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Aerobic Biodegradation of a Sulfonated Phenylazonaphthol Dye by a Bacterial Community Immobilized in a Multistage Packed-Bed BAC Reactor

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Abstract A microbial community able to aerobically degrade the azo dye Acid Orange 7 was selected from riparian or lacustrine sediments collected at sites receiving textile wastewaters. Three bacterial strains, pertaining to the genera *Pseudomonas*, *Arthrobacter*, and *Rhizobium*, constitute the selected community. The biodegradation of AO7 was carried out in batch-suspended cell culture and in a continuously operated multistage packed-bed BAC reactor. The rapid decolorization observed in batch culture, joined to a delay of about 24 h in COD removal and cell growth, suggests that enzymes involved in biodegradation of the aromatic amines generated after AO7 azo-bond cleavage (1-amino-2-naphthol [1-A2N] and 4-aminobenzenesulfonic acid [4-ABS]), are inducible in this microbial consortium. After this presumptive induction period, the accumulated byproducts, measured through COD, were partially metabolized and transformed in cell mass. At all azo dye loading rates used, complete removal of AO7 and 1-A2N was obtained in the multistage packed-bed BAC reactor (PBR).; however, the overall COD (η_{COD}) and 4-ABS (η_{ABS}) removal efficiencies obtained in steady state continuous culture were about 90%. Considering the toxicity of 1-A2N, its complete removal has particular relevance. In the first stages of the packed-bed BAC reactor (Fig. 4a–c), major removal was observed. In the last stage, only a slight removal of COD and 4-ABS was obtained. Comparing to several reported studies, the continuously operated multistage packed-bed BAC reactor showed similar or superior results. In addition, the operation of large-packed-bed BAC reactors could be improved by using several shallow BAC bed stages, because the pressure drop caused by bed compaction of a support material constituted by small and fragile particles can be reduced.

Keywords Acid Orange 7 · Sulfanilic acid · 4-aminobenzenesulfonic acid · 1-amino-2-naphthol · Packed-bed reactor · Biofilm · Biodegradation · *Arthrobacter* · *Rhizobium*

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Nomenclature and units

BAC	Biological activated carbon
$B_{V,AO7}=F\ c_{I,AO7}/V_{PBL}$	AO7 loading rate [mg L^{-1} h^{-1}]
$B_{V,COD}=F\ c_{I,COD}/V_{PBL}$	COD loading rate [mg L^{-1} h^{-1}]
$B_{V,ABS}=F\ c_{SI,ABS}/V_{PBL}$	Stoichiometric 4-ABS loading rate [mg L^{-1} h^{-1}]
$B_{V,AN}=F\ c_{SI,AN}/V_{PBL}$	Stoichiometric 1-amino-2-naphthol loading rate [mg L^{-1} h^{-1}]
$c_{I,AO7}$	Input concentration of AO7 [mg L^{-1}]
$c_{I,COD}$	Input COD concentration [mg L^{-1}]
$c_{SI,ABS}$	Input concentration of 4-ABS stoichiometrically calculated [mg L^{-1}]
$c_{SI,AN}$	Input concentration of 1-amino-2-naphthol stoichiometrically calculated [mg L^{-1}]
c_{AO7}	Output concentration of AO7 [mg L^{-1}]
c_{COD}	Output COD concentration [mg L^{-1}]
c_{ABS}	Output 4-ABS concentration[mg L^{-1}]
c_{AN}	Output 1-amino-2-naphthol concentration[mg L^{-1}]
F	Liquid flow rate [mL h^{-1}]
H_{PB}	Packed-bed height [mm]
$R_{V,AO7}=F\ (c_{I,AO7}-c_{AO7})/V_{PBL}$	AO7 removal rate [mg L^{-1} h^{-1}]
$R_{V,COD}=F\ (c_{I,COD}-c_{COD})/V_{PBL}$	COD removal rate [mg L^{-1} h^{-1}]
$R_{V,ABS}=F\ (c_{SI,ABS}-c_{ABS})/V_{PBL}$	AO7 removal rate [mg L^{-1} h^{-1}]
$R_{V,AN}=F\ (c_{SI,AN}-c_{AN})/V_{PBL}$	COD removal rate [mg L^{-1} h^{-1}]
t_D	Decolorization time in batch enrichment cultures [h]
V_{PB}	Packed-bed volume [cm^3] or [L]
$V_{PBL}=\varepsilon_B\ W_{GAC}/\rho_B$	Interstitial packed-bed volume [cm^3] or [L]
W_{GAC}	GAC weight [g]

Greeks

ε_B	Packed-bed porosity [dimensionless]
φ_{PB}	Packed-bed diameter [mm]
η_{AO7}	AO7 removal efficiency [%]
η_{COD}	COD removal efficiency [%]
ρ_B	Packed-bed density [g cm^{-3}]

Subscripts

AO7	Acid Orange 7
ABS	4-aminobenzene sulfonate
AN	1-amino-2-naphthol
CI	Input concentration
COD	Chemical oxygen demand
CSI	Input concentration stoichiometrically calculated
PB	Packed bed
PBL	Liquid content in packed bed

Introduction

Azo dyes represent 60–70% of the thousands of azo compounds used in the textile, pharmaceutical, cosmetics, and food industries. It is estimated that about 2% dyes are lost

during their manufacture and may be present in wastewater flowing out from a textile industry [1]. Many azo dyes contain potentially carcinogenic aromatic amines in their chemical formulation [2], and owing to the genotoxicity and carcinogenic potential of some byproducts released by azo reduction [3–7], azo dye discharges to water treatment facilities or water bodies constitute a serious environmental problem and a public health concern. Therefore, their removal from textile wastewater has been a big challenge over the last decades, and the application of a chemical or biotechnological process, not only for color removal but also for the complete mineralization of azo dyes, has been increased in the last years [8].

Advanced oxidation processes (AOPs) have an important decontamination potential. However, unless contaminants are not biodegradable, chemical or photochemical processes hardly compete with the cost-effective and environmentally friendly biological treatments. The main disadvantages of AOPs are the expenses associated to reagents and energy consumption [9, 10].

As azo dyes are intentionally designed to be recalcitrant under typical usage conditions, they are not adequately removed from textile wastewater in conventional sewage treatment systems. The sulfonic acid group, introduced to increase the water solubility of dyes, and the azo linkage, interfere in its microbial degradation [11, 12]. Reductive cleavage of the −N═N− bond is the first step of the bacterial degradation of an azo dye. Its decolorization could be achieved under anaerobic (methanogenic), anoxic, or aerobic conditions by different trophic groups of bacteria. Azo dyes are generally resistant to bacterial attack under aerobic conditions, nevertheless, some strains that reduce the azo linkage by aerobic or oxygen-insensitive azoreductases, and can use the released byproducts as growth substrates, have been isolated [13–17]. On the other hand, sulfonated aromatic amines derived from azo dye breakdown offer resistance to biological attack because of their highly polar sulfone group. Thus, specialized aerobic microbial consortia are required for their mineralization [12, 13, 18].

Considering the recalcitrance of azo dyes, some authors have suggested the addition of co-substrates [19–22] or the use of sequential anoxic/aerobic treatment systems [22–24] for their complete biodegradation. However, for simple and economic bioprocesses, both aspects can be avoided by using selected microbial strains able to use the azo compounds as carbon and energy sources. Additionally, to remove azo compounds from textile wastewaters, the use of packed biofilm reactors, particularly when simultaneous adsorption and biodegradation processes are carried out in the support material, could be an economic solution. It is well known that in biofilm reactors, with activated carbon as support material, the organic polluting compounds are adsorbed by carbon, and by microbial action the adsorbent is regenerated [25]. The role of bioregeneration process in renewing the adsorbent surface for further adsorption is well recognized [25, 26]. However, the use of biofilm reactors packed with powdered or granulated activated carbon presents some problems. If the biodegradation process is aerobic, the oxygen supply to a packed mass of biological activated carbon (BAC) is complicated. The pressure drop in full scale BAC columns caused by support compacting could also be a serious difficulty. For these reasons, to study the biodegradation of the azo compound Acid Orange 7 (p-[(2-Hydroxy-1-napthyl)azo]benzene-sulfonic acid), whose azo reduction leads to the formation of the toxic compound 1-amino-2-naphthol [6] and the recalcitrant compound 4-aminobenzenesulfonic acid (4-ABS) [27], an aerobic shallow-staged biofilm reactor packed with BAC particles fed with a minimal medium containing the azo dye as the sole carbon and energy source, is proposed in this work.

Materials and Methods

Chemicals

p-[(2-Hydroxy-1-napthyl)azo]benzenesulfonic acid (Orange A, Orange II, Acid Orange 7, AO7, C.I. 15510) of analytical grade and 1-amino-2-naphthol were purchased from Sigma-Aldrich Co., USA. Sulfanilic acid (4-aminobenzene sulfonate) was from Chem-Service, PA, USA. The solvents used for HPLC were acquired from J T Baker, USA.

Culture Media

The minimal salts (MS) medium contained (in g L^{-1}): $(NH_4)_2SO_4$, 0.25; KH_2PO_4, 0.20; $MgSO_4$, 0.10; $CaCl_2$, 0.02. After adjusting the pH value to 6.0 and adding Acid Orange 7 to reach the required concentration, the culture medium was autoclaved at 121 °C for 15 min. The isolated microbial strains were maintained in MS medium-agar plates supplemented with AO7 at 50 mg L^{-1} (MS-AO7).

Selection of Microorganisms

Using the microbial enrichment technique of successive transfers to fresh MS-AO7 medium, microorganisms able to use the azo dye as the sole carbon source were selected from riparian or lacustrine sediments collected from two sites receiving textile wastewaters: the Atoyac River and the Requena Dam; both located in Central México, at the Hidalgo and Tlaxcala states, respectively. Sediment samples were inoculated in Erlenmeyer flasks containing 100 mL of MS-AO7 medium. Flasks were incubated at 28±2 °C in agitation. When decolorization was observed, a 5-mL aliquot was transferred to another flask containing an increased concentration of AO7. Decolorization time (t_D) was variable. For the first transfers, t_D was about 120 h; for the last one, t_D diminished to less than 24 h. After 12 transfers, an enriched microbial community able to grow on MS-AO7 medium containing up to 200 mg AO7 L^{-1} was obtained. Along the enrichment process, the presence or predominance of bacterial strains was determined by thermal gradient gel electrophoresis (TGGE) of 16S rDNA fragments, PCR-amplified (Gene Amp PCR System 2400, USA) using U968 [5′-(GC clamp)-AAC GCG AAG AAC CTT AC-3′] and L1401 [5′-CGG TGT GTA CAA GAC CC-3′] primers [28]. The equipment used was a TGGE System (Biometra, Göttingen, Germany).

AO7 Biodegradation in Batch Culture

Batch culture was carried out at room temperature in a magnetically stirred Erlenmeyer flask containing 500 mL of MS-AO7 medium (200 mg L^{-1}). The flask was inoculated with an enriched mixed bacterial culture aliquot. Air was bubbled through a sintered glass tube at a gas flow rate of 0.05 L min^{-1} (Gas Flowmeter, Gilmont Instruments USA). Using a pH meter/controller (Chemcadet, Cole Parmer USA), the pH value was maintained at 6.25±0.25 by addition of 0.2 M Na_2CO_3. Samples taken at different times were used to estimate COD, by reflux closed method, and the azo-dye concentration by HPLC or by measuring the sample absorbance at 485 nm in a Beckman DU-650 spectrophotometer.

Multistage Packed-Bed Biofilm Reactor

The bioreactor used in this study was a lab-scale multistage column composed by three Pyrex glass modules separated by sintered glass plates (pore size of 30–40 μm). To reduce pressure drop and column compaction in the PBR, the support material was distributed in three modules. Each one was packed with granular-activated carbon. The reactor was operated with air and liquid concurrently injected into the lower part of the column. Each stage had a lateral port for liquid sampling. A cover with an air/liquid discharge outlet was located at the top of the third stage. A diagram of the reactor is shown in Fig. 1, and the main characteristics of the fixed bed are presented in Table 1.

Continuous AO7 Biodegradation in the PBR

Granular-activated carbon (Calgon Carbon Co. PA, USA) was used as PBR biofilm support; initially, it was saturated with the azo dye as follows. In aseptic conditions, GAC was soaked by immersion during 96 h in a 2.8% AO7 solution, then, each stage was loaded with saturated GAC, and the column was assembled. MS medium plus AO7 (200 mg L^{-1}) was fed at a flow rate of 16 mL h^{-1}, maintaining an airflow rate of 0.05 L min^{-1}. The outflowing medium was periodically sampled to evaluate AO7 concentration. Once a stable value of the output AO7 concentration of 200 mg L^{-1} was reached, indicating GAC

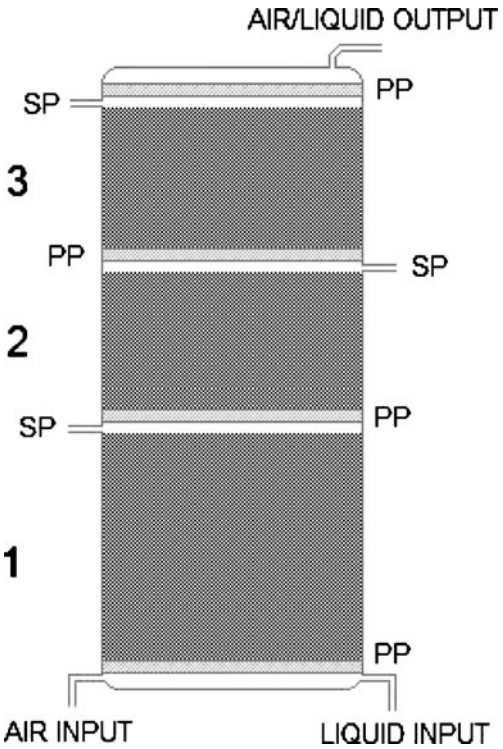

Fig. 1 Scheme of a multistage packed-bed BAC reactor. *PP* porous plate, *SP* sample port. (*1*) Lower, (*2*) middle, and (*3*) upper PBR stages

Table 1 Characteristics of the GAC support packed in the multistage packed-bed reactor.

	1st stage	2nd stage	3rd stage	Total
H_{PB} [mm]	59	28	25	112
V_{PB} [cm^3]	75.8	35	32.2	143
W_{GAC} [g]	27	12.5	11.5	51
V_{PBL} [cm^3]	55.3	25.6	23.5	104.4

φ_{PB}=42 mm; ε_B=0.73; ρ_B=0.356 g cm^{-3}

equilibrium with the azo dye solution, the reactor was inoculated with a cell suspension of the mixed microbial community. To enable microbial attachment to GAC, cells were aerobically cultivated for 24 h before starting the PBR continuous operation. Along all experiments, the same airflow rate (0.05 L min^{-1}) was maintained. The interstitial liquid volume of the PBR (VLPB) was 104.4 mL. This value was used to estimate the volumetric loading rates of the azo dye $B_{V,AO7}$. The equivalent loading rates of the constituent parts of Acid Orange 7, 4-ABS, and 1-A2N were calculated as follows.

According to the reductive cleavage of the AO7 azo bond, [$C_{16}H_{12}O_4N_2S+4H^+ \rightarrow C_6H_7O_3NS+C_{10}H_9ON$], 1 mg of Acid Orange 7 stoichiometrically renders 0.525 mg of 4-amino benzenesulfonic acid plus 0.485 mg of 1-amino-2-naphthol. On this basis, the concentrations of both components in the inflowing medium were estimated and their corresponding loading rates $B_{V,ABS}$ and $B_{V,1-A2N}$ were calculated. To operate the bioreactor with several $B_{V,AO7}$ values, liquid flow rate F was varied from 16 to 49.2 mL h^{-1}.

Each stage of the packed-bed reactor was sampled to evaluate, spectrophotometrically and by HPLC, the concentrations of Acid Orange 7, 1-amino-2-naphthol, and 4-amino benzenesulfonic acid. COD was determined as described in "Analytical Methods". Once a steady state was reached (COD and AO7 effluent concentrations remain constant), the volumetric loading rate $B_{V,AO7}$ was changed. Finally, the reactor was disassembled, and samples of the biological activated carbon BAC were used to obtain micrographs of the biofilm adhered to GAC.

Isolation and Identification of Microorganisms

With the aid of a vortex agitator, microbial cells were extracted from BAC particles suspended in water. After BAC particles settling, appropriated dilutions of the supernatant cell suspension were poured in nutrient agar, and the strains showing differences in colonial morphology were isolated. Bacterial DNA was extracted, purified, and used for 16S rDNA amplification using the oligonucleotides 8FPL-5′ and 1492RPL-5′ [29], as primers. Purity of strains was verified by gel electrophoresis of their 16S rDNA fragments. Amplicons of about 1,500 bp were sequenced (Instituto de Biología, UNAM) and compared with known sequences of bacterial 16S rDNA at NCBI GenBank by using the Basic Local Alignment Search Tool (BLAST) algorithm. Reported species showing the higher similarities were regarded as the isolated species.

Analytical Methods

Cell Concentration

In batch suspended cell culture, cell mass was gravimetrically determined by filtering sample aliquots through pre-weighted Whatman GF/F (0.7 μm) filters. Then, filters were

dried to constant weight at 100 °C. Cell mass was determined by weight difference. By microscopical observation of filtrates, it was verified that cells were retained in glass fiber filters. Filtrates were used for determination of AO7 and its biodegradation products.

Determination of AO7, 4-amino Benzenesulfonic Acid, and 1-amino-2-naphthol

Concentrations of these compounds were routinely estimated by measuring their absorbance at their respective maximum wavelengths: 485 nm for AO7, 245 nm for 4-ABS, and 286 nm for 1-A2N (Beckman DU 650 Spectrophotometer). Alternatively, a Beckman HPLC Gold System, equipped with a diode array detector (UV 243 nm) and an Alltech Econosphere C-8 column was also used to quantify the AO7 disappearance and accumulation of biodegradation products. The column was eluted with a 60:40 mixture of phosphoric acid (0.3%)/acetonitrile. Previously, a mixture of standards of AO7, 4-ABS, and 1-A2N (10 mg L^{-1} of each one), was injected to the HPLC column. The retention times were: 2.3 min for AO7, 1.7 min for 4-ABS, and 2.05 min for 1-A2N.

Chemical Oxygen Demand

Using a closed reflux method (0–150 mg L^{-1}) in a Hach reactor [30], COD values were determined in filtrates obtained after cell mass determination.

Scanning Electron Microscopy

Once the reactor was disassembled, samples of BAC particles containing the microbial biofilm were collected at each stage of the packed-bed reactor. After fixing with 2% glutaraldehyde, washing twice with phosphate buffer at pH 7, post-fixing with 1% osmium tetroxide, dehydrating with ethanol, drying, and finally covering with gold, micrographs of BAC particles were taken in a scanning electron microscope (JEOL, JSM-5800 LV, Japan).

Results and Discussion

Enrichment of Dye-Decolorizing Bacterial Community

Along the enrichment process, changes in the number of strains constituting the bacterial community were observed by TGGE analysis. After 12 successive transfers to flasks containing MS-AO7 medium, the number of DNA bands diminished from eight to three. In the last transfers, this value remained without change. Assuming that each DNA band represents a pure strain, a stable ternary bacterial community was obtained (Fig. 2)

AO7 Biodegradation in Suspended Cell Batch Culture

Figure 3 shows a fast aerobic AO7 azo reduction; however, in the first 24-h cell growth was not observed and only a slight diminution in COD occurs, indicating that the products released by azo-bond cleavage were poorly metabolized. Later, these byproducts were partially used by cells as the carbon and energy source as observed through the COD removal efficiency (η_{COD}=83.3%). The rapid decolorization observed in batch culture, joined to the small change in COD and cell concentrations observed at the same time, suggest that in this bacterial consortium, the synthesis of enzymes involved in 4-ABS and

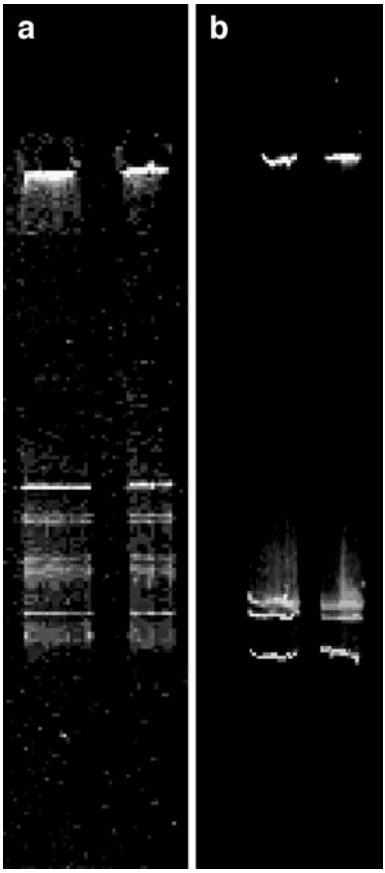

Fig. 2 TGGE of DNA obtained from the original microbial community (**a**), and after 12 successive transfers made to enrich the microbial community with bacterial strains able to degrade AO7 (**b**)

1-amino-2-naphthol catabolism is inducible. Specific information about the constitutive or inducible character of the main enzymes involved in the biodegradation pathway of the aromatic amines 4-ABS and 1-A2N is scarce [31–33]. However, the delay in COD removal observed in batch biodegradation of AO7 could be indicative of an induction period for those enzymes. A similar behavior (transient accumulation followed by biodegradation of 1-A2N and 4-ABS) was also observed in a biosulphidogenic batch reactor used for Orange II (AO7) decolorization and biodegradation [34].

At the end of the batch culture, the accumulation of 4-ABS was determined by liquid chromatography; thus, this compound could be mainly responsible for the residual COD determined. On the other hand, after 96 h, only traces of 1-amino-2 naphthol were detected by HPLC; thus, the observed cell growth could be mainly attributed to the metabolism of 1-A2N.

AO7 Biodegradation in Continuous Multistage Packed-Bed BAC Reactor

At all loading rates $B_{V,AO7}$ applied to the PBR biosystem (30.6–94 mg L^{-1} h^{-1}), complete removal of AO7 and 1-A2N was obtained. At the lowest loading rate used, the overall COD

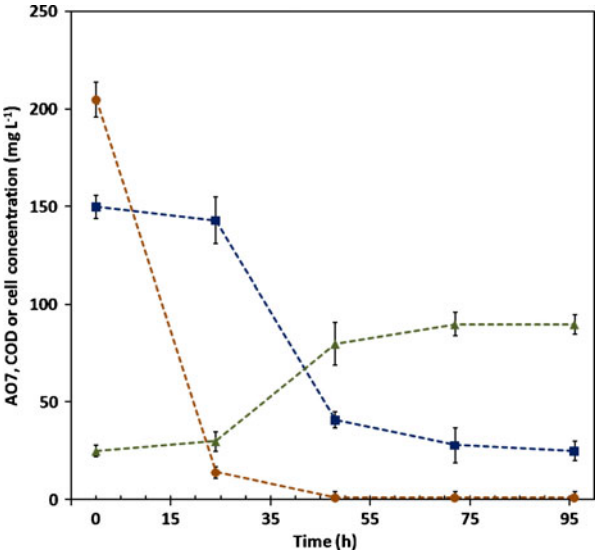

Fig. 3 Batch biodegradation of AO7 by a suspended cell culture of a ternary microbial community. AO7 (●), COD (■), and cell mass (▲) concentrations

and 4-ABS removal efficiencies obtained in steady state were about 90% (Table 2). When a continuous steady state was altered by changing the AO7 loading rate, in the first stage of the PBR, a transient accumulation of 1-A2N was detected. In the upper stages, this accumulation was not observed. Once it reached the corresponding steady state, 1-A2N was no longer detected in the reactor. In Fig. 4a–c, it can be observed that 1-A2N removal efficiencies in all bioreactors' stages rounded 100%. Although BAC adsorption of 1-A2N could contribute to its complete removal, the fact that this aromatic amine is an unstable [35, 36] and biodegradable compound [37], joined to the results obtained in batch culture (only traces of 1-A2N could be detected by HPLC at the end of the suspended cell culture) indicate its complete biodegradation in the biofilm reactor.

Major removal was observed in the first stages of the packed-bed BAC reactor (Fig. 4a–c). In the last stage, only a slight removal of COD and 4-ABS removal rates and efficiencies was obtained. The low substrate input in the last stage, and consequently, the low biofilm density observed in BAC fragments (Fig. 5c) could explain this fact.

Responding to changes in the loading rates $B_{V,AO7}$ applied, variations in removal rates R_V and removal efficiencies (η) of COD, AO7, 4-ABS and 1-amino-2-naphthol, along the PBR, can be observed in Table 2 and Fig. 4a–c. As observed, the 4-ABS removal efficiency, stoichiometrically calculated, never exceeded the value of 90%.

4-ABS is considered a compound recalcitrant to biodegradation. Although it can be used as a sulfur source by some microbial species, few of them can use it as a carbon and energy source [38–40], unless the sulfonated compound is cometabolically degraded in the presence of added co-substrates. The partial removal of 4-ABS observed in batch or continuous culture could be explained by its cometabolic biodegradation in the presence of 1-A2N released by azo dye bond cleavage. The behavior of both compounds at each PBR stage shows that once 1-A2N disappears, there is no further change in 4-ABS concentration in the second and third stages. This fact suggests that 1-A2N could be used by the microbial community as a primary energy source for biodegradation of the sulfonated compound. At all $B_{V,AO7}$ values used, the toxic amino naphthol was totally removed by the microbial community.

Table 2 Operating the multistage packed-bed reactor at several AO7 volumetric rates, the AO7, COD, 4-ABS, and 1-A2N removal rates were obtained in steady state conditions. At each loading rate used, the time to reach a steady state condition is indicated. From the stoichiometric release of 4-ABS and 1-A2N after the azo-bond cleavage of Acid Orange 7, the corresponding values of $B_{V,ABS}$ and $B_{V,1-A2N}$, were calculated.

Time to reach a steady state h	$B_{V,AO7}$ mg L^{-1} h^{-1}	$R_{V,AO7}$ mg L^{-1} h^{-1}	$B_{V,COD}$ mg L^{-1} h^{-1}	$R_{V,COD}$ mg L^{-1} h^{-1}	$B_{V,ABS}$ mg L^{-1} h^{-1}	$R_{V,ABS}$ mg L^{-1} h^{-1}	$B_{V,AN}$ mg L^{-1} h^{-1}	$R_{V,AN}$ mg L^{-1} h^{-1}
288	30.6	30.5±0.04	22.9	20.6±0.87	15.95	14.26±0.70	14.65	14.65
120	68.0	67.9±0.04	51.0	45.9±1.53	35.43	31.69±0.34	32.57	32.57
144	85.0	84.9±0.06	63.7	55.7±0.76	44.29	39.62±0.46	40.71	40.71
120	94.0	93.9±0.08	70.5	59.7±1.04	48.98	43.81±0.29	45.02	45.02

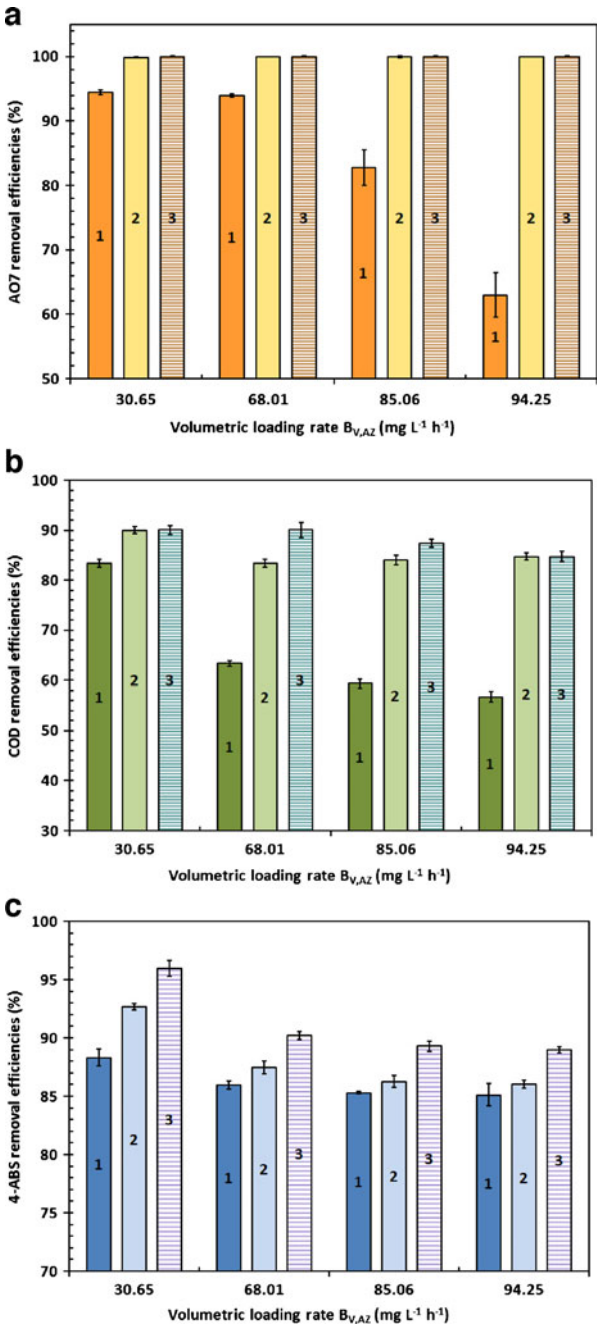

Fig. 4 Removal efficiencies of AO7 (**a**), COD (**b**), and 4-ABS (**c**) in the triple stage packed-bed biofilm reactor fed with AO7 volumetric loading rates $B_{V,AO7}$ varying from 30.65 to 94.25 mg L^{-1} h^{-1}. *Bar* numbers correspond to lower (*1*), middle (*2*), and upper (*3*) PBR stages. For 1-amino-2-naphthol, in all the stages, removal efficiencies near 100% were obtained

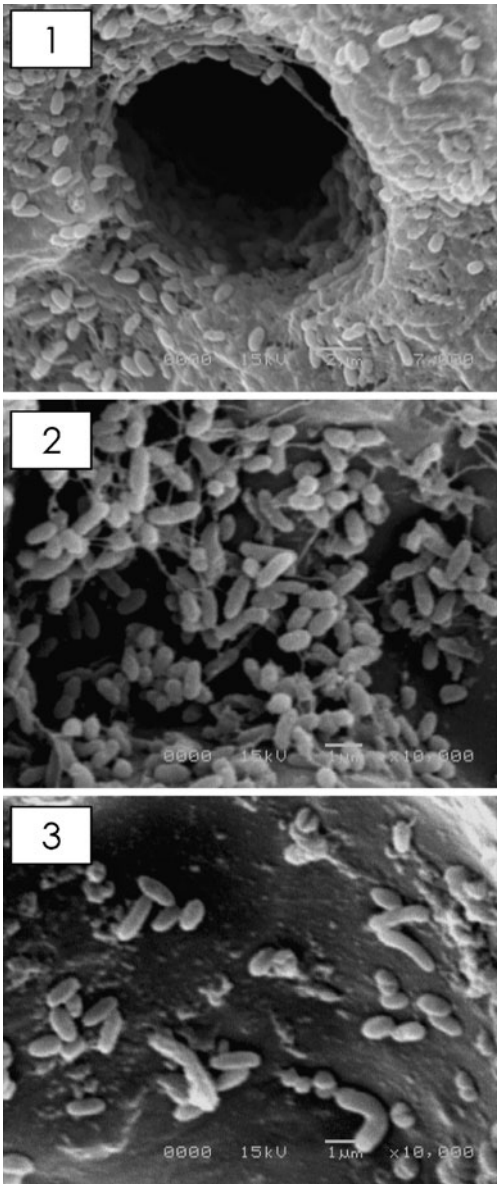

Fig. 5 Bacterial biofilm density observed in (*1*) Lower, (*2*) middle, and (*3*) upper PBR stages

In Table 3, bioprocesses carried out for AO7 decolorization or biodegradation, as well as their corresponding removal efficiencies and removal rates reported in literature, are compared with those obtained in this work. In some of the reported processes, byproduct accumulation was found; mainly, 4-ABS (sulfanilic acid), 1-A2N, or their derivatives. Although in this work, 4-ABS accumulation was observed, the 1-A2N removal efficiency always rounded 100%.

Several studies point to the carcinogenic potential of 1-amino-2-naphthol; however, the data available are inconclusive. A search in relevant databases from DIMDI (*Deutsches*

Institut für Medizinische Dokumentation und Information) and GENETOX from the National Library of Medicine's TOXNET System only sourced limited information about the carcinogenic potential of 1-amino-2-naphthol. Nevertheless, several papers point to the hazardous nature of this compound. By example, a structure-activity study of 1-amino-2-naphthol derived azo dyes using a Computer Automated Structure Evaluation system (CASE) revealed that for optimal mutagenicity reduction of the azo bond was required; thus, that activity could be related to the liberated aromatic amines [6]. The toxicity and genotoxicity of Acid Orange 7 tested with the bioluminescent bacterium *Vibrio fischeri* and *Escherichia coli* strains were attributed to the 1-amino-2-naphthol generated by hydrolysis and decolorization of the dye [41]. In texts related to clinical toxicology [42] and hazardous industrial materials [43–45], 1-A2N is included among the mutagenic compounds. It is reported that 1-A2N induced gene mutations in *Salmonella typhimurium* TA100 [46], and that it is responsible for in vitro formation of methemoglobin [47]. As a clear indication of oxidatively denatured hemoglobin, Heinz's bodies were found in the erythrocytes of mice after oral administration of 1-A2N [48]. Its possible role in carcinogenesis could be related to the generation of hydrogen peroxide and superoxide anions [49]. Additionally, 1-amino-2-naphthol is considered a tissue carcinogen that irreversibly alters the transition midpoint (T_m) of salmon sperm DNA and synthetic polyribonucleotides [50]. Because experimental evidence suggests that, at least, 1-A2N should be considered a harmful compound, its complete removal from textile effluents should be of ecological concern. In the case of 4-ABS, we could not find reports about its toxicity to aquatic or terrestrial species. Thus, it could be considered a presumptively innocuous compound to aquatic environments.

Identification of Bacterial Strains Present in the Attached Biofilm

As described in Section 2.7, three bacterial strains were isolated from BAC attached biomass. Their bacterial DNA was extracted, purified, and used for 16S rDNA amplification. The sequenced amplicons were compared with known sequences of bacterial 16S rDNA at the NCBI GenBank. The bacterial strains showing the higher similarities were identified as: *Pseudomonas* sp. (96%), *Rhizobium* sp. (96%), and *Arthrobacter* sp. (98%). Their respective NCBI GenBank accession numbers were AB088539, AY490118, and DQ628958.

Strains of the genus *Pseudomonas* able to reduce Acid Orange 7 [51], and to carry out the desulfonation of benzenesulfonic byproducts [52] have been reported. The ability of *Pseudomonas* to decolorize and degrade textile azo dyes is well documented [53, 54]. *Arthrobacter* strains are able to degrade naphthalenic compounds [55], and can either degrade 2-amino naphthyl sulfonate (2-ANS) or use it as a sulfur source [13]; thus, the role of both bacterial strains in the consortium could be explained.

On the other hand, references involving *Rhizobium* in azo reduction or biodegradation of azo dye byproducts are scarce [56]. However, it is well known that bacterial surface components such as extracellular polysaccharides (EPS) play an important role in aggregation and surface colonization of plant-associated bacteria such as *Rhizobium* [57–59]; thus, together with its possible participation in AO7 azo reduction or in biodegradation of its byproducts, the most important role of *Rhizobium* in this bacterial community could reside in biofilm formation.

In Fig. 5, the structure of the bacterial community colonizing BAC particles can be observed. Micrographs show the change in biofilm density along the different stages of the packed-bed reactor. In micrograph 5(2), the presence of biopolymers joining short bacilli is clearly observed.

Table 3 Some bioprocesses for decolorization or biodegradation of Acid Orange 7. Comparison of the byproducts accumulation, removal efficiencies, and volumetric or specific biodegradation rates of the azo dye.

Organism and system		Azo dye and cosubstrate	Byproducts accumulation	Volumetric decolorization or biodegradation rate (mg L^{-1} h^{-1})	Specific decolorization or biodegradation rate (μg (g cell)$^{-1}$ h^{-1})	AO7 removal efficiency	Reference
Suspended cells of *Candida zeylanoides*	Aerobic batch system	Methyl Orange or Acid Orange 7 as nitrogen sources	Metanilic or sulfanilic acids		15–42		[60]
Suspended cells of a mixed microbial community	Anaerobic batch system	Acid Orange 7	Orthanilic and metanilic acids, 1-amino-2-naphthol	4.16–14.58		93 to 97% decolorization, 80% COD and 75% TOC removal	[61]
GAC-immobilized bacteria	Continuous anaerobic upflow stirred BAC reactor	Acid Orange 7	Not reported			96%	[62]
Phragmites sp	Vertical-flow constructed wetland. Continuous aerobic system	Acid Orange 7	Not reported			99% decolorization and 93% TOC removal	[63]
Suspended cells of a mixed microbial community	Anaerobic batch system	Acid Orange 7 with glucose as cosubstrate	sulfanilic acid and 1,2-naphtoquinone	1.28 to 4.69			[64]

GAC-immobilized cells of a mixed microbial community	Sequential anaerobic and aerobic-sequencing batch system	Acid Orange 7				96%	[23]
Suspended cells of *Pseudomonas* sp.	Cyclic aerobic-anaerobic operation in an airlift bioreactor	Acid Orange 7 in Luria-Bertani medium or semi synthetic medium with glucose as cosubstrate	Not determined	1.1	1.3	100% decolorization	[51]
Suspended cells of a microbial community constituted by *Pseudomonas*, *Rhizobium* and *Arthrobacter* genera	Aerobic batch system	Acid Orange	4-ABS	7.95	265	100% decolorization and 83% COD removal	This work
Immobilized cells of a microbial community constituted by *Pseudomonas*, *Rhizobium* and *Arthrobacter* genera	Aerobic continuous system	Acid Orange 7	4-ABS	30.5 to 93.9		100% decolorization and 90 to 93% COD removal	This work

Conclusions

When two biodegradation systems, suspended cells batch culture and a continuously operated PBR, were compared, it was observed that the ternary bacterial consortium immobilized in the second system, significantly improved the removal rates of AO7 and the catabolic intermediaries, 4-ABS and 1-A2N, released after the AO7 azo-bond cleavage. The incomplete removal of 4-ABS observed in batch or continuous culture could be explained by its cometabolic biodegradation in the presence of 1-A2N released by azo dye bond cleavage. Results obtained in the staged biofilm reactor suggest that 1-A2N could be used by the microbial community as a primary energy source for biodegradation of the sulfonated compound. With the GAC-attached microbial community, the complete removal of 1-A2N from textile effluents was reached in this type of biofilm reactor. Bioprocesses that avoid the release of this hazardous compound to the environment have an ecological interest. Two of the bacterial strains, *Arthrobacter* sp. and *Pseudomonas* sp., have clear catabolic roles [13, 53–55]. The third, *Rhizobium* sp., could play a role in the biodegradation of azo dyes [56], joined to aggregation and cell colonization through synthesis of exopolysaccharides. After 2 months of continuous operation, flow diminution caused by column blockage was not observed, thus, we consider that the use of multiple shallow stages packed-bed columns reduces the compaction of fragile BAC support and consequently, the pressure drop. Therefore, this type of columns seems adequate for scaling up purposes.

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