

FINAL PROJECT REPORT

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Treatment of Nitrate in Groundwater with Autotrophic Bioreactors

Submitted to:

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SECTION 1 – TITLE

Treatment of Nitrate in Groundwater with Autotrophic Bioreactors

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SECTION 3 - CONGRESSIONAL DISTRICT

District 05 (Arizona)

SECTION 4 - DESCRIPTION INFORMATION

A. Problem and Research Objectives:

Statement of Critical Regional or State Water Problems

Nitrate (NO_3^-) is one of the most common groundwater contaminants in Arizona. Over 1,000 wells across the State exceed the maximum contaminant level (MCL) for nitrate in drinking water ($10 \text{ mg NO}_3\text{-N L}^{-1}$) set by the US EPA. Nitrate concentrations in groundwater in the West Salt River Valley (WSRV), including areas in Glendale, Mesa, Chandler and Phoenix, are among the highest in the Nation (9). Shallow groundwater from an agricultural area in the WSRV exceeded USEPA drinking-water standards and guidelines for nitrate in more than 78 percent of samples. In this area, groundwater samples from above the clay beds had a median nitrate concentration of $19.0 \text{ mg NO}_3\text{-N L}^{-1}$. High nitrate levels also occur in other areas in the State, including Marana, St. David, Quartzsite, Bullhead City, Lake Havasu City, among others (2).

Nitrate in groundwater originates primarily from agricultural fertilizers, septic systems, landfills, and wastewater treatment plants. Nitrate is not significantly attenuated by the soil and it is transported with the groundwater largely unchanged (9).

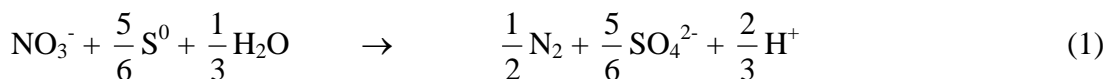
The high nitrate concentrations in Arizona groundwater resources constitute a public health concern. Nitrate at concentrations exceeding the MCL can cause methemoglobinemia, or "blue-baby disease" (18). Birth defects also have been attributed to high nitrate concentrations (5). In adults, high nitrate levels have been associated with cancer (39, 50).

Background Information:

Denitrification is an anaerobic microbial process in which nitrate (NO_3^-) is converted into dinitrogen gas (N_2) in four enzymatic steps via the intermediates nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) (12,52). The ability to respire nitrate under anaerobic conditions is widespread among several genera of heterotrophic bacteria (23,44,52). Heterotrophic denitrifiers utilize simple organic substances such as methanol, ethanol and glucose, as electron donating substrate (e-donor). Some denitrifying bacteria are chemolithoautotrophic and use reduced sulfur compounds such as elemental sulfur (S^0), sulfide (S^{2-}), thiosulfate ($\text{S}_2\text{O}_3^{2-}$), or sulfite (SO_3^{2-}) as electron donors (e-donor) (37,42,45). Under chemolithoautotrophic conditions, carbon dioxide or bicarbonate are used as a C source for microbial cell synthesis. The occurrence of denitrification coupled to the oxidation of reduced sulfur compounds has been previously reported in natural environments (27,33,36,41) and sulfur-utilizing chemolithoautotrophic denitrifiers are believed to play an important role in mineral cycling by linking sulfur and nitrogen cycles. Among these, two obligate autotrophic species are known, *Thiobacillus denitrificans* and *Thiomicrospira denitrificans*, which grow at neutral pH (29,37,46).

Denitrification has been studied for the treatment of drinking water (21,35). The process has been applied at full-scale in Europe. The main reasons for slow transfer of the technology to the USA are concerns over bacterial contamination and presence of residual organics used as electron donors. Potential problems associated with residual organics can be avoided if inorganic substances are used as e-donors. The use of hydrogen gas (H_2) has been considered (7, 30). Under practical conditions, the application of H_2 will require membrane systems (20, 30), which would involve high maintenance and operating costs. A much simpler, low-cost and low-maintenance approach would be to utilize S^0 as e-donor for denitrification.

A technology under consideration that utilizes S^0 for denitrification is the "*Sulfur – Limestone Autotrophic Denitrification (SLAD)*" process, in which elemental sulfur serves as the e-donor to support chemolithoautotrophic denitrification. The stoichiometry of the reaction indicates that acidity is produced.



Limestone serves to buffer the generated acidity as well as to supply inorganic carbon for cell synthesis by the denitrifying bacteria. Recommended ratios of S^0 :limestone range from 3:1 to 1:1 (10,32,51). The SLAD technology was first proposed by Dutch scientists in the year 1987 (38). Since then, a number of studies have reported on its applicability for the removal of nitrate in drinking water (10,16,25,47). The results demonstrate that volumetric loads up to $200 \text{ g NO}_3^- \text{N m}^{-3} \text{ reactor d}^{-1}$ can be treated effectively with 95% removal efficiencies. The SLAD process was tested at the pilot-scale in parallel with reverse osmosis and ion exchange for the removal of nitrates from drinking water (11). The physico-chemical methods provided an average nitrate removal efficiency of 85 to 90% and generated waste brines. The SLAD provided an average nitrate removal efficiency of 96% without generating waste brines. All of the previous research has been carried out with relatively high nitrate concentrations (generally $60\text{-}500 \text{ mg NO}_3^- \text{N L}^{-1}$) necessitating the use of the limestone for buffering.

S^0 is an apolar mineral, thus mass transfer is expected to be an important rate-limiting factor in the overall process. The specific surface area of S^0 is a principal factor governing the kinetics of its biological oxidation (26,43), including oxidation linked to denitrification (24). Surface colonization of S^0 particles is essential in the aerobic biooxidation of S^0 by *Thiobacillus*. In order to achieve high surface areas, S^0 particle sizes may be too small to be suitable for a continuous bioreactor due to washout. However, newly developed puffed S^0 products are now available, such as “*Popcorn sulfur*” which could provide the high surface area while maintaining a large particle size, amendable to retention in bioreactors. Biologically produced sulfur is more hydrophilic than mineral S^0 (22) and, thus, it would be expected to be more bioavailable. Therefore, different forms of S^0 varying in properties of specific surface area and hydrophobicity still need to be considered to improve the kinetics of the SLAD process.

Research Objectives

The objective of this study is to evaluate the application of elemental sulfur as an electron donor for the biological treatment of nitrate in groundwater. Novel forms of S^0 of enhanced bioavailability will be tested which are expected to provide more rapid biological conversion rates compared to conventional S^0 products.

B. Methodology

Microorganisms The chemolithoautotrophic denitrifying enrichment was obtained in a laboratory-scale anaerobic bioreactor (0.5 L) operated at a hydraulic retention time (HRT) of 8 h in a temperature-controlled chamber at 25°C for 8 weeks. The reactor was fed a medium composed of 100 mg L^{-1} S as thiosulfate ($\text{S}_2\text{O}_3^{2-}$), 20 mg L^{-1} as $\text{NO}_3^- \text{N}$, 1000 mg L^{-1} of bicarbonate (carbon and alkalinity source) and micro-/macronutrients (40). Thiosulfate was selected as electron donor to facilitate enrichment of autotrophic denitrifiers. The bioavailability of thiosulfate, a soluble compound, exceeds many-fold that of elemental sulfur. The reactor was inoculated with anaerobic sludge from a full-scale anaerobic bioreactor treating recycle paper wastewater. Earlier research has shown that a highly active autotrophic denitrifying enrichment can be obtained utilizing the latter consortium as inoculum after approx. 8 weeks of operation

(15). The influent of the bioreactor was monitored daily for pH, nitrate and thiosulfate. Parameters monitored in the effluent will include: pH value, nitrate, nitrite, thiosulfate, and sulfate. Biomass cultivated in the laboratory reactor was used as inoculum for batch- and continuous experiments. The autotrophic denitrification activity of the enrichment was determined in bioassays with thiosulfate by following the loss of thiosulfate and nitrate with an ion-chromatograph or appearance of N₂ in a helium flushed headspace. Bioassays were set up as described below. Stock cultures were maintained in the mineral medium without thiosulfate and kept under refrigerated conditions (4°C).

Batch bioassays: The effect of S⁰ on denitrification kinetics was determined at 30°C in shaken anaerobic batch bioassays. Various commercial grades of S⁰ were tested including biologically-produced sulfur and popcorn sulfur of different particle sizes. To prevent O₂ contamination, the bottles were sealed with thick butyl rubber stoppers and aluminum crimp caps and, then flushed thoroughly with helium gas. Subsequently, the flasks were supplied aseptically with a pH 7, O₂-free mineral medium (40) containing bicarbonate (1000 mg L⁻¹), nitrate (5-25 mg NO₃-N L⁻¹) and inorganic reduced sulfur (thiosulfate and/or elemental sulfur) at specific concentrations, depending on the aim of the experiment. Then, bacterial inoculum was added. Anaerobic conditions were established in each bottle by flushing with helium gas. Samples were taken periodically to determine substrate or an electron acceptor utilization and product formation. Samples of the headspace gas were analyzed for N₂ and N₂O.

Flow-through columns: A packed bed reactor (0.4 l) was filled with a mixture of S⁰ granules (120.8 ml) and limestone grit (128.4 ml) between 5 and 16 mesh. The reactor was inoculated with 1.2 g VSS of enrichment culture granular sludge. The S⁰ granule particle size was approximately 3.5 mm wide ×1 mm thick. The total mass of S⁰ added to the reactor was 141.3 g. The reactor was fed with an influent containing 7.1 mM NO₃⁻, 23.8 mM and basal mineral medium containing (g/l): KH₂PO₄, 1; MgSO₄·6H₂O, 0.2; NH₄Cl, 0.4; Na HCO₃, 2; trace element solution (described above), 2 ml/l. The column effluent was recycled back to the top of the reactor until biofilm development on the packing was noticeable. The performance of the reactor was then tested at HRTs ranging from 24 to 1.5 h. HRT are based on the empty bed volume of the reactors. Once fully operational, influent nitrate concentration was decreased to determine if treatment is feasible at concentration range that is realistic for groundwater treatment conditions. The influent was monitored periodically for pH, nitrate and bicarbonate alkalinity. The effluent was also monitored for pH value, nitrate, nitrite, sulfate, and thiosulfate. The composition of the biogas (CO₂, N₂, N₂O) was monitored weekly or as needed.

Analytical Methods: Dinitrogen gas (N₂), nitrous oxide (N₂O) and carbon dioxide (CO₂) were quantified in a GC equipped with a thermal conductivity detector. The concentration of nitrate, nitrite, thiosulfate and sulfate in liquid samples were analyzed by ion chromatography (IC) with suppressed conductivity detection using a Dionex DX-500 system equipped with a Dionex AS11-HC4 column (Dionex, Sunnydale, CA) and an eluent containing 15 mM KOH at a flow rate of 1.2 mL min⁻¹. Liquid samples were membrane-filtered (0.45 µm) prior to IC analysis. Other parameters, such as volatile suspended solids (VSS) in the biomass and bicarbonate alkalinity in liquid samples were measured according to *Standard Methods for the Examination of Water and Wastewater* (4).

C. Principal Findings and Significance

Chemolithotrophic enrichment cultures have been established that can couple denitrification to the oxidation of S⁰ (Fig. 1). The role of elemental sulfur and nitrate concentrations on the kinetics and stoichiometry of autotrophic denitrification was investigated. Results of batch bioassays indicated a continued increase in denitrification rates at concentrations far exceeding the stoichiometric requirement (Fig. 2), pointing to the occurrence of mass transfer limitations from solid phase S⁰ to the aqueous phase. Different grades of S⁰ were tested and the material

providing the best compromise between physical and electron donating properties was selected for further work. An increase in denitrification rates was observed with decreasing S^0 particle size, which can be attributed to the increase in surface area, resulting in better mass transfer.

The rates of chemolithotrophic denitrification in assays utilizing different reduced sulfur compounds as e-donors were compared. The average oxidation state of the sulfur atoms in the three compounds tested, *i.e.*, sulfide, elemental sulfur and thiosulfate, is -2 , 0 and $+2$, respectively. Fig. 3 illustrates the conversion of nitrate and the formation of nitrogen-containing products as a function of time for the various assays. Also plotted in this figure is the time course of conversion for the various reduced sulfur compounds to sulfate. Nitrogenous gas intermediates were not detected in this experiment. Thiosulfate was the most readily utilized electron donor, followed by hydrogen sulfide and elemental sulfur. The rates of nitrate degradation in assays with thiosulfate were 4.6 and 9.5 fold higher compared to sulfide and elemental sulfur, respectively. Similarly, the rates of sulfate generation in assays with thiosulfate were 4.8 and 25.3-fold higher compared to sulfide and elemental sulfur, respectively. Nitrate was recovered as N_2 gas in (near) stoichiometric proportions by the end of the experiments. Thiosulfate is readily bioavailable and non-toxic, which could partly explain the high sulfoxidation and denitrification rates detected with this compound. While, H_2S is also bioavailable, it is a well-known inhibitor of a wide variety of microorganisms, including denitrifying bacteria, and its inhibitory impact may account for lower metabolic rates compared to thiosulfate. The lowest rates were observed for chemolithotrophic denitrification of S^0 and this is most likely due to the limited mass transfer of substrate from solid phase S^0 . Elemental sulfur is an apolar mineral, thus mass transfer is expected to be an important rate-limiting factor in the overall process. The specific surface area of elemental sulfur is a principal factor governing the kinetics of its biological oxidation (26), including oxidation linked to denitrification (24).

The feasibility of removing nitrate in continuous bench-scale columns packed with S^0 as slow-release e-donor was investigated. A packed bed reactor with an approximate ratio 1:1 of sulfur:limestone ($CaCO_3$) granules was rapidly started up utilizing a chemolithotrophic denitrifying enrichment culture as inoculum. The initial start up concentration was 105 mg $N-NO_3^-/L$. Nitrate concentration in the influent was lowered stepwise to concentrations typical of highly contaminated groundwater resources in Arizona (approx. 20 mg $N-NO_3^-/L$). The performance of the reactor is illustrated in Fig. 4. Results obtained indicate that a bioreactor packed with S^0 can successfully treat nitrate with a high efficiency at high volumetric loading (Table 1) and HRTs of only 1.8 h. The maximum nitrate loading rate attained, 237 mg $NO_3^- - N/L_{\text{reactor-d}}$, is comparable to the fastest rates achieved in the literature with S^0 as e-donor. The recovery of N as benign N_2 gas was nearly stoichiometric.

The results of this study confirm the effectiveness of microbial chemolithotrophic denitrification linked to oxidation of S^0 for the removal of nitrate. In addition, these findings indicate the potential of sulfur-limestone biofilters for the low-cost, low-maintenance treatment of nitrate-contaminated groundwater.

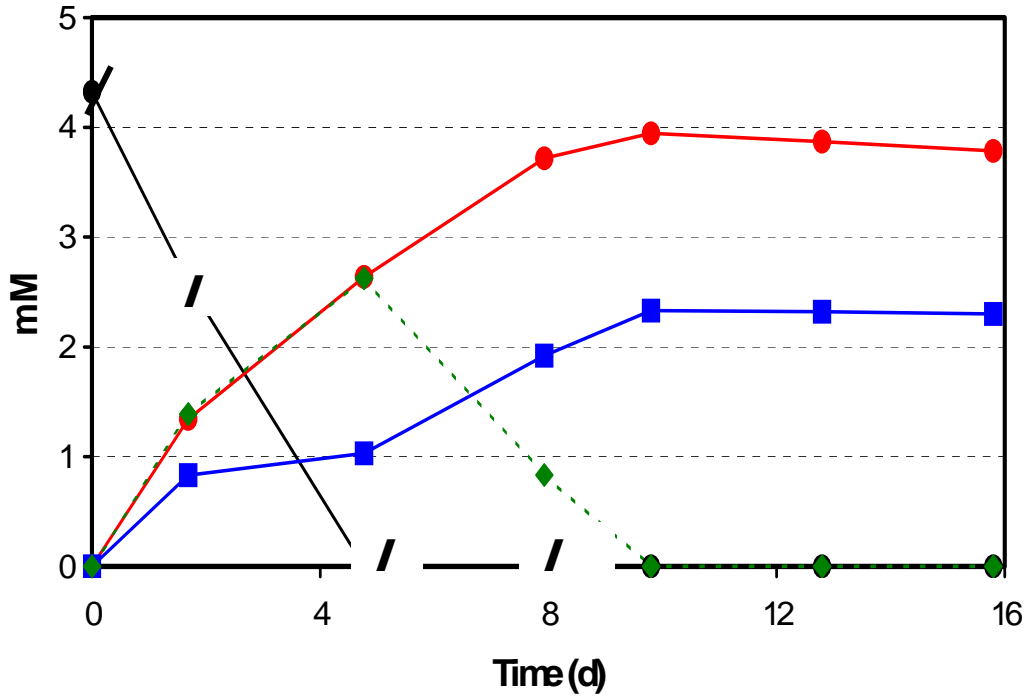


Figure 1. Time course of denitrification by a chemolithotrophic enrichment culture utilizing elemental sulfur (15.2 mM) as electron donating substrate. The initial nitrate concentration was 3.8 mM, and the sludge concentration was 0.5 g VSS/L. (●) Nitrate; (■) N₂ gas; (◆) nitrite; (●) sulfate.

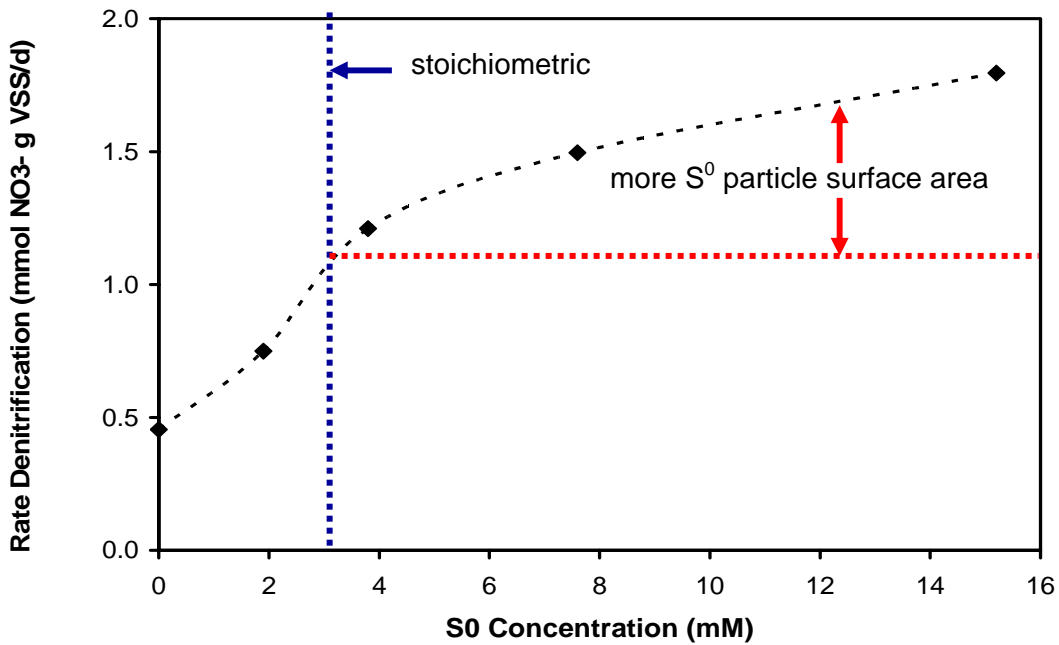


Figure 2. Effect of the sulfur concentration on the rate of denitrification determined for a chemolithotrophic denitrifying enrichment culture.

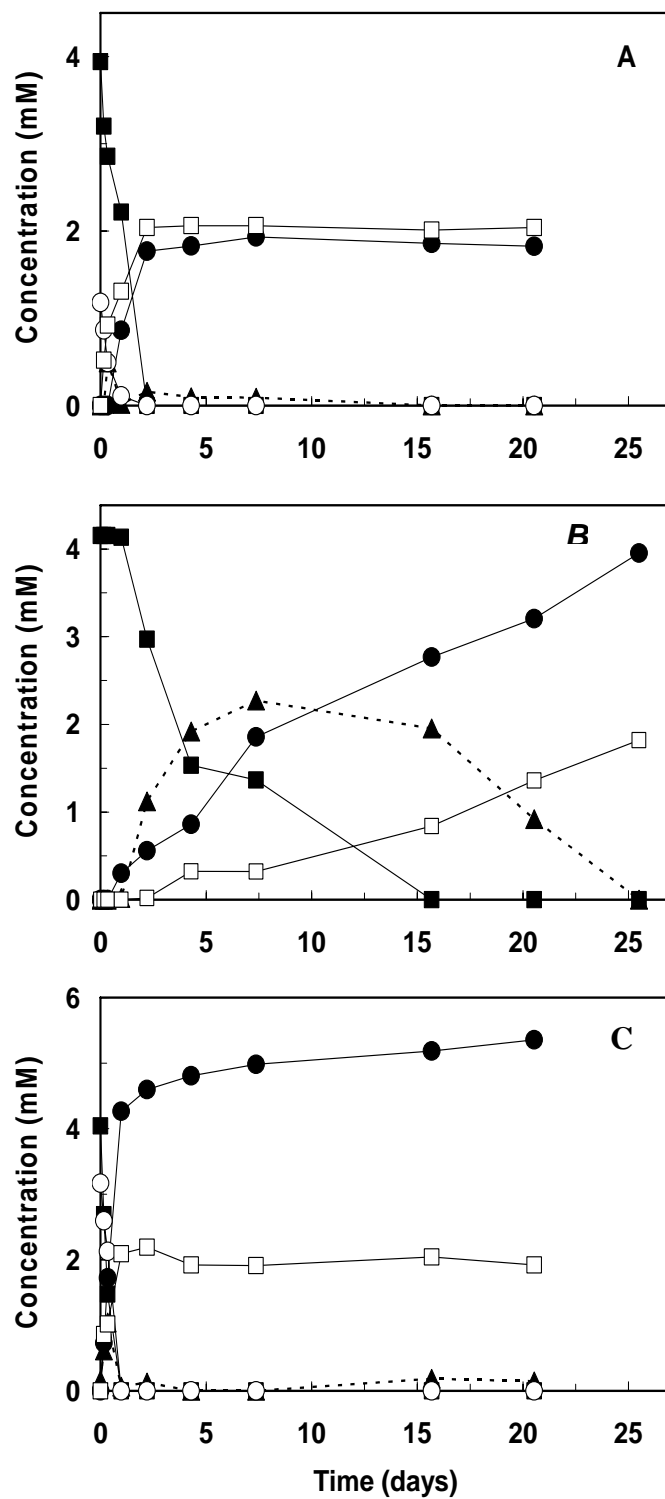


Figure 3. Time course of denitrification and sulfoxidation by a chemolithotrophic denitrifying mixed culture (0.5 g VSS/L) utilizing hydrogen sulfide (H_2S) (Panel A), elemental sulfur (S^0) (Panel B), or thiosulfate ($S_2O_3^{2-}$) (Panel C) as electron donors. Electron donor (○); sulfate (●); nitrate (■), nitrite (▲), and dinitrogen gas (□). Bioassays were supplied with 4 mM nitrate and stoichiometric concentrations of the reduced sulfur compounds.

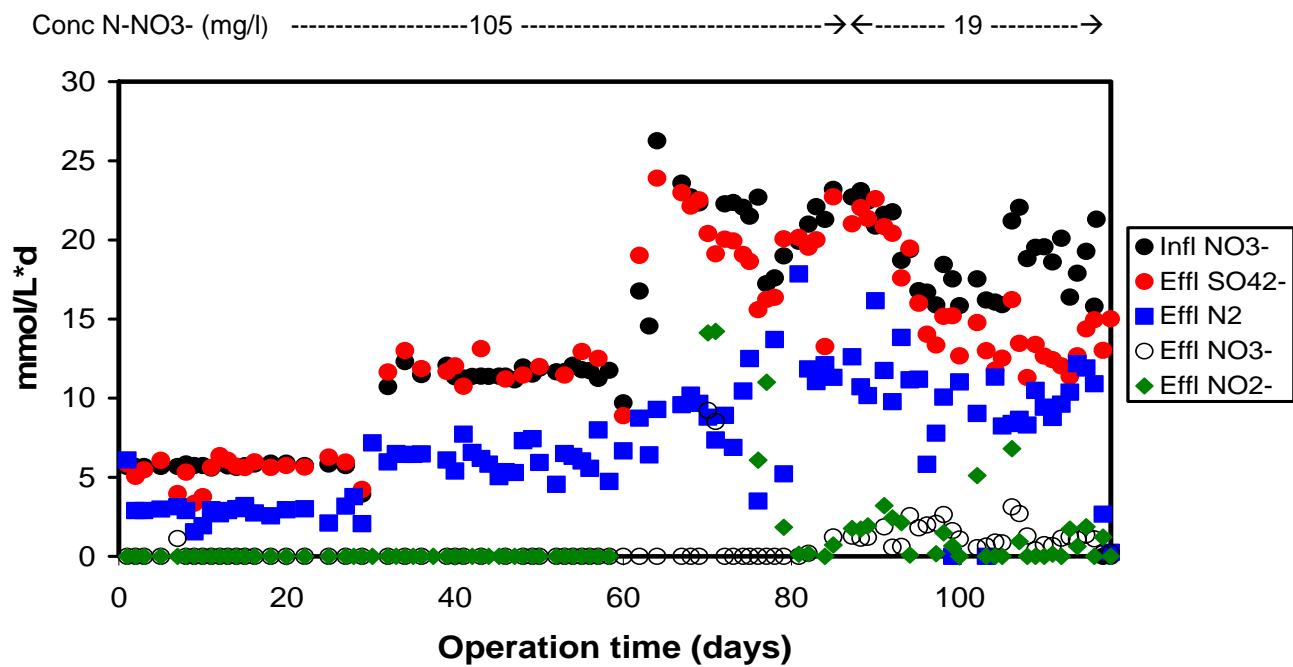


Figure 4. Nitrate and sulfur conversion in a laboratory-scale reactor packed with sulfur: limestone fed with a simulated groundwater supplied with nitrate.

Table 1. Performance of an Elemental Sulfur Packed Bioreactor Used for the Treatment of Nitrate

Parameter	Value	Units
Inlet Concentration NO_3^- -N	19.0	mg l^{-1}
Hydraulic Retention Time	1.9	h
Volumetric Load NO_3^- -N	237	$\text{mg l}^{-1}_{\text{reactor}} \text{d}^{-1}$
Removal NO_3^- -N	92.1	% NO_3^- -N _{in}
Recovery NO_2^- -N	4.5	% NO_3^- -N _{in}
Recovery N_2 -N	99.4	% NO_3^- -N _{in}

SECTION 5 - PUBLICATION INFORMATION

a. Articles in Refereed Scientific Journals

- Beristain Cardoso R, Sierra-Alvarez R, Rowlette P, Razo Flores E, Gómez J, Field JA. 2006. Sulfide oxidation under chemolithoautotrophic denitrifying conditions. *Biotechnol. Bioengr.* (Under review).
- Sanz JL, Fernández N, Gómez R, Amils R, Field, JA and Sierra-Alvarez R. 2006. Microbiological and structural aspects of granular sludge from autotrophic denitrifying reactors. *Water Sci. Technol.* (In press).

b. Book Chapters: ----

c. Dissertations: -----

d. Conference Proceedings:

- Beristain, R., Sierra-Alvarez, R., Salazar, M., Fernandez, N., Gomez, J., Razo-Flores, E., and Field, J. A. 2005. Autotrophic Denitrification with Elemental Sulfur. VIII Latin American Workshop and Symposium on Anaerobic Digestion. October 2-5, 2005. Punta del Este, Uruguay. Pp. 383-388.
- Sanz JL, Fernández N, Gómez R, Amils R, Sierra-Alvarez R and Field JA. 2005. Microbiological and structural aspects of granular sludge from autotrophic denitrifying reactors. VIII Latin American Workshop and Symposium on Anaerobic Digestion. October 2-5, 2005. Punta del Este, Uruguay. Pp. 15-20.
- Fernández, R. Gomez, R. Amils, J.L.Sanz, R. Sierra-Alvarez, J.A Field. Microbial ecology of autotrophic denitrifying reactors. Biomicroworld 2005: Int Conf on Environ, industrial and Applied Microbiology. March 15-18th, 2005. Badajoz, Spain.
- Sierra-Alvarez, R and J. A. Field. 2005. Autotrophic Denitrification For The Treatment Of Drinking Water Fall semi-annual meeting of the NSF Arizona Water Quality Center. Dec. 5th, 2005, Tucson, Arizona. (Abstract).

SECTION 6. STUDENT SUPPORT

Master 0
PhD 2
Post Doc 0
Undergraduate 1
Total 3

The project served as a research topic for exchange PhD students, Ricardo Beristain (Dept Environmental Biotechnology, Autonomous Metropolitan-Iztapalapa University, Mexico D.F.) and Nuria Fernandez (Dept of Molecular Biology, Autonomous University of Madrid, Madrid, Spain). In addition, one undergraduate students have also benefited from this project.

SECTION 7. NOTABLE ACHIEVEMENTS AND AWARDS

Findings from this research have facilitated the acquisition of additional research to continue work in the area of nitrate bioremediation. A two-year project entitled “Autotrophic Denitrification for the Treatment of Drinking Water”, which is funded by the University of Arizona Technology and Research Initiative Fund (TRIF) and by the engineering company, Hydro Geo Chem Inc (<http://www.hgcinc.com/>), is currently ongoing (June, 2005-June 2007).

Research collaboration with the Dept. Microbiology (Autonomous University of Madrid, Spain) has been established. The purpose of the joint effort is to characterize the sulfoxidizing, nitrate-reducing microorganisms in the reactor biofilms utilizing molecular ecology techniques.

Acknowledgements

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