J Ind Microbiol Biotechnol (2012) 39:1169–1177
DOI 10.1007/s10295-012-1123-z

ENVIRONMENTAL MICROBIOLOGY

# Degradation kinetics of 4-amino naphthalene-1-sulfonic acid by a biofilm-forming bacterial consortium under carbon and nitrogen limitations

**C. Juárez-Ramírez · R. Velázquez-García · N. Ruiz-Ordaz · J. Galíndez-Mayer · O. Ramos Monroy**

Received: 18 November 2011 / Accepted: 19 March 2012 / Published online: 11 April 2012
© Society for Industrial Microbiology and Biotechnology 2012

**Abstract** By decolorization of azo dyes, caused by reductive cleavage of the azo linkage, toxic or recalcitrant amines are generated. The present study deals with the effect of the inflowing medium composition (C:N ratio) on the kinetic behavior of a bacterial biofilm-forming consortium, able to use as carbon, nitrogen and sulfur source, the molecule of 4-aminonaphthalene-1-sulfonic acid (4ANS), which is one of the most recalcitrant byproducts generated by decolorization of azo dyes. All the experiments were carried out at room temperature in a lab-scale packed-bed biofilm reactor. Because environmental conditions affect the bioreactor performance, two mineral salts media containing 4ANS, with distinct C:N ratios; 0.68 (carbon as the limiting nutrient) and 8.57 (nitrogen as the limiting nutrient) were used to evaluate their effect on 4ANS biodegradation. By HPLC and COD measurements, the 4ANS removal rates and removal efficiencies were determined. The cultivable bacterial strains that compose the consortium were identified by their 16S rDNA gene sequence. With the enrichment technique used, a microbial consortium able to use efficiently 4ANS as the sole carbon source and energy, nitrogen and sulfur, was selected. The bacterial strains that constitute the consortium were isolated and identified. They belong to the following genera: *Bacillus*, *Arthrobacter*, *Microbacterium*, *Nocardioides*, and *Oleomonas*. The results obtained with this consortium showed, under nitrogen limitation, a remarkable increase in the 4ANS removal efficiency $\eta_{ANS}$, and in the 4ANS volumetric removal rates $R_{V,4ANS}$, as compared to those obtained under carbon limitation. Differences observed in bioreactor performance after changing the nutrient limitation could be caused by changes in biofilm properties and structure.

**Keywords** Biofilm reactor · 4-aminonaphtalene-1-sulfonic acid · Growth-limiting substrate · *Oleomonas* · *Arthrobacter*

C. Juárez-Ramírez · R. Velázquez-García · N. Ruiz-Ordaz · J. Galíndez-Mayer (✉) · O. Ramos Monroy
Departamento de Ingeniería Bioquímica,
Escuela Nacional de Ciencias Biológicas,
Instituto Politécnico Nacional, Carpio y Plan de Ayala,
Col Santo Tomás, CP 11340 Mexico, D.F., Mexico
e-mail: cmayer@encb.ipn.mx; juvenciogm@yahoo.com

## Introduction

Aromatic amines are major industrial chemicals used to produce detergents, dispersing and wetting agents, synthetic dyes, and pharmaceuticals [39, 58]. Degradation of azo dyes is also an important generation source of these compounds. By reductive cleavage of the azo bond, aromatic amines are formed. Many of them have been reported as carcinogens and mutagens [7]; therefore, their removal from contaminated sources is of health and environmental concern.

A group of these aromatic amines represented by aryl sulfonates (AS), aminobenzene (ABS), and amino naphthyl sulfonates (ANS). 4-aminonaphthalene-1-sulfonic acid (4ANS, 1-naphthylamine-4-sulfonic acid or naphthionic acid) is used as a diazo component in many azo dyes, e.g., Food Red 3, Food Red 7, Food Red 9, Acid Red 3, Acid Red 18, Acid Red 25, Acid Red 27, Acid Red 88, Fast Sulphon Black F (1-hydroxy-8-(2-hydroxy-1-naphthylazo)-2-(4-sulfo-1-naphthylazo)-naphthalene-3,6-disulfonic acid), and DI-S NADNS (3,6-bis(4-sulfo-1-naphtylazo)-4,5-dihydroxy-2,7-naphtalenedisulfonic acid) [10]. These derivatives can pose a health hazard and some have been designated as priority pollutants. Most are toxic or inhibitory to biological

activities; and therefore could present a severe problem in conventional wastewater treatment systems [55].

The presence of a sulfonic group enhances the water-solubility of naphtylamines; therefore, during production processes, sulfonated aromatic amines could easily reach the aquatic environment through industrial wastewaters. Sulfonated aromatic amines have been found in rivers of Italy, Germany, and Spain [15, 28, 33, 65, 66]; also in Mediterranean coastal water [17]. This means that these compounds are not efficiently removed in wastewater treatment plants (WTP). Once hazardous pollutants enter a WTP, some alternatives to remove them could be the bioaugmentation of WTP's biota with specialized microorganisms able to degrade these compounds, or the implementation of subsequent treatment processes.

In the first case, the environmental conditions prevailing in a WTP are generally hostile toward exogenous microorganisms, which must compete with the biota from the wastewater sludge; thus, bioaugmentation is not always an effective solution. Babcock et al. [4] summarize several cases of wastewater treatment processes, showing either successful or unsuccessful persistence of the inoculated microorganisms. In the second case, physicochemical or biological post-treatment processes could be used to remove naphthalene sulfonic acids or to increase their biodegradability [55]. However, the removal of diluted compounds using chemical, photochemical, or electrochemical oxidation techniques could be uneconomical [44, 49, 59], and the use of non-destructive removal procedures, such as adsorption by activated carbon, acid or basic resins, requires the disposal or reactivation of the saturated adsorbent [21].

An efficient and cheaper alternative to physicochemical processes could be the use of specialized microorganisms and simple biological processes to remove these compounds [35]; however, some problems exist. Several studies have shown the biodegradation recalcitrance and toxicity of this kind of xenobiotic compounds, and the main difficulties to find microorganisms able to use them as the sole source of carbon.

Several authors report that the polar nature of aminoarylsulfonates makes these compounds recalcitrant to biotransformation and hardly used by microorganisms as a nutrient source, requiring for their biodegradation specialized aerobic microbial consortia [38, 56]. By example, *Aeromonas* sp., is unable to degrade 4ANS, accumulating it after decolorization of the dye Bordeaux S [19]. Mixed microorganisms from various environmental samples can degrade several aminobenbenzene sulfonic acids, but not amino naphthalene sulfonic acids [61]. *Shewanella decolorantius* S12 was unable to degrade 1-aminonaphthyl-4-sulfonic acid, either in anaerobic or aerobic conditions [20]. Other authors report that naphthalene sulfonates are partially degraded by certain bacteria and algae, using them as a source of sulfur. Similarly, *Pseudomonas* sp., an *Arthrobacter* sp. and an unidentified bacterium isolated from sewage, desulfonate at least 16 aromatic compounds, although none of them served as the carbon source [68].

On the other hand, a recent study has shown that immobilized or free cells of *Alcaligenes latus* degrade the amino hydroxy naphthalene disulfonic acid [63]; however, the culture medium should be complemented with relatively expensive nutrients such as glucose and yeast extract.

The use of biofilm-forming microbial consortia, selected in response to the continuous availability of amino naphthalene sulfonic acids, could help to their rapid and complete degradation. By several reasons, the use of fixed-bed reactors containing a mixed microbial biofilm is appropriate to remove compounds toxic to cells; because the exopolymeric matrix acts as a diffusion barrier to the chemicals, or interacts with the toxic substances acting as an ion exchanger that sequesters charged substances [22]. The biofilm matrix could also inhibit washout of enzymes, nutrients, or even signaling molecules that could then accumulate locally and create favorable microenvironments within the biofilm [6]. Additionally, mixed-culture biofilms provide an ideal environment for microbial residents to coordinate metabolic activities and share genetic elements encoding beneficial traits with each other [30]. The retention of attached active bacteria provides biofilm reactors with several advantages over conventional suspended-growth reactors, including faster conversion rates due to higher concentrations of active biomass, reduced bacterial washout at high-dilution rates, enhanced reactor stability, and rapid response to varying or adverse operating conditions [9]. However, it is well known that bacterial cell composition and physiology are affected by the carbon/nitrogen ratio of the nutrient supply [12, 64] and that change in biofilm structure and properties are caused by nutrient limitation [3, 51]. Therefore, it is expected that a change in the limiting nutrient affects the performance of a biofilm removal process.

For the previous reasons, the aim of this work is the selection of a biofilm-forming microbial consortium able to use 4-amino naphthalene1-sulfonic acid as its sole source of carbon, nitrogen, and sulfur, and study its biodegradation kinetics in a laboratory-scale packed-bed-biofilm column under carbon or nitrogen limitations.

## Methods

### Chemicals

4-aminonaphthalene-1-sulfonic acid (1-naphthylamine-4-sulfonic acid, naphthionic acid), 97 % purity, was purchased from Chem-Service, PA, USA. The solvents used

for high-performance liquid chromatography (HPLC) were purchased from J. T. Baker, USA.

### Culture media

Two different mineral salts media (MS) were used. To probe the 4-aminonaphthalene-1-sulfonic acid as the sole carbon and energy source, the $MS_1$ medium, containing the following components, in mg $l^{-1}$, was used $CaCl_2$, 20; $MgSO_4 \cdot 7H_2O$, 100; $(NH_4)_2SO_4$, 100; and $K_2HPO_4$, 200. 4ANS was added to obtain a concentration of 50 mg $l^{-1}$. The C/N and C/S proportions in $MS_1$ were 0.68 and 0.29, respectively; thus, with this medium, the microbial growth was limited by the carbon source.

To probe the 4-aminonaphthalene-1-sulfonic acid as the sole carbon, energy, nitrogen, and sulfur source, the $MS_2$ medium was used. It contains the following components, in mg $l^{-1}$: $CaCl_2$, 20; $MgCl_2$, 80; and $K_2HPO_4$, 200. The 4ANS was added to obtain a concentration of 50 mg $l^{-1}$. This medium was formulated considering the C/N and C/S ratios of the 4ANS molecule (8.57 and 3.74, respectively). With $MS_2$ medium, the microbial growth was limited by the nitrogen source.

### Selection of the microbial community able to grow on 4ANS

Soil samples collected nearby a textile industry were inoculated in 250-ml flasks containing 50 ml of $MS_1$ plus 4ANS as the sole source of carbon and energy. The flasks were incubated at 30 °C in a rotatory shaker for 48 h. The consumption of the 4ANS was spectrophotometrically estimated. Aliquots of the culture medium were transferred to flasks containing new mineral salts medium, and the procedure was repeated. After 12 successive transfers, a microbial community that uses the 4ANS as the carbon and energy source was obtained. This community was maintained on agar slants containing $MS_1$ medium.

### Packed-bed column reactor

All experiments were carried out at room temperature in a lab-scale packed-bed column reactor, which, at the bottom, has a sintered glass plate diffuser with pore diameter 40–100 μm (Heraeus Quarzglas GmbH & Co., Germany). The body of the glass column has lateral ports for liquid input and air/liquid outflow. It also has a glass cover with ports for inoculation, sampling, and air venting (Fig. 1). The supply of air to the bubbling column was kept constant at 0.67 l $min^{-1}$. Air and liquid were concurrently supplied through the column, which was packed with small fragments of a porous volcanic stone named tezontle; material widely distributed in central Mexico.

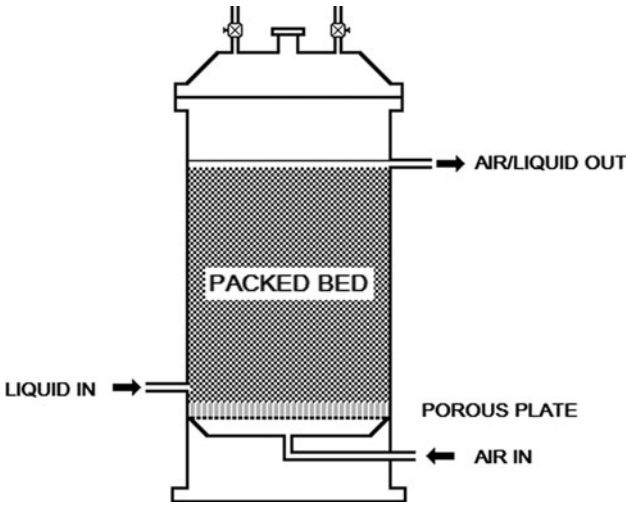


**Fig. 1** Packed-bed column reactor

The particle volume was calculated and used to estimate the equivalent diameter $d_p$ of the volcanic stone particles considering porous fragments as ellipsoidal bodies, with three characteristic radii [16]. The average $d_p$ value was 7.4 ± 2.7 mm. The column was packed with 998 g of porous stone fragments. The liquid contained in the inter-particle space corresponds to a circulating liquid volume, or drained volume, $V_L$ of 560 ml. With this value, the volumetric loading ($B_{V,ANS} = F\ C_{ANS\text{-}R}/V_L$) and removal ($R_{V,ANS} = F(C_{ANS\text{-}R} - C_{ANS\text{-}OUT})/V_L$) rates of 4ANS in the PBR operating in steady-state continuous regime were estimated. In these equations, $F$ is the medium flow rate, $C_{ANS\text{-}R}$ and $C_{ANS\text{-}OUT}$, are the concentrations of 4ANS entering or leaving the PBR. The corresponding volumetric loading and removal rates of the compound were also expressed in terms of the chemical oxygen demand ($B_{V,COD}$, and $R_{V,COD}$) entering or leaving the reactor. The general performance of the bioreactor was evaluated through these volumetric removal rates, and the biodegradation capabilities of the bacterial consortium were estimated through the values of removal efficiencies $\eta_{ANS} = R_{V,ANS}/B_{V,ANS}$, $\eta_{COD} = R_{V,COD}/B_{V,COD}$.

#### *Start-up of the bioreactor*

Before the continuous operation, the column was packed with the porous support, sterilized (121 °C for 30 min), and saturated with the 4ANS by supplying $MS_1$ medium containing 50 mg $l^{-1}$ of 4ANS. It was considered that the support was saturated when the 4ANS concentration of the outflowing medium, spectrophotometrically measured at $\lambda_{238}$, was equal to that of the inflowing medium.

The reactor was then inoculated with 25 ml of suspended cells of the selected bacterial consortium, previously cultivated in $MS_1$ medium. The reactor was operated in batch

mode for 48 h in aerobic conditions to allow the microbial colonization of the porous support. When operated in continuous regime, a constant flow of $MS_1$ medium (carbon limited) was maintained until no change in 4ANS, COD, and TOC concentrations could be observed (about four hydraulic retention times $HRT = V_L/F$). In this condition, it was considered that a steady-state was reached. Several flow rates of $MS_1$ (16 to 400 ml $h^{-1}$), corresponding to 4ANS loading rates $B_{VANS}$ ranging from 1.43 to 35.7 mg $l^{-1}$ $h^{-1}$, were probed. When a consistent decay in 4ANS removal efficiencies was evident, the medium was changed to $MS_2$ medium (nitrogen limited). The new flow rates were varied from 30 to 825 ml $h^{-1}$, with corresponding $B_{VANS}$ values ranging from 2.68 to 73.66 mg $l^{-1}$ $h^{-1}$.

### Analytical methods

#### *4ANS determination*

Two methods were used. For a rapid estimation, the absorbance of the samples was measured at $\lambda_{238}$ in a Beckman DU-620 spectrophotometer, and by HPLC, using a Shimadzu 10AT system with an UV detector. The column used was a Merck LiChrospher 100, C18 (250 mm × 4 mm i.d., particle size 5 μm). The mobile phase was a 60:40, v/v mixture of 0.3 % phosphoric acid solution, and acetonitrile 100 %.

#### *Chemical oxygen demand (COD)*

Another form to evaluate the removal of 4ANS, and particularly of some degradation byproducts that cannot be measured by HPLC, was the determination of the chemical oxygen demand in all samples using a closed reflux method 8000 [18]. The reactive kit used could determine COD levels from 3 to 150 mg $l^{-1}$.

### Estimation of bacterial diversity in the biofilm and the PBR outflowing liquid

The presence or predominance of bacterial strains in the biofilm and in the PBR outflowing liquid was determined by thermal gradient gel electrophoresis (TGGE) of 16S rDNA fragments PCR-amplified (Gene Amp PCR System 2400, USA) using U968 and L1401 primers [13]. The equipment used was a TGGE System (Biometra, Göttingen, Germany), using a thermal gradient of 39–55 °C.

### Isolation and identification of the cultivable bacteria constituting the microbial community

Once finished, the PBR's last run under nitrogen limitation and the cultivable microorganisms present in the microbial consortium were isolated. Decimal dilutions of a sample of suspended cells were prepared. Aliquots of 200 μl were distributed in nutrient agar plates and incubated at 30 °C. Daily observations were made. The number of different cultivable bacteria present in the microbial community was estimated by observing differences in colonial morphology. Bacterial isolates were cryopreserved in glycerol at −70 °C. Each isolate was propagated in Luria–Bertani medium, and DNA from harvested cells were extracted and purified. By PCR amplification of 16S rDNA with 8FPL (5′ AGT TTG ATC CTG GCT CAG 3′) and 13B (5′ AGG CCC GGG AAC GTA TTC AC 3′) oligonucleotides [45, 46], amplicons of about 1,400 bp were obtained and purified. Purity of bacterial strains was verified by gel electrophoresis of their 16S rDNA fragments. Amplicons were sequenced and compared with known sequences of bacterial 16S rDNA at NCBI GeneBank. Reported species showing the higher similarities were regarded as the isolated species.

## Results

### Selection of a microbial consortium able to degrade 4ANS

As described in the Methods section, an aliquot of soil suspension was inoculated and incubated in $MS_1$ medium. After successive transfers, the cell population was enriched with microorganisms able to use 4ANS as the sole carbon and energy source. At the eleventh transfer, after incubating the flaks for 24 h, the 4ANS (spectrophotometrically measured at $\lambda_{238}$) showed a diminution of 95 %.

This microbial community was seeded in agar slants containing $MS_1$ medium. The biomass harvested from the slants was suspended in liquid $MS_1$ medium. Part of the cell suspension was used to isolate the constituents of the microbial consortium. The other part was used as inoculum for the bioreactor start-up. Following the procedure described in the Methods section, the bacterial isolates were identified (Table 1).

**Table 1** Identification of the bacterial strains constituting the microbial consortium, which degrades 4ANS

| Microorganism | Similarity (%) | NCBI accession number |
|---|---|---|
| *Oleomonas* (*sagarenensis*) | 98 | D45202 |
| *Arthrobacter* (*chlorophenolicus*) | 97 | CP001341 |
| *Arthrobacter sp.* | 97 | FJ449635 |
| *Bacillus sp* | 97 | FJ601659 |
| *Nocardioides sp* | 95 | AB373748 |
| *Microbacterium* (*oxydans*) | 92 | FJ009389 |

Degradation of 4ANS in the packed-bed column reactor operating in steady-state continuous regime

*Carbon-limited continuous culture*

Using the $MS_1$ medium, 4ANS volumetric removal rate was evaluated at eight dilution rates $D$, 0.029–0.714 $h^{-1}$, corresponding to hydraulic retention times *HRT* varying from 35.0 to 1.4 h, and 4ANS volumetric loading rates $B_{V,ANS}$ from 1.43 to 35.7 mg $l^{-1}$ $h^{-1}$. The results are shown in Fig. 2a. At $B_{V,ANS}$ values lesser than 14.7 mg $l^{-1}$ $h^{-1}$, the 4ANS removal efficiencies $\eta_{ANS}$, measured by HPLC, rounded the 100 %. At higher $B_{V,ANS}$ values, a gradual decay in $\eta_{ANS}$ was observed. In carbon limited conditions, at the highest loading rate probed ($B_{V,ANS} = 35.7$ mg $l^{-1}$ $h^{-1}$) $\eta_{ANS}$ diminished in 63 % (Fig. 3a).

When the xenobiotic removal was measured by the diminution in the chemical oxygen demand COD, a similar trend in $R_{V,COD}$ was observed (Fig. 4a), although with smaller values, rendering $\eta_{COD}$ values as low as 49 % (Fig. 5a).

Accumulation of aromatic byproducts, detected by HPLC, was less than 0.7 %; thus, the difference in the 4ANS removal efficiencies could not be explained by this fact. Thus, accumulation of non-aromatic intermediaries could be responsible of the low values of $\eta_{COD}$ obtained at the smallest loading rates probed in carbon-limited continuous culture.

*Nitrogen-limited continuous culture*

The bioreactor was fed with $MS_2$ medium containing 4ANS (50 mg $l^{-1}$) as the sole source of carbon, nitrogen, and sulfur. In this case, the nitrogen contained in the 4ANS

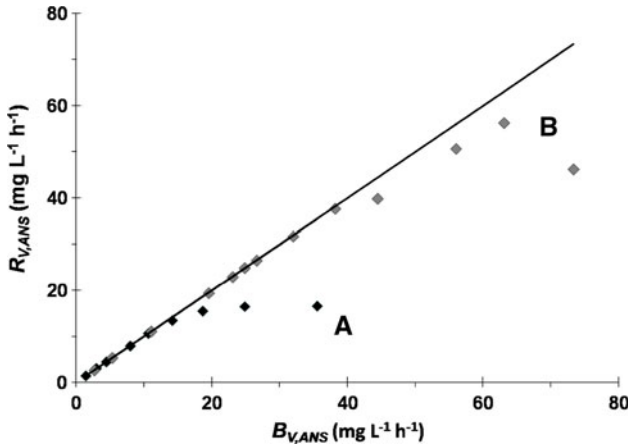


**Fig. 2** Volumetric removal rate of 4ANS ($R_{V,ANS}$) in the PBR operating in steady-state continuous regime. **a** Under carbon-limiting conditions. **b** Under nitrogen-limiting conditions

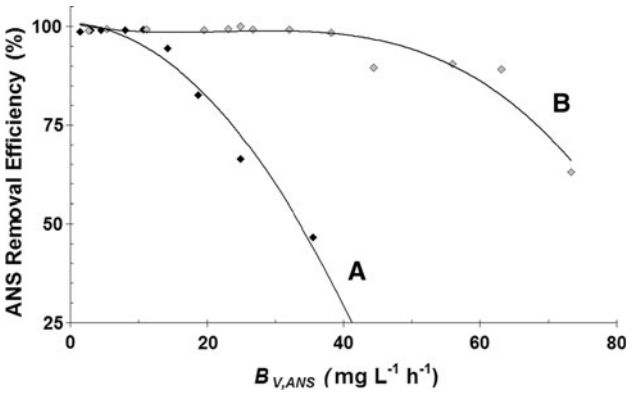


**Fig. 3** Removal efficiency of 4ANS ($\eta_{ANS}$) in the PBR operating in steady-state continuous regime. **a** Under carbon-limiting conditions. **b** Under nitrogen-limiting conditions

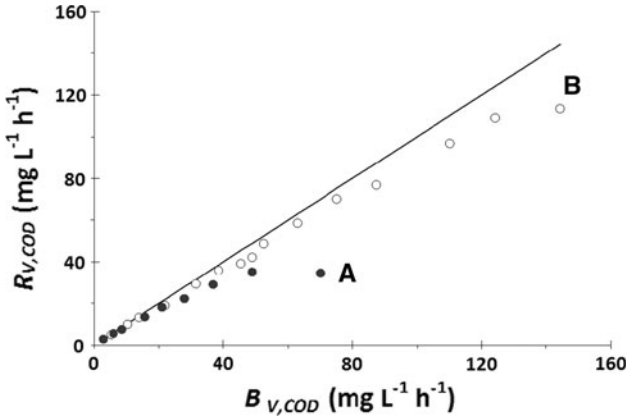


**Fig. 4** Volumetric removal rate of 4ANS, measured as COD ($R_{V,COD}$) in the PBR operating in steady-state continuous regime. **a** Under carbon-limiting conditions. **b** Under nitrogen-limiting conditions

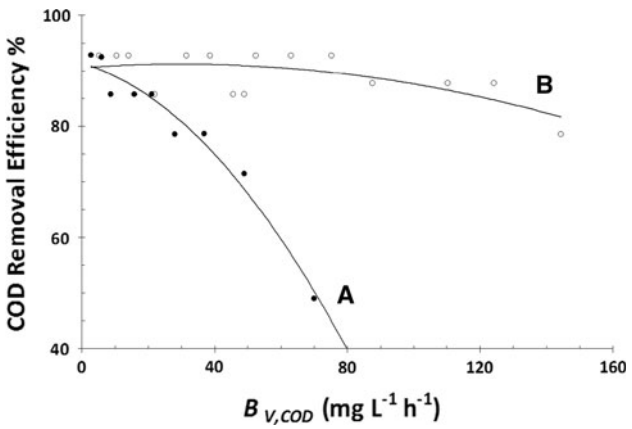


**Fig. 5** Removal efficiency of 4ANS, measured as COD ($\eta_{COD}$) in the PBR operating in steady-state continuous regime. **a** Under carbon-limiting conditions. **b** Under nitrogen-limiting conditions

molecule limited the cell growth. Sulfur and carbon were in excess. The $R_{V,ANS}$ was evaluated at dilution rates ranging from 0.0054 to 1.43 $h^{-1}$, corresponding to *HRT* values varying from 18.6 to 0.7 h and 4ANS loading rates $B_{V,ANS}$ from 2.6 to 73 mg $l^{-1}$ $h^{-1}$.

In Figs. 2b and 4b, it can be appreciated that when the microbial consortium was limited by nitrogen (carbon excess), the bioreactor supported much higher 4ANS loading rates than when cell growth was limited by the carbon source. Under nitrogen limitation, it was determined that at $B_{V,ANS}$ values less than 38 mg $l^{-1}$ $h^{-1}$, the 4ANS removal efficiencies, measured by HPLC rounded the 100 % (Fig. 3b), and 93 % when measured by COD (Fig. 5b). At higher $B_{V,ANS}$ values, the 4ANS removal rates and efficiencies diminished noticeably.

### Identification of the isolated bacteria constituting the microbial community

The bacterial isolates were identified by 16S rDNA amplification, sequencing, and comparison with known sequences of bacterial 16S rDNA at NCBI GeneBank. The results are presented in Table 1.

### Estimation of bacterial diversity in the biofilm and in the PBR outflowing liquid

TGGE profiles of 16S rDNA fragments of the bacterial consortium grown on 4ANS under carbon or nitrogen limiting conditions (carbon-excess conditions) are shown in Fig. 6. A shift in the microbial diversity corresponding to the change in substrate limiting conditions was observed. Assuming that one DNA band correspond to one bacterial strain, a higher abundance of some bacterial strains in the consortium that grew under nitrogen limitation could be observed. In this case, six well-defined DNA bands in the biofilm and in the PBR-outflowing liquid are evidenced. In contrast, only four sharp DNA bands appear under carbon limitation. It was evident that the consortium structure was altered by the environmental changes that occurred in the bioreactor.

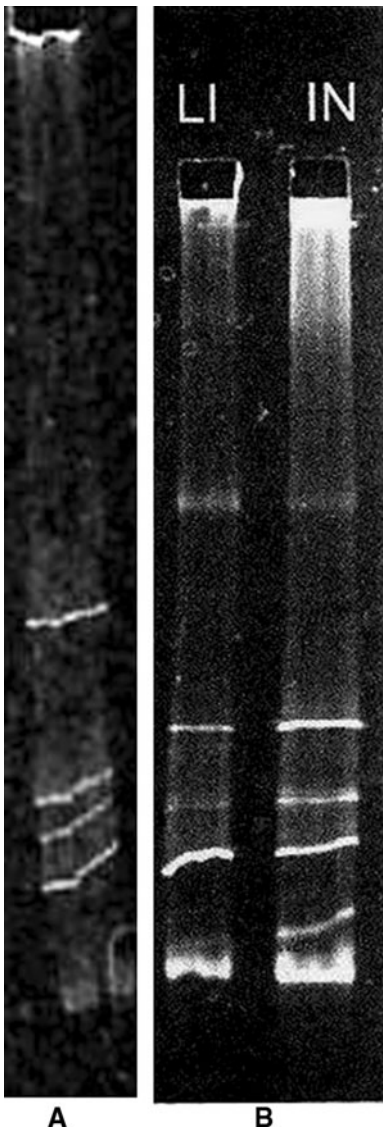


**Fig. 6** Thermal gradient gel electrophoresis of amplicons obtained from 16SrDNA of planktonic cells (*LI*) and sessile cells *(IN)* of the microbial community that degrades 4ANS

## Discussion

Differences observed in bioreactor behavior after changing the nutrient limitation could be explained by changes in biofilm structure [3], and in the viscoelastic properties of the biofilm, that difficult its detachment caused by shear stress [27, 48]. From an evolutionary viewpoint, microorganisms have acquired a high degree of plasticity in their physiological responses to changes in environmental conditions; thus, changes in the growth environment can result in a wide variation in morphology, composition, and enzyme activities [31]. Cells growing under carbon excess or carbon limitation could present important differences in gene expression and physiological differences; thus, biofilms formed in carbon-excess conditions (e.g., C/N =8.57) could show reduced shear removal and minor susceptibility to sloughing due to a more cohesive structure supported on its greater exopolysaccharides (EPS) production.

Oxygen and nutrient limitation may be key factors that trigger enhanced resistance mechanisms of sessile communities to hydrodynamic shear forces [52]. This phenomenon was observed when the effects of carbon and oxygen limitations on *Pseudomonas putida* biofilm removal processes were studied [3]. Additionally, the

viscoelastic properties of bacterial biofilms produced in carbon excess conditions allow the biofilm deformation, resisting its detachment due to increased fluid shear, while remaining attached to a surface [48]. Other authors, studying the effects of growth conditions on the production of *Xanthomonas campestris* exopolysaccharides [60], or on biofilm formation of a *P. putida* strain [47] also observed that high C:N and C:P ratios in the medium were associated with high levels of polymer production.

Additionally, it is a commonly accepted observation that due to the concerted activity of a multimember consortium, often the biodegradation rate of a compound is faster in nature than in pure cultures or in cultures with microbial communities constituted by few members [57]. The concerted metabolic activity of a higher number of bacterial strains could have contributed to the remarkable increase in the biodegradation rate of 4ANS determined under carbon-excess conditions (nitrogen-limiting conditions).

### Cultivable constituents identified in the bacterial consortium

Searching for the characteristics of the species that compose the bacterial biofilm it can be observed that with the exception of *Oleomonas*, all the bacterial genera found in the microbial consortium have been reported as xenobiotic degraders; however, some strains of this genus could be involved in the initial stages of the biofilm formation. For example, Fernández et al. [14] reports that the initial community involved in biofilm formation is mainly comprised by *Oleomonas sagaranensis*, an α-proteobacteria [25] that produces extracellular polymers, facilitating its adhesion to the surface of the support material. Few reports exist about the degradation capabilities of *Oleomonas*, one of them reports that *O. sagarensis* is involved in the biodegradation of alophanate [24].

Strains of the genus *Arthrobacter* degrade several organophosphorous pesticides such as Malathion [5], parathion [34], glyphosate [40, 41], monochrotophos [8], and fenitrothion [36].

*Bacillus* is a bacterial genus with broad metabolic capabilities, able to degrade several xenobiotic compounds. Parathion [34], methyl parathion [54], monocrotophos [43], glyphosate [42], chlorpyrifos [2], pentachlorophenol [26], azo dyes [37, 50], *p*-aminobenzene [67], and *p*-nitrophenol [23]. Additionally, it has been reported that the surfactines produced by *Bacillus subtilis* enhance the biodegradation of hydrocarbon in wastewater bioremediation [32].

Species of the genus *Microbacterium* have been reported as keratinolytic bacteria [62], as degraders of the pesticides lindane [1], and fenamiphos [11], and able to carry out the desulfurization of dibenzothiophene and other organic sulfur compounds [29], and finally, some species of *Nocardioides* have been reported as novel crude-oil-degrading bacteria [53].

## Conclusions

With the enrichment technique used, a microbial community of six bacterial strains, able to use efficiently 4ANS as the sole carbon source and energy, nitrogen and sulfur, was selected. The N-limited system used for the biodegradation of the compound yielded removal rates and efficiencies, measured by HPLC and COD, much higher than those obtained in a C-limited system at comparable 4ANS loading rates and *HRT* values. The concerted metabolic activity of a higher number of bacterial strains could have contributed to the remarkable increase in the biodegradation rate of 4ANS determined under carbon-excess conditions (nitrogen-limiting conditions).

**Acknowledgments** The authors thank the Secretaría de Investigación y Posgrado, Instituto Politécnico Nacional (SIP-IPN), and Comisión de Fomento de las Actividades Académicas (COFAA-IPN) for fellowships to C. Juárez-Ramírez, N. Ruiz-Ordaz, and J. Galíndez-Mayer, to SIP-IPN for financial support of R. V-G, and to the Consejo Nacional de Ciencia y Tecnología for a graduate scholarship to O. R-M.

## References

1. Abhilash PC, Srivastava S, Singh N (2011) Comparative bioremediation potential of four rhizospheric microbial species against lindane. Chemosphere 82:56–63
2. Anwar S, Liaquat F, Khan QM, Khalid ZM, Iqba S (2009) Biodegradation of chlorpyrifos and its hydrolysis product 3,5,6-trichloro-2-pyridinol by *Bacillus pumilus* strain C2A1. J Hazard Mater 168:400–405
3. Applegate DH, Bryers JD (1991) Effects of carbon and oxygen limitations and calcium concentrations on biofilm removal processes. Biotechnol Bioeng 37:17–25
4. Babcock RW, Ro KS, Hsieh C–C, Stenstrom MK (1992) Development of an offline enricher-reactor process for activated sludge degradation of hazardous wastes. Water Environ Res 64:782–791
5. Barik S, Munnecke DM, Fletcher S (1982) Enzymatic hydrolysis of Malathion and other dithioate pesticides. Biotech Lett 4:795–798
6. Beloin C, Roux A, Ghigo J-M (2008) *Escherichia coli* biofilms. In: Romeo T (ed) Bacterial biofilms. Current topics in microbiology and immunology, vol 322. Springer, Berlin, pp 249–290
7. Benigni R, Passerini L (2002) Carcinogenicity of the aromatic amines: from structure-activity relationships to mechanisms of action and risk assessment. Mutat Res 511:191–206
8. Bhadbhade BJ, Sarnik SS, Kanekar PP (2002) Biomineralization of an organophosphorus pesticide, monocrotophos, by soil bacteria. J Appl Microbiol 93:224–234
9. Bolte JP, Hill DT (1990) A Monod-based model of attached-growth anaerobic fermenters. Biol Wastes 31:275–289
10. Booth G (2005) Naphthalene derivatives. In: Ullmann's Encyclopedia of Industrial Chemistry, Electronic Edition, Wiley-VCH, Weinheim

11. Cabrera JA, Kurtz A, Sikora RA, Schouten A (2010) Isolation and characterization of fenamiphos degrading bacteria. Biodegradation 21:1017–1027
12. Durmaz B, Sanin FD (2001) Effect of carbon to nitrogen ratio on the composition of extracellular polymers in activated sludge. Water Sci Technol 44:221–229
13. Felske A, Engelen B, Nübel U, Backhaus H (1996) Direct ribosome isolation from soil to extract bacterial rRNA for community analysis. Appl Environ Microbiol 62:4162–4167
14. Fernández N, Díaz EE, Amils R, Sanz JL (2008) Analysis of microbial community during biofilm development in an anaerobic wastewater treatment reactor. Microbial Ecol 56:121–130
15. Fichtner S, Lange FT, Schmidt W, Brauch H-J (1995) Determination of aromatic sulfonates in the river Elbe by on-line ion-pair extraction and ion-pair chromatography. Fresen J Anal Chem 353:57–63
16. Galindez-Nájera SP, Llamas-Martínez MA, Ruiz-Ordaz N, Juárez-Ramírez C, Mondragón-Parada ME, Ahuatzi-Chacón D, Galíndez-Mayer J (2009) Cyanuric acid biodegradation by a mixed bacterial culture of *Agrobacterium tumefaciens* and *Acinetobacter* sp. in a packed-bed biofilm reactor. J Ind Microbiol Biot 36:275–284
17. Gimeno RA, Marcé RM, Borrull F (2001) Determination of aromatic sulfonates in coastal water by on-line ion-pair solid-phase extraction/ion-pair liquid chromatography with UV detection. Chromatographia 53:22–26
18. HACH-Wastewater and biosolids analysis manual (1999) Hach Company. Colorado, USA
19. Hayase N, Kouno K, Ushio K (2000) Isolation and characterization of *Aeromonas* sp B-5 capable of decolorizing various dyes. J Biosci Bioeng 90:570–573
20. Hong Y, Guo J, Zhincheng X, Cuiyun M, Meiying X, Guoping S (2007) Reduction and partial degradation mechanisms of naphthylaminesulfonic azo dye amaranth by *Shewanella decolorantionis* S12. Appl Microbiol Biot 75:647–654
21. Jia D, Li C, Zhao B, Sun S (2010) Studies on the adsorption of 2-naphthalenesulfonic acid on basic resin from effluents. J Chem Eng Data 55:5801–5806
22. Jiang X, Pace JL (2006) Microbial biofilms. In: Pace JL, Rupp M, Finch RG (eds) Biofilms, infection, and antimicrobial therapy. CRC Press, Taylor & Francis Group, Boca Raton, pp 1–20
23. Kadiyala V, Spain JC (1998) A two component monooxygenase catalyzes both the hydroxylation of *p*-nitrophenol and the oxidative release of nitrite from 4-nitrocatechol in *Bacillus sphaericus* JS905. Appl Environ Microbiol 64:2479–2484
24. Kanamori T, Kanou N, Kusakabe S, Atomi H, Imanaka T (2005) Allophanate hydrolase of *Oleomonas sagaranensis* involved in an ATP-dependent degradation pathway specific to urea. FEMS Microbiol Lett 245:61–65
25. Kanamori T, Rashid N, Morikawa M, Atomi H, Imanaka T (2002) *Oleomonas sagaranensis* gen. nov., sp. nov., represents a novel genus in the α-*Proteobacteria*. FEMS Microbiol Lett 217:255–261
26. Karn SKr, Chakrabarty SK, Reddy MS (2010) Characterization of pentachlorophenol degrading *Bacillus* strains from secondary pulp-and-paper-industry sludge. Int Biodeter Biodegr 64:609–613
27. Klapper I, Rupp CJ, Cargo R, Purvedorj B, Stoodley P (2002) Viscoelastic fluid description of bacterial biofilm material properties. Biotechnol Bioeng 80:289–296
28. Lange FT, Brauch H-J (2006) Analysis, occurrence, and fate of aromatic sulfonates in the Rhine and its tributaries. In: Knepper TP (ed) The Rhine. The handbook of environmental chemistry, vol 5L. Springer, Berlin, pp 185–210
29. Li W, Zhang Y, Wang MD, Shi Y (2005) Biodesulfurization of dibenzothiophene and other organic sulfur compounds by a newly isolated *Microbacterium* strain ZD-M2. FEMS Microbiol Lett 247:45–50
30. Manuzon MY, Wang HH (2007) Mixed culture biofilms. In: Blaschek HP, Wang HH, Agle ME (eds) Biofilms in the food environment. Blackwell Publishing and the Institute of Food Technologists, Ames Iowa, pp 105–126
31. Melling JT (1977) Regulation in enzyme synthesis in continuous culture. In: Wiseman A (ed) Topics in enzyme and fermentation biotechnology, vol 1. Ellis Horwood Ltd., Sussex England, pp 10–42
32. Moran AC, Olivera N, Commendatore M, Esteves JL, Sineriz F (2000) Enhancement of hydrocarbon waste biodegradation by addition of a biosurfactant from *Bacillus subtilis* O9. Biodegradation 11:65–71
33. Navarro-Ortega A, Barceló D (2011) Persistent organic pollutants in water, sediments, and biota in the Ebro River Basin. In: Barceló D, Petrovic M (eds) The Ebro River Basin. The handbook of environmental chemistry, vol 13. Springer, Berlin, pp 139–166
34. Nelson ML, Yaron B, Nye PH (1982) Biologically induced hydrolysis of parathion in soil: kinetics and modeling. Soil Biol Biochem 14:223–228
35. Nicolella C, Zolezzi M, Furfaro M, Cattaneo C, Rovatti M (2007) High-rate degradation of aromatic sulfonates in a biofilm airlift suspension reactor. Ind Eng Chem Res 46:6674–6680
36. Ohshiro K, Kakuta T, Sakai T, Hidenori H, Hoshino T, Uchiyama T (1996) Biodegradation of organophosphorus insecticides by bacterial isolated from turf green soil. J Ferment Bioeng 82: 299–305
37. Oturkar CC, Nemade HN, Mulik PM, Patole MS, Hawaldar RR, Gawai KR (2011) Mechanistic investigation of decolorization and degradation of Reactive Red 120 by *Bacillus lentus* BI377. Bioresource Technol 102:758–764
38. Pandey A, Singh P, Iyengar L (2007) Bacterial decolorization and degradation of azo dyes. Int Biodeter Biodegr 59:73–84
39. Pereira R, Pereira L, van der Zee FP, Alves MM (2011) Fate of aniline and sulfanilic acid in UASB bioreactors under denitrifying conditions. Water Res 45:191–200
40. Pipke R, Amrhein N (1988) Isolation and characterization of a mutant of *Arthrobacter* sp. strain GLP-1 which utilises the herbicide glyphosate as its sole source of phosphorus and nitrogen. Appl Environ Microbiol 54:2868–2870
41. Pipke R, Amrhein N, Jacob GS, Kishore GM, Schaefer J (1987) Metabolism of glyphosate in an *Arthrobacter* sp. GLP-1. Eur J Biochem 165:267–273
42. Quinn JP, Peden JMM, Dick RE (1989) Carbon-phosphorus bond cleavage by Gram-positive and Gram-negative soil bacteria. Appl Microbiol Biot 31:283–287
43. Rangaswamy V, Venkateswaralu K (1992) Degradation of selected insecticides by bacteria isolated from soil. B Environ Contam Tox 49:797–804
44. Ravera M, Musso D, Gosetti F, Cassino C, Gamalero E, Osella D (2010) Oxidative degradation of 1,5-naphthalenedisulfonic acid in aqueous solutions by UV-photolysis in the absence and presence of $H_2O_2$. Chemosphere 79:144–148
45. Relman DA (1993) Universal bacterial 16S rDNA amplification and sequencing. In: Persing DH, Smith TF, Tenover FC, White TJ (eds) Diagnostic molecular microbiology: principles and applications. American Society for Microbiology, Washington, pp 489–495
46. Reysenbach A-L, Wickham GS, Pace NR (1994) Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus Spring, Yellowstone National Park. Appl Environ Microbiol 60:2113–2211
47. Rochex A, Lebault J-M (2007) Effects of nutrients on biofilm formation and detachment of a *Pseudomonas putida* strain isolated from a paper machine. Water Res 41:2885–2892
48. Rupp CJ, Fux CA, Stoodley P (2005) Viscoelasticity of *Staphylococcus aureus* biofilms in response to fluid shear allows

resistance to detachment and facilitates rolling migration. Appl Environ Microbiol 71:2175–2178
49. Santos V, Diogo J, Pacheco MJ, Ciríaco L, Morão A, Lopes A (2010) Electrochemical degradation of sulfonated amines on SI/BDD electrodes. Chemosphere 79:637–645
50. Saratale RG, Saratale GD, Chang JS, Govindwa SP (2011) Bacterial decolorization and degradation of azo dyes: a review. J Taiwan Inst Chem Eng 42:138–157
51. Sauer K, Cullen MC, Rickard AH, Zeef LAH, Davies DG, Gilbert P (2004) Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. J Bacteriol 186:7312–7326
52. Schembri MA, Kjærgaard K, Klemm P (2003) Global gene expression in *Escherichia coli* biofilms. Mol Microbiol 48:253–267
53. Schippers A, Schumann P, Spröer C (2005) *Nocardioides oleivorans* sp. nov., a novel crude-oil-degrading bacterium. Int J Syst Evol Microbiol 55:1501–1504
54. Sharmila M, Ramanand K, Sethunathan N (1989) Effect of yeast extract on the degradation of organophosphorus insecticides by soil enrichment and bacterial cultures. Can J Microbiol 35:1105–1110
55. Shiyun Z, Xuesong Z, Daotang L (2002) Ozonation of naphthalene sulfonic acids in aqueous solutions. Part I: elimination of COD, TOC and increase of their biodegradability. Water Res 36:1237–1243
56. Singh S, Singh P, Awasthi SK, Pandey A, Iyengar L (2008) Mineralization of 2-aminobenzenesulfonate by a bacterial consortium. World J Microbiol Biot 24:841–847
57. Slater JH, Lovatt D (1984) Biodegradation and the significance of microbial communities. In: Gibson DT (ed) Microbial degradation of organic compounds. Marcel Dekker, NY and Basel, pp 439–485
58. Song Z, Edwards S, Burns R (2005) Biodegradation of naphthalene-2-sulfonic acid present in tannery wastewater by bacterial isolates *Arthrobacter* sp 2AC and *Comamonas* sp. 4BC. Biodegradation 16:237–252
59. Szabó-Bárdos E, Markovics O, Horváth O, Töro N, Kiss G (2011) Photocatalytic degradation of benzenesulfonate on colloidal titanium dioxide. Water Res 45:1617–1628
60. Tait MI, Sutherland IW, Clarkesturman AJ (1986) Effect of growth conditions on the production, composition and viscosity of *Xanthomonas campestris* exopolysaccharide. J Gen Microbiol 132:1483–1492
61. Tan NCG, van Leeuwen A, van Voorthuizen EM, Slenders P, Prenafeta-Boldu FX, Temmink H, Lettinga G, Field JA (2005) Fate and biodegradability of sulfonated aromatic amines. Biodegradation 16:527–537
62. Thys RCS, Lucas FS, Riffel A, Heeb P, Brandelli A (2004) Characterization of a protease of a feather-degrading *Microbacterium* species. Lett Appl Microbiol 39:181–186
63. Usha MS, Sanjay MK, Gaddad SM, Shivannavar CT (2010) Degradation of H-acid by free and immobilized cells of *Alcaligenes latus*. Brazilian J Microbiol 41:931–945. Online at http://www.scielo.br/pdf/bjm/v41n4/12.pdf, consulted 5 Nov 2011
64. Vrede K, Heldal M, Norland S, Bratbak G (2002) Elemental composition (C, N, P) and cell volume of exponentially growing and nutrient-limited bacterioplankton. Appl Microbiol Biot 68:2965–2971
65. Zerbinati O, Salomone S, Ostacoli G (1994) Sulfonated derivatives of naphthalene in water samples of an Italian river. Chemosphere 29:2639–2643
66. Zerbinati O, Vincenti M, Pittavino S, Gennaro MC (1997) Fate of aromatic sulfonates in fluvial environment. Chemosphere 35:2295–2305
67. Zissi US, Kornaros ME, Lyberatos GC (1999) Kinetics of *p*-Aminoazobenzene degradation by *Bacillus subtilis* under denitrifying conditions. Water Environ Res 71:323–331
68. Zurrer D, Cook AM, Leisenger T (1987) Microbial desulfonation of substituted naphthalene sulphonic acids and benzene sulphonic acids. Appl Microbiol Biot 53:1459–1463