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Effect of dissolved oxygen concentration on nitrate removal from groundwater using a denitrifying submerged filter

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Abstract

A unidirectional submerged filter system was employed to purify groundwater contaminated with nitrate by biological denitrification. The influence of the concentration of dissolved oxygen (DO) in the process was tested using ethanol, methanol and sucrose as carbon sources. Inorganic-nitrogen removal, growth of the biofilm, platable denitrifying bacteria and nitrate reducing bacteria in biofilm were studied. With regard to the type of electron donor used, the presence of oxygen decreased the removal efficiency of inorganic nitrogen and caused an increase of nitrite concentration in the treated water. These negative effects depended on utilised carbon source. Biological denitrification with alcohols such as ethanol and methanol was less affected by DO than with sucrose. The development of the biofilm was also influenced by the DO concentration as excess O_2 caused reduced biofilm growth. These biofilms developed in oxygen presence had a smaller bacterial density and a lower denitrifying bacteria versus nitrate reducing bacteria ratio, which led to an unfavorable inorganic nitrogen removal and presence of nitrite in the treated water. All these effects are more pronounced when sucrose is used as carbon source.

Keywords: Dissolved oxygen; Biofilm; Denitrification; Nitrate; Nitrite; Denitrifying bacteria; Nitrate reducing bacteria

1. Introduction

Nutrients removal from wastewater and drinking water sources has become one of the main problems throughout Europe. Eutrophication or groundwater contamination by

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nitrates, which cause serious social and economical problems, are related to an increase of nutrient concentration in the aquatic environment [1,2].

Several nitrate removal methods such as ion exchange, reverse osmosis, electrodialysis, distillation, chemical reduction or bio-denitrification have been employed, with different rates of efficiency [3,4,5]. Bio-denitrification has proved to be one of the most feasible, advanced, selective and cost effective processes for removing nitrate by disassimilatory reduction [6,7], which transforms it into nitrogen gas [8]. This process has been applied to nitrogen removal of wastewater [9] and contaminated groundwater [10], with biofilm technology achieving the highest nitrogen removal rate per volume [11].

Different types of biofilm reactors have been used for biological treatment of water and wastewater [12], from which submerged filter technology seems to be particularly appropriate for application to freshwater treatment [13]. For this process, a biofilm is developed around an inert substrate and water to be treated passes through the biofilter consisting of the inert substrate and biofilm. The biofilter is always full of water.

To apply submerged filter bio-denitrification to contaminated water like groundwater, besides pH, temperature and carbon source concentration controls, effluent dissolved oxygen (DO) concentration must be eliminated [10].

DO to be treated in the water negatively affects biological denitrification [14]. Certainly, denitrification was believed to be a strictly anaerobic process [15] as many investigators had found that very low DO concentrations could cause complete cessation of denitrifying activity [16]. However, the effect of oxygen on denitrifies, depends on the microbial strains [17]. Denitrification produces less energy yield than oxygen respiration [18], therefore, a bacterial cell growing in aerobic conditions will choose to use oxygen as terminal electron acceptor. In addition to this competitive effect, oxygen controls denitrification at two levels: reversible inhibition of the activities of the denitrification enzymes and regulation of gene expression [17].

Nitrate reductase or nitrous oxide reductase, which catalyse the first and fourth step of the denitrification process respectively, are inhibited by different oxygen concentrations [19,20]. This inhibitory effect can stop the enzymatic process, giving rise to a progressive reduction of molecular nitrogen production and the consequent accumulation of the different intermediates, principally nitrite, a highly toxic compound [21] and nitrous oxide.

Due to the fact that oxygen has to be transported into the biofilm by diffusion, oxygen negative effect could be minimized. The penetration of oxygen result in a decrease in concentration corresponding with the depth of biofilm, meaning that oxygen will be used as an electron acceptor in the outer zone. Nitrate diffuses through the aerobic zone and serves as electron acceptor for oxidation of organic matter in an inner anoxic zone [14].

In addition, the aim of this paper is to study the influence of the concentration of DO on groundwater nitrogen removal with a denitrifying submerged filter using different types and concentrations of carbon sources (Ethanol, methanol and sucrose). Comparison is made up of inorganic nitrogen removal, nitrite accumulation, biomass production and presence of denitrifying and nitrate reducer bacteria in the biofilm.

2. Experimental section

2.1. Pilot plant description

The pilot scale plant used in this study consisted of a methacrylate cylindrical (3.0 m high and 0.3 m diameter, Fig. 1) column of the anoxic submerged biofilter type, operating with an upward flow of the groundwater and an upward flow of rinsing water and air for filter cleaning. Residual clayey schists from a brick factory (Cerámicas Siles, Granada, Spain) were used as support media for biofilm growth, packing the column up to a height of 2.0 m. The average diameter of the particles was 2–4 mm with a density of 1.75 g/cm^3. A communicating-vessels system was employed for its operation.

2.2. Experimental procedure

The water to be treated was groundwater from La Vega aquifer (Granada, Spain). The following water characteristics were determined daily for 1 month, according to the Standard

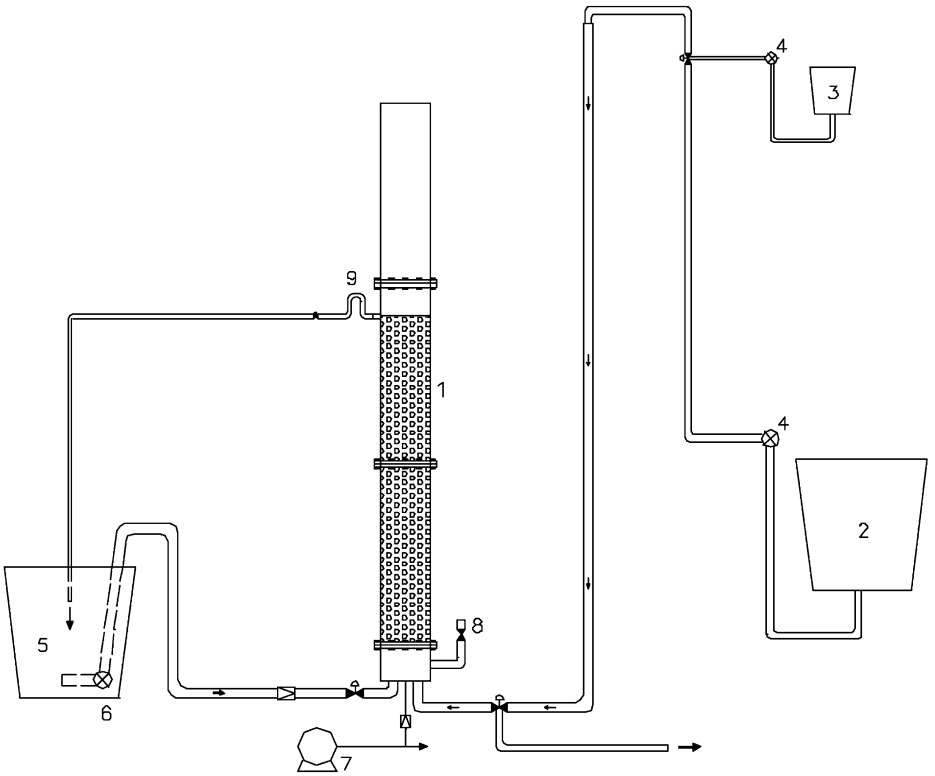

Fig. 1. The pilot scale plant (1) submerged filter ($V = 0.21\,m^3$); (2) influent tank ($V = 1.7\,m^3$); (3) carbon source tank ($V = 0.03\,m^3$); (4) piston pump; (5) effluent tank ($V = 0.5\,m^3$); (6) rinsing pump; (7) air compressor; (8) safety valve; (9) U-bend.

Methods [22]: 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; COD, 0.0–0.02 mg O_2/l and pH 7.0–7.5. The water to be treated was pumped at 0.5 l/min flow rate (10.2 m^3/m^2) using a piston pump. Water temperature in the system was in the range of 15–20 °C. The reactor was operated at an influent nitrate concentration of 100 mg/l. The nitrate was supplemented by the addition of an appropriate volume of a concentrated stock solution of $NaNO_3$.

Three carbon sources were selected for these experiments (sucrose, ethanol and methanol), on the basis of their widespread availability, easy handling and low specific cost. The system was operated under continuous organic carbon addition, applying the minimum concentration necessary to accomplish total nitrogen removal of the water to be treated. A concentrated stock solution of carbon source was stored in a tank from which it was pumped to the influent pipe.

The submerged biofilter substrate was inoculated with an activated sludge amended with nitrate (1 g/l) and ethanol (0.5 g/l), which was recirculated for 7 days, after which the water to be treated was pumped in together with the adequate carbon source concentration. We considered that the reactor had reached state conditions when the nitrogen in the effluent was completely removed in total absence of DO.

In order to achieve the desirable oxygen concentration in the water to be treated, the groundwater was aerated strongly, obtaining a final DO concentration of about 8.0 mg/l. A stoichiometric quantity of sodium sulphite (Na_2SO_3) was added to reduce DO concentration until the desirable concentration was reached.

After each test with different oxygen concentrations, the system was cleaned using an upward flow of air (70 m/h) for 1 min, rising water (50 m/h) and upward flow of air (70 m/h) for 10 min. A flow of water was applied for 5 min to eliminate the remaining biofilm [23]. Steady state conditions were reached before each test. For the experiments to be carried out with different carbon source, a new biofilm was developed.

Biomass production and growth of denitrifying and nitrate-reducing bacteria was tested with (4.5 mg/l) and without DO. The system was operated under continuous organic carbon addition (ethanol, methanol and sucrose), with a concentration range from 0 mg/l to the minimum necessary to accomplish total nitrogen removal of the water to be treated.

2.3. Analytical determinations

The influent and effluent (200 ml) of the submerged filter were taken out every 24 h, obtaining three replicates for each carbon source and oxygen concentrations assayed. These water samples were routinely tested for nitrate, nitrite, and carbon source concentration.

Water samples were filtered through 0.45 μm membrane filters (Millipore HAWP). Nitrate and nitrite were measured by an ion-chromatography system using conductivity detection (Dionex® DX-300). Separation and elution of the anions were carried out on an anion analytical column (Ionpac® AS14) using a carbonate/bicarbonate eluent and a sulphuric regenerant. Before measuring, the filtered samples were diluted to achieve nitrate and nitrite concentrations lower than 10 mg/l. Ethanol and methanol concentrations were measured in the effluent and influent by gaseous phase chromatography (Perkin-Elmer® Autosystem GC). Sucrose concentration was determined by Roe and Papadopoulos method

[24]. pH and DO levels were followed continuously in the effluent using an pH-meter and an OXI 921 electrode (CRISON®), respectively.

For biomass production and growth of denitrifying and nitrate reducing bacteria in the biofilm assays, the biofilm covered media were removed from the reactor through a special sampling port located along the column, using a cylindrical sampler. Biofilm samples (1 g) were taken from three different heights (16, 64 and 123 cm) and were thoroughly mixed before to estimate the biomass production. 1 g of mixed clayey schists was introduced in sterile glass bottles with 100 ml of sterile saline solution (NaCl 0.9%). The biofilm was separated from inert substrate by sonication (5 min) and the suspended solids obtained were determined by vacuum filtration of the 100 ml of saline solution through a pre-weight and pre-ignited glass fibre filter (0.45 μm), then dried for 24 h at 105 °C. The recoverable dry weight (mg/g substrate) was used as an estimation of biomass production.

2.4. Quantification of denitrifying and nitrate reducing bacteria

The number of denitrifying bacteria was obtained by viable counts on NSA agar plates (nitrate–sucrose–agar). The composition of the NSA medium (per litre of distilled water) was the following : $NaNO_3$ 2.0 g, K_2HPO_4 1.0 g, $MgSO_47H_2O$ 0.5 g, KCI 0.5 g, $FeSO_47H_2O$ 0.01 g, yeast extract 1.0 g, sucrose 30.0 g and agar 20.0 g. Biofilm was separated from substrate as previously described for biomass production estimation, although samples were sonicated only for 1 m. The separated biofilm was homogenised using a magnetic stirrer at maximum speed for 1 h. Dilution series (1:10) were made in sterile saline solution (NaCl 0.9%). From each dilution, 0.1 ml was spread on sterile NSA agar plates (three replicates of each dilution) and incubated anaerobically (Anaerogen system, OXOID) at 30 ± 1 °C for 2 weeks. Colony forming units (cfu) were counted on plates of the series featuring aproximately 10–100 cfu. Colonies were counted separately according to their morphology. Five different strains were isolated and purified for each morphology.

All the isolated strains were tested for their capacity of reducing NO_3^- to N_2O or N_2 (Denitrification) or to NO_2^- (Nitrate reduction). 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 an substituted by helium. Inside the vial, 10% of acetylene was introduced to inhibit any existing oxide nitrate reductase activity, according to Yoshinari and Knowles [25]. 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 were incubated in the dark for 24 h at 30 ± 1 °C. After the incubation, sulphanilic acid plus α naphthylamine was added [26]. Positive tests developed a pink or red color. Strains that gave mainly a positive N_2O function were considered denitrifying and those which gave a negative N_2O function, while having a positive NO_2^- presence were considered NO_3^- reducers. The strains which did not show any of those positive results were not counted.

2.5. Statistic study

Data obtained from this study were analysed by computer assisted statistics, using Statgraphics Plus for Windows 3.0 by (Statical Graphics Corp, 1997). Linear regressions were used to compare the influence of DO over inorganic nitrogen removal using several carbon sources. Biofilm composition with (4.5 mg/l) and without oxygen was compared by linear regressions using different carbon sources.

3. Results and discussion

Oxygen influence on denitrification developed in biofilm is different from that in batch culture. Bishop et al. [27] noticed that the biofilm structure is highly stratified and the effective diffusion of the DO decreases with the biofilm depth. Different efficiencies were observed in a denitrifying submerged filter when the influent DO was increased, decreased the inorganic nitrogen removal independently of carbon source assayed. The concentration of carbon source used was in all cases the optimal for total removal of inorganic nitrogen in the complete absence of oxygen (137.0 mg/l for sucrose, 68.3 mg/l for methanol and 46.8 mg/l for ethanol). Negative linear correlation between DO and nitrogen removal yields was shown when ethanol was used as carbon source (Fig. 2), similar to methanol and sucrose assays. However, the magnitude of the negative effect varies depending on the oxygen concentration and the type of electron donor used. Low DO showed small influence but in high DO concentrations the influence was greater. Nitrogen removal was almost constant

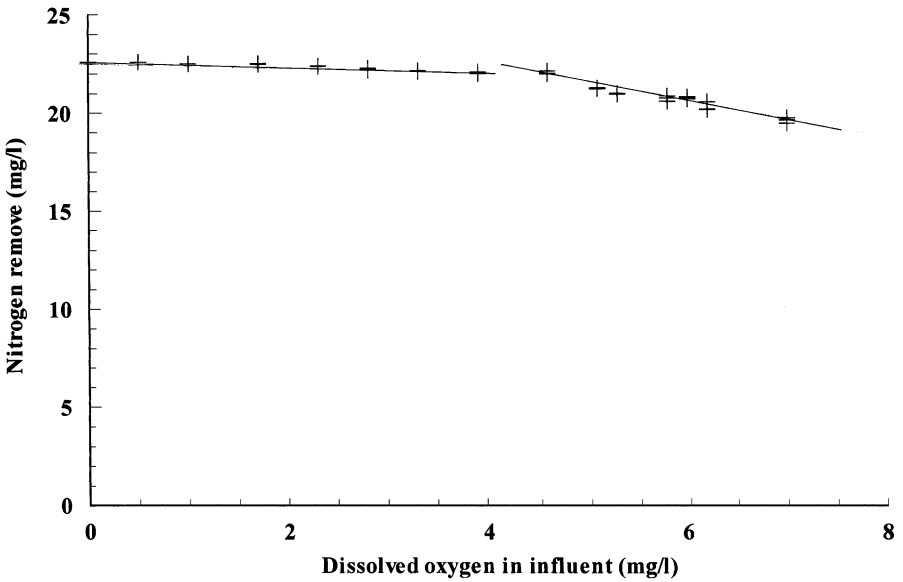

Fig. 2. Linear regression between DO concentration in the influent (100 mg/l NO_3^-) and nitrogen removal with ethanol as carbon source (50 mg/l ethanol).

Table 1
Regression analysis (linear model) between influent DO (low and high concentration) and nitrogen removal yields with ethanol, methanol and sucrose assays.

	Low DO (<4.5 mg O_2/l)			High DO (>4.5 mg O_2/l)		
	Ethanol	Methanol	Sucrose	Ethanol	Methanol	Sucrose
Correlation coefficient	−0.96	−0.89	−0.9	−0.97	−0.98	−0.83
Slope	−0.14	−0.3573	−0.5694	−0.93	−0.78	−2.29

for DO concentration below 4.5 mg/l. However, statistical differences were noticed between tests using different carbon sources (Table 1), with the ethanol test showing least influence. Statistical differences were also for DO concentration over 4.5 mg/l (Table 1). Sucrose was shown to be the least favorable substrate while the loss in inorganic nitrogen removal was less pronounced for methanol and ethanol.

The negative effect of the increased DO concentration could be overcome with an increment in the concentration of carbon source added, as a higher amount of electron acceptor is needed for a higher concentration of electron donor added. Thus, increasing the carbon source dose, all NO_3^- must be reduced even in the presence of oxygen [23]. Alternatively, the elimination of oxygen can be achieved by addition of a reagent such as Na_2SO_3. Both processes will increase system running cost. According to the results presented here, ethanol added as a carbon source offers a viable alternative, as it is less affected by low DO concentration.

Apart from the negative effect of DO on inorganic nitrogen removal, the presence of O_2 also causes an increase in the concentration of nitrite in the treated water. Fig. 3 shows the

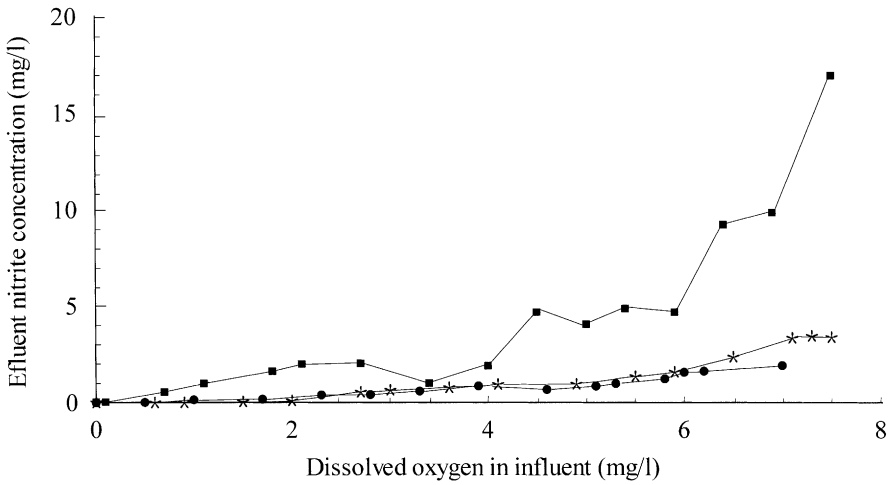

Fig. 3. Residual nitrite concentration in contaminated (100 mg/l NO_3^-) groundwater treated by a denitrifying submerged filter, with different DO concentration and amended with different carbon sources: (●) methanol (68.3 mg/l), (■) sucrose (137 mg/l) and (∗) ethanol (46.8 mg/l). Values are means of five samples.

increase in NO_2^- concentration in the treated water as DO concentration increases. At high DO concentration, further treatment of the water is needed, as concentration of NO_2^- in drinking water cannot exceed 0.1 mg/l [28]. The effect is more pronounced when sucrose is used as carbon source, while similar effect was observed with alcohols. Nitrites can be easily removed from outlet water by addition of oxidant reagents also used for disinfection [29]; however, a higher dosage should be applied for high DO concentration especially when sucrose is used as carbon source.

As with the previous test, the biofilm assays were performed with high DO concentration (4.5 mg/l) and without oxygen. The influence on biofilm growth of carbon utilization, both in the presence or absence of DO, is shown in Fig. 4. After submerged filter inoculation phase, a biofilm was obtained with an average recoverable dry weight of 13.5 ± 0.5 mg/g support. The average recoverable dry weight of the biofilm increased with carbon source utilization, showing a different behavior for each of the substrates tested. When oxygen is absent, a significant positive linear correlation was observed between the rate of carbon consumed and biofilm growth ($r = 0.98$ for sucrose, $r = 0.97$ for methanol and $r = 0.96$ for ethanol). Biofilm size is directly related to clogging, which is one of the main problems of submerged filter technology [30]. In our study, this problem was more considerable when sucrose was used as carbon source, needing a higher rate of filter cleaning. In the test with 4.5 mg O_2/l water, a significant positive linear correlation was also found between carbon utilization and biofilm growth ($r = 0.98$ for sucrose, $r = 0.97$ for methanol and $r = 0.98$ for ethanol). Nevertheless, for a same rate of carbon consumed, biofilm growth was lesser when compared with the values obtained in an anoxic system (Fig. 4).

Fig. 5. shows the linear correlation between the rate of consumed carbon and density of denitrifying bacteria on the biofilm (cfu/mg recoverable dry weight). A strong positive linear correlation is shown for all the carbon sources tested, regardless of the presence or absence of DO in the influent ($r = 0.92$ for sucrose, $r = 0.93$ for methanol and $r = 0.94$ for ethanol. With DO and $r = 0.94$ for sucrose, $r = 0.96$ for methanol and $r = 0.92$ for ethanol, without DO). The slope of the curves is higher in absence of DO for alcohols, and in presence of DO for sucrose. In all cases, a significant difference was found in the bacterial densities obtained with alcohols as carbon source, when compared to sucrose. This difference could explain the inorganic nitrogen removal, which is greater in the absence of DO for alcohols.

In absence of DO the increase in biofilm recoverable dry weight is more probably due to an increase in exopolysaccharide production, as reported by Jahn et al. [31] for biofilms formed by *Pseudomonas* spp. Exopolysaccharide production also varies depending on availability of carbon sources, and is by sugar such as sucrose [32]. This fact explains the formation of big-sized biofilms with low bacterial density when sucrose is employed.

Spatial variations in biofilm physical properties affect the populations of microbial species directly. Zhang and Bishop [33] observed that facultative bacteria increased from 10^7–10^8 in the top layers to 10^9–10^{10} (cfu/cm^3 biofilm) in the bottom layers for both heterothrophic and autothrophic biofilms. This stratification is due to oxygen limitation in the bottom layers. The stratified structure and nutrient diffusion are also affected by biofilm thickness. For this reason, oxygen diffusion and developed microbial populations vary for different thickness of biofilms [34].

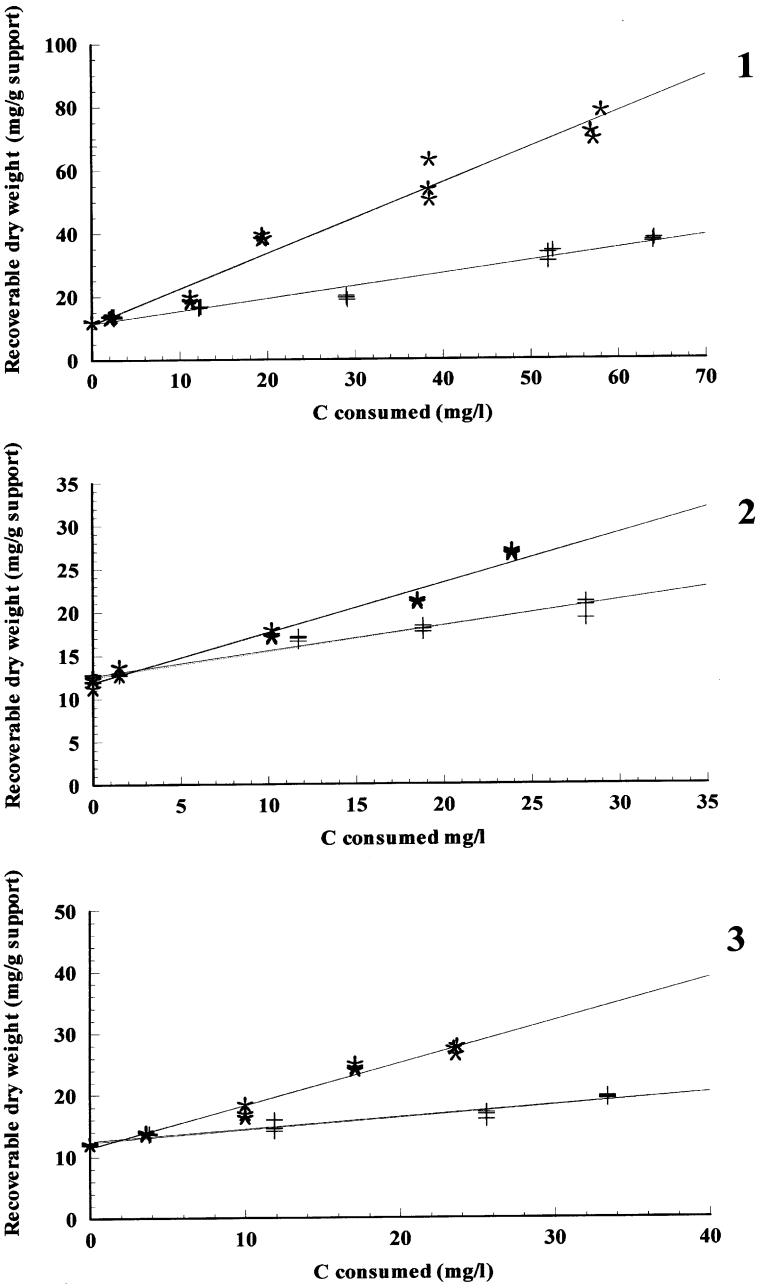

Fig. 4. Linear regression between biofilm growth (mg of recoverable dry weight/g support) and consumed carbon with (+) and without (∗) DO in influent (100 mg/litre NO_3^-) for different carbon sources: (1) sucrose, (2) methanol and (3) ethanol.

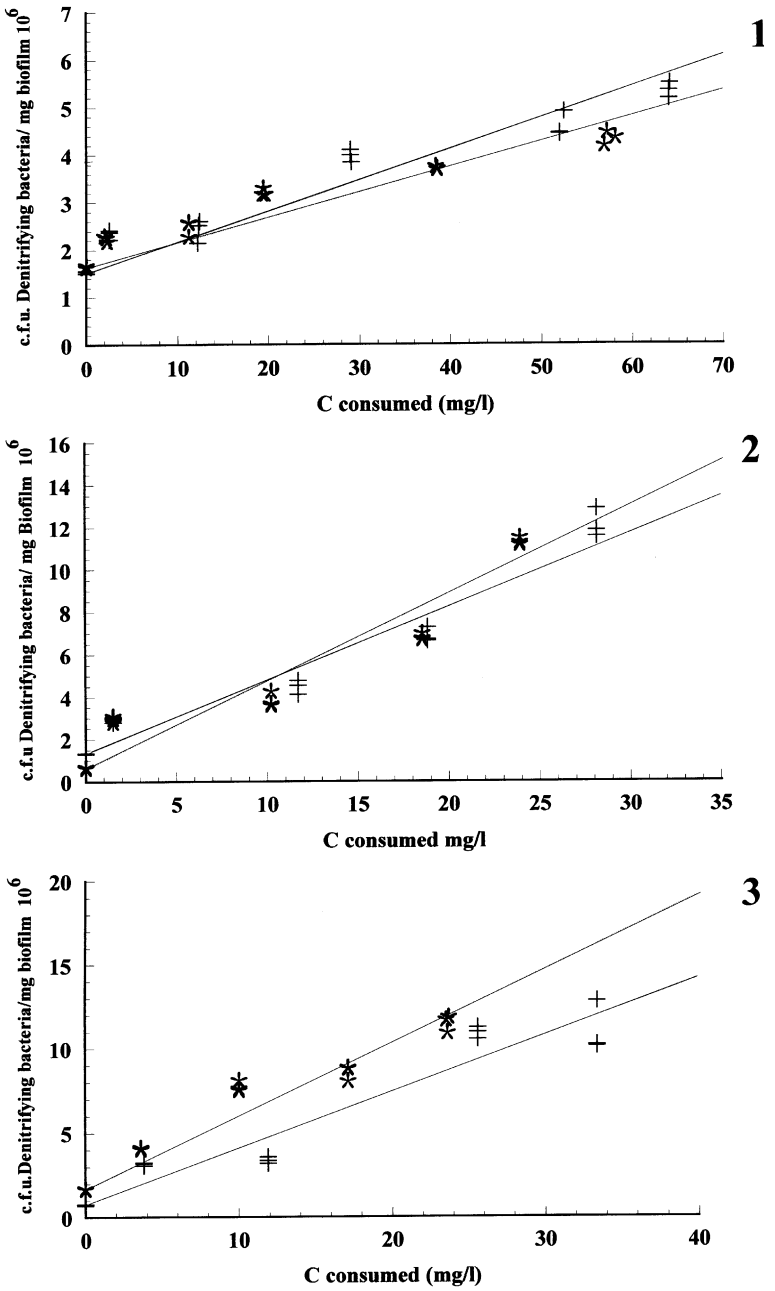

Fig. 5. Linear regression between denitrifying bacteria in biofilm (cfu/mg recoverable dry weight) and consumed carbon with (+) and without (∗) DO in influent (100 mg/litre NO_3^-) for different carbon sources: (1) sucrose, (2) methanol and (3) ethanol.

Oxygen diffusion could be more limited through biofilm developed without oxygen than through biofilm developed in presence of high DO concentration (4.5 mg/l), due to the higher biofilm formed. This would explain the different influence on inorganic nitrogen removal for low and high DO concentration.

High nitrite levels have been observed in bioreactors with fixed microorganisms treating drinking water by denitrification with heterotrophic cultures [35]. Oxygen concentration is one of the main factors responsible for nitrite accumulation, together with the nitrate reducing bacteria and the selection of the denitrifying microorganisms [36]. Denitrifying bacteria versus nitrate reducing bacteria ratio was also linearly and positively correlated to the consumption of carbon source in all the tests carried out, both in presence or absence of DO, similar to density of denitrifying bacteria. This demonstrates that raising the consumption of carbon source increases the population of denitrifying bacteria more than the nitrate reducing bacteria. The increments were more pronounced in tests run in absence of DO. The type of carbon source used also influenced the composition of the biofilm, with a lesser ratio for the test using sucrose as carbon source. Increase of nitrate reducing bacteria could be related to the presence of nitrites in the outlet water. The effect is more pronounced with sucrose as carbon source and in the presence of DO.

4. Conclusions

The application of biofilms as biological processes for the treatment of groundwater contaminated with nitrates, allows for the obtaining of fresh water suitable for further processing into drinking water without presenting any public health problems related with these anions. These systems require the control of different factors which affect the process, being one of the most important constraints to be considered DO.

This presence of O_2 as electron acceptor in the water makes the inorganic nitrogen removal decrease, and increases the concentration of nitrite, the anion mainly responsible for nitrate-related diseases. This negative effect is increased with the rise in DO concentration and varies depending on the nature of the electron donor applied in the process.

The use of sugars such as sucrose involves the formation of large biofilms (recoverable dry weight), whose size increases in the absence of DO, producing frequent clogging of the system. Bacterial density in these biofilms is low, particularly in the presence of oxygen, because of which yields are very low. Bacterial composition of the biofilm also varies with the oxygen concentration, there is an increase of the denitrifying bacteria versus nitrate reducing bacteria ratio when the DO concentration is low. This ratio is very low when sucrose is employed, giving rise to an immediate presence of nitrites in the treated water because of the increase in DO concentration.

The effects caused by oxygen with alcohols such as ethanol and methanol used as carbon sources are similar, although in these cases smaller biofilms were obtained, with higher bacterial density and superior rates of denitrification versus nitrate reduction, causing a descent in the DO negative effect. These carbon sources are more appropriate for the biological denitrification of water contaminated with nitrate and containing DO.

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