate nitrogen and incubated with shaking to evaluate the nitrate-nitrogen removal, ammonia-nitrogen removal, and repeated removal performance. Finally, nitrate-nitrogen removal tests were conducted using groundwater from two locations in Kumamoto City.

2. Materials and Methods

2.1. Materials

R. gra was used after thawing a lyophilized product purchased from the Biological Resource Center of the National Institute of Technology and Evaluation (NITE), Japan. Glucose was used as the carbon source due to the low concentration of organic carbon in groundwater. First, 0.6 g of sodium nitrate and 2 g of glucose were dissolved in 200 mL of Czapek liquid medium and sterilized in an autoclave at 110 °C for 10 min. After sterilization, the inoculating loop of *R. gra* was dipped into this medium. Shaking the culture was performed at 20 °C and 50 rpm for 3–4 days. The free organisms of *R. gra* were obtained by repeatedly centrifuging the pre-cultured suspension at 3000 rpm for 15 min and then added to the Czapek liquid medium. For immobilized yeast, *R. gra* suspension was washed by centrifugation with sterilized water and added to 0.5, 1.0, 1.5, and 2.0% sodium alginate solution, and then, 1% CaCl₂·H₂O solution was added dropwise. The beads solidified into a spherical shape at all concentrations of sodium alginate except 0.5%. After the sample was allowed to stand for 30 min, the immobilized yeasts were collected by filtering and added to Czapek liquid medium.

Model water containing 10 mg/L nitrate nitrogen was prepared using ion-exchange water and NaNO₃. In the previous study [22], we used a nitrogen concentration of 25–200 mg/L and a glucose carbon concentration of 500–4000 mg/L to remove nitrate nitrogen by *R. gra* yeast. In the process of removing nitrate ions while growing this yeast, we confirmed that a C/N ratio of 20 was sufficient, and therefore, the glucose concentration was set at 200 mg/L in this study. Two types of groundwater were collected from Kumamoto city, Japan. These waters had been sterilized in an autoclave at 110 °C for 10 min for use in the experiments.

2.2. Methods

R. gra suspension (1 mL) or immobilized yeast suspension (10 mL) was added to 100 mL of the water medium containing 10 mg/L nitrate nitrogen, and the nitrate assimilation reaction was carried out. The turbidity of the medium was assessed using a UV-vis

spectrometer at 660 nm. It was confirmed that there was a direct proportional relationship between the value of turbidity and the number of *R. gra* yeast counted by the microscopic method. The sample solution was centrifuged at 3000 rpm for 10 min, and the supernatant liquid was filtered through a 0.45 μm membrane filter to measure the nitrate-nitrogen concentration and ammonia-nitrogen concentration. Determination of nitrate nitrogen was performed by ion chromatography (IA-100, DKK-TOA Corporation, Tokyo, Japan). Ammonia nitrogen and residual sugar concentrations were determined using a UV-vis spectrophotometer by the indophenol blue method (630 nm) and Somogyi–Nelson method (660 nm), respectively.

3. Results

3.1. Leak of *R. gra* Yeast from Alginate Hydrogel Beads

Figure 1 shows the change in turbidity caused by *R. gra* leaked from alginate beads during the nitrate assimilation reaction. At a sodium alginate concentration of 2.0%, the turbidity was slightly higher than at 1.0 and 1.5%, suggesting that the leakage of *R. gra* slightly increased due to the volume expansion during the preparation of alginate hydrogel beads. However, the turbidity increased in the early stage of the nitrate assimilation reaction, but it did not change significantly in the subsequent reaction period. This suggests that the initial increase is due to the leakage of *R. gra* attached to the surface of alginate beads. Compared to the turbidity of the free bacterial suspension, the increase in turbidity of the alginate beads was about 5% or less. In the subsequent experiments, alginate beads were prepared with a concentration of 1.5% sodium alginate and 1% calcium chloride.

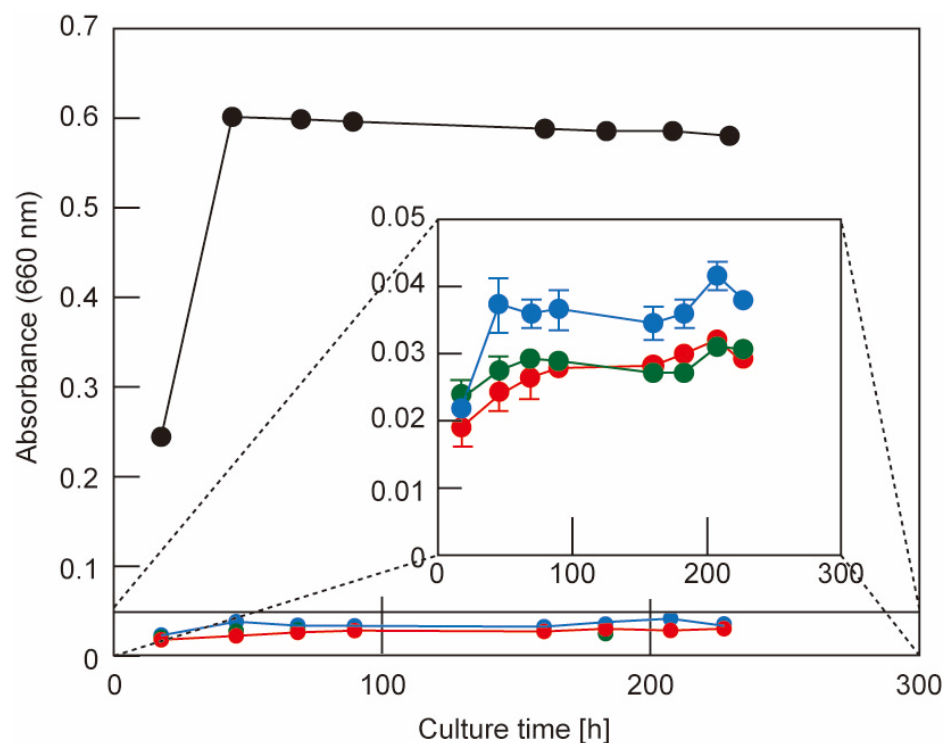


Figure 1. Change of turbidity (absorbance at 660 nm) of the medium caused by *R. gra* leaked from alginate hydrogel beads during the nitrate assimilation reaction: *R. gra* suspension (1 mL) or immobilized yeast suspension (10 mL) was added to 100 mL of the water medium containing 10 mg/L nitrate nitrogen. Sodium alginate concentration in the preparation of alginate beads with *R. gra*, red circle, 1.0%, green circle, 1.5%, blue circle, 2.0%, black circle free *R. gra* suspension.

3.2. Effect of Yeast Density on Nitrate-Nitrogen Removal

Figure 2 shows the effect of yeast density in alginate beads on nitrate-nitrogen removal. The yeast density in the beads was adjusted by changing the volume of 1.5% sodium

alginate solution and the volume of *R. gra* suspension during the preparation of alginate beads. The yeast density in alginate beads prepared by adding 1 mL of *R. gra* suspension to 10 mL of sodium alginate solution is equivalent to 1×10^7 cells/mL. To eliminate the effect of yeast leakage, the alginate beads that were re-cultured after one week of nitrate assimilation reaction were used for this experiment. As shown in Figure 2, there was no change in the removal rate of nitrate nitrogen by changing the density in the alginate beads. This suggests that the reaction might be conducted on the surface without diffusing into the beads at low nitrate-nitrogen concentration. In all conditions, the nitrate-nitrogen concentration was below 0.2 mg/L, the detection limit by the ion chromatography method, after 24 h of incubation. There are no data on the growth of *R. gra* in alginate beads, but the beads became darker in pink color due to *R. gra* as the reaction progressed. In this study, the effect of yeast density on the nitrogen assimilation reaction was small. However, the activity of *R. gra* tended to decrease gradually for the high yeast density in alginate beads and might be affected by miscellaneous germs for the low yeast density. Therefore, as a standard preparation condition for alginate beads, the liquid volume of sodium alginate to be mixed with 1 mL of *R. gra* suspension was set at 10 mL.

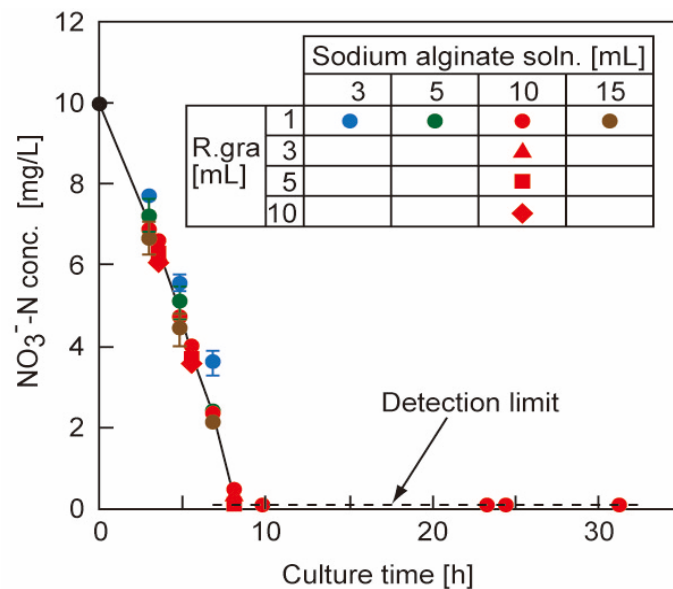


Figure 2. Change in nitrate-nitrogen concentration at various yeast densities in alginate hydrogel bead. The detection limit of nitrate nitrogen by the ion chromatography method is 0.2 mg/L: Red symbols, Volume of *R. gra* suspension was varied from 1, 3, 5, and 10 mL to 1 mL of sodium alginate of 1.5% concentration. Circular symbols, Volume of sodium alginate at a concentration of 1.5% was varied from 3, 5, 10, and 15 mL to 1 mL of *R. gra* suspension.

Residual carbon in drinking water is undesirable because it is a precursor for trihalomethane formation [24]. In these experiments with a C/N ratio of 20, the glucose concentration decreased from 200 mg/L to less than 10 mg/L, which is the qualitative limit of the Somogyi–Nelson method, in 24 h. As a result, it was confirmed that the amount of glucose at C/N ratio 20 was sufficient for *R. gra* immobilized alginate beads as well as for the removal of nitrate nitrogen in *R. gra* suspensions [22].

Zen et al. [4] used a red yeast, *Sporidiobolus pararoseus* Y1, for the denitrification reaction of nitrate nitrogen. The results showed that the nitrogen removal efficiency with glucose was 92.2% at a nitrate-nitrogen concentration of 14 mg/L for 72 h, and the average removal rate was $0.39 \text{ mg} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$. The results shown in Figure 3 indicate that the immobilized *R. gra* has a high performance in nitrogen removal efficiency and the removal rate of more than 98% and $1.0 \text{ mg} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$, respectively.

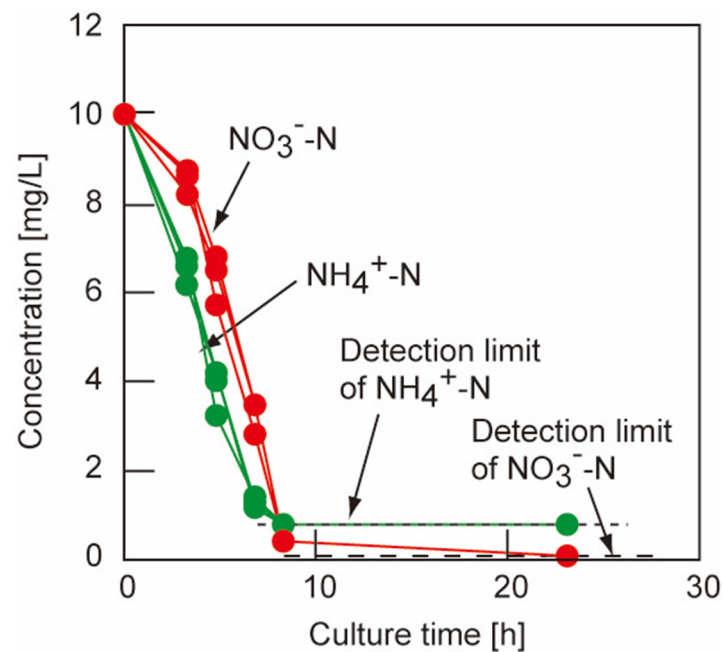


Figure 3. Comparison of nitrogen removal between denitrification of nitrate nitrogen and nitrification of ammonia nitrogen by alginate beads: red circle, nitrate-nitrogen concentration, green circle, ammonia-nitrogen concentration. The detection limit of nitrate nitrogen by the ion chromatography method is 0.2 mg/L. The alginate beads were prepared by adding 1 mL of *R. gra* suspension to 10 mL of 1.5% concentration sodium alginate solution. The detection limit of nitrate nitrogen by the ion chromatography method is 0.2 mg/L, and that of ammonia nitrogen by the indophenol blue method is 0.8 mg/L.

3.3. Simultaneous Removal of Nitrate Nitrogen and Ammonia Nitrogen

Nitrate-nitrogen removal and ammonia-nitrogen removal experiments were performed independently using *R. gra*-immobilized alginate beads prepared under standard conditions (10 mL of 1.5% concentration sodium alginate solution and 1 mL of *R. gra* suspension). As shown in Figure 3, ammonia nitrogen was removed at the same rate as nitrate-nitrogen removal, and it reached 0.8 mg/L, the detection limit by the indophenol blue method, in 8 h. In the ammonia-nitrogen removal experiment, no nitrite nitrogen could be detected in the samples. Similar results have been obtained with ammonia-nitrogen removal by the red yeast *Sporidiobolus pararoseus* Y1 [4]. In this study, the removal rate of ammonia nitrogen was almost equal to that of nitrate nitrogen, suggesting that the removal of nitrate nitrogen is the rate-determining step.

Figure 4 shows the performance of *R. gra* yeast in removing ammonia nitrogen and nitrate nitrogen under coexisting conditions. In this case, the total concentration of ammonia nitrogen plus nitrate nitrogen was 20 mg/L, but the elemental ratio of glucose to nitrogen was set at C/N = 20. Comparing Figures 3 and 4, it was found that the removal rate of ammonia nitrogen did not change much. The concentration of nitrate nitrogen decreased simultaneously with ammonia nitrogen in the early stage of the reaction, but it increased at 5 h of incubation due to the formation of nitrate nitrogen by nitrification of ammonia nitrogen. Then, after the ammonia nitrogen reached the detection limit, it gradually decreased and reached the detection limit of nitrate nitrogen in about 20 h. In these experiments, the initial glucose concentration of 400 mg/L was reduced to less than 10 mg/L, the detection limit by the Somogy–Nelson method, after 24 h.

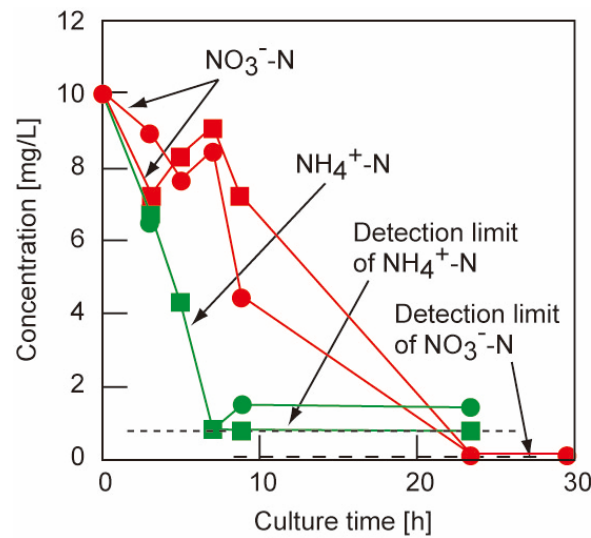


Figure 4. Simultaneous nitrogen removal of nitrate nitrogen and ammonia nitrogen by alginate beads: red symbols, nitrate-nitrogen concentration, green symbols, ammonia-nitrogen concentration, Circle and square symbols indicate first run and second run, respectively. The detection limit of nitrate nitrogen by the ion chromatography method is 0.2 mg/L, and that of ammonia nitrogen by the indophenol blue method is 0.8 mg/L.

3.4. Repeated Use of Alginate Beads Immobilized with *R. gra*

To examine the durability of *R. gra* immobilized alginate beads, the nitrate assimilation reaction was first carried out. The concentration of nitrate nitrogen reached below the detection limit in 24 h, as shown in Figure 5, and then, the beads were left to stand for 1 week. After one week, the immobilized beads were transferred to a new medium containing nitrate nitrogen and glucose, and the nitrate assimilation reaction was performed again. In total, five nitrate assimilation reactions were performed over a period of 5 weeks. Excess glucose (2000 mg/L) was added for the first incubation, but 200 mg/L glucose concentration was added for the second and subsequent incubations. As shown in Figure 5, there was no significant difference in the removal rate of nitrate nitrogen even after five repetitions. Even when excess glucose was added in the first reaction, the removal rate did not change. These results showed that the activity of the *R. gra*-immobilized alginate gel was maintained even after the glucose in the solution was completely consumed in one week.

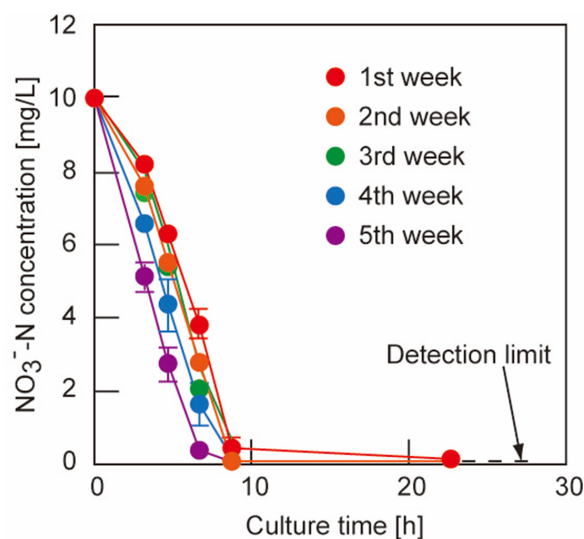


Figure 5. Repeated denitrification properties of alginate hydrogel beads with *R. gra*.

3.5. Denitrification of Ground Water

In the water treatment experiments of groundwater in Kumamoto City, glucose with a mass ratio C/N = 20 was dissolved in 100 mL of the sterilized groundwater, and then, 5 mL of immobilized *R. gra* was added. The mixed solution was incubated at 20 °C and 50 rpm for 5 days with shaking. The ion concentrations were measured after the denitrification reaction.

Table 1 summarizes the ion concentrations in groundwater collected from two wells in Kumamoto City and treated with immobilized *R. gra*. These groundwaters contain relatively high concentrations of nitrate ions, 36.4 mg/L and 32.1 mg/L. These values were converted to nitrate-nitrogen concentrations of 8.22 mg/L and 7.25 mg/L, which are close to the equivalent Japanese environmental quality standard of 10 mg/L. Water treatment with *R. gra* reduced the nitrate ion concentration to below the detection limit for well #1 and to 0.60 mg/L for well #2 at a culture time of 120 h. In actual groundwater, more than 98% of nitrate ions were removed. In the treatment of groundwater, the Ca²⁺ and Cl⁻ ion concentrations increased due to leaching from the alginate hydrogel beads after 5 days of treatment, but in the actual process, the increase in these concentrations could be controlled by optimizing the denitrification time. In drinking water treatment plants that use groundwater as their source of water, the addition of glucose does not significantly affect the overall cost because the cost of coagulants and other chemicals required for advanced treatment is not required.

Table 1. Changes in water quality due to denitrification of groundwater.

	Well #1 (Tensuiko) ^{a)}		Well #2 (Myoukensan) ^{b)}	
	Before Water Treatment	After Water Treatment	Before Water Treatment	After Water Treatment
[NO ₃ ⁻] [mg/L]	36.4	<0.2	32.1	0.60
[Cl ⁻] [mg/L]	6.5	22.8	9.0	43.2
[SO ₄ ²⁻] [mg/L]	14.6	13.9	24.0	22.6
[PO ₄ ³⁻] [mg/L]	<1	<1	<1	<1
[NH ₄ ⁺] [mg/L]	<0.1	0.15	<0.1	<0.1
[Na ⁺] [mg/L]	10.5	10.4	17.4	17.1
[K ⁺] [mg/L]	2.9	3.6	9.4	10.3
[Mg ²⁺] [mg/L]	6.2	5.0	5.8	4.8
[Ca ²⁺] [mg/L]	14.2	26.7	17.8	37.0
pH	6.7	6.9	6.5	6.8

^{a)} Kawautimachi, Nishi-ku, Kumamoto, ^{b)} Mitsugumachi, Kitaku, Kumamoto, The alginate beads were prepared by adding 0.5 mL of *R. gra* suspension to 5 mL of 1.5% concentration sodium alginate solution. Experimental condition (Shaking culture was performed at 20 °C and 50 rpm for 5 days., groundwater 100 mL, *R. gra* immobilized alginate hydrogel beads 5 mL, C/N = 20).

4. Conclusions

The leakage of *R. gra* immobilized on alginate beads was evaluated by turbidity; the leakage increased after 50 h of incubation, but thereafter, the change in turbidity was small, indicating that *R. gra* was stably immobilized. The nitrate-nitrogen removal experiments were conducted at an initial nitrate-nitrogen concentration of 10 mg/L, assuming groundwater treatment. The density of *R. gra* in alginate beads was varied by changing the amount of 1.5% sodium alginate solution and the amount of *R. gra* suspension. However, it had little effect on the removal behavior of nitrate nitrogen. The immobilized *R. gra* showed high performance in nitrogen removal efficiency and the removal rate of more than 98% and 1.0 mg.L⁻¹.h⁻¹, respectively. Ammonia-nitrogen removal by *R. gra* immobilized in the alginate beads alone in a solution with an initial ammonia-nitrogen concentration of 10 mg/L showed almost the same nitrogen removal property as the nitrate nitrogen. However, in a mixed solution containing 10 mg/L of both nitrate nitrogen and ammonia nitrogen, the removal of ammonia nitrogen proceeded regardless of the presence of nitrate nitrogen at the beginning of the reaction, and then, the concentration of nitrate nitrogen slightly increased due to the nitrification reaction, and then the concentration decreased

to the detection limit of nitrate nitrogen within 24 h. Even after the same treatment was repeated five times, the denitrification performance of the *R. gra* immobilized alginate hydrogel beads was maintained. Finally, when the treatment method of this study was applied to actual groundwater in Kumamoto City, the nitrate ion concentration was reduced to below the detection limit for well #1 and to 0.60 mg/L for well #2 at a culture time of 120 h. In actual groundwater, more than 98% of nitrate ions were removed. In addition, the Ca^{2+} and Cl^{-} ion concentrations increased due to leaching from the alginate hydrogel beads after 5 days of treatment.

In order to apply the removal of nitrate nitrogen by *R. gra* immobilized alginate beads in a drinking water treatment plant, the removal rate of nitrate nitrogen in a packed bed bioreactor must be considered. The post-treatment of treated water containing residual *R. gra*, etc. by filtration, adsorption, or disinfection should be investigated in the future.

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