Influence of Fe^{3+} Ions on Nitrate Removal by Autotrophic Denitrification Using *Thiobacillus denitrificans*

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The sulphur-based autotrophic denitrification process utilizing *Thiobacillus denitrificans* was studied experimentally as an alternative method of removing nitrates from industrial wastewater. The objective of the work was to examine the effect of ferric iron addition to the reaction mixture and determine optimal dosage for specific conditions. All experiments were carried out in anoxic batch bioreactor, and elemental sulphur was used as an electron donor. Compared to the control operation without ferric iron addition, significant increases in nitrates removal were demonstrated for the concentration of ferric iron equal to 0.1 mg L^{-1}. However, under these conditions, increased nitrite content was detected in the reaction mixture which exceeds the limits for drinking water.

Key words:

autotrophic denitrification, nitrates removal, nitrites removal, batch reactor, *Thiobacillus denitrificans*, water treatment

Introduction

Nitrate is commonly regarded as a contaminant due to its impact on the environment and public health. It is one of the major contaminants responsible for the eutrophication of aquatic ecosystems and degradation of surface water quality. A high concentration of nitrate in drinking water may cause methemoglobinemia,[1] congenital malformations, and other diseases.[2] Nitrates may also play a role in the development of some cancers.[3–6]

Recently, biological treatment processes have been widely used to remove nitrates from industrial wastewater.[7–9] Also, several other techniques, such as ion exchange, reverse osmosis, nanofiltration, electrodialysis and chemical precipitation,[2,10–14] are available for both wastewater and drinking water treatment. However, the utilization of these physical/chemical processes has been limited due to their poor selectivity, expensive operation, and subsequent disposal problem of the generated nitrate waste brine.

Biological methods for the removal of nitrates are based on using a microorganism's metabolism. An estimated nitrate reduction scheme during denitrification is shown in Eq. 1.[15]

The process has the advantage of harmless nitrogen gas being the major end product. Many species of bacteria are capable of denitrification. These species can be ordered into two groups according to electron donor substrate. In the first case, denitrifying bacteria use an organic substrate. This method is called heterotrophic denitrification and includes *Bacillus*, *Escherichia*, *Pseudomonas,* etc.[2,16,17] In the second case, inorganic compounds are used as bacterial electron donors in autotrophic denitrification. One of the representatives of autotrophic denitrification bacteria is *Thiobacillus denitrificans.*[18]

T. denitrificans is a widely distributed microorganism found in soil and water habitats. It is not toxic or pathogenic, and belongs to the group of gram-negative, chemolithoautotrophic and facultative anaerobic bacteria. This group can obtain energy by the oxidation of elemental sulphur and other sulphur-containing inorganic compounds while electrons are released. Under anoxic conditions, nitrate and nitrite ions are reduced by these electrons, and molecules of nitrogen gas are formed. The denitrification process of *T. denitrificans* is catalysed by bacterial enzymes. The energy that is obtained is used for the utilization of carbon dioxide as a carbon source.[19,20]

$$NO_3^- \xrightarrow{\text{Nitrate reductase}} NO_2^- \xrightarrow{\text{Nitrite reductase}} NO \xrightarrow{\text{Reductase of nitric oxide}} N_2O \xrightarrow{\text{Reductase of nitrous oxide}} N_2 \quad (1)$$

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The heterotrophic denitrification process is applied most extensively today because of its high efficiency and the simplicity of the reactors required. However, some industrial wastewaters lack sufficient quantities of organic carbon that are required by the bacteria. An inadequate amount of organic electron donor may result in a high level of nitrate and nitrite in the effluent, whereas overloading can cause secondary pollution by the organic substrate, which necessitates post-treatment.[16] Thus, as an alternative, the autotrophic denitrification process has been receiving more attention recently due to its two major advantages: (1) no external organic substrate needs to be added, thus decreasing the operating costs; and (2) less sludge is produced, thus reducing the handling of sludge.[21,22]

The optimal function of autotrophic denitrification using *T. denitrificans* is influenced by a number of agents and factors. Important factors include the type of electron donor (elemental sulphur, sulphide or thiosulphate), and the pH value. Nitrate removal in autotrophic denitrification is accompanied by the production of hydrogen ions, thus lowering the pH. For example, with elemental sulphur it can be described by equation[23]

$$5S + 6NO_3^- + 2H_2O \rightarrow 5SO_4^{2-} + 3N_2 + 4H^+ \quad (2)$$

Adjustment of pH is therefore necessary to keep the pH above 5.5 for optimal bacterial activity. Therefore, limestone granules are usually added to sulphur to maintain the pH during the denitrification process. The optimum ratio of sulphur to limestone was found to be approximately 1:1[24]. Higher efficiencies of autotrophic denitrification with sulphide as the electron donor at 35 °C were observed by Fajardo *et al.*[25] Authors[24,26] also studied the influence of both C/N and S/N ratios. The nitrite-nitrogen concentrations in the range of 36 to 60 mg L^{-1} as well as nitrate-nitrogen concentrations above 660 mg L^{-1} may cause inhibition of sulphur-driven autotrophic denitrification.[18,25] Some studies have reported a sulphide inhibitory effect on the reduction of nitrous oxide to nitrogen gas (the last step of denitrification).[27,28] The high level of water hardness and the existence of sulphide in the effluent might also be limiting factors in denitrifying nitrate by using sulphur and limestone because of the precipitation crust formed by calcium sulphide on the sulphur surface.

The presence of biogenic elements and other compounds in autotrophic denitrification process was also evaluated.[22,29] Authors[30] studied the ability of autotrophic nitrate reduction in the presence of ferrous iron. They have shown that pure cultures of nitrate-reducing bacteria can grow under anaerobic conditions with ferrous iron as the only electron donor, or as a member of a mixture of electron donors and acetate.

However, there is no detailed information on the advantages and limitations of the process for treatment of nitrate contaminated waters containing ferric iron. Ferric iron is one of the biogenic elements usually present in bacterial cells and their components, and it is also a part of growth media recommended for *T. denitrificans* cultivation in the form $FeCl_3$. Also, the initial attack by oxidant Fe^{3+} was proved to be crucial for the complete sulphur biological oxidation.[31,32] Dissolved iron is mainly present in water as Fe^{3+} under acidic and neutral, oxygen-rich conditions. The amount varies strongly, and is different in rivers (usually 0.5–1 mg L^{-1}) and groundwater (up to 100 mg L^{-1}). Potential sources for autotropic denitrification, for example, industrial wastewaters from leather, fertilizer-processing, and surface finishing industries, can even contain considerably higher amounts of ferric iron. Thus, the objective of this work was to study experimentally the influence of ferric iron on nitrate and nitrite removal in autotrophic denitrification using *T. denitrificans* in suspension batch bioreactors, where elemental sulphur was used as electron donor.

Material and methods

Microorganism cultivation

The *T. denitrificans* strain (DSM 12475) was obtained from Leibnitz Institute DSMZ – German Collection of Microorganisms and Cell Structures. The culture was maintained at 33 °C in a sterile liquid growth medium composed of $Na_2S_2O_3$ (10 g L^{-1}), KH_2PO_4 (11.8 g L^{-1}), Na_2HPO_4 (1.2 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.1 g L^{-1}), $(NH_4)_2SO_4$ (0.1 g L^{-1}), $CaCl_2$ (0.03 g L^{-1}), $FeCl_3$ (0.02 g L^{-1}) and $MnSO_4$ (0.02 g L^{-1}).[33] The seed culture was incubated for four weeks as a shaken culture (150 rpm), and the fresh sterile growth medium was added every week. After four weeks, a *T. denitrificans* suspension with optical density (OD = 0.03) was obtained.

Suspension batch bioreactors

Glass bottles (volume 1 L) with a polypropylene screw cap and septum were purchased from Fisher Scientific (CR) and used as batch bioreactors in all experiments. Bioreactors were sterilized by autoclaving and filled with sterile crushed sulphur (100 g, size fraction 2.5–5.0 mm), sterile crushed limestone (100 g, size fraction 3.0–4.0 mm, Aqua Medic, Germany), sterile solution of $NaNO_3$ (volume 1 L, initial concentration 100 mg L^{-1} NO_3^-, e.g. 22.26 mg L^{-1} of N-NO_3^-), 1 mL of *T. denitrificans* suspension, and a solution of $FeCl_3$ at various concentrations of Fe^{3+} equal to 0.05, 0.1, 0.5, and

1.0 mg L^{-1}. No other particular nutrients for the microorganisms were added.

The total reaction volume was adjusted by demineralized water to 1 L. During the experiments, samples were taken every 7 days using sterile needles through the septum in the screw cap of each bioreactor.

The oxidation of sulphur using *T. denitrificans* during the denitrification process led to the formation of sulphuric acid, and the environment in bioreactors was potentially acidified. In our experiments, the pH was kept in the range of 7.0 to 7.3, because the limestone present in the bioreactors reacted with the sulphuric acid and formed carbon dioxide and calcium sulphate. The resulting carbon dioxide was utilized by *T. denitrificans* as a carbon source.[24]

Analytical methods

The concentrations of nitrate and nitrite ions in the reaction mixture were determined by spectrophotometric methods (European Standard ISO 7890-3: 1988 and European Standard EN 26777:1993) using a Spectrophotometer UV/VIS DR 6000 (Hach, CR). The nitrate ions reacted with sulphosalicylic acid (in the presence of sodium salicylate and sulphuric acid) followed by alkalization by sodium hydroxide. The final mixture was measured spectrophotometrically at 415 nm. The nitrite ions were reacting with 4-aminobenzenesulphonamide in the presence of phosphoric acid (at pH 1.9) to create diazonium salt, which further reacted with N-(1-naphtyl)ethylenediamine in the presence of 4-aminobenzenesulphonamide. The absorbance was measured at 540 nm. The values of total nitrogen were counted as the sum of nitrogen-nitrate and nitrogen-nitrite ions. The acidity and redox potential of samples were measured by a potentiometric method using a combined probe connected to a Multimeter HQ 30d (Hach, CR). The values of dissolved oxygen were determined by an electrochemical method (ISO 10523:2008, EN ISO 5814:2012) by means of an electrochemical cell, which was isolated from the sample by a gas permeable membrane probe (Cybersan DO 300, Eutech Instruments, USA). All analyses were performed in duplicate.

Microorganisms monitoring

The bioreaction was run for 70 days, and every 7 days, the presence of microorganisms was verified by the cultivation using two different solid growth media. The first one was composed of agar (15 g L^{-1}), $Na_2S_2O_3$ (10 g L^{-1}), KNO_3 (5 g L^{-1}), KH_2PO_4 (1.8 g L^{-1}), NaH_2PO_4 (1.2 g L^{-1}), $NaHCO_3$ (0.5 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.1 g L^{-1}), $(NH_4)_2SO_4$ (0.1 g L^{-1}), $CaCl_2$ (0.03 g L^{-1}), $FeCl_3$ (0.02 g L^{-1}) and $MnSO_4$ (0.02 g L^{-1}) [33] and used standardly for *T. denitrificans* cultivation. The second solid growth medium was R-2A Agar (HiMedia Laboratories), which enabled the cultivation of potential contaminating microorganisms. Cultures on solid media were grown in 33 °C and under both aerobic and anoxic conditions. Also, samples for identifying the microbial population by polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) were collected. These analyses were done in the laboratory of the Department of Environmental Protection Engineering (Tomas Bata University in Zlín, Faculty of Technology, Czech Republic).

Results and discussion

First collected were samples of reaction mixture for identification of *T. denitrificans* in bacterial suspension used. Comparing the sequencing result with existing gene sequences in the database, our sequence shares the highest identity of 97 % with the sequence of *T. denitrificans*. The samples were then subjected to single colony isolation on agar plates. Small opalescent colonies were observed after 10 days of anoxic incubation on the thiosulphate/nitrate solid growth medium, indicating presence of bacteria *T. denitrificans*. On the other hand, the samples incubated under aerobic conditions in the same medium, as well as the samples incubated using R-2A medium under both aerobic and anoxic conditions showed no growing colonies of microorganisms. To verify both the presence of *T. denitrificans* and absence of other contaminating microorganisms, the cultivation experiments were repeated every seventh day. The number of cells remained quite constant during the whole experiment. These results strongly suggest that *T. denitrificans* was responsible for denitrification and sulphur-oxidation under the applied reactor operating conditions.

Nitrate reduction

The concentrations of nitrate ions in the reaction mixture were monitored for 70 days. The results are given in Fig. 1. During the first 40 days or so, a minimal decline of nitrate ion concentration could be observed. There are two possible explanations for this phenomenon. Firstly, there was an adaptation phase of the bacterial cells to the environment (lag phase), and secondly, some portion of oxygen still remained in the system, and oxygen has precedence over nitrate and nitrite ions as a terminal electron acceptor in the energy metabolism of *T. denitrificans*.[20] The values of dissolved oxygen concentration higher than 0.5 mg L^{-1} were determined in the initial phase of the process (see Fig. 1), indicating a considerable contribution of the latter mechanism.

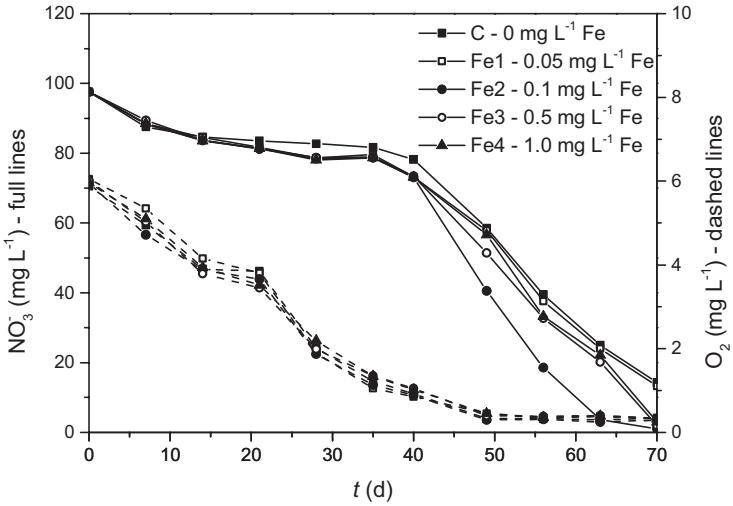

Fig. 1 – *Time dependence of nitrate and dissolved oxygen concentrations in the reaction mixture of autotrophic cultivation in batch bioreactors for various concentrations of ferric iron. The standard deviation of individual nitrate concentration values was 0.48 mg L^{-1} NO_3^-.*

As can be seen from Fig. 1, after 40 days of stabilization, the nitrate concentrations rapidly declined in all cases. The trends in the concentration decline were similar, indicating that in the treatment with ferric ion concentrations of higher than 0.05 mg L^{-1}, nearly complete nitrate removal occurred in 70 days. The nitrate concentration trends followed pseudo first-order kinetics, which can be described by equation

$$\ln\frac{c}{c_0} = -k\cdot(t-t_0) \qquad (3)$$

where c is the concertation in time t, subscript 0 indicates initial value, and k is the reaction constant. The values of k were determined from experimental data using least squares method, and are summarized in Table 1.

Generally, compared with the reaction rate of heterotrophic denitrification, the reaction rate of sulphur-based autotrophic denitrification is low. The reaction constant k in the bioreactors with added Fe^{3+} was, in some cases, higher than that in the bioreactor without ferric iron. This shows that Fe^{3+} salts minimally benefit the metabolic activity of *T. denitrificans* in the first step of nitrate decomposition. Compared to the control operation without ferric iron addition, significant increases in nitrate removal were demonstrated for the ferric iron concentration of 0.1 mg L^{-1}. A higher dosage of ferric iron had a lower impact on nitrate removal.

Table 1 – *Values of reaction constants of nitrate reduction*

System	t_0 (d)	c_0 (mg L^{-1})	k (d^{-1})	R^2
C	40	78.2	0.0569	0.9724
Fe1	40	73.2	0.0575	0.9619
Fe2	40	73.1	0.1462	0.9464
Fe3	40	73.5	0.0565	0.9829
Fe4	40	73.1	0.0535	0.9653

Because of pH value higher than 7, the soluble ferric iron was not detected in the reaction mixture. In our opinion, most of the ferric iron added into the reactor precipitated on the surface of sulphur and limestone. This should have resulted in the several phenomena. For low concentrations, the ferric iron acts as an accelerator of sulphur biological oxidation.[31,32] However, with the increase in ferric iron loading, the amount of precipitated matter increased, and the limit in the mass transfer rate of sulphur in the reaction may have led to the deficiency of electron donors, thus becoming one of the limiting factors for the reaction rate of denitrification.

Nitrite accumulation

Nitrite is an intermediate in the denitrification process. The initial nitrite concentration in the systems investigated was undetectable. However, the experimental results show that nitrite ions were formed and accumulated in the reaction mixture during the ferric-iron-influenced autotrophic denitrification using *T. denitrificans*. As shown in Fig. 2, nitrite concentrations in the reaction mixture first increased and then decreased. Nitrite accumulation in the system was the most significant at the Fe^{3+} concentration of 0.1 mg L^{-1}, giving maximum nitrite concentration of 3.2 mg L^{-1}. For an Fe^{3+} concentration equal to 0.05 mg L^{-1}, it was slightly lower, but higher in the case of 0.5 and 1 mg L^{-1} ferric concentrations. In operations without ferric iron addition, the production of nitrite was the lowest, approaching the detection limit of the molecular absorption spectrometric method used for the analysis. Nitrite is much more toxic than nitrate. It is difficult to establish an exact level at which nitrite concentrations in water are safe or unsafe, but the maximum nitrite concentrations shown in Fig. 2 significantly exceeded the nitrite content limit for drinking water, which is usually 0.5 mg L^{-1}.[34]

As follows from Eq. 1, four key enzymes induced sequentially under anoxic conditions are responsible for reductions in the autotrophic denitrification process. In this study, nitrite is an intermediate formed in the first step and used up in a subsequent step. The subsequent nitrite decomposition is probably the rate-determining step, and the overall reaction cannot proceed any faster than this slowest step. If the presence of ferric ions accelerates the first reaction step, i.e. nitrate decomposition, the result is the nitrite accumulation in the reaction mix-

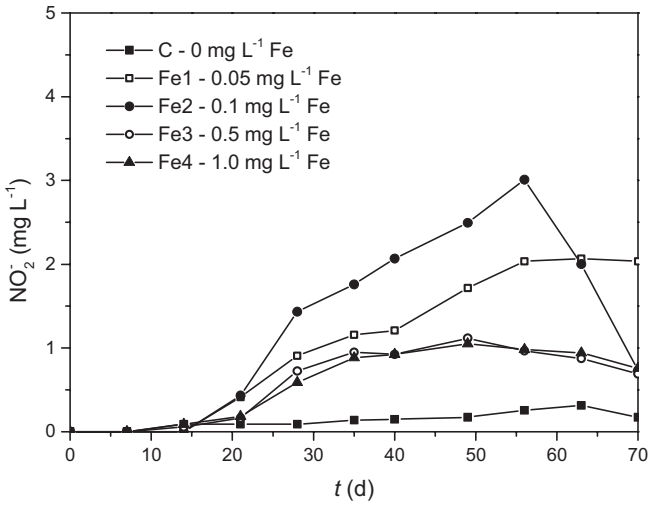

Fig. 2 – *Time dependence of nitrite concentrations in the reaction mixture of autotrophic cultivation in batch bioreactors for various concentrations of ferric iron. The standard deviation of individual concentration values was 0.13 mg L^{-1} NO_2^-.*

ture, as seen in Fig. 2. The lack of nitrates in the final stage of the process is the reason for the drop in the nitrite concentration after 60 days.

The existence of nitrite accumulation in the reaction mixture can also be used as a reliable judgment index for the occurrence of sulphur fouling, and deterioration by ferric iron precipitate.

Total nitrogen removal and sulphate accumulation

Also, the concentrations of total nitrogen in the reaction mixture were determined as the sum of nitrate-nitrogen and nitrite-nitrogen. The course of dependence obtained is similar to that for nitrates (see Fig. 1). This means that the contribution of nitrite to the total nitrogen content in the reaction mixture is virtually negligible.

The stoichiometric Eq. 2 shows that, theoretically, 1.27 mg SO_4^{2-} will be produced when 1 mg NO_3^- is consumed. The experimental values of this ratio obtained by many authors[35–38] are close to this theoretical value. The contamination of produced water by sulphates is obvious, and it has a negative effect on the quality of water. In many countries, the maximum possible concentration of sulphates in water is specified, because large amount of sulphates in water can cause the formation of sulphate-aluminate complexes, and swell and crack concrete, which is made from certain types of cement. On the other hand, sulphates in water do not represent a direct danger to the environment – they are chemically inert, non-volatile, and non-toxic compounds.[39]

Conclusions

The results indicate that Fe^{3+} ions can influence the denitrifying activity of *T. denitrificans* in sulphur-based autotrophic denitrification. The highest increase in nitrate removal was found for the concentration of ferric iron equal to 0.1 mg L^{-1}. However, under these conditions, increased nitrite content was detected in the reaction mixture which exceeded the limits for drinking water. The nitrite decomposition was evaluated as the reaction rate-determining step.

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