PN-ACH-421
104544

ANNUAL REPORT

Covering Period: January 1999 - December 1999

Submitted to the Center for Economic Growth
U.S. Agency for International Development

Hydrogen-dependent denitrification of drinking water

Principal Investigator: M. Inês M. Soares
Grantee Institution: Ben-Gurion University of the Negev

Collaborator: Kornel Kovacs
Institution: Institute of Biophysics - Biological Research Center
Hungarian Academy of Sciences

Grant Number: TA-MOU-C15-045

Project Duration: 1 January 1996 - 30 June 2000

Executive Summary

The objective of this study is to develop an efficient and cost effective microbial process for removal of nitrate from polluted drinking water. A small-scale biological system is being developed in which denitrification is achieved by autotrophic bacteria using hydrogen as their energy source.

Generation of the required hydrogen is an integral part of the denitrification setup and is achieved in an electrolysis cell by electrolysis of the water to be treated. The cell is divided into two compartments by a cation-exchange membrane which prevents oxygen formed in the anode from reaching the cathode and the water to be treated (oxygen inhibits denitrification); water is pumped through the cathodic compartment where it is enriched with hydrogen.

In continuation of a comparative study started in the previous year, two system configurations were tested: (1) a single-reactor with the biomass in the cathodic compartment of the electrochemical cell and (2) a system consisting of two distinct units, an electrochemical cell and a bioreactor. In both systems granulated activated carbon served as physical support for the biomass. The two-reactor configuration was found to be the most suitable.

The long-term performance and stability of the two-reactor system was analyzed, as well as the effects of its main operational parameters, water velocity and current intensity. The system was operated for one year and was very stable.

A study of the composition and dynamics of the microbial population in the bioreactor is in progress: bacteria present at various levels are analyzed immediately after inoculation, and after various periods of operation. Classical and modern molecular methods are used.

In Hungary, a two-step denitrification system has been developed consisting of ion-exchange removal of nitrate followed by hydrogen-dependent biological denitrification of the brine.

Table of contents

Executive summary	page	2
Section I		
Research objectives		3
Research accomplishments		3
Scientific impact of collaboration		4
Description of project impact		4
Strengthening of developing country institutions		4
Future work		4
Section II		
Managerial Issues		5
Budget		5
Special concerns		5
Collaboration, travel, training and publications		5
Request for A.I.D. or BOSTID Actions		6
Appendix A		
Appendix B		
Appendix C		

Section I

A) Research Objectives

The overall objective of this project is to develop and standardize a simple, small-scale biological system for the treatment of nitrate contaminated drinking water. This will be achieved by denitrifying bacteria using electrochemically produced hydrogen as energy source. The specific research objectives during the last year were:

(a) To compare the performance of two different system configurations: (1) a single reactor where both the generation of hydrogen and denitrification took place, and (2) a two-reactor system where water was first enriched with hydrogen in an electrolysis cell prior to entering a packed-bed bioreactor. To select the configuration most suitable for further use.

(b) To test the long-term performance and stability of the system selected under (a).

(c) To study the microbial population present in the bioreactors, and to obtain an understanding of its temporal and spatial changes.

B) Research Accomplishments

The main research accomplishments during the last year were:

(a) Selection of the two-reactor system as the most suitable configuration for the hydrogen-dependent bio-electrochemical denitrification process. This work is summarized in a manuscript accepted for publication in *Water Science and Technology*, a copy of which is attached (Appendix A).

(b) The long-term performance and stability of the two-reactor system was analyzed, as well as the effects of its operational parameters (water velocity and current intensity). The system was found to be very stable. This work is summarized in the attached draft of a paper to be submitted to *Water Research* (Appendix B).

(c) A study of the composition and dynamics of the microbial population in the bioreactor is in progress: bacteria present at various levels are analyzed immediately after inoculation, and after various periods of operation. Classical and modern molecular methods are used. The results obtained will be presented in the final report to be submitted in June 2000.

(d) A two-step denitrification system consisting of ion-exchange removal of nitrate followed by hydrogen-dependent biological denitrification of the brine has been developed and is described in detail in a manuscript submitted to *Acta Microbiol. Immunol.* (Appendix C).

C) Scientific Impact of Collaboration

At the end of March, Istvan Kiss, a Hungarian Ph.D. student concluded one-year training in Israel and returned to his home laboratory. Another Hungarian Ph.D. student, Szilvia Szekeres, joined the Israeli group at the beginning of April 1999, and will stay until June 2000. On his return to Hungary Istvan Kiss set up reactors similar to the ones built in the Israeli laboratory. Thus the Hungarian group has taken full advantage of the studies carried out in Israel and of the services of Dr. T. Bejerano, the electrochemist adviser to the Israeli group.

D) Description of Project Impact

As described under E) the project has upgraded the research capacity of the Hungarian institution. It has introduced a new line of applied research and has allowed training of young scientists. By the end of project, a blueprint will be drawn for the immediate application of the denitrification technology which is being developed.

E) Strengthening of Developing Country Institutions

The project investment has allowed the Hungarian institution to undertake the basic research required to back up the practical solution of a very pressing local problem. It has provided the funds to support two Ph.D. students in the Hungarian institution. Two other Ph.D. students have joined the Israeli team where they had the opportunity to work and interact with a large and interdisciplinary group of scientists with long experience on water treatment. In our view, this has been one the most important and lasting contributions to the developing country institution.

F) Future work

The next six months will be devoted to the characterization of the bacterial population present in the reactors. This study is already in progress. Columns of different ages are dismantled, and bacteria present at different levels of the packed bed are isolated. Classical and modern molecular

(PCR) techniques are used. This is an attempt to go beyond the "black-box" approach, and to gain a deeper understanding of the processes taking place in the bioreactors. Such information is important for the reliable operation of a system for the treatment of potable water.

Section II

A) Managerial Issues

Following our request to end the project on 30 June 2000, the extension was granted in July 1999.

B) Budget

The Hungarian PI requested to transfer $4,893 from the allocation "Training" to "Other Direct Costs". The increase in direct cost expenses was due to computing costs (buying and licensing of software and user fee to networking), and to contributions to the subscription of scientific journals. On the other hand, funds had accumulated in the item "Training", as additional funding became available for this purpose. This request has been submitted to Mr. Boaz Ayalon, American Embassy, Tel Aviv.

C) Special concerns

No change in the protocols was or is required.

D) Collaboration, Travel, Training and Publications

Two Hungarian Ph.D. students have been in the Israeli laboratory: Istvan Kiss left at the end of March 1999, after one year training, and Szilvia Szekeres arrived at the beginning of April 1999 and will stay until the end of June 2000.

The Israeli PI visited the Hungarian group in May 1999, and another visit is planned to take place in May 2000.

Project publications are as follows:

Kiss, I., S. Szekeres, T. T. Bejerano and M. I. M. Soares. 1999. Hydrogen-dependent denitrification of drinking water. The 7th International Conference of the Israel Society for Ecology and Environmental Quality Sciences on Environmental Challenges for the Next Millennium, Jerusalem, 13-18 June. Abstracts, p. 129.

Soares, M. I. M. 1999. Biological denitrification of groundwater. *Water, Air, and Soil Pollution* (Accepted).

Kiss, I., S. Szekeres, T. T. Bejerano and M. I. M. Soares. 1999. Hydrogen-dependent denitrification: preliminary assessment of two bio-electrochemical systems. *Water Science and Technology* (Accepted).

Polyák, B., P. Agócs, I. Kiss, K. Perei, I. Soares, P. Kesserû, Zs. Rácz, G. Rákhely, and K. L. Kovács. 2000. A combination of chemical removal and biological elimination of nitrate. *Acta Microbiol. Immunol.* (Submitted).

Szekeres, S., Kiss, I., T. T. Bejerano and M. I. M. Soares. 2000. Hydrogen-dependent denitrification in a two-reactor bio-electrochemical system (In preparation for submission to *Water Research*).

6) Request for American Embassy Tel Aviv, A.I.D. or BOSTID Actions

There are no new requests for assistance of the A.I.D. or BOSTID staff.

Appendix A

HYDROGEN-DEPENDENT DENITRIFICATION: PRELIMINARY ASSESSMENT OF TWO BIO-ELECTROCHEMICAL SYSTEMS

Istvan Kiss, Szilvia Szekeres, T. Tony Bejerano and M. Inês M. Soares

Department of Environmental Hydrology and Microbiology, The Jacob Blaustein Institute for Desert Research, Ben-Gurion University of the Negev, Sede Boqer 84993, Israel

ABSTRACT

An autotrophic biological process for the treatment of nitrate-contaminated drinking water was studied in the laboratory, with the objective of developing a continuous system which would be simple, stable and amenable to upscaling. Hydrogen generated by electrolysis of the water to be treated was the source of energy for denitrifying microorganisms. Two main process configurations were compared: (1) a single reactor where both the generation of hydrogen and denitrification took place, and (2) a two-reactor system where water was first enriched with hydrogen in an electrolysis cell prior to entering a packed-bed bioreactor. The reactors were operated in a continuous mode and granulated activated carbon served as physical support for the biomass. Although the highest rates of denitrification (0.45 kg N m^{-3} d^{-1}) and the shortest residence times (removal of 18 mg N l^{-1} in 17 min) were obtained in the single-reactor system, the two-reactor system was more stable and more suitable for upscaling.

KEYWORDS

Denitrification; hydrogen; water electrolysis; water treatment

INTRODUCTION

Biological denitrification is a mechanism by which certain bacteria use nitrate as terminal electron acceptor in their respiratory process, in the absence of oxygen. Denitrification consists of a sequence of enzymatic reactions leading to the evolution of nitrogen gas. The process involves the formation of a number of nitrogen intermediates and can be summarized as follows:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \qquad (1)$$

Denitrifying bacteria are ubiquitous in nature (Gamble *et al.*, 1977; Zumft, 1992), and biological denitrification treatment consists of the provision of suitable carbon and energy sources which may be organic or inorganic compounds. Hydrogen gas is one of the possible sources of energy for denitrifying bacteria although growth under autotrophic anaerobic conditions is thought to be restricted to *Paracoccus denitrificans* and closely related strains (Aragano and Schlegel, 1992)

Hydrogen gas is an ideal energy source for biological denitrification in the sense that it is completely harmless to potable water, and no further steps are required to remove either excess substrate or its derivatives which is the case with other organic (e.g., methanol, acetate, ethanol) and inorganic (e.g., sulfur) substrates. In addition, since only a few species can carry out H_2-dependent denitrification, the production of biomass is low. However, the use of H_2 has been limited by two of its inherent properties: formation of flammable and explosive mixtures with oxygen, and very low solubility in water (1.6 mg l^{-1} at 20°C).

A number of denitrification systems have been described in which compressed hydrogen gas was used. Kurt *et al.* (1987) studied a bench-scale fluidized bed reactor in which a residence time of 4.5 h was required for complete denitrification of water containing 25 mg nitrate-N l^{-1}. A commercial-scale process known as DENITROPUR was developed by various authors and operated in Monchengladbach, Germany (Gros and Treutler, 1986; Gros *et al.*, 1986). The process incorporated a hydrogen saturator, addition of phosphate and carbon dioxide, a number of packed-bed reactors in series, postaeration, floculant addition, filtration, and UV filtration; residence times of 1 – 2 h were required to remove 50 mg l^{-1} nitrate. Dries *et al.* (1988) used a two-

column system in which nitrate was removed in the first column, and in the second column removal of excess hydrogen and oxidation of residual nitrite to nitrate took place.

More recently, Sakakibara and Kuroda (1993) reported a different approach: hydrogen was generated in the denitrification reactor by electrolysis of the water to be treated. They studied a batch system consisting of two interconnected reactors, cathodic and anodic. Prior to start up, a biofilm was allowed to develop on the surface of the cathode, by batch cultivation in a rich organic medium. When a biofilm was visible, the electrode was transferred to the reactor and connected to the power supply; hydrogen generated at the cathode was immediately taken up by the microorganisms on its surface.

In the study presented here hydrogen was also generated in the water to be denitrified, with the objective of developing a continuous system which would be simple, stable and amenable to upscaling. Important design features are: (a) a cation permeable membrane separating the electrodes to prevent O_2 generated at the anode from reaching the water to be treated while allowing transfer of H^+, (b) a large surface area for biofilm establishment to be provided by a bed of granulated activated carbon (GAC), and (c) biomass development under operating (autotrophic) conditions. Two configurations were studied: a single reactor (ECBR) with the biomass in the cathode compartment of the electrochemical cell (EC), and a system consisting of two distinct units (EC+BR), an electrochemical cell and a bioreactor (BR).

The net chemical reaction describing the hydrogen-dependent denitrification reaction is:

$$2\ NO_3^- + 5\ H_2 + 2\ H^+ \rightarrow N_2 + 6\ H_2O \qquad (2)$$

The hydrogen is provided by the cathodic reaction:

$$10\ e + 10\ H_2O \rightarrow 5\ H_2 + 10\ OH^- \qquad (3)$$

Combining equations (2) and (3) and rearranging:

$$10\ e + 2\ NO_3^- + 12\ H^+ \rightarrow N_2 + 6\ H_2O \qquad (4)$$

Assuming that the sole anodic reaction is:

$$5\ H_2O \rightarrow 21/2\ O_2 + 10\ H^+ + 10\ e \qquad (5)$$

and that there is no separation between the anode and cathode (undivided electrochemical cell) then the net reaction is given by:

$$2\ NO_3^- + 2\ H^+ \rightarrow N_2 + 21/2\ O_2 + H_2O \qquad (6)$$

In this case, one equivalent of acid is needed for each mole of nitrate. If the EC is separated by a cation-exchange membrane and dilute sulfuric acid is used as an anolyte, then most of the current, about 95%, will be carried through the membrane by protons. Thus, similar demand for acid is expected in both the divided and undivided electroreactors, and only minor pH changes are expected at the concentrations of nitrate tested.

MATERIALS AND METHODS

Experimental set up

The two systems used, ECBR and EC+BR, are schematically represented in Fig. 1. The EC is shown in detail Fig. 2 and was identical in both systems, except for the differences in dimensions presented in Table 1. A Nafion cation-exchange membrane separated the two electrodes and Hypalon rubber spacers (6 mm thick in the cathode side of ECBR, 3 mm thick in all other cases) intercalated between the membrane and the electrode created cathodic and anodic chambers. Plates of rubber (3 mm thick), PVC (1 cm thick) and stainless steel (1 cm thick), in this order, followed each electrode, and the different layers were held together by four stainless steel bolts. The anodes were cut from a 2 mm thick plate of titanium coated with platinum-iridium oxide, and the cathodes were made from 5 mm thick graphite plates. The electrodes were connected to a DC power

Table 1. Characteristics of the single-reactor (ECBR) and of the two-reactor (EC+BR) systems

		ECBR	EC+BR
Electrodes active area	Dimensions (cm)	14 x 7	9 x 5
Bed	Dimensions (cm)	14 x 7 x 0.6	4.5 (Ø) x 38.5
	Volume (cm^3)	59	610
	Void Volume (cm^3)	17	270
	GAC (g)	35	550

supply. The EC was kept in the vertical position, and peristaltic pumps regulated the flow of solutions: 0.1 N H_2SO_4 was recirculated in the anodic chamber and the feed solution (tap water amended with 0.29 g H_3PO_4 l^{-1}, 70 mg $NaHCO_3$ l^{-1} and nitrate) flowed through the cathodic chamber. The feed reservoir was gently sparged with N_2.

Packed beds of pre-washed 0.85 - 1.70 mm matrix granulated activated carbon (GAC) served as physical support for the biomass; their characteristics are summarized in Table 1. In the EC+BR system the feed solution was enriched in the EC with hydrogen prior to entering the BR. All experiments were carried out at 26±1 °C.

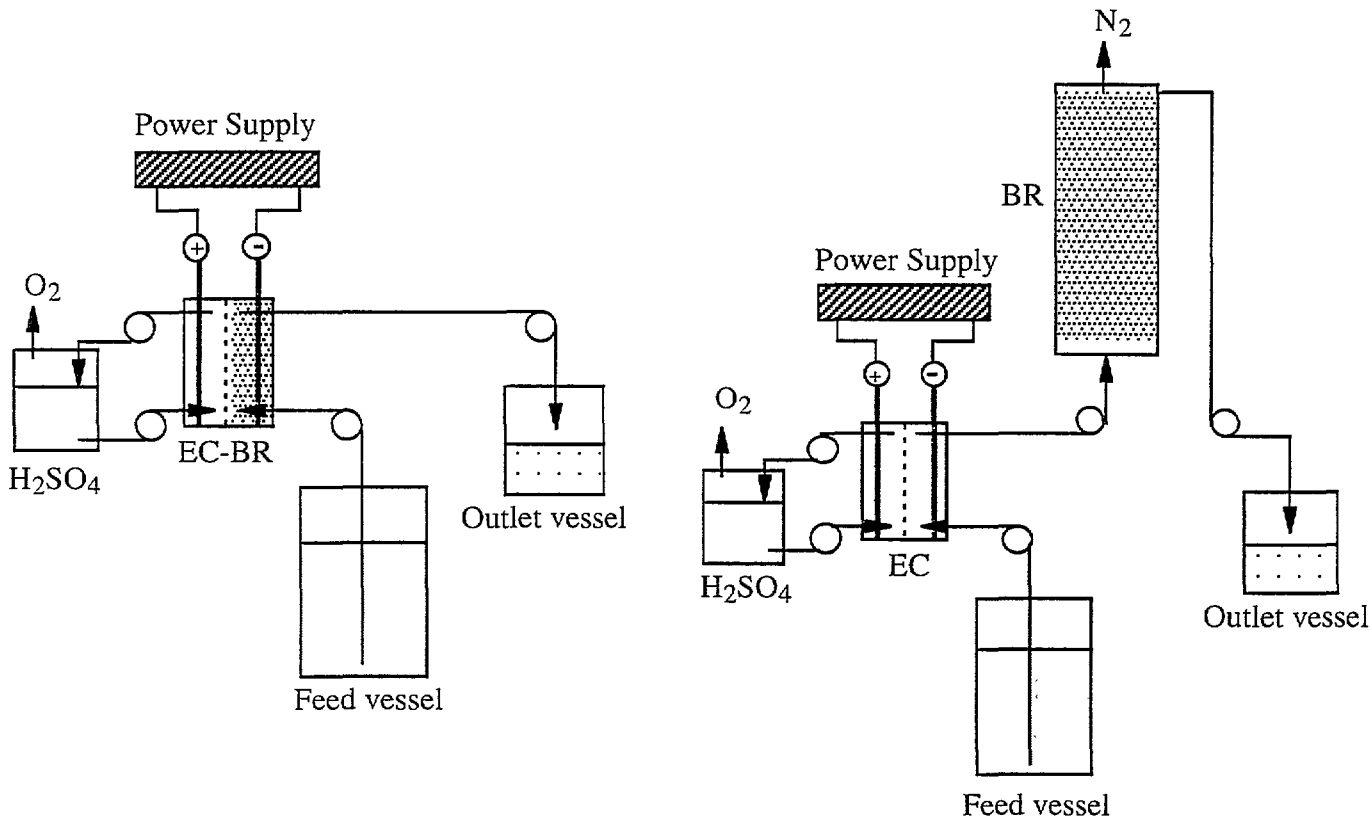


Figure 1. Schematic representation of the ECBR (left) and EC+BR (right) denitrification systems.

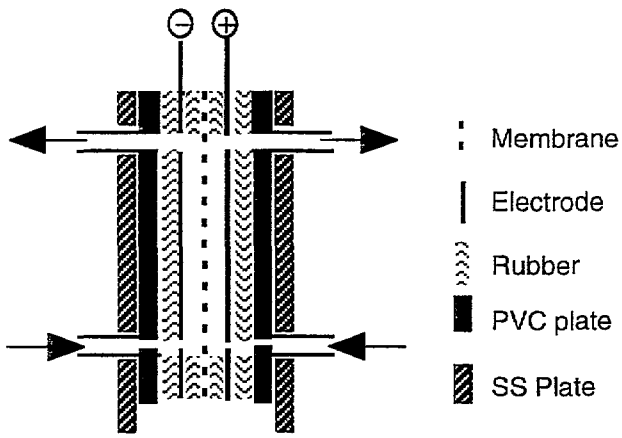


Figure 2. Schematic representation of the electrochemical cell.

The bacterial inoculum consisted of a mixture of four denitrifying strains isolated from a previous reactor and was prepared by cultivation in nutrient broth for 48 h, harvesting by centrifugation and washing, and resuspension in mineral medium (Schlegel *et al.*, 1961); the suspension was recirculated through the packed bed for 3-5 days. The original inoculum was an enrichment culture (in mineral medium and under an atmosphere of 6% H_2 and 94% N_2) of sediment from an oxidation pond.

The systems were operated at various water velocities and current intensities. A water velocity of 1 m d^{-1} corresponded to a feed rate of approximately 0.29 ml min^{-1} in the ECBR and 1.1 ml min^{-1} in the EC+BR.

Analytical determinations and bacterial counts

Nitrate was determined by the colorimetric method of Cataldo *et al.* (1975), and nitrite and ammonia were assayed according to Standard Methods (1992). Colony forming units (CFU) in the influent and the effluent were counted by standard plating techniques on R2A agar (Difco Laboratories).

RESULTS

The EC+BR system was operated for 3 months at nitrate load of approximately 23 mg N l^{-1}, and at water velocities and current intensities of 1.06 - 4.12 m d^{-1} and 40 - 80 mA respectively (Fig. 3). Total removal of nitrate was achieved during most of the time (Fig. 3a). Breakthrough of significant concentrations of nitrate (up to 11 mg N l^{-1}) occurred only on days 32-69, while the water velocity was raised from 1.45 to 4.12 m d^{-1}, at 50 and 80 mA current intensities (Fig. 3 a and b).

In general, the concentrations of nitrite in the effluent were low (Fig. 3a). The highest breakthrough of nitrite, 7 mg nitrite-N l^{-1}, occurred during the start up period as the water velocity was raised to 3 m d^{-1}. The highest rate of denitrification achieved was 0.18 kg N m^{-3} d^{-1} when 22 mg N l^{-1} were removed in 1.4 h (Fig. 3a and b).

Denitrification had little effect on the water pH, and it increased the number of bacteria (CFU) by approximately 10^2, from the order of 10^3 in the influent to the order of 10^5 ml^{-1} in the effluent (results not shown).

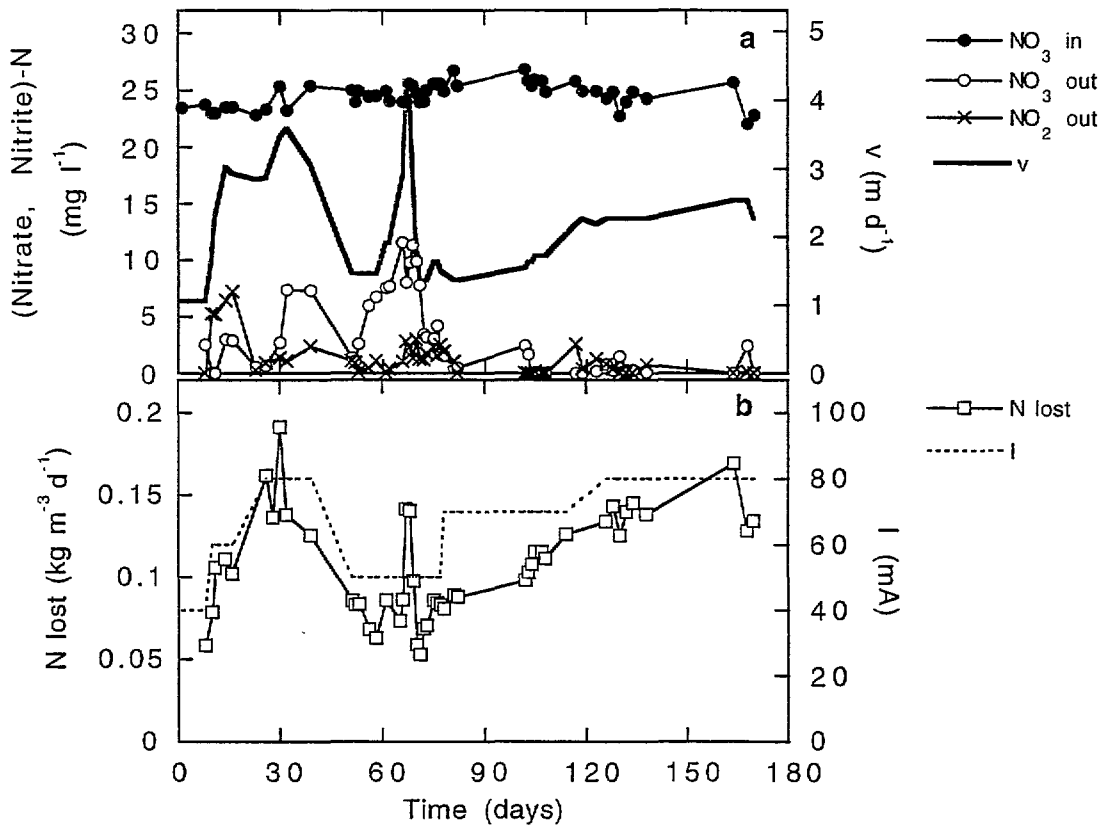


Figure 3. Rates of denitrification and changes in concentrations of nitrate and nitrite in the EC+BR system operated at various water velocities and current intensities.

The ECBR system ran for three months at current intensities of 20 and 40 mA, while the water velocity varied from 1.61-3.43 m d^{-1} in order to allow the breakthrough of at least 5 mg nitrate-N l^{-1} (Fig. 4). Nitrite was usually not detected in the effluent, and only once was its concentration above 0.5 mg N l^{-1} (Fig. 4a). The highest rates of denitrification achieved were around 0.45 kg N m^{-3} d^{-1} (Fig. 4b, days 25 - 40), when a

retention time of only 17 min was needed for the removal of 18 mg N l^{-1}. However, this was followed by an increase in nitrate washout and a decline in the removal rates which remained close to 0.25 kg N m^{-3} d^{-1} until the end of the experiment (Fig. 4b). In contrast to what was observed before, changes in water velocity had little effect on the overall performance of the system. The deterioration observed after day 40 appeared to be due to the formation of scale which by the end of the experiment was found to cover the cathode and bind the GAC particles in a hard block in the lower half of the bed. This reduced the effective surface area of the cathode, diminished the biofilm area and limited mass transfer. Changes in pH and bacterial counts in the effluent water were in the order of magnitude of those in the EC+BR system.

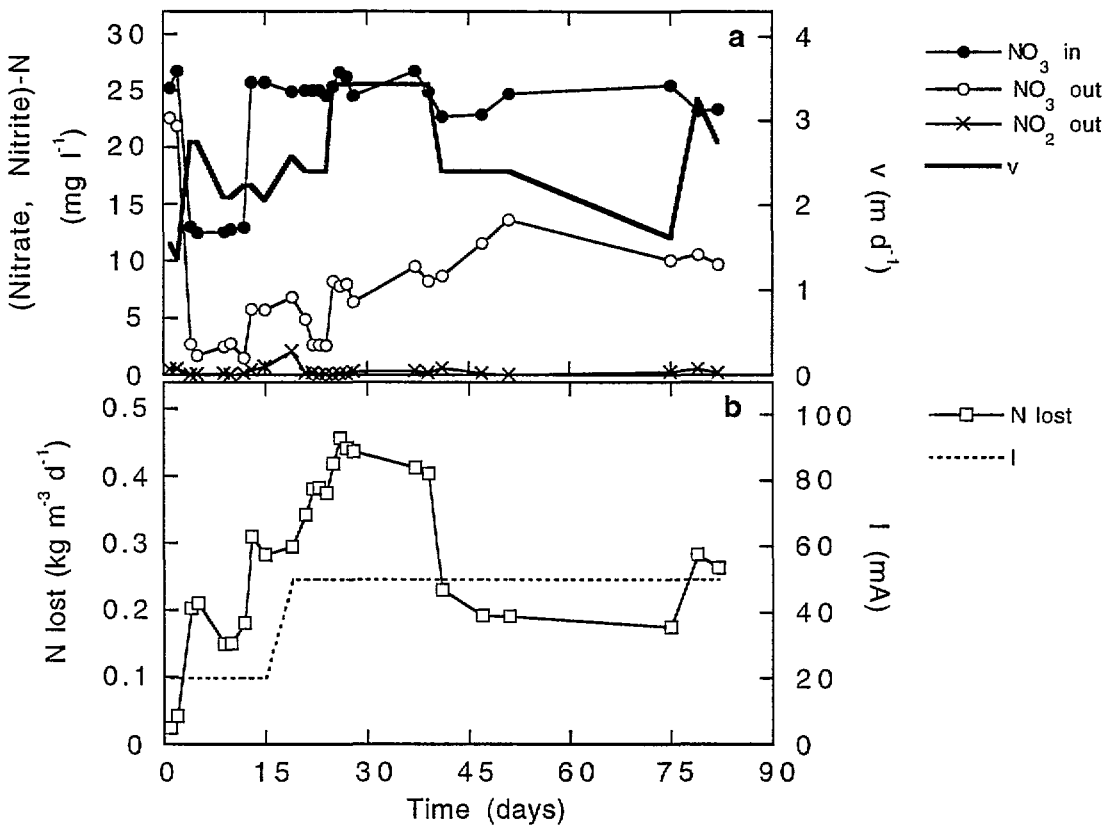


Figure 4. Rates of denitrification and changes in concentrations of nitrate and nitrite in the ECBR system operated at various water velocities and at current intensities of 20 and 40 mA.

DISCUSSION

The first objective of this study was to determine the feasibility of our basic design of a bio-electrochemical denitrification process for potable water. The second objective was to compare the efficiency, stability and convenience of operation of two configurations, ECBR and EC+BR, with a view to future upscaling. Work is still in progress in order to determine (under conditions of oversupply of nitrate) the effects of water velocity and current intensity on the rates of denitrification; the results obtained will allow the optimization of each system and the well grounded comparison between them. However, the preliminary studies presented above have already yielded important information.

Both systems required short start-up periods. Elution of nitrite was usually negligible or undetected in the ECBR (Fig. 4a). Nitrite was detected more frequently in the EC+BR, albeit at low concentrations (Fig. 3a). Low accumulation of nitrite is an important consideration in drinking water denitrification. It is sufficient to lower the concentration of nitrate-N to 11.29 mg l^{-1} (WHO, 1984), but in practice breakthrough of relatively high concentrations of nitrate is frequently accompanied by high concentrations of nitrite.

As discussed above, with the exception of short periods between days 32 and 69, the EC+BR system operated under limiting supply of nitrate. During these stages a 22 mg N l^{-1} were removed in 1.4 h (Fig. 3), a residence time lower than those reported by Gros *et al.* (1986) and Kurt *et al.* (1987).

The ECBR system ran under non-limiting supply of nitrate, and its performance was closer to its true capacity, although the current intensity was not optimized. Denitrification rates of up to 0.45 kg N m^{-3} d^{-1} were measured when a residence time of 17 minutes only was required for the removal of 18 mg N l^{-1} (Fig. 4).

The overall performance of the ECBR appeared to be superior, but formation of scale on and around the cathode was a problem. In the EC+BR system, deposits accumulated on the cathode were not in direct contact with the biomass and could be easily removed by disconnecting the BR from the EC for a few minutes while flushing the cathodic compartment with a diluted acid solution. A possible strategy to prevent formation of scale in the EC+BR is to periodically disconnect the BR, and switch the polarity of the electrodes for a few minutes.

The EC is the most complex and expensive component, and increasing the volume of the bed will be more costly than using a small EC with a larger BR. This makes the EC+BR more suitable for upscaling.

In conclusion, the bio-electrochemical process tested is feasible, economical and simple. The overall quality of the denitrified water is good, requiring only desinfection, as is usual in other biological treatments of drinking water.

ACKNOWLEDGMENTS

This research was supported under Grant No. TA-MOU-C15-045, U.S.-Israel Cooperative Development Research Program, U.S. Agency for International Development.

REFERENCES

Aragano M. and Schlegel H. (1992). The mesophilic hydrogen-oxidizing (Knallgas) bacteria. In: *The Prokaryotes*, A. Balows, H. C. Trupper, M. Dworkin, W. Harder and K.-H. Schleifer (eds), vol 1, Springer-Verlag, New York, pp. 344-384.

Cataldo D. A., Haroon M., Schrader L. E. and Youngs V. L. (1975). Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Commun. Soil Sci. Plant Anal.* **6**, 71-80.

Dries D., Liessens J., Verstrate W., Stevens P., de Vos P. and Ley, J. (1988). Nitrate removal from drinking water by means of hydrogenotrophic denitrifiers in a polyurethane carrier reactor. *Water Supply* **6**, 181-192.

Gamble T. N., Betlach, M. R. and Tiedje, J. M. (1977). Numerically dominant denitrifying bacteria from world soils. *Appl. Environ. Microbiol.* **33**, 926-939.

Gros H. and Treutler K. (1986). Biological denitrification process with hydrogen-oxidizing bacteria for drinking water treatment. *Aqua* **5**, 288-290.

Gros H., Schnoor G. and Rutten P. (1986). Nitrate removal from groundwater by autotrophic microorganisms. *Wat. Supply* **4**, 11-21.

Kurt M., Dunn I. J. and Bourne J. R. (1987). Biological denitrification of drinking water using autotrophic organisms with H_2 in a fluidized-bed biofilm reactor. *Biotechnol. Bioengrg.* **29,** 493-501.

Sakakibara Y. and Kuroda M. (1993). Electric prompting and control of denitrification. *Biotechnol. Bioeng.* **42,** 535-537.

Schlegel H. G., Kaltwasser H. and Gottschalk G. (1961). Ein Submersverfahren zur Kultur wasserstoffoxydierender Bakterien: Wachstumsphysiologische Untersuchungen. *Arch. für Mikrobiologie* **38,** 209-222.

Standard Methods for the Examination of Water and Wastewater (1992). 18th edn, American Public Health Association / American Water Works Association / Water Environmental Federation, Washington, DC, USA.

WHO (1984). *Guidelines for Drinking Water Quality. vol. I, Recommendations.* World Health Organization, Geneva.

Zumft W. G. (1992). The denitrifying prokaryotes. In: *The Prokaryotes*, A. Balows, H. C. Trupper, M. Dworkin, W. Harder and K.-H. Schleifer (eds), vol 1, Springer-Verlag, New York, pp. 554-582.

Appendix B

HYDROGEN-DEPENDENT DENITRIFICATION IN A TWO-REACTOR BIO-ELECTROCHEMICAL SYSTEM

SZILVIA SZEKERES, ISTVAN KISS, T. TONY BEJERANO AND M. INÊS M. SOARES*

Department of Environmental Hydrology and Microbiology, The Jacob Blaustein Institute for Desert Research, Ben-Gurion University of the Negev, Sede Boqer 84990, Israel

*Author to whom all correspondence should be addressed

Tel : (972)-7-6596834

Fax : (972)-7-6596831

E-mail: soares@bgumail.bgu.ac.il

Running head: Hydrogen-dependent denitrification

Abstract

An autotrophic biological process was developed for the treatment of nitrate-contaminated drinking water. The system comprised two steps: the water to be treated was first enriched with hydrogen (energy source) in the cathodic chamber of an electrochemical cell, and then denitrified in the bioreactor. The bioreactor was a packed bed of granulated activated carbon, and the water flow was directed in an upwards continuous mode. The system was operated for one year, at various water velocities and current intensities. Denitrification rates of up to 0.25 kg N m^{-3} d^{-1} were obtained at the hydraulic residence time of 1 h. The system was stable. When detected, breakthrough of nitrite was low, even under conditions which resulted in the elution of very high concentrations of nitrate.

Key words : biological denitrification, drinking water, water treatment, hydrogen, water electrolysis.

INTRODUCTION

Biological denitrification is a mechanism by which certain bacteria use nitrate as terminal electron acceptor in their respiratory process, in the absence of oxygen. Denitrification consists of a sequence of enzymatic reactions leading to the evolution of nitrogen gas. The process involves the formation of a number of nitrogen intermediates and can be summarized as follows:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \qquad (1)$$

Denitrifying bacteria are ubiquitous in nature (Gamble *et al.*, 1977; Zumft, 1992), and biological denitrification treatment consists of the provision of suitable carbon and energy sources which may be organic or inorganic compounds. Hydrogen gas is one of the possible sources of energy for denitrifying bacteria although growth under autotrophic anaerobic conditions is thought to be restricted to *Paracoccus denitrificans* and closely related strains (Aragano and Schlegel, 1992)

Hydrogen gas is an ideal energy source for biological denitrification in the sense that it is completely harmless to potable water, and no further steps are required to remove either

excess substrate or its derivatives. However, H_2 forms flammable and explosive mixtures with air, and is poorly soluble in water (1.6 mg l^{-1} at 20°C), properties which have limited its use in water treatment.

A number of denitrification systems have been described in which H_2 was sparged in the water to be treated. Kurt *et al.* (1987) studied a bench-scale fluidized bed sand reactor in which a residence time of 4.5 h was required for complete denitrification of water containing 25 mg nitrate-N l^{-1}, at rates of up to 0.55 kg N m^{-3} d^{-1}. Dries *et al.* (1988) tested a two-column system with removal of nitrate in the first column using polyurethane as support medium, and removal of excess hydrogen and oxidation of residual nitrite to nitrate in the second column. Water flowed downwards in the first column while hydrogen entered from the bottom; the water then passed through the second column in a upflow mode. Denitrification rates of 0.5 kg N m^{-3} d^{-1} were obtained, at 20°C.

A full-scale process known as DENITROPUR was developed by various authors and operated in Monchengladbach, Germany (Gros and Treutler, 1986; Gros *et al.*, 1986). The process incorporated a hydrogen saturator, addition of phosphate and carbon dioxide, a number of packed-bed reactors in series, post-aeration, floculant addition, filtration, and UV filtration; the reactor operated at a

loading rate of 0.25 kg N m^{-3} d^{-1} and residence times of 1 to 2 h were required to remove 11.29 mg N l^{-1}.

More recently Sakakibara and Kuroda (1993) reported a different approach: H_2 was generated in the denitrification reactor by electrolysis of the water to be treated. A batch system with two interconnected reactors, cathodic and anodic, was used. Prior to start up, a biofilm was allowed to develop on the surface of the cathode, by batch cultivation in a rich organic medium. When the biofilm was visible, the electrode was transferred to the reactor and connected to the power supply; the microorganisms immediately took up hydrogen generated at the cathode. In a follow-up study Sakakibara *et al.* (1994a) used a single-reactor continuous system consisting of a tubular cathode with a concentrical carbon rod (the anode); the biomass was immobilized with sodium alginate on the surface of the cathode. Approximately 10 mg N l^{-1} were removed at an applied electric current of 2.5 mA and a hydraulic retention time of 9 h (Sakakibara *et al.*, 1994a).

Recently, we carried out a preliminary laboratory comparison of two configurations of a bio-electrochemical continuous denitrification process: a single reactor where both the generation of hydrogen and denitrification took place, and a two-reactor system where water was enriched with hydrogen in an electrolysis cell (EC)

prior to entering a packed-bed bioreactor (BR) (Kiss *et al.*, 2000). Special design characteristics in both systems were: (1) a cation-permeable membrane separated the electrodes preventing O_2 generated at the anode from reaching the water to be treated while allowing transfer of H^+, and (2) granulated activated carbon (GAC) served as physical support for the biomass. Although the highest rates of denitrification per cubic meter of reactor were obtained in the single-reactor system, the two-reactor configuration was found to be the most suitable for upscaling (Kiss *et al.*, 2000). In the study presented here the long-term performance of the two-reactor system was evaluated and two of its main operational parameters, water velocity and current intensity, were optimized.

The net chemical reaction describing the hydrogen-dependent denitrification reaction is:

$$2\ NO_3^- + 5\ H_2 + 2\ H^+ \rightarrow N_2 + 6\ H_2O \qquad (2)$$

and the hydrogen is provided by the cathodic reaction:

$$10\ e + 10\ H_2O \rightarrow 5\ H_2 + 10\ OH^- \qquad (3)$$

Combining equations (1) and (2) and rearranging:

$$10\ e + 2\ NO_3^- + 12\ H^+ \rightarrow N_2 + 6\ H_2O \qquad (4)$$

Assuming that the sole anodic reaction is:

$$5\ H_2O \rightarrow 21/2\ O_2 + 10\ H^+ + 10\ e \qquad (5)$$

and that there is no separation between the anode and cathode (undivided electrochemical cell) then the net reaction is given by:

$$2\ NO_3^- + 2\ H^+ \rightarrow N_2 + 21/2\ O_2 + H_2O \qquad (6)$$

In this case, one equivalent of acid is needed for each mole of nitrate. If the EC is separated by a cation-exchange membrane and dilute sulfuric acid is used as an anolyte, then most of the current, about 95%, will be carried through the membrane by protons. Thus, similar demand for acid is expected in both the divided and undivided electroreactors, and only minor pH changes are expected at the concentrations of nitrate tested.

MATERIALS AND METHODS

Experimental set-up

The denitrification system is schematically represented in Fig. 1, and is similar to that described before (Kiss *et al.*, 2000). It consisted of an EC and a BR. In the EC, shown in detail in Fig. 2, a Nafion® 417 (Aldrich) cation-exchange membrane separated the two electrodes, and Hypalon rubber spacers (3 mm thick) intercalated between the membrane and the electrode created cathodic and anodic chambers. Plates of rubber (3 mm thick), PVC (1 cm thick) and stainless steel (1 cm thick), in this order, followed each electrode, and the different layers were held together by four stainless steel

bolts. The anode was cut from a 2 mm thick plate of titanium coated with platinum-iridium oxide, and the cathode was made from a 5 mm thick graphite plate. The electrodes were connected to a Zico DC power supply (model Zi 6013) set at fixed current which was accurately monitored by means of a digital multimeter. The EC was kept in the vertical position, and peristaltic pumps regulated the flow of solutions: 0.1 N H_2SO_4 was recirculated in the anodic chamber and the feed solution (tap water amended with 0.29 g H_3PO_4 l^{-1}, 70 mg $NaHCO_3$ l^{-1} and nitrate) flowed through the cathodic chamber. The feed reservoir was usually sparged with N_2. The feed solution was pumped through the cathodic chamber of the EC where it was enriched with H_2 prior to entering the BR which was a glass column packed with pre-washed 0.85-1.70 mm matrix GAC. Further details of the system are summarized in Table 1. Experiments were conducted at 25-27 °C, and at various water velocities (v) and current intensities (I); a water velocity of 0. 1 m h^{-1} corresponded to a feed rate of 2.65 ml min^{-1}.

Bacterial inoculum

The bioreactor was inoculated with a mixture of four denitrifying strains isolated from a previous reactor. Cells were cultivated in nutrient broth for 48 h, harvested by centrifugation,

washed twice and resuspended in mineral medium (Schlegel *et al.*, 1961). The suspension was recirculated through the column for 3 days. The original inoculum was an enrichment culture (in mineral medium and under an atmosphere of 6% H_2 and 94% N_2) of sediment from an oxidation pond.

Analytical methods and bacterial counts

Nitrate was determined by the colorimetric method of Cataldo *et al.* (1975), and nitrite and ammonia were assayed according to Standard Methods (1992). Colony forming units (CFU) were determined by standard plating techniques on R2A agar (Difco Laboratories).

RESULTS AND DISCUSSION

The long-term performance of the denitrification system was assessed. After inoculation of the BR for 3 days in a recirculation mode, the set-up was operated in a continuous mode for one year, at approximately constant inlet nitrate concentration (21-27 mg N l^{-1}), and at various water velocities (v) and current intensities (I).

The start up period was short and 90% removal of nitrogen had already been reached after one week at v and I values of 0.04 m h^{-1}

and 40 mA respectively (Fig. 3a,b). Increasing *v* to 0.14 m h^{-1} and *I* 80 mA (days 10-30) had little effect on the breakthrough of nitrate and nitrite, showing that the system was operating under nitrogen limitation. On the other hand, the increase in nitrogen breakthrough observed on days 50-90 showed that hydrogen became the limiting factor at 50 mA and *v* around 0.06 m h^{-1}; latter on, hydrogen again became limiting at *I* 40-60 mA (Fig. 3a,b). The effect of *I* on denitrification was studied by maintaining *v* at 0.11 m h^{-1} while varying *I* between 40 and 100 mA, and the correlation between *I* and nitrogen removal is summarized in Fig 4. At 40 and 50 mA, most nitrate washed out from the BR (Fig. 4a). A marked effect of *I* was observed between 50 and 60 mA, when the rates of denitrification more than doubled, and a further increase (up to around 0.18 kg N m^3 d^{-1} occurred at 70 mA (Fig. 4b). The rate of denitrification was somewhat lower at 80 mA, and increasing the current to 90 and 100 mA appeared to have little effect on the overall amount of nitrogen removal. Nitrite in the effluent was either undetected or in very low concentrations as the current varied between 50 and 70 mA; at higher and lower currents, breakthrough up to 2-3 mg N l^{-1} was observed (Fig. 4a).

The reason for the decline in denitrification rates at the highest values of *I* tested is not clear. In reactors with the biomass

attached to the cathode, the decline of denitrification rates at relatively high currents was attributed to the forced migration of nitrate to the bulk liquid, brought about by the potential gradient (Sakakibara *et al.*, 1994b). However, this was not the case in our system where the biomass was not exposed to the electric field. At currents of 80-100 mA, gas bubbles were clearly visible at the EC outlet, and the concentration of H_2 in the gases evolved at the top of the BR increased from 70 mA up (results not shown). Thus a possible explanation for the decrease in denitrification rates observed at the highest currents tested is the presence of H_2 bubbles along the bioreactor. These bubbles may have caused channeling in the GAC bed (Soares *et al.*, 1991) and created dry patches where transfer of nitrate to the biofilm was affected.

Water velocity had a marked effect on the overall performance of the system. A study carried out at I 80 mA is presented in Fig. 5. The rates of denitrification increased with v until it reached 0.15 m h^{-1}, when approximately 0.23 kg N m^{-3} d^{-1} were removed with a breakthrough of nitrite below 1 mg N l^{-1} (Fig. 5b). This represented a loss of more than 85% of the original N with a hydraulic retention time of approximately 1 h. At higher water velocities, however, nitrate removal was drastically reduced.

Denitrification slightly increased the water pH (Fig. 3c), and increased the number of bacteria (CFU) by approximately 10^2, from the order of 10^3 in the influent to the order of 10^5 ml^{-1} in the effluent (results not shown).

Increasing *I* from 20 to 100 mA brought about a change in electrical potential of 1 V (from 2.3 to 3.3 V). The electrical energy used to remove approximately 20 mg nitrate-N l^{-1} (1 h contact time) at a current intensity of 70 mA and a potential difference of 3 V was 1.17 KWh m^{-3} water.

The denitrification system described here was simple and easy to operate. Maintenance requirements were limited to descaling of the cathode. The tap water was hard and a layer of calcium carbonate could gradually build up on the surface of the cathode. As a preventive measure, cleaning of the cathode was carried out once a week: the EC was disconnected from the BR for a few minutes during which the polarity of the electrodes was reversed or a diluted acid solution was flushed through the cathodic chamber.

The system was capable of sustaining the effect of the extreme settings of *v* and *I* tried throughout the year, and its performance was predictable and stable.

CONCLUSIONS

- The process presents the simplicity of operation of a plug-flow denitrification reactor. The use of H_2 as energy source does not require storage and sparging of gas and the risk of forming dangerous mixtures with air is eliminated.
- The system consists of two modules in series, electrochemical cell and bioreactor, a configuration which allows great flexibility of operation and simplifies maintenance.
- The process is economical. Since H_2 generation and biomass are separated, a small electrochemical cell (the most expensive component) can be combined with a relatively large bioreactor.
- The quality of the treated water is good: changes in pH are small and the concentration of nitrite is low, even in the presence of very high nitrate breakthrough.

Acknowledgments

This research was supported under Grant No. TA-MOU-C15-045, U.S.-Israel Cooperative Development Research Program, U.S. Agency for International Development.

REFERENCES

Aragano M. and Schlegel H. (1992). The mesophilic hydrogen-oxidizing (Knallgas) bacteria. In: *The Prokaryotes*, A. Balows, H. C. Trupper, M. Dworkin, W. Harder and K.-H. Schleifer (eds), vol 1, Springer-Verlag, New York, pp. 344-384.

Cataldo D. A., Haroon M., Schrader L. E. and Youngs V. L. (1975). Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Commun. Soil Sci. Plant Anal.* **6**, 71-80.

Dries D., Liessens J., Verstrate W., Stevens P., de Vos P. and Ley, J. (1988). Nitrate removal from drinking water by means of hydrogenotrophic denitrifiers in a polyurethane carrier reactor. *Water Supply* **6**, 181-192.

Gamble T. N., Betlach, M. R. and Tiedje, J. M. (1977). Numerically dominant denitrifying bacteria from world soils. *Appl. Environ. Microbiol.* **33**, 926-939.

Gros H. and Treutler K. (1986) Biological denitrification process with hydrogen-oxidizing bacteria for drinking water treatment. *Aqua* **5**, 288-290.

Gros H., Schnoor G. and Rutten P. (1986) Nitrate removal from groundwater by autotrophic microorganisms. *Wat. Supply* **4**, 11-21.

Kiss I., Szekeres S., Bejerano T. T. and Soares M. I. M. (2000). Hydrogen-dependent denitrification: preliminary assessment of two bio-electrochemical systems. *Wat. Sci.Technol.* (In Press).

Kurt M., Dunn I. J. and Bourne J. R. (1987) Biological denitrification of drinking water using autotrophic organisms with H_2 in a fluidized-bed biofilm reactor. *Biotechnol. Bioengrg.* **29,** 493-501.

Sakakibara Y., Araki K., Tanaka T., Watanabe T. and Kuroda M. (1994a). Denitrification and neutralization with an electrochemical and biological reactor. *Wat. Sci. Tech.* **30,**151-155.

Sakakibara Y., Flora J. R. V., Suidan M. T. and Kuroda M. (1994b). Modeling of electrochemically-activated denitrifying biofilms. *Wat. Res.* **28,** 1077-1086.

Sakakibara Y. and Kuroda M. (1993). Electric prompting and control of denitrification. *Biotechnol. Bioeng.* **42,** 535-537.

Schlegel H. G., Kaltwasser H. and Gottschalk G. (1961) Ein Submersverfahren zur Kultur wasserstoffoxydierender

Bakterien: Wachstumsphysiologische Untersuchungen. *Arch. für Mikrobiologie* **38,** 209-222.

Soares M. I. M., Belkin S. and Abeliovich A. (1991). Denitrification in laboratory sand columns: carbon regime, gas accumulation and hydraulic properties. *Wat. Res.* **25,** 325-332.

Standard Methods for the Examination of Water and Wastewater (1992). 18th edn, American Public Health Association / American Water Works Association / Water Environmental Federation, Washington, DC, USA.

Zumft W. G. (1992). The denitrifying prokaryotes. In: The Prokaryotes, A. Balows, H. C. Trupper, M. Dworkin, W. Harder and K.-H. Schleifer (eds), vol 1, Springer-Verlag, New York, pp. 554-582.

Table 1. Characteristics the of the bio-electrochemical denitrification system

Electrodes active area	Dimensions (cm)	9 x 5
Bed	Height (cm)	38.5
	Diameter (cm)	4.5
	Void Volume (cm^3)	270
	GAC (g)	550

Figures legends

Fig. 1. Schematic representation of the bio-electrochemical denitrification system.

Fig. 2. Schematic representation of the electrochemical cell.

Fig. 3. Changes in concentrations of nitrate and nitrite (a) and of pH (c), and percentage of nitrogen removal (b) in the denitrification system operated at various water velocities and current intensities.

Fig. 4. Effect of the electric current on the breakthrough of nitrate and nitrite (a), and on the rate of denitrification (b) nitrogen in the bioreactor operated at water velocity of 0.11 m h^{-1}.

Fig. 5. Effect of water velocity on the breakthrough of nitrate and nitrite (a), and on the rate of denitrification (b) in the denitrification system operated at the current intensity of 80 mA.

Fig. 1

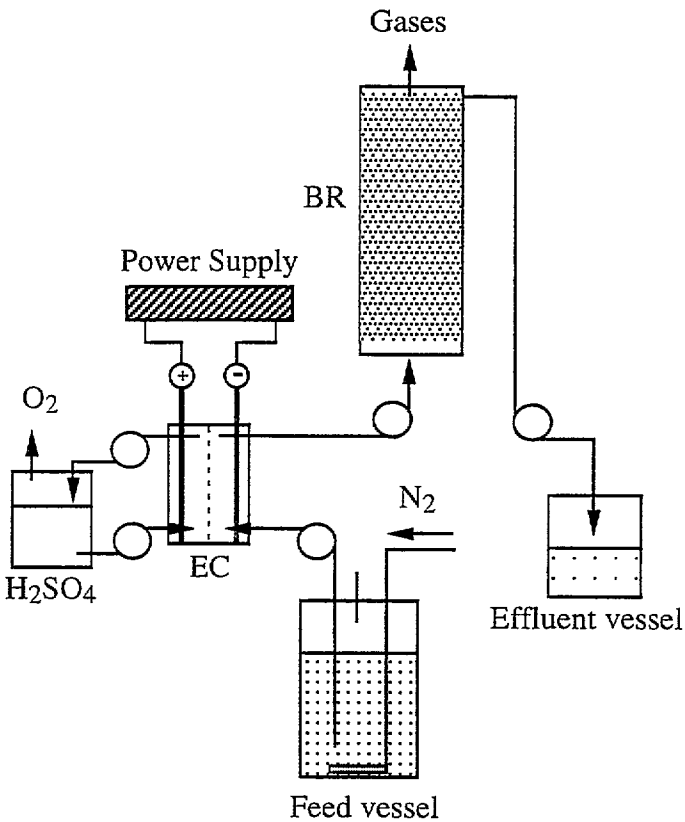

Gases
BR
Power Supply
+
-
O_2
N_2
EC
H_2SO_4
Effluent vessel
Feed vessel

Fig. 2

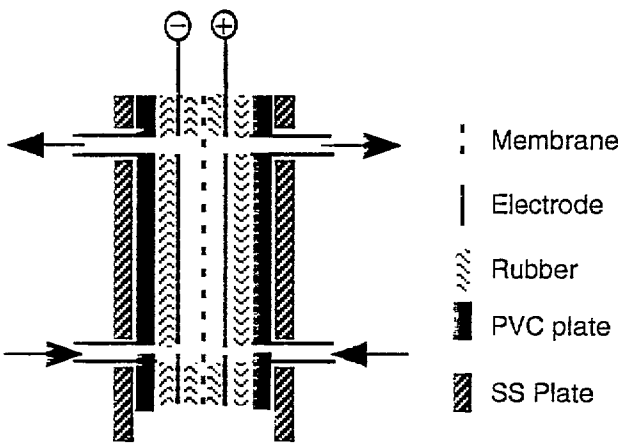


Fig.3

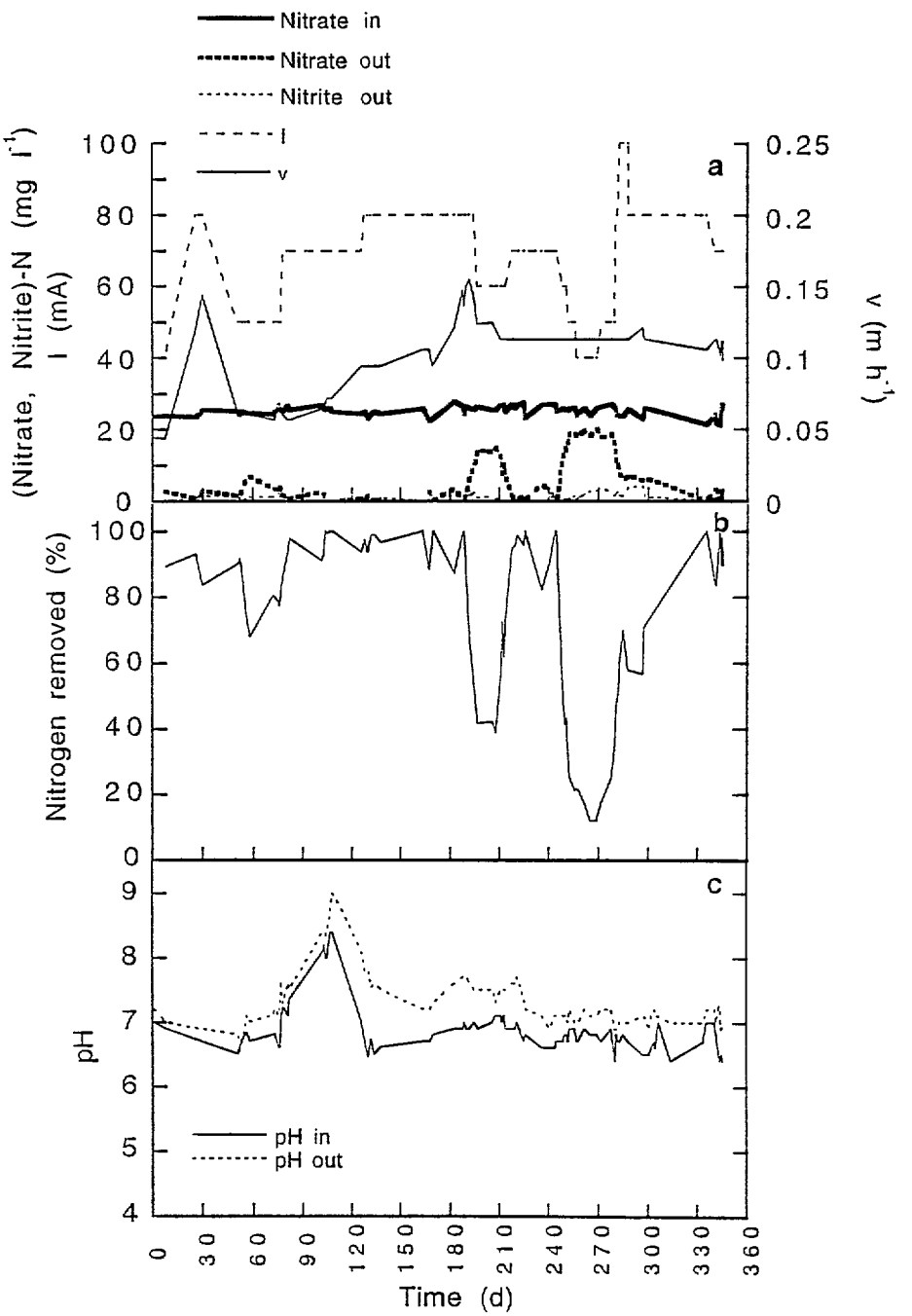

Nitrate in
Nitrate out
Nitrite out
I
v
a
b
c
(Nitrate, Nitrite)-N (mg l-1)
I (mA)
v (m h-1)
Nitrogen removed (%)
pH
pH in
pH out
Time (d)

Fig. 4

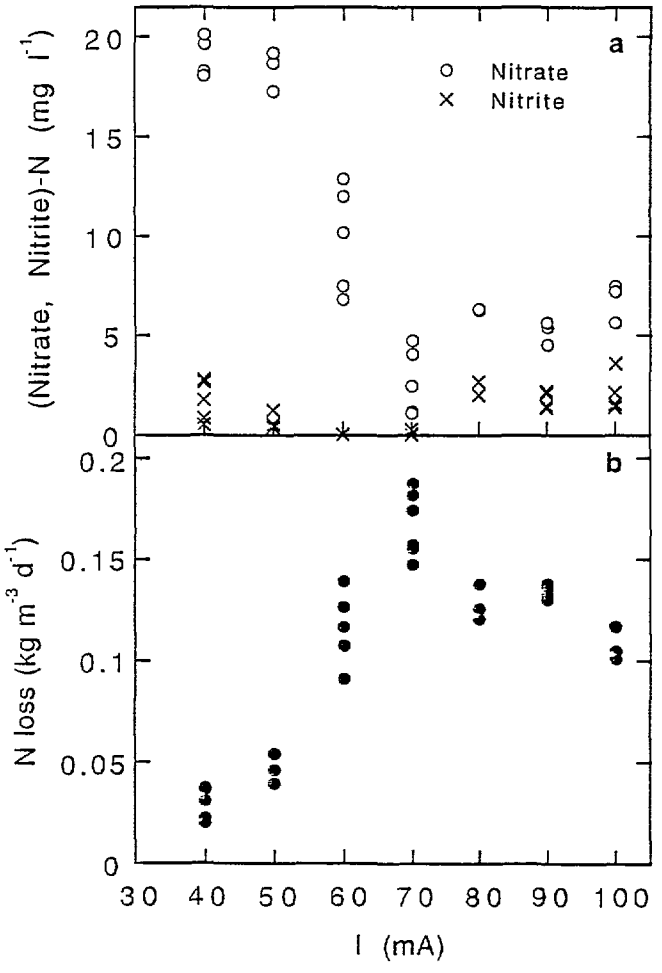
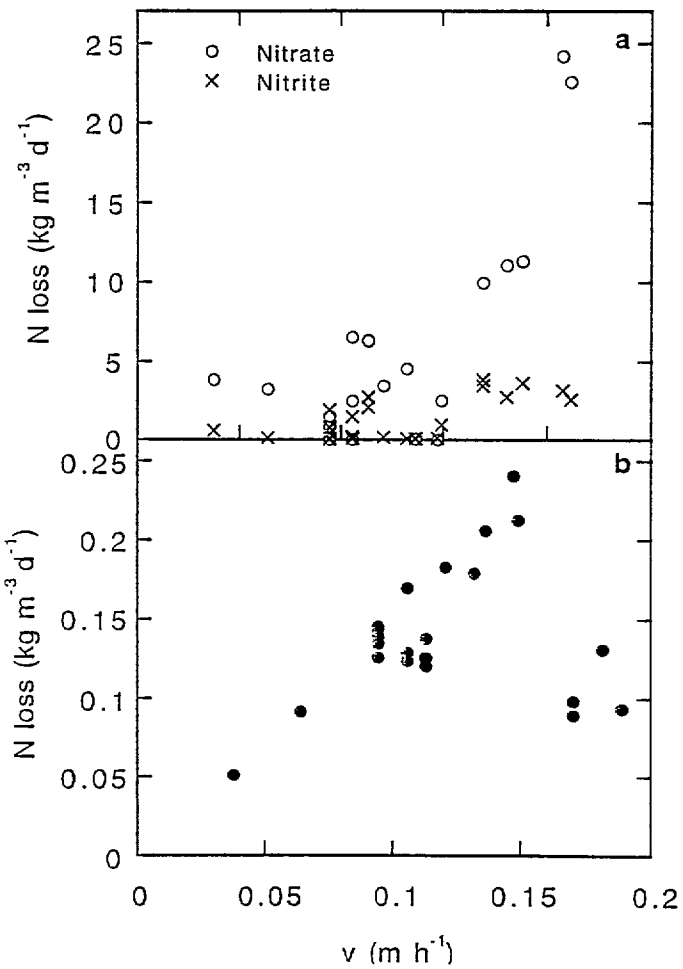

(Nitrate, Nitrite)-N (mg l-1)
20
15
10
5
0
a
Nitrate
Nitrite
b
N loss (kg m-3 d-1)
0.2
0.15
0.1
0.05
0
30 40 50 60 70 80 90 100
I (mA)

Fig. 5


Nitrate
Nitrite
a
b
N loss (kg m^{-3} d^{-1})
N loss (kg m^{-3} d^{-1})
v (m h^{-1})
0
5
10
15
20
25
0
0.05
0.1
0.15
0.2
0.25
0
0.05
0.1
0.15
0.2

Appendix C

Acta Microbiol. Immunol.
Submitted: January 26, 2000

A Combination of Chemical Removal and Biological Elimination of Nitrate

B. Polyák[3], P. Agócs[4], I. Kiss[3], K. Perei[2], M. I. M. Soares[5], P. Kesserű[3], Zs. Rácz[4], G. Rákhely[2], and K. L. Kovács[1-3]

[1]Department of Biotechnology, József A. University of Szeged, Hungary

[2]Institute of Biophysics, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

[3]Institute for Biotechnology, Bay Z. Foundation, Szeged, Hungary

[4]Nitrokémia Chemical Works, Inc., Fűzfőgyártelep, Hungary

[5] The Jacob Blaustein Institute for Desert Research, Ben Gurion University of the Negev, Sede Boqer, Israel

Running title: Water denitrification

Abstract

A novel type of nitrate selective ion exchange resin has been developed and combined with a bioreactor containing immobilized bacteria which can effectively convert nitrate to dinitrogen. In the bacterial system hydrogen is generated *in situ* by facultative anaerobic hydrogen producer microorganisms and it is safely and effectively delivered to the denitrifying bacteria. The hydrogen producers are fed by oligosacharides produced by co-immobilized cellulose and cellulolytic bacteria.

The purified water is not contaminated with the employed bacteria and the biotechnological system operates in a wide range of nitrate concentrations. Laboratory performance characteristics of an experimental biological regeneration column that treated 10 L brine solution (200 mg nitrate/L) per day with *in situ* hydrogen production have been studied.

Key words: denitrification, ion-exchange, microbiology, immobilized bacteria, cellulose, hydrogen

Introduction

Until 1945, nitrate in drinking water was considered to be entirely harmless to human beings; in that year it was recognized that a form of anaemia in infants was caused by nitrate in well water[1]. Oxygen deprivation due to nitrate intake may be lethal, primarily for infants. Continuous hypoxia has severe effects in adults, too. Since brain is the most sensitive organ demanding a constant supply of oxygen, brain damage, mental disorders, mental retardation, imbecility are the commonly observed diseases in the population that consumes nitrate contaminated water for an extended period of time [1,2]. High nitrate consumption is also harmful [3] because of the formation of nitrosamines which are known for their strong carcinogenic effect.

In 1970 the World Health Organization (WHO) set a standard of less than 50 mg/L nitrate as NO_3^- (11.3 mg/L as N) as a level above which health hazards might arise [4]. Accumulation of nitrate in groundwater directly correlates with the degree of human interference into the nitrogen cycle in the environment.

A combination of physico-chemical and biotechnological principles is presented in this study for treatment of dissolved nitrate. Due to the flexible nature of the system it is appropriate for nitrate elimination in a wide concentration range covering the needs of small settlements for drinking water and those of industrial wastewater purification. The results demonstrate the feasibility of an efficient and economical system based on the combination of ion-exchange and bioreactor techniques.

Materials and methods

Organisms and cultivation conditions. *Acetivibrio cellulolyticus* (DSM 1870) was grown anaerobically in a Bactron IV (Sheldon) anaerobic chamber. *Enterobacter cloaceae* (DSM 3264) was cultivated under heterotrophic conditions in Luria Broth. *Pseudomonas*

denitrificans (DSM 1650) was grown in a medium recommended by the supplier under semianaerobic conditions. Pure inocula were maintained on Petri dishes, biomass for immobilization was cultured in liquid media. Individual pure cultures were blended before use.

Ion exchange. For ion exchange, VARION BBM nitrate selective resin, a product of Nitrokémia Ltd. (Balatonfûzfõ, Hungary) was used. The material of the resin matrix, the type and synthesis of the functional groups are subjects of a pending patent application [Hungarian Patent P 9403029]. The resin was saturated with a solution containing 1 % (w/v) KNO_3 either in a batch mode on a shaker (250 rpm) at room temperature for two days or in a column mode by circulating the nitrate containing solution through a column filled with the resin by a peristaltic pump (200 mL/h). Bound nitrate was removed by a solution containing 3-5 % (w/v) NaCl or $NaHCO_3$ at a rate of 4 bed volume per hour. For the biological denitrification this solution was diluted 2-10 times with distilled water.

Immobilization of bacteria. The separately grown pure cultures were collected by centrifugation under non-sterile conditions (8,000 rpm, Sorvall 5 centrifuge, GSA rotor) at 10 °C and resuspended in small volume of 0.85 % (w/v) NaCl solution. The suspensions of the pure cultures were blended, centrifuged and rinsed with 0.85 % (w/v) NaCl solution. The final total cell count was adjusted to 10^9 cells/mL. This suspension was then mixed with an equal volume of 2 % (w/v) solution of Na-alginate (Protanal HF). The alginate solution was sterilized in 0.85 % (w/v) NaCl solution and 50 mg/L Na_2EDTA. An equal volume of separately sterilized 2 % (w/v) solution of Phytagel (Sigma) in 0.85 % (w/v) NaCl and 50 mg/L Na_2EDTA was mixed with the Na-alginate solution at 80 °C and with cellulose powder (5g/100mL, Sigma). The mixture was cooled to 40-45 °C before the addition of the bacteria. The suspension was immediately extruded through a glass vacuum filtering device funnel, fitted with a bank of Teflon tubes, into excess of 1 % (w/v) $CaCl_2$ solution [Hungarian patent

42

PCT/HU97/00047]. The hardened beads were then rinsed in distilled water and dropped into a solution of chitosan [5].

Reactor operation

The beads were stored at 4 °C in 1.2 % (w/v) $CaCl_2$ solution before filling into columns of 2.5-5 cm diameter. The flow direction was maintained against gravity by a multichannel peristaltic pump (Gilson) in order to avoid compression of the beads and free exit of gas bubbles. The columns were kept at ambient temperature during the operational period. Immobilization stress affected *A. cellulolyticus*, therefore, for the first few days of operation the columns were run on 100 mg/L KNO3 and 200 mg/L cellobiose mixture. Samples were taken from the effluent regularly for the analysis of nitrate, nitrite, and dissolved gas composition.

Analytical methods

Nitrate was determined as described by[6], nitrite and ammonia were assayed according to [7], and direct Nesslerization [8], respectively. The concentration of dissolved organic carbon and dissolved inorganic carbon were determined with a high temperature TOC analyser (Dohrmann DC-190, Rosemount Analytical Inc.,).

Nitrous oxide determinations were carried out with gas chromatograph (GOW-MAC) equipped with a thermal conductivity detector and Porapak Q (Supelco) column. The detector temperature was 50 °C and the flow rate of the carrier gas, helium, was 25 mL/min. The gas chromatograph was connected to a Hewlett-Packard 3390A integrator.

Hydrogen was measured in a Hitachi 263-50 gas chromatograph fitted with a 5 Å molecular sieve column and a thermal conductivity detector. Ultrapure nitrogen was used as carrier gas and the column and detector temperature was set to 120 °C.

43

Results

Ion exchange. The VARION BBM resin material had been developed by Nitrokémia Ltd., for this special purpose and was strongly selective for nitrate. It is evident from Figure 1 that it preferentially binds nitrate also in the presence of sulfate and this selectivity is retained regardless of the number of charging-regeneration cycles up to 20 cycles. In a separate study the performance characteristics of VARION BBM was compared with that of IMAC-HP-555 (Rohm and Haas), A-520 (Purolite), and SN-36-L (Wofatit). When measured against the ion exchange resins of the leading manufacturers, VARION BBM was superior in the nitrate to sulfate binding capacity ratio. It was similarly important that the levels of sulfate, chloride, and hydrocarbonate ions, which maintained the taste and quality of potable water, were only slightly changed by the ion-exchange treatment when compared to the performance of leading brand products (Figure 2).

Laboratory size ion exchange reactor volumes were in the range of 1 L, which allowed a water flow rate of about 35 L/h. The rinse flow rates were varied between 10-12 L/h and regenerant concentration of 30-50 g NaCl/L at pH around 8.0 was used. The process in its simplest form contained an ion exchange column producing potable water while the effluent from another ion exchange column was regenerated through a biological denitrification reactor (Figure 3). The stream of regeneration solution was filtered through an activated carbon filter in order to withhold any bacteria, cell debris or organic compounds and biopolymers excreted by the bacteria.

Biological denitrification. The system included two novel features, that, to our knowledge, have not been applied in denitrification technologies yet [Hungarian Patent PCT/HU97/00047]. We exploited mixotrophic growth conditions for denitrifying bacteria and the benefits of interspecies hydrogen transfer in a system using mixtures of well defined

44

pure bacterial cultures on the one hand and a novel immobilization technology on the other hand [5,9].

Separation of the energy production and denitrification and confinement of these tasks to distinct species allowed the use of a wide range of organic substrates. As an example, a cellulose based system is presented below in details (Figure 4). *Acetivibrio cellulolyticus* was selected as cellulolytic bacterium, which could grow on cellulose as well as on cellobiose. Cellulose was converted by these bacteria to end products such as reducing sugars, acetate, CO_2, and H_2. The cellulolytic microorganism was co-immobilized with cellulose, a fermentative hydrogen producer bacterium, e.g., *Enterobacter cloaceae*, which evolved hydrogen from reducing sugars and acetate obtained from the cellulolytic microorganisms, and a denitrifying bacterium. Alternatively, *A. cellulolyticus* could serve alone as an energy and reductant producer from cellulose, although the operational stability of these systems appeared less than the ones constructed of three bacterial strains. It was assumed that the extreme oxygen sensitivity of *A. cellulolyticus* is balanced by the co-immobilized facultative anaerobe, which consumed the oxygen before it could exert its toxic effect on *A cellulolyticus*.

From the hydrogen producer helper bacteria H_2 was delivered to denitrifying bacteria by interspecies hydrogen transfer. Because of the close spatial proximity, hydrogen was effectively transferred from the helper microorganisms to the denitrifying bacteria immobilized together with the H_2 producer species. Interspecies hydrogen transfer brought about synergistic effects for the denitrifying bacteria. From operational safety point of view *in situ* generation and utilization of hydrogen is evidently superior to bubbling the system with highly explosive hydrogen gas.

Immobilization of the participating bacteria in alginate or carragean are normally employed in whole cell immobilization because they are not toxic and are available at low cost [10, 11]. In

45

order to get rid of the disadvantages of alginate immobilization an improved version of the technology was employed [5]. The experimental bioreactor containing the immobilized microorganism population of three strains was self sufficient and operated for several months without failure.

Such a biological denitrification system has been found to be able to handle easily 1000-2000 mg nitrate/L (100 mmol $NaNO_3$/L) at a conversion rate of 10 mg NO_3^-/g bead/h. Nitrite was not detectable in the effluent of our experimental bioreactor even after continuous operation [9].

Discussion

Several methods have been attempted for the removal of nitrate from water [12], but only ion exchange and/or biological denitrification [9, 13, 14] offer a potential of practical solution to the problem. Ion exchange is the single effective technique used in full-scale treatment facilities to remove nitrate from drinking water [15, 16]. There are two major difficulties associated with this technique: the selectivity of the ion exchange resin for nitrate and the disposal of the waste brine solution containing the removed nitrate in a concentrated form. The resins appear to preferentially bind sulfate over nitrate. The performance of these ion exchange systems therefore depends on the sulfate concentration in water, which changes from location to location. The VARION BBM resin is superior in this respect to the leading brand ion-exchange materials.

The carbon filter extended the operational lifetime of the ion exchange resin, prevented its contamination and alleviated the need for the preparation of a new regeneration solution for each regeneration cycle. Thus the regeneration of ion exchange material could be performed in a closed circuit without creating excess amount of wastewater loaded with high concentration of salt.

Since denitrification is a respiratory process, an oxidizable substrate or electron donor is needed to drive the biological reduction of nitrate to nitrogen [4, 16]. A denitrification system may utilize *in situ* produced hydrogen, e.g., one species of an artificial microbial ecosystem produces hydrogen from added organic substrate (certain industrial wastewaters, sugars, or cellulose can serve as organic substrate). The advantage of employing heterotrophic hydrogen producers and mixotrophic denitrifying bacteria instead of the traditional heterotrophic denitrification is in the flexibility of the microbiological system. Denitrifying bacteria are most frequently found among heterotrophs. Some denitrifying bacteria are autotrophs, which utilize hydrogen or reduced sulfur compounds as reductants [10, 16, 17].

The technique as practised today in pilot plants consists of the use of denitrifying microorganisms in a fixed-film, packed bed, or biofilter arrangements [13, 14, 18-20].

Cellulose is an inexpensive and inert substrate and is available in practically unlimited quantity. Many fungal and bacterial cellulose degrading enzymes, cellulases, exist in complexes or are located on the surface of the microorganism [5, 21]. Alternatively, hydrogen can be evolved locally by water electrolysis on a special electrode surface harbouring the denitrifying bacteria on the electrode [22].

A general difficulty in biotechnological approaches is operational stability of the system. In addition, the excess biomass formed during the biological step needs to be disposed of. In packed-bed denitrification reactors trapped gases can also cause operational difficulties [23].

Entrapment of bacteria in a calcium alginate matrix is a widely used immobilization technology [24-26]. There are, however, a few disadvantages of the commonly used alginate method as applied for water denitrification. First, the immobilization is simple to carry out, but the beads remain stable only in the presence of relatively high concentration of calcium ions (30-100 mM, depending on the concentration of other divalent cations in the water), which are needed to stabilize the immobilization matrix. Second, the bacteria can grow and multiply in the matrix which leads to biomass leaking into the environment. Application of a separate coating layer is advantageous [5].

The possibility to combine ion exchange with biological denitrification, and several benefits of such combined systems, has been recognised by van der Hoek and coworkers [13, 14, 20]. They used commercially available ion-exchange resin which appeared not selective enough for nitrate and the biological denitrification system was based on using an upflow sludge blanket reactor. Stable operation of such a poorly defined biological system is difficult to maintain and due to varying nitrate load of the bioreactor, nitrite accumulation can occur. The

48

system presented here eradicate most of the above difficulties and a combination of ion exchange and biological denitrification becomes functionally feasible.

In conclusion, the following new elements have been included in an improved experimental denitrification system:

1. A new ion-exchange resin that was truly nitrate selective had been developed and incorporated into the system.

2. The biological phase was based on using mixotrophic denitrifying bacterial metabolism. The bacteria were supplied with acetate (carbon source) and hydrogen (reductant source). Thus the dosage and control of organic substrate as well as the clean up of residual organic contaminants in drinking water could be eliminated. In addition, undesired excess biomass formation did not complicate system operation.

3. Biologically produced hydrogen was used as reductant and the benefits of interspecies hydrogen transfer was exploited instead of supplying reductant for denitrification in the form of hydrogen gas. To accomplish this task, well defined and properly managed mixtures of co-immobilized pure strains of bacteria were employed.

4. The bacteria used in the process were immobilized with a novel technique: essentially sterile fermentation conditions were maintained at low cost, which allowed a strict control of the microbiological events. Further, because of the close spatial proximity, hydrogen was effectively transferred from the helper microorganisms to the denitrifying bacteria immobilized together with the hydrogen producer species.

Immobilization of the participating bacteria in beads of high physical resistance provided improved performance due to the dramatically increased bacterial population density, and high flow rate. The technique employed a substantially elevated concentration of active cells without invoking the difficulties encountered by high biomass production in the system.

Precise control of biomass and flexible adjustment to operational loading demands within the bioreactor were routine tasks if immobilized cells were used.

5. Activated carbon filters were incorporated into the system. As extra safety devices these filters trapped the bacteria which might accidentally escape from the bioreactor and thus safeguarded the ion exchange resin from biological contamination. The activated carbon filters also bound any organic material released by the bacteria, e.g., enzymes, metabolic by-products, etc., and rendered costly special disinfecting measures unnecessary.

References

[1] Kostraba JL, Gay EC, Rewers M, Hamman RF. 1992. Nitrate levels in community drinking waters and risk of IDDM. Diabetes Care **15**:1505-1508.

[2] Kleinjans JCS, Albering HJ, Marx A, van Maanen JMS, van Agen B, ten Hoor F, Swaen GMH, Mertens PLJM. 1991. Contamination of drinking water: evaluation of genotoxic risk in human populations. Environ Health Perspectives **94**, 189-193.

[3] Fandrem SI, Kjuus H, Andersen A, Amlie E. 1993. Incidence of cancer among workers in a Norwegian nitrate fertilizer plant. British J Ind Med **50**:647-652.

[4] Hochstein LI. 1988. The enzymes associated with denitrification. Ann Rev Microbiol **42**:231-261.

[5] Martins dos Santos VAP, Vasilevska T, Kajuk B, Tramper J, Wiffels RH. 1997. Production and characterization of double-layer beads for coimmobilization of microbial cells. Biotechnol Ann Rev **3**:227-244.

[6] Cataldo DA, Haroon M, Schrader LE, Youngs VL. 1975. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Commun Soil Sci Plant Anal **6**:71-80.

[7] Snell FD, Snell CT. 1949. Colorimetric methods of analysis. D. van Nostrand Co., New York. pp. 802-807.

[8] APHA 1992. Standard methods for the examination of water and wastewater, 18th ed. American Public Health Association. Washington, D.C.

[9] Kovács KL, Polyák B. 1992. Hydrogenase reactions and utilization of hydrogen in biogas production and microbiological denitrification systems. Gas, Oil, Environ. Biotechnol. Proc. IV PAP Int. Symp., pp. 445-459.

51

[10] Kokufuta E, Shimohashi M, Nakamura I. 1988. Simultaneously occurring nitrification and denitrification under oxygen gradient by polyelectrolyte complex-coimmmobilized *Nitrosomonas europea* and *Paracoccus denitrificans* cells. Biotechnol Bioeng **31**:382-384.

[11] Sanchez JM, Arijo S, Munoz MA, Morinigo MA, Borrego JJ. 1994. Microbial colonization of different support materials used to enhance the methanogenic process. Appl Microbiol Biotechnol **41**:480-486.

[12] Knowles, R. 1982. Denitrification. Microbiol Rev **46**:43-70.

[13] van der Hoek JP, Klapwijk A. 1988a. The use of nitrate selective resin in the combined ion exchange/biological denitrification process for nitrate removal from ground water. Water Supply **6**:57-62.

[14] van der Hoek JP, Griffioen A, Klapwijk A. 1988b. Biological regeneration of nitrate-loaded anion-exchange resins by denitrifying bacteria. J Chem Technol Biotechnol **43**:213-222.

[15] Kurt, M, Dunn IJ, Bourne JR. 1987. Biological denitrification of drinking water using autotrophic organisms with H_2 in a fluidized-bed biofilm reactor. Biotechnol Bioeng **29**, 493-501.

[16] Zumft WG. 1997. Cell biology and molecular basis of denitrification. Microbiol Mol Biol Rev **61**:533-616.

[17] Robertson LA, Kuenen JG. 1984. Aerobic denitrification: a controversy revived. Arch Microbiol **139**:351-354.

[18] Lemoine D. Jouenne T, Junter G-A. 1988. Reduction of nitrate by *Pseudomonas putrefaciens* entrapped in composite agar layer/microporous membrane structures. Biotechnol Lett **10**:399-402.

[19] Lemoine D, Jouenne T, Junter G-A. 1991. Biological denitrification of water in a two-chambered immobilized-cell bioreactor. Appl Microbiol Biotechnol **36**:257-264.

[20] van der Hoek JP, Latour PJM, Klapwijk A. 1987. Denitrification with methanol in the presence of high salt concentrations and at high pH levels. Appl Microbiol Biotechnol **27**:199-205.

[21] Marvin-Sikkema FD, Richardson AJ, Stewart CS, Gottschal JC, Prins RA. Influence of hydrogen-consuming bacteria on cellulose degradation by anaerobic fungi. 1990. Appl Environ Microbiol **56**:3793-3797.

[22] Sakakibara Y, Kuroda M. 1993. Electric prompting and control of denitrification. Biotechnol Bioeng **42**:535-537.

[23] Soares MIM, Braester C, Belkin S, Abeliovich A. 1991. Denitrification in laboratory sand columns: carbon regime, gas accumulation and hydraulic properties. Water Res **25**:325-332.

[24] Kierstan M, Bucke C. 1977. The immobilization of microbial cells, subcellular organelles, and enzymes in calcium alginate. Biotechnol Bioeng **19**:387-397.

[25] Tosa T, Sato T, Mori T, Yamamoto K, Takata I, Nishida Y, Chibata I. 1979. Immobilization of enzymes and microbial cells using carragean as matrix. Biotechnol Bioeng **21**:1697-1709.

[26] Smidsrod O, Skjak-Braek G. 1990. Alginate as immobilization matrix for cells. TIBTECH **8**:71-78.

Corresponding author:

Prof. Kornél L. Kovács
Department of Biotechnology, JATE
Temesvári krt. 62
H-6726 Szeged,
Hungary
Tel: +36 62 432 434, +36 62 544 351

Fax: +36 62 544 352

E-mail: Kornel@nucleus.szbk.u-szeged.hu

Authors:

Dr. Béla Polyák, István Kiss, Péter Kesserű,

Institute for Biotechnology, Bay Z. Foundation,

H-6726 Szeged, Derkovits fasor 2, Hungary

Dr. Gábor Rákhely, Katalin Perei and Dr. Kornél L. Kovács

Department of Biotechnology, University of Szeged, and

Institute of Biophysics, Biological Research Center, Hungarian Academy of Sciences, H-6726 Szeged, Temesvári krt. 62, Hungary

Dr. Pál Agócs, and Zsuzsanna Rácz,

Nitrokémia Chemical Works, Inc., Fűzfőgyártelep, Hungary

Dr. M. Ines M. Soares,

The Jacob Blaustein Institute for Desert Research, Ben Gurion University of the Negev, Sede Boqer, Israel

Figure legends

Figure 1. **Performance characteristics of the VARION BBM ion exchange resin.** The resin was packed into a glass column and water containing 500 mg/L sulfate and 250 mg/L nitrate was passed through the column. The breakthrough curves are indicated by the open and filled dots, data were collected during 10 cycles of regeneration and loading. No change in the binding capacity was observed. The nitrate selectivity was apparent from the shift of breakthrough curves towards higher volumes.

Figure 2. **Ion exchange resin selectivity**. Comparison of VARION BBM [shaded columns] with IMAC-HP-555 (Rohm and Haas), A-520 (Purolite), and SN-36-L (Wofatit) [clear columns]. VARION BBM shows somewhat lower binding capacity than the leading brand ion exchangers. An improved binding capacity of sulfate and hydrocarbonate and decreased chloride binding contribute to the preservation of taste of drinking water. Binding capacity was expressed as the inflection point of the breakthrough curves using 20 mg/L of the given ion.

Figure 3. **Schematic illustration of the combined system.** The ion-exchange column (left hand side) efficently removes the contaminating nitrate providing potable water. The resin is regenerated in a separate step (right hand side) by salt wash. The effluent containing high concentration of nitrate is fed directly into the bioreactor. The bioreactor contains immobilized microbes utilizing either in situ generated hydrogen or organic source (sugar or polymeric sugar) for reductant.

Figure 4. **Performance of the biological regeneration column reactors.** Hydrogen is administered either externally (a) or produced *in situ* by coimmobilized bacteria (b). Nitrate is measured by its absorption at 216 nm in a flow-trough specrophotometer cell.

55

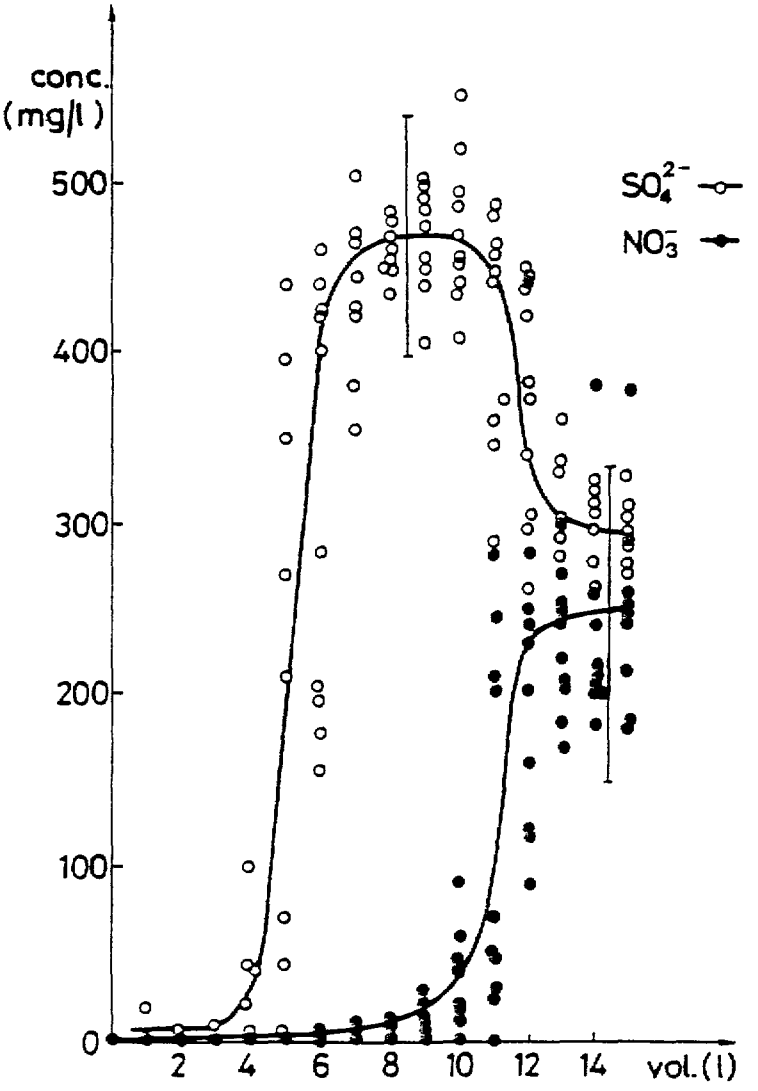


Figure 1. Polyak et al.

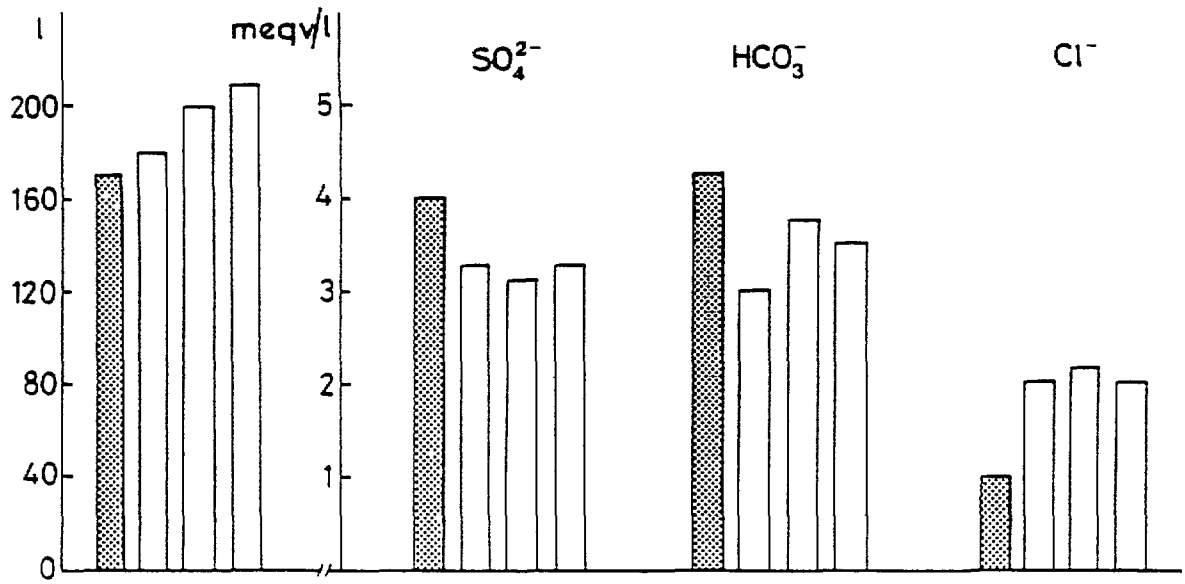


Figure 2. Polyak et al.

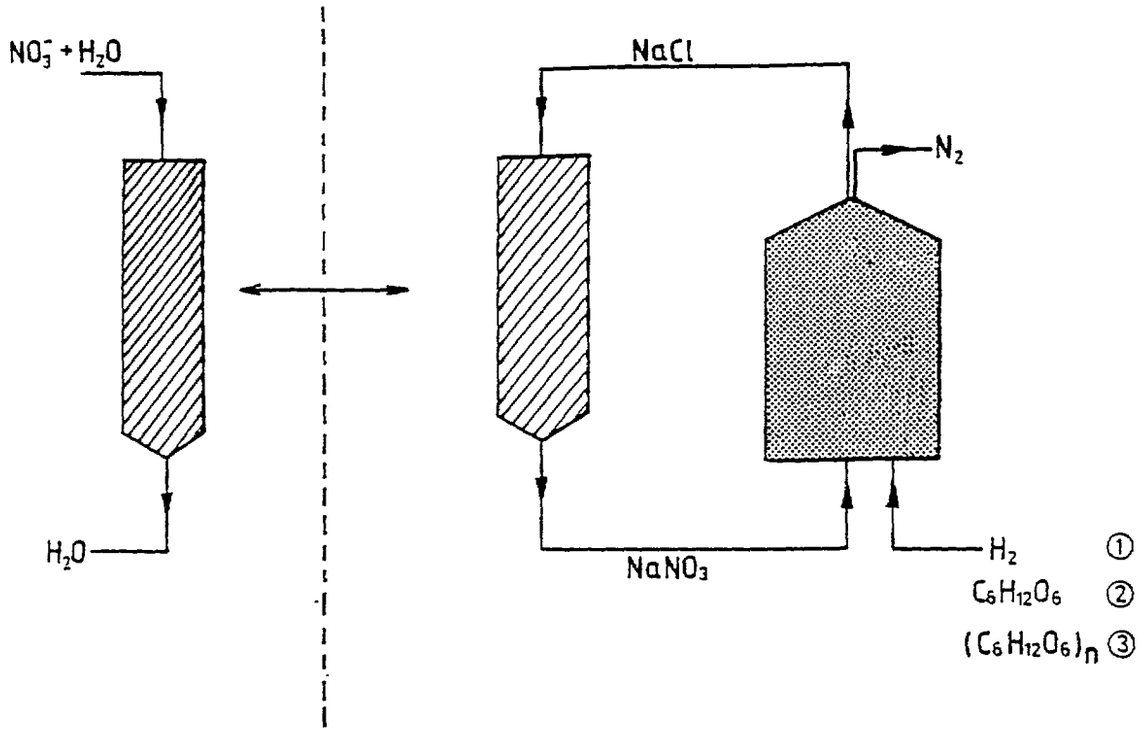


Figure 3. Polyak et al.

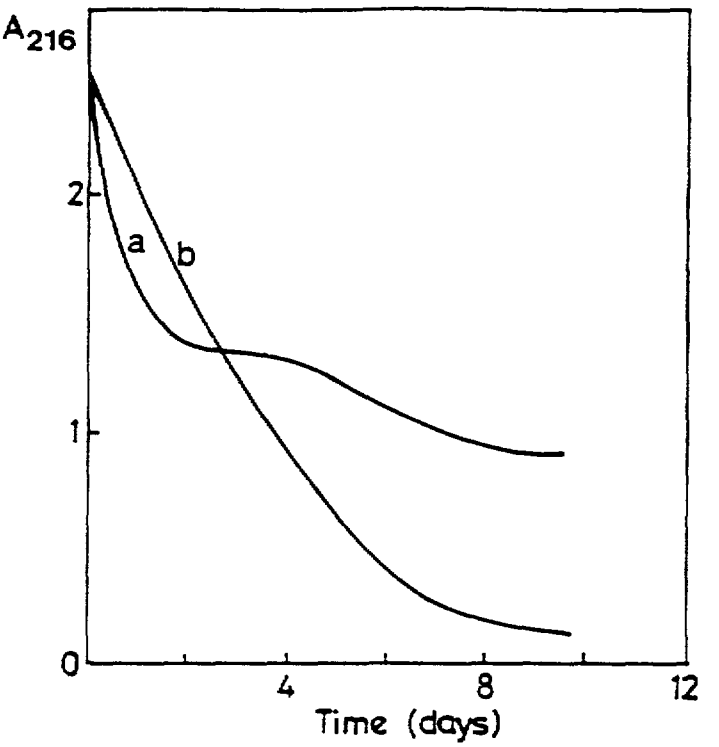


Figure 4. Polyak et al.