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Influence of inocula over start up of a denitrifying submerged filter applied to nitrate contaminated groundwater treatment

B. Moreno [a,b,*], M.A. Gómez [a,b], A. Ramos [a,b], J. González-López [b], E. Hontoria [a,b]

[a] *Department of Civil Engineering, E.T.S.I.C.C.P., Campus de Fuentenueva s/n, University of Granada, 18071 Granada, Spain*
[b] *Institute of Water Research, University of Granada, 18071 Granada, Spain*

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Abstract

Pure culture of denitrifying bacteria isolated from heterogeneous biofilm was applied as inocula to a submerged filter for removing nitrogen from contaminated groundwater. Five highly denitrifying bacteria were used, comparing their attachment, nitrogen-removal ability and final water quality in the start up of the system. Our experiments showed that inocula selection is the crucial step when the submerged filter is applied to obtain drinking water, since selected strains vary in their ability to colonise support material and to remove nitrogen, and in their effect on treated water quality during start up phase. *Hidrogenophaga pseudoflava* strain proved to be the most suitable inoculum out of the five tested, under the experimental conditions.

Keywords: Biological denitrification; Groundwater; Biofilm; Submerged filter start up; Selective inoculation

1. Introduction

Nitrate contamination of groundwater is a problem recognized throughout the world. Among the various methods for dealing with the problem, biological denitrification is of particular interest since it is the only selective method for removing nitrate by transforming it into nitrogen, the inert gas which makes up most of the atmosphere. Today denitrification is a highly advanced method both technically and scientifically [1–3].

Submerged filter technology has proved effective for biological treatment of groundwater [4,5]. In this process, the bacterial biofilm grows on a support through which the water passes, and the biofilm always remains under water. To set up a process of submerged filter denitrification it is necessary to control a series of parameters such as pH, temperature and dissolved oxygen concentration [6], and also to supply the biofilter with concentrations of carbon source [4]. Once this has been done, one of the most important factors for the process to function successfully is the microbial activity of the biofilm [7].

A major drawback of groundwater denitrification is the fact that formation of biofilm from its own microbiota in the influent is necessarily an extremely slow process, owing to the low presence of microorganisms in the water. For this reason it is necessary to inoculate the submerged filter previously, bearing in mind that the type of inoculation will determine the initial microbial activity in the biofilm. Experiments have been undertaken using various inocula, typically mix liquor of activated sludge [1,4,6]. However, this type of inoculation presents certain drawbacks such as the presence of pathogens in the treated water and the formation of heterogeneous biofilms composed of various microorganisms with different levels of denitrifying activity and even non-denitrifying bacterial groups [8]. In consequence problems arise such as the presence of nitrate-reducing bacteria, responsible for concentrations of nitrite in the effluent, and sulphate-reducing bacteria, which may lead to the presence of sulphide acid in the treated water. Additionally, other non-denitrifying bacterial groups may consume the added carbon source.

* Corresponding author. Tel.: +34 958 249462; fax: +34 958 246138.
E-mail address: bgmoreno@ugr.es (B. Moreno).

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These problems arising from the bacterial composition of the biofilm result in a deterioration in the quality of the treated water and are affected both by the carbon source employed [9,10] and by the concentrations of carbon source added [8]. A possible solution lies in selective inoculation using bacteria with high denitrifying activity as inocula. A previous comparative study between a submerged filter inoculated with mix liquor of activated sludge and a pure culture [5] showed the advantages of selective inoculation, improving design parameters and running of the process.

The start up of a submerged filter is a crucial phase when it is applied to nitrate contaminated groundwater treatment, obtaining treated water to use as drinking water. This pressing need to obtain high quality water requires a technology with a fast response time. However, several factors such as nitrite generation, low nitrogen removal capacity and biofilm microbial detachment, prolong the necessary time for reaching steady state conditions. The addition of pure cultures reduces the start up period [11].

Given the existence of many bacterial species with high denitrifying activity, selective inoculation should ideally test different microorganisms isolated from heterogeneous denitrifying biofilms. The aim of the present study is therefore to compare the denitrifying performance of submerged filters in the start up phase employing different microorganisms as inocula, in order to determine the most suitable selective inoculum for use in this process. Suitability depends not only on the inoculum's denitrifying activity, but also on the capacity to colonise inert support and on the immediate quality of water obtained.

2. Materials and methods

2.1. Isolation of denitrifying strains

Different bacterial strains were isolated from a denitrifying biofilm used in submerged filter treatment of groundwater contaminated with nitrate [5]. Total platable bacteria in the denitrifying biofilm were counted by the dilution-plate technique, using nitrate–sucrose-agar (NSA) medium [12]. In order to extract the biofilm, 1 g of support material, previously mixed in 100 ml of sterile saline solution (0.9% NaCl), was sonicated for 1 min and homogenised with a magnetic stirrer (700 rpm, 30 min). The inoculated agar plates (three replicates) were incubated anaerobically (Anaerogen System, OXOID) at $30 \pm 1\,^{\circ}C$ for 2 weeks.

All the isolated and purified strains from biofilms were tested for denitrifying activity (capacity of reducing NO_3^- to N_2O or N_2). Every single isolated strain was inoculated in a hermetically sealed vial containing 5 ml of nitrate–sucrose-broth (NSB). Previous to inoculation the vial inner air was removed and substituted by helium. Inside the vial, 10% of acetylene was introduced to inhibit any existing nitrous oxide reductase activity, in accordance with Yoshinari and Knowles [13]. The inoculated vials were incubated in the dark for 24 h at $30 \pm 1\,^{\circ}C$. After this time, N_2O presence was determined inside each vial by employing a gas chromatograph Varian CX3400 equipped with a thermal conductivity detector and pure N_2O (Air–Liquid) as control.

2.2. Identification of the strains

Denitrifying strains selected using the above method were identified by analysis of the sequence of the encoding 16S rRNA (16S rDNA). Primers fD1 and rD1 [14] were synthesized by Amersham Pharmacia (Sweden) and were used to amplify almost the full length of 16S rRNA gene from strains. A fresh cultured colony of each strain was lysed by the addition of 20 μl of a mixture of NaOK ($0.05\,mol\,l^{-1}$)–SDS ($0.25\%\,MV^{-1}$) and then boiled for 15 min. The lysate was adjusted to 200 μl with sterile water and centrifuged at $10\,000 \times g$ for 5 min. The cleared lysate (4 μl) was used as template for amplification. PCR was done adding to the lysate $1\times$ PCR buffer (GeneCraft, Germany) $1.5\,\mu mol\,l^{-1}$ $MgCl_2$ (GeneCraft, Germany), $200\,\mu mol\,l^{-1}$ dNTPs (Roche Molecular Brochemicals, Germany), 20 pmol of each primer, and 1 U of Taq polymercase (GeneCraft, Germany). Final volume of the reaction tubes was adjusted to 50 μl. Reactions were run in a Perkin-Elmer Gene Amp PCR System 2400 (Perkin-Elmer, Norwalk, USA). The temperature profile was the one previously described by Vinuesa et al. [15]. PCR products were run on 1% agarose gels and the bands were purified using the Quiaex II kit (Quiagen, Germany). The nucleotide sequence of the purified bands was determined by the dideoxy chain terminator method, using the ABI-PRISM Big Dye Terminator Cycle Sequence Ready Reaction Kit (Perkin-Elmer, USA) and automated sequencer Applied Biosystems ABI 373 (Perkin-Elmer, USA). Sequence data were analysed using the GCG Wisconsin Package v. 10.1. program (Genetics Computing Group, Madison, WI, USA), and were compared to sequences in EMBL bank using FASTA v. 33t07 [16].

2.3. Inoculation of the submerged filter reactors

The lab-scale plant used in this study consisted of a glass cylindrical column (50 cm high and 6 cm diameter), operating with a downward flow of nitrate contaminated groundwater ($22.58\,mg\,N\text{-}NO_3^-/l$). The anoxic reactor was packed with clayey schists of 2–4 mm average size and $1.75\,g/cm^3$ density, up to 15 cm high. A communicating vessels system was employed for its operation (Fig. 1).

Selected denitrifying microorganisms were grown in NSB medium [12] at $30\,^{\circ}C$ for 48 h. Two litres of cell culture ($10^{11}\,cfu\,ml^{-1}$ approximately) of each selected strain were recirculated at a flow rate of 0.24 l/h for 24 h. Subsequently, groundwater contaminated with nitrate was pumped in at a flow rate of 0.48 l/h (hydraulic loading $4\,m^3/(m^2\,day)$). The composition of NSB medium (per litre of distilled water) was the following: $NaNO_3$ 2.0 g, K_2HPO_4 1.0 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, KCl 0.5 g, $FeSO_4{\cdot}7H_2O$ 0.01 g, yeast extract 1.0 g, sucrose 30.0 g. Groundwater to be treated was previously

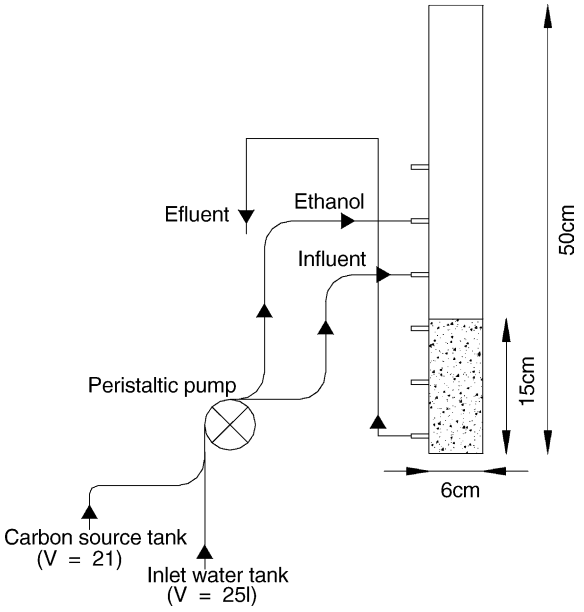

Fig. 1. Lab-scale plant used for the experiments.

sterilized in order to avoid interferences in the comparative study due to the presence in the system of other uncontrolled microorganisms. Groundwater characteristics were the following: NO_3^- 100.0 mg/l, NO_2^- 0.0 mg/l, PO_4^{3-} 0.4 mg/l, SO_4^{2-} 180.0 mg/l, COD 0.0 mg/l and pH 7.3. After each experiment, the plant and support material were then cleaned manually and sterilized prior to the next test. A concentrated stock solution of ethanol was stored in a tank from which it was pumped continuously to the influent pipe. An excess of carbon source (0.5 mg ethanol/mg NO_3^-) was added to avoid interferences. Complete anoxic conditions were monitored by means of stoichiometric quantity of sodium sulphite (Na_2SO_3) added to the water to be treated. The reactor was incubated in a thermostated chamber (Bioblock Scientific) at 20 °C.

2.4. Electron microscopy studies

Scanning electron microscopy observations of the biofilm produced in the submerged filter plant were made after inoculation. The cells of the biofilm growth on the inert support were immediately fixed with glutaraldehyde (3%) in PBS (130 mM NaCl and 10 mM Na_2HPO_4/NaH_2PO_4, pH 7.4) for 2 h, then rinsed and treated with 1% osmium-oxide for 3 h. Subsequent dehydration included rinse and retention in a graded ethanol series (30, 50, 70, 90 and 100%). Finally, the samples were dried to the critical point and mounted on support stubs. The samples were viewed by a scanning electron microscope (Hitachi) without gold coating.

2.5. Sampling and analytical determination

Every 24 h after inoculation, water samples (50 ml) were collected from the inlet and the outlet of the column obtaining five replicates per day for each inoculum assayed. All the samples were monitored for nitrate and nitrite concentration in order to determine capacity to eliminate nitrogen from the influent in each case. Turbidity and recount of microorganisms in the treated water were also studied to determine the final water quality.

Nitrate and nitrite concentrations were determined by ionic chromatography (Dionex® DX-300). Separation and dilution of the anions was carried out on an Ionpac® AS14 column using a solution of carbonate–bicarbonate as eluent, and sulphuric acid as regenerant. Before the analysis, all samples were filtered through 0.22 μm membrane filters (Millipore HAWP) and diluted to achieve nitrate and nitrite concentrations lower than 10 mg/l. Turbidity was determined by the spectrophotometic method at 650 nm [17]. Total platable microorganisms in treated water were determined on plates (three replicates) using the NSA medium [12] according to Gómez et al. [4].

2.6. Statistical analysis

Data obtained through this study were analysed by computer-assisted statistics, using Statgraphics Plus for Windows 3.0 (Statistical Graphics Corp., 1997). The least significant differences test (LSD-test) was used to measure the differences among the selected strains for the various parameters analysed both in the support material and in the effluent. An analysis of variance (ANOVA) test was used to assess the homogeneity of variance with significance level of 1% ($p < 0.01$).

3. Results and discussion

3.1. Denitrifying bacteria isolation

More than 250 different strains able to grow on NSA medium were isolated and purified from a stable denitrifying biofilm formed starting from mixed liquor. However, only five strains showed high levels of denitrification activity (Table 1) and consequently were selected for further studies.

The strategy used to sequence the 16S rDNA gene of selected denitrifying strains produced a continuous stretch of nucleotides representing >95% of the primary 16S rDNA sequence. Percentage identity values obtained after pairwise alignment of the sequences of 16S rDNA of the isolated denitrifying strains against the sequences at the EMBL database are shown in Table 1.

3.2. SEM of biofilm formation

Selected strains with high denitrification activity were used as inocula in the lab-scale submerged filter system. Before the system was set in process, the submerged filter was inoculated with each denitrifying strain for 24 h. The presence of biofilms on the support material after inoculation with each

Table 1
Denitrifying activity and identity of selected strains 16S rDNA sequence with sequences in the EMBL database

Isolated strain	Denitrifying activity (mg N-N_2O/h 10^{10} cfu)	EMBL sequence of 16S rDNA from strain	Identity (%)	Nucleotides overlap
1	0.28 ± 0.03	*Pseudomonas mandelii* (AF058286)	99.72	1442
		Pseudomonas borealis (PBO012712)	99.66	1477
2	0.23 ± 0.03	Bacterium RRP-E4 (AJ536690.1)	100.00	559
		Afipia sp. (AJ300771.1)	100.00	559
		Alpha proteobacterium (AJ508612.1)	99.28	555
3	0.23 ± 0.02	*Hidrogenophaga pseudoflava* (AF078770.1)	99.60	500
		Uncultured bacterium clone oc52 (AY491593.1)	99.20	500
		Uncultured bacterium clone oc18 (AY491564.1)	99.00	500
4	0.16 ± 0.02	*Pseudomonas kilonensis* (AJ292426)	99.72	1429
		Pseudomonas fluorescens (AF134704)	99.65	1432
5	0.15 ± 0.01	*Pseudomonas syringae* pv. tomato (AE016875)	99.02	1423
		P. syringae pv. morsprunorum (AB001445)	99.02	1426

denitrifying strain was confirmed by electronic microscopy (Fig. 2). In each case colonisation of the inert material was observed after 24 h showed that the inoculated bacteria were able to colonise the support.

Biofilms produced by strain 3 (Fig. 2c) and strain 4 (Fig. 2d) showed densely colonised areas grouped together to cover the entire surface of the clayey schists. Protuberant cell mass (colonies) were observed, grouped and surrounded by connecting material forming channelled biofilms, in line with the model defined by Beer and Stoodley [18] as water-channel-model. However, strains 1, 2 and 5 (Fig. 2a, b and e, respectively) showed a partial covering of the surface of the inert surfaces.

Wimpenny and Colasanti [19] reported the effects of several environmental factors on the biofilm structure, concluded that the concentration of nutrients can decisively affect biofilm growth. However, under our experiment conditions the environmental factors were maintained stable (nutrients, pH, etc.) suggested that differences in inert support colonisation and biofilm formation depend on the denitrifying strain inoculated, which show greater or lesser capacity for colonisation of the inert material used in our experiments. Similar observations have been reported by Sich and Van Rijn [20].

3.3. Nitrogen removal

Once the system had been inoculated, treatment of contaminated water (22.58 mg N-NO_3^-/l) began, and differences in the performance of each inoculum became apparent. Our data shows statistically significant differences ($p = 0.000$) between inocula and days in process were noted for the nitrogen concentration (N-NO_3^- plus N-NO_2^-) of outlet water (Fig. 3). The submerged filters inoculated with strain 3 showed increased nitrogen removal capacity compared the submerged filters inoculated with the other strains. Thus, strain 3 was used for the formation of biofilm in the filters 86.14% of the nitrogen was removed from the contaminated groundwater, while the inoculation of the filters with strains 1, 2, 4 and 5 produce a nitrogen removal of 39% (approximately) in the treated water.

In this context, strain 3 showed a high denitrifying activity (0.23 ± 0.02 mg N-N_2O/h 10^{10} cfu) and also formed complex biofilm structures containing significant numbers of water channels and high cell density. However, both characteristics were not observed in the other inocula used in our experiments. Our data suggest that the inoculation of submerged filters with denitrifying bacteria must be performed on the basis not only of their denitrifying activity but of their capacity for colonisation of the specific inert material used in the filter as support.

The presence of high concentrations of nitrite in treated water complicates the use of effluent obtained in the start up phase as drinking water. High nitrite levels can be expected in the start up phase of denitrifying bioreactors with heterogeneous biofilm. Denitrifying bacteria as well as nitrate reducers are responsible for the production of nitrite and only denitrifying bacteria reduce nitrite to dinitrogen (N_2). So, the ratio denitrifying bacteria versus nitrate reducers influence nitrite accumulation [21,8]. Our data clearly showed that submerged filters inoculated with strain 3 produced during start up phase effluent treated with very low amounts of nitrite (Fig. 4), while when the bioreactors were inoculated with strains 1, 2 or 4 produced effluents with increased concentrations of nitrite. No nitrite accumulation during start up phase can be expected in homogeneous denitrifying biofilm. However, selection of the denitrifying microorganisms is other of the factors responsible for nitrite accumulation.

3.4. Turbidity and platable microorganisms in the effluent

One of the main disadvantages of the submerged fixed film reactors for treating groundwater destined for human consumption is the presence of cell biomass in the treated effluent. Under our experiment conditions, significant differences ($p = 0.000$) were observed in the turbidity of the treated effluent according to the inoculated strain applied and also this parameter was affected with the working time (Fig. 5).

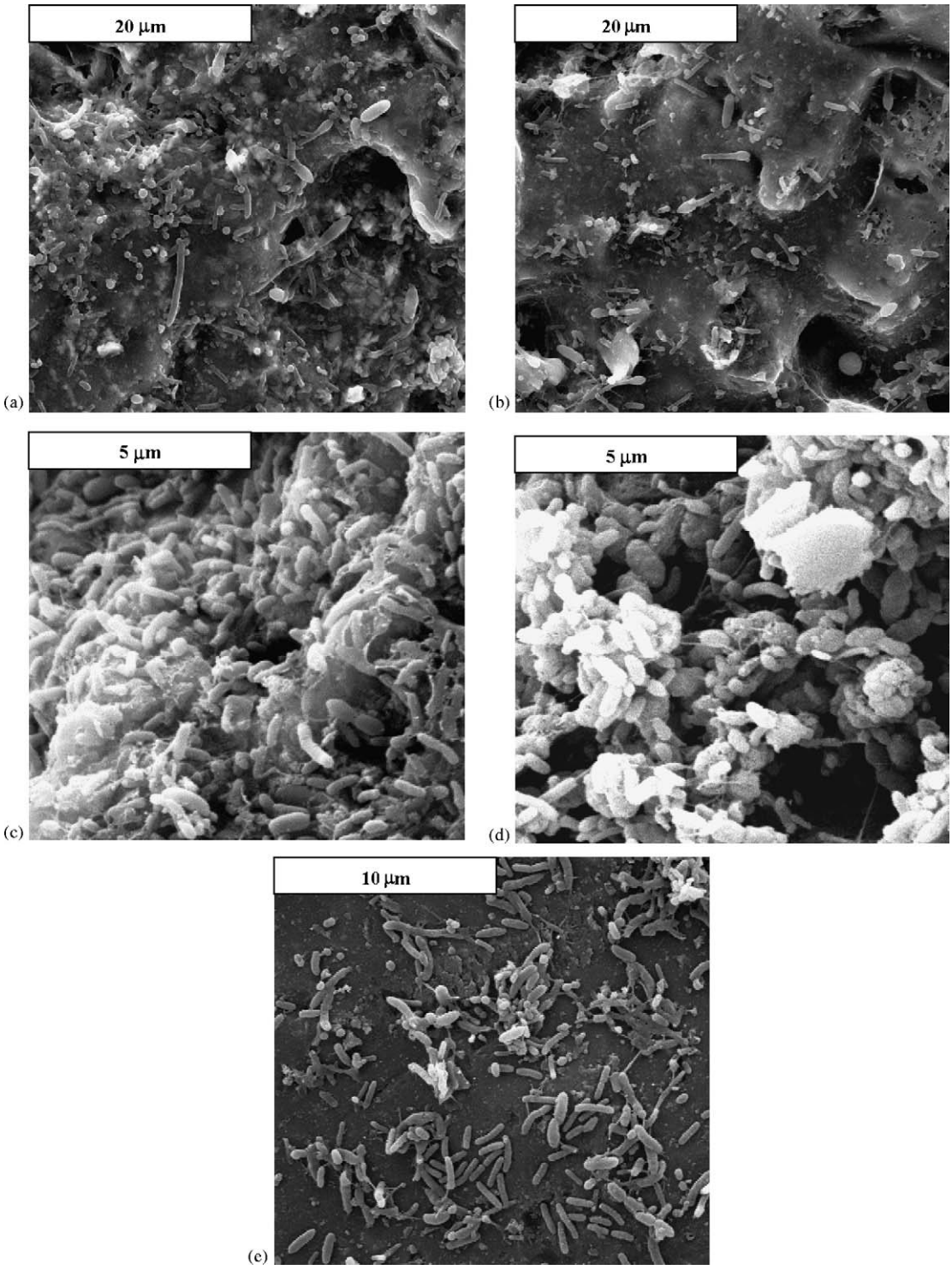

Fig. 2. Scanning electron micrograph of the biofilms produced in the submerged filters inoculated with strain 1 (a), strain 2 (b), strain 3 (c), strain 4 (d) and strain 5 (e).

When strain 4 and strain 5 were used as inocula only a slight increase of turbidity was detected. However, when strain 3 was applied as inocula the turbidity was increased.

In spite of differences observed for turbidity, there were no statically significant differences ($p = 0.017$) for total platable microorganisms in the treated water were detected independently of the denitrifying strain utilised for the inoculation of the bioreactors (Fig. 6). Given the varying turbidity valves, we might have expected a higher presence of microorganisms in the treated water obtained from submerged filters inoculated with strain 3. However, our data showed similar numbers of microorganisms when compared with effluents

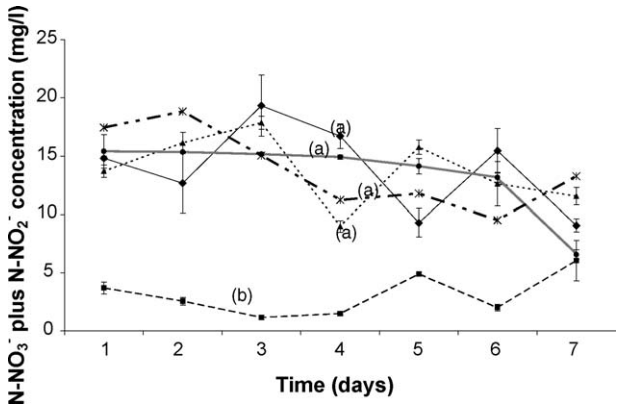

Fig. 3. Total nitrogen concentration in outlet water in the submerged filters inoculated with strain 1 (▲), strain 2 (♦), strain 3 (■), strain 4 (*) and strain 5 (●). Values are the mean of five replicates. a and b: homogeneous groups with significant differences ($p < 0.01$) from other letter.

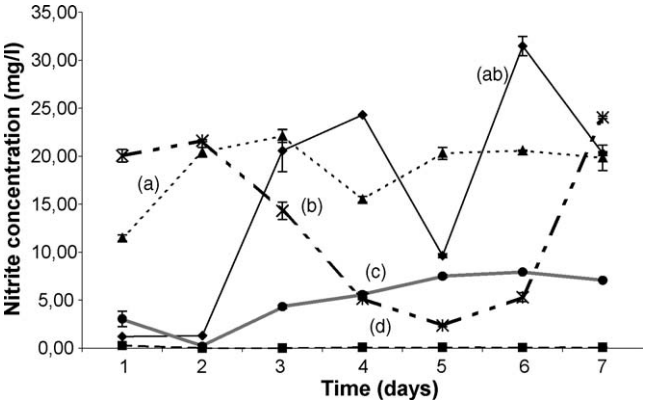

Fig. 4. Nitrite concentration in outlet water in the submerged filters inoculated with strain 1 (▲), strain 2 (♦), strain 3 (■), strain 4 (*) and strain 5 (●). Values are the mean of five replicates. a–d: homogeneous groups with significant differences ($p < 0.01$) from other letter.

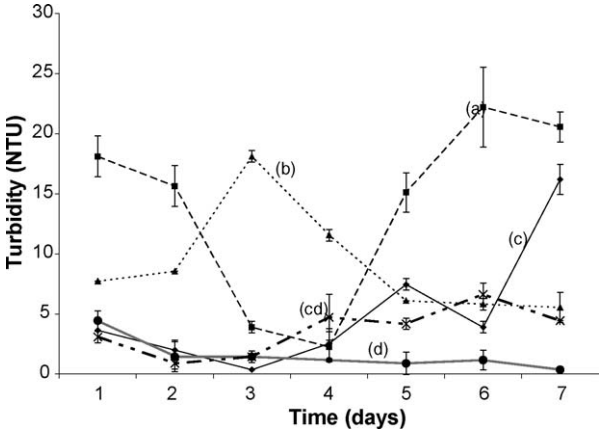

Fig. 5. Daily value of turbidity in outlet water in the submerged filters inoculated with strain 1 (▲), strain 2 (♦), strain 3 (■), strain 4 (*) and strain 5 (●). Values are the mean of five replicates. a–c: homogeneous groups with significant differences ($p < 0.01$) from other letter.

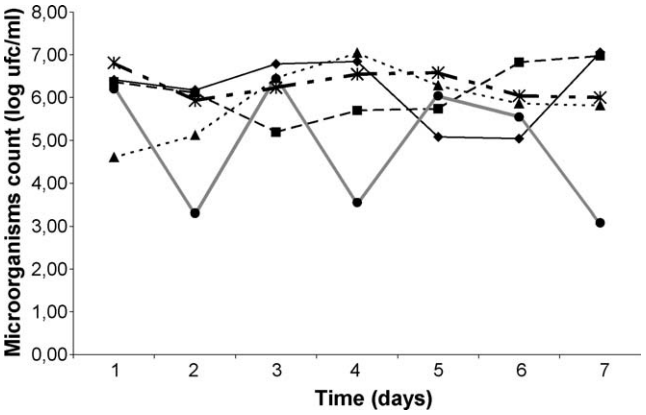

Fig. 6. Microorganisms count in outlet water in the submerged filters inoculated with strain 1 (▲), strain 2 (♦), strain 3 (■), strain 4 (*) and strain 5 (●). Values are the mean of five replicates.

obtained from bioreactor inoculated with strains 1, 2, 4 or 5. So, the increase of turbidity observed when strain 3 was utilised as inocula could be a consequence of some biofilms components including unviable cells or extracellular components such as proteins and exopolysacharides (EPS). In this context, further studies are in progress in order to understand more clearly the effects of these extracellular components in the turbidity of the effluents generated after submerged fixed film reactors treatment.

4. Conclusions

Selective inoculation of denitrifying submerged filter applied to groundwater treatment for use as drinking water avoids the problem of traditional inoculation with mix liquor from activated sludge, since it creates a homogeneous biofilms in which the inoculated bacteria are the predominant microorganisms. In the light of these results we may state that the microorganisms employed in the selective inoculation affect stability and denitrifying activity of the bioreactors conditioning process yield and water quality during start up phase. Capacity to colonise the inert support, nitrite accumulation and a high denitrifying activity of the selected inocula are the main factors to take into account in order to applied these microorganisms in contaminated groundwater with nitrate. Our data suggest that strain 3 (identified as *Hidrogenophaga pseudoflava*) proved to be a suitable inoculum for nitrate removal from water using submerged fixed film reactors.

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