Autotrophic denitrification of synthetic nitrate-contaminated groundwater in up-flow fixed-bed bioreactor by pumice as porous media

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Abstract

Background: Increasing nitrate concentrations in groundwater resources is considered a common environmental and public health problem worldwide. In this research, an autotrophic up-flow bioreactor with pumice as media was used to study the effects of the sulfur-to-nitrogen (S/N) ratio and empty bed contact time (EBCT) on nitrate removal efficiency and byproducts.

Methods: Experiments were carried out in a 3.47 L up-flow, fixed-bed reactor with 3 sampling ports. To evaluate the overall impact of S/N ratio and EBCT on the performance of the bioreactor, several phases with different S/N ratios and EBCTs were applied.

Results: At a constant S/N ratio of 3.85 g/g, as EBCT decreased from 24 hours to 2 hours, the nitrate removal efficiency decreased from 98% to 64%. On the other hand, at the desired EBCT of 4 hr, as S/N ratio decreased from 3.85 to 1.51 g/g, nitrate removal efficiency was reduced from 85% to 32%. Changing the EBCT and S/N ratio also affected the effluent nitrite and sulfate concentrations as byproducts. At the S/N ratio of 3.85 g/g and EBCT of 24 hours, effluent nitrite and sulfate concentrations were 0.1 mg NO₂⁻/L and 463 mg SO₄²⁻/L, respectively. Decreasing the S/N ratio to 1.51 g/g and the EBCT to 4 hours caused drastic changes in effluent nitrite and sulfate concentrations.

Conclusion: The results indicated that the autotrophic denitrification with thiosulfate as electron donor and pumice as media was feasible and applicable for nitrate contaminated groundwater.

Keywords: Autotrophic Denitrification, Pumice, Nitrate, Nitrite, Thiosulfate

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Introduction

Inorganic nitrogen compounds, such as nitrite, nitrate, and ammonium, are abundant contaminants in ground and surface water. High NO₃⁻⁻N concentrations (>10 mg/L) have been recorded in numerous aquifers in many countries (1). Nitrate that is used mainly in inorganic fertilizers leaks into the aquifer and surface waters (2, 3). It has been estimated that more than 70% of all nitrate existing in surface and groundwater comes from fertilizers and other materials used in agricultural activities. However, emissions from septic tanks and seep from sewers, atmospheric deposition, and the usage of sewage sludge as fertilizer can all be effective (4,5). In some cases, high nitrate concentrations are caused by natural sources (6). In rural and urban areas of Iran, the growth in agriculture and industry has led to groundwater nitrate concentrations over the acceptable thresholds (7).

Causing eutrophication, nitrate has adverse effects on water ecosystems (8). According to Sun et al (2) and Migeot et al (9), health implications of nitrate in humans include but are not limited to methemoglobinemia, diminished vitality, low weight in newborns, higher chance of fetal death, and formation of carcinogens in the digestive system. In livestock, nitrate contamination can cause reduced weight gain rates. Among different physical, chemical, and biological nitrate removal processes, biological denitrification is an efficient microbial process that changes nitrate to N₂ under anoxic conditions (1,10,11). In this process, four enzymatic steps produce intermediates such as nitrite (NO₂⁻), nitric oxide (NO), and nitrous oxide (N₂O) (12). Nitrate can be exploited as a terminal electron acceptor by a variety of heterotrophic and autotrophic bacteria. While the former uses organic matters, the latter relies...
on inorganic compounds such as molecular hydrogen, sulfur, sulfide, thiosulfate, and Fe$^{2+}$ as electron donors (7,13). Heterotrophic denitrification has been widely applied to wastewater treatment due to its high efficiency and economical aspects (14), but for natural bodies of water, it needs the organic carbon as the electron donor to reduce nitrate and metabolism of heterotrophs. The most important problem of using this process is the existence of residual organic matter such as methanol, ethanol, or acetic acid that may create several problems in the treated water (15).

Compared with heterotrophic treatment, autotrophic treatment is an attractive alternative because of 2 notable advantages. First, since autotrophic denitrification does not need any external organic carbon such as methanol and ethanol, it is very cost effective with a lower risk of secondary contamination compared to heterotrophic denitrification. Second, autotrophic denitrification has a lower cell yield of autotrophic bacteria and, therefore, less sludge production, which minimizes the handling of sludge (16). In autotrophic denitrification, inorganic carbon compounds such as carbon dioxide (CO$_2$) and bicarbonate (HCO$_3^-$) are used as carbon sources (13,17,18). The produced H$^+$ consumes alkalinity to balance the charge; therefore, some alkaline sources such as limestone are needed to retain the pH in the best condition for autotrophic denitrifiers (19,20).

In sulfur-based autotrophic denitrification, sulfur-oxidizing bacteria such as *Thiobacillus denitrificans* and *Thiomicrospira denitrificans* are agents for reducing nitrate to N$_2$ (21-24). A variety of solid materials has been applied as biofilm carriers for attached growth autotrophic denitrification processes like granular-activated carbon (25,26), polystyrene bead (27), elemental sulfur (28,29), zeolite (30), ceramsite (10), and polyurethane foam (31). The aims of this study were to determine the feasibility of using pumice as a biofilm cost-effective carrier, and the effects of S/N ratio, EBCT, and height of bioreactor on nitrate removal efficiency and obtained byproducts.

**Methods**

An up-flow bioreactor system, as shown in Figure 1, was used for the experiments. The column bioreactor was made of steel with an inner diameter of 8 cm, height of 80 cm, and total volume of 3.78 L. Three sampling ports were placed at intervals of 23, 46, and 69 cm from the inlet valve. The pumice particles used as biofilm carriers had diameters ranging from 2.36 mm to 4.75 mm and a porosity of 60%. Due to the high porosity of pumice, this media has a high specific surface that increases the attached growth. In addition, pumice has some other advantages such as low cost and availability in all parts of Iran. The bioreactor was fed continuously in the up-flow mode using an adjustable peristaltic pump with the minimum flow rate of 0.5 L/min. Influent groundwater was taken from one of the Tehran’s Qanats (Iran). The chemical properties of the water of the Qanat are shown in Table 1. To achieve the desired influent nitrate concentration (150 mg/L), KNO$_3$ was added to the influent (32).

The inoculum was obtained from the returned anoxic activated sludge of the A2O process treating municipal wastewater in the Shahrah-e-Ekbatan wastewater treatment plant in Tehran (Iran). To pre-enrichment the autotrophic denitrifying biomass, a synthetic medium consisting of 3.0 g KNO$_3$/L, 0.6 g Na$_2$S$_2$O$_3$.5H$_2$O/L, 1.5 g NaHCO$_3$/L, 0.3 g KH$_2$PO$_4$/L, 0.4 g MgSO$_4$.7H$_2$O/L, and 1 ml/L of trace element containing 5.74 g NH$_4$Cl/L, 5.6 g K$_2$HPO$_4$/L, 1 g MgCl$_2$/L, 1 g FeCl$_3$.6H$_2$O/L, 1 g MnSO$_4$.H$_2$O/L, and 1 g CaCl$_2$/L was added to the inoculum in a master culture reactor. After sufficient bacterial growth, the bioreactor was inoculated with one liter of the culture reactor. The rest of the bioreactor volume was filled with synthetic groundwater. After a few hours, the bioreactor was operated continuously at an empty bed contact time (EBCT) of 24 hours with synthetic groundwater as influent. The autotrophic denitrification process needs an electron donor to participate in the oxidation/reduction mechanism. Sodium thiosulfate was added to the influent as the electron donor. At first, the bioreactor was operated

![Figure 1. Experimental set-up.](image-url)
with the maximum electron donor concentration (500 mg/L Na$_2$SO$_4$·5H$_2$O) and different EBCTs (2, 4, 8, 16, and 24 hours). In this step, the desired EBCT was determined. Then, in this desired EBCT, the effects of electron donor concentration and column height on denitrification efficiency were studied. To determine the effect of the S/N ratio on the bioreactor performance, different S/N ratios (3.85, 3.05, 2.29, and 1.51 g/g) were tested. All experiments were conducted at 25±2°C.

Analysis methods
All chemical analyses were performed according to the APHA's Standard Methods for Examination of Water and Wastewater (33). Concentrations of nitrate, nitrite, and sulfate were determined using a HACH DR5000 spectrophotometer (USA). Alkalinity was measured by the titration method, and pH values were monitored using a standard digital pH meter (691 Metrohm). In each analysis, at least one in 3 samples was duplicated, and the deviation between the two samples was always less than 5%.

Results
The effects of flow rates on the denitrification of synthetic groundwater was investigated by increasing the flow rate from 3.47 L/d to 41.62 L/d with a corresponding EBCT of 24 to 2 hours at the S/N ratio of 3.85 g/g. Figure 2 shows the variations of nitrate removal efficiency and effluent nitrate concentration during the operation. The maximum nitrate removal efficiency of 98% was obtained with an EBCT of 24 hours. As EBCT was decreased from 24 to 2 hours, the nitrate removal efficiency decreased from 98% to 64% and the effluent nitrate concentration increased from 3 to 54 mg/L. According to Figure 2, changes in nitrate removal efficiency in different EBCTs are not the same. In lower EBCTs, these changes were severe. However, an increase in the nitrate-nitrogen removal rate was observed during EBCT reduction; the nitrate-nitrogen removal rate increased from 33 mg/L/d NO$_3$-N in an EBCT of 24 hours to 260 mg/L/d NO$_3$-N in an EBCT of 2 hours. In an EBCT of 4 hours, since the effluent nitrate concentration of 23 mg/L was lesser than the regulatory maximum contaminant level of 50 mg/L for drinking water (according to Iranian standards), this EBCT was selected as the desired EBCT for subsequent experiments (34).

The effects of EBCT on the effluent nitrite and produced sulfate concentrations are shown in Figure 3. Nitrite concentration in the effluent represented the incomplete denitrification.

The optimal range of pH for autotrophic denitrifiers is 6.8-8.2 (35), and maximum denitrification activity is observed at pH 7.5-8.0 (36). During the experiments, pH was controlled by adding buffer. Autotrophic denitrifiers consume alkalinity. According to the current results, the ratio of alkalinity (as CaCO$_3$) consumed per NO$_3$-N removed (g/g) was 2.92, which was similar to the theoretical ratio (37), and the results found by Chung et al (38) indicated that autotrophic denitrification consumed 3-4 mg CaCO$_3$/mg NO$_3$-N.

Effects of S/N ratio on nitrate removal and byproducts
After determining the EBCT of 4 hours as the desired EBCT, the effect of S/N ratio on nitrate removal efficiency was investigated. S/N ratios of 3.85, 3.05, 2.29, and 1.51 (g/g) were tested. The stoichiometric S/N ratio for complete denitrification is 3.84. Previous studies have shown that in S/N ratios between 3.70 and 6.67, no significant changes in denitrification rate were observed (3).

In Figure 4 the effect of S/N ratio on nitrate removal efficiency is shown. At an EBCT of 4 hours, decreasing these conditions would lead to a higher nitrate removal efficiency. The results of this study are shown in Figure 3. The nitrate removal efficiency is shown. At an EBCT of 2 hours, the nitrate removal efficiency decreased from 98% to 64% and the effluent nitrate concentration increased from 3 to 54 mg/L. According to Figure 2, changes in nitrate removal efficiency in different EBCTs are not the same. In lower EBCTs, these changes were severe. However, an increase in the nitrate-nitrogen removal rate was observed during EBCT reduction; the nitrate-nitrogen removal rate increased from 33 mg/L/d NO$_3$-N in an EBCT of 24 hours to 260 mg/L/d NO$_3$-N in an EBCT of 2 hours. In an EBCT of 4 hours, since the effluent nitrate concentration of 23 mg/L was lesser than the regulatory maximum contaminant level of 50 mg/L for drinking water (according to Iranian standards), this EBCT was selected as the desired EBCT for subsequent experiments (34).

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the S/N ratio from 3.85 to 1.51 (g/g) reduced nitrate removal efficiency from 85% to 32% and increased nitrite accumulation.

Figure 5 illustrates the effect of S/N ratio on nitrate, nitrite, and sulfate concentrations. As the S/N ratio was reduced, a decrease in the amount of produced sulfate was observed. At the S/N ratio of 3.85, the nitrate concentration in the effluent was 3.95 mg NO$_3^-$/L. By decreasing the S/N ratio, nitrite accumulation was increased so that at the S/N ratio of 1.51, about 25 mg/L nitrite was observed in the effluent. Figures 6, 7 and 8 show the profile of effluent nitrate and nitrite concentrations and sulfate production versus reactor height (23, 46, and 69 cm), at different EBCTs and an S/N ratio of 3.85 g/g, respectively. High rates of nitrate removal and sulfate production at the lower sampling port (23 cm) were observed in all cases. Generally, the 46 cm and 69 cm sampling ports were associated with lower removal and production rates. Increased cell growth at the lower sampling port was anticipated considering the high nitrate and thiosulfate concentration in the feed.

It was observed that at an EBCT of 4 hours, more than 90% of nitrate removal efficiency occurred in the first 23 cm of the column. The reduction of EBCT increased the effect of higher parts on the removal of nitrate removal, so that at an EBCT of 2 hours, 50% of total nitrate removal occurred in the mentioned part of the column (Figure 6). As shown in Figure 7, a rapid accumulation of nitrite was observed in the first 23 cm of the column at EBCTs of 24, 16, and 8 hours and an S/N ratio of 3.85 g/g. However, upon completion of denitrification at the height of 46 cm, an intensive nitrite decrease was observed.

Figure 8 shows the effect of column height on effluent sulfate concentration at different EBCTs. The sulfate production profile was very similar to that of nitrate; therefore, a fast increase in sulfate concentration was observed in the first 23 cm of the column. In upper ports, the increase in sulfate concentration occurred at a slower rate.

**Discussion**

Effects of EBCT on nitrate removal and byproducts

According to Zhou et al (16), low influent concentration and longer EBCTs improve nitrate removal efficiency. In addition, they reported that the nitrogen in groundwater and effluent from municipal WWT can obtain removal rates of up to 90% with a four-hour EBCT. However, longer EBCTs are required for water with a nitrate contamination over 70 mg/L. According to this study, reducing the EBCT from 24 hours to 2 hours led to the reduction of nitrate removal efficiency from 98% to 64% (with a constant S/N ratio of 3.85 g/g).

As shown in Figure 3, at EBCTs of 24, 16, and 8 hours, the effluent nitrite concentrations were less than the maximum allowable level of 3 mg/L. As EBCT decreased to 4 and 2 hours, a drastic nitrite accumulation occurred in the effluent due to incomplete denitrification. Although molecular analysis associates the same microorganisms to denitrifications of nitrate and nitrite into nitrogen (16), the nitrite accumulation in autotrophic denitrification is associated with different causes. High specific utilization rate of nitrate, the reduction in induction time of NO$_2^-$ reducing enzymes caused by nitrate, the variety in...
saturation rates and affinities of electron acceptors, and the type of bacteria are all considered to be contributors to nitrate accumulation in this process (39).

When thiosulfate is used as the electron donor, sulfate ion is produced as a byproduct (31). Figure 9 shows the relationship between the produced sulfate and removed nitrate for different S/N ratios at various EBCTs. It was found that for each mg NO$_3^-$ removed, 2.43 mg/L SO$_4^{2-}$ was produced, which corresponds closely with the stoichiometric ratios of 2.62 and 2.24 derived from Equations (1) and (2), respectively (37,40). The difference between these values is caused by several factors such as feed composition, microbial population, pure culture, temperature, pH, etc.

\[ 0.844S_2O_3^{2-} + NO_3^- + 0.434H_2O + 0.347CO_2 + 0.0865HCO_3^- + 0.0865NH_4^+ \rightarrow 0.0865C_2H_3NO_2 + 0.5N_2 + 1.689SO_4^{2-} + 0.697H^+ \]  
(1)

\[ NO_3^- + 0.844SO_4^{2-} + 0.347CO_2 + 0.0865HCO_3^- + 0.0865NH_4^+ \rightarrow 0.036C_2H_3NO_2 + 0.48N_2 + 1.455SO_4^{2-} + 0.045H^+ \]  
(2)

As shown in Figure 9, the produced sulfate had a linear relationship with removed nitrate, and the EBCT had no significant effect on the stoichiometric ratio.

**Effect of S/N ratio on Nitrate removal and byproducts**

As shown in Figure 4, decreasing the S/N ratio to 1.51 caused a notable decrease in the nitrate removal efficiency. This reduction occurred because of the electron donor shortage. In this case, increasing EBCT did not cause any significant changes in the nitrate removal efficiency; so, it seems that the concentration of electron donor is the controller agent. Campos et al (39) showed that when S/N ratio was less than 2.44, an increase in nitrate concentration occurred during denitrification.

It can be observed from Figure 5 that, in the denitrification process, a rapid initial decrease in thiosulfate concentration resulting in sudden nitrite accumulation occurred (38). In this case (low S/N ratio), the electron donor shortage caused incomplete denitrification and led to the ultimate nitrite accumulation. The results of this study correspond with those of previous studies and show that at S/N ratios lower than 4.35 (g/g), the accumulation of nitrite is inevitable (39). In this condition, even in greater EBCTs, nitrite accumulation occurred. Also, 2.43 mg of SO$_4^{2-}$ was produced for each milligram of NO$_3^-$ removed, and this ratio did not change with different S/N ratios.

Campos et al (39) also showed that sulfate concentrations greater than 500 mg/L induced inhibitory effects on the nitrate removal efficiency. According to their study, autotrophic denitrifying activity was inhibited to 85% of that in the control test at a concentration of 5000 mg SO$_4^{2-}$/L. Oh et al reported that sulfate inhibition began at concentrations above 2000 mg SO$_4^{2-}$/L using a mixed culture (41), while Claus and Kutzner found that this compound started to inhibit at 1600 mg SO$_4^{2-}$/L (36). However, in this study, according to the low concentrations of nitrite and sulfate, the inhibitory effects were negligible.

**Effects of column height on nitrate removal and byproducts**

Moon et al (42) showed that a larger portion of nitrate removal took place in the bottom part of the column. Their findings are very similar to the results of this study that show more than 90% of nitrate removal occurred in the first 23 cm of the column (Figure 6). They observed that almost all the nitrate removal occurred in the first 25 cm of the column, and the effect of S/N ratio was negligible. Figure 7 shows the effluent nitrite concentrations at different EBCTs and heights. The results showed a great nitrite accumulation in the first 23 cm, similar to the findings of Moon et al (42) which showed nitrite accumulation at the height of 17 cm. Incomplete denitrification caused primary nitrite accumulation, but in upper heights, nitrite concentration decreased because of complete denitrification. In shorter EBCTs, such as 2 hours and 4 hours, nitrite accumulation continued, so that a great nitrite concentration in the effluent was observed. The behavior of the sulfate concentration was very similar to revers trend nitrate removal (Figure 8). The results showed a notable accumulation of sulfate concentration in the first 23 cm due to the high rate of nitrate removal in this section of the bioreactor.

**Conclusion**

Continuous bioreactor tests showed that pumice granules can be used as neutral media in autotrophic bio-filters and have a significant performance in reducing the nitrate concentration of groundwater. However, parameters such as EBCT, S/N ratio, and column height play important roles in nitrate removal efficiency. While using autotrophic microorganisms for denitrification, byproducts such as nitrite and sulfate (if sulfur-oxidizing bacteria are used) should always be monitored. In fact, parameters that control the efficiency of nitrate removal can also control the byproducts.
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Ethical issues
It is confirmed that this manuscript is the original work of the authors. It has not been published, nor is it under review in another journal, and it is not being submitted for publication elsewhere.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
All authors contributed equally and participated in the collection, analysis, and interpretation of the data. All authors critically reviewed, refined, and approved the manuscript.

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