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Inoculation of a submerged filter for biological denitrification of nitrate polluted groundwater: a comparative study

B. Moreno[a,b,*], M.A. Gómez[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

Activated sludge from a wastewater treatment plant and pure culture of *Hydrogenophaga pseudoflava* were utilized for the development of a denitrifying biofilm in a submerged filter in order to remove nitrate from polluted groundwater. Nitrate removal efficiency, nitrite accumulation, turbidity, COD and faecal indicators persistence in the treated water were determined at different superficial hydraulic loading (10, 20 and 30 m^3/m^2 d) and superficial nitrate loading rates (1, 2, 3, 6 and 9 Kg NO_3^-/m^2 d) in the submerged filter. The application of *H. pseudoflava* as inocula allowed better results in terms of system stability, higher superficial hydraulic loading and superficial nitrate loading rates (30 m^3/m^2 d and 9 kg NO_3^-/m^2 d, respectively). These values improve those obtained when the system was inoculated with activated sludge. In addition, the pure microbial inocula improved design parameters and running of the process due to its biofilm homogeneity, obtaining treated water with better characteristics to its final use as drinking water than that obtained with an activated sludge inocula.

Keywords: Denitrification; Groundwater; Biofilm; Submerged filter; Selective inoculation

1. Introduction

High nitrate and nitrite concentrations in groundwater reduce the use of this hydric resource due to the fact that, according to the World Health Organization [1] the intake of water with a high nitrogen concentration in nitrate and nitrite may cause diseases such as methahemoglobinemia [2] or stomach cancer [3]. This situation requires to create a nitrate and nitrite concentration guideline for the resources intended to be used as drinking water, establishing the following parameters 50 mg/l NO_3^- and 0.1 mg/l NO_2^- in order to avoid public health damages [4]. So, the removal of nutrients from wastewater and drinking water sources has become one of the main problems throughout Europe.

Among the possible technologies to be applied for nitrogen removal, submerged filter biodenitrification stands out [5]. Biofilm technology has been proved to be one of the most advanced methods to remove in a selective way nitrate and nitrite ions by dissasimilatory reduction, showing low cost [6]. Denitrifying bacteria use nitrate and nitrite as ending electron acceptors in the electron transport chain obtaining energy and transforming nitrogen oxides into dinitrogen at the same time [7]. To apply biodenitrification of nitrate contaminated groundwater, anoxic conditions and a carbon source dosage is required [8]. Previous research works have established a *C*/*N* ratio of 1.08 with ethanol as carbon source [5] and a dissolved oxygen concentration below 4.5 mg O_2/l [8], using submerged filters inoculated with activated sludge for a total nitrogen removal of contaminated groundwater.

Influence of microbial composition and denitrifying microbiota in the biofilm are significant factors that affect the application of this technology for water treatment [9]. In this context, biofilms are very complex habitats where the microbial cells responsible for the treating process are embedded in a polymer matrix. The main components of the biofilm

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

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are different physiological and morphological bacterial cells and their composition depends on environmental conditions [10]. Therefore, the presence of one or another microorganism in the biofilm will directly affect the quality of the effluent. A denitrifying biofilm is formed mainly by denitrifying bacteria but other physiological groups can develop affecting the activity of the biofilm and the quality of the effluent [11,12].

Water treatment by submerged filter technology requires a biofilm formation around an inert substrate. The water to be treated passes through a biofilter consisting of the inert substrate and the biofilm. The biofilter is always full of water [13]. The formation of a biofilm to treat a determined effluent is carried out by developing its own microbiota or by a previously isolated microbial inoculum. When the water to be treated has a very reduced microbial loading, the start up of the biofilter is accelerated by a previous microbial inoculation.

Biofilm formation in submerged filter biodenitrification for nitrate removal of contaminated groundwater required a previous inoculation, usually achieved by means of activated sludge [14]. This biofilm is very heterogeneous affecting the denitrifying activity and the quality of the effluent [12], so another inocula is necessary, in order to increase the nitrate removal of contaminated groundwater.

Taking all this into consideration, the objective of this paper is to compare process yields in groundwater nitrate removal by a submerged filter classically inoculated with activated sludge and a submerged filter inoculated by a selective inoculum. A comparative study can be achieved between a heterogeneous biofilm and an homogeneous biofilm in order to establish the influence of microbial biofilm composition in nitrogen removal capacity, as well as the performance of both systems at different superficial hydraulic loadings and superficial nitrogen loadings.

2. Material and methods

2.1. Pilot-plant submerged filter

The pilot-scale plant used in this study consisted of a biological filter made of a plexiglass cylindrical column (2.5 m high and 0.3 m diameter) forming an anoxic submerged filter. To keep the process submerged a communicating vessel connected the cylinder to a PVC inlet tube system (Fig. 1). The anoxic reactor was packed to 1.5 m high [15], the support material was a ceramic residue whose granulometry was between 2.0 and 5.0 mm and whose density was 1.75 g/cm^3. The system was run upflow with nitrate contaminated groundwater; backwashing consisted in water and air in co-current. For biofilter washing, the packing was loosened, firstly by a constant flow of air (70 m/h) for 1 min. and secondly by a constant rising flow of water (50 m/h) and air (70 m/h) for 10 min. A steady rising flow of water was applied for 5 min to eliminate the remaining biofilm [16].

2.2. Experimental procedure

Due to the low microbial population presented in the water to be treated, the production of a biofilm was necessary before treating the polluted groundwater. This initial phase was

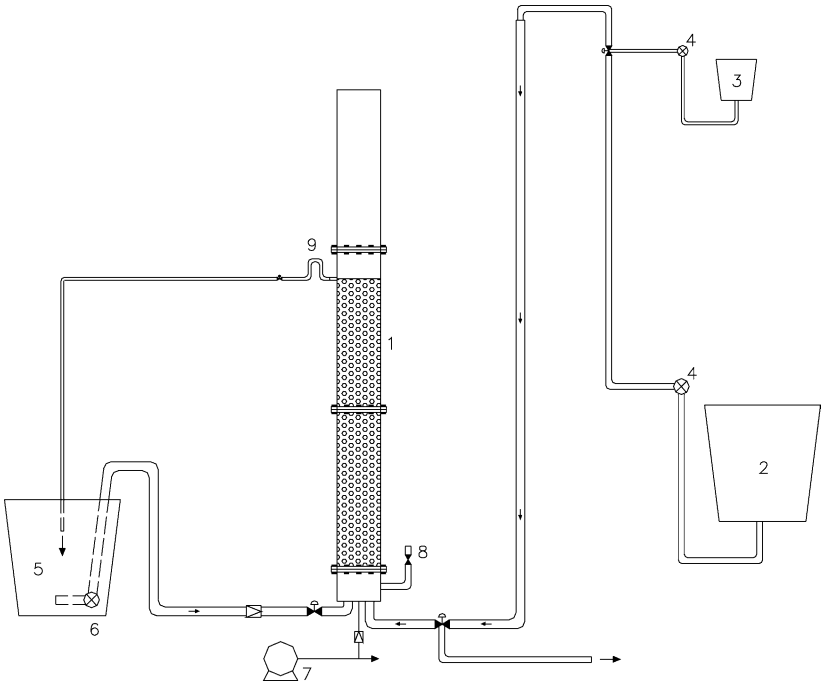


Fig. 1. Pilot scale plant (1) submerged filter (V = 0.21 m^3); (2) inlet water tank (V = 1.7 m^3); (3) carbon source tank (V = 0.03 m^3); (4) piston pump; (5) outlet water tank (V = 0.5 m^3); (6) backwashing pump; (7) air compressor; (8) safety valve; (9) siphon outlet water.

accomplished with activated sludge from the aerobic biological reactor of an urban wastewater treatment plant (Granada-Spain), or with a pure culture of *Hydrogenophaga pseudoflava* strain isolated from a denitrifying biofilm.

In order to get the biofilm formation, a highly dense microbial culture of *H. pseudoflava* (10^{12} colony forming units/milliliter, cfu/ml) or mix liquor of activated sludge, both amended with nitrate (1 g/l) and ethanol (0.5 g/l), were recirculated at low flow (2 m^3/m^2 d) for 24 h through the submerged filter. After the inoculation phase, the influent water was pumped in.

Different water flowrates (29.5, 58.9 and 88.4 l/h) and nitrate concentrations in the influent (100–300 mg NO_3^-/l) were assayed; reaching different superficial hydraulic loadings (10, 20 and 30 m^3/m^2 d) and superficial nitrate loadings (1, 2, 3, 6 and 9 kg NO_3^-/m^2 d), allowing to study their influence on the process. For the experiments carried out with different superficial hydraulic loadings or superficial nitrate loadings, a new biofilm was developed, so, after each test, the system was strongly rinsed and support material was dried. Each test was run for 10 days after reaching steady state conditions (nitrogen removal was stable) in order to maintain the initial characteristics of the biofilm.

The groundwater to be treated was from La Vega aquifer (Granada, Spain). The following water characteristics were daily determined during 1 month, according to the standard methods [17]: NO_3^-, 50–70 mg/l; NO_2^-, 0.0–0.01 mg/l: PO_4^{3-}, 0.4–0.8 mg/l; SO_4^{2-}, 180–210 mg/l; O_2, 2.0–4.5 mg/l; and pH 7.0–7.5. Nitrate was supplemented by the addition of an appropriate volume of a concentrated stock solution of $NaNO_3$. In order to obtain the suitable conditions for denitrification, the system was run with a continuous ethanol addition, maintaining always a *C*/*N* ratio of 1.08 [5]. A concentrated stock solution of carbon source was stored in a tank from where it was pumped to the influent pipe. Complete anoxic conditions were monitored by means of the stoichiometric quantity of sodium sulphite (Na_2SO_3) added to the water to be treated. Water temperature in the system was in the range of 15.0–20.0 °C.

2.3. Analytical determinations

Inlet and outlet water samples were taken every 24 h (200 ml). Nitrate and nitrite concentrations were set regularly to establish the nitrogen removal capacity from polluted water. Turbidity, COD and presence of faecal indicators in the treated water were studied to determine the necessity of further treatment.

Nitrate and nitrite concentrations were determined by ionic chromatography (Dionex® DX-300). Separation and dilution of anions was realised by a Ionpac® AS14 column using a solution of carbonate-bicarbonate as eluent, and sulphuric acid as regenerant. Before the analysis, all samples were filtered through 0.45 μm membrane filters (Millipore HAWP) and diluted to achieve nitrate and nitrite concentrations lower than 10 mg/l. Chemical oxygen demand (COD) was determined using closed reflux micro method [17]. Absorbance of the digestate was measured colorimetrically at 600 nm wavelength and COD concentration was calculated from a calibration curve, prepared with potassium acid phthalate. Turbidity was determined by the spectrophotometic method at 650 nm. Among the different indicators of faecal pollution, total coliforms method was selected using a membrane method on m-Endo agar as culture medium [17].

2.4. Biofilm determinations

Once the inoculation step was finished, the formed biofilm was studied by scanning electron microscopy, as well as its microbial composition related to denitrifiers and other microbial groups, such as nitrate reducers or sulphate reducers. Once the tests were finished, the microbial composition of the biofilm, inoculated with activated sludge and *H. pseudoflava* was studied. For these analyses, support material samples were taken at three different column heights, mixing the three samples before the analysis was done.

Scanning electron microscopy observations of the biofilm were made after bioparticles treatment. The cells of the biofilm 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.

Total platable bacteria in the biofilm were counted by dilution-plate technique, using nitrate–sucrose–agar medium [18], before biofilm extraction. 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 anaerobically incubated (Anaerogen system, OXOID) at 30 ± 1 °C for 2 weeks. Colony forming units were counted on plates of the series featuring approximately 10–100 cfu. Colonies were counted separately according to their morphology. Five different strains were isolated and purified for each colony type. All the isolated strains were tested for their capacity of reducing NO_3^- to NO_2^- (nitrate reduction) or to N_2O (denitrification).

In order to discover the ability to reduce NO_3^- to N_2O gas, every single isolated strain was inoculated in a hermetically closed vial containing 5 ml of NSB (Nitrate–Sucrose–Broth). 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 oxide nitrate reductase activity, according to Yoshinari and Knowles [19]. The inoculated vials were incubated in the dark for 24 h at 30 ± 1 °C. After this time, N_2O presence was determined inside each vial employing a gas chromatograph Varian CX3400 equipped with a thermal conductivity detector.

In order to determine the ability to reduce NO_3^- to NO_2^-, the isolated strains were inoculated in bacto nitrate broth (DIFCO) and then incubated in the dark for 24 h at 30 ± 1 °C. After incubation, sulphanilic acid and α-naphthylamine were added to the growth media and the production of NO_2^- was detected when a pink or red colour was observed [18].

Sulphate reducing bacteria were counted according to Rodina [18]. The selected strains were incubated in Sturm media which contained an indicator paper saturated with lead acetate and incubated at 30 °C for 1 week. Tubes that darkened the indicator were considered positive.

3. Results and discussion

3.1. Biofilm formation

The generation of an active biofilm from the microbiota normally present in groundwater is a very slow process due to the low microbial population and nutrient concentration in these oligotrophic environments. In this sense, many authors reported [20,21,5] that a previous inoculation of submerged filters is required before starting the systems in order to obtain an adequate yield. Under our experimental conditions two different inocula were used, activated sludge from a wastewater treatment plant and pure culture of a denitrifying bacterium (*H. pseudoflava*) isolated from a denitrifying biofilm.

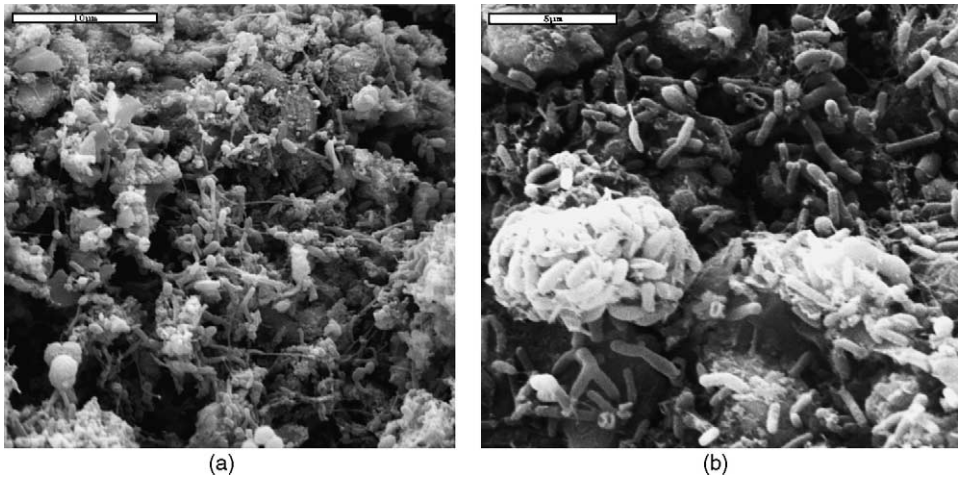
Biofilm formation in the submerged filter after inoculation was confirmed by scanning electron microscopy. When activated sludge was used as inoculum, an heterogeneous biofilm was obtained, showing several bacterial morphologies and microbial types (Fig. 2a). However, when *H. pseudoflava* was used as inoculum an homogeneous biofilm was produced. The predominant microorganism observed in the biofilm was the inoculated bacteria (Fig. 2b). In both cases, and despite of the differences, it is observed a water channel type biofilm [22] being the low nutrient concentration the main cause for this canalised structure [23].

Properties of both biofilms were not significantly modified during the operation time of the bioreactor (10 days after reaching steady state conditions). However, Gómez et al. [12] observed that properties of a denitrifying biofilm formed by inoculation with activated sludge were modified during operation of the bioreactor for a longer time of 10 days.

The presence of one or another physiological bacterial group in a biofilm applied for groundwater treatment affect the quality of the treated effluent. So, selective inoculation for biofilm formation previous to groundwater treatment tries to obtain the best running conditions. However, the presence of other microorganisms in the influent to be treated (groundwater) can modify the microbial biofilm composition and then the activity of biofilm. More experiments are in progress in order to understand in detail the evolution of denitrifying biofilms formed by selective inoculation and the influence of alteration in microbial composition over quality of treated effluent.

3.2. Performance according to loading rates

Once the system was operating under running conditions, different superficial hydraulic loading and superficial nitrate loading rates were tested to compare the results according to nitrate removal yield as well as other water characteristics for both inocula assayed. Nitrogen concentrations in treated water at different superficial nitrate loading (1–9 kg NO_3^-/m^2 d) are shown in Fig. 3. In both assays a convex polynomial correlation was found ($r^2 = 0.9932$ for pure culture assay and $r^2 = 0.9761$ for activated sludge assay). Values for superficial nitrate loading higher than 4 kg NO_3^-/m^2 d increased nitrogen concentration over legal limits (11 mg N/l) when activated sludge was applied, however the system inoculated with *H. pseudoflava* easily adapted to increments in the superficial nitrate loading.



(a) (b)

Fig. 2. Scanning electron microscopy of support material with biofilm formed after inoculation using (a) activated sludge and (b) *H. pseudoflava* strain-inocula.

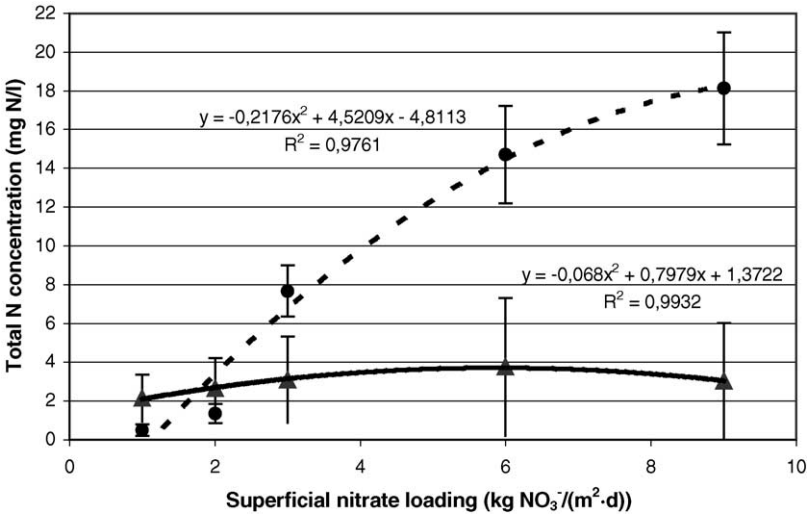


Fig. 3. Total nitrogen concentration in treated water at different superficial loadings using (●) activated sludge and (▲) *H. pseudoflava* as inocula. Water flowrate was constant.

Nitrogen concentration in the outlet water at different superficial hydraulic loadings (10–30 m^3/m^2 d), applying 100 mg NO_3^-/l in the inlet water are represented in order to know the global behaviour of the system for both inocula tested. A linear correlation was obtained ($r^2 = 0.94$) when activated sludge was applied and a convex polynomial correlation was found ($r^2 = 0.99$) in the case of *H. pseudoflava* inoculation (Fig. 4).

Total nitrogen concentration in the outlet water increases proportionally when superficial hydraulic loading rises, using activated sludge as inoculant. For *H. pseudoflava* strain-inocula, nitrogen concentration increases with the superficial hydraulic loading, but starts to decrease when the superficial hydraulic loading is higher than 24 m^3/m^2 d.

Activated sludge inoculation allowed values for superficial hydraulic loading rates lower than 20 m^3/m^2 d, while *H. pseudoflava* strain-inocula total nitrogen showed values lower than the legal limit established (11 mg N/l) for all the superficial hydraulic loadings tested. Similar results were observed for superficial nitrate loading. The same *C*/*N* ratio (*C*/*N* = 1.08) was maintained for all the experiments performed, which suggested that initial inoculation affects nitrogen removal yield in the submerged filter technology. In this context, our results suggest that the formation of heterogeneous biofilms in denitrifying submerged filters drastically affects the quality of the treated water compared to submerged filters containing homogeneous biofilms induced after a selected inoculation with denitrifying bacteria such as *H. pseudoflava*.

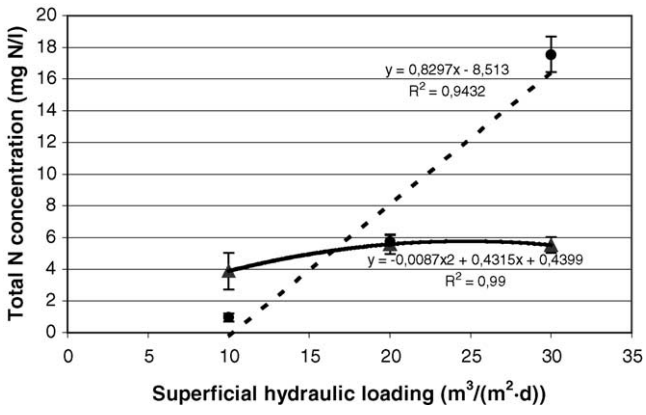


Fig. 4. Total nitrogen concentration in treated water at different hydraulic loadings using (●) activated sludge and (▲) *H. pseudoflava* as inocula. Nitrate concentration in the inlet water was 100 mg/l.

Nitrite concentration in the treated water increased considerably as superficial hydraulic loading raised when activated sludge was applied as inocula (Fig. 5). However nitrite accumulation was lesser when submerged filter inoculation was achieved with *H. pseudoflava* strain-inocula (Fig. 6). Accumulation of nitrite in submerged filter technologies for water denitrification has been previously reported. Several factors such as oxygen concentration, pH, biofilm composition and available carbon source, influence nitrite accumulation [6,9,24]. Our experiments clearly show that the superficial hydraulic loading may affect the nitrite concentration in the treated water by this biological procedure regardless to biofilm characteristics. However, procedures carry out by a submerged filter inoculated with activated sludge showed high nitrite concentration in treated water.

Data obtained for COD, turbidity and coliforms in the treated water, for the different superficial hydraulic loadings tested, are shown in Table 1. As above reported the inoculation with activated sludge presented difficulties not detected with inoculation of *H. pseudoflava* strain-inocula. In this sense, our results show significant concentrations of faecal bacterial indicators (total coliforms) in the treated water. This faecal pollution was contributed by the activated sludge used for inoculation of the denitrifying column.

When activated sludge was used as inoculum an increase in the COD values was observed according to Table 1. These

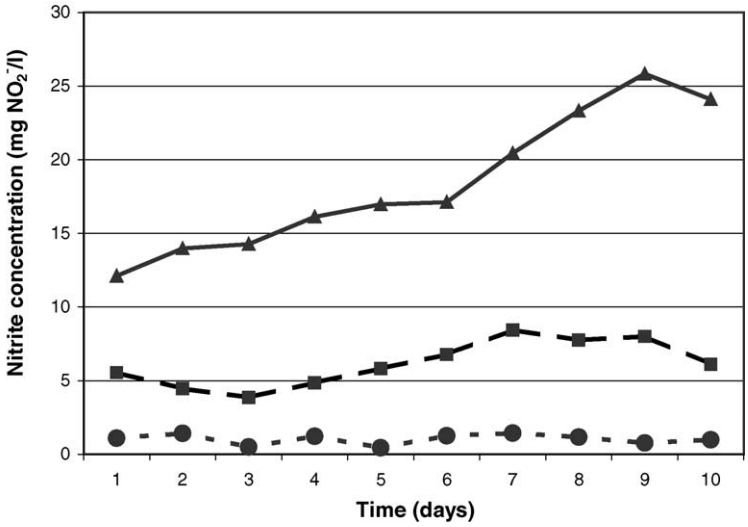


Fig. 5. Nitrite concentration in the outlet water at (●) 10, (■) 20 and (▲) 30 m^3/m^2 d of superficial hydraulic loadings using activated sludge as inocula. Nitrate concentration in the inlet water was 100 mg/l.

Table 1
Mean values for COD, turbidity and coliforms in the treated water at different hydraulic loadings (HL) using activated sludge and *H. pseudoflava* as inocula

Parameter	Activated sludge (HL (m^3/m^2 d))			*H. pseudoflava* (HL (m^3/m^2 d))		
	10	20	30	10	20	30
COD (mg O_2/l)	0.5 ± 0.1[a]	12 ± 7	25 ± 7	15 ± 6	4 ± 2	8 ± 3
Turbidity (NTU)	2 ± 1.1	3.7 ± 1.6	4.5 ± 2	1.5 ± 0.3	3 ± 1	2 ± 0.5
Coliforms (cfu/ml)	10000 ± 6200	11500 ± 8000	13600 ± 2600	0	0	0

Nitrate concentration in the inlet water was 100 mg/l.
[a] Values are mean ± E.D. of five replicates.

results were not observed when *H. pseudoflava* strain-inocula was used as inocula. Biofilm loosening produce COD increase, what suggests that the stability of biofilm can be measured by COD values in the treated water. No differences were observed respect to turbidity between both assays.

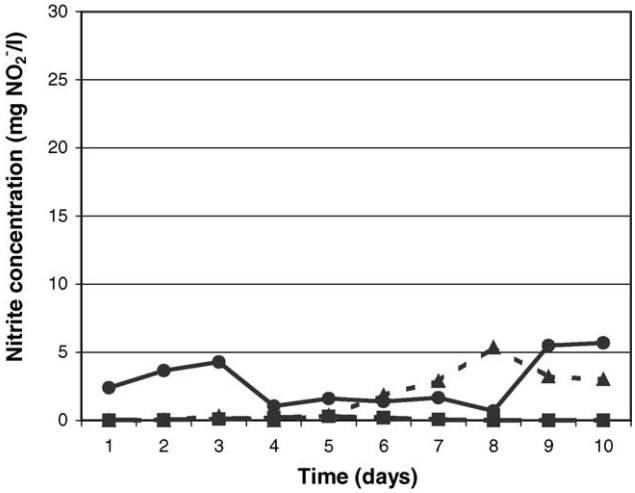


Fig. 6. Nitrite concentration in the outlet water at (●) 10, (■) 20 and (▲) 30 m^3/m^2 d of superficial hydraulic loadings using *H. pseudoflava* as inocula. Nitrate concentration in the inlet water was 100 mg/l.

3.3. Influence of biofilm composition

Studies developed over biofilm composition make clear that a different biofilm bacterial composition alter inoculation, mainly with respect to non-denitrifying bacteria, present in the biofilm developed with activated sludge and totally absent in the biofilm developed with selective inocula. After running conditions both biofilms were enriched in denitrifying bacteria, however a significant non-denitrifying bacteria were present in biofilm developed with activated sludge (Table 2).

When the submerged filters were inoculated with activated sludge a significant population of nitrate reducing bacteria was detected. This phenomenon was not detected in biofilters inoculated with *H. pseudoflava* where the denitrifying microbiota (mainly the inoculated bacteria) was predominant during the period of time of the present study. Similar results were observed in sulphate reducing bacteria, although it had a smaller effect on the total microbiota.

Increase in superficial loadings (hydraulic and nitrate) imply an increase in dosage of carbon source in order to maintain *C*/*N* ratio. With regard to this, the heterogeneous biofilms produced in submerged filters inoculated with activated sludge showed a lower ability for nitrogen dissimilatory removal in terms of carbon source (ethanol) consumed versus

Table 2
Biofilm bacterial composition formed with activated sludge and *Hydrogenophaga pseudoflava* after inoculation and running conditions (cfu/mg biofilm 10^5)

Microbial group	Activated sludge		*H. pseudoflava*	
	After inoculation phase	After running conditions (10 d)	After inoculation phase	After running conditions (10 d)
Nitrate reducers	34.1 ± 6.0[a]	45.1 ± 3.23	nd	0.005 ± 0.0001
Denitrifiers	37.2 ± 5.8	115.1 ± 4.9	63.4 ± 2.1	288.1 ± 16.2
Sulphate reducers	2.81 ± 0.73	5.7 ± 0.28	nd	0.0001

nd, not detected.
[a] Values are mean ± E.D. of five replicates.

nitrogen removal, when this yield was compared with submerged filters inoculated with *H. pseudoflava*. These results are a consequence of the different microbial groups detected in the heterogeneous biofilms, since a significant fraction of the ethanol added to the system inoculated with activated sludge was consumed by microorganisms without denitrification activity (i.e. nitrate reducing bacteria). Obviously, this effect was not detected in the filters containing homogeneous biofilms mainly formed by denitrifying bacteria (*H. pseudoflava*). Similar results had been previously reported by Prescott et al. [25].

Denitrifying bacteria as well as nitrate reducing bacteria are responsible for the production of nitrite, an anion which is employed by denitrifiers as electron acceptor [7]. Significant differences were observed with respect to the concentration of this anion in treated water, with an important increase when superficial hydraulic loading rises in submerged filter inoculated with activated sludge. The biofilms developed with activated sludge produced an increase in nitrate reducing bacteria during the biological process, suggesting that this microbial group are responsible of nitrite accumulation.

Another important bacterial group identified in the biofilm formed with activated sludge was sulphate reducing bacteria. These microorganisms can use SO_4^{2-} as an ending electron acceptor to originate H_2S, under anaerobic conditions and absence of nitrate [25]. Obviously, an increase in sulphate reducing bacteria affects the organoleptic characteristics of the treated water.

4. Conclusions

H. pseudoflava strain-inocula was used to induce an homogeneous biofilm in a submerged filter for the treatment of nitrate polluted groundwater. This procedure involves an efficient process which allows submerged filters to work with high superficial hydraulic loading and superficial nitrate loading rates. When *H. pseudoflava* was used as inocula the system tolerated loading rates of 30 m^3/m^2 d and 9 kg NO_3^-/m^2 d. These values double those obtained when the system was inoculated with activated sludge from a wastewater treatment plant. Consequently, the selection of a bacterial strain with high denitrifying activity and stable biofilm-forming ability for its use as inocula in a submerged filter enhances design parameters and running of the process, obtaining a better quality for treated water.

Acknowledgements

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