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Characterization and performance of constructed nitrifying biofilms during nitrogen bioremediation of a wastewater effluent

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Abstract Constructed ammonium oxidizing biofilms (CAOB) and constructed nitrite oxidizing biofilms (CNOB) were characterized during the bioremediation of a wastewater effluent. The maximum ammonium removal rate and removal efficiency in CAOB was 322 mg $N\text{-}NH_4^+$ m^{-3} d^{-1} and 96%, respectively, while in CNOB a maximum removal rate of 255 mg $N\text{-}NH_4^+$ m^{-3} d^{-1} and a removal efficiency of 76% was achieved. Both constructed biofilms on low-density polyester Dacron support achieved removal efficiencies higher than that of the concentrations normally present in reactors without constructed biofilms ($P < 0.05$). Nitrifying bacteria from the constructed biofilms cultures were typed by sequencing 16S rRNA genes that had been amplified by PCR from genomic DNA. Analysis of enrichment biofilms has therefore provided evidence of high removal of ammonium and the presence of *Nitrosomonas eutropha*, *N. halophila* and *N. europaea* in CAOB, while in CNOB *Nitrobacter hamburgensis, N. winogradskyi and N. alkalicus* were identified according to 16S rRNA gene sequences comparison. The biofilm reactors were nitrifying over the whole experimental period (15 days), showing a definite advantage of constructed biofilms for enhancing a high biomass concentration as evidenced by environmental electron microscopic analysis (ESEM). Our research demonstrates that low-density polyester Dacron can be effectively used for the construction of nitrifying biofilms obtaining high removal efficiencies of nitrogen in a relatively short time from municipal effluents from wastewater treatment plants. CAOB and CNOB are potentially promissory for the treatment of industrial wastewaters that otherwise requires very large and expensive reactors for efficient bioremediation of effluents.

Keywords Nitrification · Constructed biofilm reactors · Wastewater effluent · Bioremediation

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Introduction

Municipal wastewater effluents is one of the largest sources of pollution, by volume, being discharged to surface water bodies in coastal countries. New and more efficient wastewater treatment biotechnologies for pollutant removal are therefore needed to maintain water quality standards into receiving water bodies and to meet regulatory requirements.

Biofilm systems are prototypes of highly stratified and dense microbial communities. It is accepted that boundary layers protect the microorganisms of biofilms against losses by flushing, shear stress, and inhibitory substances. That is why microorganisms structured in support matrix are preferred than in a system with suspended biomass and a high flow-through rate [5, 28]. Biofilm systems allow slow growing nitrifying bacteria to remain in the reactors by their attached growth, which improves nitrogen removal from wastewater [24].

However, difficulties often arise in establishing stable nitrification in support matrix sometimes due to the

low substrate affinities. Although extensive studies through a combination of microcopy, selective culture, microprobes, and molecular methods have been carried out on nitrifying biofilms in different supports [6, 10, 28], little information is available on the molecular characterization and performance of constructed nitrifying biofilms on low-density polyester Dacron support during the bioremediation of a wastewater effluent.

Much of what makes life in a constructed microbial biofilm different from life in a free aqueous suspension or from natural biofilm is the support matrix. Our main premise of this research was based on the fact that nitrification carried out by the ammonia-oxidizing bacteria and the nitrite-oxidizing bacteria, could exhibit contrasting results when artificially structured in biofilms. The objective was to characterize the populations of constructed nitrifying biofilms in low-density polyester Dacron support and to reveal which biofilm population (CAOB, CNOB) contribute to which part of the nitrogen bioremediation (ammonium and nitrite oxidation) of the effluent of a municipal wastewater treatment plant. Advantages of using low-density polyester include improved effluent quality, reduced system volume, improved oxygen transfer and greater surface contact area (approximately 90%) per unit volume than other media as well as low cost. These advantages can be applied for the treatment of industrial wastewater that would otherwise require very large, expensive and impractical bioreactors to meet regulatory requirements [16].

Materials and methods

Enrichment of autotrophic nitrifying bacteria

Mixed liquor from El Naranjo municipal wastewater treatment plant in Ensenada BC, Mexico was used for the development of ammonium and nitrite oxidizing bacteria by inoculating samples in two set of batch cultures. One set containing selective medium for the development of ammonia-oxidizing bacteria contained in g l^{-1} distilled water: Na_2HPO_4 13.5; KH_2PO_4 0.7; $MgSO_4 \cdot 7H_20$ 0.1; $NaHCO_3$ 0.5; $(NH_4)_2SO_4$ 2.5; $FeCl_3 \cdot 6H_2O$ 7; $CaCl_2 \cdot 2H_2O$ 0.12; pH 7.8 ± 0.2. A second set with medium for nitrite-oxidizing bacteria contained in g l^{-1} glass distilled water: $NaNO_2$ 2.0; K_2HPO_4 1.0; $MgSO_4 \cdot 7H_20$ 0.1; NaCl 0.5; $MnSO_4 \cdot 4H_2O$ trace; $Fe_2(SO_4)_3 \cdot 9H_2O$ trace; pH 7.8 ± 0.2. The media were sterilized by autoclaving at 1 kg cm^2 (15 psi), 121°C and then cooled. In both cases nitrogen and phosphate enrichment concentrations were similar to the in situ concentrations. Each culture was subcultured several times in batch columns containing enrichment medium for isolation of ammonium- or nitrite-oxidizing bacteria to exert a strong selection for bacteria prior to the construction of the nitrifying biofilms.

Nitrifying activity and characterization of ammonium and nitrite oxidizing Proteobacteria were determined by analyzing the presence of ammonium, nitrite and nitrate concentrations and pH level in enrichment cultures following the methodology of analysis described in the section of chemical analysis.

Construction of nitrifying biofilms and bioreactors

The support for biofilm construction was low-density Polyester Dacron, a commercially available insulating and domestic polymer distributed by DuPont. Disks of this polymer were prepared with a cork boring tool (50-mm diameter). Nitrifying biofilms were constructed by adding 1 ml (2.5×107 cells ml–1 and 2.8×107 cells m–1) of ammonia and nitrite oxidizing bacteria culture, respectively, into circular surfaces of low-density polyester Dacron support (20 mm thickness × 50 mm diameter). Constructed ammonia oxidizing biofilms (CAOB) and constructed nitrite oxidizing biofilms (CNOB) were packed into their respective bioreactors separately. Three bioreactors were packed by treatment (CAOB, CNOB and control respectively). The bioreactors made of glass were 50 mm in diameter and 300 mm tall for a maximum functional volume of 400 ml; Circular acrylic disks (profusely perforated) were placed inside the reactors in order to overlay the constructed biofilms (three per bioreactor) in the experimental bioreactors (Fig. 1). Air was bubbled continuously into each culture via a manifold system connected to an air compressor at a flow rate of 300 ml of sterile air per minute, corre-

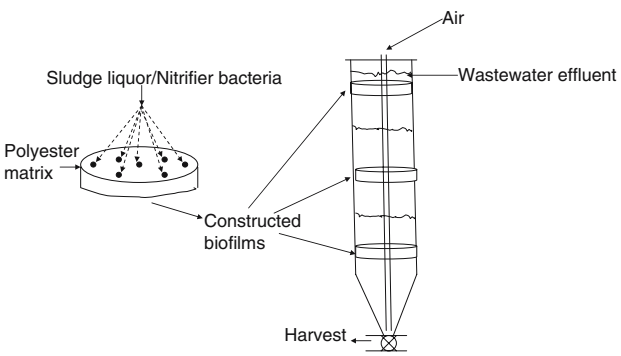

Fig. 1 Construction of nitrifying biofilms and set-up in bioreactors

sponding to a dissolved oxygen level of 6 mg l^{-1}. Each bioreactor had a lid to minimize aerial contamination and aerosol production.

Wastewater and assays of bioremediation

Water samples taken from the combined effluent of the two largest municipal wastewater treatment facilities, El Gallo and El Naranjo (Ensenada BC, Mexico) were fed to bioreactors. The effluent wastewater had the following composition (mg l^{-1}): PO_4–P 6.5, NH_4–N 6, NO_2–N 3.2, NO_3–N 0.3, BOD_5 50. pH 7.8. Effluents from El Gallo and El Naranjo discharge to the Todos Santos Bay (Ensenada BC Mexico) at a flow rate of approximately 30,000 m^3 per day.

The reactors were continuously operated for 15 days in batch mode and were harvested for analysis every 2 days unless otherwise specified. The temperature was maintained at 21°C under dark conditions. Ammonia and nitrite enrichment conditions were set up in reactors independently. Control bioreactors were prepared with support lacking inoculation liquor. Neither carbonate for pH nor carbon source was added into reactors for pH stability. Levels of oxygen higher than 6 mg l^{-1} were kept in all the biofilm treatments. Samples of treated wastewater were harvested after passing through the biofilms in reactors in down flow direction (Fig. 1).

Chemical analysis

Ammonium (N-NH_4^+), Nitrite (N-NO_2^-) and nitrate (N-NO_3^-) in the constructed biofilm reactors with wastewater were determined colorimetrically by spectrophotometry (Hewlett Packard model 8453 with diode array) following methods described in [8]. Briefly, filtered samples were obtained by filtering the wastewater through Millipore filters (pore size 0.22 μm). Ammonium was heated with a solution of fenol-nitroprusside and an oxidant reagent (sodium citrate and sodium hypochlorite in an alkaline medium). Nitrite was determined by diazotizing with sulfanilamide followed by coupling with N-(1-naphthyl) ethlyenediaminie dihydrochloride. Nitrate was analyzed by reduction to nitrite via a copperized cadmium column. Dissolved oxygen concentrations were measured with a Yellow Spring electrode model 52 and pH was measured with an Orion pH meter. Results reported herein are the arithmetic mean of triplicate analysis performed in each constructed biofilm reactors during the bioremediation process. A one-way analysis of variance was applied to detect differences in ammonium and nitrite removal rate between both constructed biofilms reactors and control reactors . Means were compared by treatment and ranked using Tukey's multiple range tests [35]. The critical value in all test was $P = 0.05$ unless tested otherwise.

Effluent treatment efficiency

The effluent treatment efficiency was calculated as a percentage of element (X) removed, using the formula: removal (%) = $(C_{inf} - C_{eff})/C_{inf} \times 100$, where C_{inf} and C_{eff} are the concentrations of the element X in the influent and effluent wastewater in the biofilm reactors. Removal rate (R, mg X m^{-3} day^{-1}) was evaluated in each bioreactor by the formula:

$$R = (S_0 - S)/\mathrm{HRT}$$

where, S_0 and S are the initial (day 0) and sample substrate concentration as mg l^{-1} calculated at hydraulic retention time (HRT).

DNA extraction

Genomic DNA was extracted at the end of the experiment according to a standard protocol [32]. Samples of activated sludge and constructed biofilms were homogenized by vigorous vortexing. The bacterial cells recovered after centrifugations were resuspended in saline-EDTA at pH 8 and incubated at 37°C for 1.5 h with lysozyme (15 mg ml^{-1}), proteinase K (20 mg ml^{-1}). The cell lysate was extracted with hexadecyltrimethyl ammonium bromide (10%/0.7 M NaCl). Nucleic acids were precipitate with isopropanol, washed with ethanol and resuspended in distilled water. The quality of the nucleic acids was visualized by agarose gel electrophoresis (1%, wt/vol).

Previously described oligonucleotides primers using the 16S rRNA gene sequences of several species specific for certain ammonia-oxidizing [34] and nitrite-oxidizing [11] bacteria were used (Table 1). An extra set of primers were used to amplify 16S rRNA gene sequences by universal primers described by [2]. 16S rRNA gene fragments from DNA samples of liquor sludge and constructed biofilms were amplified with Taq DNA polymerase using a programmable thermal cycler (Hybaid model HBP × 110) as follows: 3 min of initial denaturation at 94°C followed by 30 cycles of amplification at 94°C for 45 s, 58 and 55°C for 1 min for ammonia oxidizing and nitrite oxidizing bacteria, respectively, and 72°C for 1 min. An extra extension step for 5 min at of 72°C was added. PCR amplification of the complete 16S rDNA gene using universal primers was performed under the following conditions:

Table 1 Primer sequences used for PCR

Primer	Sequence (5′–3′)	Specificity amplification	Size (bp)
BONF	5′TTTTTTGAGATTTGCTAG3′	NO 16S rRNA gene	397
NonsR	5′CTAAAACTCAAAGGAATTGA3′	NO 16S rRNA gene	397
NITA	5′CTTAAGTGGGGAATAACGCATCG3′	AO 16S rRNA gene	1,080
NITB	5′TTSCGTGTGAAGCCCTACCCA3′	AO 16S rRNA gene	1,080

NO nitrite oxidizing belonging to the α subclass of Proteobacteria, *AO* ammonia oxidizing belonging to the β subclass of Proteobacteria

5 min of initial denaturation at 95°C followed by 25 cycles of amplification at 95°C for 1 min, 55°C for 1 min and 72°C for 2 min. An extra extension step for 10 min at 72°C was added. The PCR products were electrophoresed on a 1% (wt/vol) agarose gel.

Cloning of the 16S rRNA gene fragments

The PCR products were ligated with the pCR 2.1 plasmid vector and transferred into TOP10 one shot competent *Escherichia coli* cells (TA cloning Invitrogen, Carlsbad, Calif). Twenty viable, white colonies were picked and grown aerobically overnight at 37°C in sterile Luria-Bertani broth, supplemented with 100 μg ml^{-1} ampicillin, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for plasmid selection. Plasmid DNA was extracted and purified by using the Wizard Plus Minipreps DNA purification system (Promega, Madison, Wis.). Plasmids were digested with *EcoR*I restriction enzyme (Roche Biochemicals) and the digested products were separated by 1.2% agarose gel electrophoresis in TRIS-boric acid-EDTA to confirm the presence of the DNA insert. Seven and five clones selected from CAOB and CNOB, respectively, were sequenced. The obtained clones were identified as pCALX3 for ammonia oxidizing group; pCALX1 for nitrite oxidizing group; pCALX5 for ammonia oxidizing biofilms using universal primers (Table 4). Plasmid DNA containing the PCR fragments was subjected to cycle sequencing and analysis. The sequences of the clones were determined in the Applied Biosystems sequencer Model 3100 (Foster City, CA USA) at the National University of Mexico (Institute of Biotechnology at Cuernavaca). DNA sequences were examined and checked for chimera formation using the CHECK-CHIMERA program [21] of the Ribosomal Database Project.

Analysis of sequence data

Resulting sequences (450 bp from CAOB, 381 bp from CNOB, and 850 bp from CAOB using universal primers) were compared with sequences in publicly accessible databases by using the program Basic local alignment search tool (BLAST, [1]). Analysis of sequence homology was performed by comparing the sequences of the clones from constructed biofilms with sequences of strains from NCBI database. The sequences obtained in this study are available in NCBI GenBank under accession numbers AY912447 and DQ011851.

Electron microscopy

Bacterial cells embebed into constructed nitrifying biofilms (CAOB and CNOB) were analyzed in order to evaluate colonization of the low-density polyester Dacron support by microorganisms in these biofilms. Analysis was performed without prior sample preparation using a Philips XL30 environmental scanning electron microscope (ESEM) in dry operating mode. Biofilms were carefully removed from bioreactors, a section of each biofilm was cut and were carefully placed facedown over the slides for viewing.

Results

Effluent nitrite removal efficiency in CAOB and CNOB peaked on day 12 (50 and 25%, respectively), but a maximum removal rate of 145 mg-NO_2 m^{-3} day^{-1} and 297 mg-NO_2 m^{-3} day^{-1} was registered on day 2 and 4, respectively.

Bioremediation with CAOB and CNOB

Air flow rate was kept constant in all bioreactors and routine checking of dissolved oxygen showed consistent levels of 6 mg l^{-1}. Therefore, oxygen may have not been limiting nitrification. The biofilm thickness was about 200 μm. In both biofilm reactors pH started at 7.6, achieving 8.5 in CAOB and 8.3 in CNOB. Temperature was kept at 21 ± 2°C, the range for collected water samples, and for supporting nitrifying bacterial growth [18].

Table 2 Ammonium removal rate in biofilm bioreactors and in control bioreactors

HRT (days)	Removal rate (mg N-NH_4^+ m^{-3} d^{-1})		
	Control	CAOB	CNOB
0	0.00[a]	0.00[a]	0.00[a]
2	< 0.00[c]	40.00 ± 3.82[a]	20.00 ± 2.86[b]
4	< 0.00[c]	50.00 ± 4.41[a]	22.50 ± 1.25[b]
6	10.00 ± 0.42[c]	53.33 ± 2.92[a]	18.33 ± 0.83[b]
8	11.25 ± 0.13[c]	197.50 ± 15.15[a]	67.50 ± 4.77[b]
10	4.00 ± 0.10[c]	306.00 ± 11.53[a]	99.00 ± 10.53[b]
12	35.00 ± 1.15[c]	303.33 ± 12.10[a]	187.50 ± 8.50[b]
15	44.29 ± 1.37[c]	322.14 ± 8.72[a]	255.71 ± 12.12[b]

Different letters in columns indicate significant differences ($P < 0.05$)

The treatment performance of bioreactors during the 15 days of experiment in terms of ammonium removal rates is shown in Table 2. A significant difference in ammonium removal rate ($p < 0.05$) was observed in both biofilms with respect to control. In CAOB the removal rate of ammonium reached up to 322 mg N-NH_4 m^{-3} d^{-1}. Similar trends were observed in CNOB, the maximum ammonium removal rate was 255 mg N-NH_4 m^{-3} d^{-1} registered at the end of the bioremediation process. However, the conversion of ammonium went slowly compared to CAOB (Table 3). Higher removal rates of nitrite were achieved at the beginning of the bioremediation process in CNOB (297 mg-NO_2 m^3 day^{-1}) than in CAOB (145 mg-NO_2 m^3 day^{-1}) relative to control bioreactors. Nitrite removal rates decreased during the experiment as a result of bioconversion to nitrate in both biofilm reactors enhancing the nitrification process by nitrite-oxidizing bacteria after day 8 of the experiment. This effect is corroborated by a more substantial increase of nitrate built-up in CNOB than in CAOB. Figure 4 showed that denitrification occurs in CAOB and CNOB up until day 6. The evolution in nitrate production (Fig. 4) from day 8 onwards mostly suggests

Table 3 Nitrite removal levels in biofilm bioreactors and in control bioreactors

HRT (days)	Removal rate (mg N-NO_2^- m^{-3} d^{-1})		
	Control	CAOB	CNOB
0	0.00[a]	0.00[a]	0.00[a]
2	5.00 ± 1.44[c]	145.00 ± 5.00[b]	235.00 ± 7.64[a]
4	42.50 ± 1.25[c]	107.50 ± 4.60[b]	297.50 ± 10.60[a]
6	41.67 ± 0.96[c]	83.33 ± 7.86[b]	236.67 ± 12.55[a]
8	47.50 ± 1.91[b]	38.75 ± 0.83[c]	163.75 ± 10.25[a]
10	24.00 ± 0.50[b]	< 0.00[c]	128.00 ± 11.75[a]
12	17.50 ± 0.87[c]	70.83 ± 11.25[b]	140.00 ± 13.70[a]
15	< 0.00[c]	29.29 ± 1.15[b]	86.43 ± 8.76[a]

Different letters in columns indicate significant differences ($P < 0.05$)

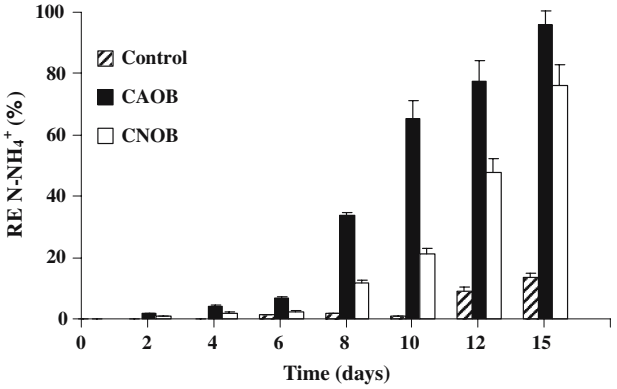

Fig. 2 Ammonium removal efficiency in constructed ammonium-oxidizing biofilms (CAOB); in constructed nitrite-oxidizing biofilm (CNOB) and in control reactor. The standard deviation indicated is from the mean of at least three independent experiments

that nitrifying activity were dominant over denitrifying bacteria. The heterotrophic role of certain aerobic bacteria such as Nitrobacter cannot be discarded. Control bioreactors showed little nitrification activity relative to either set of CAOB and CNOB, respectively. In general it can be seen that nitrogenous pollutant removal, however, was more sensitive to hydraulic retention time.

For both sets of bioreactors, a 5-day lag period was observed before efficient nitrification activity could be measured as ammonium consumption (Fig. 2) and nitrate production. In CAOB, ammonium removal efficiencies of 96% were registered at the end of the experimental period. Similar trends were observed in CNOB which reached removal efficiencies of 76% (Fig. 2). Between days 10 and 12 nitrite concentrations decreased to a minimu value in CAOB, with an overall removal efficiency of 12% (Fig. 3). In contrast, CNOB

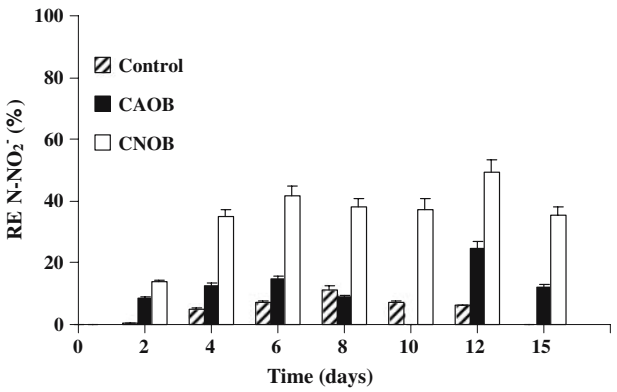

Fig. 3 Nitrite removal efficiency in constructed ammonium-oxidizing biofilms (CAOB); in constructed-nitrite oxidizing biofilms (CNOB) and in control reactor. The standard deviation indicated is from the mean of at least three independent experiments

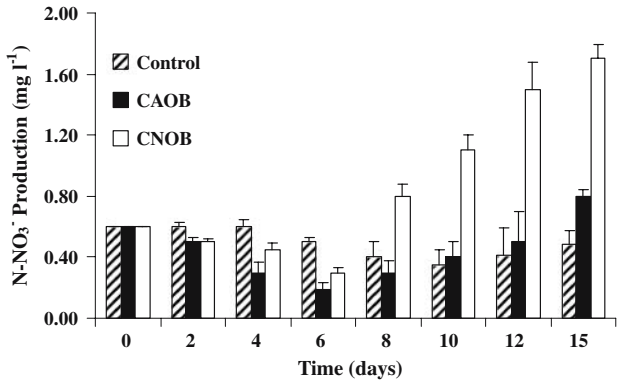

Fig. 4 Build up profiles of nitrate in constructed ammonium-oxidizing biofilms (CAOB), in constructed nitrite-oxidizing biofilms (CNOB) and in control reactor. The standard deviation indicated is from the mean of at least three independent experiments

showed average nitrite removal efficiencies of 35% (Fig. 3). High rates of nitrite conversion to nitrate were also noted for CNOB. Unpacked control bioreactors showed little nitrification activity relative to either set of packed bioreactors.

Therefore, differences in nitrification activity between CAOB- and CNOB- were likely due to differences in microbial community composition developed according to the wastewater enrichment and attachment to the low-density polyester Dacron support (Fig. 4).

Microscopy

Bacterial colonization of the polyester material was examined by ESEM at the end of a 15-day experiment. Adsorption of biomass onto the fibrous polyester material was easily imaged on both the inner and outer surfaces of the hollow fibers. Vertical thin sections of biofilms in CAOB (Fig. 5a) and the ammonium removal rates indicated the presence of members of ammonia oxidizing bacteria (suclass β-Proteobacteria), which were present in the form of spherical cells forming dense micro colonies. In the case of the bioreactor previously enriched with nitrite, some of the microcolonies in the constructed biofilms had a spherical appearance, but most of them were shaped irregularly (Fig. 5b) which is a characteristic feature of nitrite-oxidizing bacteria (*Nitrospira)* [9]. It seems that microcolonies of different sizes were developing in the inner part than in the surface of the constructed biofilm. In both cases (CAOB and CNOB), bacterial communities were found occupying all available surface area in contact with the wastewater in bioreactors.

Molecular characterization of nitrifying bacteria in constructed biofilms

PCR products were cloned and sequenced in order to better characterize bacterial composition in low-density polyester support during the performance of bioreactors. Agarosa gel electrophoresis of the resulting products, revealed amplification of a fragment of *ca.* 1,080 bp in samples of both biofilms CAOB and CNOB as well as in a sample of liquor of activated sludge. The size of this band was similar to that obtained from the *Nitrosomonas europaea* ATCC reference strain. Specific primers were used to detect nitrite-oxidizing bacteria in the nitrifying biofilms (Table 1). After PCR amplification, one band of *ca* 397 bp was observed in CAOB and CNOB biofilms. A set of universal primers were used in order to amplify the 16S rDNA. One band of *ca* 1,500 bp was amplified for samples of constructed biofilms (Fig. 6, lane 2 CAOB and lane 3 CNOB), the same band was obtained for DNA of *Nitrosomonas europaea* (lane 1) and DNA of *Bacillus* sp. (lane 4). Partial sequences of the 16S rDNA (between 400 and 600 nucleotides in length) were determined and compared to published sequences in order to determine the similarity of the clones to the closest matching group of bacteria (Table 4). Sequence-based characterization of the reactors was showed that β-Proteobacteria were found domi-

Table 4 Identity of partial rRNA genes from PCR products

Phylum	Closest match	% similarity with closest match
Proteobacteria		
Beta subclass	*Nitrosomonas eutropha*	97
	Nitrosomonas halophila	97
(pCALX3)	*Nitrosomonas europaea*	97
	Nitrosomonas multiformes	96
	Nitrosomonas briensis	96
Alpha subclass	*Nitrobacter hamburgensis*	97
	Nitrobacter winogradskyi	97
(pCALX1)	*Nitrobacter sp*	97
	Nitrobacter alkalicus	97
	Rhodopseudomonas palustres	78
	Afipia broomeae	78
Gamma subclass	*Pseudomonas corrugata*	98
	Pseudomonas gingeri	87
(pCALX5)	*E. coli*	90
	Vibrio parahemolyticus	90
	Vibrio vulnificus	90

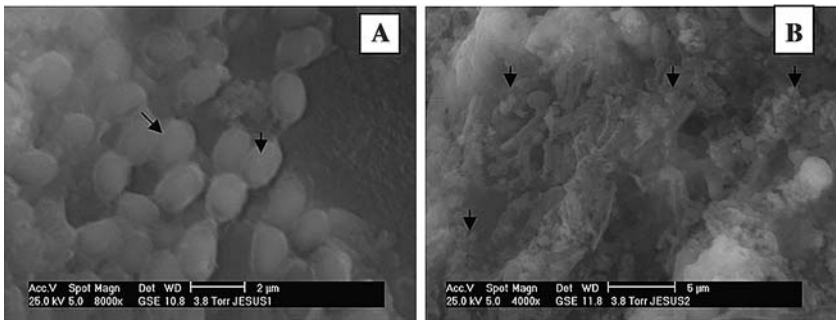

Fig. 5 Environmental scanning electron micrograph (×8,000) of bacterial biofilms on polyester support at the end of the bioremediation process. The *arrows* indicate ammonium- oxidizing (**a**) and nitrite-oxidizing bacteria (**b**) developed on polyester surface in the in the constructed biofilm

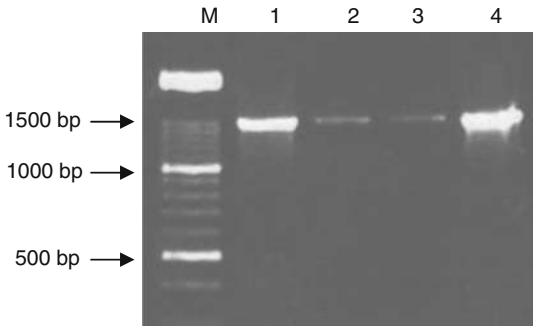

Fig. 6 PCR amplification of complete 16S rDNA gene for the detection of nitrifying bacteria from constructed biofilms in bioreactors. *Lanes*: *M*, DNA molecular size marker (base pairs); *1*, *Nitrosomonas europaea* DNA; *2* and *3*, DNA from ammonium and nitrite enriched biofilms, respectively; *4*, DNA from *Bacillus sp* (positive control). The approximate size of fragments is indicated

nant in CAOB. β-Proteobacteria from CAOB included sequences sharing high similarities to *Nitrosomonas eutropha, Nitrosomonas halophila, Nitrosomonas europaea, N. multiformis* and *Nitrosomonas briensis*, whereas alpha-proteobacteria from CNOB were related to *Nitrobacter hamburgensis, Nitrobacter winogradoskyi, Nitrobacter sp, Nitrobacter alkalicus, Rhodopseudomonas palustres* and *Aphia broomeae* (Table 4). Thus, CAOB was inhabited by β-Proteobacteria related to genera of ammonia oxidizing bacteria, whereas α-Proteobacteria in CNOB were related to nitrite-oxidizing bacteria. Different results also occurred for gamma-Proteobacteria, which were present in both reactors as determined by 16S rDNA analysis.

Discussion

This research is of practical importance because the construction of microbial biofilms on low-density polyester Dacron support is a promissory relatively new alternative for the advanced treatment of industrial and municipal wastewaters, but there is relatively little information regarding the performance and characterization of nitrifying populations in constructed biofilm reactors, particular on this kind of support media during nitrogen bioremediation of municipal wastewater effluents.

The removal rates of both ammonium and nitrite as well as nitrate formation obtained in our study follows the trends of biofilms developed under aerobic conditions and are on the order of magnitude of those calculated for aggregates of nitrifying bioreactors [3, 30].

The experimental results shown that a HRT of 15 days was appropriate for efficient ammonium removal and an operating capacity of 5 mg l^{-1} N-NH_4^+. Both constructed biofilm reactors achieved ammonium and nitrite removal rates higher than that of the concentrations normally present in the control reactors (without constructed biofilms) ($P < 0.05$). This removal proficiency is indicative of the dominance of metabolically active ammonia-oxidizing bacteria in CAOB and nitrite-oxidizing bacteria in CNOB, respectively, because nitrite-oxidizing bacteria exhibits slower growth rate than ammonium-oxidizing bacteria [20]. Nevertheless, the removal efficiencies of ammonium attained in CAOB (96%) are similar to the efficiencies obtained in a membrane coupled bed bioreactor by [16, 22] and in polystyrene beds [26] but with longer hydraulic residence time [14, 27]. Constructed biofilms on low-density polyester Dacron support avoids the clogging problems of membrane bioreactors and are highly resistant when compared natural polymers for the treatment of wastewater.

Products obtained from PCR amplification of nitrifying bacteria from the constructed biofilms with the set of specific primers of 16S rDNA for ammonia-oxidizing and nitrite-oxidizing species (Table 1), revealed a band at ca. 1080 bp with homologies greater than 95% for *Nitrosomonas*-like bacteria as the predominant ammonia-oxidizing species in CAOB. Ammonium oxidation to nitrite and subsequent built-up of nitrate in our system was related and represented by *Nitrosomonas halophila* and *N. eutropha* members of ammonia-oxidizing Proteobacteria. We found that the group of *Nitrosomonas* were colonizing biofilms at concentrations of ammonium in the range of 5–6 mg l^{-1},

similar approaches were registered by [6, 19] who showed that ammonium oxidizing bacteria varies according to ammonium concentrations.

Low-ammonia environments will likely produce Nitrosomonas marina-like such as in our results in CAOB, while at enhanced ammonia levels, Nitrosomonas europaea-like become more dominant [33]. Our results suggest that the CAOBs developed in wastewater effluent are members of *Nitrosomonas* in which concentrations rarely exceed 10 mg l^{-1} of ammonium. A second band at 397 bp was indicative of the presence and nitrite-oxidizing bacteria in CNOB with homologies greater than 95% which was closely related to previously identified *Nitrobacter* spp. [9, 34]. Cultivated nitrite-oxidizing bacteria have been assigned to the genera *Nitrobacter, Nitrospira, Nitrococcus, and Nitrospina* [4, 6, 13], however undetected groups, viz., *Nitrospira*, or *Nitrococcus* is not a proof of their absence [30]. However in other studies, Nitrobacter could not be detected, and it was speculated that Nitrospira was likely responsible for nitrite oxidation [17]. We hypothesized that the predominance of *Nitrobacter* spp. on *Nitrospira* could be associated to environments lower in nitrite concentrations such as in biofilm bioreactor systems which can explain the growth strategy in each strain (k or r strategist) mentioned by Schramm et al. [30, 33]. [6, 7] suggested that in processes where nitrification is poor, Nitrospira is absent.

Nitrite-xidizing Proteobacteria, *Nitrobacter winogradsky, N. hamburgensis* have been associated with nitrate build-up and phosphate removal from wastewater [9, 31]. The possibility of a marginal heterotrophic growth due to the presence of organic nutrients present in domestic wastewater cannot be discarded. *Nitrobacter* (α subclass of the class *Proteobacteria)* can grow heterotrophically, while the remaining known nitrite-oxidizers, *Nitrospina* (δ subclass), *Nitrococcus* (γ subclass), and *Nitrospira* (*Nitrospira* phylum) are unable to grow heterotrophically [6, 18]. The presence of heterotrophic components such as α- and γ- Proteobacterial populations in our constructed biofilms as an autotrophic biofilms have been reported previously [23]. It has been suggested [18] that α- and γ-Proteobacteria primarily utilize low-molecular-weight fatty acids from degradation of soluble microbial compounds produced by nitrifiers.

Excellent colonization of the low-density polyester Dacron support by the identified ammonium- and nitrite-oxidant Proteobacteria following the 15 days experiments exemplified their utility in supporting high, continuous, rates of nitrification relative to our less successful bioremediation trials without low-density polyester Dacron support. This material was well suited for immobilization because of its fibrous structure and easy diffusion of nutrients into it [25]. The low-density structure and continuous fibers of polyester offers high surface area to volume ratio, and also a high strength to weight ratio enhances substrate affinity for bacterial attachment and growth. A high voidage network of polyester fibers with high consistency, durability and low cost were additional attributes.

Electron microscopy revealed that the constructed biofilm on the feedwater effluent was composed of filaments of polyester covered by several layers of compacted ammonia oxidizing bacteria (Fig. 5a). In the case of nitrite-oxidizing biofilms, the microcolonies showed irregular distribution and shapes (Fig. 5b), viz., rod-shaped, ellipsoidal, spherical, spirillar or lobate [30]. Polyester (Dacron) fibers located on the internal part as well as on the surface of biofilm were sparsely colonized, suggesting bacterial regrowth in the constructed biofilm system during the bioremediation of wastewater. This approach can accommodate slow-growing nitrifying bacteria better than conventional activated sludge processes and other nitrifying biofilm systems [15, 30]. This finding supports the fact that treatment efficiency in bioreactors is limited by biomass separation instead of process microbiology [12, 16]. Many techniques using stronger synthetic polymers involve chemical polymerization initiation which causes loss of cell viability; this factor also gives rise to safety concerns and can complicate the immobilization procedure [29].

In conclusion, it appears that the performance of constructed biofilms on low-density polyester Dacron support showed promise for the advanced treatment and nitrogen bioremediation of wastewater effluents from municipal treatment plants. The fibrous structure of the polyester matrix provides an environment for nitrifiers protection and regrowth when structured in biofilms in which active nitrifying bacteria are embedded and oxygen transfer can be enhanced by convection. Additionally, constructed biofilms are easier to fluidize in breakdown of aeration and exhibits lower sensitivity to adverse environmental conditions. Biofilms constructed on low-density polyester Dacron support leads towards more compact and inexpensive treatment of wastewater due to the availability of biofilm support matrix with high specific surface area for nitrifiers for enhanced nitrifying activity.

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