FINAL REPORT

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CELLULOSE-DEPENDENT BIOLOGICAL RESTORATION OF NITRATE POLLUTED AQUIFERS

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2) Table of Contents

Cover Sheet	page	1
Table of Contents		2
Executive summary		3
Research objectives		4
Methods and results		5
Impact relevance and technology transfer		13
Project activities/outputs		14
Project productivity		15
Future work		15
Literature cited		17

3) Executive Summary

The objective of this project was to develop a cost effective and simple microbial process for the removal of nitrate from contaminated drinking water by using cellulose-rich substrates as the sole carbon and energy source The project comprised three years of laboratory work and a final year of fieldwork in Hungary

In the laboratory, columns were packed with newspaper, wheat straw, corn husks or unprocessed short-fiber (low quality) cotton, inoculated and then continuously supplied with nitrate-amended tap water Various operational parameters (water velocity, amount of substrate, temperature) and the quality of the effluent water (bacterial numbers, concentration of nitrate, nitrite and dissolved organic carbon (DOC)) were studied in order to select the most adequate substrate for the field experiments The highest rates of denitrification were observed in the cotton reactors Second in efficiency was wheat straw although in this case the denitrification rates declined more rapidly with time Color and DOC washed out from fresh straw, but these could be removed by activated carbon, a process routinely used in most water plants

Cotton is not grown in Hungary, the Hungarian partners decided to use wheat straw in the field study The field reactor was housed in a small building adjacent to a private well in the village of Bugyi The performance of the field reactors was similar to that observed in the laboratory Close to the end of the project a new substrate, cellulose waste from a baby diaper factory, became available and was tried in the field This substrate supported the highest denitrification rates observed in the project

Bacterial isolates were studied in an attempt to understand the complex microbial processes taking place in the reactors No single organism was found which could carry out both degradation of cellulose and denitrification Cellulose-dependent denitrification was due to the combined action of different groups of organisms some bacteria degraded cellulose to simpler compounds which were in turn used by the denitrifying bacteria

4) Research Objectives

Nitrate contamination of drinking water is rapidly reducing the potable water resources in many parts of the world The process is particularly accelerated in developing countries where rapid demographic and economic changes are taking place and intensive human activity in fast growing urban settlements threatens the quality of the water supplies The establishment of intensive agriculture as well as the use of effluents in irrigation poses an increasing problem of nitrate contamination of the aquifers Excessive levels of nitrate in some countries have required the capping of drinking water wells which in turn forces people to resort to contaminated surface water Nitrate contamination of drinking water resources is a serious ecological problem Although it is generally accepted that the level of nitrate in drinking water should not exceed 11 29 mg nitrate-N per liter (WHO, 1984), this recommendation is often not implemented due to economic reasons In Hungary, serious nitrate contamination of drinking water affects *ca* 1000 settlements and hundreds of nitrate related methaemoglobinaemia (frequently fatal) have been reported in recent years Even so, denitrification of drinking water is not practiced and the population in affected areas is expected to resort to bottled water or to install long and expensive pipelines

The adverse health effects associated with nitrate in drinking water have led to the development of a number of nitrate removal technologies among which the microbial approach is considered to be the most economical and environmentally sound as well as feasible on a large scale

The microbial method relies on the capacity of certain bacteria, in the absence of oxygen, to use nitrate as a terminal electron acceptor in their respiratory processes This is achieved through a series of enzymatic steps (denitrification) and results in the reduction of nitrate to N_2 as follows

$$NO_3 \rightarrow NO_2 \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

Denitrifiers (bacteria capable of carrying out denitrification) comprise both heterotrophs and autotrophs Most biological denitrification processes are based on heterotrophic bacteria utilizing organic carbon in the form of a simple compound (e g , ethanol, methanol or acetate) However complex carbon sources such as cellulose-rich materials can also be used The capacity of cellulose to support water denitrification has been shown by Boussaid *et al* (1988) using field and laboratory reactors packed with wheat straw mixed with sand or with sand and maerl in the treatment groundwater, and by Avnimelech *et al* (1993) who observed removal of nitrate in wheat straw filters designed to remove particulate matter from effluents prior to their application in drip irrigation

Cellulose is a basic component of all plant materials and constitutes the most abundant renewable resource in the world It is a linear glucose polymer with hydrogen bonding between hydroxyl groups of neighboring parallel chains, and is organized in fibers in close association with lignin and hemicellulose (cotton is an exception being the purest form of naturally occurring cellulose) Microorganisms capable of degrading cellulose are widely distributed in nature and usually occur in mixed culture with organisms that degrade associated polymers Initial cellulose degradation requires direct physical contact between the enzyme molecules and the surface of the cellulose, and complete degradation depends on the concerted action of various enzymes (cellulases) which may act in synergism (Beguin and Aubert, 1994)

The overall objective of this project was to develop a system for the microbial removal of nitrate from contaminated drinking water by using cellulose wastes as the energy source This denitrification strategy is simple and cost-effective and, to the best of our knowledge, neither cotton nor cellulose waste from diaper factories have been used in water treatment These substrates are degradable and "clean" and after post-treatment (a process required after any biological treatment) the denitrified water will meet all the hygienic requirements The use of the carbon substrate as the sole physical support for bacterial growth is innovative and allows high filling ratio of the bulky substrate and simplifies packing/refilling of the reactors

5) Methods and Results

Below is a brief summary of the work carried out Most of the reactor studies carried out by the Israeli group have been published and full experimental details and discussion of the results obtained with newspaper (Volokita *et al*, 1996b), cotton (Volokita *et al*, 1996a) and straw (Soares and Abeliovich, 1998 Preprint) can be found in the papers attached Submitted (Volokita *et al*, 1988) or in preparation for publication (see Project Activities/outputs) are data from detailed studies carried out with bacteria isolated from the laboratory reactors

Methods

Laboratory experimental apparatus

In Israel the laboratory reactors were PVC columns (50/55 cm high and 8/10 cm dia) packed with wheat straw newspaper or unprocessed low-grade (short fiber) cotton In Hungary PVC columns (60 cm high and 8 cm dia) were also used and were packed with wheat straw, newspaper or corn husks The columns were inoculated with a small amount of matrix removed from an active denitrification column (the original inoculum was a mixture of forest and garden soils) The freshly packed columns were filled with feed solution (tap water amended with at least 22 6 mg nitrate-N l^{-1}

and 3 mg l^{-1} phosphate) which was recirculated for two days After this inoculation period, the reactors were started up (day 0) in an upflow mode Water velocities v ($v = Q/A$, where Q is the measured flow rate and A is the cross section of the column) were calculated in m h^{-1} or m d^{-1} Influent and effluent samples were tested for nitrate, nitrite, ammonia, pH, dissolved organic carbon (DOC) or COD (chemical oxygen demand) and bacterial counts Up to three columns were connected in series in some experiments with straw in Hungary, either to increase the contact time or as a convenient strategy to periodically replace spent substrate In Israel, additions of fresh straw were carried out at the inlet of the reactor

Laboratory adsorption experiments

The removal of dissolved organic carbon (DOC) by powdered activated carbon (PAC) was investigated in a preliminary experiment in which 20 g of fresh straw were soaked overnight at 4 °C in 3 l tap water Constant volumes of water containing 141 mg DOC l^{-1} were then supplemented with a series of known weights of PAC in glass Erlenmeyer flasks, and the slurries obtained were agitated at a constant stirring velocity (100 rpm) and temperature (25±1 °C) Prior to use, the PAC was oven-dried at 105 °C for 2 h and then cooled in a dessicator The procedure adopted for the treatment of column effluents consisted of 1 h contact with 4% PAC

Field pilot

The field pilot was operated in the village of Bugyi near Budapest and was housed in a small building adjacent to a private nitrate-contaminated well in a vegetable garden Raw water was first pumped from the well to a 1 m^3 reservoir (where it was amended with phosphate) and then through the reactor in an upwards mode Two substrates were studied in the field straw and cellulosic waste from a baby diaper factory Straw was packed into 3 PVC columns (40 cm high and 29 cm dia) which could be operated in series or in parallel The factory waste filled half of a cylindrical (125 cm high 29 cm dia) reactor which was mixed for a few seconds at 30 min intervals by means of an overhead stirrer The influent was pumped at the rate of 1m^3 d^{-1} (water velocity, 15 m d^{-1}) and the effluent was post-treated in a sand filter (60 cm high and 12 cm dia) and/or an activated carbon filter (60 cm high and 4 cm dia)

Analytical procedures

In Israel, nitrate was determined by the method of Cataldo *et al* (1975), nitrite and ammonia were assayed according to Standard Methods (APHA, 1989) and DOC was determined by a TOC analyzer (Dohrmann DC-190, Rosemount Analytical Inc , Santa Clara, CA, USA) In Hungary, nitrate and nitrite were determined with an ion chromatograph (Metrohom-690,

Metrohom, Herisau, Suitzerland), and ammonia and COD were assayed according to Standard Methods (APHA, 1989)

Bacterial counts

Colony forming units were counted in the reactor influent and the effluent by standard plating techniques on R2A agar (Difco Laboratories)

Detection of overall cellulase activity

A simplified method was developed (Volokita *et al*, 1988), based on the classical detection method in which bacteria with total cellulase activity form clearing zones in agar plates with ground filter paper (Kluepfel, 1988) Sterile lens paper (Schleicher & Schuell, Dassel, Germany, product no 2478) discs were carefully laid on the surface of the freshly inoculated plates which were then incubated at 30 °C Colonies of microorganisms with overall cellulolytic activity formed visible holes in the paper The perforations were usually observed within 3 to 7 days and could be made more conspicuous by dying the paper, on the plate or after its removal, with 0 2% Irgalan Black BGL 200% (Ciba-Geigy) in 2% acetic acid

Isolation of bacteria from the reactors

The methods used in the isolation, characterization and identification of the bacteria colonizing the reactors have been described in detail in a previous report (Annual report, 1997)

Batch cultures of Comamonas sp and Cellulomonas uda

Single cultures were grown overnight in a Luria broth and harvested in 250 ml sterile plastic bottles by centrifugation in a Sorval 5415 C centrifuge (15 min at 10,000 rpm for *Cellulomonas uda*, and 5 min at 10,000 rpm for *Comamonas* sp) The cells were washed twice and resuspended in minimal medium containing (w/v) 0 1% K_2HPO_4, 0 01% KH_2PO_4, 0 05 % of each KNO_3 and $MgSO_4(7H_2O)$, and 0 5 ml of sterilized microelements solution (Vishniac and Santer, 1957) Triplicate batches prepared in 250 ml Erlenmeyer flasks containing 120 ml of sterile minimal medium and 0 25 g of sterile medical (100% pure, absorbent) cotton wool were then inoculated with a suspension of *Cellulomonas uda*, of *Comamonas,* or with both The cultures and triplicate uninoculated controls were maintained at 27 °C in an anaerobic chamber (Forma Scientific Inc , Marietta, Ohio, USA) Samples were removed periodically, the cells were precipitated by centrifugation and aliquots of the supernatant were assayed for nitrate, nitrite, DOC and pH

Results

In laboratory experiments, the efficiencies of newspaper (Annual Report, 1996, Volokita *et al*, 1996b), unprocessed low grade cotton (Volokita *et al*, 1996a), wheat straw (Annual Report, 1996, Soares and Abeliovich, 1998), and corn husks (Annual Report, 1996) were assessed with the objective of selecting the most suitable substrate for the field trial to be carried out in Hungary during the last year of the project A system was developed in which reactors were packed with the cellulose-rich substrate only, which functioned as carbon source as well as the sole physical support for microorganisms This design was chosen in order to (1) minimize clogging of the reactor due to entrapment of N_2 bubbles (a process more acute in fine matrixes) (Soares *et al*, 1989, 1991), (2) allow a high filling ratio of substrate (important when dealing with the bulky, non-diffusible carbon sources which were slowly, and in some cases incompletely, degraded), and (3) simplify packing of reactors and removal and disposal of spent substrate

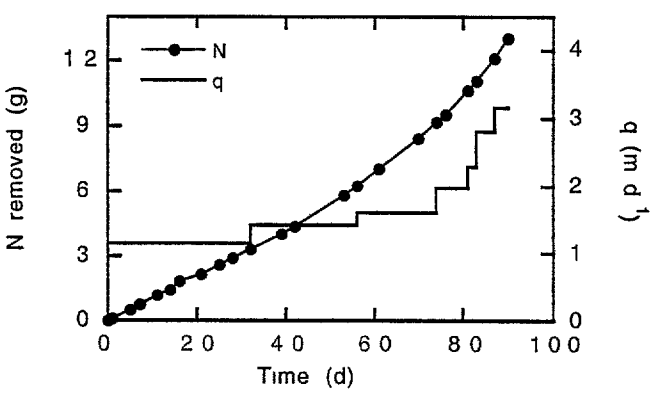

Fig 1 Removal of nitrogen in a column packed with 184 g of unprocessed cotton and operated at various flow rates (Volokita *et al* (1996a)

Among the substrates tested in the laboratory cotton was by far the most efficient it was completely degraded supported the highest denitrification rates and the treated water never showed color or high concentrations of dissolved organic carbon (DOC)(Volokita *et al* 1996a) The denitrification rates observed with newspaper, wheat straw and cotton were in the order of 0 037, 0 048 and 0 215 kg N removed $m^3 day^{-1}$ However the latter was an underestimation of the capacity of the cotton-packed reactor since it represents the last stages of the experiment illustrated in Fig 1, when the initial weight of cotton had decreased by 60%, and the bed had been reduced to the top third of the column with the lower two thirds occupied by water only (Volokita *et al*, 1996a)

In the experiment presented on Fig 1 the flux was varied so that an approximately constant breakthrough of nitrate was maintained Colonization of the substrate by bacteria was slow, and this is reflected in the lower rates of denitrification observed during the first half of the experiment Cotton was entirely consumed, and its replacement strategy should consist of small and relatively frequent additions This will assure consistently high rates of denitrification and will minimize reduction of activity during the initial stages of bacterial colonization of fresh substrate Long-term continuous operation will not require emptying and disposal of waste or spent substrate

Table 1 Cost of cotton as electron donor for denitrification

Electron donor	Cost/kg ($)	kg electron donor needed to remove 1 kg NO_3 -N	Cost/kg NO_3 -N ($)
Methanol	0 70[a]	1 9[c]	1 33
Acetate	1 67[a]	2 7[c]	4 37
Cotton (short fiber)	0 53[b]	2 8[d]	1 48

[a]Bulk price (Frutarom, 1995)
[b]Bulk price (Kibbutz Mishmar Haemek, 1995)
[c]Gomers (1987)
[d]Volokita *et al* (1996a)

In Table 1 the cost of cotton is compared to that of two simple carbon sources acetate and methanol Methanol is the cheapest simple organic carbon substrate for denitrification of

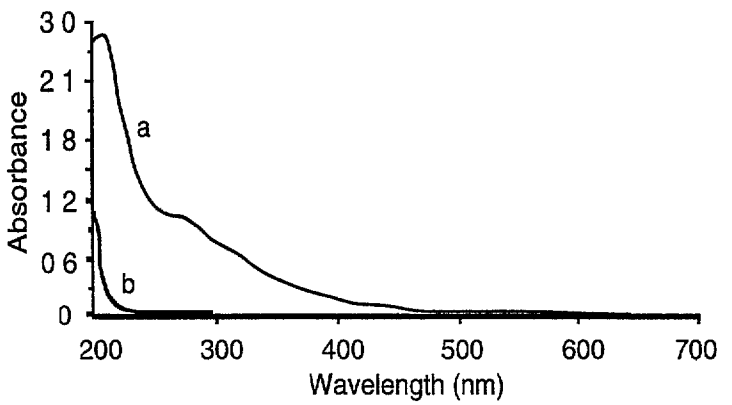

Fig 2 UV and visible spectra of effluent collected 4-6 h after the addition of 20 g of fresh straw to the reactor on Fig 3 (a) and of the same effluent after treatment with powdered activated carbon (b) (Soares and Abeliovich, 1998)

wastewater (its use in the treatment of drinking water is not permitted), and acetate is generally considered to be the lowest priced substrate for denitrification of drinking water The C/N ratio for cotton was calculated from the data in Fig 1 and, as mentioned above, those rates can be improved, even so, cotton comes out as a very attractive option, its cost being only one third that of acetate

Compared to cotton, wheat straw supported lower denitrification rates which deteriorated rapidly, it was less degradable (due to the presence of lignin, and other recalcitrant components) and when fresh brought about a temporary elution of color and high DOC although these could be removed by activated carbon (Fig 2) (Annual Report, 1996, Soares and Abeliovich, 1998)

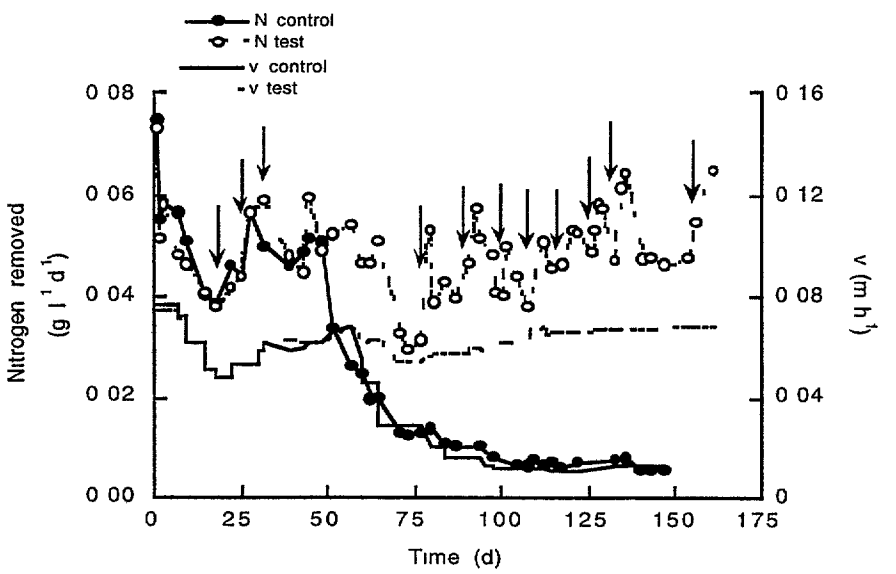

Fig 3 Removal of nitrogen in two reactors, control and test, packed with 177 g of wheat straw and operated at the flow rates indicated Arrows indicate the addition of 20 g of fresh straw (Soares and Abeliovich, 1998)

The decay in the performance of the reactors was prevented by periodic additions of fresh substrate (Fig 3) (Annual Report, 1996, Soares and Abeliovich 1998) Thus, if the strategy of substrate replenishment is optimized, wheat straw can support consistent denitrification rates for several months

Newspaper was the least efficient substrate tested Although the denitrified water was of good quality (e g , low nitrate and DOC), the rates of denitrification declined sharply while the weight of the substrate decreased only slightly (Volokita et al , 1996b) The physical

characteristics of the newspaper which has high lignin content appear to hamper the access of microorganisms to the cellulose, although pretreatment by heat (autoclave) or with diluted acid or base failed to improve the denitrification rates (Volokita *et al*, 1996b)

In spite of the advantages presented by cotton, the Hungarian partners decided that a long-term process based on cotton would not be practical in their country (where cotton is not cultivated) and opted for using wheat straw in the field pilot The performance of these field reactors was similar to that of the laboratory reactors, and no special difficulties or observations were found

During the last months of the field experiment the Hungarian partners came across a different substrate cellulose waste from a baby diaper factory The material appeared to present the advantages associated with cotton (e g , chemical homogeneity, absence of color and DOC) and was tested in a fluidized bed reactor

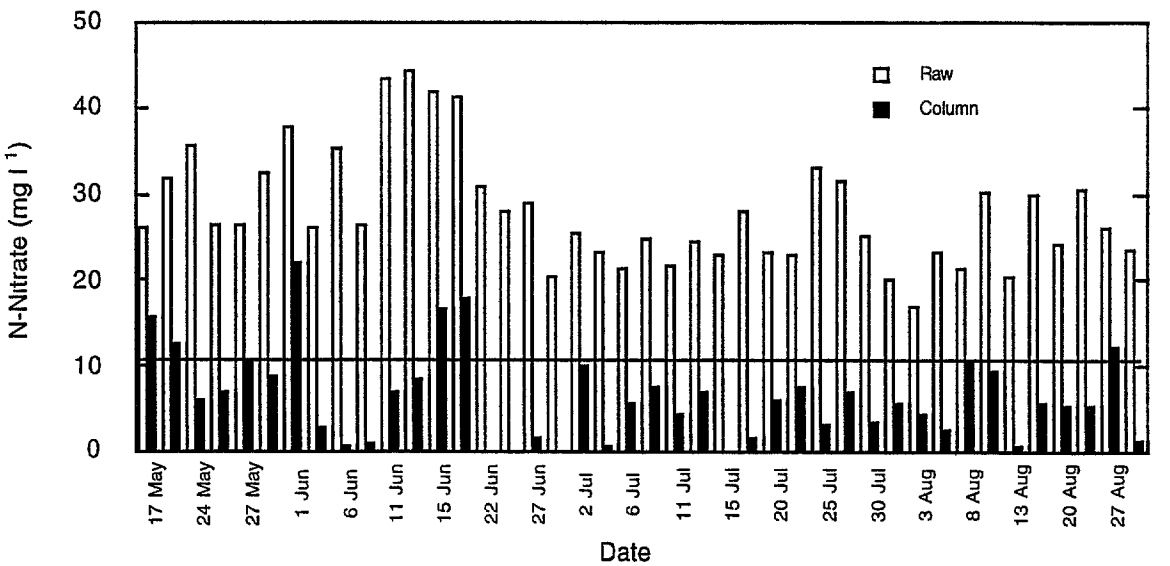

Fig 4 Concentrations of nitrate in the influent and effluent of the field reactor The horizontal line represents the WHO standard for nitrate in drinking water

The results obtained in this field study are summarized in Figs 4-6 While the concentration of nitrate in the raw water varied between approximately 21 and 44 mg N l^{-1}(the variation was due to meteorologic factors and to seepage of the reactor effluent into the well) the concentration of nitrate in the effluent rarely exceed the WHO standard (WHO, 1984) (Fig 4) Furthermore, post-treatment lowered the concentrations of nitrite (Fig 5) and organic carbon (Fig 6) in the denitrified water to levels acceptable in drinking water Denitrification rates of

up to 0 4 kg N m^{-3} d^{-1} were observed It should be stressed that at this late stage of the project none of the operational parameters was optimized and the performance of the system can most certainly be improved

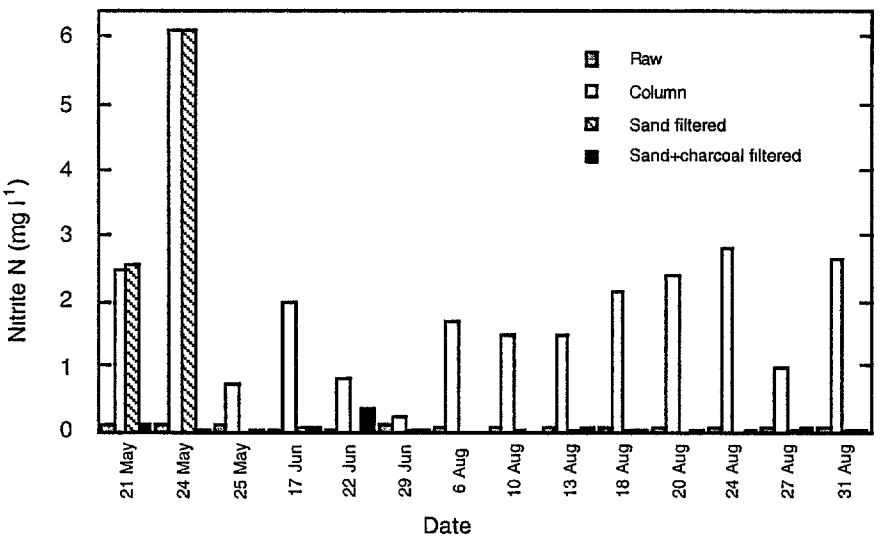

Fig 5 Concentrations of nitrite in the influent and effluent of the field reactor, and in the effluent after sand and charcoal filtration

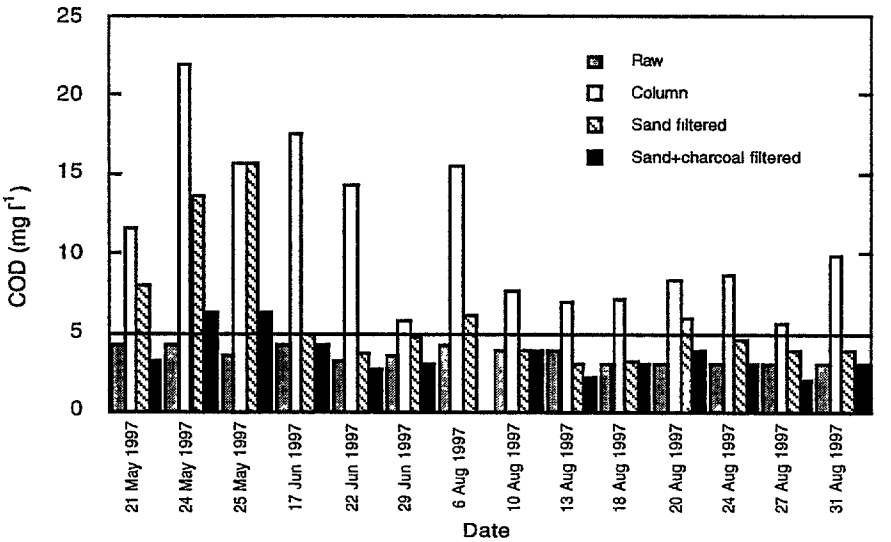

Fig 6 Values of COD in the influent and effluent of the field reactor and in the effluent after sand and charcoal filtration The horizontal line represents the Hungarian standard for drinking water

An interesting question in this project was to determine how the two relevant pathways, cellulose degradation and denitrification, were distributed in the biomass were there bacteria with both pathways or was the overall process the result of a concerted performance in which

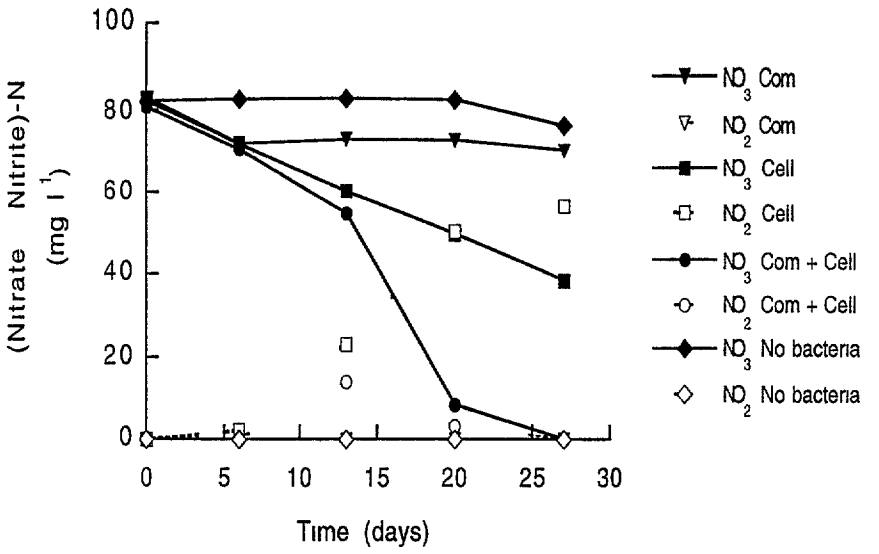

Fig 7 Concentrations of nitrate-N and nitrite-N in single and mixed cultures of *Comamonas* sp and *Cellulomonas uda*, and in controls without bacteria

non-denitrifying bacteria broke down cellulose to products which were in turn used by the denitrifiers? To answer this question, bacteria were isolated from the cotton bed of active reactors and studied No single organism was found which could degrade cotton while reducing nitrate to N_2, although some of the cellulose degraders could reduce nitrate to nitrite (Volokita, 1998) Among the denitrifying bacteria, was a new species of *Comamonas* (Annual Report, 1997, Soares *et al*, 1998) The most common cellulolytic bacterium was *Cellulomonas uda* which also reduced nitrate to nitrite (Volokita, 1998) As shown in Fig 7, mixed batch cultures of these bacteria were a model for the overall process taking place in the reactor removal of nitrate was observed only with the combined action of the two bacteria (Volokita, 1998, Volokita *et al*, 1998)

6) Impact Relevance and Technology Transfer

The findings of this project will be used in Hungary in the reclamation for human consumption of groundwater which is now unfit for human consumption As shown above, the process is efficient and economical and will have an immediate and significant impact in many rural areas where the local water is not potable The project brought to Hungary an original and

practical approach to an important local problem Optimization and upscaling of the denitrification process based on waste cellulose should be completed before the findings can be fully implemented, and a detailed progam has been proposed to US-AID (Proposal C19-030 to be submitted in August 1998) The administrative and scientific skills acquired in the project will have lasting effect The scientific approach used to remove nitrate from drinking water can be extrapolated to the biological removal of other contaminants The equipment purchased is versatile and upgraded the capabilities of the Hungarian laboratory Of particular importance was the ion analyzer The apparatus was used not only for the determination of nitrate an nitrite in this project but also for many other routine chemical determinations in the laboratory, cutting down manpower costs

7) Project Activities/outputs

Papers published/in preparation

Volokita, M , Belkin, S , Abeliovich, A and Soares, M I M (1996) Biological denitrification of drinking water using newspaper Water Research 30 965-971

Volokita M , A Abeliovich and M I M Soares (1996) Denitrification of groundwater using cotton as energy source J Water Science and Technology 34 379-385

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Volokita, M , A Abeliovich and M I M Soares (1998) Detection of cellulolytic microorganisms (Submitted)

Soares, M I M , Volokita, M , A Abeliovich and F Rainey (1998) A new species of *Comamonas* isolated from a denitrification reactor packed with cotton (In preparation)

Volokita, M , M I M Soares and A Abeliovich (1998) Microbial consortium degrades cellulose under denitrifying conditions (In preparation)

Participation in conferences

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8) Project Productivity

The proposed project consisted of a laboratory phase in which newspaper, cotton and wheat straw were to be tested as substrates for denitrification, the most efficient substrate would then be used in a field trial to be carried out in Hungary during the last year of the project The proposed laboratory studies were concluded, and an additional substrate, corn husks, was also tested The best substrate was cotton, followed by wheat straw At the beginning of the field stage the Hungarian group decided to use wheat straw as cotton is not produced in their country The pilot was operated according to plan and included post treatment of the denitrified water An unplanned addition to the project was the use in the field of cellulosic waste from a diaper factory, a substrate which appeared to present the advantages of cotton As described above, this happened close to the end of project in a reactor which was not optimized and the rates of denitrification obtained (up to 0 4 kg N m^{3} d^{1}) can be improved (for instance, by increasing the substrate filling ratio)

In conclusion, the proposed goals and work plan were accomplished, even if at the end the most efficient substrate in the laboratory was not tested in the field, and the best substrate tested in the field was not studied in the laboratory We have shown beyond any doubt that cotton and the cellulose waste from diaper factories are economic and efficient substrates for denitrification of drinking water

9) Future work

Although cotton and waste cellulose from the diaper factory appeared to be equally suitable as substrates for denitrification the former is cultivated in many warm climate countries while the latter is easily obtainable in Hungary but is not available in many other developing countries These two carbon sources differ markedly in their physical characteristics unprocessed cotton consists of fibers covered by a fine layer of wax which makes it somewhat hydrophobic during the early stages of degradation, and the factory waste is in the form of low density flakes/particles Furthermore, a field trial carried out in Israel

(outside the scope of this project) showed that simple upscale of the reactor used in the laboratory is not suitable for a large-scale treatment

On the other hand the Hungarian group used a fluidized bed reactor which was not optimized or upscaled due to time and financial limitations since, as mentioned above, the opportunity to use the new substrate arose at the end of the project Thus, future work is required before a large-scale denitrification system based on either of the two cellulose substrates can be implemented This should concentrate on selecting, optimizing and field testing the reactors suitable for each substrate

For the factory waste, two configurations should be studied

(1) A continuous flow/completely mixed reactor similar to the operation of an activated sludge system and requiring an additional separation device (settling tank or a membrane filter) should be installed to separate the flakes and recycle them back to the process

(2) A batch reactor (it may treat a continuous flow of raw water if at least two reactors are operated in parallel) operated as a sequencing batch reactor (SBR) to enable treatment of several batches of raw-contaminated water per day, without the need for a separate clarifier

For cotton, two types of reactor should also be studied

(1) A continuous flow/plug-flow tubular reactor One of the characteristics of cotton (observed by the Israeli group in a preliminary study in large field reactor) is that it tends to absorb water and to be compressed (by the water flow) in such a way that high pressure is formed towards the reactor head Small tubular reactors in series should enable convenient maintenance The laboratory and pilot-scale units of the tubular plug flow regime, should be fed by a continuous upflow through a compacted cotton bed

(2) A batch reactor In such a batch system cotton would be kept in partial suspension by hydraulic means, i e , by recirculation of water This is a simple form of the sequencing batch reactor (SBR) where pumps serve for raw water introduction and for reactor content mixing The cotton clods formed in the reactor should not be completely mixed since water recirculation causes continuous contact with the cotton clods where the bacterial mass develops The operation cycle of the system should include a short separation and decant period without introducing raw water

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DENITRIFICATION OF GROUNDWATER USING COTTON AS ENERGY SOURCE

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ABSTRACT

Microbial removal of nitrate from drinking water was studied in laboratory columns packed with unprocessed short fiber (low quality) cotton (*Gossypium hirsutum*) Cotton served as the sole chemical and physical substrate for the microbial population Removal of nitrate was rapidly achieved without the formation of nitrite Cotton (cellulose)-dependent denitrification was affected by changes in temperature denitrification rates at 14°C were approximately half of the rates observed at 30°C The cotton was entirely consumed in the process In a fresh reactor, colonization of substrate by bacteria appears to be the rate limiting factor in the removal of nitrate Copyright © 1996 IAWQ Published by Elsevier Science Ltd

KEYWORDS

Cellulose, cotton, denitrification, microbial water treatment, nitrate contamination

INTRODUCTION

Increased nitrate contamination of available and future drinking water sources constitutes an important and rapidly growing environmental problem in many countries Concern for possible health consequences has led to the recommendation of an upper limit of 50 mg nitrate l^{-1} in water for human consumption (WHO, 1984), and to the development of a number of techniques for lowering nitrate concentration to acceptable levels Among these techniques, microbial removal (denitrification) stands out as being the most economical and environmentally sound, as well as being feasible on a large scale

Denitrification is the reduction of nitrate to a gaseous product, usually nitrogen gas, through a sequence of enzymatic reactions Bacteria capable of denitrifying are ubiquitous in nature (Gamble *et al*, 1977) and have the capacity, under anaerobic conditions, to use nitrate in place of oxygen as terminal electron acceptor in their respiratory processes Microbial nitrate removal treatments take advantage of this anaerobic respiratory process and aim to maximize the rates of nitrate consumption by assuring a steady supply of carbon and energy sources which are usually simple organic compounds (inorganic carbon and energy sources are used less frequently)

Lately, we have studied the use of various cellulose-rich sources as substrates for denitrification (Volokita *et al*, 1996) Cellulose is the most abundant renewable resource in the world (Coughlan, 1985) and may offer an inexpensive alternative to the refined carbon compounds commonly used in the treatment of nitrate-polluted water Cellulose is a linear glucose polymer with hydrogen bonding between hydroxyl groups of neighboring parallel chains It is organized in fibers imbedded in lignin and hemicellulose, an association which confers resistance to enzymatic degradation This is not the case, however, with cotton which is the purest form of naturally occurring cellulose with only a small percentage of impurities, mostly

18

in the form of wax (0 3-1%), pectin in the outer layers (0 5-1 2%), and protein residues (Wood, 1988)

In the study presented here, short fiber (low commercial value) unprocessed cotton served as the sole chemical and physical substrate for denitrification in laboratory reactors

MATERIALS AND METHODS

Experimental set up Laboratory reactors were cylindrical PVC columns, 55 cm high and 10 cm dia, except for one experiment in which a transparent perspex column, 50 cm high and 8 cm diameter, was used In the latter, side ports placed at 5 cm intervals allowed the removal of liquid samples Columns were packed with short fiber unprocessed cotton (*Gossypium hirsutum*) previously weighed and inoculated, a thin layer of glass wool was placed at each end of the cotton bed Inoculation was carried out by mixing the preweighed cotton with a small amount of cotton from an active denitrification column (initial inoculum obtained as described by Volokita *et al*, 1996), and nitrate- and phosphate-supplemented (200 and 3 mg l^{-1} respectively) tap water This inoculation lasted 4-5 days with daily replenishments of nitrate Columns were kept in the vertical position and fed with tap water amended with nitrate and 3 mg l^{-1} phosphate Flow of water through the columns was directed upwards and regulated by peristaltic pumps Flow rates are presented as Darcy's flux ($q = Q/A$ in m d^{-1}, where Q is the measured flow rate and A is the section area of the column)

Samples were usually collected twice a week from the inlet and the outlet, and routinely assayed for nitrate, nitrite, ammonia, pH and DOC (dissolved organic carbon) Unless otherwise indicated, the ambient temperature was maintained at 24±1°C

Analytical determinations Nitrate was determined by the colorimetric method of Cataldo *et al* (1975), and nitrite and ammonia were assayed according to Standard Methods (APHA, 1989) Organic carbon was determined by means of a high-temperature TOC analyzer (Dohrmann DC-190, Rosemount Analytical Inc, Santa Clara, CA USA)

Bacterial counts Colony forming units in the effluent were counted by standard plating techniques on R2A agar (Difco Laboratories)

RESULTS

A column packed with 380 g of cotton was operated for approximately seven months during which the flow rate, temperature and concentration of nitrate varied (Fig 1)

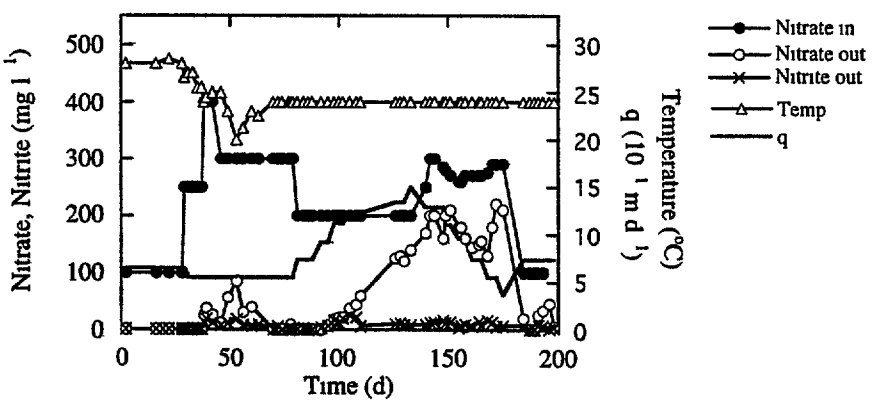

Fig 1 Concentrations of nitrate, nitrite and ~~DOC~~ in the effluent, and of nitrate in the influent of a column packed with 380 g of unprocessed cotton and operated at various temperatures and flow rates

From the first day of operation, complete removal of 100 mg nitrate l^{-1} was achieved, at average daily

19

temperature of 28°C and flow rate of 0 64 m d^{-1}, nitrite was not detected The concentration of nitrate in the influent was then increased to 250 mg l^{-1} on day 27 and subsequently, on day 37, to 400 mg nitrate l^{-1} Breakthrough of nitrate was observed when the influent nitrate concentration reached 400 mg l^{-1} By then, the average daily temperature had decreased to 24°C and continued to decrease until it reached 20°C on day 53 From day 70 onwards the temperature was controlled and maintained at 24°C At this temperature, 300g nitrate l^{-1} were reduced without accumulation of nitrite (days 70-79) Higher flow rates were then tried while the concentration of nitrate in the influent was maintained at 200 mg nitrate l^{-1} Breakthrough of nitrate started at 1 12 m d^{-1} and increased as the flux was raised up to 1 50 m d^{-1} breakthrough of nitrite was also observed

On day 140 the concentration of nitrate in the influent was raised to approximately 300 g l^{-1} and the flux was gradually lowered, with the purpose of comparing the existing denitrification capacity to that observed two months earlier, when complete removal of 300 mg nitrate l^{-1} had taken place without accumulation of nitrite (days 70-79) By now, the efficiency of the column had deteriorated and nitrate breakthrough was observed even when the flux and the inlet nitrate concentration were equal to those of day 1 The column was then emptied and the remaining cotton was dried and weighed From the initial 380 g only 150 g were left, representing a weight loss of 60%

An experiment was set up with the objectives of (1) studying the distribution of denitrification along the column and (2) estimating the approximate ratio of cotton used to nitrogen removed A transparent perspex column with side sampling ports was packed with 184 g of cotton and supplied with approximately 100 mg nitrate l^{-1}, fluxes varied between 1 15 and 3 16 m d^{-1} and the temperature varied from 24-27°C Removal of nitrogen per day was calculated as follows

$$\text{N lost (mg day}^{-1}) = [\text{nitrate-N in} - (\text{nitrate-N out} + \text{nitrite-N out})]\ (\text{mg l}^{-1}) \times \text{Volume day}^{-1}(\text{l})$$

and from the daily rates the cumulative loss of nitrogen was computed

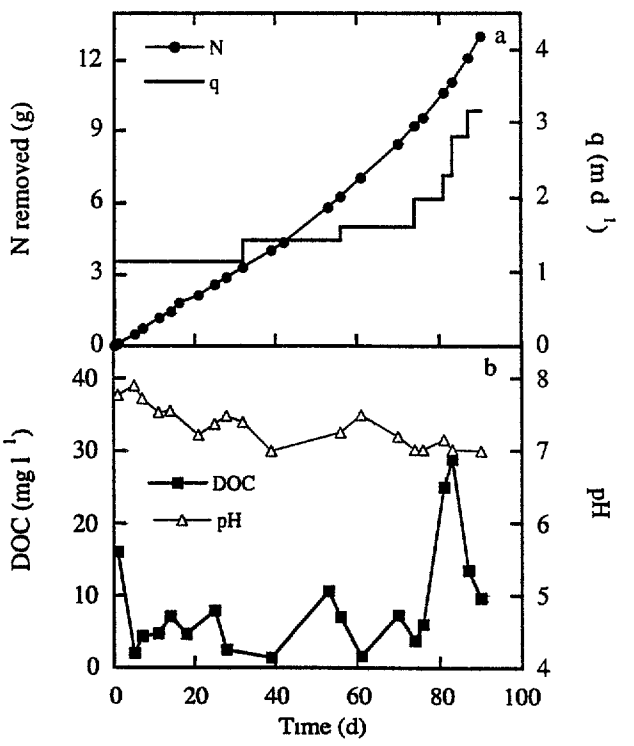

Fig 2 Removal of nitrogen (a), and pH and concentration of dissolved organic carbon in the effluent (b) in a column packed with 184 g of unprocessed cotton and operated at various flow rates Temperature, 24-27°C

20

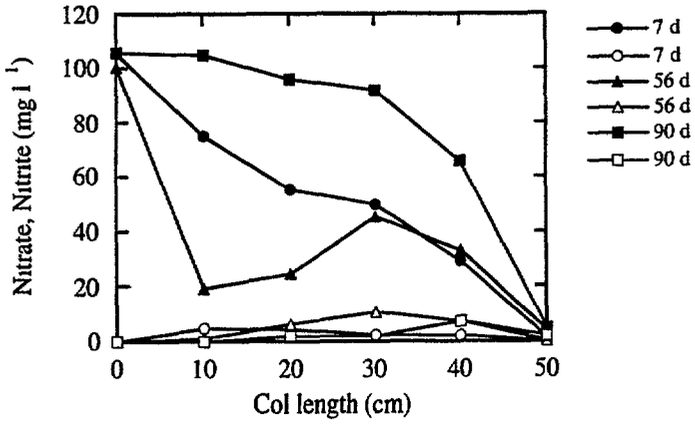

Fig 3 Concentrations of nitrate and nitrite in the column in Fig 2, on days 7, 56 and 90 Closed symbols, nitrate, open symbols, nitrite

During the initial 40 days, the rates of nitrogen removal were relatively constant at around 100 mg d^{-1} From then on the rates increased significantly and had reached 330 mg nitrogen d^{-1} by day 90 (Fig 2) The overall amount of nitrogen removed during the 90 days of operation amounted to 13 0 g, during the same period, the dry weight of cotton was reduced by 75 g From these data, a ratio of cotton used to nitrogen removed can be calculated However, the value obtained, 5 8, represents the average over the 3 months period and may be taken as a very broad estimation only between days 2 and 90 the rate of nitrogen removal per day increased by a factor of 3 3 while the weight of substrate decreased by a factor of 1 7, which represents a cotton to nitrogen ratio 5 6 times higher at the beginning than at the end of the experiment

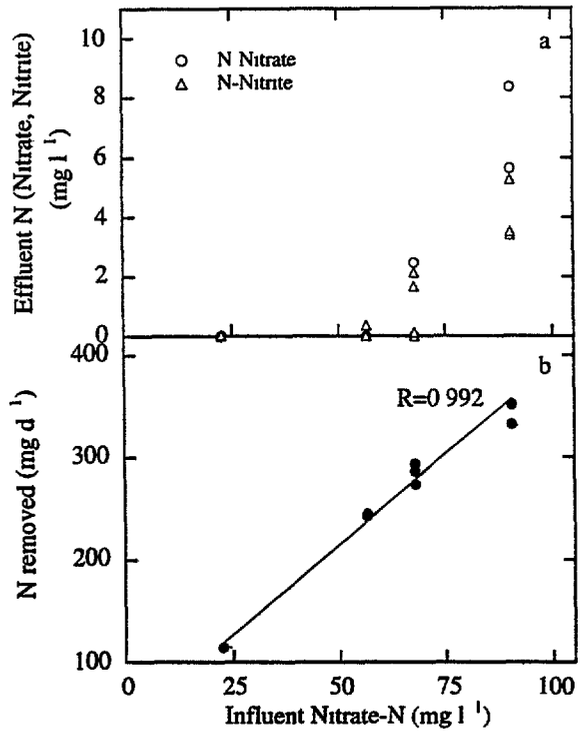

Fig 4 Effect of influent nitrate concentration on the removal of nitrogen in a column packed with 380 g of unprocessed cotton a, breakthrough of nitrate and nitrite, b, nitrogen removed per day Temperature, 24°C

21

The distribution of denitrification activity along the column varied with time (Fig 3) During the first two weeks, nitrate reduction was evenly distributed throughout the column, nitrite accumulated in the middle section but was removed by the time the flowing water (4 ml min^{1}, equivalent to a flux of 1 16 m d^{1}) reached the outlet Later, oscillations were observed in the concentrations of nitrate and nitrite, suggesting channeling in the cotton bed, this was confirmed by visual observations The extent and location of the oscillations varied from day to day and an example (day 56) is shown in Fig 3 By day 76, the cotton bed was pushed upwards, the lower third of the column was occupied by water only, and removal of nitrate took place in a narrow section of the column At this stage, the flow rate was 6 9 ml min^{1} (1 98 m d^{1}) and the rates of denitrification continued to increase rapidly in spite of the decreasing contact times (Fig 2a)

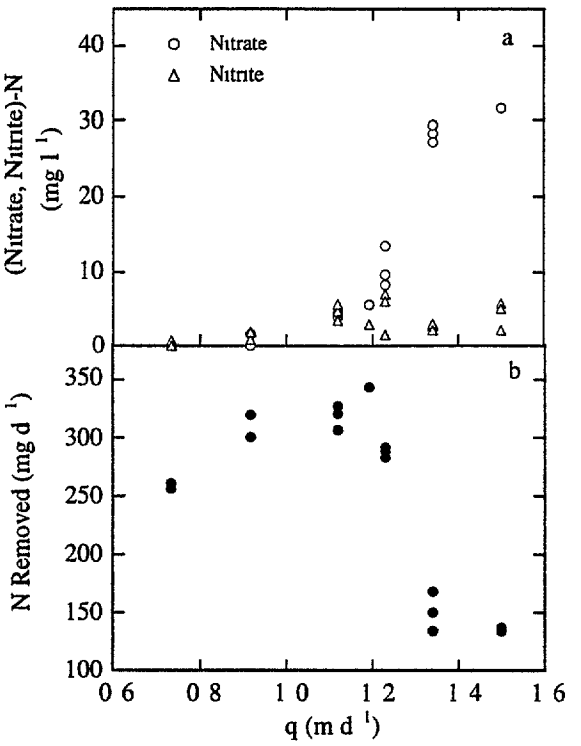

Fig 5 Effect of flow rate on the removal of nitrogen in a column packed with 380 g of unprocessed cotton and kept at 24°C a, concentrations of nitrate and nitrite in the effluent, b, nitrogen removed per day

By the end of the experiment, marked changes were also visible (naked eye observation) in the composition of the cotton bed the bulk of the remaining material was in an advanced state of degradation, between a thin lower layer of less degraded cotton and a thicker upper layer of apparently well preserved cotton fibers

Ammonia was never detected in the effluent The concentrations of dissolved organic carbon were usually below 10-15 mg l^{1} (Fig 2 and results not shown) Odors were detected only when, due to technical problems, flow was stopped for a number of hours (e g , overnight), at all other times, no color or odor could be detected in the treated water The number of colony forming units in the denitrified water was in the order of 10^{5} ml^{1}

The effect of the concentration of nitrate on the rate of denitrification was studied in a column identical to that described in Fig. 1, maintained at the flow rate of 0 55 m d^{1} at the temperature of 25°C Influent nitrate concentrations varied from 100 to 400 mg l^{1} (22 58 - 90 32 mg nitrogen l^{1})

A linear relationship existed (R= 0 99) between the concentration of nitrate in the influent and the loss of nitrogen per day (Fig 4b) Breakthrough of nitrate and nitrite started around 67 74 mg nitrate-N l^{1} (300 mg nitrate l^{1}) and at the highest nitrogen load (90 32 mg l^{1}), less than 10% was washed out (Fig 4a)

The relationship between flow rate and removal of nitrogen was studied in a column packed with 380 g

22

cotton and fed with tap water amended with 200 mg nitrate l^{-1} (45 2 mg nitrogen l^{-1}) Breakthrough of nitrate (about 20 mg l^{-1}) appeared at the flow rate of 1 12 m d^{-1} and gradually increased as the flow rate was increased up to 1 23 m d^{-1} Increasing the flow rate further to 1 34 m d^{-1} resulted in a sharp increase in nitrate washout, with less than 60% of the influent nitrate being removed In contrast, breakthrough of nitrite did not increase it started at 1 12 m d^{-1} and was approximately constant at the higher flow rates tested (Fig 5a)

Calculation of the amount of nitrogen removed per day shows that maximal denitrification efficiency was achieved at flow rates of 0 92 - 1 23 m d^{-1} (Fig 5b) A sharp decline in removal of nitrate followed as the flow rate was raised to 1 34 m d^{-1} (Fig 5b) coinciding with the burst in nitrate breakthrough (Fig 5a)

The effect of temperature on the cellulose degradation/denitrification process was studied in a column packed with 400 g of cotton and allowed to stabilize for 4 weeks at 24°C before the following temperatures were tested in sequence 30, 28, 26, 23 19 and 15°C, two weeks per setting Flow rates were in the order of 0 88 - 0 94 m d^{-1} and the concentrations of nitrate in the influent varied from 150 to 220 mg l^{-1}, to allow an approximately constant breakthrough of nitrate (around 40 mg l^{-1})

Increasing the temperature from 14 to 19°C had little effect on the amount of nitrogen removed per day (Fig 6) In contrast raising the temperature from 19 to 24°C brought about an increase of 60% in the rate of denitrification Higher temperatures continued to stimulate denitrification and the rate at 30°C was more than double the rate observed at 14°C

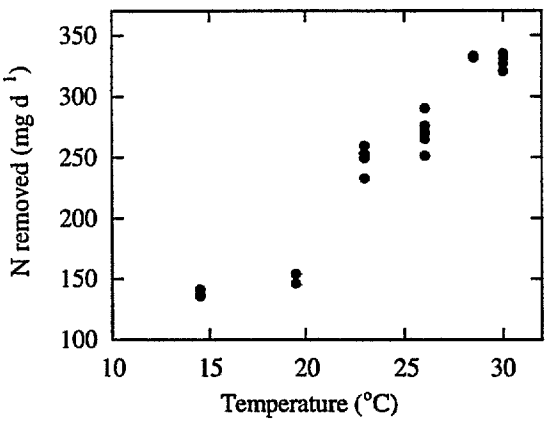

Fig 6 Effect of temperature on the removal of nitrogen in a column packed with 400 g of unprocessed cotton and operated at flow rates of 0 88 - 0 94 m d^{-1}

DISCUSSION AND CONCLUSIONS

The microbial process described here for the removal of nitrate from contaminated groundwater uses cotton as the sole physical and chemical substrate for denitrifying microorganisms Cotton is an abundant crop in many developed and developing countries and the relatively low price of the short fiber product makes it a very attractive substrate for denitrification

Unprocessed cotton is an effective substrate for the removal of nitrate from contaminated water The treated water contained low concentrations of DOC and was without detectable color or odor, bacterial counts (colony forming units) were in the order of 10^5 ml^{-1} Post treatment in the form of sand filtration and chlorination will be required before the denitrified water reaches drinking quality

Denitrification rates in a freshly packed column increased steadily with time and continued to do so even when only 60% of the initial substrate remained (Fig 2a) This suggests that colonization of the substrate by bacteria was the rate-limiting factor in the removal of nitrate However, a stage is reached beyond which substrate appears to be limiting In the experiment represented in Fig 1, the denitrification capacity of the column had declined when 40% of the initial substrate remained. This deterioration might be due to lack of substrate surfaces for bacterial colonization Bacteria isolated from denitrifying reactors are being studied in an attempt to understand this and other points of the complex microbial processes

23

taking place, namely the concerted activity of cellulose degraders and denitrifies and whether some of the denitrifying bacteria are also cellulose degraders

Increasing nitrate loading by means of increasing flow rates improved the efficiency of the column until a threshold was reached (at around 1 23 m d^{-1}) beyond which the removal of nitrate was markedly reduced (Fig 5) The reason(s) for this sharp decline are not clear yet and may include wash out of soluble substrate or wash out of bacteria, although the latter could not be detected by counts of colony forming units in the effluent (results not shown)

Cellulose-dependent denitrification is affected by changes in temperature (Fig 6) Although the temperature of groundwater is quite stable (around 22°C in Israel), the residence time in an above ground reactor may be such that significant changes in temperature occur During the winter, the concentrations of nitrate in groundwater are often lower but ambient temperatures are also lower and the two effects may cancel each other In summer, both the concentration of nitrate and the demand for treated water are higher but these may be compensated, at least partially, by the faster denitrification rates at the higher temperatures

Clogging of denitrification reactors due to entrapment of N_2 bubbles can be a serious problem and we have encountered this phenomenon in sand packed columns (Soares et al , 1989 1991) Clogging by N_2 appeared not to be a problem in the current study, however, a new reactor should not be tightly packed Cotton fibers are covered by a thin layer of wax which makes them somehow hydrophobic and although this changes with time, a too compressed bed will remain mostly dry and water will flow thorough a few channels only

Cotton was entirely consumed and this will simplify a long-term continuous operation since emptying and disposal of waste or spent substrate will not be required The substrate replacement strategy should consist of small and relatively frequent additions of fresh cotton This will assure consistently high rates of denitrification and will minimize reduction in activity during the initial stages of bacterial colonization of fresh substrate

ACKNOWLEDGMENTS

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24

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BIOLOGICAL DENITRIFICATION OF DRINKING WATER USING NEWSPAPER

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Abstract—Microbial denitrification of drinking water was studied in laboratory columns packed with shredded newspapers Newspaper served as the sole carbon and energy substrate as well as the only physical support for the microbial population Complete removal of nitrate (100 mg l^{-1}) was readily achieved without accumulation of nitrite The treated water contained low dissolved organic carbon (4–10 mg l^{-1}) The cellulose dependent denitrification process was sensitive to changes in temperature nitrate removal rates at 14°C were approximately one third of the rates observed at 32°C Pretreatment of newspaper with diluted NaOH or diluted HCl or by autoclave did not improve the efficiency of the process A time dependent decay in denitrification rate was noticeable after several months of operation The reasons for this phenomenon which may be due to weakened adhesion of the bacteria to the substrate are under investigation

Key words—nitrate contamination denitrification microbial water treatment newspaper cellulose

INTRODUCTION

Nitrate pollution of drinking water constitutes an important and rapidly growing environmental problem The most common causes of nitrate pollution are the excessive use of fertilizers in intensive agriculture, and irrigation with ammonia-rich effluents discharged by wastewater treatment plants Concern for possible health consequences has led to the adoption of a stringent nitrate standard (50 mg nitrate per liter) in water for human consumption (WHO, 1984) Removal of nitrate from drinking water is, however, an expensive process and for this reason the nitrate standard is often not implemented, even in countries where it has been officially adopted Therefore, inexpensive and easy to apply nitrate removal technologies are urgently required

Among the techniques available for the removal of nitrate from drinking water, microbial denitrification stands out for being the most economical and environmentally sound, as well as for being feasible on a large scale Denitrification is carried out by facultative aerobic bacteria which in the absence of oxygen utilize nitrate as a terminal electron acceptor in respiration (Payne, 1981)

The majority of microbial denitrification treatments rely on heterotrophic bacteria which require an organic carbon substrate Usually, a simple carbon compound such as ethanol, methanol or acetate is used, however, cheaper readily available carbon sources should be explored Among these possible substrates is cellulose, a basic component of all plant materials and the most abundant renewable resource in the world (Couglan, 1985)

The use of cellulose in the restoration of nitrate-contaminated ground water has been described by Boussaid *et al* (1988) In their study denitrification reactors were packed with a mixture of straw, sand, and maerl, or with straw and maerl, post-treatment was carried out by reinfiltration of the effluent back into the aquifer

Cellulose is a linear glucose polymer with hydrogen bonding between hydroxyl groups of neighboring parallel chains Native cellulose is organized in fibers and occurs in close association with lignin and hemicellulose Although all the above characteristics confer resistance to enzymatic attack specific bacterial groups have the capacity to degrade cellulose Even so, one or more forms of pretreatment (chemical, physical or physicochemical) are often applied to cellulose-rich materials in order to increase their susceptibility to degradation (Millet *et al*, 1975, Fan *et al* 1981, Wood and Saddler, 1988)

In the study described here, a cellulose-rich waste, newspaper, was used as the sole carbon and energy substrate as well as the only physical support for bacteria in laboratory denitrification reactors

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25

MATERIALS AND METHODS

Selection of inoculum

Various inocula of cellulose degrading bacteria (e g forest and garden soil wood infested by termites) were studied for the selection of the most efficient cellulose dependent denitrification Enrichment cultures were prepared in 150 ml Erlenmeyer flasks containing 75 ml of tap water supplemented with 100 mg l^{-1} nitrate (as KNO_3) 3 mg l^{-1} phosphate (as K_2HPO_4), 2 5 mg l^{-1} cyclohexemide (to prevent fungal growth) and one of the following cotton wool (0 4 g), straw (0 5 g) or shredded newspapers (1 g) All substrates were sterilized (autoclave) Cultures were kept on a Labquake shaker (Labindustries Inc, Marietta Ohio) inside an anaerobic chamber (Forma Scientific Inc Marietta Ohio) at 27°C Aliquots were drawn 2–3 times a week and assayed for nitrate and nitrite Nitrate was supplemented by addition of an appropriate volume of a concentrated stock solution

The performance of the various inocula was monitored for 4 weeks The inoculum obtained from wood infested by termites consistently brought about the highest rates of denitrification with all three cellulose substrates and was chosen for further studies

Experimental apparatus

The substrate used was newspaper shredded to ribbons (0 4 cm width) by an office type paper shredder Laboratory reactors were cylindrical PVC columns 55 cm high and 10 cm dia Smaller glass columns (44 5 cm height 4 5 cm dia) were used to study the effect of various pretreatments applied to paper The amount of newspaper packed into the columns varied as described under Results glass wool plugs were placed at both ends of the packing material Columns were fed with tap water amended with nitrate at the desired concentration and 3 mg l^{-1} phosphate Flow rate was regulated by peristaltic pumps and was directed to create an up-flow through the columns, flow rates are presented as Darcy s flux (q in m d^{-1})

$$q = Q/A$$

where Q is the measured flow rate (m^3 d^{-1}) and A is the cross section of the column (m^2) In the PVC reactors a q value of 1 m d^{-1} corresponds to a flow rate of 5 5 ml min^{-1} in the smaller glass columns 0 1 m d^{-1} corresponds to 1 1 ml min^{-1}

After packing, columns were dosed with medium and the void volume was replaced by a suspension prepared by blending a small amount of material from an active denitrification column (original inoculum described above) The inoculated columns were allowed to stand for 2–3 d before flow was initiated Samples were usually collected twice a week from the inlet and the outlet of the column and routinely assayed for nitrate nitrite, ammonia, pH, dissolved organic carbon (DOC) and bacterial counts One experiment was carried out at room temperature and the average (minimal/maximal) daily temperatures are indicated, all other experiments were carried out at 25 ± 1°C unless indicated otherwise

Pretreatment of newspaper

Milling was carried out with a ball mill (Pascal Eng U K) the other treatments are described in Table 1

Analytical determinations

Nitrate was determined with a nitrate-selective electrode (Orion Research Inc Boston Mass) or by colorimetry (Cataldo *et al*, 1975) Nitrite and ammonia were assayed according to Standard Methods (APHA 1989) Carbon was determined by means of a high-temperature TOC analyzer (Dohrmann DC 190 Rosemount Analytical Inc Santa Clara, Calif)

The chemical composition of newspaper was determined in samples ground to pass a 40 mesh screen with a Wiley intermediate mill (Thomas Scientific Apparatus Swedesboro N J) Extractive components acid detergent fiber lignin and ash were determined by the Goering-Van Soest method as described by Kirk and Obst (1988) Fresh newspaper (dried at 105°C) contained 25 49 ± 0 15% extractives, 43 11 ± 0 71% cellulose 29 59 ± 0 32% lignin and 2 59 ± 0 10% ash

The extractive fraction includes protein hemicellulose and other labile carbon fractions Ink contains vegetable oil mineral oil resins and carbon black (lamp black) and probably accounts for a significant proportion of the extractable fraction

Bacterial counts

Colony forming units were counted by standard plating techniques on R2A agar (Difco Laboratories)

Electron microscopy

Samples for scanning electron microscopy studies were fixed with glutaraldehyde Examination was carried out in a Jeol JFM 35C microscope

RESULTS

Nitrate removal and water quality

Figure 1 illustrates the performance of a column packed with 600 g of shredded newspaper and supplied with nitrate at a concentration of approx 100 mg l^{-1} (22 58 mg N l^{-1}) The ambient temperature was not constant and the values presented are daily averages

Complete removal of nitrate without accumulation of nitrite was achieved after onset of flow (0 55 m d^{-1}) A temporary breakthrough of nitrate was observed after day 19, this may be due to a sharp drop in temperature (6–10°C) during this period

Stable denitrification followed until the flow rate was raised to 0 77 m d^{-1} and later up to 0 95 m d^{-1} These changes were accompanied by breakthrough of nitrate up to a maximum concentration of 54 mg l^{-1} nitrite also appeared although at much lower concentrations (the highest concentration measured was 8 4 mg l^{-1}) Upon decrease of flow rate to 0 4 m d^{-1} (day 148) nitrate and nitrite disappeared from the effluent Ammonia was never detected

Washout of significant amounts of DOC was observed at the beginning of operation (up to day 12),

Table 1 Treatments applied to newspaper prior to packing into the columns During treatment the ratio of newspaper to liquid was 100 g/4 l Treatments were followed by rinsing with tap water the acid- and base treated paper was washed until no change in pH was observed the volume of water used was measured and an equal volume was used for rinsing the control and the autoclave treated paper

	Treatment
Control	Water for 24 h in a shaker at room temperature
Autoclave	2 h
Acid	1N HCl for 24 h in a shaker at room temperature
Base	1% NaOH for 24 h in a shaker at room temperature

26

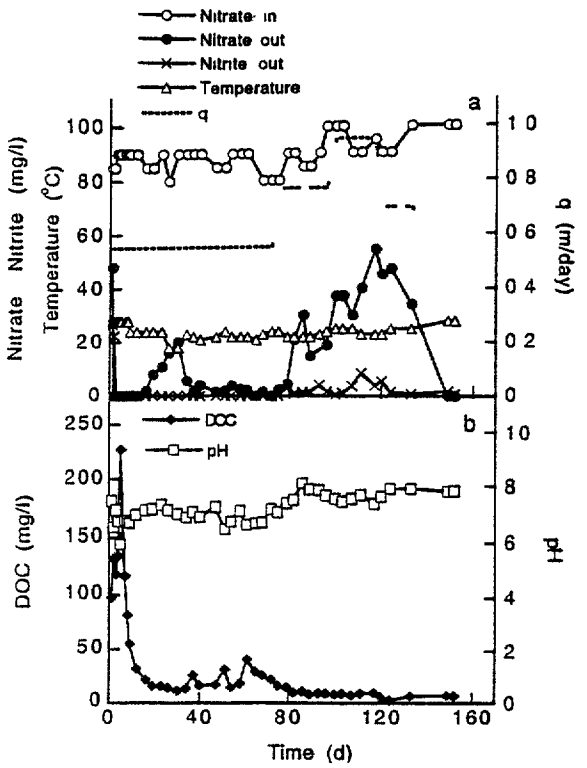

Fig 1 Concentrations of nitrate nitrite and DOC and pH of water treated in a column packed with 600 g of shredded newspaper and operated at room temperature (daily averages are shown)

the concentrations decreased rapidly and stabilized at 4–10 mg l^{-1}

The treated water never exhibited noticeable color or odor The number of colony forming units was in the order of 10^5 ml^{-1}

Effect of temperature

A column packed with 400 g of shredded newspapers was inoculated and left to stand for one week while the soluble fraction of OC [the fraction eluted during the first week in Fig 1(b)] was depleted After flow initiation, the process was allowed to stabilize at 25°C for 2 weeks before the following temperatures were tested in sequence 32, 29, 26, 23, 19 and 14°C, with 2 weeks per setting Denitrification was calculated as the amount of N lost per day as follows

$$\text{N lost}_{(\text{mg day}^{-1})} = [\text{nitrate-N in} - (\text{nitrate-N out} + \text{nitrite-N out})]_{(\text{mg l}^{-1})} \times \text{volume d}^{-1}{}_{(l)}$$

As shown in Fig 2, cellulose-dependent denitrification was markedly affected by temperature denitrification rates at 25–32°C were approximately three times higher than at 14°C

Effect of flow rate

The effect of flow rate on denitrification was studied in a column packed with 400 g newspaper and kept at 25°C, inlet nitrate concentration was 100 mg l^{-1} and the flow rate was varied between 0 5 and 1 5 m d^{-1} (Fig 3) Complete removal of nitrate was achieved at flow rates up to 0 8 m d^{-1} Breakthrough of nitrate occurred at higher flow rates, and a linear relationship (R = 0 96) was observed between the degree of nitrate removal and q At the highest flow rate tested, the concentration of nitrate decreased by 30% only [Fig 3(a)]

When the amount of nitrate removed per d was plotted [Fig 3(b)] it became evident that the column operated below capacity at the lower fluxes, and that maximal efficiency was achieved at approx 1 2 m d^{-1} A very sharp decline was observed at higher flow rates, and this may be due to washout of bacteria, enzymes or substrate

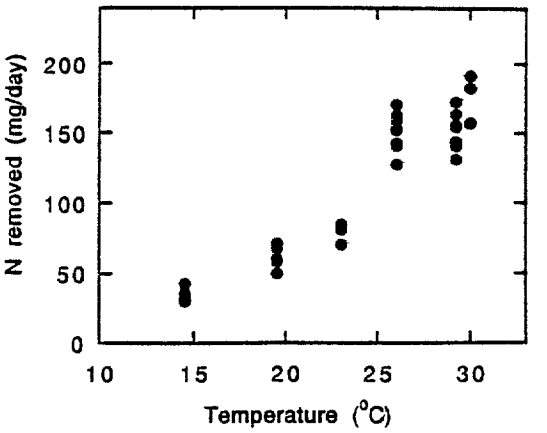

Fig 2 Effect of temperature on the removal of nitrate in a column packed with 400 g shredded newspaper

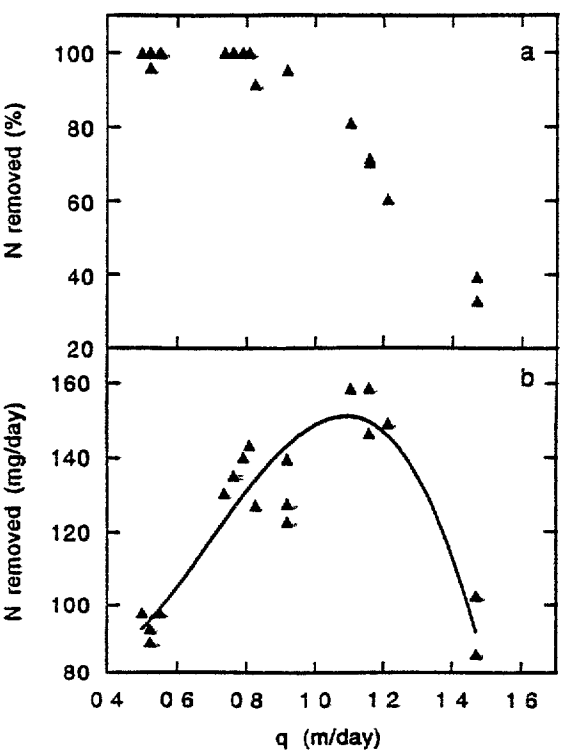

Fig 3 Effect of flow rate on the removal of N in a column packed with 400 g shredded newspaper and kept at 25°C (a) Percentage of N loss, (b) N lost per day

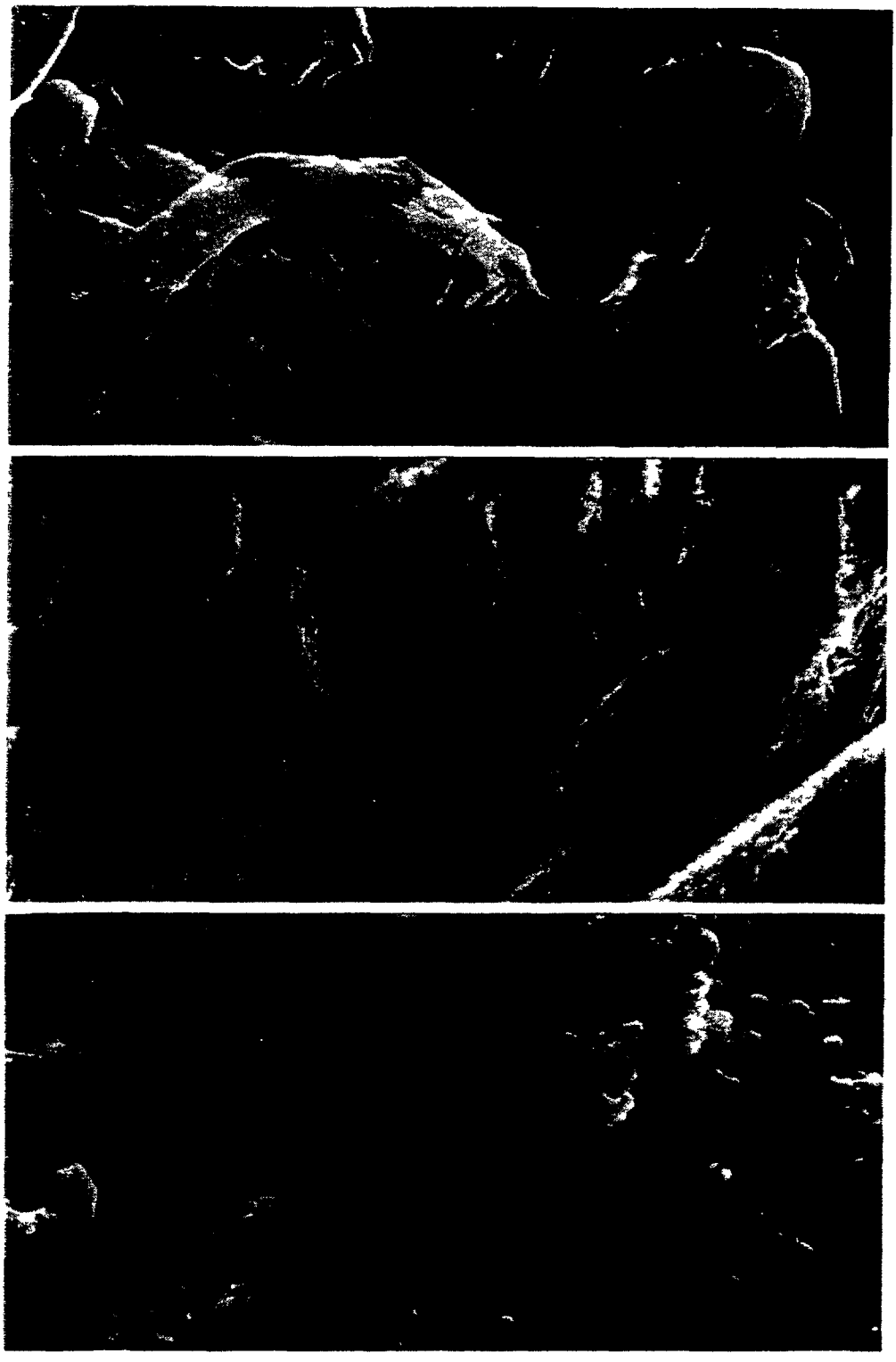

Fig 4 Appearance of fresh unprinted newspaper (top) and of printed (middle) and unprinted (bottom) newspaper removed from a denitrification column after 6 months of continuous operation Bar represents 10 μm

Effect of ink

As considerable surface area of newspaper is covered by ink its effect on colonization by bacteria was investigated with the aid of electron microscopy In paper removed from active denitrification columns the printed areas appeared flattened' and without attached bacteria (Fig, 4, middle) Thus ink apparently decreased the availability of cellulose surfaces bv occlusion and/or prevention of bacterial colonization

In order to assess the effect of ink in the performance of the denitrification system, the unprinted margins of newspapers were separated from the printed portions and two columns were packed with 400 g of printed or unprinted paper The columns operated for three months with oversupply of nitrate During the first month of operation, the

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28

unprinted newspaper supported higher rates of denitrification (Fig 5) This apparent advantage diminished with time and the cumulative N removal in the column packed with printed newspaper remained at about 75% of that in the column packed with unprinted newspaper The decrease in denitrification rates observed in both columns on day 20 is at least in part due to the decline in average temperature however the original rates of denitrification were not recovered when temperatures were elevated again

Effect of newspaper pretreatment

Treatments which facilitate degradation of cellulose were expected to increase the denitrification efficiency of the system Among the methods described in the literature four appeared to be feasible under our experimental conditions and were studied ball milling treatment with 1% (w/v) NaOH treatment with 1 N HCl and heat treatment in an autoclave

Ball milling of newspaper is not a viable option for large water treatment systems the process is slow and too expensive in terms of energy requirement Milling gradually pulverized the paper fragments and the fine matrix of the final product is not suitable as solid support for a flowthrough-type reactor

The experiment described on Table 1 aimed at assessing the effects of cellulose pretreatments on denitrification Four small columns were packed with one of the following types of newspaper untreated autoclave-treated acid-treated and base-treated The columns were operated at identical flow rates (Fig 6)

Neither acid nor base pretreatment improved the denitrification capacity of the columns On the contrary a decline in the denitrification rate was observed after the first week of operation with lower removal of nitrate and higher accumulation of nitrite The high efficiency during the first week may probably be attributed to the available excess of soluble organic matter during this period [see Fig 1(b)]

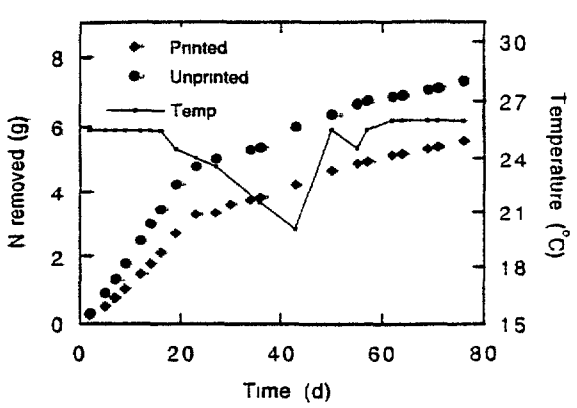

Fig 5 Cumulative N removal in two columns packed with 400 g of printed or unprinted newspaper

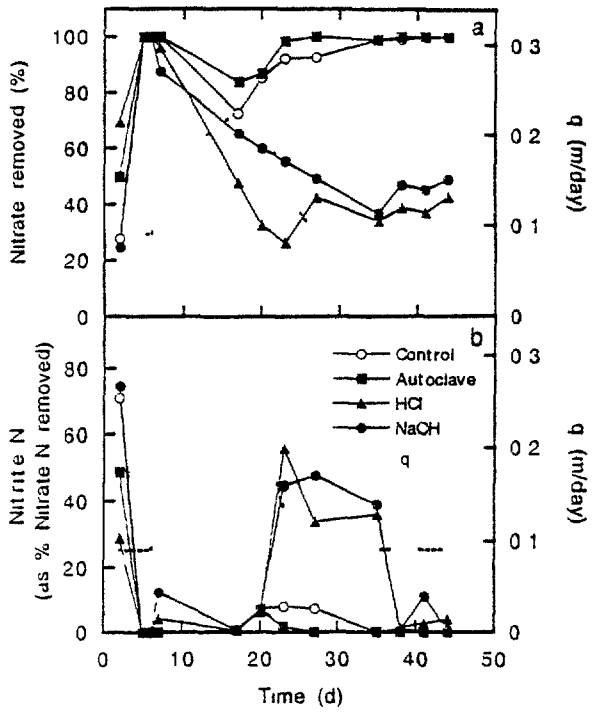

Fig 6 Removal of nitrate and accumulation of nitrite in small columns packed with 154 g of newspaper pretreated as described in Table 1 and kept at 25 C

The difference between control and autoclave treated paper was not clear A transient slight improvement of denitrification rates with lower nitrite accumulation may have occurred between days 13 and 27 but from then on both columns operated below capacity To clarify this point two fresh columns with control or autoclave-treated paper were operated at flow rates allowing breakthrough of nitrate autoclave pretreatment did not improve nitrate removal (data not shown)

Except for a decrease (approx 5%) in the extractable fraction of the paper treated by alkali or acid the pretreatments appeared to cause no change in the chemical composition of newspaper (data not shown) Print was faded and illegible suggesting that acid and alkali removed ink and this may explain the lower proportion of extractables

Changes in newspaper during denitrification

Efficient nitrate removal in the newspaper-packed columns could usually be maintained up to 3–4 months With time however an irreversible decrease in denitrifying capacity was observed Addition of micronutrients or a fresh inoculum failed to improve the performance of spent columns On the other hand paper samples removed from these columns and transferred to batch experiments in an anaerobic chamber were capable of sustaining denitrification at rates 8–13 times higher than those measured in the columns at the time of sampling (an example is presented on Table 2)

In an attempt to identify the cause(s) for this decline in denitrification capacity changes in the

29

composition, appearance and weight of newspaper were investigated

Weight loss At the end of the operation, the packing paper was removed from the columns, dried and weighed As expected, the extent of weight loss depended on the duration and regime of operation of the columns During 6 months of continuous operation, a loss of 20% dry weight was observed, columns operated for 3–4 months showed lower weight losses (10–12%) Washout of paper fragments was never detected and the weight loss appeared to be due to microbial degradation

Composition On a dry weight basis, the microbial processes taking place in the columns should result in a decrease in the relative percentage of cellulose accompanied by an increase in the relative percentage of lignin These changes, however, were apparently too small to be consistently detected by the methods employed statistically significant differences were not always found between the chemical composition of fresh newspaper and that of newspaper removed from denitrification columns

Appearance The appearance of the paper was studied by scanning electron microscopy Unless covered by ink, fresh newspaper had a loose appearance with thin flat structures at the surface (Fig 4, top) In contrast, the surface of ink-free spent newspaper (Fig 4, bottom) appeared devoid of fine fibers, numerous bacteria were visible

DISCUSSION AND CONCLUSIONS

The results described above demonstrate that newspapers can effectively support denitrification of nitrate-contaminated water The treated water is characterized by low DOC and no detectable flavor, odor or color Washout of bacteria was relatively high, requiring further disinfection of the treated water

Temperature had a marked effect on the cellulose degradation-denitrification process (Fig 2) The diminished capacity at lower temperatures should be taken into account when planning a field reactor In order to compensate for the slower rates of denitrification at lower temperatures, longer contact times might be necessary This can be achieved by increasing the length of the reactor or by decreasing the rate of water flow through the reactor

Table 2 Nitrogen removal in batch mode with fresh newspaper or with paper from a spent 10 month-old column and removal of nitrogen by the column at the time of sampling (temperature 30°C) Batches were maintained in an anaerobic chamber at 27°C average daily rates during the second week are shown

		Nitrogen removed (mg g^{-1} paper d^{-1})
Batch	Fresh paper + fresh inoculum	0 875
	Column paper	0 730
	Column paper + fresh inoculum	0 660
	Control (no paper no inoculum)	0
Column		0 056

The amount of N removed per day was higher when the columns were maintained at overcapacity, i e when a certain level of nitrate breakthrough was allowed (Fig 3) Often, complete removal of nitrate is not required in a denitrification treatment, and the system can be operated at flow rates allowing the highest efficiency while maintaining acceptable concentrations of nitrate and nitrite

The contact times required for the complete removal of nitrogen (as 100 mg nitrate l^{-1}) were approx 1 5 times (1 3–1 8) longer than those needed for the total removal of the same nitrogen concentration when the columns were packed with sand and supplied with a simple carbon source (sucrose acetate or ethanol) at a C N molecular ratio of 2 (Soares *et al* 1989 and unpublished results)

The use of newspaper as sole physical matrix in our view contributes to the reliable performance of the system Our previous denitrification studies both *in situ* and in above ground sand-packed reactors showed that entrapment in pore spaces of N_2 bubbles released during denitrification causes severe clogging (Mercado *et al* 1988 Soares *et al* 1989 1991) In a clogged reactor water migrates at increased velocity along preferential canals and the efficiency of the system is impaired In the experiments described here the matrix formed by the packed paper ribbons allowed the free release of gas and no clogging was observed Furthermore, reactors filled with paper only are easy to operate packing is facilitated as well as emptying and disposal of the spent newspaper

However during operation, a marked deterioration in the overall performance of the reactors was observed while a relatively small weight fraction (10–12%) was lost The reason(s) for this phenomenon are not well understood and are under investigation Prevention of bacterial colonization or occlusion of cellulose fibers by ink are not the main reasons since the decay in denitrification capacity was also observed with unprinted newspaper (Fig 5) This might suggest that the overriding factor is an intrinsic characteristic of the paper (e g high content of lignin, low proportion of easily degradable amorphous fraction) However 'spent" paper was capable of supporting high rates of denitrification and for long periods of time (one experiment lasted six months) anaerobically in batch mode This in turn suggested that the adhesion of bacteria to paper might be impaired, and the cellulose degrading bacteria had been washed out from the columns, although the addition of a fresh inoculum to the spent column failed to improve the rate of nitrate removal A possible explanation is that the shearing forces of water flowing through the column slowly removed the fine fibers, thus decreasing available microbial colonization sites The physical appearance (electron microscopy) of "spent" paper (Fig 4 bottom) is consistent with this hypothesis

Newspapers are the largest single item in landfills and paper products constitute as much as 30–50% of

solid refuses (Wigfall and Gregory, 1991, Baver and Lamed 1992) Disposal of municipal solid waste poses great economical and ecological problems While the amounts of newspaper required as substrate for denitrification are too modest to have an impact in solid waste disposal it should be emphasized that the system described here not only avoids the use of an expensive refined carbon source but replaces it with a refuse which is burdening our environment

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31

WR 1838 [MB 8/6/98]

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RESEARCH NOTE

WHEAT STRAW AS SUBSTRATE FOR WATER DENITRIFICATION

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Abstract—Biological denitrification of drinking water was studied in up flow laboratory reactors packed with wheat straw which served as the sole carbon source as well as the only physical support for the microorganisms The highest rates of denitrification (0 053 g N removed l^{-1} d^{-1}) were observed in fresh reactors during their first week of operation and the efficiency of the process declined thereafter The addition of fresh wheat straw brought about a temporary improvement of the denitrification performance and a regime of one weekly addition prevented the deterioration of a reactor which was operated for 5 months The rate of denitrification was affected by the water velocity and decreased at velocities above 0 054 m d^{-1} Colour and soluble organic carbon associated with fresh straw were removed by adsorption on powdered activated carbon © 1998 Elsevier Science Ltd All rights reserved

INTRODUCTION

Decrease of nitrate concentration is often required for drinking water in order to meet the standard of 11 29 mg nitrate-N per l in water for human consumption (WHO 1984) Among various methods available (physical chemical physico-chemical and biological) for the removal of nitrate biological removal (denitrification) is considered to be the most economical and environmentally sound and to be feasible on a large scale Denitrification is the reduction of nitrate to N_2 carried out by aerobic bacteria which in the absence of dissolved oxygen can use nitrate nitrogen as a terminal electron acceptor (Payne, 1981)

Most biological denitrification processes are based on heterotrophic bacteria utilizing organic carbon in the form of a simple compound (e g ethanol methanol acetate) However complex carbon sources such as cellulose-rich materials can also be used and we have recently studied the feasibility of using newspaper (Volokita *et al* 1996b) and cotton (Volokita *et al* 1996a) as carbon sources for the remediation of nitrate polluted groundwater

Microorganisms capable of degrading cellulose (cellulolytic microorganisms) are widely distributed in nature and usually occur in mixed culture with organisms which degrade associated polymers Initial cellulose degradation requires direct physical contact between the enzyme molecules and the surface of cellulose and complete degradation depends on the concerted action of various enzymes (cellulases) which may act in synergism (Beguin and Aubert 1994)

Cellulose is a basic component of all plant materials and constitutes the most abundant renewable resource in the world with an estimated production rate of 4 10^{10} ton per year (Couglan 1985) It is a linear glucose polymer with hydrogen bonding between hydroxyl groups of neighboring parallel chains and is organized in fibers in close association with lignin and hemicellulose (cotton is an exception being the purest form of naturally occurring cellulose) Thus wheat straw is a complex mixture of cellulose hemicelluloses (including xylan), pectins and lignins of which xylans and other xylose polymers constitute about 25% (Buchala and Wilkie 1973)

The capacity of wheat straw to support water denitrification has been shown by others Boussaid *et al* (1988) used field and laboratory reactors packed with wheat straw mixed with sand or with sand and maerl in the treatment groundwater and Avnimelech *et al* (1993) observed removal of nitrate in wheat straw filters designed to remove particulate matter from effluents prior to their application in drip irrigation

The formation of gases and the special characteristics of the carbon substrate were taken into consideration in the development of this water denitrification system In order to minimize clogging of the reactor due to the entrapment of N_2 bubbles

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32

WR 1838 [MB 8/6/98]

(Soares *et al* 1989 1991) a coarse matrix should be used On the other hand a high filling ratio of substrate would be necessary because wheat straw is a bulky non-diffusible carbon source which is slow and incompletely degraded Furthermore packing of the reactors as well as the removal and disposal of spent substrate should be easily performed Filling up the reactors with straw only appeared to be the most suitable process Thus the aim of this study was to determine if wheat straw can serve as the sole carbon substrate for the denitrification of drinking water as well as the sole physical support for bacterial growth

MATERIALS AND METHODS

Experimental apparatus

The reactors routinely used were PVC columns 50 cm high and 8 cm diameter packed with 103 g wheat straw with a thin layer of glass wool placed at each end Slightly larger columns (55 cm high and 10 cm diameter) were used when additions of 20 g of fresh straw were carried out because their design made it easier to open at the bottom where the new substrate was added they were packed with 177 g of straw so that the same filling ratio (41 g straw per l) was used in all experiments The columns were inoculated with a small amount of straw removed from an active denitrification column and the original inoculum was a mixture of forest and garden soils The freshly packed columns were filled with feed solution (tap water amended with 22 6 mg nitrate N l^{-1} and 3 mg l^{-1} phosphate) which was recirculated for 2 days After this inoculation period the reactors were started up (day 0) in an upflow mode (Fig 1) Water velocities v ($v = Q/A$ where Q is the measured flow rate and A is the cross section of the column) were calculated in m h^{-1}

Unless otherwise indicated the ambient temperature was maintained at 25 ± 1 C Influent and effluent samples were tested for nitrate nitrite ammonia pH dissolved organic carbon (DOC) and bacterial counts

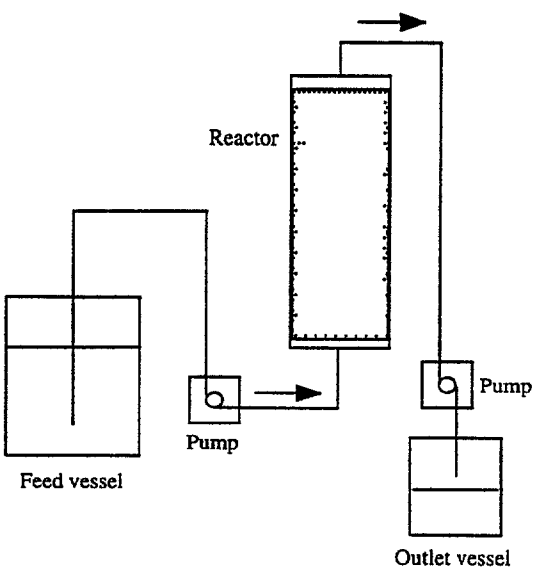

Fig 1 Schematic representation of the experimental set up

Adsorption experiments

The removal of dissolved organic carbon (DOC) by powdered activated carbon (PAC) was investigated in a preliminary experiment in which 20 g of fresh straw were soaked overnight at 4 C in 3 l tap water Constant volumes of water containing 141 mg DOC l^{-1} were then supplemented with a series of known weights of PAC in glass Erlenmeyer flasks and the slurries obtained were agitated at a constant stirring velocity (100 rpm) and temperature (25 ± 1 C) Prior to use the PAC was oven dried at 105 C for 2 h and then cooled in a dessicator The procedure adopted for the treatment of column effluents consisted of 1 h contact with 4% PAC

Analytical procedures

Nitrate was determined by the method of Cataldo *et al* (1975) and nitrite and ammonia were assayed according to APHA (1989) DOC was determined by a TOC analyzer (Dohrmann DC 190 Rosemount Analytical Santa Clara CA)

Bacterial counts

Colony forming units were counted by standard plating techniques on R2A agar (Difco Laboratories)

RESULTS AND DISCUSSION

Nitrate removal and water quality

A freshly inoculated reactor was operated for 2 months during which the water velocity was regulated to allow breakthrough of nitrate up to 6 mg N l^{-1} (Fig 2) High rates of nitrogen elimination were observed during the first days of operation

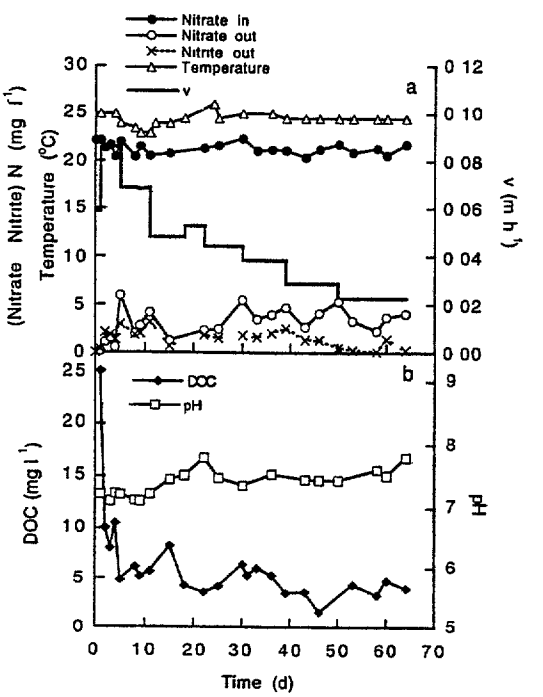

Fig 2 Concentration of nitrate N in the influent and pH and concentrations of nitrate N nitrite N and DOC in the effluent of a reactor packed with 103 g of wheat straw and operated at the temperatures and water velocities indicated

WR 1838 [MB 8/6/98]

when little nitrate and nitrite washed out at a water velocity of 0 086 m h^{-1} [Fig 2(a)] The soluble fraction of carbon present in the fresh straw allowed rapid microbial growth and the fast colonization of the substrate so that high removal of nitrate was observed at the start-up The DOC not utilized during the inoculation period was rapidly eluted from the reactor and from then on its concentration (corrected for the concentration in the influent) was consistently below 10 mg l^{-1} [Fig 2(b)]

Breakthrough of nitrite was always lower than that of nitrate and was never observed in its absence [Fig 2(a)] Ammonia seldom reached detection limits and its concentration never exceeded 0 3 mg l^{-1}

The water velocity was gradually lowered to 0 023 m h^{-1} in order to keep the breakthrough of nitrate within the set limit of 6 mg N l^{-1} (the ratio of N eliminated to N applied was usually within the 75–90% range) This indicated a continuous deterioration of the denitrification performance of the reactor which can be attributed to quantitative and qualitative changes in the substrate 40% of the initial weight was lost and 11 g of straw were consumed per g of N eliminated Due to the physical and chemical heterogeneity of the substrate this ratio should be considered as a very broad estimation only It can be assumed that all water soluble components and a good proportion of the cellulose and hemicellulose had been lost by the end of the experiment while lignin and mineral components remained unchanged

The number of colony forming bacteria in the effluent was in the order of 10^{-6} bacteria ml^{-1} and this is within the range found in denitrification with simple carbon sources

Effect of water velocity

The water velocity was lowered in a 13 day-old reactor until complete removal of nitrogen was achieved and then gradually increased up to 0 117 m h^{-1} (days 14 to 34 Fig 3) Breakthrough of nitrate and nitrite started at 0 054 m h^{-1} followed by a continuous increase of the former (up to approximately 12 mg N l^{-1}) while the latter stabilized below the concentration of 5 mg N l^{-1} [Figs 3 and 4(a)]

The highest rate of denitrification (0 053 g N removed l^{-1} d^{-1}) was observed at the water velocity of 0 054 m d^{-1} and the lowest (approximately 0 032 g N l^{-1} d^{-1}) at 0 092 m h^{-1} and thereafter [Fig 4(b)] The decrease in efficiency was somewhat exaggerated by changes taking place in the substrate This could be deduced from the breakthrough of nitrite and nitrate when the water velocity was lowered again while complete removal of nitrogen had been achieved earlier at the same water velocity (Fig 3) By day 40 the weight of straw had been reduced by 42%

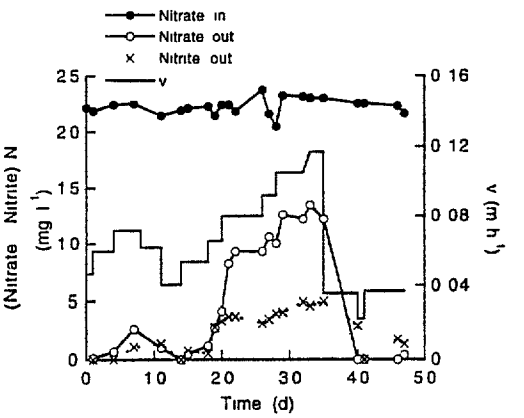

Fig 3 Concentration of nitrate N in the influent and concentrations of nitrate N and nitrite N in the effluent of a reactor similar to that in Fig 2 and operated at various water velocities and at the temperature of 25 ± 1 C

Thus the water velocity plays an important role in the denitrification performance of the system and the reasons for the sharp decrease in efficiency at the higher velocities may include wash out of bacteria wash-out of extracellular enzymes and wash out of solubilized substrate

Long term operation

In an attempt to maintain the initial high rates of denitrification in fresh reactors two identical reactors control and test, were started and operated under similar conditions except for the addition of 20 g of straw to the test reactor on the days marked

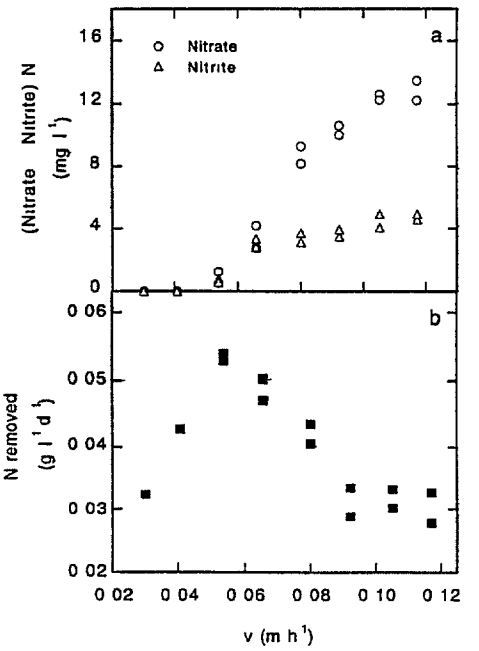

Fig 4 Effect of water velocity on the removal of nitrogen in the reactor described in Fig 3 (a) Effluent concentrations of nitrate N and nitrite N and (b) rates of nitrogen removal

34

WR 1838 [MB 8/6/98]

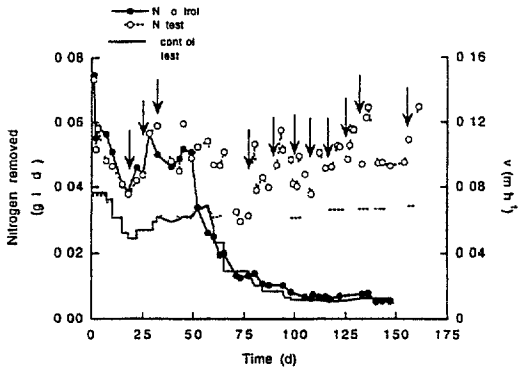

Fig 5 Removal of nitrogen in two reactors control and test packed with 177 g of wheat straw and operated at the flow rates indicated Arrows indicate the addition of 20 g of fresh straw to the test reactor

by an arrow (Fig 5) During the first 7 weeks both reactors removed approximately the same amount of nitrogen and the addition of fresh substrate had no immediate effect From then on the control reactor declined rapidly (the water velocity was lowered so that breakthrough of nitrate would not exceed 6 mg N l^{-1}) The replenished reactor maintained its performance for 2 weeks longer before also showing clear signs of decline At this stage the additions of substrate were resumed and brought about a short term increase (while the fresh soluble carbon washed out) as well as a lasting effect which prevented the deterioration of the system

Thus if the strategy of substrate replenishment is optimized wheat straw can support consistent denitrification performances for several months

In order to minimize the breakthrough of soluble carbon in the fresh substrate, additions were carried out at the base of the column Even so a considerable amount of DOC washed out (Fig 6) Most of the eluted DOC was removed by treatment with PAC (Fig 6) which also removed the colour associated with the fresh substrate (Fig 7) This is

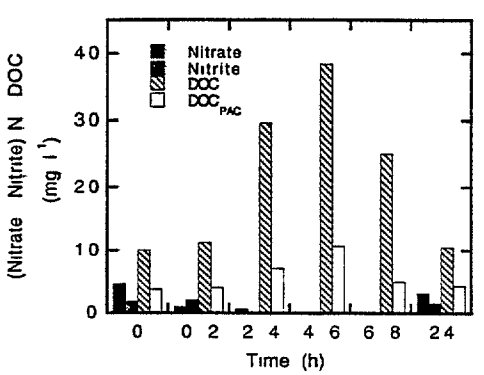

Fig 6 Elution of nitrate N nitrite N and DOC before and following the addition of 20 g of fresh straw on day 127 to the test column shown in Fig 5 DOC_{PAC} represents the DOC remaining after treatment of the effluent with powdered activated carbon

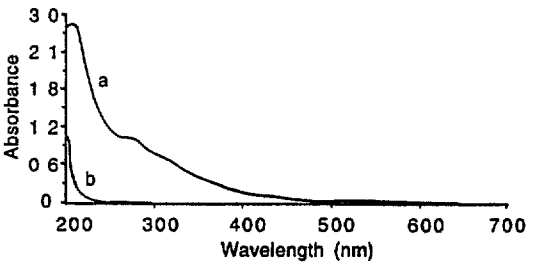

Fig 7 UV and visible spectra of effluent collected 4–6 h after the addition of 20 g of fresh straw to the reactor on Fig 5 (a) and of the same effluent after treatment with powdered activated carbon (b)

an important requirement for the implementation of the system in the field

CONCLUSIONS

(1) The denitrification performance of the system is affected by qualitative and quantitative changes of the carbon source The addition of fresh wheat straw temporarily improves the performance

(2) Water velocity has a marked effect on the denitrification performance of the system

(3) Wheat straw is a suitable carbon source for water denitrification and at the same time can serve as the sole support for bacterial growth

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