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HYDROGEN-DEPENDENT DENITRIFICATION OF DRINKING WATER

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3) Executive Summary

The objective of this study was to develop a novel, efficient and cost effective microbial process for the removal of nitrate from polluted drinking water. A small-scale system was designed in which denitrification was achieved by autotrophic bacteria using hydrogen as their energy source. Generation of the required hydrogen was an integral part of the denitrification set-up and was achieved by electrolysis of the water to be treated, in an electrolysis cell. The cell was divided into two compartments by a cation-exchange membrane which prevented oxygen formed in the anode from reaching the cathode and the water to be treated (oxygen inhibits denitrification); water was pumped through the cathodic compartment where it was enriched with hydrogen.

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 for upscaling.

The long-term performance and stability of the two-reactor system was studied, as well as the effects of its main operational parameters, namely water velocity and current intensity. The system was operated for one year and was very stable. The electrical energy used to remove approximately 20 mg nitrate-N l^{-1} was 1.17 KWh m^{-3}. The microbial population in the bioreactor consisted of four different strains which were studied, using classical and molecular methods.

Two Hungarian Ph.D. students, Istvan Kiss and Szilvia Szekeres, spent fruitful periods of 12 and 16 months in the Israeli laboratory; they are co-authors of two papers in press, and a third one is in advanced preparation.

All the project objectives have been fulfilled and the denitrification process developed presents the following advantages: (1) simplicity of operation, (2) long-term stability and reliability, (3) low running costs, compared to those in systems based on other energy sources, (4) safety of operation (there is no danger of forming explosive mixtures of hydrogen with air), and (5) minimal post-treatment requirements, since organic carbon is absent and washout of bacteria from the autotrophic bioreator is relatively low. The process is now ready for field upscaling. This type of technology is urgently needed in many developing countries around the world where nitrate-contamination of drinking water sources is a serious environmental problem.

4) Research Objectives

Widespread pollution of drinking water sources by nitrate is an important environmental problem in many parts of the world. The main causes for increasing levels of nitrate are increased usage of nitrogenous fertilizers, increasing irrigation with domestic wastewater and changes in land-use patterns. Nitrogenous compounds are transformed in soils via microbiologically mediated reactions. As a result, nitrate is formed and, being a very mobile ion, it readily passes through the soils and reaches the aquifer.

Concern over nitrate contamination is due to the link found between nitrate and methaemoglobinaemia, the blue-baby syndrome, in bottle-fed infants (under six months of age), and to the possible formation of n-nitroso compounds which are known to be potent carcinogens in the digestive tract (Mirvish, 1985). For these reasons, standards have been set for nitrate in water for human consumption: the European Community Drinking Water Directive (EC, 1980) incorporated into the legislation of most European countries stipulates a Maximum Admissible Concentration and Guide Level for nitrate-N of 11.30 mg l^{-1} and 5.65 mg l^{-1} respectively, and the same upper limit is also recommended by the World Health Organization (WHO, 1984).

Conventional drinking water treatment does not remove nitrate, and special treatment processes are required for lowering the concentration of nitrate to acceptable levels. The simplest solution, blending with low nitrate water, is often not viable, either because low nitrate water is not available or because of the expense of transferring low nitrate water over long distances. Removal of nitrate, then, has to be carried out, and a number of processes have been developed which can be chemical (chemical reduction), physical (reverse osmosis, electrodialysis), chemical-physical (ion exchange) or biological. Among these, only ion exchange and biological denitrification are feasible on a large scale. Furthermore, the biological process is the most environmentally sound as nitrate is completely eliminated, while ion exchange generates a waste of highly concentrated nitrate (and sulfate in least selective resins) and regenerating chemicals, chloride or bicarbonate. The total cost of biological treatment is usually reported to be lower than that of ion exchange (Hall *et al.*, 1985; Dahab, 1987). Some estimates place the cost of the two processes at the same level or lower for ion exchange, although the high price of disposal of the brine is often not included in the costs indicated for ion exchange (Rogalla *et al.*, 1990; Kapoor and Viraraghavan, 1997). Biological denitrification is a mechanism by which certain bacteria use nitrate as terminal electron acceptor in their respiratory processes, in the absence of oxygen. Denitrification consists of a sequence of enzymatic reactions leading to the evolution of nitrogen gas.

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 (Aragno 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 an 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 disinfection; 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.* (1994) used a single-reactor continuous system consisting of a tubular cathode with a concentric carbon rod (the anode); the biomass was immobilized with

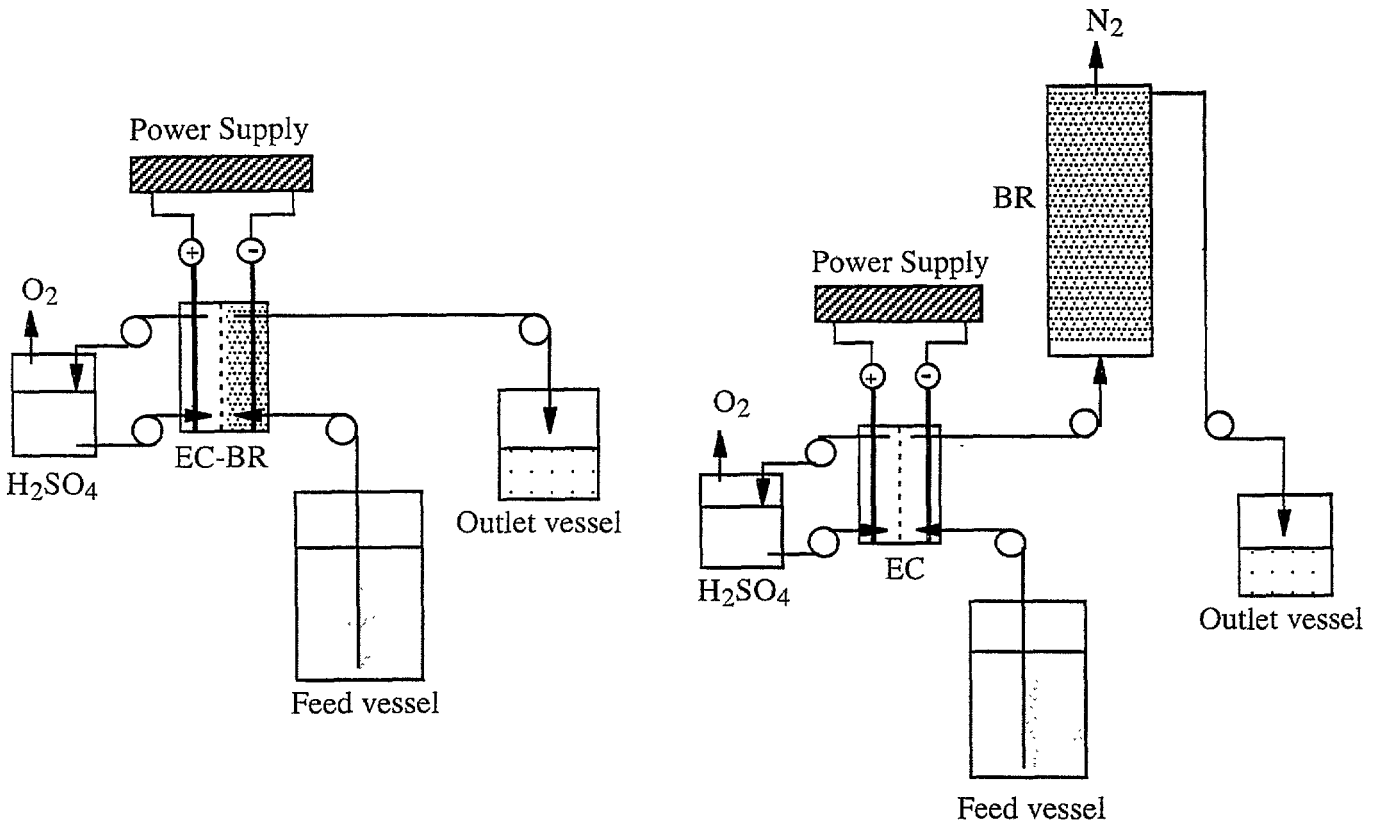

Figure 1. Schematic representation of the ECBR (left) and EC+BR (right) denitrification systems.

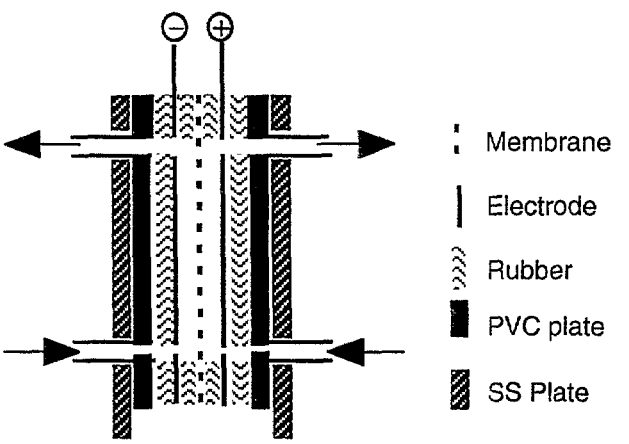

Figure 2. Schematic representation of the electrochemical cell.

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.*, 1994).

In our system, hydrogen was also generated in the water to be denitrified. However, our aim was to develop a continuous system which would be simple, stable and amenable to upscaling. Important design features were: (a) a cation permeable membrane separating the electrodes to

prevent O_2 generated at the anode from reaching the water to be treated (O_2 inhibits denitrification) while allowing transfer of H^+, (b) a large surface area for biofilm establishment provided by a bed of granulated activated carbon, and (c) biomass development under operating (autotrophic) conditions. Two configurations were studied: a single reactor with the biomass in the cathode compartment of the electrochemical cell, and a system consisting of two distinct units, an electrochemical cell and a bioreactor (Figs 1 and 2).

A comparative study of the two configurations showed that the two-reactor system was the most stable and suitable for long-term operation and upscaling (Kiss *et al.*, 2000). Its main advantages were (Szekeres *et al.*, 2000):

1) The simplicity of operation of a plug-flow denitrification reactor. The use of H_2 as energy source did not require storage and sparging of gas and the risk of forming dangerous mixtures with air was eliminated.
2) The system consisted of two modules in series, an electrochemical cell and a bioreactor, a configuration that allowed great flexibility of operation and simplified maintenance.
3) The process was 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 electrical energy used to remove approximately 20 mg nitrate-N l^{-1} (0.11 m h^{-1} water velocity) at a current intensity of 70 mA and a potential difference of 3 V was 1.17 KWh m^{-3}).
4) The quality of the treated water was good: changes in pH were small and the concentration of nitrite was low, even in the presence of very high effluent nitrate concentrations.

Significant support to this project was received from The Blaustein International Center, in the form of fellowships to two Hungarian Ph.D. students, Istvan Kiss and Szilvia Szekeres.

5) Methods and Results

All work originally proposed had been concluded by December 1999, and was discussed in detail in the report presented then. An extension of the project until the end of June 2000 was requested to allow for a study on the microbial population present in the bioreactors.

Thus, only this last study is now presented in detail (Appendix A), in the form of a draft of a paper in preparation.

The main research accomplishments were:

a) A comparative study of two system configurations, 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 prior to entering a packed-bed bioreactor. The two-reactor system was selected as the most suitable configuration for upscaling and long-term operation. This work was summarized in a paper published in *Water Science and Technology*; a copy of the accepted manuscript was included in the 1999 yearly report.

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 was summarized in a paper now in press in *Water Research*; an advanced draft of the manuscript was included in the 1999 yearly report.

c) A study of the composition and dynamics of the microbial population in the bioreactor. Bacteria present at various levels of the column were analyzed immediately after inoculation, and after one and three months of continuous operation. The isolates obtained were studied using classical and modern molecular methods. This study will be submitted for publication, and a draft of the manuscript in preparation is presented in Appendix A.

d) A two-step denitrification system consisting of ion-exchange removal of nitrate followed by hydrogen-dependent biological denitrification of the brine was developed and was described in detail in a manuscript submitted to *Acta Microbiol. Immunol.*, a copy of which was included in the 1999 yearly report.

6) Impact Relevance and Technology Transfer

During the project, K. Kovacs (Hungarian PI) became chairman of the newly founded Department of Biotechnology at the University of Szeged. The chemicals, labware and equipment purchased with funds from the project were of great help in the establishment of the new laboratory. No less important were the fellowships allocated to graduate students at the new department.

In the framework of the project, two Hungarian Ph.D. students, Istvan Kiss and Szilvia Szekeres, spent periods of 12 and 16 months at the Israeli laboratory, where they carried out a significant part of the experimental work. Their full integration in the Israeli team is shown in their participation in an international conference, in a published paper, a paper in press, and another in advanced stages of publication. These training periods had and will continue to have a great impact in the two young scientists. Their language skills improved considerably, as did their

capacity to tackle a concrete problem and search for a viable, practical solution. They learned new techniques and their interaction with other scientists at the Israeli institution is a good basis for future collaboration. The water treatment process developed in the project is ready for upscaling and field trials, and attempts to obtain suitable funding are in progress. As mentioned above, the process can benefit many people all over the world.

7) Project Activities/outputs

Meetings:

The two Hungarian partners visited the Israeli laboratory: Bela Polyak, in 1996, and Kornel Kovacs in 1997.

The Israeli PI visited the Hungarian group in May 1999, and in May 2000. Meetings were also held in 1996 and 1998 when the Israeli PI traveled to Hungary, in the framework of an Israel/Hungary exchange program and of an AID/CDR project with J. Bitskey, Budapest.

Training:

Two Hungarian Ph.D. students worked in the Israeli laboratory: Istvan Kiss, during the period April 1998-March 1999, and Szilvia Szekeres from April 1999 to June 2000.

Project publications:

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.

Kiss, I., S. Szekeres, T. T. Bejerano and M. I. M. Soares. 2000. Hydrogen-dependent denitrification: preliminary assessment of two bio-electrochemical systems. *Water Science and Technology* 24, 373-379.

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8) Project Productivity

The project accomplished all the proposed goals.

F) Future work

The denitrification system developed in the project needs to be upscaled and implemented in the field. This has to be done in collaboration with a process electrochemist. Further studies will be conducted on the physiology of the bacterial isolates.

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Appendix A

MICROBIAL POPULATION IN A HYDROGEN-DEPENDENT DENITRIFICATION REACTOR

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Running head: Bacteria in a hydrogen-dependent denitrification reactor

Abstract

The bacterial population responsible for the removal of nitrate in an H_2-dependent denitrification system was studied. The laboratory system was designed for the treatment of potable water and consisted of an electrochemical cell and a bioreactor (Szekeres, *et al.*, 2000). The bioreactors (columns packed with granulated active carbon) were inoculated with equal amounts of four different strains of denitrifying bacteria, then sampled immediately after inoculation, or after one or two months of continuous operation. The total number of bacteria (CFU) and the number of each different strain were determined at various levels of the bioreactor. The four strains present in the inoculum were identified as *Ochrobactrum anthropi*, *Pseudomonas stutzeri*, *Paracoccus panthotrophus* and *Paracoccus denitrificans*. From these, *Ochrobactrum anthropi*, *Pseudomonas stutzeri* and *Paracoccus panthotrophus* became predominant, and were responsible for denitrification in the reactor.

Key words -- bacteria, biological denitrification, drinking water, water treatment, hydrogen.

INTRODUCTION

Nitrate contamination of drinking water sources is an important environmental problem in many countries. Microbial removal of nitrate, denitrification, is the most environmentally sound and may be the most economical strategy for the reclamation for human consumption of nitrate polluted water (Soares, 2000).

Through biological denitrification, nitrate is reduced to nitrogen gas by certain bacteria which in the absence of oxygen are capable of using nitrate as terminal electron acceptor in their respiratory processes.

Denitrifiers are widespread in nature (Gamble *et al.*, 1977; Zumft, 1992), and in a denitrification system for drinking water, a suitable source of energy has to be supplied to the microorganisms, which can be an organic (heterotrophic system) or inorganic (autotrophic system) compound. The choice of substrate depends on a number of considerations such as cost, capacity and configuration of reactor, and postreatment of the denitrified water.

Hydrogen gas is one of the possible sources of energy for denitrification of potable water. It has the advantage of being completely harmless to water, and no further steps are required to

remove either excess substrate or its derivatives. The use of H_2 has been limited, due to its low solubility in water, and to the formation of flammable and explosive mixtures with air. Even so, a number of laboratory and field systems have been reported, in which H_2 was sparged in the water to be treated (Gros and Treutler, 1986; Gros *et al.*, 1986; Kurt *et al.*,1987; Dries *et al.,* 1988). More recently, Sakakibara and coworkers (Sakakibara and Kuroda, 1993; Sakakibara *et al.*,1994a) devised a different approach by which H_2 was generated in the denitrification reactor, by electrolysis of the water to be treated.

Lately, we have developed a continuous two-reactor denitrification system in which the water to be treated passed through the cathodic chamber of an electrochemical cell where it was enriched with H_2, prior to entering the biorector which consisted of a packed bed of granulated activated carbon (GAC) (Kiss *et al.*, 2000; Szekeres *et al.*, 2000). The system was found to be stable, simple to operate and economical (Szekeres *et al.*, 2000).

Although H_2 can serve as electron donor to many bacteria, growth under autotrophic conditions with nitrate as terminal electron acceptor is thought to be restricted to *Paracoccus denitrificans* and closely related strains (Aragno and Schlegel, 1992). Preliminary isolation of bacteria from our bioreactors showed that the number of distinct

strains present was very small. Reported here is an attempt to characterize the microorganisms responsible for denitrification in our water treatment system, how were they distributed along the column and what changes occurred with time, aspects which maybe of great importance for the efficiency and stability of the system.

Materials and Methods

Denitrification reactors

The denitrification set-up is schematically represented in Fig. 1, and has been described in detail elsewhere (Kiss *et al.*, 2000; Szekeres *et al.*, 2000). It consisted of an electrochemical reactor and a biological reactor. The bioreactor was a glass column with a 38.5 cm by 4.5 cm bed of 370 g of pre-washed 0.85-1.70 mm matrix GAC. The feed solution entered the bioreactor in an upward mode at the velocity of approximately 0.1 m h^{-1}; it consisted of tap water amended with inorganic carbon (70 mg $NaHCO_3$ l^{-1}), phosphorous (0.29 g H_3PO_4 l^{-1}) and nitrate (0.163 g KNO_3 l^{-1}). Prior to entering the bioreactor, the feed solution was pumped through the cathodic chamber of the electrochemical reactor where it was enriched with H_2. The system was maintained at the temperature of 25-27°C, and at the current intensity of 70 mA.

Inoculation of denitrification reactors

The bioreactors were inoculated as described before (Kiss *et al.*, 2000; Szekeres *et al.*, 2000), with a mixture of four denitrifying strains (A4, M2, M3 and P11). These strains had been isolated from our first H_2-dependent denitrification reactor, which in turn had been inoculated with an enrichment culture of sediment from an oxidation pond treating domestic wastewater, in mineral medium (Schlegel *et al.*, 1961) and under an atmosphere of 6% H_2 and 94% N_2. The enrichment was obtained in an anaerobic chamber, under autotrophic conditions, and with nitrate as electron acceptor and H_2 as electron donor. This first reactor was a column with a 10 cm^3 bed of GAC, and was operated for a few weeks, after which the biomass was detached from the substratum, and the resulting suspension was serially diluted and plated. The resulting colonies were tested for denitrifying capacity and the four distinct denitrifying isolates were found. These strains were grown separately in nutrient broth containing 5 g KNO_3 l^{-1}, for 48h. Cells were harvested by centrifugation, washed three times with 0.85% NaCl, resuspended in mineral medium, and the concentration of bacteria in each suspension was determined with a Petroff-Hausser counter. Aliquots of the four single-strain suspensions were mixed so that the final concentration of each strain was $6.5x10^8$ cells ml^{-1}

($2.6x10^9$ total cells ml^{-1}); 750 ml of this mixed suspension were recirculated for 3 days, at a rate of 1 ml min^{-1}.

Sampling of bacteria from denitrification reactors

Three parallel denitrification systems were set up and inoculated simultaneously as described above. One of the bioreactors was sampled immediately after inoculation; of the remaining two, one was stopped and sampled after 1 month, and the other was sampled after 3 months of operation. The GAC bed was carefully removed from the glass column so that 9 different sections were obtained, at 5 cm intervals. Samples of GAC weighing approximately 1 g (wet weight) were transferred to test tubes containing 10 ml 0.85% NaCl with 0.01% (v/v) Tween 80, and vortexed for 2 min. The resulting suspensions were serially diluted in 0.85% NaCl, and tripiclate aliquots were plated on R2A medium. After incubation for four days at 25 °C, all plates containing 30-150 discreet colonies were selected.

Characterization of bacterial isolates

Most morphological and biochemical studies were conducted according to standard procedures. Colony appearance was studied on R2A (Difco Laboratories) plates. Cell morphology was examined by phase-contrast microscopy. Cell dimensions were determined by using

an ocular micrometer calibrated with a stage micrometer. Motility was assessed by direct microscopic observation during growth, and by testing the ability of the strain to migrate from the point of inoculation through semisolid (0.3%) agar plates containing 20 mM succinate (Adler, 1966). Gram staining was determined as described by Gerdhart *et al.*, 1994) in cells grown in nutrient agar, at 30°C for 2 days. The presence of poly-β-hydroxybutyrate (PHB) granules was detected by microscopic examination of nitrogen-limited cells stained with Sudan Black. Spore formation was determined by malachite green staining of cells grown in nutrient agar. Catalase activity was detected on fresh colonies by the production of bubbles after addition of 3% hydrogen peroxide. Oxidase activity was determined by monitoring the oxidation of a 1% aquous solution of N,N,N',N'-tetramethyl-*p*-phenylenediamine on filter paper at room temperature. Production of hydrogen sulfide from cystein was detected by darkening of the lower portion of a lead acetate impregnated paper strip suspended over the culture. Salt tolerance was tested on LB liquid medium containing 0.5, 1 and 1.5 M NaCl, at 30°C. Growth at 7°C was tested on LB plates, and growth at 37, 40 and 45°C was determined in liquid LB medium. Denitrification activity was detected using Durham tubes and yeast extract medium supplemented with nitrate or nitrite and confirmed by assaying for nitrate and nitrite as described by (Tiedje, 1982). Autotrophic growth

with H_2 was tested in liquid mineral medium under agitation and an atmosphere similar to that recommended by Aragno and Schlegel (1992), and nitrogen fixation was also tested as described by the same authors, on plates of nitrogen-free mineral medium. The assimilation of α-ketoglutarate, propionic acid, L-alanine, L-arginine, caprylic acid, tryptophan, ethylene glycol and propylene glycol was studied by the addition of 0.2% (wt/vol) of one of these compounds (sterilized by filtration) to autoclaved standard mineral medium of Stanier *et al.* (1966). Further assimilation and metabolic tests were carried out using API 20NE strips (BioMerieux SA, Marcy, l'Etoile, France) and Biolog microplates (Biolog, Inc., Hayward, Calif.).

RAPD-PCR analysis

When counting colonies on R2A agar, differences in the appearance of fresh small colonies of strains A4 and P11 were difficult to detect; for this reason, they were distinguished by randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) analysis.

DNA for use as template was prepared directly from plate-grown colonies. A single colony was picked with a sterile toothpick and the cells were suspended in 10 µl of 0.85 % NaCl followed by heating at 97°C for 10 min. Among a number of PCR primers tested, two generated distinct DNA-banding patterns for each of the two strains;

they were the 10-mer primer RAPD26 (5'-CTCGGACTAT-3') and the 10-mer primer RAPD35 (5'-TCATAGGTGC-3') (Fig. 2). The PCR assay medium (25 µl) contained the following: 10 mM Tris-HCl (pH 8.3), 4 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, 0.4 µM of primer, 5 U *Taq* DNA polymerase, and 1 µl of the denaturated cell suspension. The assay buffer was covered with 25 µl of sterile mineral oil. The DNA fragments were amplified in a programmable DNA thermocycler (Model PTC-150 Minicycler™, MJ Research, Watertown, MA) by 2 min at 94°C for denaturation, five cycles of 30 s at 94°C (denaturation), 30 s at 36°C (annealing), and 1 min at 72°C (extension). These were followed by 35 cycles of 30 s at 94°C, 30 s at 46°C, and 1 min at 72 °C. The final extension was 4 min at 72°C. The PCR products were analysed by gel electrophoresis on 1.5% agarose gels and visualised by staining with ethidium bromide.

Assay of denitrification activity

Rates of denitrification activity were measured in single strain suspensions prepared by growing the cells to mid-logarithmic phase in LB medium, harvesting by centrifugation (4,000 rpm for 15 minutes), washing three times in 15 ml of 0.85% NaCl, and resuspending in 10 ml of mineral basal medium supplemented with 0.65 g KNO_3 l^{-1} and 0.2% (w/v) sodium acetate. The suspensions were transferred to two 25 ml

glass bottles. These reaction bottles were closed with butyl rubber septa in screw caps, flushed with N_2 to remove all traces of O_2, and incubated in a shaking water bath, at 30°C. At 15 min intervals, a liquid sample was removed for the determination of nitrate, nitrite and ammonia. Nitrite and ammonia were not detected in the assay medium.

Analytical methods and bacterial counts

Nitrate was determined as described by Cataldo *et al.* (1975), nitrite was assayed according to Snell and Snell (1949) and ammonia was determined by nesslerization (Standard Methods, 1992).

Protein was determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as standard. Samples were prepared for determination by centrifugation of 1ml aliquots of cell suspensions for 5 min at 14,000 rpm, followed by addition of 0.2 ml 1N NaOH to the pellets, and boiling for 10 min; after centrifugation, aliquots of the supernatant were analyzed.

The weight of GAC samples was determined by drying to constant weight at 105°C.

Colony forming units (CFU) were determined by standard plating techniques on R2A agar.

RESULTS AND DISCUSSION

As described above, the bioreactors were inoculated with a mixture of four strains. Preliminary observations of bacteria isolated from the reactors showed a very small number of distinct colonies. This was not surprising as it is generally believed that only *Paracoccus denitrificans* and closely related strains can grow autotrophically with nitrate as terminal electron acceptor (Aragno and Schlegel, 1992).

A more detailed study was then carried out in order to elucidate the composition of the bacterial population present in the reactors, its distribution along the column, and its changes with time, factors which may be important for the reliable operation of a potable water treatment system. Three parallel columns were inoculated, one was sampled immediately after inoculation, another was operated for one month prior to sampling, and the remaining one was sampled three months after inoculation. Bacteria on GAC samples removed from different levels of the columns were serially diluted and cultivated on agar plates.

Immediately after inoculation, total counts of bacteria were approximately constant at all levels of reactor, and in the order of 10^8 CFU g^{-1} GAC (Fig. 3). One month later, the concentration of bacteria had increased markedly, and was higher in the lower 15 cm, at approximately $2.5*10^{10}$ CFU g^{-1} GAC. From this level up, a gradual

decline was observed, until a minimum of $1.7*10^9$ CFU g^{-1} GAC in the upper 5 cm of the column. Two months later, no further changes could be detected in the total number of bacteria (Fig. 3).

A less constant pattern emerged from the qualitative analysis of the microbial population present in the column (Fig. 4). After inoculation, strain M3 was the less abundant while the other three strains appeared in approximately equal proportions (Fig. 4a). After one month of operation, however, strain A4 had become the most abundant and the proportion of strain P11 had declined drastically at all levels of the column, to below 5% of the total CFU (Fig. 4b). In the three month old column (Fig. 4c), the proportion of CFU of strain M3 was still the highest, that of strain A4 was somewhat higher than in the one-month old column, P11 had declined further, and up to 1% CFU of other strains was found. These "new" strains showed no denitrification capacity.

The reason for the changes observed in time in the relative numbers of the four inoculating strains was not clear. The inoculum contained equal numbers of each strain and immediately after inoculation strains M2, A4 and P11 were present at approximately similar concentrations, while that of strain M3 was somewhat lower. This suggested that the three first strains were more successful than M3 in attaching to the GAC particles. One month later, however, strain

M3 had become the most abundant, a position which it still maintained two months later. On the other hand, strain P11 constituted more than 25% of the bacteria present after inoculation, but its numbers declined drastically within the first month of operation.

A possible reason for the marked temporal changes observed in the numbers of bacteria of strains M3 and P11 could be differences in their capacity to use nitrate as terminal electron acceptor. Therefore, the denitrification capacity of the four strains was tested (Table 1). Strain M3 showed the highest specific rate of denitrification, at 22.4 mmoles N lost h^{-1} g^{-1} protein; the denitrification rate of strain P11 was the lowest, at 9.0 mmoles N lost h^{-1} g^{-1} protein. The rates of denitrification of strains M2 and A4 were 18.9 and 15.6 mmoles N lost h^{-1} g^{-1} protein.

Thus, the relatively low specific rate of denitrification appeared to be at least one of the reasons for the decline of P11. Similarly, the relatively high specific rates of denitrification found in strain M3 may have conferred a competitive edge, allowing it to become the dominant biomass component in the reactor.

The environment in the bioreactor was highly selective for organisms that had the capacity to simultaneously carry out the following: utilize nitrate as nitrogen source, grow with inorganic carbon, utilize H_2 as electron donor and use nitrate as terminal (respiratory)

electron acceptor. As can be seen in Fig. 4, these conditions imposed a severe restriction to the establishment of exogenous, opportunistic bacteria. In practical terms, this confers stability to the system and assures the success of a long-term operation. A one-year study reported elsewhere showed that high rates of denitrification were reached during the first week of operation and were maintained until the end of the experiment (Szekeres *et al.*, 2000).

A comprehensive study was then carried out in order to identify the four strains. It included analysis of their morphological, cultural and metabolic characteristics, and culminated with the analysis of cellular fatty acids and of partial sequencing of the 16S rDNA. The results obtained are summarized as follows:

Strain M2. Cells were rods, measuring 0.5-0.8 μm in width and 1.5-5 μm in length, gram negative, motile and spores were not formed. They grew at 7°C and at 41°C. Colonies on R2A were circular, transparent, yellowish, spreading and very fast growing. The strain was catalase, oxidase and aminopeptidase positive, denitrified nitrate to N_2 and grew autotrophicaly with H_2. Negative reactions were observed for urease, indole production, arginine dehydrolase, glucose acidification, gelatine hydrolysis, β-galactosidase and glucose acidification. Gluconate, malate, citrate, propionic acid, malate, acetate, citrate, caprylic acid, sebacinate, azelate, caprate,

propionic acid, caprylic acid, L-valine, trehalose, glucose and maltose were utilized. Tryptophan, L-histidine, L-arginine, L- alanine, N-acetyl-glucosamine, α-ketogluterate, phenylacetate, geraniol, adipate, xylose, arabinose, mannose and mannitol were not utilized.

The partial sequencing of the 16S rDNA showed similarities of more than 99% to different strains *of Pseudomonas stutzeri.* This identification was in agreement with that suggested by the profile of cellular fatty acids (Table 2), and by the physiological and cultural characteristics.

Strain A4. Cells were gram negative rods measuring 0.5-0.7 μm in width 1.5-2 μm in length. They grew at 37°C but could not grow at 7°C and at 40°C. Colonies on R2A were medium size, circular with entire borders, convex, milky-white and translucent. Nitrate was reduced to N_2 and autotrophic growth with H_2 was present. The strain was positive for catalase, oxidase, aminopeptidase, urease and production of H_2S. It was negative for arginine dehydrolase, indole production, β-galactosidase, acidfication of glucose, and for the hydrolyis of starch, DNA, escoline, casein and gelatin. Acid was produced from D-glucose, D-fructose, D-xylose, arabinose, ethanol, rhamnose and raffinose. The following carbon substrates were utilized: citrate, malate, gluconate, acetate, caprylic acid, caprate, propionic acid, β-hydroxybutyrate, N-acetylglucosamine, L-arginine,

tryptophan, arabinose, mannose, glucose, mannitol and maltose. Phenylacetate, α-ketogluterate, adipate, L-alanine and methanol were not utilized. The strain could grow on MacConkey medium.

The partial sequencing of the 16S rDNA showed a similarity of more than 99.5% to the type strain of the *Ochrobactrum anthropi.* The profile of the cellular fatty acids (Table 2) was typical for *Ochrobactrum anthropi.*

Strain P11: Cells were short rods 0.6-0.8 μm in width and 1.5-3.0 μm in length, non-motile, gram negative and occurring singly or in pairs. PHB was stored. Colonies on R2A were small, circular, smooth, white, shiny and with entire margins. Cells grew at pH 6, at 40°C and at 3% NaCl. They reduced nitrate to N_2 and grew autotrophicaly with H_2. The strain was positive for oxidase, catalase, aminopeptidase and production of H_2S; it was negative for indole production, arginine dihydrolase, urease, β-galactosidase, glucose acidification, and for the hydrolysis of gelatine and escoline. The following carbon sources were utilized: glucose, arabinose, mannitol, maltose, N-acetylglucosamine, methylamine, L-alanine, L-arginine, gluconate, caprylic acid, acetate, malate, propionic acid, phenylacetate and trehalose, Tryptophan, α-ketogluterate, adipate, citrate, caprate, mannose and xylose were not utilized.

The partial 16S rDNA sequence analysis showed a similarity of 100% to the Type strain of the species *Paracoccus denitrificans.* The profile of cellular fatty acids (Table 2) and the physiological characteristics also pointed to this species.

Strain M3. Cells were short rods with dimensions identical to those of cells of strain P11. Their cultural and metabolic characteristics were similar to those of P11, except for the utilization of tryptophan which was positive. However, colonies on R2A differed markedly: they were undulate, transparent and dull, with a shiny center. The partial 16S rDNA showed a similarity of 100% to the type strain of the species *Paracoccus panthotrophus* (and 97.8% to *Paracoccus denitrificans*). The profile of the cellular fatty acids (Table 2) was typical for the genus *Paracoccus.*

Thus, according to current taxonomic database, the four main strains present in the reactors, A4, M2, M3 and P11, were identified as *Ochrobactrum anthropi*, *Pseudomonas stutzeri, Paracoccus panthotrophus* and *Paracoccus denitrificans,* respectively.

CONCLUSIONS

- Although the set-up and the solutions used were not sterile, the highly selective conditions in the bioreactor prevented the establishment of strains other than those present in the inoculum.

In practical terms, this confers stability to the system and assures the success of a long-term operation.

- Bacteria in the inoculum were identified as *Ochrobactrum anthropi*, *Pseudomonas stutzeri*, *Paracoccus panthotrophus* and *Paracoccus denitrificans*. From these, *Ochrobactrum anthropi*, *Pseudomonas stutzeri* and *Paracoccus panthotrophus* became predominant, and were responsible for denitrification in the reactor.

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Table 1. Rates of denitrification activity of the four strains

Strain	mmoles N lost h^{-1} g^{-1} protein
A4	15.6
M2	18.9
M3	22.4
P11	9.0

Table 2. Cellular fatty acid composition (%) of strains A4, M2, M3 and P11.

Strain	10:0 3OH	12:0	12:0 3OH	14:0	16:0	15:0 iso 3OH	17:0 cyclo	17:1 w8c	18:0	18:1 2OH	18:1 w7c	19:0 Cyclo w8c	Summed Feature 2[a]	Summed feature 3[b]	Summed feature 7[c]	Others[d]
A4					5.52		1.60		2.49	3.20	77.06	8.38		1.75		
M2	2.51	7.73	1.39	1.33	17.4		1.69				32.88			35.03		
M3	3.19				6.61					1.74					83.73	
P11	2.29				6.77			1.15	3.08		83.30		1.30			2.12

[a] Summed feature 2 comprises 14:0 3OH and 16:1 iso I.
[b] Summed feature 3 comprises 16:1 w7c and15:O iso 2OH.
[c] Summed feature 7 comprises 18:1 w7c/w9t/w12 and 18:1 w9c/w12t/w7c.
[d] Unknown 11.798.

Figures legends

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

Fig. 2. Ethidium bromide stained 1.5% agarose gel of PCR-amplified DNA. Lanes 1 and 3 are DNA of strain A4 amplified by primers RAPD26 and RAPD35 respectively. Lanes 2 and 4 are DNA of strain P11 amplified by primers RAPD26 and RAPD35 respectively. M: 100 bp ladder, C: control without DNA template.

Fig. 3. Total number of cells (CFU) at different highs of bioreactors, immediately after inoculation (a), and after one month (b) and three months (c) of operation.

Fig. 4. Strain distribution at different highs of bioreactors, immediately after inoculation (a), and after one (b) and three months (c) of operation.

Fig. 1

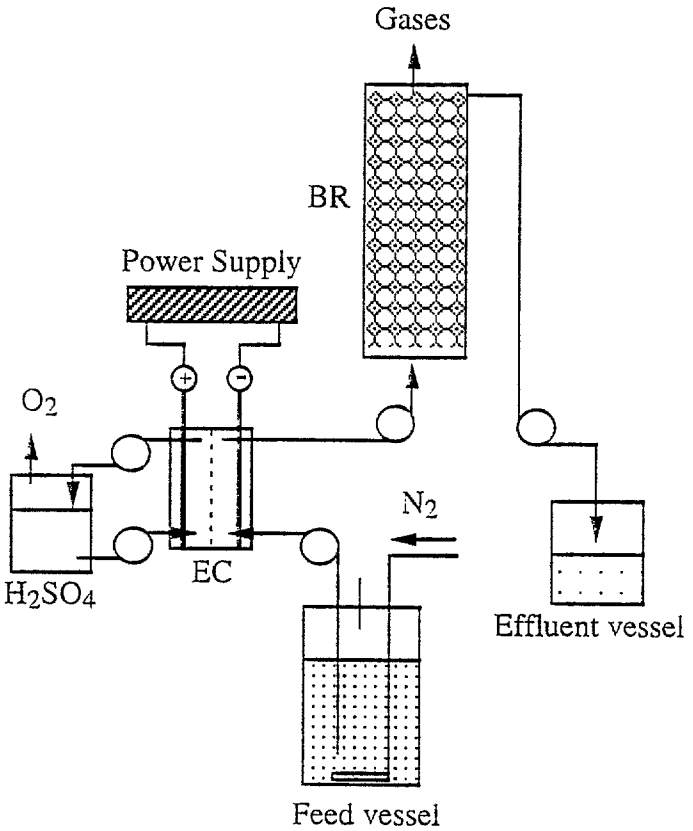
Gases
BR
Power Supply
O2
N2
H2SO4
EC
Effluent vessel
Feed vessel

Fig. 2

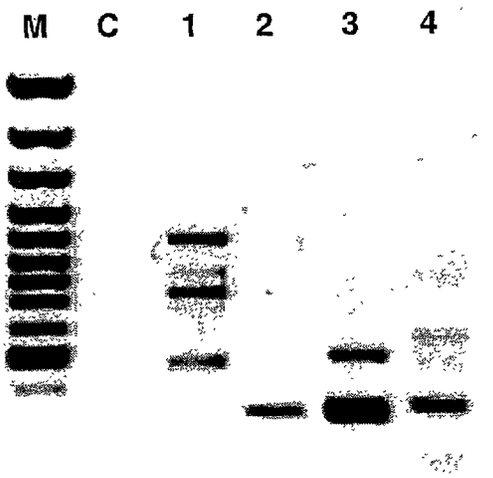

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Fig. 3

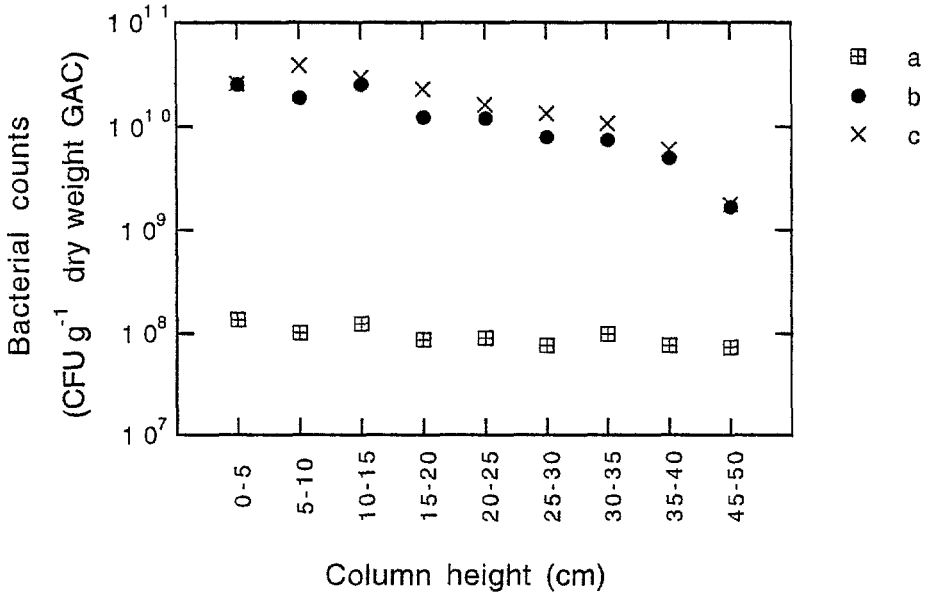
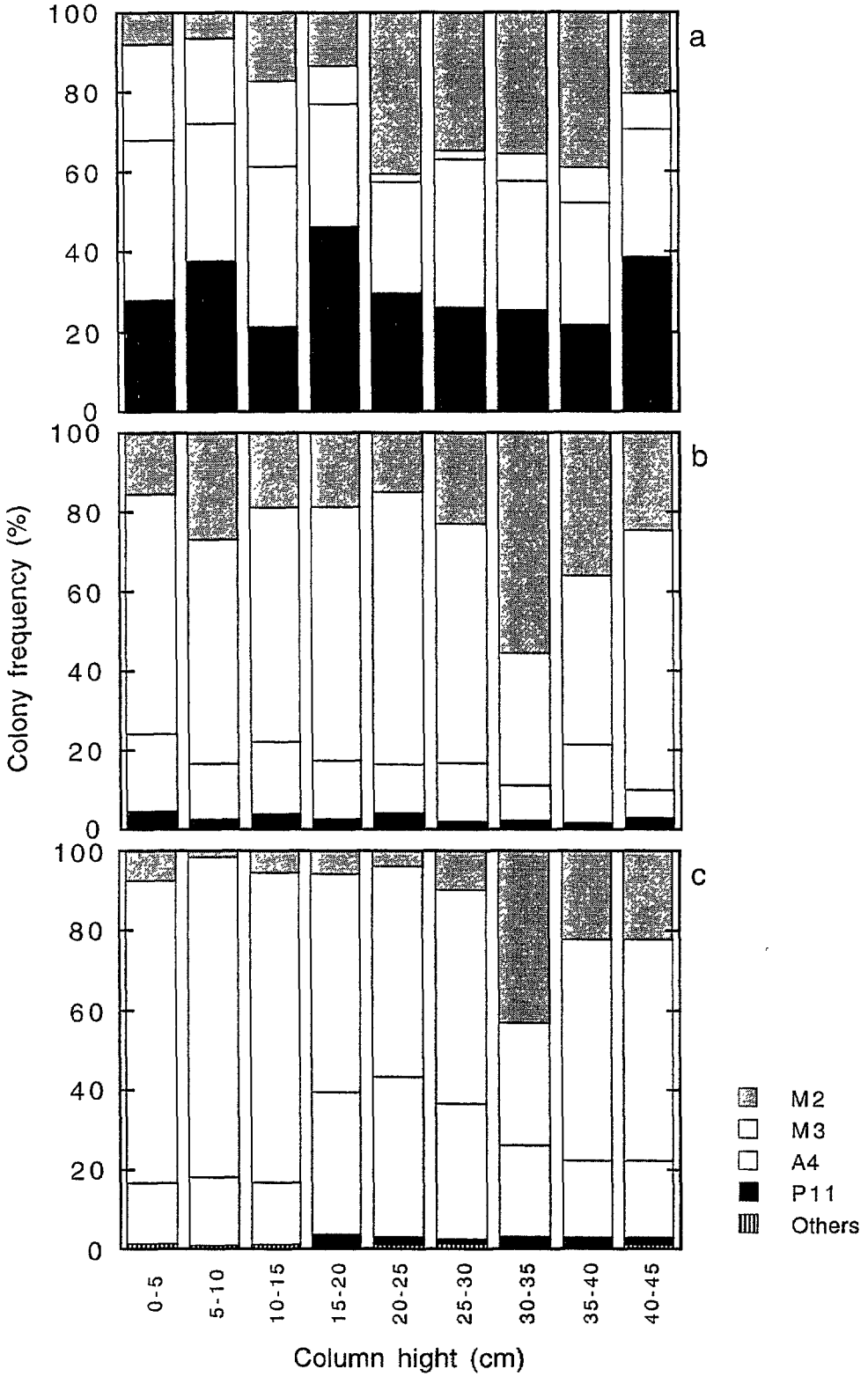

Bacterial counts
(CFU g-1 dry weight GAC)
10^11
10^10
10^9
10^8
10^7
0-5
5-10
10-15
15-20
20-25
25-30
30-35
35-40
45-50
Column height (cm)
a
b
c

Fig. 4


a
b
c
100
80
60
40
20
0
Colony frequency (%)
0-5
5-10
10-15
15-20
20-25
25-30
30-35
35-40
40-45
Column hight (cm)
M2
M3
A4
P11
Others