Evaluation of a Sulfur-*Thiobacillus denitrificans* Nitrate Removal System[1]

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ABSTRACT

An evaluation of sulfur-*Thiobacillus denitrificans* nitrate removal system as a means of denitrifying nitrified septic tank effluent was conducted. Duplicate 10 by 64 cm columns were filled with a 1/1 mixture (wt/wt) of elemental S (>2 mm) and dolomite chips (1 cm), and were pretreated by recycling an enrichment culture of *T. denitrificans* ATCC 23642 through the columns for 3 days. Continuous passage of the nitrified septic tank effluent (column influent) containing 40 μg NO_3^--N/ml through the columns resulted in nearly complete NO_3^- removal in 3.3 hours at steady-state conditions (23C). Statistical analyses indicated that the denitrification kinetics closely resemble first order in the range of NO_3^- concentrations employed. Oxidation-reduction potentials (Pt black electrodes) ranged from +160 to +300 mV. Analysis of the gases produced showed that N_2 was present in highest concentration. Sulfate was the major S end product and was present at relatively high concentrations (90 μg SO_4^{2-}-S/ml). A significant decrease in inorganic C occurred with depth in the columns while changes in organic C content were insignificant. A significant linear relationship was obtained between decreases in $(NO_3^- + NO_2^-)$-N and production of SO_4^{2-}-S. Passage of column effluent through 10 by 60 cm Plainfield sand columns did not significantly decrease SO_4^{2-} levels. While use of this NO_3^- removal system appears promising, SO_4^{2-} contamination of the ground water may limit its applicability.

Additional Index Words: first order kinetics, oxidation-reduction potentials, gas analysis, sulfate, denitrification.

The number of individual home waste disposal systems in the United States has increased markedly in the last three decades. This increase is primarily due to the trend of Americans to choose homes in suburban and rural communities where municipal waste treatment is absent. Populous eastern states such as Connecticut and Maryland have approximately 50% of their total population served by septic tanks (25). Recent studies of the septic tank-soil disposal field (seepage bed) have indicated that when these systems are properly sited, installed, and maintained, they are efficient in removing pathogens, organic carbon (C), and phosphorus (P) (15, 29). Nitrogen (N) is not removed as completely, especially when the systems are located in well-drained, aerated soils (28). In these instances, nitrification of the septic tank effluent N occurs in the subcrust portion of the seepage bed yielding the highly mobile nitrate (NO_3^-) ion. Since conditions are generally unfavorable for denitrification, the probability of NO_3^- movement into ground water with the percolating effluent is high.

In response to concerns of NO_3^- pollution problems, studies were initiated to test the feasibility of onsite denitrification for home sewage disposal. The difficulties in designing a denitrifying system to perform in conjunction with the usual septic tank-soil disposal field system have been discussed previously (20). The general characteristics of such a system must be: (i) that it have a relatively long lifetime; (ii) relatively inexpensive; (iii) require low maintenance; (iv) function so as to not disrupt the performance of the seepage bed; and (v) not add pollutants to the environment in exchange for NO_3^- removal. Although still in the design stages, a system basically as diagrammed in Fig. 1 is proposed. Here, effluent from the seepage bed would be held by an impermeable barrier in an anaerobic environment for a designated length of time during which the denitrification would occur. The effluent would then be forced into the surrounding soils by the incoming liquid from the seepage bed. This design is similar to that Erickson et al. (10) used for removing P and N from animal wastes. Of course, such a system would need to be proven in field studies to ensure that the listed requirements are attained. Preliminary studies were undertaken to test various energy sources for denitrification systems in which heterotrophic bacteria would predominate. Exogenous energy sources are necessary to stimulate denitrification becasue of the high degree of C removal under the aerobic conditions resulting in nitrified effluent (15, 21). Methanol has been found to be a suitable energy source yielding a system with rapid denitrification rates and minimal, nonpolluting end products (20).

Dissimilatory nitrate removal can also be accomplished by employing a system of reduced S compounds and the ubiquitous obligate chemolithotroph *Thiobacillus denitrificans* which obtains energy by oxidizing reduced S compounds and passing electrons to NO_3^- in the absence of O_2. When elemental S is the electron source, a reaction similar to Eq. [1] is obtained.
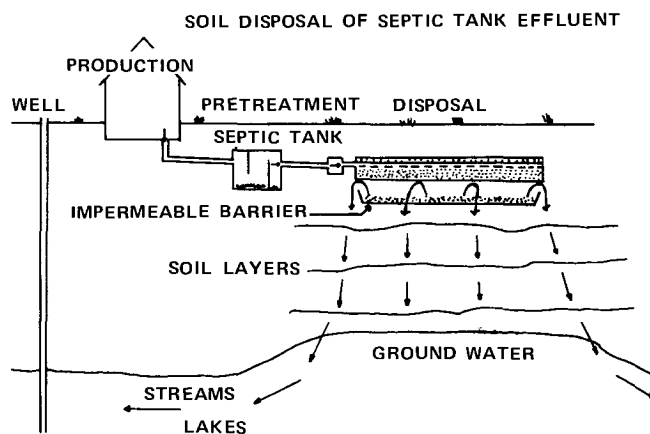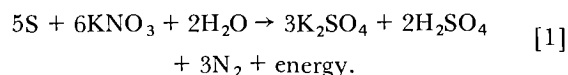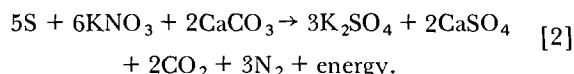
$$5S + 6KNO_3 + 2H_2O \rightarrow 3K_2SO_4 + 2H_2SO_4 \quad [1]$$
$$+ 3N_2 + \text{energy.}$$

SOIL DISPOSAL OF SEPTIC TANK EFFLUENT

Fig. 1—Diagram of septic tank-soil disposal field with proposed denitrification system.

This system can be buffered to neutralize the resulting acid by using calcium carbonate ($CaCO_3$) in which case the reaction would be similar to Eq. [2].

$$5S + 6KNO_3 + 2CaCO_3 \rightarrow 3K_2SO_4 + 2CaSO_4$$
$$+ 2CO_2 + 3N_2 + \text{energy.} \quad [2]$$

In this study an evaluation of the sulfur-*T. denitrificans* nitrate removal system for septic tank seepage bed effluent was performed. Denitrification rates were determined and analyses of the end products formed and their possible effects on the environment were conducted by using continuous flow column studies.

MATERIALS AND METHODS

Continuous Flow Columns

Duplicate 10 by 64 cm Plexiglas columns (Fig. 2, disregard mixing coil used in heterotrophic studies) were filled with a 1:1 mixture (wt/wt) of dolomitic limestone (1 cm) and elemental S (crude, lump sulfur, Stauffer Chemical Co., > 2 mm) and sealed. The columns were equipped with sampling ports and Pt black oxidation-reduction electrodes at 12, 32, and 52 cm from the top of the columns. The electrodes were prepared according to Magdoff et al. (14) and checked according to Quispel (19). Readings were adjusted to pH 7 by using the -0.059 V/pH correction factor (4). A silver-silver chloride reference electrode was used. Residence times were determined by continuous addition of chloride and analysis of the effluent for chloride using a Buchler-Cotlove chloridometer (9). The influent used was a mechanically aerated septic tank effluent which is comparable to seepage bed effluent in C and N content. For this study the influent was diluted 1:36 with tap water and NO_3^- was added as potassium nitrate (KNO_3) to equal a final concentration of 40 μg N/ml. Diluted influent was used for two reasons. First, the supply of nitrified influent available for our use was limited. Second, to insure that the denitrification rates observed were due to the autotroph *T. denitrificans*, the endogenous C was diluted to low levels (< 5 μg C/ml). In initial studies, using undiluted nitrified influent, denitrification rates similar to those presented were obtained. However, steady-state conditions were
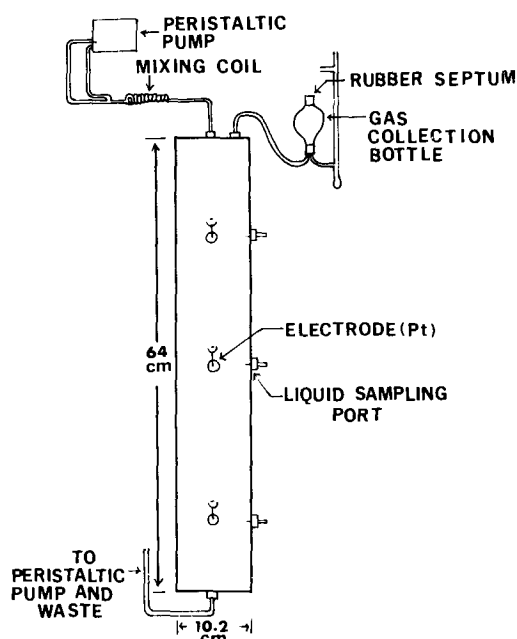

Fig. 2—Diagram of continuous flow column apparatus.

never attained and direct comparisons were not attempted. The influent flow was controlled by a peristaltic pump and the effluent flow was adjusted using screw clamps to equal the influent flow.

Inorganic Nitrogen Analyses

Nitrate plus nitrite nitrogen was determined using steam distillation methods of Bremner and Keeney (6). Nitrite was determined using the modified Griess-Ilosvay method (5).

Sulfur Analyses

Sulfate (SO_4^{2-}) was determined by turbidometric assay (23). Sulfides (S^{2-}) were determined by methylene blue method (2). Polythionates were determined according to the method of Starkey (22).

Gas Analyses

Gas samples were collected by a water displacement method (Fig. 2). Gas syringes were flushed with helium prior to sampling the gas collection bottles. Samples were taken and transported to the gas chromatographs with the needles embedded in rubber to prevent contamination. Samples were immediately analyzed for N_2, O_2, nitric oxide (NO), nitrous oxide (N_2O), carbon dioxide (CO_2), methane (CH_4), and hydrogen sulfide (H_2S). A 406 Series Packard dual column gas chromatograph equipped with Molecular Sieve 5A column (0.64 by 366 cm) and Porapak Q column (0.64 by 366 cm) was used in connection with an Autolab 6300 digital integrator for analyses of all gases except CH_4. The injection port temperature was 120C and column temperature was 70C. Helium was the carrier gas. Methane was determined on a 407 Series Packard gas chromatograph with a flame ionization detector according to the method of MacGregor et al. (13).

Carbon Analyses

Soluble C content (total organic and total inorganic) of filtered (0.45 μm) samples was estimated with a Beckman Model 915 Total Organic Carbon Analyzer.

T. denitrificans Culture

Thiobacillus denitrificans ATCC 23642 was grown in the medium described in Taylor et al. (24). For column preparation, the stock culture was inoculated into 500-ml aliquots of medium and incubated under helium at 30C for 7-14 days. After incubation the medium was recycled through the sulfur-limestone columns for 3 days for establishment of the organism within the columns.

RESULTS AND DISCUSSIONS

Nitrogen Analyses

Establishment of the *T. denitrificans* on the S within the columns depends upon such factors as the growth stage of the culture, concentration of organisms in the enrichment medium, and surface area of the S. In these studies the enrichment culture was recycled through the columns for 3 days and approximately 2 weeks of influent continuous flow had occurred before significant denitrification was observed. By 8 weeks, denitrification in the columns reached steady-state when the NO_3^- concentration at all sampling ports did not vary by more than 10% for 3 consecutive days. Under steady-state conditions, essentially all of the NO_3^--N had been removed from the influent in 3.3 hours residence time (Fig. 3). Nitrite increased and then decreased with time to approximately 5 μg/ml NO_2^--N. Accumulation of NO_2^-, a characteristic of *T. denitrificans* (3), was observed in the columns using
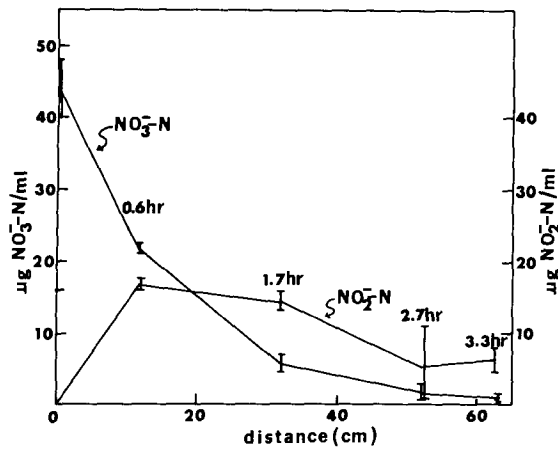
Fig. 3—Nitrate and nitrite levels (mean μg N/ml and standard error) and residence times in continuous flow columns at steady-state conditions (23C).

shorter residence times ($<$ 2 hours). However, with longer residence times ($>$ 3.5 hours) NO_2^- levels decreased to near zero.

Significant immobilization of the NO_3^--N by heterotrophic activity was considered negligible because of the low soluble C content (C/N ratio of 0.15). However, some N must be assimilated into growing cells, but this amount would be nearly impossible to quantitate because the organisms were attached to the colloidal sulfur. While Aleem (1) reported quantative NO_3^- reduction to N_2 gas with *T. denitrificans*, results from heterotrophic denitrification studies indicate 3 to 8% of the NO_3^- is assimilated into biomass (12). Applying this percentage to our study would indicate an assimilation of 1-3 μg NO_3^--N/ml of influent.

Kinetics

The kinetics of denitrification in soil have been equated to first order primarily due to the low levels of NO_3^- normally occurring in soils (11, 17). Accordingly, a test was made to determine if a first order similarity in this study also occurred. A regression analysis was performed on the logarithm of NO_3^- concentration vs. residence time (from Fig. 3) and a significant linear relationship was
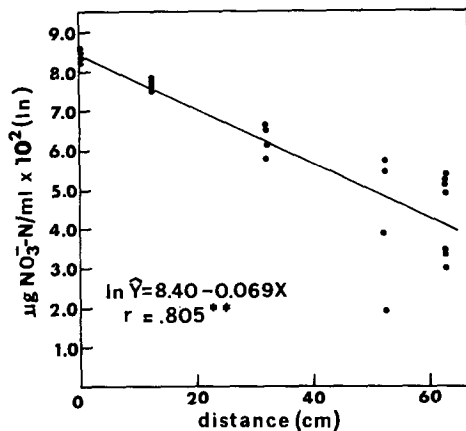

Fig. 4—Regression analysis of decrease in NO_3^- vs. residence time (data in Fig. 3) in continuous flow columns (steady-state, 23C).

obtained (Fig. 4). The enzymatic reactions involved are probably Michaelis-Menten type (31). However, due to the relatively low NO_3^- concentrations normally occurring in septic tank systems (40-60 μg NO_3^--N/ml), the portion of the curve observed resembles first order kinetics.

Oxidation-reduction Potentials

Oxidation-reduction (redox) potentials (Eh_7), taken during steady-state conditions ranged from +160 to +300 mV (Table 1). The readings were highest at the top of the column and decreased with depth. The redox potential normally associated with an actively denitrifying soil system is about +225 mV (18) which is approximately the midpoint in the range of readings obtained. Mann et al. (16) recorded Eh values ranging from −156 to +55 mV in their packed columns which contained a mixture of soil, $CaCO_3$, and S. The difference in potentials observed in our system and those measured by Mann et al. (16) are probably due to the fact that their system was far more heterogeneous and thus would be affected by more ionic species such as Mn^{2+} and Fe^{2+} (4).

Gas Analyses

Gas bubbles originated on the elemental S, rose to the top of the columns, and collected in the gas collection bottles. The data for gases detected during steady-state conditions (Table 2) indicate that N_2, the end product of denitrification, was present in highest concentration. Small amounts of O_2 were also detected which may have resulted from trace contamination by air entering the columns when the influent bottles were changed. Accordingly, N_2 would also be a contaminant in a concentration of approximately 9 μmoles/cm^3 (equal to the N_2/O_2 atmospheric ratio). The remaining 34 μmoles N_2/cm^3 were considered to be of microbial origin. The CO_2 detected probably was the result of the neutralization reaction between the resulting sulphuric acid and limestone (8).

Nitrous oxide, a recognized intermediate involved in *T. denitrificans* nitrate reduction (1, 3, 7), was not detected during steady-state conditions, probably because it was rapidly transformed to N_2. Nitric oxide, also a suspected intermediate, was never detected.

Table 1—Oxidation-reduction potentials (Eh_7 mV) at three levels in continuous flow columns (steady-state conditions, 23C)

Level	Mean	Range
cm		
12	+278	+250-+300
32	+241	+227-+250
52	+175	+160-+195

Table 2—Concentration of gases (μmoles/cm^3 gas produced) detected in continuous flow columns (steady-state conditions, 23C)

Gas	Mean	Range
O_2	2.40	1.20- 3.60
N_2	43.10	41.30-44.70
CO_2	0.30	0.20- 0.40

From 0.2–0.5 μmoles/cm³ of H₂S was produced after approximately 4 months continuous flow operation. This concentration was about the detection limit of the gas chromatographic method. Thus, earlier production of H₂S would not have been detected.

Sulfur End Products

Sulfate was the primary end product in effluent from the columns (Table 3) and originated from the oxidation of the S by *T. denitrificans*. Heterotrophic S oxidation was considered negligible because little soluble organic C was present in the influent and no change in organic C content in the columns occurred with depth (Table 3). Sulfate contribution from the dolomite was also considered negligible since the dolomite used contained only about 10 mg SO_4^{2-}-S per 1,000 g of limestone, and very little SO_4^{2-} was leached from limestone columns in studies where methanol rather than S was used as the energy source.

Sulfide was detected at low levels only at the bottom of the columns. These findings correlate with the positive redox potentials observed which were considerably higher than the −150 mV normally associated with SO_4^{2-} reduction (18). Only low levels of polythionates were present, most of which were probably thiosulfate. Under optimal growth conditions, no sulfur intermediates are known to accumulate during *T. denitrificans* nitrate reduction but many external factors such as pH, O₂ concentration, and phosphate concentration may affect the accumulation of intermediates (27). In these studies conditions appear to be such that intermediates of S oxidation accumulate in significant amounts.

A regression equation relating the production of SO_4^{2-} to the decrease in $(NO_3^- + NO_2^-)$-N (Fig. 5) gave a slope of 2.13 which is close to the theoretical value of 1.90 (160/84) for SO_4^{2-}-S produced per NO_3^--N reduced (Eq. [2]). The difference in ratios may be due to the MgSO₄ formation from the dolomitic limestone as well as any other cations which exist in the influent and are unaccounted for as SO_4^{2-} in Eq. [2]. Precipitation should be negligible in this system since CaSO₄ has a solubility of 1.93 g/liter at 20C (30). The other probable sulfates have higher solubilities.

Carbon Analyses

Significant decreases in inorganic C occurred with depth in columns while changes in organic C were insignificant (Table 3). Although Starkey (22) warns against the assumption that inorganic C assimilation indicates an autotrophic metabolic activity, these data, in conjunction with the NO_3^- decrease, gas analyses data, and S data in-
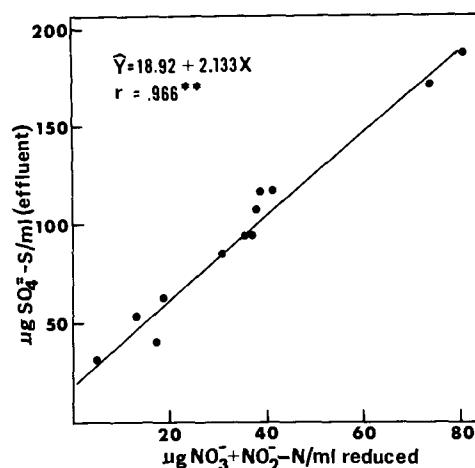

Fig. 5—Regression analysis of reduction of $(NO_3^- + NO_2^-)$-N with SO_4^{2-}-S production in continuous flow columns (steady-state, 23C).

dicate that the autotroph, *T. denitrificans*, is the dominant species within the columns. Also, smears of column effluent organisms (concentrated by 0.45 μm Millipore filtration and resuspended in sterile deionized water) indicated a dominant gram negative short rod identical to smears made from the stock culture of *T. denitrificans*.

Nitrate removal by algae was never indicated through visual inspection of the columns. Also, when the columns were covered with foil to inhibit light entrance for 2 weeks, no noticeable change in rate was observed.

Passage of S-limestone Column Effluent through Sand Columns

Home sewage disposal systems located in areas having highly permeable sands to the ground water offer the highest potential for significant NO_3^- pollution. Therefore, an experiment was conducted to evaluate mobility of resulting SO_4^{2-} from a sulfur-*T. denitrificans* nitrate removal system. Effluents from each column (500 ml/day equal to 6 cm unit area⁻¹ day⁻¹) were applied to the top of 10 by 60 cm Plainfield sand columns daily and the percolate was analyzed for SO_4^{2-} and S^{2-}. Sulfate was not retained by the sandy soil (Fig. 6). Sulfate concentrations

Table 3—Mean concentrations of sulfur and carbon products (μg/ml) in continuous flow columns (steady-state conditions, 23C)

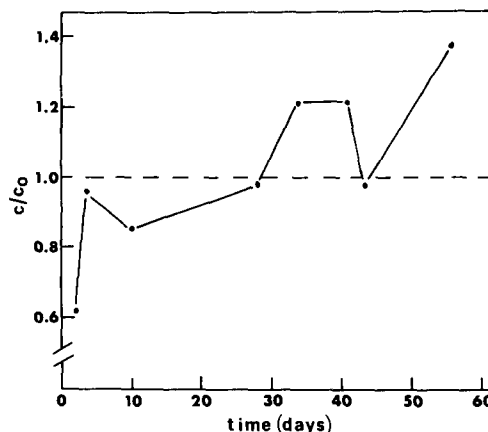| Port	SO_4^{2-}-S	S^{2-}	Polythionates	Organic C	Inorganic C
Top	6.2	<0.1	<0.5	6.3	65.3
12 cm	36.4	<0.1	<0.5	5.5	56.8
32 cm	69.5	<0.1	<0.5	5.8	51.3
52 cm	93.9	<0.1	<0.5	5.0	50.0
Bottom	93.9	0.1	<0.5	6.5	50.5

Fig. 6—Ratios of SO_4^{2-} concentration in column effluent (*C*) to influent concentration (*C₀*, S-limestone column effluent) of Plainfield sand columns.

equal to or even greater than that in the S-limestone column effluent (C/C_0 ratios > 1.0) were obtained from the sand. The higher SO_4^{2-} levels from the sand columns were probably due to evaporation taking place in the columns between doses. Upon subsequent dosing, higher SO_4^{2-} concentrations occurred in the percolate. Sulfates are retained in acid soils mainly by kaolinitic clay minerals and hydrous oxides of iron and aluminum (32), and it is obvious from the data in Fig. 6 that the Plainfield sand contains very little of these components.

EVALUATION

The results indicate that a sulfur-*T. denitrificans* nitrate removal system would fulfill most requirements for a denitrification system. The rate of nitrate removal is rapid with complete denitrification occurring in < 4 hours at 23C. Assuming that the denitrification rate at 5C is approximately 10% of that at 23C, complete denitrification at extreme winter temperatures could be obtained in approximately 1.5–2 days residence time.

The sulfur-*T. denitrificans* system would also be relatively inexpensive. The amount of S needed for a family of four for 10 years can be estimated from Eq. [2] as approximately 450 kg of crude, lump S (approximately $125). Sufficient limestone to mix with the S to obtain a reactor bed size holding 2.0 days effluent would be about $90. Depending upon the type of septic system installed initially, this denitrification system would add approximately 20% to the initial cost ($1,200 ± $200). Dividing the total cost by the number of years in use (estimated lifetime of 10 years), the additional cost would be only $20 per year.

The effect of the sulfur-*T. denitrificans* NO_3^- removal system upon the performance of the overlying seepage bed as well as the amount of maintenance necessary for proper functioning of the system can only be determined in field studies. It appears that, after establishment of the *T. denitrificans* on the S, little maintenance would be needed.

The resulting level of SO_4^{2-}, however, limits the application of the *T. denitrificans* NO_3^- removal system. The permissible level of SO_4^{2-} in drinking water is 250 μg SO_4^{2-}/ml (83 μg S/ml) (26). Whether or not this level of SO_4^{2-} is reached in ground water below a sulfur-*T. denitrificans* NO_3^- removal system depends upon the density of systems per unit area, and the volume and recharge characteristics of the aquifer or watershed.

Adapting the sulfur-*T. denitrificans* nitrate removal system for municipal sewage systems may be feasible, especially, if the resulting SO_4^{2-} could be recovered and reused elsewhere, either in the plant or sold commercially. At this time, recovery as alum ($Al_2[SO_4]_3$) or transformation of SO_4^{2-} to another S form on an industrial basis appears uneconomical (W. W. Gilbert, E. I. du Pont de Nemours & Co., personal communication). However, with the 1985 goal of zero discharge into navigable waterways, a recycling system of NO_3^- removal and SO_4^{2-} recovery may be financially fruitful.

ACKNOWLEDGMENT

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