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ORIGINAL ARTICLE

Effect of herbicide adjuvants on the biodegradation rate of the methylthiotriazine herbicide prometryn

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Abstract A microbial community, selected by its ability to degrade triazinic herbicides was acclimatized by successive transfers in batch cultures. Initially, its ability to degrade prometryn, was evaluated using free cells or cells attached to fragments of a porous support. As carbon, nitrogen and sulfur sources, prometryn, (98.8 % purity), or Gesagard, a herbicide formulation containing 44.5 % prometryn and 65.5 % of adjuvants, were used. In batch cultures, a considerable delay in the degradation of prometryn, presumptively caused by the elevated concentration of inhibitory adjuvants, occurred. When pure prometryn was used, volumetric removal rates remarkably higher than those obtained with the herbicide formulation were estimated by fitting the raw experimental data to sigmoidal decay models, and differentiating them. When the microbial consortium was immobilized in a continuously operated biofilm reactor, the negative effect of adjuvants on the rate and removal efficiency of prometryn could not be detected. Using the herbicide formulation, the consortium showed volumetric removal rates greater than 20 g m^{-3} h^{-1}, with prometryn removal efficiencies of 100 %. The predominant bacterial strains isolated from the microbial consortium were *Microbacterium* sp., *Enterobacter* sp., *Acinetobacter* sp., and *Flavobacterium* sp. Finally, by comparison of the prometryn removal rates with others reported in the literature, it can be concluded that the use of microbial consortia immobilized in a biofilm reactor operated in continuous regime offer better results than batch cultures of pure microbial strains.

Keywords Methylthiotriazine herbicides · Prometryn biodegradation · Biofilm reactor · Microbial consortium · Herbicide adjuvants

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Introduction

Prometryn is a persistent methylthiotriazine herbicide acting as a PSII inhibitor, frequently found in aquatic ecosystems (Schuler and Rand 2008), mainly as a result of terrestrial runoffs or land drainage. Prometryn is listed in the Pesticide Action Network (PAN) Bad Actor Pesticides as a chemical of special concern, in the US EPA Toxics Release Inventory (TRI) List, as a reproductive and developmental toxic compound, and in the prioritization list of the European Union as an endocrine disruptor (PAN Pesticides Database 2013). Because prometryn is a PSII inhibitor, it has a direct impact on photosynthetic aquatic microorganisms. Although microbial communities in freshwater ecosystems are not direct targets, they can be affected by

herbicides as a result of the interactions that occur between aquatic microorganisms (Villeneuve et al. 2011). Notwithstanding that herbicides could be found in aquatic environments in low concentrations, a potential hazard of persistent organic pollutants is their bioaccumulation and biomagnification in aquatic organisms essentials in the food web (Geyer et al. 2000; Jin et al. 2012). In addition to pesticides, some adjuvants (solvents, dispersants, surfactants) have been detected in runoffs from irrigated fields. Commonly, these xenobiotics are inappropriately perceived as inert compounds (Pedersen et al. 2003); however, by their chemical characteristics or their potential toxicity, they can have significant effects on the environment. Thus, in remediation processes of soil or water contaminated by pesticides, the removal of adjuvants should also be considered. To reduce the contamination of water bodies by xenobiotic compounds, the use of bio-barriers could be a feasible option to reduce the environmental risk represented by these contaminants. For these reasons, the aims of this work were: (a) to determine the effect that adjuvants have on prometryn biodegradation by free and attached cells of a bacterial consortium growing in batch culture, and (b) to evaluate the performance of a continuously operated biofilm reactor, devised to remove prometryn, together with the adjuvants present in the commercial herbicide Gesagard.

Materials and methods

Chemicals

The herbicide Gesagard was acquired from Syngenta. It contains not less than 44.4 % of prometryn (2,4-*Bis*(isopropylamino)-6-(methylmercapto)-s-triazine) as the active ingredient; the rest of components are adjuvants (diluents, wetting and dispersing compounds). Standards of prometryn (98.8 %) and cyanuric acid (98.0 %) were acquired from Sigma-Aldrich Inc., Steinheim Germany. The solvents used for HPLC were purchased from J. T. Baker, USA.

Culture media

Along the experimental process, a minimal-mineral-salts medium (MS) was prepared, and its formulation was (in g L^{-1}); K_2HPO_4, 0.4; $MgSO_4$, 0.1; NaCl, 0.1; $CaCl_2$, 0.02. Five mL of an oligo-elemental solution (in mg L^{-1}, $FeSO_4{\cdot}7H_2O$, 0.55; $ZnSO_4{\cdot}7H_2O$, 0.23; $MnSO_4{\cdot}H_2O$, 0.34; $CoCl_2{\cdot}6H_2O$, 0.065; $Na_2MoO_4{\cdot}2H_2O$, 0.34) were added to reach the final concentration.

MS medium plus 32 mg L^{-1} of equivalent prometryn contained in the commercial herbicide Gesagard (MSG) was used for the selection and batch tests of the microbial consortium able to grow on prometryn. The same medium was fed to the biofilm reactor. The MS medium containing 32 mg L^{-1} of pure prometryn (MSP) was utilized exclusively for batch tests of the microbial consortium. MSG-Agar (2 %) was used as selective medium for maintenance of the bacterial consortium and the isolated bacterial strains. To assess the growing ability of the bacterial isolates on cyanuric acid, MS medium, plus 50 mg L^{-1} of the catabolic intermediary was used (MSC). Peptone-yeast extract-agar medium (PYA) was chosen for viable cell counting, and for observation of the morphological diversity of the cultivable microorganisms. For DNA extraction from isolated bacterial strains, they were cultivated in Luria–Bertani medium.

Microorganisms

By successive transfers of soil samples incubated on mineral salt medium complemented with an amount of Gesagard equivalent to 32 mg prometryn L^{-1}, a microbial community, able to grow on this herbicide, was obtained from agricultural soil samples collected at the surroundings of Mexico City. Erlenmeyer flasks containing 50 mL of MSG medium and fragments of volcanic rock were incubated under stirring at room temperature for 96 h. After that time, 45 mL of the medium were drained and substituted with fresh MSG medium. The rock fragments were maintained in the flasks. To verify the herbicide degradation, flasks were periodically sampled, and the remaining prometryn was spectrophotometrically measured. When the removal of the herbicide was evident, suspended cells and cells detached from a sample of the rock fragments were harvested by centrifugation at 13,000 rpm for 5 min. To preserve the microbial community, the pellets obtained after decanting the culture medium were resuspended on 200 μL of glycerol and cryopreserved at −70 °C in a Revco ultra-low freezer (General Signal Laboratory Equipment, Inc., USA). To define the amount of cells to be used as inoculum in

batch cultures, the viable cell counting of the colonized porous support and the suspended culture was determined (Macías-Flores et al. 2009).

Prometryn biodegradation in batch culture

To observe the effect of herbicide adjuvants on the prometryn removal kinetics, using free or biofilm-forming microorganisms, the selected consortium was inoculated in 500 mL Erlenmeyer flasks containing 100 mL of MSG or MSP media (for growing free cells). For growing of biofilm-forming microorganisms, the same volume of medium was used, but, fragments of colonized porous support were added to the Erlenmeyer flasks. The flasks were incubated at room temperature under constant agitation in a rotatory shaker (60 rpm). Flasks were periodically sampled for determination of prometryn concentration, using UV spectrophotometry and liquid chromatography (HPLC).

Biofilm reactor

Figure 1 shows a column with a porous glass base (pore diameter of 40–100 μm) packed with fragments of volcanic rock ($\phi = 12.16 \pm 4.8$ mm) that was utilized as biofilm reactor. The total operating capacity of the bioreactor was 1,670 cm^3, and the drained liquid volume was 570 cm^3. The column has ports for sampling, input of liquid medium, and output of exhausted air and liquid medium. The column has a glass cover sealed with a neoprene gasket for hermetic operation. Air and liquid medium were concurrently supplied at the base of the packed column. To avoid channeling and redistribute the air along the reactor, a porous plate was inserted in the middle of the column.

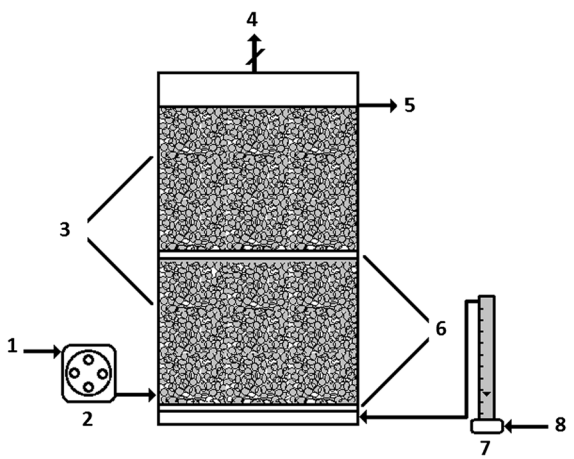

Fig. 1 Packed bed biofilm reactor. Inflowing liquid (*1*), peristaltic pump (*2*), packed-bed support material (*3*), air vent (*4*), outflowing liquid (*5*), sintered glass plates for air dispersion (*6*), air rotameter (*7*), air input (*8*)

Continuous biodegradation of prometryn in the biofilm reactor

To assess the degradation of the herbicide prometryn, the packed bed column was used. Prior to the inoculation, an abiotic test was conducted. To saturate the support material with the herbicide, the reactor was fed with MSG medium at a flow rate of 0.013 L h^{-1}; concurrently, the reactor was aerated at a gas flow rate of 0.15 L min^{-1}. Changes in prometryn concentration, in the outflowing liquid, were spectrophotometrically measured at $\lambda = 222$ nm. It was considered that the support was saturated when the value of absorbance in the effluent reached the value of the MSG medium supplied. At this moment, the reactor was inoculated with the microbial consortium. After 4 days of batch culture, it was considered that the porous support was colonized by the microbial consortium, and the bioreactor begun to operate in continuous regime at different herbicide loading rates ($R_V = F\ C_i/V_L$). In this expression, C_i is the input concentration of prometryn or COD, and V_L is the liquid volume of the reactor. Along the reactor operation, the outflowing liquid was periodically sampled to measure the chemical oxygen demand (COD) and the concentrations of prometryn and cyanuric acid (1,3,5-triazine-2,4,6-triol; [OOOT]), which usually is the main intermediary of the catabolism of prometryn. Once a steady state was reached, samples of liquid medium were taken, and a new loading rate was probed.

Identification of bacterial isolates

Once finished the operation of the reactor, it was dismantled, and samples recovered from colonized fragments of the porous support were used for the isolation of the main constituents of the biofilm-forming microorganisms. After recovering the attached cells, they were resuspended in distilled water. The cell suspension obtained was diluted, and the higher decimal dilutions were plated in PYA medium. Once growth was

observed, the isolated colonies showing morphological differences were selected and conserved in agar slants of MSG medium. To obtain a cell package suitable for DNA extraction, the bacterial isolates were propagated in Luria–Bertani liquid culture medium. The extracted and purified DNA was used for PCR amplification of the 16S rDNA, using the 8FPL and 149RPL primers (Relman 1993). The amplicons were purified (Wizard Genomic DNA Purification Kit, Promega Co., USA). After amplicons sequencing (Macrogen Inc., Seoul, South Korea), and comparison with the sequences deposited in the NCBI gene bank, the isolated strains were identified. 16S rDNA sequences were registered at the NCBI GenBank. The assigned accession numbers were KF619443 for *Enterobacter* sp., KF619444 for *Acinetobacter* sp., KF619445 for *Microbacterium* sp. and KF619446 for the *Flavobacteriaceae* bacterium. The partial sequences of the 16S rDNA genes for these bacterial isolates were aligned using Clustal X (Larkin et al. 2007), and the corresponding phylogenetic trees were constructed using the neighbor-joining method (Tamura et al. 2011).

Analytical methods

Spectrophotometric determination of prometryn

For rapid evaluation of transient changes in the bioreactor after a loading rate shift, the concentrations of prometryn were determined by measuring the absorbance of the outflowing medium at $\lambda = 222$ nm in a Beckman DU650 spectrophotometer.

Determination of cyanuric acid

A turbidimetric Hach method 8139 (Hach 2012) was used for rapid determination of cyanuric acid. The reactive kit used could determine cyanuric acid levels of 5–50 mg L^{-1}.

Determination of prometryn and cyanuric acid by HPLC

From sample filtrates, prometryn and cyanuric acid were determined by liquid chromatography using a Shimadzu HPLC System (Shimadzu LC-10AT). For prometryn determination, the system was equipped with a LiChrospher C18 column (5 µm, 150 × 4.6 mm), and a UV detector (222 nm). The flow rate of the mobile phase was 1.0 mL min^{-1}. The chromatographic separation was performed using a linear gradient of 10 mM phosphate buffer (pH 7.0) with acetonitrile, increasing from 30 to 70 % in 12 min (Marja et al. 2008). For cyanuric acid determination, the system was equipped with an an Inerstil column (5 µm, 150 × 4.6 mm) and a UV detector (280 nm). An isocratic mobile phase (octan-sodium sulfonate 5 mmol L^{-1} in 0.05 % H_3PO_4, pH 2.8) was fed at a flow rate of 1.0 mL min^{-1} (Galíndez-Nájera et al. 2009).

Chemical oxygen demand (COD)

A closed reflux method 8000 (Hach 2012) was used for COD determination. The reactive kit used could determine COD levels from 0.7 to 40 mg L^{-1}.

Results and discussion

Prometryn biodegradation in batch culture

Figure 2 shows the removal kinetics of prometryn in submerged batch cultures using free cells or cells attached to fragments of the porous support immersed in the culture medium. The graph shows a fast degradation of pure prometryn, which disappears in 9.0 h. The possible effect of the adjuvants of the herbicide formulation on prometryn degradation can be observed in the same graph. Although the culture

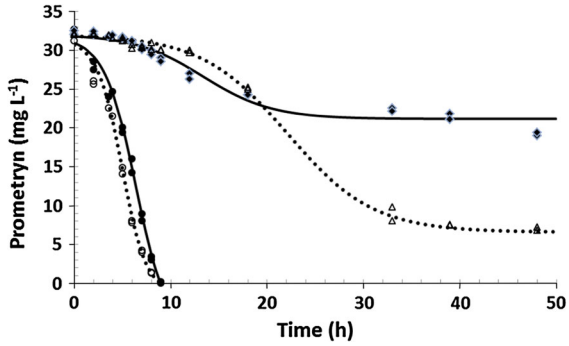

Fig. 2 Effect of adjuvants on prometryn removal in a submerged batch culture using free or immobilized cells. Removal of pure prometryn by free (*filled circles*) or immobilized cells (*void circles*). Prometryn removal in the Gesagard formulation containing adjuvants by free (*filled diamonds*) or immobilized cells (*void triangles*). Curves described by the logistic Eq. 1, using the parameters shown in Table 1. Immobilized cells (*dotted lines*). Free cells (*solid lines*)

was extended to more than 100 h, prometryn was not entirely removed. On the other hand, differences in the viable cell count along the batch cultures using Gesagard or pure prometryn are shown in Table 1. Notwithstanding that the initial cell count was greater when Gesagard was used as substrate, a clear delay in prometryn degradation took place.

After fitting the batch experimental data to the empirical logistic decay model (1), and then, by differentiation of the mathematical function, the behavior of the volumetric removal rates of prometryn $R_{V,P}$ can be estimated.

$$p(t) = p_i - \frac{a}{z(1 + e^{b-ct^m})} \quad (1)$$

In this case, the model (1) is viewed as a purely empirical function, meaning that the equation parameters a, b, c, m, and z shown in Table 2, lack of biological or physical implications. Under these circumstances, the model was used only as a differentiable intermediate function (Marrón-Montiel et al. 2006; Arino et al. 2006). In this model, p_i is the initial prometryn concentration, and $p(t)$ is the prometryn concentration at the time t.

This equation was differentiated to obtain the transient behavior of the volumetric removal rate $R_{V,P}(t)$ of prometryn along the distinct batch cultures (Eq. 2)

$$p'(t) = \frac{\partial p}{\partial t} = R_{V,P}(t) = -\frac{(ace^{b-ct^m})mt^{m-1}}{z(1 + e^{b-ct^m})^2} \quad (2)$$

Figure 3 shows the curves described by Eq. (2). In this figure, differences in the degradation kinetics of prometryn by free or attached cells can be observed. When the bacterial consortium grew on the MSP medium, the prometryn removal rates reached maximal values of 5.8 and 6.3 mg L^{-1} h^{-1}, for free and attached cells, respectively. When the consortium was

Table 1 Viable cell counting in batch cultures of free cells growing on pure prometryn or in Gesagard, herbicide containing prometryn plus adjuvants

t (h)	Prometryn CFU ($\times 10^6$) mL^{-1}	Gesagard CFU ($\times 10^6$) mL^{-1}
0	0.84 ± 0.3	10.2 ± 3.1
2	0.94 ± 0.2	–
3	1.1 ± 0.2	–
5	1.24 ± 0.2	–
7	2.16 ± 0.4	–
9	8.7 ± 2.0	18.3 ± 2.4
16	–	38.1 ± 4.2
29	–	49.8 ± 4.8
36	–	59.3 ± 6.1
45	–	93.0 ± 7.2

In both cases, the initial prometryn concentration was 32 mg L^{-1}

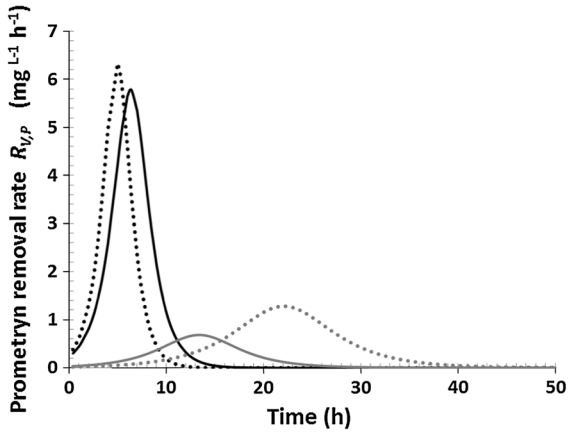

Fig. 3 Effect of adjuvants on the instantaneous removal rates of prometryn ($R_{V,P}$) in submerged batch culture using free or immobilized cells. Curves described by Eq. 2, using the parameters shown in Table 1. Change in $R_{V,P}$ of pure prometryn by free (*black solid line*) or immobilized cells (*black dotted line*). Change in $R_{V,P}$ of prometryn in the Gesagard formulation containing adjuvants by free (*gray solid line*) or immobilized cells (*gray dotted line*)

Table 2 Values of the parameters of Eqs. 1 and 2, and coefficients of determination R^2 for the data fitted to Eq. 2

Batch culture	a	b	c	m	z	R^2
Prometryn (IC)	5.73 ± 0.424	3.54 ± 0.240	0.467 ± 0.050	1.1	0.15 ± 0.01	0.998
Prometryn (FC)	4.96 ± 0.242	3.17 ± 0.400	0.474 ± 0.069	1.2	0.15 ± 0.01	0.996
Gesagard (IC)	37.02 ± 0.016	3.96 ± 0.640	0.336 ± 0.066	0.95 ± 0.002	3.41 ± 0.18	0.999
Gesagard (FC)	5.97 ± 0.01	4.94 ± 0.323	0.224 ± 0.19	1.5 ± 0.01	0.235 ± 0.01	0.999

IC Immobilized cells, *FC* free cells

grown in the herbicide Gesagard, which contains prometryn plus adjuvants, the kinetic behavior of the consortium changed. In addition to the delay in prometryn degradation, a noteworthy decay in the top removal rates of free and attached cells, 0.78 and 1.22 mg L^{-1} h^{-1}, respectively, was observed.

Table 3 shows the final removal efficiencies of prometryn (η_P) determined by HPLC, COD (η_{COD}), and the catabolic intermediary, cyanuric acid (η_{OOOT}). These values were obtained in batch cultures of free or immobilized cells when the microbial consortium was grown on prometryn pure ($t_f = 9.0$ h), or in the commercial formulation of the herbicide Gesagard, containing prometryn and adjuvants ($t_f = 104$ h). Notwithstanding the larger time used for prometryn degradation contained in the Gesagard medium, the removal efficiencies of cyanuric acid were lower than those obtained when prometryn pure was totally degraded in a shorter time. Possibly, adjuvants were preferentially used as carbon sources; and mainly prometryn, not the refractory cyanuric acid, was used as nitrogen source for growth of the microbial consortium. In some cases, adjuvants could serve as co-substrates, facilitating the cometabolic degradation of recalcitrant compounds; also, non-ionic surfactants could augment the bioavailability of hydrophobic compounds increasing their biodegradation rates, and removal efficiencies. However, as occurred in this case, adjuvants negatively affect the biodegradation of recalcitrant compounds (Krogh et al. 2003; Ma et al. 2004; Ostroumov 2006).

Biodegradation of prometryn in the continuously operated biofilm reactor

When the concentration of presumptively inhibitory adjuvants was relatively high, at the beginning of the batch cultures, a delay in the degradation of prometryn was observed. In continuous regime, the substrate concentration, in steady-state operation, is certainly smaller than the concentration in the inflowing medium; therefore, it was considered that the operation of the packed bed reactor in continuous regime should lessen the negative effect that the adjuvants present in the medium MSG have on prometryn removal rates end efficiencies.

Table 3 Final removal efficiencies of prometryn (η_P), determined by HPLC, COD (η_{COD}) and the cyanuric acid (η_{OOOT}) in batch cultures of immobilized (IC) and free cells (FC)

Batch culture	η_P (%)	η_{COD} (%)	η_{OOOT}
Prometryn (IC)	100.0	100.0	44.6
Prometryn (FC)	96.6	100.0	42.3
Gesagard (IC)	88.3	94	38.45
Gesagard (FC)	88	91	28.72

The microbial consortium was grown on prometryn pure or in the commercial formulation of the herbicide Gesagard, containing prometryn and adjuvants

When the packed-bed biofilm reactor was continuously fed with MSG medium, eight flow rates were probed. Table 4 shows the changes in the operational characteristics of the continuously operated biofilm reactor along 6,000 h of operation.

The initial flow rate used was 0.06 L h^{-1}, corresponding to a hydraulic retention time $HRT = V_L/F = 9.50$ h and volumetric loading rates (B_V) of prometryn, COD and OOOT of 3.37, 14.79 and 1.80 g m^{-3} h^{-1}, respectively. This flow rate was maintained until no appreciable change in concentrations was observed, meaning that a steady state was reached in the bioreactor. Then, different *HRT* values were probed. In Fig. 4 the chronological variations in the input and output concentrations of prometryn, COD and detached cells are shown for the different loading rates and HRTs used. The biofilm dynamics is relatively complex; it includes biomass attachment, growth, decay, lysis and detachment. Even when biofilm is weakened by lysis and biomass detachment, which is often caused by combined fluid shear and pressure forces acting on the biofilm, the biofilm growth remains balanced (Bottero et al. 2013).

The major variations in prometryn and COD were observed at the start-up and the end of the degradation process. At the highest loading rates, a decrease in the detached cells concentration was observed, possibly caused by the interstitial dilution rate in the packed bed.

Although at all the $B_{V,P}$ values tested the prometryn was totally removed from the liquid medium, part of the herbicide was biotransformed to cyanuric acid (OOOT). At low prometryn loading rates and high HRT values, about 44 % of the heterocyclic ring was degraded; this fraction gradually diminished to less than 10 % when $B_{V,P}$ values were increased, and HRT values were lowered. Evidently, an increase in the herbicide loading rate brings an increase in the supply of inhibitory adjuvants. This fact could help to explain the increase in OOOT accumulation and the lowering in COD removal at high loading rates in the PBR.

Table 4 Operational characteristics of the biofilm reactor, and removal rates of prometryn, COD and cyanuric acid obtained along 6,000 h of continuous operation at eight different hydraulic retention times (HRT)

HRT (h)	Prometryn		Chemical oxygen demand		Cyanuric acid	
	$B_{V,P}$ (mg L^{-1} h^{-1})	$R_{V,P}$ (mg L^{-1} h^{-1})	$B_{V,COD}$ (mg L^{-1} h^{-1})	$R_{V,COD}$ (mg L^{-1} h^{-1})	$B_{V,OOOT}$ (mg L^{-1} h^{-1})	$R_{V,OOOT}$ (mg L^{-1} h^{-1})
9.50	3.37 ± 0.08	3.37 ± 0.08	14.79 ± 0.60	14.79 ± 0.35	1.80 ± 0.04	0.79 ± 0.02
8.77	3.65 ± 0.09	3.65 ± 0.09	16.02 ± 0.66	16.02 ± 0.38	1.95 ± 0.05	0.86 ± 0.02
7.60	4.21 ± 0.10	4.21 ± 0.10	18.49 ± 0.76	18.49 ± 0.44	2.25 ± 0.05	0.87 ± 0.02
6.00	5.33 ± 0.13	5.33 ± 0.13	23.42 ± 0.96	23.42 ± 0.56	2.85 ± 0.0.07	1.10 ± 0.02
4.29	7.47 ± 0.18	7.47 ± 0.18	32.78 ± 1.34	31.07 ± 0.75	3.99 ± 0.10	1.36 ± 0.03
3.31	9.66 ± 0.23	9.66 ± 0.23	42.40 ± 1.73	38.17 ± 0.92	5.17 ± 0.12	1.54 ± 0.04
2.11	15.16 ± 0.36	15.16 ± 0.36	66.55 ± 2.72	59.92 ± 1.44	8.11 ± 0.19	1.10 ± 0.03
1.58	20.21 ± 0.49	20.21 ± 0.49	88.74 ± 3.63	76.11 ± 1.83	10.81 ± 0.26	0.89 ± 0.02

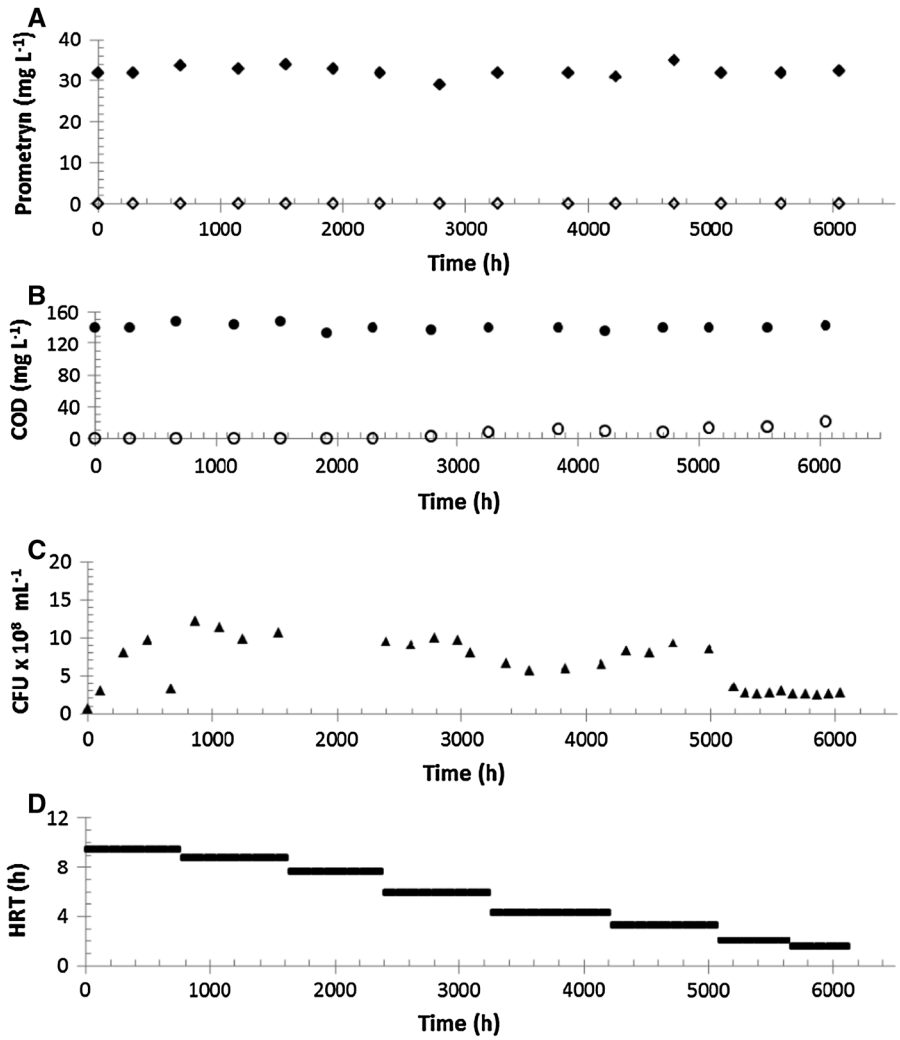

Fig. 4 Influent and effluent concentrations of prometryn (**a**), COD (**b**) and detached cells (**c**) along the operational history of the biofilm reactor at different hydraulic retention times (**d**)

Although the PBR was continuously fed with the herbicide Gesagard, containing inhibitory adjuvants, the prometryn removal rates obtained were significantly higher than those reached in batch culture, even when the microbial consortium was batch cultivated in pure prometryn (Fig. 3)

To compare the behavior of the volumetric removal rates of prometryn, COD and OOOT ($R_{V,P}$, $R_{V,COD}$ and $R_{V,OOOT}$), at the different operational conditions, the corresponding volumetric removal rates were expressed in relative terms. The relative volumetric removal rates ($R_{VR} = R_V/R_{VMAX}$) were plotted as a function of the relative loading rates ($B_{VR} = B_V/B_{VMAX}$) (Fig. 5, upper graph). The highest loading rates probed were 20.2, 88.7 and 10.8 g m^{-3} h^{-1} for prometryn, COD and OOOT, respectively. The removal efficiencies of prometryn, COD, and the catabolic intermediary OOOT obtained in eight runs, are also shown in Fig. 5, lower graph. It is clearly appreciated that at all the HRTs probed prometryn was completely removed, though, at the lowest $B_{V,P}$ values, about 56 % of the prometryn was biotransformed to cyanuric acid. This fraction is increased to more than 90 % at the highest loading rates probed. The behavior of the COD removal rate observed in Fig. 5 denotes that besides prometryn, other compounds able to be chemically oxidized, presumptively adjuvants, were efficiently removed. In contrast, as a result of the recalcitrant nature of the triazinic ring species (Watanabe et al. 2005), the cyanuric acid that was not removed could not be determined by the COD technique.

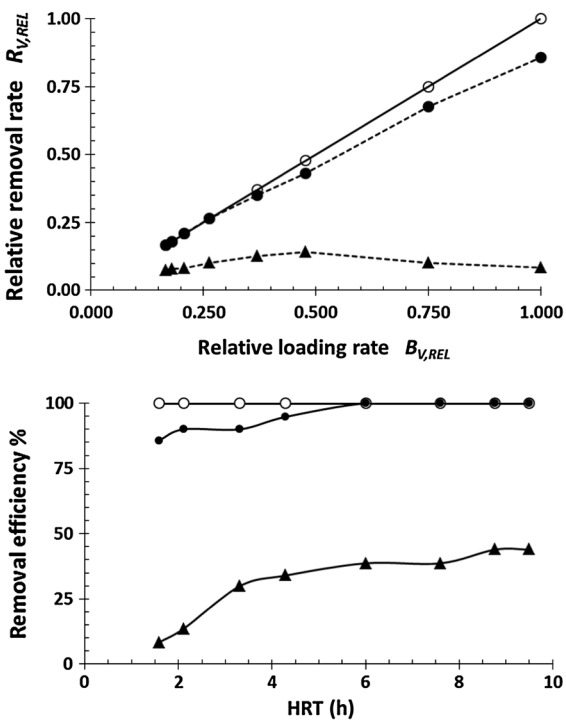

Fig. 5 Relative removal rates (*upper graph*), and removal efficiencies (*lower graph*) of prometryn (*void circles*), COD (*filled circles*) and cyanuric acid (*filled triangles*) in the biofilm reactor operating at increasing loading ($B_{V,REL}$) and hydraulic retention times (HRT)

Few processes regarding prometryn biodegradation can be found in the literature. Some of them were carried out in batch culture using free cells of *Streptomyces* sp. (Shelton et al. 1996), *Arthrobacter aurescens* (Strong et al. 2002), *Rhodococcus* sp. (Fujii et al. 2007) and *Nocardioides* sp. (Satsuma 2010). Generally, the best form to evaluate the microbial capacity to degrade a compound is through its specific removal rate ($R_{V,X} = R_{V,P}/X$), which is the decay rate expressed per unit of cell mass (X). Unfortunately, this rate could not be estimated with the kinetic data reported for prometryn degradation. Nevertheless, some works report removal efficiencies (η_P) or volumetric removal rates of prometryn ($R_{V,P}$), that can be compared with the present work. The highest prometryn removal rate reported, for a strain of *Streptomyces* sp., was 3.3 mg L^{-1} h^{-1}, with a η_P value of 100 % (Shelton et al. 1996). In the present work, the maximum $R_{V,P}$ value obtained in the continuously operated biofilm reactor containing an acclimated microbial consortium was 20.2 mg L^{-1} h^{-1}, with a η_P value of 100 %. On this basis, the use of a microbial community immobilized in a biofilm reactor operated in continuous regime offer better results than batch cultures of pure microbial strains.

Identification of the bacterial isolates

Four cultivable bacterial strains were isolated from the biofilm formed in the support material at the end of acclimatization in batch culture. They were identified by sequencing and comparison with known 16S rDNA sequences at the NCBI GenBank. The identified bacteria were *Enterobacter* sp. (FJ472852.1, 98 % similarity), *Acinetobacter* sp. (HM246137.1, 98 % similarity), *Microbacterium* sp. (HM234007.1, 97 % similarity), and an uncultured *Flavobacteriaceae* bacterium (JQ328000 0.1, 96 % similarity).

Based on the results shown in Fig. 6, it was verified that the isolates KF619443, KF619444, KF619445

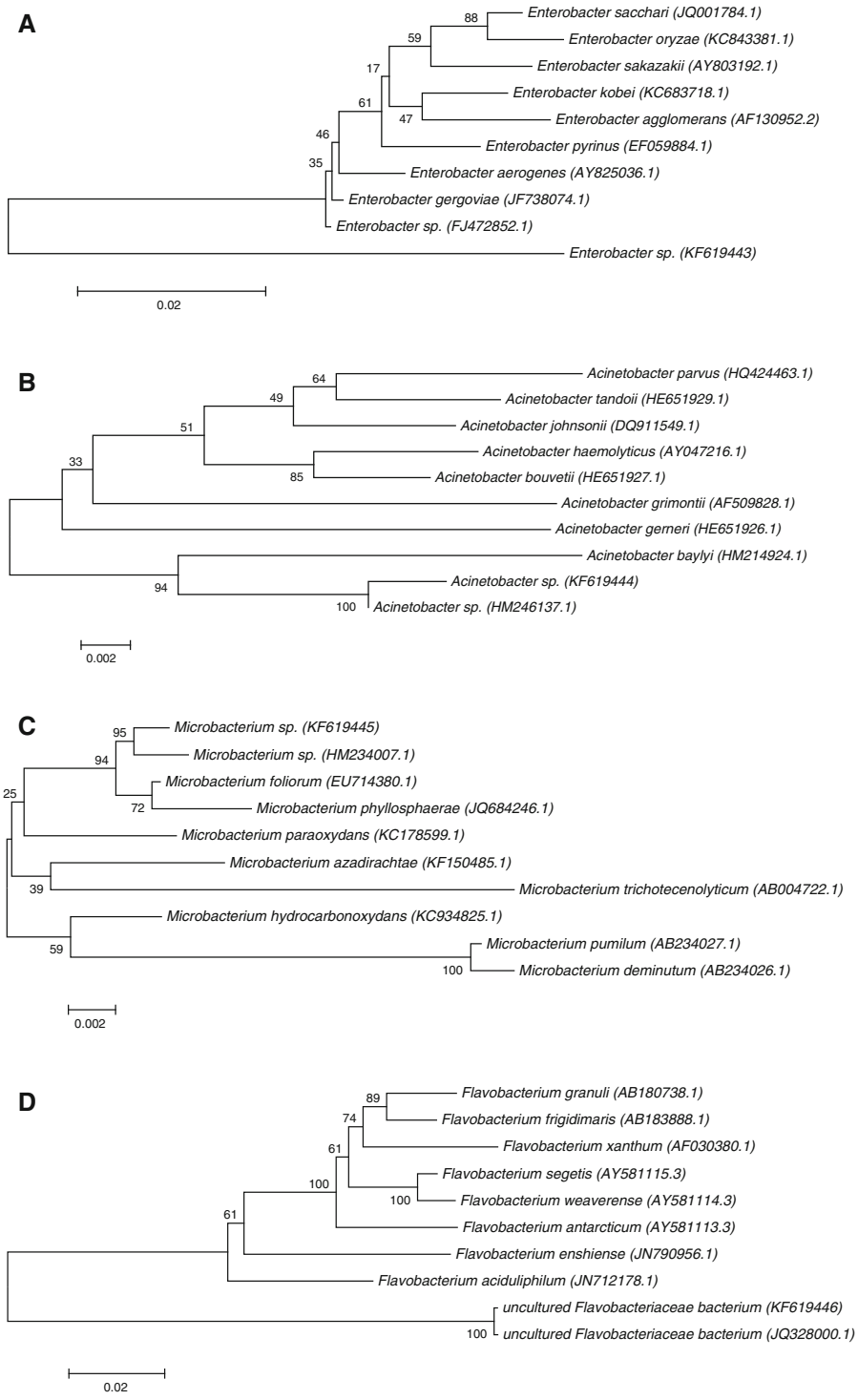

Fig. 6 Phylogenetic trees of *Enterobacter* sp. KF619443 (**a**), *Acinetobacter* sp. KF619444 (**b**), *Microbacterium* sp. KF619445 (**c**) and *Flavobacterium* sp. KF619446 (**d**)

and KF619446 belong phylogenetically to the genera *Enterobacter, Acinetobacter, Microbacterium* and *Flavobacterium*. Since bacterial strains presenting 16S rDNA gene similarities between 97 and 99.5 % may belong to different species (Ramani et al. 2012), definitive identification of these isolates may require

additional biochemical tests, or sequencing of additional gene loci.

Growth on prometryn of the bacterial isolates

The ability of the individual bacterial strains to grow on MSG medium, containing the herbicide Gesagard, on MSP medium, containing pure prometryn, and MSC medium, containing the metabolic intermediary cyanuric acid was evaluated.

Enterobacter and *Acinetobacter* had the ability to use all substrates for growth. *Microbacterium* grew well on MSG and MSP media, but not in the medium containing cyanuric acid, and the bacterium of the *Flavobacteriaceae* family could not grow in any of the tested substrates. However, it is known that microorganisms belonging to the family *Flavobacteriaceae* are able to form biofilms (Basson et al. 2008; Jacobs and Chenia 2011); thus, a possible role of this bacterium in the microbial consortium is biofilm formation.

Several xenobiotic compounds are degraded by members of the genera *Microbacterium* (Wang et al. 2009; Plotnikova et al. 2006), *Enterobacter* (French et al. 1998; Kryuchkova et al. 2013), and *Acinetobacter* (Lee et al. 2010; Singh et al. 2004; Sánchez-Sánchez et al. 2013), but, to our best knowledge, no strains of these genera have been reported as able to degrade prometryn.

Conclusions

In batch cultures, a considerable delay in the degradation of prometryn, presumptively caused by the elevated concentration of inhibitory adjuvants, was estimated by fitting raw experimental data to sigmoidal decay models, and differentiating them. This procedure could be a practical tool to analyze the kinetics of transient biodegradation processes.

In comparison with batch cultures, the substrate concentration, in steady-state continuous culture, is certainly smaller than the concentration in the inflowing medium; therefore, when the packed bed reactor was operated in continuous regime the negative effect of the adjuvants on the rates and removal efficiencies of prometryn was not observed.

Finally, by comparison of the prometryn removal rates reported in the literature, it can be concluded that the use of microbial communities immobilized in a biofilm reactor operated in continuous regime offer better results than batch cultures of pure microbial strains.

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