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# Assessment of the addition of *Thiobacillus denitrificans* and *Thiomicrospira denitrificans* to chemolithoautotrophic denitrifying bioreactors

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**Summary.** The aim of the present study was to assess the impact of adding cultures of *Thiobacillus denitrificans* and *Thiomicrospira denitrificans* to two upflow anaerobic sludge bed (UASB) reactors: one inoculated with granular sludge and the other filled only with activated carbon (AC). The performances of the bioreactors and the changes in biomass were compared with a non-bioaugmented control UASB reactor inoculated with granular sludge. The reactors inoculated with granular sludge achieved efficiencies close to 90% in nitrate and thiosulfate removal for loading rates as high as 107 mmol-$NO_3^-$/l per day and 68 mmol-$S_2O_3^{2-}$/l per day. Bioaugmentation with *Tb. denitrificans* and *Tm. denitrificans* did not enhance the efficiency compared to that achieved with non-bioaugmented granular sludge. The loading rates and efficiencies were 30-40% lower in the AC reactor. In all the reactors tested, *Tb. denitrificans* became the predominant species. The results strongly suggest that this bacterium was responsible for denitrification and sulfoxidation within the reactors. We additionally observed that granules partially lost their integrity during operation under chemolithoautotrophic conditions, suggesting limitations for long-term operation if bioaugmentation is applied in practice. [**Int Microbiol** 2008; 11(3):XXX-XXX]

**Key words:** *Thiobacillus denitrificans* · *Thiomicrospira denitrificans* · chemolithotropic denitrification · bioaugmentation · granular sludge · UASB reactors

## Introduction

Nitrate contamination in groundwater is a problem in Europe. Of the contributing factors, agricultural sources are the largest contributor since nitrogen fertilizers and manure are used in excessive amounts to increase crop productivity. Consequently, non-consumed nitrogen leaches through the soil, polluting fresh waters. According to the guidelines of the European Union (EU) and World Health Organization (WHO), nitrate concentrations in drinking water should not exceed 50 mg-$NO_3^-$/liter. However, this limit is surpassed in approximately one-third of the groundwater bodies in the EU, especially those in southwestern Europe [Council Directive 98/83/EC (1998). Official Journal L 330, 05/12/1998, pp. 0032-0054; EC Report COM (2007), 120 final, 20]. In the case of surface waters, nitrate concentrations are usually lower but in 47% of surface water bodies the level set by WHO guidelines for short-term exposure to nitrate (10 mg/l) is exceeded [20]. High nitrate concentrations in either surface waters or ground waters have been associated with several environmental and health problems. Low levels of nitrate contamination are apparently nontoxic, but the toxicity of nitrate greatly increases when bacteria commonly found in the upper gastrointestinal tract reduce it to nitrite. Nitrite can undergo nitrosation reactions to produce *N*-nitroso compounds, which are some of the most powerful carcinogens known [3,9]. Moreover, nitrate is one of the main contaminants responsible for the eutrophication of surface waters.

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To eliminate nitrate as a contaminant, physicochemical methods (ion exchange and reverse osmosis) can be used but they present different problems that limit their application [13]. The most promising approach is biological denitrification. Heterotrophic denitrification—in which organic matter provides electron donors to reduce nitrate to di-nitrogen gas—has been broadly used for decades in wastewater treatment. Many bacterial genera (*Pseudomonas*, *Alcaligenes*, *Bacillus*, etc.) include species capable of denitrification [13]. Autotrophic denitrification is an alternative to heterotrophic denitrification in the treatment of water with low chemical oxygen demand, such as ground water. It produces less sludge and avoids the limitations related to the need for organic matter. Some bacteria can use hydrogen, ferrous iron, or reduced sulfur compounds as electron donors and carbon dioxide as the carbon source. Sulfoxidizing denitrification (SOD) is very promising because it allows the removal of two contaminants (such as $H_2S$ and $NO_3^-$) in a single step, converting them into environmentally benign compounds ($SO_4^{2-}$ and $N_2$). Only two species are able to perform SOD under neutral and non-salt conditions: *Thiobacillus denitrificans* and *Thiomicrospira denitrificans*. This new technique has been studied at laboratory-scale mainly using sulfur packed-bed reactors and, in a few cases, fluidized bed or upflow anaerobic sludge bed (UASB) reactors [18].

Bioaugmentation is based on the introduction of non-indigenous microbial strains (natural or genetically engineered) to improve microbial treatment of polluted environments. The advantages of this technique include decrease in the retention time of solids and an increase in degradation capability as well as resistance to toxic compounds and extreme conditions [10,17,19]. Bioaugmentation has been used recently to improve nitrification [10,11,12,14] but only a few studies have assessed denitrification [8]. Therefore, the aim of this study was to assess the feasibility of bioaugmentation to increase nitrate removal in SOD bioreactors. With this goal, a UASB reactor filled with anaerobic granular sludge and an activated carbon packed-bed reactor were inoculated with *Tb. denitrificans* and *Tm. denitrificans*. Scanning electron microscopy (SEM) and fluorescence in situ hybridization (FISH) were used to follow the changes into granules and the microbial population of SOD bacteria.

## Materials and methods

**Sludge and microorganisms**. The methanogenic granular sludge used as inoculum was obtained from a full-scale UASB reactor from a paper factory (Eerbeek, the Netherlands) and maintained at 4°C over 4 months until its use. The concentration of volatile suspended solids (VSS) in the sludge was 11.8%. Pure cultures of *Tb. denitrificans* (Kelly and Harrison, 1989) DSM807 and *Tm. denitrificans* (Timmer-ten Hoor, 1975) DSM125 were purchased from the DSMZ (Braunschweig, Germany). The following mineral media were used to incubate the microorganisms at 30°C (g/l): 1.05 $K_2HPO_4{\cdot}3H_2O$, 0.3 $KH_2PO_4$, 0.18 $MgCl_2{\cdot}6H_2O$, 0.4 $NH_4Cl$, 2 $NaHCO_3$, 3.16 $Na_2S_2O_3{\cdot}5H_2O$, 3.79 $KNO_3$, plus 2 ml of a mixture of trace elements/l. The trace element solution was prepared as follows (mg/l): 500 EDTA, 39 $ZnSO_4{\cdot}7\ H_2O$, 55 $CaCl_2$, 51 $MnCl_2{\cdot}4\ H_2O$, 11 $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 16 $CuSO_4{\cdot}5H_2O$, 16 $CoCl_2{\cdot}6\ H_2O$; the pH was adjusted to 7.3 ± 0.2.

**Experimental design**. Three laboratory-scale UASB reactors (0.9 l) were operated for 4.5 months at 30 ± 1°C. Reactors 1 and 2 were inoculated with 47 g-VSS/l of methanogenic granular sludge. Reactor 3 was filled with 133 g/l of granular activated carbon (Chemviron F-400, Aguas de Levante, Barcelona, Spain) without granular sludge. The basal mineral media described above were used to feed the reactors. $KNO_3$ served as electron acceptor and $Na_2\ S_2O_3 \cdot 5\ H_2O$ as electron donor in a redox equimolecular ratio according to Eq 1. No other carbon or energy source was added.

$$8\ NO_3^- + 5\ S_2O_3^{2-} + H_20 \rightarrow 4\ N_2 + 10\ SO_4^{2-} + 2\ H^+ \qquad \text{(Eq. 1)}$$

After a period of 45 days of adaptation to chemolithotrophic conditions, reactors 2 and 3 were inoculated with a 1:1 mixture 1:1 of *Tb. denitrificans* (240 ml, $OD_{660}$ = 0.055) and *Tm. denitrificans* (320 ml, $OD_{660}$ = 0.041) and operated in batch mode for 15 days. Subsequently, the reactors were operated in continuous mode with a hydraulic retention time (HRT) of 1 day. The $S_2O_3^{2-}$- and $NO_3^-$-loading rates were increased from 40 mmol $NO_3^-$/l per day and 20 mmol $S_2O_3^{2-}$/l per day up to 107 mmol $NO_3^-$/ l per day and 68.5 mmol $S_2O_3^{2-}$/l per day. This experimental set-up allowed the comparison of: (i) the effect of bioaugmentation on UASB reactors inoculated with granular sludge and (ii) the difference between granular sludge and activated carbon as packed material for bioaugmentation.

**Catalyzed reporter deposition–fluorescent in situ hybridization (CARD–FISH).** Granular sludge was fixed with formaldehyde (4% in phosphate saline buffer, PBS) for 12 h. Afterwards, the samples were washed twice in PBS, and stored in PBS:ethanol (1:1) at –20°C. Samples were hybridized according to a procedure described previously [15] and using the following probes: EUB338, universal for the *Bacteria* domain [2]; TBD121, specific for *Tb. denitrificans* [7]; and TMD131, specific for *Tm. denitrificans* [7]. Oligonucleotide probes were supplied by Genotek (Barcelona, Spain). Hybridized and DAPI (4′,6′-diamin-2-phenylindole)-stained cells were quantified using an epifluorescent microscope (Zeiss Axioskop) equipped with filters for Cy3 (G-2A, λ = 550–570) and DAPI (UV-2A, λ = 359–461).

**Scanning electron microscopy (SEM).** Sludge and activated carbon granules were studied by SEM as described elsewhere [1]. Samples were taken after 0, 23, 90, and 132 days of operation, fixed by immersion in glutaraldehyde (2.5%) for 2 h, and then washed twice in sodium cacodylate buffer (0.2 M, pH 7.1). The granules were dehydrated in a graded series (10, 30, 50, 70, 90, and 100%) of ethanol/water mixtures for 20 min each. After dehydration, the samples were critical-point dried and mounted on stubs. After gold shadowing, the samples were examined in a Phillips XL30 EDAX DX4i SEM.

**Chemical analyses.** Nitrate, nitrite, sulfate, and thiosulfate were determined by ion chromatography with a suppressed conductivity detector, using a Metrohom system equipped with an AS11-HC Dionex column (Dionex, Sunnydale, CA). The eluent was a carbonate/bicarbonate buffer and the flow rate 0.7 ml/min. Liquid samples were membrane-filtered (0.22 μm) prior to chromatographic analysis. The pH was determined immediately after sampling with an Orion model 310 PerpHecT pH-meter with a PerpHecT ROSS glass combination electrode. VSS content was determined according to Standard Methods APHA [4] (method 2540 E).

## Results and Discussion

**Reactor performance.** Figure 1 shows the nitrate and thiosulfate loading rates and removal efficiencies. The pH remained stable throughout the first 100 days. After the thiosulfate-loading rate had increased to 40 mmol/l per day (day 106), the pH of reactors R1 and R2 drastically dropped to values of 6 and 5, respectively (data not shown). This fall was probably due to increased production of sulfate and protons (see Eq. 1).

As seen in Fig. 1, the performances of reactors R1 and R2 were very similar. With respect to nitrate and thiosulfate removal, the addition of SOD bacteria did not improve the effectiveness of the UASB containing granular sludge. The bioaugmented UASB and the control UASB had similar removal efficiencies, which were close to 90% for both contaminants even at the maximum volumetric loads tested. The loading rates of the reactor containing only activated carbon and inoculated with SOD bacteria were 70% of those measured in the reactors with granular sludge. Despite the lower loading rates, the SOD-bioaugmented activated carbon reactor reached $NO_3^-$ and $S_2O_3^{2-}$ removal efficiencies of only 60%. Flores et al. [10] compared the performance of an enriched sludge augmented with *Alcaligenes defragrans* with that of non-bioaugmented ones. No advantage of the bioaugmented system over the enriched sludge system without *A. defragrans* was observed regarding the overall efficiency of denitrification. Unfortunately, the efficiency of bioaugmentation is difficult to predict; however, when effective, the results are often very encouraging. The difficulties arise from the diversity of the microorganisms used, environmental heterogeneity, and variations in the influence of critical parameters (e.g., reactor operational conditions) which are not always identified. Moreover, even though some knowledge about the optimal conditions on pure cultures is available, this information does not necessarily apply to real or bioreac-

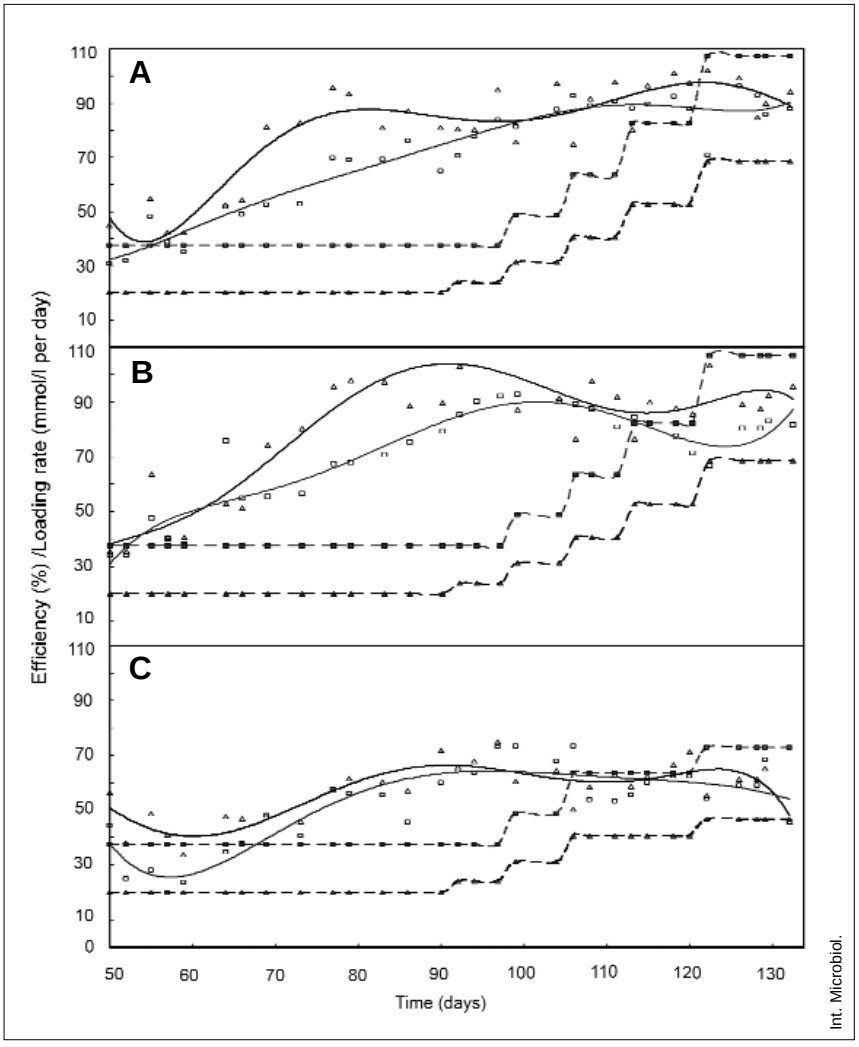


**Fig. 1**. Effect of thiosulfate (full squares) and nitrate (full triangles) loading rates on efficiencies in thiosulfate (empty squares) and nitrate (empty triangles) removal. (**A**) Reactor 1, control; (**B**) reactor 2, bioaugmented; (**C**) reactor 3, activated carbon.

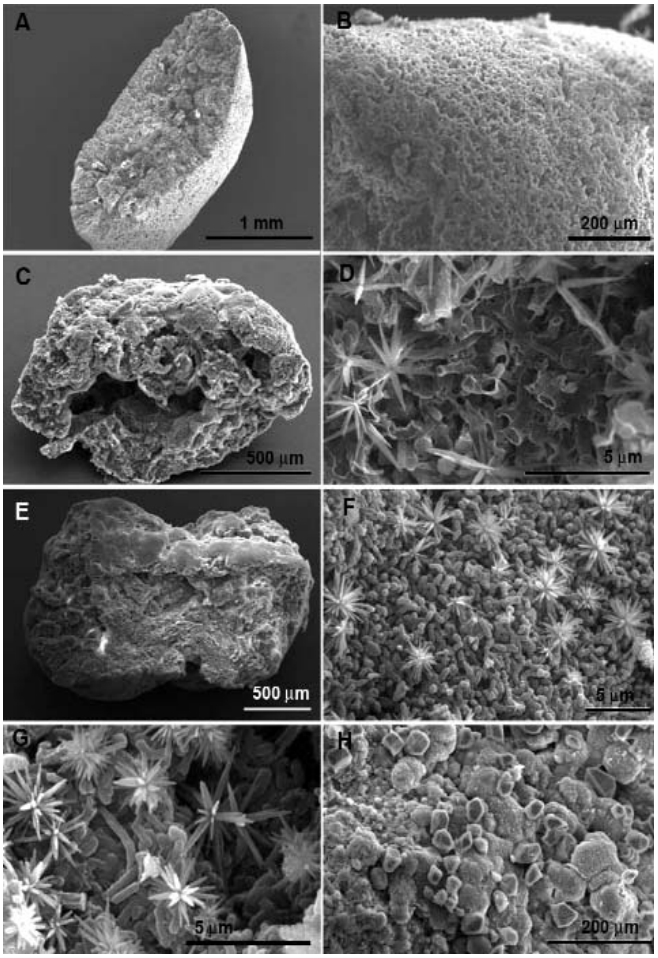


**Fig. 2**. SEM images. (**A**,**B**) Methanogenic granules, used as inoculum; (**C**–**H**) chemolithotrophic granules.

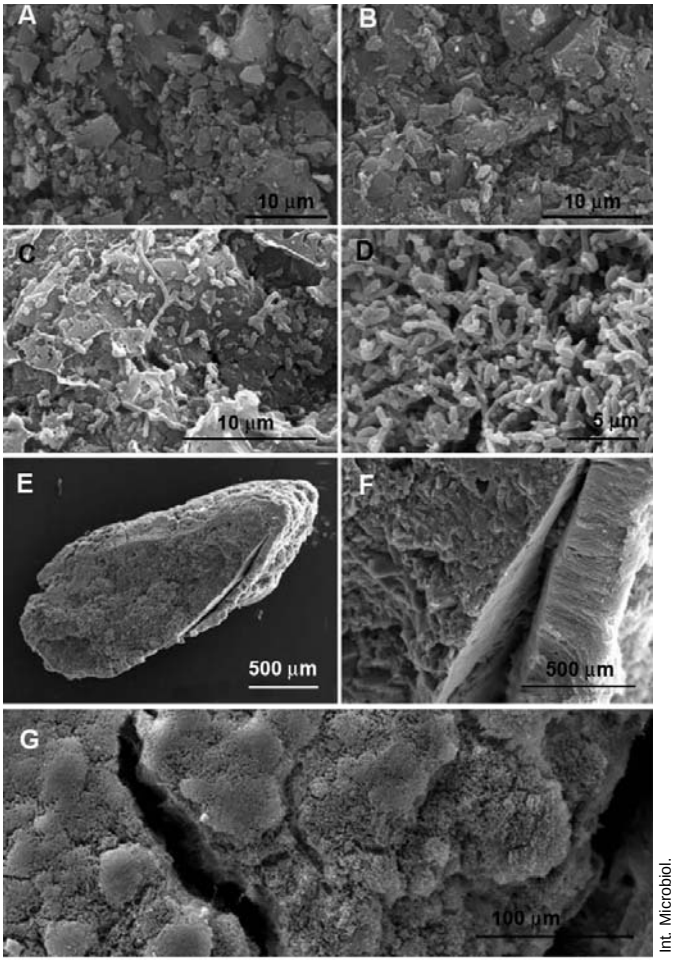


**Fig. 3.** Scanning electron micrographs of a biofilm that developed over time on granules of activated carbon. (**A**) Granule of carbon used as inoculum; (**B**) after 23 days; (**C**,**D**) after 90 days; (**E**,**F**) after 132 days. Panels C and D correspond to carbon particles from the top and bottom of the reactor, respectively. Panels E and F show the section of a granule. Details of the surface of the biofilm can be appreciated in panel G.

tor environmental conditions, in which there is competition with other bacteria. Chemolithoautotrophic denitrifiers have not been investigated in detail, and greater efforts are needed to define optimal conditions for their use in the field.

**Macroscopic and microscopic changes in the granular sludge.** Distinct differences between the granular sludge used as inoculum and the granules after completion of the reactor operation under chemolitotrophic conditions were observed. The color of the granules shifted from black to brown and the granules lost their spherical shape, becoming amorphous. At the same time, large clusters or flocks formed and individual/isolated granules were not seen. Based on the operational conditions, the theoretical $N_2$ release during the first 3 months of operation, in terms of nitrate removal, was approximately 300 ml-$N_2$/l per day. The amount of biogas generated by a similar methanogenic reactor is 25-50 times higher. The low-level mixing of the sludge bed due to the low gas release together with the low upflow velocity achieved in the UASB reactors at laboratory scale could have caused granule aggregation by the welding of outer-layer slime polysaccharides.

Structural changes in the granules were analyzed by SEM (Fig. 2). Methanogenic granules used as inoculum were spherical-elliptical and compact (Fig. 2A,B). The chemolitotrophic granules were characterized by large voids (Fig. 2C), and sheaths of dead cells (Fig. 2D). Shift from methanogenic to chemolitoautotrophic conditions most likely decrease the activity and viability of the majority of heterotrophic and methanogenic microorganisms, increasing the decay rate. The consequent endogenous biomass digestion results in the formation of inner voids in the granules. These results were confirmed by determination of the VSS content of the granular sludge, which decreased during performance of the reactors,

**Table 1.** Microbial composition of the three reactors determined by CARD-FISH using specific probes for the domain Bacteria (EUB338), *Thiobacillus denitrificans* (TBD121), and *Thiomicrospira denitrificans* (TMD131)

| | Cells hybridized (% of DAPI-stained cells) | | |
|---|---|---|---|
| Time (days) | EUB338 (mean ± SD) | TBD121 (mean ± SD) | TMD131(mean ± SD) |
| | Control (R1) | | |
| 0 | 23.8 ± 7.1 | 6.4 ± 4.4 | 2.2 ± 3.6 |
| 41 | 25.8 ± 11.4 | 10.1 ± 6.8 | 2.0 ± 3.8 |
| 69 | 22.2 ± 12.7 | 3.8 ± 3.1 | 0.3 ± 0.6 |
| 103 | 45.4 ± 7.7 | 10.9 ± 3.7 | 2.5 ± 1.7 |
| 132 | 81.1 ± 14.6 | 36.4 ± 10.7 | 1.3 ± 1.4 |
| | Bioaugmented (R2) | | |
| 0 | 23.8 ± 7.2 | 6.4 ± 4.4 | 2.3 ± 3.7 |
| 41 | 24.0 ± 6.9 | 10.9 ± 7.8 | 1.0 ± 0.7 |
| 69 | 30.4 ± 12.7 | 27.1 ± 11.3 | 2.6 ± 4.8 |
| 103 | 43.9 ± 12.0 | 43.9 ± 17.0 | 1.8 ± 2.0 |
| 132 | 96.9 ± 4.61 | 93.1 ± 5.8 | 1.0 ± 2.3 |
| | Activated carbon (R3) | | |
| 69 | 99.3 ± 1.6 | 25.3 ± 8.3 | 0.8 ± 1.4 |
| 103 | 97.0 ± 9.2 | 83.4 ± 0.6 | 0.5 ± 0.6 |
| 132 | 98.3 ± 2.1 | 100.0 ± 0.1 | 0.3 ± 0.4 |

from 11.8 ± 0.5% in granules used as inoculum to 10.0 ± 0.7% in R1 and 10.8 ± 0.1% in R2 at the end of the experiment. These values agree with those obtained in an endogenous digestion observed by SEM (Fig. 2B,C). Other granules were less affected (Fig. 2E), showing large microbial diversity (Fig. 2F,H). As an intermediate step in thiosulfate oxidation, crystals of elemental sulfur appear (rhomboedrical shapes in Fig. 2G), as determined by EDAX. With time, granules partially lose their integrity under chemolithoautotrophic conditions, which could cause long-term problems in the operation of a full-scale reactor. A potential alternative is to use reactors packed with granular activated carbon, although the loading rates and efficiencies are lower.

The development of a biofilm on the activated carbon granules was also followed by SEM. Figure 3 shows the sequence of colonization over time, from inoculum up to day 132, at which point the granule of carbon was completely covered by a biofilm.

**CARD-FISH analyses.** In the reactor filled with granular activated carbon, all microorganisms present corresponded to those of the domain Bacteria (Table 1). The number of *Tb. denitrificans* cells increased with time such that at the end of the experiment all bacteria present belonged to this species. Colonization by *Tm. denitrificans* was minimal, close to 2% of the total bacteria in the carbon.

In the reactors inoculated with granular sludge, 24% of the DAPI-stained cells in the inoculum hybridized with the universal probe for Bacteria (Table 1). This low value is in the range reported by other authors, 37% for granular sludge in the treatment of paper-mill wastewater [19], and 22–50% for granular sludge in the treatment of brewery wastewater [5]. The percentage of cells in reactors R1 and R2 that hybridized with the Bacteria probe increased with time, reaching 80% (R1) and 95% (R2) of the total microorganisms (Table1). This was in accordance with the operational conditions, which were unfavorable to methanogenic archaea such that their relative numbers decreased throughout reactor operation.

As in the reactor filled with activated carbon, the number of *Tb. denitrificans* in the reactor increased gradually. The number of cells remained quite constant (R1) or increased slightly (R2) during the first 100 days, when the loading rates and the efficiencies were almost constant. After that, the number of *Tb. denitrificans* rose sharply, in parallel with the increased loading rates (Fig.1); after 4.5 months of operation, the bacterium accounted for 93% of the total cells in R2 and 37% of those in R1. This latter value is in accordance with

the percentage of *T. denitrificans* measured by cloning (30%) and CARD-FISH (35%), reported for a chemolitotrophic reactor after 6 months of operation [11]. These values contrast with the low and constant values obtained for *Tm. denitrificans*: 2–3% without significant changes. In the operational conditions of our reactors, *Tb. denitrificans* outperformed *Tm. denitrificans*, although these chemolitotrophic bacteria compete for the same substrate and electron acceptor. The difference was probably due to the fact that *Tm. denitrificans*, a marine bacterium, suffered saline stress in the reactors. Consequently, these results strongly suggest that *Tb. denitrificans* was responsible for denitrification and sulfur-oxidation under the operational conditions of our reactors; however it cannot be excluded that other species may predominate in these reactions under incubation and process conditions other than those applied here.

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