EVALUATION OF SULFUR-BASED AUTOTROPHIC DENITRIFICATION

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ABSTRACT Sulfur-based autotrophic denitriﬁcation processes utilize autotrophic denitriﬁers, such as Thiobacillus denitrificans and Thiomicrospira denitrificans, to reduce nitrate to nitrogen gas. Bench-scale experiments were conducted to evaluate the sulfur-based autotrophic denitriﬁcation process. This paper addresses three issues: (1) start-up and operation of a CSTR to provide an adequate seed source of autotrophic denitriﬁers, (2) optimum ratios of elemental sulfur to limestone for various hydraulic loading rates to determine effectiveness of above-ground treatment of nitrate-contaminated ground water, and (3) feasibility studies on in situ remediation of nitrate-contaminated surface water using pond sulfur-based systems. Thiosulfate in a CSTR system was chosen over elemental sulfur in a batch system to provide a sufﬁcient seed source of autotrophic denitriﬁers. Four individual upﬂow ﬁxed-bed reactors with sulfur:limestone ratios of 1:3, 1:1, 3:1, and sulfur only were operated at empty bed hydraulic loading rates of 17.0 hours, 9.6 hours, and 3.1 hours. Initial concentrations of nitrate-nitrogen were 35 mg/l. Results indicate that optimal conditions occurred with a sulfur:limestone ratio of 3:1 and that limestone buffering is critical. A nitrate-nitrogen removal rate of 17.8 mg/l/hr was achieved. Studies on batch pond reactors with complex sediment media reveal denitriﬁcation occurring under both aerobic and anaerobic conditions. The addition of sulfur and limestone enhances the denitriﬁcation rates in the system and further addition of a seed of autotrophic denitriﬁers accelerates the processes even more.

KEYWORDS: autotrophic denitriﬁcation, nitrate, sulfur, bioremediation

INTRODUCTION Nitrate contamination of ground and surface waters has become an increasingly serious problem in the U.S. The United States Environmental Protection Agency has set the maximum contaminant level (MCL) at 10 mg/l for nitrate-nitrogen. In Nebraska alone, more than 20% of nitrate-nitrogen concentrations, measured in 5,826 wells, exceed the maximum contaminant level [1]. Most of the samples exceeding the MCL have concentrations between 10 and 19.9 ppm. Nebraska is not alone in its concern for nitrate-contaminated waters. Since ground water serves as the primary domestic water supply for about 90% of the rural population and 50% of the total population in North America [2], nitrate removal from ground water becomes an extremely important practice due to the adverse health affects associated with nitrate.

Treatment methods for the removal of nitrates from water resources include (1) physical/chemical treatment, such as the ion-exchange process, reverse osmosis, electrodialysis, chemical precipitation, distillation, and (2) biological denitrification by heterotrophic and autotrophic denitriﬁers. The physical/chemical methods only separate nitrates from one liquid phase to concentrate them in another. The further treatment of the concentrated nitrate solution can be very expensive [3]. Moreover, it is difficult to use the

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physical/chemical methods for *in situ* remediation. Heterotrophic biological denitrification is very effective in nitrate removal as long as there is sufficient organic carbon in the system. However, in many situations, such as in ground water, lakes, and stabilization ponds for tertiary treatment, insufficient organic carbon may limit the application of *in situ* heterotrophic denitrification unless organic substances [e.g., methanol (CH₃OH), glucose, and glycerol] are added as external carbon sources [3].

Autotrophic denitrification processes utilize autotrophic denitrifiers, such as *Thiobacillus denitrificans* and *Thiomicrospira denitrificans*, to reduce nitrate to nitrogen gas. The energy source of the autotrophic denitrifying microorganisms is derived from inorganic oxidation-reduction reactions with elements such as hydrogen or various reduced-sulfur compounds (H₂S, S, S₂O₃²⁻, S₄O₆²⁻, SO₃²⁻) as the electron donor. Autotrophic denitrifiers utilize inorganic carbon compounds (e.g., CO₂, HCO₃⁻) as their carbon source. Therefore, compared with heterotrophic denitrification, autotrophic denitrification has two advantages: (1) no need for an external organic carbon source, i.e., methanol and ethanol, which lowers the cost and risk of the process; and (2) less sludge production, which minimizes the handling of sludge.

Studies on autotrophic denitrification processes have been divided into two major directions—that is hydrogen-based and sulfur-based autotrophic denitrification. Because it is difficult to handle hydrogen gas, and generating hydrogen (e.g., from methanol) is very expensive, much more attention has been concentrated recently on sulfur-based autotrophic denitrification. Beijerinck [4], Lieske [5], and Baalsruud and Baalsruud [6] are among the first researchers to establish physiological and biochemical studies on sulfur-oxidizing microorganisms. Recently, considerable research on sulfur-based autotrophic denitrification has been conducted in Europe. Claus and Kutzner [7, 8] investigated continuous cultures of *Thiobacillus denitrificans* to determine kinetics using thiosulfate as an electron donor. They also employed an upflow packed bed reactor with lava stones as the media and thiosulfate as an electron donor. Schippers, *et al.* [9], van der Hoek, *et al.* [10-12], and Kruithof, *et al.* [13], have conducted sulfur/limestone research on the bench-scale, pilot-scale, and demonstration-plant level. Justin and Kelly [14, 15] have performed work using *Thiobacillus denitrificans* in aerobic and anaerobic chemostats with thiosulfate. In the U.S., the sulfur-based autotrophic denitrification has been studied seriously only by very few researchers [16-20]. Batchelor and Lawrence [17-19] have conducted kinetic studies with elemental sulfur. The stoichiometric equation for the reduction of nitrate using elemental sulfur proceeds as follows [17]:

\[
55S + 20CO_2 + 50NO_3^- + 38HO + 4NH_4^+ \rightarrow 4C_5H_7O_2N + 25N_2 + 55SO_4^{2-} + 64H^+
\]

A sulfur-based fluidized bed reactor and a sulfur slurry reactor were tested in Orange County, CA [20-21]. It has been concluded that the sulfur-based autotrophic denitrification process is a reliable and simple process, able to produce bacteriologically- and chemically-safe water that can meet the guideline. However, detailed information about sulfur-based autotrophic denitrification for above-ground treatment is still unavailable, including the process kinetics, biomass distribution in the system, optimal operation conditions, and the procedures for controlling H₂S gas.
produced in the upper part of the fixed bed of the process. Moreover, no successful studies on \textit{in situ} remediation of nitrate-contaminated ground and surface water using the sulfur-based autotrophic denitrification process have been reported.

The overall objective of this study is to develop practical procedures for designing and operating sulfur-based autotrophic denitrification processes for above-ground treatment and remediation of nitrate-contaminated ground and surface waters. The objective of this paper is to present the preliminary evaluation of the start-up and performance of sulfur-based autotrophic denitrifiers at the bench-scale level to determine their effectiveness in above-ground treatment and \textit{in situ} remediation of nitrate-contaminated ground and surface waters. Specifically, the following issues will be addressed: (1) start-up and operation of a continuously stirred tank reactor (CSTR) capable of providing an adequate seed source of autotrophic denitrifiers; (2) evaluation of the performance of a fixed-bed reactor with respect to varying hydraulic retention times and sulfur:limestone ratios; and (3) feasibility studies on \textit{in situ} remediation of nitrate-contaminated surface water using pond sulfur-based autotrophic denitrification systems.

\section*{Materials and Methods}

\subsection*{CSTR and start-up}

Enrichment techniques have been utilized to establish a microbial population of \textit{Thiobacillus denitrificans} to be used in mixed microbial environments. The seed for enrichment was taken from a 1:1 mixture of sludge from the primary and secondary anaerobic digesters at a local municipal waste water treatment plant. From this mixture, a 500 ml seed was added to a 3.4 liter anaerobic CSTR with sampling and gas collection systems to acclimate the denitrifiers (Figure 1). Acclimation was pursued using powdered elemental sulfur, nitrate, buffers, and trace nutrients, and the reactor was run as a semi-batch reactor with a feeding of sulfur powder and other substrate and nutrients once a day.

Difficulties were encountered with this system. Because of the low specific gravity of elemental sulfur, uniform mixing of the powdered sulfur became problematic. Although nearly 100\% nitrate removal could be obtained at concentrations below 60 mg/l, higher concentrations yielded a build-up of nitrite and sulfate, which inhibit the denitrification process. Furthermore, the system provided an inadequate microbial population for use as a seed source.

Due to the difficulties encountered, the anaerobic batch reactor system was changed
to an anaerobic CSTR with thiosulfate to replace elemental sulfur as the electron donor. The feed solution composition for the CSTR is a slight modification of that used by Baldensperger and Garcia [22]: KNO₃, 3.0 g/l; Na₂S₂O₃·5H₂O, 6.0 g/l; NaHCO₃, 1.5 g/l; Na₂HPO₄, 1.5 g/l; KH₂PO₄, 0.3 g/l; MgSO₄·7H₂O, 0.4 g/l; and 1 ml of stock trace nutrient solution per liter of feed solution. The stock trace nutrient solution used was derived from Batchelor and Lawrence [19] and contained the following chemical compounds: NH₄Cl, 5.74 g/l; K₂HPO₄, 5.60 g/l; MgCl₂·6H₂O, 1.00 g/l; FeCl₂·6H₂O, 1.00 g/l; MnSO₄·H₂O, 1.00 g/l; and CaCl₂, 1.00 g/l. The constituents of both feed and trace nutrient solutions were prepared using tap water.

A Masterflex L/S peristaltic pump with standard pump head was used to deliver the feed solution to the reactor. The CSTR reactor was sealed from the atmosphere to allow anaerobic conditions to prevail, and gas was collected via a gas collection system. A saturated sodium chloride solution containing 5% sulfuric acid was used as the retaining fluid in the gas collection system [23]. This system provided the seed source of autotrophic denitrifiers, specifically *Thiobacillus denitrificans*, for the fixed-bed reactors, the batch pond reactor tests, and was also used to evaluate CSTR performance data.

**Fixed-bed reactor**

As shown in Figure 2, four anaerobic upflow fixed-bed reactors were constructed from 2.5-inch I.D. acrylic tubing with four sampling ports. The empty bed reactor volume is 1.11 liters. The sulfur and limestone grain sizes ranged from 2.38 mm to 4.76 mm. Gas collection and monitoring systems have been included in this design.

Start-up of the upflow anaerobic fixed-bed reactors began with the inoculation of 250 ml mixed liquor sampled from the CSTR. The remaining volume in the column was filled with the feed solution used to culture autotrophs in the CSTR. One-day standstill operation (without influent and effluent flow) was allowed for the denitrifiers to acclimate to the granular sulfur and limestone media to form biofilms.

After the first day, a high flow rate operation with a recirculation ratio of 100% began. For the first week, a daily inoculation of 250 ml of mixed liquor from the CSTR and 750 ml of nitrate feed solution went on. At the time of inoculation, 1,000 ml of feed solution was introduced daily and 1,000 ml of mixed liquor continued to be wasted daily.

After approximately 10 days of the initial inoculation, signs of attached growth appeared. At 14 days the attached growth was considered substantial enough to stop the recirculation. The system was then

**Figure 2. Upflow fixed-bed column reactor.**
operated as individual units without recycling. After 28 days from the initial inoculation, nitrate-nitrogen concentrations in the effluents and gas production rates had stabilized, showing that quasi-steady-state conditions had been reached and further testing could begin.

**Batch-pond system**

Two conditions were analyzed to evaluate the feasibility of using batch-pond systems for *in situ* remediation of nitrate-contaminated surface water. The first condition, an open environment, was evaluated using standard 400 ml griffin beakers. The second condition, an anaerobic environment, was evaluated using 160 ml milk dilution bottles with a screw-on septum cap. Anaerobic conditions were established in the dilution bottles by flushing them with nitrogen gas for 5 minutes.

Table 1 shows the experimental conditions in pond batch reactor systems. As shown in Table 1, batch-pond reactors contained either one or all of the following items: (1) sediment A or B with wet mass 50.0 g for open beakers and 20.0 g for anaerobic batch reactors; (2) granular sulfur and limestone grains (total mass = 25.0 g; sulfur/limestone ratio = 3:1 or 1:0) with the sizes ranging from 2.38 to 4.76 mm, (3) 250 ml (for batch-pond reactors) or 120 ml (for anaerobic batch reactors) nutrient solution (type 1 or type 2) spiked with nitrate, (4) 10 ml (for batch-pond reactors) or 4 ml (for anaerobic batch reactors) (about 200 mg/l of

<table>
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<th>Condition</th>
<th>Media</th>
<th>Sediment Wet Mass (g)</th>
<th>S:L Mass (g)</th>
<th>Feed Solution Type</th>
<th>Feed Solution Volume from CSTR (ml)</th>
<th>Seed Volume from CSTR (ml)</th>
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<td>25.0</td>
<td>2</td>
<td>250</td>
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**Open Environment Pond System (Aerobic Conditions)**

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<th>S:L Mass (g)</th>
<th>Nutrient Solution Type</th>
<th>Nutrient Solution Volume from CSTR (ml)</th>
<th>Seed Volume from CSTR (ml)</th>
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<tr>
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<td>0</td>
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<tr>
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</tr>
<tr>
<td>5</td>
<td>Sediment A + S:L + Seed</td>
<td>20.0</td>
<td>10.0</td>
<td>2</td>
<td>120</td>
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</table>

**Closed Environment Pond System (Anaerobic Conditions)**

*a* Type 1: See CSTR feed solution; Type 2 - 3 g/l KNO₃, 0.5 g/l KH₂PO₄, CSTR trace nutrients, tap water.
suspended solids) seed sludge from the CSTR for inoculating autotrophic denitrifiers. However, control tests were conducted using the batch-pond system without limestone, sulfur stone, or seed sludge, depending on the experimental design.

Eight to twelve batch reactors were run at the beginning of each condition in Table 1. After the selected time interval, we took one batch reactor to provide the sample and measured the corresponding items such as nitrate, nitrite, sulfate, pH, DO, etc. After the measurement, the batch reactor was wasted. In other words, the time courses of any item in the batch reactors results from a series of batch reactors instead of from only one batch reactor. For open environment batch pond reactors, distilled water was injected into the reactors to compensate for the loss of water due to evaporation. Dissolved oxygen was measured by inserting an oxygen macroelectrode 2 cm above the surface of the sediment or sulfur/limestone layer.

Sediment A was taken from a runoff pond that displayed wetland characteristics including duckweed and long stem grasses. Water depth in the area of sampling was 8-9 inches and the sample depth was 3-8 inches below the ground surface. The sediment contained a high percentage of observed organic material such as dead grass and fallen leaves. The watershed that contributes to the runoff is approximately 80% row crops and 20% grassland and trees. Sediment B came from a small pond that lacked the qualities found in wetlands similar to that of sediment A. The total depth of the pond appeared deeper than that of sediment A. The surface of the pond was devoid of aquatic vegetation. Plant growth, however, did continue to grow up to the shallows of the pond, and amphibians and waterfowl thrived in the surroundings. Water depth in the area of sampling was 12-15 inches and the sample depth was 3-6 inches below the ground surface. A lower percentage of organic material was observed in the sediment compared to that of sediment A. Run-on to the pond came from a watershed of approximately 85% pasture and 15% row crops. The pasture was used for cattle grazing. Waste excreted from the cattle are a source of nitrogen in the lake.

**Analytical methods**

**Nitrate-nitrogen**

A HACH method was used to determine nitrate-nitrogen concentrations. The method is a spectrophotometric method and is modified from the cadmium reduction method by replacing 1-naphthylamine with gentisic acid and using a wavelength of 500 nm with a 1 cm wavelength path [24]. In using this method, nitrite is an interference. To compensate for interferences caused by nitrite, bromine water, 30 mg/l, is added dropwise until a yellow color persists. The bromine will convert any nitrite in solution to nitrate. The yellow color is eliminated from the sample before testing with the addition of one drop of 30 mg/l phenol solution. During the test the total nitrate/nitrite concentration is measured. By determining nitrite-nitrogen and subtracting it from the total nitrate/nitrite, the concentration of nitrate can be obtained. In this study, samples were filtered with a 0.45 µm membrane filter before analysis.

**Nitrite-nitrogen**

Two ranges were used to determine nitrite-nitrogen. Both methods were spectrophotometric methods (HACH method). A low-range determinant employed the diazotization method with a wavelength of 507 nm, and the high range method utilized a ferrous sulfate method at a
wavelength of 585 nm [24]. These samples were filtered as mentioned above.

**Sulfate**

Sulfate was determined spectro-photometrically by the SulfaVer 4 Method at a wavelength of 450 nm [24], adapted from *Standard Methods for the Examination of Water and Wastewater* [25]. These samples were filtered as mentioned above.

**pH and dissolved oxygen**

pH was measured using a standard-sized Fisher Scientific Accu-pHast polymer combination electrode with an Accumet 925 pH meter. The dissolved oxygen was determined from a Leeds and Northrup 7932 Portable dissolved oxygen meter with probe.

**Biomass**

Biomass for the CSTR was measured by filtering a known amount of mixed liquor through a preweighed 0.45 µm membrane filter. The filter was dried at 105°C for 24 hours and reweighed [25].

**RESULTS AND DISCUSSION**

**Start-up and CSTR**

The seed reactor was first run as a semi-batch reactor with a feeding of sulfur powder and other substrate and nutrients once a day. Although nearly 100% nitrate removal could be obtained at nitrate loading rates below 120 mg/l/d, higher nitrate loading rates resulted in a build-up of nitrate. The suspected cause of this is a high nitrite and sulfate concentration in the semi-batch reactor which can inhibit the denitrification process. The hydraulic retention time for this reactor was 15 days. A shorter retention time, and therefore a higher loading rate, would allow more nutrients for the denitrifiers and also relieve the culture of toxic chemical buildup. A CSTR system could also decrease any effects of shock loading that may occur with the batch system. In addition to the performance dissatisfaction, the semi-batch reactor system yielded an inadequate microbial population for use as a seed source.

Powdered elemental sulfur has a specific gravity of 2.07 and under normal circumstances is considered an insoluble material. Although it was possible to rapidly pump the feed solution into the anaerobic batch reactor, getting all of the preweighed powdered sulfur into the reactor proved difficult. Once pumped into the reactor, much of the sulfur floated to the top of the water surface or the particles conglomerated. Complete mixing was not taking place within the reactor, thereby lessening the surface area from which the denitrifiers could affix themselves to or take advantage of the nutrients. Because of this, elemental sulfur was not considered a practical constituent for a feed solution.

Due to the two reasons mentioned previously, the semi-batch reactor was changed to be a CSTR by using thiosulfate as the electron donor. Thiosulfate dissolves in water and therefore mixes readily and is compatible with a continuous reactor system. The thiosulfate-based, continuously stirred tank reactor permits a constant loading rate that can be easily adjusted.

Under the CSTR system, nitrate-nitrogen removal efficiencies were greater than 99% at hydraulic retention times of 26.6 hours, 21.3 hours, and 16.4 hours. Nitrite-nitrogen concentrations were less than 0.1 mg/l at all three hydraulic retention times. The influent sulfate concentration was 500 mg/l. Sulfate effluent concentrations ranged from 4,290 to 5,190 mg/l and the pH ranged from 6.48 to 6.88 a drop of about 2 pH units from the influent pH. Biomass in the reactor declined
with lower HRTs from 208 mg MLSS/l to 151 mg MLSS/l. The seed source for the fixed-bed reactors was taken during an average HRT of 26.6 hours. At this rate, the MLSS was highest, and performance of the reactor remained stable.

**Fixed-bed reactor**

Tests were conducted to identify (1) the optimum ratio of elemental sulfur volume to limestone volume and (2) optimum hydraulic loading rates of feed solution. Four individual column reactors with sulfur:limestone ratios of 1:3, 1:1, 3:1, and sulfur only were operated. The four reactors were simultaneously being fed with feed solution of 35 mg NO₃⁻-N/l which represents ground water nitrate concentration that might be expected under natural conditions. The four reactors were run at empty bed hydraulic retention times (HRT) of 17.0 hours, 9.6 hours, and 3.1 hours.

Figure 3 shows the effect of HRT on effluent nitrate-nitrogen concentrations for fixed bed reactors with different sulfur:limestone ratios. Optimum conditions for the removal of nitrate-nitrogen occurred at a hydraulic retention time of 9.6 hours for the four sulfur:limestone ratios. As the flow rate increased, the ratio of sulfur:limestone became increasingly important. At a hydraulic retention time of 3.1 hours, the fixed-bed reactor with sulfur only and that with a sulfur:limestone ratio of 1:3 and 1:1 yielded a reduction in nitrate removal efficiency. Ultimately, a sulfur:limestone ratio of 3:1 yielded the best results at the lowest hydraulic retention time tested.

With an average HRT of 17.0 hours, nitrate reductions were 93%, 99%, 99%, and 42% for sulfur:limestone ratios of 1:3, 1:1, 3:1, and sulfur only, respectively. Sulfate concentrations increased with higher proportions of limestone with a maximum of 5,250 mg SO₄²⁻/l. Nitrite was only detected in the column without limestone at 4.4 mg NO₂⁻-N/l. The pH was 4.96 for the sulfur only column, 6.8 with a sulfur:limestone ratio of 3:1, 7.12 for a ratio of 1:1, and 7.09 for a sulfur:limestone ratio of 1:3.

With an average HRT of 9.6 hours, nitrate reductions were 99%, 99%, 99%, and 59% for sulfur:limestone ratios of 1:3, 1:1, 3:1, and sulfur only, respectively. Sulfate concentrations increased with higher proportions of limestone with a maximum of 4,140 mg SO₄²⁻/l. Nitrite was again detectable in the sulfur only column but increased to 5.7 mg NO₂⁻-N/l. The pH increased slightly to 5.04 for the sulfur only column but decreased slightly for the other columns with pHs of 6.78, 7.10, and 7.04 for sulfur:limestone ratios of 3:1, 1:1, and 1:3, respectively.

An average HRT of 3.1 hours was the breaking point for the performance of the reactors. Nitrate reductions were 32% for the sulfur only column and 85%, 41%, and 8% for sulfur:limestone ratios of 3:1, 1:1, and 1:3, respectively. Sulfate concentrations fell with decreased nitrate-nitrogen efficiencies as expected from the stoichiometric equation. The maximum concentration of sulfate produced was found...
in the column with a sulfur:limestone ratio of 3:1. An incompletion of the denitrification process was evident in columns with sulfur:limestone ratios of 1:1, 1:3, and sulfur only. In the column with a 3:1 sulfur:limestone ratio, the nitrite-nitrogen remained essentially zero. The pH in the sulfur only column increased significantly to 5.89 while the pH decreased in columns with sulfur:limestone ratios of 3:1 and 1:1. The pH in the column with a sulfur:limestone ratio of 1:3 remained about the same.

Results indicate that at a hydraulic retention time of 3.1 hours the sulfur:limestone ratio of 3:1 is best suitable for further research and applicability. It should be noted again that the hydraulic retention time is based on the empty bed reactor volume. If the average working volume of 817 ml is used, the true hydraulic retention time is 1.7 hours for a nitrate-nitrogen loading rate of almost 21 mg/l/hr. For these operating conditions the sulfate concentration in the effluent was 366 mg/l and the pH was 6.51. Nitrite-nitrogen concentrations were less than 0.2 mg/l.

Open environment batch pond reactor

Open beaker batch reactors were tested under several conditions to analyze the effectiveness of the system for lab-scale simulation of a pond system. Figure 4 shows the nitrate-nitrogen reduction in batch pond reactors under either aerobic or anaerobic conditions. Nitrate-nitrogen removal rates for the various conditions in an open beaker were 0.21 mg/l/hr for sediment A only (condition 2 in Table 1), 0.42 mg/l/hr for sediment A with granular sulfur and limestone (condition 3 in Table 1), 0.34 mg/l/hr for sediment B with granular sulfur and limestone (condition 4 in Table 1), 0.63 mg/l/hr for sediment A with granular sulfur and limestone plus the addition of a seed of autotrophic denitrifiers (condition 5 in Table 1), and a maximum of 1.80 mg/l/hr for granular sulfur with additional nutrients and a seed of autotrophic denitrifiers (condition 1 in Table 1). It can be understood that the addition of elemental sulfur into an open pond system will facilitate the occurrence of autotrophic denitrification while addition of a seed of autotrophic denitrifiers into the system will accelerate the process. It was found that the nitrate removal was less than 30% if the open beaker batch reactor contained sediment only without addition of element sulfur (condition 2); the nitrate removal, however, was found to be about 60% if the system contained sediment and element sulfur (see Figure 4). We observed that the system with the addition of sulfur and limestone produced some bubbles on the surface of sulfur stones after 3-5 days, indicating the autotrophic denitrification occurred within the system. Different sediments, such as sediments A and B, had similar results, which shows that the autotrophic denitrifiers widely exist in natural sediments. Therefore, our experiments demonstrate that it is possible to use the sulfur-based pond system for in situ remediation of nitrate-contaminated surface.
It should be noted that the open beaker batch pond system is a very complicated system since sediment was used to serve as inoculation of autotrophic denitrifiers. Sediments may contain different substrate, nutrients, and trophs of microorganisms. Nitrification and denitrification may occur in the system. For this reason, nitrate may be produced due to nitrification occurring in the system. That is why the nitrate reducing rates in the systems spiked with sediment, sulfur/limestone, and seed is much lower than that in the system with sulfur and seed only. Additionally, in the open beaker systems, both heterotrophic and autotrophic denitrification can occur, which explains why the system with sediment only can also have 30% removal of nitrate in the open beaker batch pond system.

The open beaker batch pond system was also tested for sulfate, nitrite-nitrogen, pH, and dissolved oxygen. Figure 5 shows the sulfate production in batch pond reactors. Sulfate production rates in an open beaker environment ranged from 0.2 mg/l/hr for condition 2 to 8.2 mg/l/hr for condition 1. By comparison, it can be seen that the addition of sulfur will facilitate the autotrophic denitrification in the systems and, therefore, the sulfate will be produced as time increases. It was found that the final sulfate concentrations under conditions 1 and 5 were as high as 1,800 mg/l. However, it took only 160 h for the reactor under condition 1 to reach this sulfate concentration, while it took about 400 h for the reactor under condition 5 to reach the same concentration. The reason for this is not clear at this stage.

Figure 6 shows the time courses of nitrite-nitrogen in aerobic batch reactors. Nitrite-nitrogen concentrations and patterns varied significantly for the different medium conditions, yielding a maximum concentration of 24.5 mg/l at 450 hours for sediment A with granular sulfur and limestone medium (condition 3) and 0.2 mg/l at 168 hours with granular sulfur, additional nutrients, and seed of autotrophic denitrifiers (condition 1). Comparing curves 1, 3-5 with curve 2, it can be understood that autotrophic denitrification did occur in the open beaker systems and that the addition of elemental sulfur could facilitate the denitrification. There was no accumulation of nitrite in the reactor under condition 2 in Table 1, which does not mean that the denitrification in the system under condition 2 was faster than systems under conditions 1, 3, and 5; instead, it simply means that the denitrification in the system under condition 2 did not occur very rigorously. From Figure 6, we can see that the system under condition 1 is the most efficient system due.
to the fact that there was no accumulation of nitrite occurring in the system.

Figure 7 shows the time courses of pH in aerobic batch reactors. The pH under various conditions decreased up to 1 unit for conditions 3 (curve 3), 4 (curve 4), and 5 (curve 5). In contrast, for the sediment A only (condition 2), the pH increased almost 1 unit after 385 hours (curve 2 in Figure 7). These results on pH changes in the systems further demonstrate that the addition of elemental sulfur will facilitate the occurrence of autotrophic denitrification in the open beaker batch systems because, according to Batchelor and Lawrence [19], H⁺ will be produced as the autotrophic denitrification processes take place.

Dissolved oxygen testing was not performed for the sulfur and nutrient addition case. Figure 8 shows the dissolved oxygen in open beaker batch pond reactors. We found a decrease and then recovery of dissolved oxygen for the conditions 3 (curve 3), 4 (curve 4), and 5 (curve 5). Increases of dissolved oxygen in systems under conditions 3 and 5 (curves 3 and 5 in Figure 8) several days after beginning may be the reason for nitrite concentrations in these systems to accumulate with time due to the fact that high dissolved oxygen concentration will inhibit denitrification processes in the systems. The dissolved oxygen trend for the sediment A only (condition 2) was just the opposite, showing a sharp decrease initially but increasing with time and then falling again, which indicates that after 5-10 days, autotrophic denitrifiers increased their number and, therefore, the denitrification process within the system was accelerated so that the rate of dissolved oxygen consumption was even faster than that of reoxygenation.

**Anaerobic batch pond reactor**

Anaerobic batch pond reactors were tested under three conditions to analyze the effectiveness of the system for lab-scale simulation of a pond system under an anaerobic environment. In addition, the results from anaerobic conditions can also provide information without nitrification occurring in the pond system due to the fact that nitrification is an oxic process. Anaerobic conditions can be compared with those of the open beaker to help understand the behavior of the systems regarding nitrate reduction, nitrite formation, and sulfate production.

Nitrate-nitrogen, sulfate, nitrite-nitrogen, and pH were measured in the anaerobic batch pond reactors. However dissolved oxygen was not measured. Nitrate-nitrogen
removal rates were 0.24, 0.49, and 0.66 mg/l/hr for anaerobic batch reactor conditions 2, 3, and 5, respectively (see Figure 4). From Figure 4, it can be seen that nitrate reduction is more efficient in anaerobic batch systems than that in aerobic batch systems. At this stage, however, the reason for this difference is not clear. One possible reason is dissolved oxygen in anaerobic batch systems is much lower than that in aerobic batch systems so that the autotrophic denitrification process is well protected. The other possible reason can be the inhibition of the nitrification process with the anaerobic batch system so that there is no production of nitrate or nitrite transformed from ammonium due to the nitrification process.

Sulfate production rates were 0.6, 2.2, and 3.4 mg/l/hr in the same conditions order (see Figure 5). Figure 9 shows the time courses of nitrite-nitrogen concentration in anaerobic batch reactors. In all three cases, a increase in nitrite-nitrogen concentration appeared within 150 hours. After the increase, the sediment A only condition (condition 2) showed a decreased in nitrite-nitrogen, while the anaerobic batch reactors with conditions 3 and 5 showed a gradually increasing trend (Figure 9). It is difficult to understand at this stage why nitrite concentrations in reactors under conditions 3 and 5 in Table 1 increased after 150 h. Since these reactors were under anaerobic conditions, dissolved oxygen could not be a reason for the nitrite-increasing tendency observed. The inhibition of autotrophic denitrification by its intermediate or end products such as nitrite, sulfate, etc., may be one of the reasons for nitrite accumulation. The exhaustion of nutrients in the systems may be the other reason. Obviously, more detailed studies are needed. The pH for all three conditions dipped at approximately 150 hours, made a recovery or surpassed the original pH by 250 hours, and then began to drop again after 450 hours (Figure 10). Comparison of the results from sediment A only (condition 2) with those from conditions 3 and 5 demonstrates that the sulfur-based batch system has the potential to be used for in situ remediation of nitrate-contaminated surface water.

The results of this study on sulfur-based autotrophic denitrification in pond systems under both aerobic and anaerobic conditions demonstrate a new technology that can be used for in situ remediation of nitrate-contaminated surface water. The use of constructed wetlands or stabilization ponds for the treatment of municipal, industrial, and agricultural waste water has been the subject of a considerable amount of investigation over the past decade. Much
work has focused on the ability of these systems to remove nitrogen from waste waters. The potential nitrogen removal mechanisms in these systems are storage in living biomass, detritus, and sediments; ammonia volatilization; and denitrification. Unless plants are harvested and the biomass removed from the system (which is costly), denitrification is the only significant long-term mechanism for nitrogen removal. However, nitrogen removal may be limited by insufficient organic carbon if heterotrophic denitrification processes are involved [26]. The preliminary experiments conducted in this study indicated that the sulfur-based autotrophic denitrification may be a replacement of heterotrophic denitrification in these pond systems due to the fact that no organic carbon source is needed in sulfur-based autotrophic denitrification processes.

**CONCLUSION**

Start-up procedures using powdered elemental sulfur as the electron donor in an anaerobic batch reactor proved futile, from which it was determined that thiosulfate in a CSTR system would provide an adequate feed source. Performance data of the CSTR reactor indicates that nitrate-loading rates as high as 30 mg/l/hr are capable of nearly 100% removal of nitrate-nitrogen.

The potential for an upflow anaerobic fixed-bed reactor is viable. Our results also indicate that limestone addition is a effective contributor to the buffering capacity within the reactor. The optimal ratio of sulfur:limestone is 3:1 based on this study.

The open beaker batch pond reactor system is a system with many complex system elements and chemical reactions taking place. This study shows that denitrification will occur in batch pond systems under either aerobic or anaerobic conditions and that nitrate-nitrogen removal rates will increase with the addition of granular sulfur and limestone. The actual rate varies depending on the pond sediment’s buffering capacity and nutrient contents.

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