



Denitrification performance and microbial community variation during reverse osmosis concentrate treatment by sulfur denitrification process

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ABSTRACT

Heterotrophic denitrification is widely used as a method for removing nitrate, but incomplete denitrification was reported in the case of wastewater having a low C/N ratio. Specifically, the reverse osmosis concentrate (ROC) generated during the water reuse process contains a high concentration of nitrate but a low amount of biodegradable organic carbon for heterotrophic denitrification. In this study, a sulfur denitrification process was considered as an alternative method to eliminate nitrate in ROC. We studied the possibility of microbial community formation in a continuous process and the period required for culture and stabilization. To this end, in this study, a denitrification efficiency according to the initial cultivation methods was compared to determine the effect of the reactor start-up strategy, and the optimal hydraulic retention time was obtained. The denitrification efficiency and the microbial content were very similar in both a continuous reactor and a batch reactor after 30 d. We thus concluded that in-situ cultivation in the continuous reactor without batch cultivation was possible. The optimal hydraulic retention time was then determined as 2 h. In order to evaluate the applicability of the ROC, the change of denitrification efficiency was monitored during reactor operation with simulated ROC containing organic matter. The denitrification efficiency significantly decreased due to the growth of heterotrophic microorganisms. However, the efficiency could be restored after 25 d of operation without organic matter. Moreover, the microbial community change was monitored for a better understanding of microbial actions in the sulfur denitrification process. Increased abundance of *Thiobacillus thioparus*, *Sulfurimonas denitrificans*, and *Sulfurimonas paralvinellae* were mainly associated with denitrification performance in the cultivation process.

Keywords: Sulfur denitrification; Autotrophic denitrification; Reverse osmosis concentrate; Water reuse; in-situ cultivation

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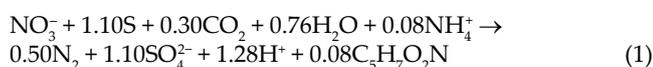
1. Introduction

High levels of nitrogen are continuously introduced into the water system due to high industrialization and human activities, and this is exacerbated by population growth [1]. Nitrogen introduced into the water system causes eutrophication and depletion of dissolved oxygen, which prevents stable water resources [2]. It is also known that drinking water containing nitrate nitrogen of 10 mg/L or more can cause cyanosis in newborns and can even lead to cancer [3,4]. Therefore, the removal of nitrogen during wastewater treatment is a critical process.

Nitrogen removal is also one of the major concerns in the application of wastewater reuse processes. The most widely applied technology for wastewater reuse as a quaternary treatment is combined membrane processes such as microfiltration/ultrafiltration together with reverse osmosis (RO) [5]. This technology employs semi-permeable membranes that allow separation of a solution into two streams: permeate (ROP), containing the purified water that passes through the membrane, and RO concentrate (ROC), the portion that contains salts and retained compounds and therefore requires a suitable and environmental friendly management option [6]. The characteristics of the waste stream, ROC, depend on the quality of the feed water and the quality of the produced water (recovery varies from 35% to 85%) [7,8]. Generally, ROC contains a high concentration of nitrate while the low amount of biodegradable organic carbon for heterotrophic denitrification.

Heterotrophic denitrification is widely used as a method for removing nitrate, which is considered to be a very economical and effective nitrate removal method. This approach is beneficial if a sufficient amount of organic matter is contained in the water. In the literature on the organic matter and nitrogen concentration of ROC generated in wastewater reuse processes, the C/N ratio is reported as 0.67 [9], 1.8 [10]. These C/N ratios are significantly lower than the C/N ratio in general wastewater, and incomplete denitrification at a COD/NO₃-N ratio below 7 was reported [11]. It has also been reported that organic matter remaining in the ROC is challenging to be biologically degraded because most of the biologically degradable organic matter is consumed through the sewage treatment process [9,12]. If the C/N ratio is low, an external carbon source such as methanol, ethanol, or acetic acid must be injected during the denitrification process, thereby incurring additional costs [13].

In this study, a sulfur denitrification process was considered as an alternative method to eliminate nitrate in ROC. A few species of sulfur utilizing autotrophic denitrifiers, such as *Thiobacillus denitrificans* and *Thiomicrospira denitrificans*, have been found to reduce nitrate to nitrogen gas [14]. The following is a stoichiometric equation that provides an example of elemental-sulfur-utilizing autotrophic denitrification [15].



Energy for autotrophic denitrifying microorganisms is derived from the oxidation of elemental sulfur while they

utilize inorganic carbon compounds (e.g., CO₂, HCO₃⁻) as their carbon source. In this process, hydrogen ions are produced, indicating that alkalinity is consumed by the reaction. Therefore, an alkaline material, such as limestone, is usually added to the sulfur-based autotrophic denitrification reactors [14,16]. This process does not require the injection of an additional external organic carbon source, and sulfur is abundant and cheap, and thus it is possible to reduce the cost, and sulfur is also easy to store and easy to handle [17,18]. The above process also has the advantage of low sludge production because it uses an autotrophic microorganism [14]. Studies on domestic [19,20] and industrial wastewater [21,22], groundwater [23], leachate [24], and surface water [25] have been actively carried out to exploit the various advantages of the above mentioned autotrophic denitrification. The kinetic models for the sulfur-based autotrophic denitrification have also been reported [26,27].

Many of the aforementioned studies were carried out in a continuous batch reactor after culturing the microorganisms in a batch-type incubator more than 10 d before the experiment [16,26]. However, when the cultivation was performed separately, it was speculated that the microbial activity might be lowered, and the need for an additional reaction tank might arise. Therefore, we sought to shed light on the possibility of microbial community formation in a continuous process and the period required for culture and stabilization. To this end, in this study, the denitrification efficiency according to the initial culture methods was compared to determine the effect of the reactor start-up strategy, and the optimal hydraulic retention time was obtained. In order to evaluate the applicability of the ROC, the change of denitrification efficiency was monitored during reactor operation with simulated ROC. Moreover, the microbial community change was monitored to shed light on the microbial actions during the sulfur denitrification process.

2. Materials and methods

2.1. Materials

Elemental sulfur (3–5 mm, purity > 99.9%, Samwoo Chemical, Korea) and aragonite (3–5 mm, Nature's Ocean, USA) were mixed at a mass ratio of 9:11 as a carrier for microbial culture. The activated sludge collected from the 'U' wastewater treatment plant was settled for 24 h, and then the concentrated sludge was used as an inoculum.

The following chemicals were used for preparing culture media for microbial cultivation and synthetic wastewater for the denitrification experiment. KNO₃ (potassium nitrate, 99.0%), NaHCO₃ (sodium bicarbonate, 99.0%), NH₄Cl (ammonium chloride, 98.5%), and MgCl₂·6H₂O (magnesium chloride hexahydrate, 98.0%) were purchased from Samchun Pure Chemical (Korea). K₂HPO₄ (dipotassium hydrogen phosphate) was purchased from Junsei Chemical (Japan). FeSO₄·7H₂O (iron (II) sulfate heptahydrate) was purchased from TOYO Chemical Co. (Japan). Na₂S₂O₃·5H₂O (sodium thiosulfate pentahydrate) was purchased from SHOWA Chemicals (Japan). Humic acid and dextran were purchased from Sigma-Aldrich (USA).

2.2. Microbial cultivation and denitrification experiments

2.2.1. Composition of culture media and synthetic wastewater

Culture media for microbial cultivation and synthetic wastewater for the denitrification experiment were prepared using the compositions reported in the literature [26] (Table 1). The synthetic ROC was prepared by adding organic matter, humic acid, and dextran into the synthetic wastewater. The ratio of organic matter was selected, as reported in the literature [28]. Dextran has been frequently used as a good surrogate for polysaccharide-like substances present in secondary effluent [29], and humic-like substances are typically refractory to microbial degradation, biogenic, and yellow-colored organic acids. The ratio between dextran and humic acid was determined as 3:1 based on the findings reported by Vakondios et al. [30], where they determined the compositional distribution of organic carbon in secondary effluent.

2.2.2. Continuous denitrification reactor

An upflow fixed bed reactor was used for the continuous denitrification experiment. The reactor was made of a transparent acrylic tube with an inner diameter of 5 cm and a total height of 35 cm, as shown in Fig. 1. A perforated plate was installed at the height of 2 cm from the bottom of the tube, and 600 g of a sulfur-aragonite composite media having a depth of about 27 cm was filled thereon.

2.2.3. Microbial cultivation methods

In order to evaluate the denitrification performance according to the culture method of the sulfur-utilizing autotrophic denitrifying microorganism, a continuous reactor and a batch reactor were compared. In each reactor, 600 g of the 9:11 sulfur-aragonite complex, as described in Section 2.1.1, 100 mL of seeding sludge, and 600 mL of culture medium were injected, and the cultivation was started at the same time.

The continuous type reactor was operated by circulating the culture medium at a flow rate of 30 mL/min. The batch

type reactor was operated by stirring with a shaking incubator. The cultures of the continuous and batch reactors were replaced on the same day once every 5 d. In the case of the batch type reactor, the supernatant was discarded, and then a new culture was added due to the possibility of sludge runoff. Cultivation was carried out for a total of 50 d.

2.2.4. Continuous denitrification experiments

After the cultivation process described in Section 2.2.3, the denitrification experiment was continued using synthetic wastewater. In the cultivation process outlined in Section 2.2.3, the effluent of the continuous reactor was circulated to the influent water again, whereas in the denitrification experiment, the effluent was discharged without circulation. As the experiment progressed, the HRT was decreased from 12 h to 6, 3, and 1 h, and the denitrification efficiency was observed.

Additional experiments were conducted to determine the applicability to ROC. The optimal HRT derived from the above experiment was maintained, and the denitrification efficiency was continuously monitored with the simulated ROC prepared by mixing humic acid and dextran (1:3) in the simulated wastewater (Table 1), as suggested in a previously reported study [28].

2.2.5. Analysis

A sampling of influent and effluent was conducted daily and analyzed according to the Standard Method [31]. The pH value was measured by a pH meter (Istek, Korea), and the optical density value was measured at a wavelength of 600 nm using a UV-visible spectrophotometer (Libra S22, Biochrom, UK). For ionic substances such as SO_4^{2-} and NO_3^- , the sample was filtered with a membrane filter (0.45 μm), and then the concentration was measured using ion chromatography (Aquion, Thermo Fisher Scientific, USA). In the evaluation of the applicability of RO concentrated water, dissolved organic carbon (DOC) and UV254 were analyzed to measure changes of organic matter. The DOC was filtered with a membrane filter (0.45 μm) and measured using a TOC-V CPN (SHIMADZU, Japan). The morphology of fresh and spent sulfur granules was monitored by a scanning electron microscope (SEM, SU8010, Hitachi, Japan).

2.3. Microbial community analysis

The microbial community was analyzed by observing changes in the community during reactor operation. A total of three samples were taken during the overall operation, including the seeding sludge, after cultivation using a culture medium, and after reactor operation with synthetic ROC containing organic matter. At each sampling event, a portion of the sulfur/aragonite carrier was sampled to collect the biofilm formed on the surface.

The DNA was extracted and purified using an Ultraclean Soil DNA Kit (Mo Bio Laboratories, Solana Beach, CA, USA) and an Ultraclean Microbial DNA Isolation Kit (Mo Bio Laboratories, Solana Beach, CA, USA) according to the manufacturer's instructions. A 20-ng aliquot of each DNA sample was injected for PCR reaction. 16S universal

Table 1
Characteristics of the culture medium, synthetic wastewater, and synthetic ROC

Component	Culture medium (mg/L)	Synthetic wastewater (mg/L)	Synthetic ROC (mg/L)
KNO_3	2,000	433.3	433.3
NaHCO_3	1,000	1,000	1,000
K_2HPO_4	2,000	11.25	11.25
NH_4Cl	500	3.82	3.82
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	500	1	1
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	100	1	1
$\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$	5,000	–	–
Humic acid	–	–	6.67
Dextran	–	–	20

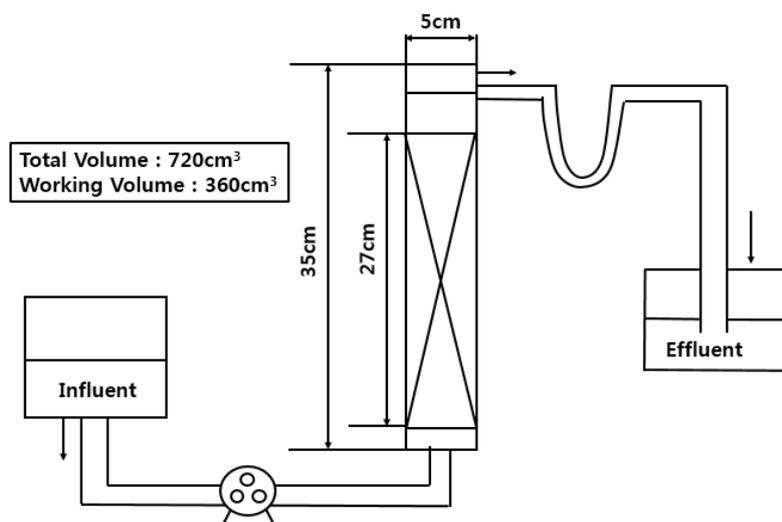


Fig. 1. Schematic diagram of a continuous denitrification reactor.

primers of 27F (5-GAGTTTGATCMTGGCTCAG-3) and 518R (5-WTTACCGCGGCTGCTGG-3) were employed for amplification of the 16S ribosomal RNA (16S rRNA) genes [32]. The obtained products from PCR were then purified by using AMPure beads (Beckman Coulter, CA, USA), and sequencing was conducted using a MiSeq PE300 (Illumina, CA, USA) by a commercial sequencing facility (Macrogen, Seoul, Korea). The results of sequences were obtained with software (QIIME) for pre-processing identification of operational taxonomic units and taxonomic assignment [33]. Filtration of poor sequences and barcode trimming were performed by following the method reported in Yun et al. [34].

3. Results and discussion

3.1. Evaluation of denitrification performance by microbial cultivation methods

After the seeding sludge was inoculated in the continuous reactor, the effect of microbial cultivation through the circulation of the culture medium was evaluated in comparison with the batch type reactor. The overall denitrification efficiency was evaluated by measuring nitrate-nitrogen ($\text{NO}_3\text{-N}$) and sulfate ion (SO_4^{2-}) concentration during the operation.

Fig. 2 shows the denitrification patterns of (a) sulfate ions and (b) nitrate-nitrogen in the continuous and batch reactor for 50 d starting from the initial stage of culture. In the case of sulfate ions, the discharging sulfate ion concentration decreased to 91 mg/L in the continuous type reactor and 192 mg/L in the batch type reactor during the initial 2–3 d after the start of the cultivation. However, after 3–4 d, the sulfate concentration in the batch reactor increased rapidly and peaked at 9,430 mg/L after 12 d. The rate of increase of sulfate ions in the continuous system was slower than that of the batch system, but it nonetheless increased continuously, and after 25 d, the concentration of sulfate ions was similar to that of the batch system. The huge fluctuation of sulfate was observed in both systems. In the cultivation experiment,

the cyclic operation was applied. The supernatant in both systems was changed to the new culture medium in every cycle; therefore, it was repeated that the sulfate ion was discharged and produced it again by denitrification. After 40 d, the batch system stabilized at about 4,000 mg/L and the continuous system at about 4,800 mg/L.

Similar results were obtained for nitrate removal. As shown in Fig. 2b, in the case of the batch reactor, all of the nitrate that was added in the first batch cycle (5 d) were removed. From the second cycle, it was confirmed that all of the added nitrate were effectively removed during the first 24 h, which is between when the culture medium is changed and the first sampling after 1 d. In the case of the continuous reactor, the nitrate removal efficiency was lower than that of the batch reactor. The concentration of nitrate in the effluent was measured to be more than 150 mg/L during two cycles (10 d). This result indicated the low denitrification efficiency of the continuous system. It might be due to the low contact time between microbes and culture medium. In the continuous system, only a small portion of culture medium can have direct contact with microbes, because the culture medium was recirculated. The nitrate concentration started to decrease significantly from the third cycle. From the fourth cycle, all of the added nitrate were effectively removed during the first 24 h.

The absorbance of the effluent measured at 600 nm is plotted in Fig. 3 to determine the concentration of microorganisms. In the case of the continuous reactor, the absorbance was close to zero. As a result, it can be seen that there is almost no sludge runoff in continuous operation. On the other hand, the absorbance value of the batch reactor continuously increased, and after 20 d, the absorbance was stabilized at about 2.7. Therefore, it was confirmed that a large number of microorganisms existed in a suspended state without adhering to the sulfur-aragonite carrier. This was confirmed by the effect of continuous stirring provided in the batch reactor.

From Fig. 2, it was confirmed that the efficiency of the continuous reactor after approximately 20 d was similar to that of the batch reactor. At the end of the fifth cycle, some

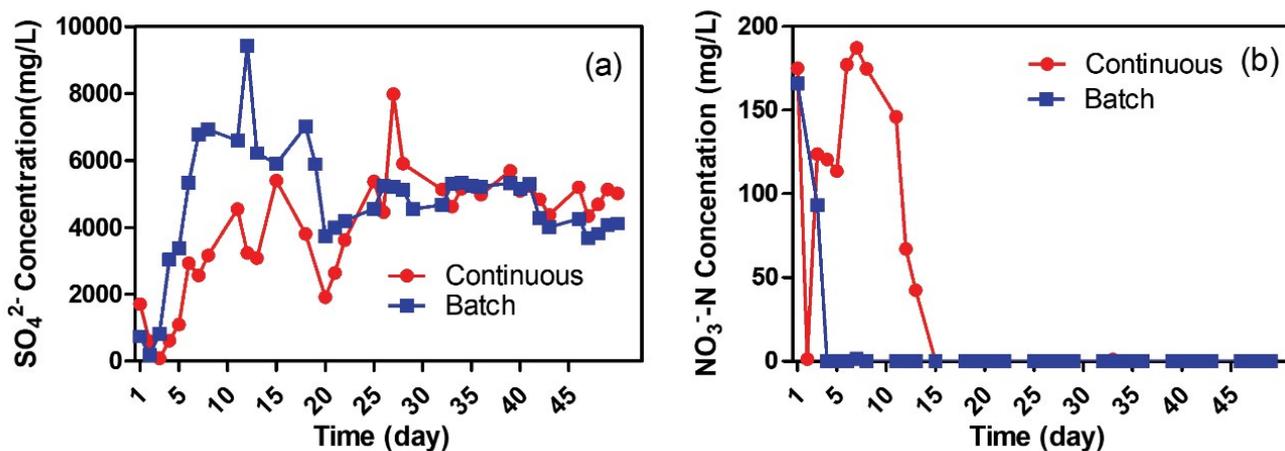


Fig. 2. Concentration of (a) sulfate ions (SO_4^{2-}) and (b) nitrate ions (NO_3^- -N) under different cultivation methods.

of the sludge and carrier of the continuous-type reactor was sampled and stirred under the same conditions as the batch reactor. The absorbance of the supernatant after stirring was similar to that of the batch culture, as shown in Fig. 3. The above results show that the microbial adaptation rate in the batch reactor is slightly faster, but after 20 d of incubation, the microbial growth and denitrification efficiencies are similar in the continuous and batch systems. Based on these results, further experiments were carried out using a method of directly culturing the cells in a continuous reactor without culture in a batch reactor. A similar approach has been widely accepted for cultivating slowly growing microorganisms, for example, anaerobic ammonium oxidation (anammox) bacteria [35,36].

In the case of pH, both the batch reactor and the continuous reactor were maintained at about 7.50, which was not significantly different from the pH of the culture solution, 7.78. It was concluded that the buffering effect of aragonite was beneficial because the pH was well maintained when the concentration of sulfate ions was increased from 4,000 to 5,000 mg/L.

3.2. Optimal HRT in the continuous denitrification process

After the cultivation of the microorganisms was completed in the continuous reactor described in Section 3.1, denitrification experiments were conducted using synthetic wastewater. The characteristics of the synthetic wastewater are shown in Table 1. The denitrification efficiency was evaluated by reducing the HRT from 6 to 1 h during the experiment. The concentration changes of nitrate and sulfate during the experiment are shown in Figs. 4a and b, respectively.

Fig. 4a shows the nitrate concentrations of influent and effluent, and the overall nitrate removal efficiency. The nitrate concentration of the influent was maintained at about 52 mg-N/L, and the removal efficiency was above 97% when the HRT was 6 and 3 h. The average nitrate concentration in the effluent during these periods was 1.44 mg/L. However, when the HRT was shortened to 1 h, nitrate started to flow out immediately, and the maximum nitrate concentration in the effluent was measured to be 14.7 mg/L, and the efficiency

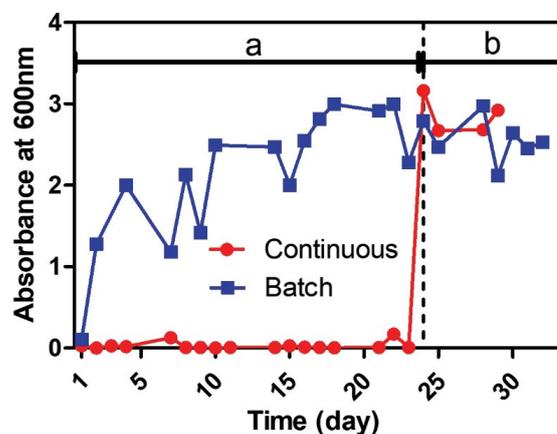


Fig. 3. Change of optical density under different cultivation methods (at the end of the fifth cycle, a portion of the sludge and carrier of the continuous reactor were sampled, and the optical density was measured; period b).

was reduced to as low as 70%. The reactor was operated at HRT 1 h for 10 d, but the concentration of nitrate in the effluent did not decrease but instead increased. After adjusting the HRT to 2 h, the nitrate concentration in the effluent was reduced to less than 1.5 mg/L, and the denitrification efficiency was measured to be more than 97%. Therefore, the optimal HRT was determined to be about 2 h, and the nitrate loading rate was 720 mg-N/L/d, which is comparable with recent reports on the sulfur-denitrification process [16,37]. In the aforementioned reports, 104–300 mg-N/L/d of nitrate loading rate was achieved in a fixed-bed reactor [37], and 800 mg-N/L/d of nitrate loading rate was achieved in a fluidized bed reactor [16].

Fig. 4b shows the concentration of sulfate ions. The sulfate ions are produced in the course of the conversion of nitrate nitrogen into nitrogen gas and show a tendency to decrease according to the HRT decrease. When the HRT was 6, 3, 1, and 2 h, the average concentrations of sulfate ions were measured as 544, 451, 344, and 440 mg/L, respectively.

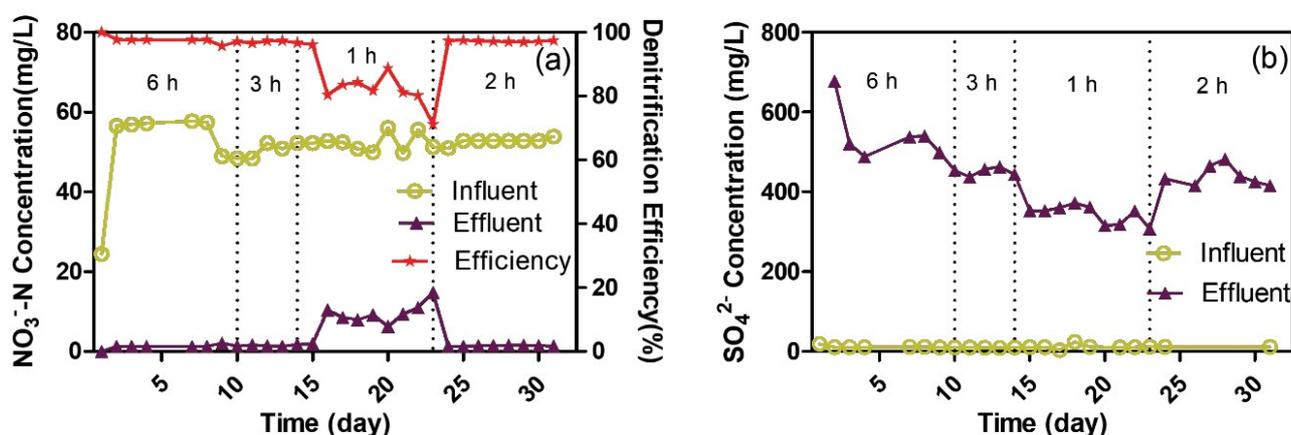


Fig. 4. Concentration profiles of (a) nitrate and (b) sulfate in the influent and effluent.

3.3. Application of ROC in the denitrification process and its inhibitory effect

The reactor was stabilized under the optimum HRT of 2 h condition derived from Section 3.2, and simulated ROC water was injected. The characteristics of the simulated ROC water are listed in Table 1. The simulated ROC is basically the addition of organic matters, humic acid, and dextran, into the synthetic wastewater used in Section 3.2. Changes in nitrate concentration, sulfate ion concentration, and dissolved organic matter concentration during the ROC test are shown in Figs. 5a–c.

First, Fig. 5a shows that the denitrification efficiency rapidly decreased to 72% immediately after 24 h of injection of the simulated ROC. The denitrification efficiency was maintained at around 80% for 1 week. However, the denitrification efficiency rapidly decreased after two weeks of operation, and denitrification did not proceed at all. As shown in Fig. 5b, the amount of sulfate produced after the ROC water injection continued to decrease, which is consistent with the nitrate results. In the case of the DOC, after the ROC injection, the measured DOC of the effluent was lower than that of the influent, and it further decreased to 40% of the injected DOC. This result confirmed that the DOC was consumed by the microbial reaction.

Based on the above results, the heterotrophic microorganisms that were present in the reactor rapidly proliferated and appeared to consume organic matter due to the organic substances of ROC water. Heterotrophic microorganisms became dominant in the competition with existing sulfur-denitrifying autotrophic microorganisms. It was reported that the presence of organic matter, especially propionate, inhibits autotrophic denitrification, as indicated by a lower sulfate production rate [38]. For accurate analysis, microbial communities were analyzed by the method outlined in Section 2.4.

The denitrification did not progress further, and thus the influent was changed to synthetic wastewater, which did not contain organic matter in order to determine whether denitrification could be recovered. Denitrification efficiency gradually increased to about 80% after 3 weeks, and it further increased to 95% after 25 d. It was confirmed that the denitrification efficiency was almost equal to that before

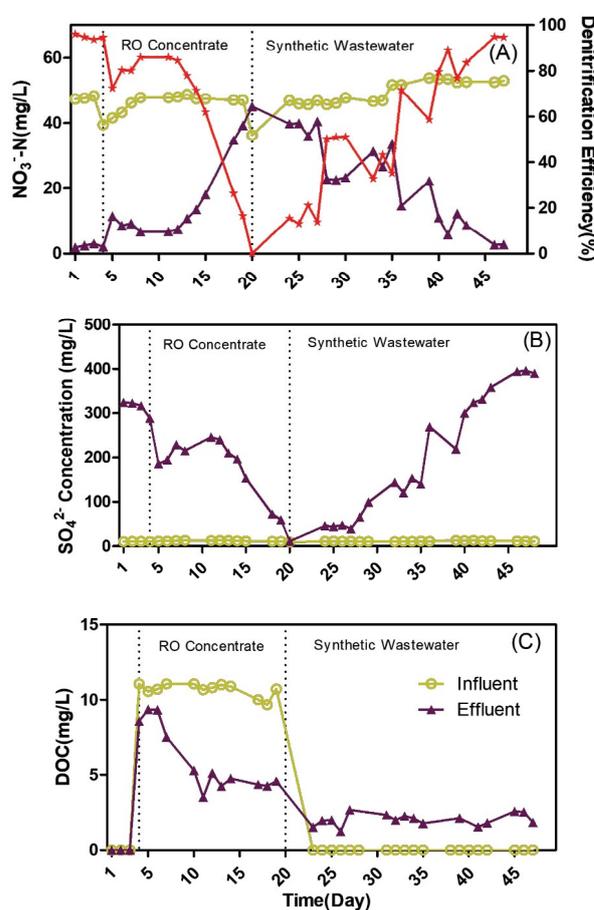


Fig. 5. Effect of RO concentrate injection and recovery in a continuous reactor. (a) nitrate concentration and denitrification efficiency, (b) sulfate concentration, (c) DOC concentration.

the ROC water injection. The sulfate ion concentration gradually increased as well, and after 22 d of synthetic wastewater injection, the concentration was 330 mg/L, which is similar to the level of sulfate ion concentration before the ROC water application. In the case of the DOC, an average DOC value of 2 mg/L was continuously detected in the effluent.

This is due to leaking out of the heterotrophic microorganisms generated by the previous RO water injection and the ROC remaining in the reactor.

3.4. Morphology change of sulfur granule

A scanning electron microscope (SEM) analysis was performed to observe the changes in the morphology of granular sulfur before and after the denitrification experiment. Figs. 6a–c show the surface of fresh granular sulfur at 100, 300, and 1,000 times magnification, respectively. It was confirmed that the sulfur granule showed a smooth appearance without special impurities.

On the other hand, the images in Figs. 6d–f showing the granular sulfur collected after the denitrification experiment reveals a different shape. From Figs. 6d and e, it was confirmed that many holes were formed on the surface of the granular sulfur. These holes can be ascribed to the oxidation of sulfur to sulfate ions through the sulfur oxidation mechanism involved in the denitrification process. Moreover, the formation of a biofilm on the granular sulfur surface could also be observed in Figs. 6e and f. The biofilm is expected to be composed of autotrophic sulfur-oxidizing bacteria.

In order to analyze the microbial community of the formed biofilm, the biofilm was separated through washing and centrifugation of the granular sulfur surface and used for microbial community analysis, as described in the upcoming section.

3.5. Microbial community analysis

Three samples collected during overall operation, including the seeding sludge, after cultivation, using the culture

medium, and after adding synthetic ROC containing organic matter, were analyzed by 16S rRNA targeted gene NGS to reveal how the performance of the sulfur denitrification process varied in response to the operating conditions was related with microbial ecology.

Fig. 7 shows apparent shifts in the diversity of key microorganisms at the species level among the three samples. The dominant species in the seed sludge were *Zoogloea ramigera*, *Rudaea cellulosilytica*, *Simplicispira psychrophila*, *Zoogloea resiniphila*, *Phaeodactylibacter xiamenensis*, and *Haliangium ochraceum*, accounting for 29.3%, 17.0%, 8.9%, 5.4%, 4.9%, and 4.2% of the total number of sequences, respectively. However, their abundance decreased while a new microbial structure appeared in the sample taken after the cultivation period; *Thiobacillus thioparus* (43.2%) became the most dominant microorganism, followed by *Sulfurimonas denitrificans* (34.2%) and *Sulfurimonas paralvinellae* (8.0%). A gradual increase of species *Thiobacillus thioparus* is correlated with the increase in the denitrification performance during the cultivation period noted in Section 3.2. *Thiobacillus thioparus* is known as one of the chemolithoautotrophic microorganisms growing on nitrate with thiosulfate as the electron donor [39]. In addition, both *Sulfurimonas denitrificans* and *Sulfurimonas paralvinellae* have been recognized as denitrifying microorganisms [40,41]. On the other hand, *Sulfurimonas denitrificans* and *Annwoodia aquaesulis* were distinctly dominant, increasing to 43.0% and 34.2% when synthetic RO was injected, but *Thiobacillus thioparus* decreased to 11.1%, and *Sulfurimonas paralvinellae* disappeared. *Annwoodia aquaesulis* is responsible for denitrification in the presence of ammonium as a nitrogen source but cannot use nitrate [42]. However, the reason for the increase of *Sulfurimonas denitrificans* in synthetic RO remains unclear. A recent study revealed that *Sulfurimonas*

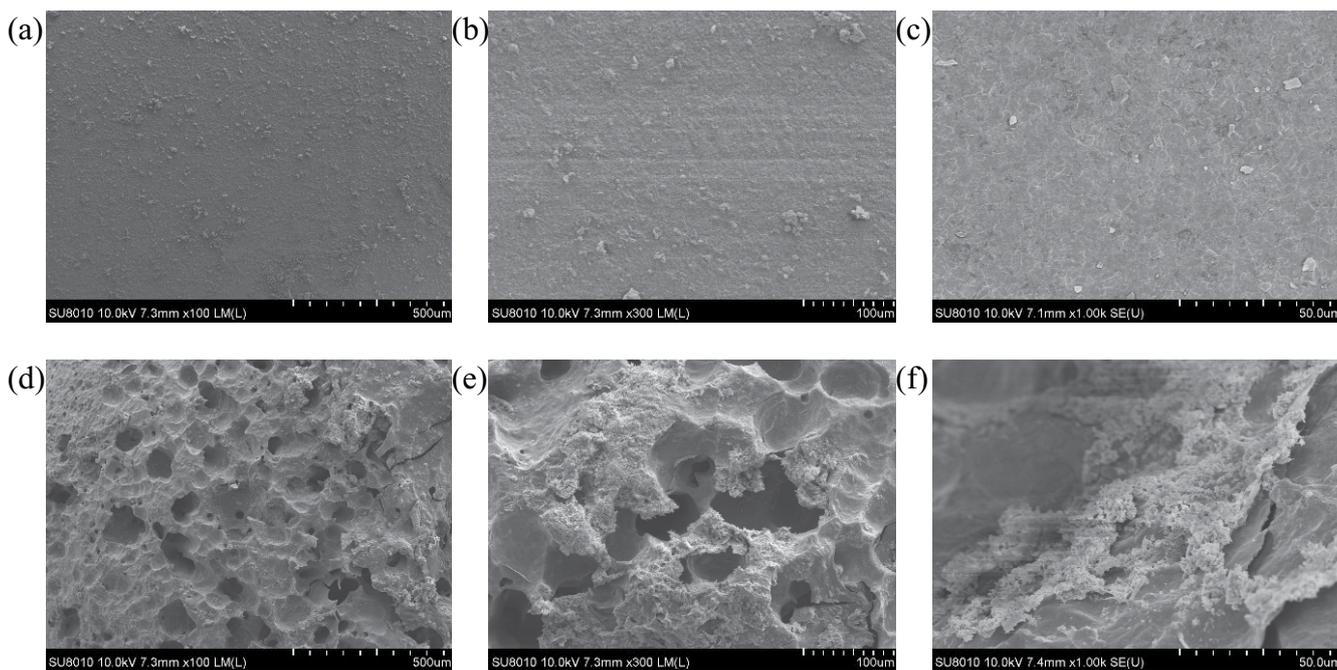


Fig. 6. Scanning electron microscope (SEM) images of a fresh granular sulfur ((a)–(c)) and spent granular sulfur ((d)–(f)).

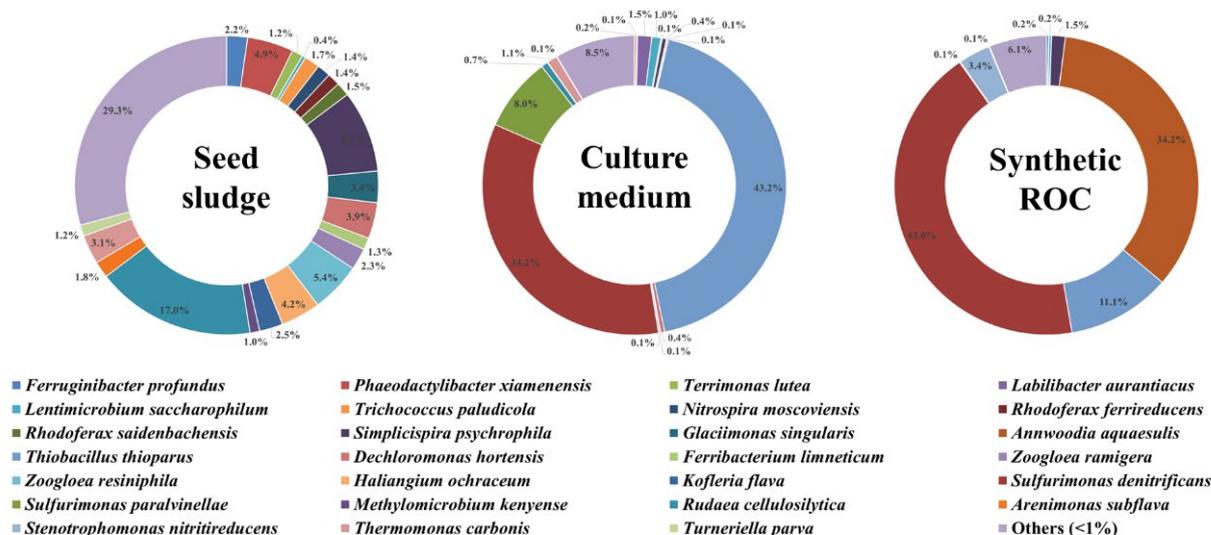


Fig. 7. Changes in the relative abundance of bacterial community at the species level.

denitrificans could grow through the oxidation of hydrogen in the absence of reduced sulfur compounds [39]. Future work thus should be conducted to verify whether the increase of the species *Sulfurimonas denitrificans* in the presence of organic matter in an anoxic condition was associated with oxidizing hydrogen, which was likely produced through the acidification of added humic acid and dextran.

4. Conclusion

In this study, the denitrification performance according to the initial culture method of autotrophic denitrifying microorganisms was studied. The hydraulic retention time in a continuous upflow reactor was optimized, and the following results were obtained.

- In the microbial cultivation, the microbial growth in the batch reactor was comparatively fast during the initial 20 d. However, the denitrification efficiency and the microbial content were very similar in the continuous reactor and batch reactor after 30 d. Therefore, we concluded that in-situ cultivation in the continuous reactor without batch cultivation was possible.
- Experiments on hydraulic retention time showed that the denitrification efficiency decreased rapidly from 100% to 70% when the HRT was gradually decreased to 1 h. When the HRT was increased to 2 h, the denitrification efficiency was immediately recovered. The optimal hydraulic retention time was then determined as 2 h.
- The pH remained constant despite the increase of sulfate ions up to 9,430 mg/L due to the denitrification process. As a result, it was confirmed that the granular aragonite carrier for the buffering action acted as an excellent alkaline source.
- When the organic matter was added to simulate RO concentration water, the denitrification efficiency significantly decreased due to the growth of heterotrophic microorganisms. However, the efficiency could be restored after 25 d of operation without organic matter.

- Increased abundance of *Thiobacillus thioparus*, *Sulfurimonas denitrificans*, and *Sulfurimonas parvalvinellae* were mainly associated with denitrification performance in the cultivation process.

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