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Biodegradation of Endocrine Disruptors in Solid-Liquid Two-Phase Partitioning Systems by Enrichment Cultures

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Naturally occurring and synthetic estrogens and other molecules from industrial sources strongly contribute to the endocrine disruption of urban wastewater. Because of the presence of these molecules in low but effective concentrations in wastewaters, these endocrine disruptors (EDs) are only partially removed after most wastewater treatments, reflecting the presence of these molecules in rivers in urban areas. The development of a two-phase partitioning bioreactor (TPPB) might be an effective strategy for the removal of EDs from wastewater plant effluents. Here, we describe the establishment of three ED-degrading microbial enrichment cultures adapted to a solid-liquid two-phase partitioning system using Hytrel as the immiscible water phase and loaded with estrone, estradiol, estriol, ethynylestradiol, nonylphenol, and bisphenol A. All molecules except ethynylestradiol were degraded in the enrichment cultures. The bacterial composition of the three enrichment cultures was determined using 16S rRNA gene sequencing and showed sequences affiliated with bacteria associated with the degradation of these compounds, such as *Sphingomonadales*. One *Rhodococcus* isolate capable of degrading estrone, estradiol, and estriol was isolated from one enrichment culture. These results highlight the great potential for the development of TPPB for the degradation of highly diluted EDs in water effluents.

The release of endocrine disruptors (EDs) in the environment has emerged as a major concern over the past decade. These molecules induce the "feminization" of male tissues and/or the presence of female reproductive organs in males, along with other dysfunctions of the reproductive systems of many aquatic animals (1, 2). Since the 1940s, an increasing incidence of testicular cancer has been observed (3–5), suggesting that EDs might also induce the apparent decline in semen quality.

Numerous studies indicate that naturally occurring (estrone [E1], estradiol [E2], estriol [E3]) and synthetic (ethynylestradiol [EE2], used as a contraceptive) estrogens strongly contribute to the endocrine disruption of urban wastewater (6, 7). These molecules affect the hormonal systems of fish, even at low concentrations (<1 ng/liter). These estrogens have been detected at 1 to 500 ng/liter in influents and effluents of municipal wastewater treatment plants (7). Other molecules from industrial sources, such as nonylphenol (NP; a degradation product of a surfactant) and bisphenol A (BPA; a precursor of polycarbonate plastics), display estrogenic activity or other undesirable biological activities. For instance, BPA can act as a receptor antagonist of the thyroid hormone (8), causing developmental problems. The estrogenic potential of many of these compounds is often several thousand times lower than that of natural estrogens (9). However, these compounds, such as NP and BPA, are encountered at much higher concentrations (on the order of μg/liter or μg/kg) in water and sediments, and their presence depends on the intensity of the industrial activities surrounding these environments (9).

These estrogens are only partially removed after most wastewater treatments (7, 10–13), reflecting the presence of these compounds in rivers in urban areas. A survey of 18 Canadian wastewater treatment plants showed that only three treatments completely removed the overall estrogenicity of the incoming effluent (12). Despite their biodegradability, the persistence of estrogens through wastewater treatments is likely a consequence of low concentrations. Thus, the development of cost-effective alternatives for the removal of EDs from wastewater plant effluents is needed.

One alternative is the development of a two-phase partitioning bioreactor (TPPB). In this system, a water-immiscible phase extracts and concentrates compounds, such as EDs, which are highly diluted in water. After concentration in the water-immiscible phase, these substances gradually diffuse to the aqueous phase, in which degrading microorganisms metabolize these molecules at various rates (14–16). Solid polymers, such as Hytrel, polystyrene butadiene, and poly(ethylene-covinyl acetate), have been used efficiently as immiscible phase in solid-liquid TPPBs for the biodegradation of aromatic compounds, such as mono- and polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and phenolic compounds (16–24), for the transformation of carveol to carvone (25), and for the production of microbial metabolites (26–28).

Recently, we assessed the use of Hytrel 8206 as an immiscible phase in the development of a TPPB for the most common EDs identified at wastewater treatment plants: E1, E2, E3, EE2, BPA, and NP (29). The results showed that Hytrel has high affinity for these EDs, with coefficients of partition for Hytrel/water ranging from 2.21 to 5.10 at the log scale. Hytrel also showed an increased capacity to absorb these EDs at several hundred micrograms per gram. Because EDs are present at low concentrations in wastewa-

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ter, a possible strategy would be to concentrate these substances on Hytrel in a contact vessel and transfer the ED-charged Hytrel to a bioreactor in which an adapted biomass would metabolize these pollutants.

Pure bacterial cultures have been used in solid-liquid TPPBs for the degradation of phenolic compounds, PCBs, or benzene (17, 21, 23, 30, 31). However, microbial consortia (also referred to as enrichment cultures or mixed cultures) acclimated to the degradation of a specific pollutant(s) can achieve superior performance and a more complete biodegradation without accumulation of potentially toxic or inhibitory intermediates. Furthermore, consortia can more readily adapt to environment changes or variations in bioprocess operating conditions (32–34). Microbial consortia were used for the degradation of PAHs, phenolic compounds, and benzene, toluene, ethylbenzene, and xylene (BTEX) in solid-liquid TPPBs (19, 20, 22, 24, 33, 35–39). However, the biodegradation adaptations of these consortia were performed in the absence of the immiscible phase. This phase may exert an important influence on the adaptation of the microbial community through selection of microorganisms with properties more suited to the context of solid-liquid TPPBs, such as optimal adherence to the solid phase and biofilm formation. Such a consortium acclimated to the solid phase should be more suitable for the development of solid-liquid TPPBs.

Here, we describe three microbial enrichment cultures acclimated to the degradation of E1, E2, E3, EE2, BPA, and NP. Acclimation was performed in a solid-liquid TPP system in the presence of ED-loaded Hytrel as the immiscible solid phase. The microbial growth and the ED-degrading performance were measured, and the composition of the bacterial populations was determined by pyrosequencing and libraries of the 16S rRNA genes. We also attempted to isolate ED-metabolizing bacterial strains from one of the enrichment cultures.

MATERIALS AND METHODS

Chemicals. Estrone (E1), 17β-estradiol (E2), estriol (E3), 17α-ethynylestradiol (EE2), bisphenol A (BPA), and 4-*n*-nonylphenol (NP) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Deuterium-labeled analogs of these substances, purchased from CDN Isotopes (Pointe-Claire, QC, Canada), were used as internal standards. Concentrated solutions of each ED, including the deuterated standards, were prepared in high-performance liquid chromatography (HPLC)-grade methanol (Fisher Scientific Co., Ottawa, ON, Canada). These solutions were stored in the dark at −20°C in glass vials with a Teflon stopper. Hytrel 8206, a copolymer of poly(butylene terephthalate) and butylene ether glycol terephthalate, was kindly provided by the DuPont Co. (Wilmington, DE). Hytrel was obtained in the form of rice-shaped granules (5 by 3 by 1.5 mm) weighing ca. 20 mg.

Gas chromatography-mass spectrometry analysis. The ED concentrations were determined using gas chromatography-mass spectrometry (GC-MS) as previously described (29). Briefly, the EDs extracted from Hytrel were first derivatized using silylation. The ED-trimethylsilyl derivatives were injected (1 μl) into a Thermo Scientific Trace GC Ultra gas chromatograph interfaced with a PolarisQ MS. The mass spectrometer was operated in positive electron impact ionization and full-scan modes with a scan range of *m/z* 100 to 600 (see Ouellette et al. [29] for the theoretical molecular ions of the various silylated EDs and the ions monitored for quantification).

Methanol concentrations were measured using a gas chromatograph-flame ionization detector (GC-FID; Hewlett Packard 5890) equipped with a ZB-WAX$_{PLUS}$ polyethylene glycol column (30 m by 0.32 mm by 0.25 μm). The oven temperature was programmed at 40°C for 5 min, rising from 40°C to 150°C at 5°C/min, and kept isothermal at 150°C for 5 min. The temperature was then raised to 220°C at 20°C/min and kept for 2 min. Helium was the carrier gas (linear velocity, 3 ml/min). The injection was carried out at 150°C with a 10:1 split ratio. Isopropanol was used as a standard.

TABLE 1 Description of the TPP enrichment cultures[a]

Enrichment culture[b]	EDs present[c]	Biomass inoculated	YE
AHyYE	E1, E2, E3, EE2	Yes	Yes
AHy	E1, E2, E3, EE2	Yes	No
BHyYE	BPA, NP	Yes	Yes
BHy	BPA, NP	Yes	No
DHyYE	E1, E2, E3, EE2, BPA, NP	Yes	Yes
DHy	E1, E2, E3, EE2, BPA, NP	Yes	No
MHyYE	None	Yes	Yes
MHy	None	Yes	No
Hy	None	No	No
CtAHy	E1, E2, E3, EE2	No	No
CtBHy	BPA, NP	No	No
CtDHy	E1, E2, E3, EE2, BPA, NP	No	No

[a] Enrichment cultures with yeast extract (YE) were carried out from the first to the 15th transfer. The enrichment cultures without the addition of YE in the culture medium were carried out after the 13th transfer.
[b] "Ct" at the start of a name indicates an abiotic control. These controls were carried out at the 20th and 23rd transfers.
[c] E1, estrone; E2, 17β-estradiol; E3, estradiol; EE2, 17α-ethynylestradiol; BPA, bisphenol A; NP, 4-*n*-nonylphenol.

Mineral medium. For each liter, the mineral medium (40) comprising 9 g $Na_2HPO_4 \cdot 12H_2O$, 1.5 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 1 g NH_4Cl, 0.005 g ferric ammonium citrate, 0.01 g $CaCl_2 \cdot 2H_2O$, and 3 ml of trace solution and supplemented with 1 ml of Wolfe's vitamin solution (10 mg/liter pyridoxine HCl, 5.0 mg/liter thiamine-HCl, 5.0 mg/liter riboflavin, 5.0 mg/liter nicotinic acid, 5.0 mg/liter Ca-pantothenate, 5.0 mg/liter *p*-aminobenzoic acid, 5.0 mg/liter thioctic acid, 2.0 mg/liter biotin, 2.0 mg/liter folic acid, and 0.1 mg/liter vitamin B_{12}, pH 7.5; filter sterilized) and 25 mg yeast extract (BBL, Becton, Dickinson and Company, Sparks, MD) was used, except where indicated. The trace solution comprised (per liter) 10 mg $ZnSO_4 \cdot 7H_2O$, 3 mg $MnCl_2 \cdot 4H_2O$, 30 mg H_3BO_3, 20 mg $CoCl_2 \cdot 6H_2O$, 0.79 mg $CuCl_2 \cdot 6H_2O$, 2 mg $NiCl_2 \cdot 6H_2O$, and 3 mg $Na_2MoO_4 \cdot 2H_2O$. The medium was autoclaved, and a filter-sterilized $NaHCO_3$ solution was added at a 0.5-g/liter final concentration.

Preparation of the ED-charged Hytrel. Seven grams of Hytrel was mixed with 10 ml methanol containing 200 μg/ml of each ED, corresponding to the AHy, BHy, and DHy enrichment cultures (Table 1) and their respective controls (CtAHy, CtBHy, and CtDHy), for 2 days at room temperature and 150 rpm. The methanol was discarded, and the Hytrel granules were washed once with water and dried at 70°C for 16 h.

Residual methanol concentration in Hytrel. Methanol (10 ml) with no EDs was mixed with Hytrel (7 g) and then incubated and dried as described previously. The residual methanol in Hytrel was extracted with water, and the concentration was measured by GC-FID.

Enrichment cultures. The original microbial inoculum consisted of a mixture, in equal proportions, of three different sources of biomass: (i) the activated sludge from a municipal wastewater treatment plant (Vaudreuil, QC, Canada), (ii) the activated sludge from a laboratory-scale bioreactor used to treat swine manure (41), and (iii) the biomass from a laboratory-scale silicone oil/water TPPB containing a polycyclic aromatic hydrocarbon (PAH)-degrading microbial consortium (42). For each enrichment culture (Table 1), six 250-ml Erlenmeyer flasks, each containing 7 g of ED-charged Hytrel and 35 ml mineral medium, were prepared. These flasks were inoculated with 5 ml of the microbial inoculum. In the MHy enrichment cultures, 35 ml of mineral medium was added to 7 g of untreated Hytrel and inoculated with 5 ml of the microbial inoculum.

Abiotic controls (CtAHy, CtBHy, and CtDHy) comprised 40 ml mineral medium containing 7 g of ED-charged Hytrel (Table 1). No biomass was added.

Three flasks were immediately used to determine the ED concentrations in the Hytrel at time zero. The other three cultures were shaken in the dark at room temperature and 150 rpm for 4 weeks. For each corresponding triplicate, two five-milliliter samples of the aqueous phase were transferred into fresh TPP medium; one sample was used for time zero, and the other sample was cultured for 4 weeks. These enrichment cultures were cultured under the same conditions. Residual biomass was centrifuged and used for DNA extraction or measuring the protein concentration. The total residual ED concentrations in the Hytrel were measured. The TPP enrichment cultures were transferred into fresh medium every 4 weeks. At the 14th and 15th transfers, two sets of enrichment cultures were carried out in triplicate, one set with yeast extract and the second without yeast extract. After the 15th transfer, yeast extract was no longer included. The DNA extracted from the 19th transfer's biomass was used in denaturing gradient gel electrophoresis (DGGE), for deriving the 16S rRNA gene libraries, and for pyrosequencing.

Extraction of EDs from Hytrel. All the Hytrel contained in a flask was recovered from the TPP enrichment cultures and dried for 16 h at 70°C. The EDs absorbed in the Hytrel were extracted twice using 5 ml of methanol and shaking at 150 rpm in capped vials for 2 days in the dark at 25°C. The two extracts were combined, and the methanol was evaporated using a stream of nitrogen gas. The extracts were dissolved in 5 ml of methanol and filtered through a polytetrafluoroethylene 0.2-μm filter. One hundred microliters of the filtered extract was mixed with 5 μl of the corresponding deuterated standard (1,000 μg/ml master solution; 50-μg/ml final concentration). The methanol was evaporated, and the EDs were silylated in 100 μl and quantified using GC-MS as described above. The ED concentrations in the aqueous solutions were consistently less than 1% of the ED concentrations in Hytrel, as predicted by the coefficient of partition (29) (data not shown).

Growth measurements. The bacterial growth was measured using the Bradford protein assay according to the manufacturer's protocol (Bio-Rad, Mississauga, ON, Canada) with a 1-ml aliquot of the aqueous phase of the TPP enrichment cultures and bovine serum albumin as a standard.

DNA extraction. Plasmid DNA from 1.5 ml of *Escherichia coli* cultures (clones from 16S rRNA gene libraries) was extracted as previously described (43). For total DNA extraction of the enrichment cultures, the remaining aqueous phase (20 to 25 ml) was centrifuged and dispersed in 0.5 volumes of TEN (50 mmol/liter Tris-HCl, pH 8.0, 100 mmol/liter EDTA, and 150 mmol/liter NaCl). Subsequently, 250 mg of glass beads (0.25- to 0.50-μm diameter) was added, and the biomass was disrupted using a FastPrep FP120 homogenizer (MP Biomedicals, Santa Ana, CA) twice for 20 s (at a speed setting of 4.0) and stored on ice. The homogenate was centrifuged for 15 min at 13,000 × *g*. The supernatant was treated with 1 μl RNase (10 mg/ml) for 15 min at room temperature and extracted once with 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1). The DNA was precipitated using ammonium acetate (2-mol/liter final concentration) and 2 volumes of 100% ethanol and incubated for 15 min at −20°C. After centrifugation at 13,000 × *g* for 15 min, the DNA pellet was washed with 70% ethanol and dissolved in 100 μl of 0.22-μm-filtered, double-autoclaved, demineralized water.

Pyrosequencing. Total DNA samples from each enrichment culture were sequenced (Research and Testing Laboratory, Lubbock, TX). A region of the 16S rRNA genes was PCR amplified using 28F-519R primers (covering the V1-V2-V3 variable regions) and subjected to pyrosequencing using a Roche 454 FLX genome sequencer system. The reads were processed on the Ribosomal Database Project (RDP) website (release 10; http://rdp.cme.msu.edu/) (44). The tag and primer sequences were trimmed, and only sequences longer than 299 nucleotides (nt) (see Table S1 in the supplemental material) with a quality of at least 20 (out of 40) were kept. The sequences were aligned; sequences with ≤3% differences were clustered as unique operational taxonomic units (OTUs). The representative sequences were realigned at the RDP, and a phylogenetic tree was generated using FastTree version 2.2.4 software (45) with the generalized time-reversion model option. Principal component statistical analysis and P test significance were performed using Fast UniFrac (46), with parameters set for each pair of sequences and 500 permutations.

PCR-denaturing gradient gel electrophoresis and 16S rRNA gene libraries. The PCR protocols, the DGGE, and the construction of the 16S rRNA gene libraries were performed as described by Lafortune et al. (42). The DGGE profiles were analyzed for similarity using the GelComparII 6.0 software (Applied Maths, Sint-Martens-Latem, Belgium) for clustering. The 16S rRNA gene libraries were screened through the restriction digestion analysis of randomly selected colonies using the restriction enzyme AfaI (New England BioLabs, Whitby, ON, Canada). The clones were grouped according to their migration profiles. At least one representative clone from each group was sequenced.

All sequences were aligned against the core set of alignment templates at the Greengenes website (http://greengenes.lbl.gov/) using NAST (47). The aligned sequences were subsequently examined for chimeras using Bellerophon (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl) (48). Using *E. coli* (U00096) as a reference sequence, the sequences were examined for chimeras through Mallard (49) with a 99.9% cutoff. The putative chimeric sequences determined using the 2 methods were all compared against the sequences in the gene databases using BLASTN (National Center for Biotechnology Information) (50), and the most closely related sequences were selected and used for the Pintail analysis (51). Only sequences with positive scores from the Pintail analyses were considered chimeras.

Isolation of bacterial isolates. The AHy enrichment cultures were vortexed, and 100 μl of the aqueous phase was serially diluted (10^0 to 10^{-6}) in mineral medium. Ten microliters was spread onto four plates containing the following agar media: R2A agar (BD, Mississauga, ON, Canada), tryptic soy agar (TSA) (BD), and the mineral medium with 1 mg/liter of E1, E2, E3, and EE2 and 1.5% agar with (ESM-V) or without (ESM) the addition of vitamins. The agar plates were incubated for 5 days at room temperature. Representative colonies were picked and restreaked three times on the corresponding medium. The representative colonies were cultured overnight at 37°C and 150 rpm in tryptic soy broth (TSB) (DB) containing 1 mg/liter of E1, E2, E3, and EE2. These precultures were used to inoculate, in triplicate, 12 ml of TSB with 1 mg/liter of E1, E2, E3, and EE2 at an optical density at 600 nm (OD_{600}) of 0.01. These cultures were incubated at room temperature and 150 rpm in the dark for 4 days. A 10-μl sample of deuterated standards (final concentration, 1 mg/ml) was added to 10 ml of the culture samples, and the estrogens were extracted using 2.5 ml ethyl acetate. Subsequently, 100 μl of the estrogens was analyzed using GC-MS. The most-probable affiliations were based on partial 16S rRNA gene sequences. The 16S rRNA sequences of the EMS-1 and TSA-1 strains showed 100% identity with several *Rhodococcus* or *Bacillus* species and strains.

Nucleotide sequence accession numbers. The partial 16S rRNA gene sequences were deposited in GenBank under accession numbers KC502970 to KC502975.

RESULTS

Establishment of ED-degrading microbial enrichment cultures in a TPP system. Two of the three sources of biomass (the activated sludge from a municipal wastewater treatment plant and a bioreactor used to treat swine manure) used in the original inoculum were chosen based on the assumption that they contain ED-degrading microorganisms. Municipal wastewater treatment plants are known to reject EDs (6, 9), and the untreated swine manure contained significant amounts of EDs. The third source consisted of a microbial enrichment culture derived from a PAH-degrading TPPB (42). The choice of this culture was based on the premise that the presence of microorganisms adapted in a TPPB

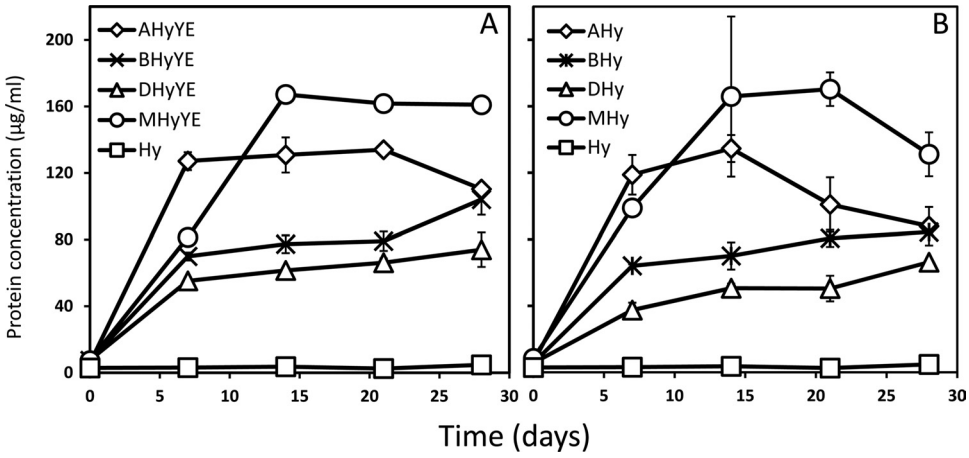

FIG 1 Impact of yeast extract (YE) on the microbial growth of the enrichment cultures. At the 15th transfer, the enrichment cultures were added into fresh medium containing yeast extract (A) or not (B). One milliliter of each flask was collected, and the protein concentration was determined. See Table 1 for nomenclature and composition. All enrichment cultures were performed in triplicate.

environment (e.g., adherence to the immiscible phase) and involved in the degradation of aromatic molecules may favor the establishment of the ED-degrading enrichment cultures in the TPP system.

Three enrichment cultures were performed (Table 1) in a TPP system containing Hytrel granules loaded with EDs. Because the EDs studied were of two different types, estrogens (E1, E2, E3, and EE2) and phenolic compounds (BPA and NP), one enrichment culture was established for each type of ED: the AHy enrichment cultures with E1, E2, E3, and EE2 and the BHy enrichment cultures with BPA and NP. The third enrichment culture, DHy, contained the six EDs (Table 1). This strategy allowed assessment of the influence of the respective types of EDs on the enriched microbial populations. Every 4 weeks, 5 ml of each culture was transferred into fresh medium containing new Hytrel granules. Yeast extract was initially added as a small source of carbon to support growth until the 15th transfer. The fourth enrichment cultures, MHy, were established with Hytrel containing no EDs (Table 1). This control was performed initially to determine the impact of the yeast extract on the microbial populations.

Microbial growth was used first as an indication of ED degradation since we expected that Hytrel would not be a carbon source and that yeast extract would be consumed rapidly in the first days. Significant growth was observed in the AHy, BHy, and DHy enrichment cultures, for which a plateau was reached during the first week (Fig. 1A), suggesting that ED degradation occurred. Unexpectedly, growth also occurred in the MHy enrichment cultures, achieving a 20 to 50% higher biomass than that in the ED-charged TPP enrichment cultures (Fig. 1A). To assess the influence of yeast extract, two sets of cultures were performed at the 14th and 15th transfers: one with the addition of yeast extract and the second without it. The absence of yeast extract in the medium did not affect the growth of the respective enrichment cultures (Fig. 1B). These results suggest that Hytrel was used as a carbon source.

Degradation of the EDs by the enrichment cultures. Each enrichment culture was performed in six flasks. To determine the capacity of ED degradation in these cultures, three flasks were collected on day 0 immediately after the transfer and the other three flasks were collected 4 weeks later. The 4-week interval was set arbitrarily, with the objective to give sufficient time to substantially degrade EE2, the most recalcitrant molecule. More than 90% of E1, E2, and E3 was degraded in 4 weeks from the 13th transfer, the first time we measured ED degradation by GC-MS, and from the 14th transfer with BPA (Table 2). More fluctuations in the percentage of NP degradation were observed in the BHy and DHy enrichment cultures (Table 2). Unfortunately, none of the enrichment cultures were able to significantly degrade EE2.

Abiotic controls containing ED-charged Hytrel without inoculated biomass were also performed under the same conditions at the 20th transfer. In the AHy enrichment cultures, E1, E2, and E3 were more than 98% degraded whereas EE2 was barely degraded compared with the abiotic controls (Table 2). In the BHy enrichment cultures, BPA was totally degraded while approximately 5% NP remained. Still, the abiotic controls showed 57% and 90% losses of BPA and NP, respectively (Table 2). For the DHy enrichment cultures, more than 98% of E1, E2, E3, and BPA and 88% of NP were degraded (Table 2). No degradation of EE2 occurred in the DHy enrichment cultures. Also, no sign of growth was observed in the abiotic controls, showing that no contamination occurred.

We noticed that the ED concentrations in the abiotic controls were reduced between 34 and 90% after 4 weeks (Table 2). To further investigate this phenomenon, we measured the concentration of EDs after 1 week using the 23th transfer (Fig. 2). Except for BPA in the BHy enrichment cultures, a slight reduction of the ED concentrations occurred in the abiotic controls during this period. An increase in the E1 concentrations was observed in the AHy (75 nmol/g) and DHy (45 nmol/g) enrichment cultures. Thus, this increase likely reflected the oxidation of the alcohol function of E2 at the C-17 position into a ketone (52, 53). There was a >85% reduction in the E2 concentrations in the AHy and DHy enrichment cultures, corresponding to 126 nmol/g and 143 nmol/g, respectively. These concentration losses were higher than the corresponding increases in the E1 concentrations in the AHy and DHy enrichment cultures, suggesting the presence of E1- and/or E2-degrading microorganisms. E3 was 52% and 64% degraded in the AHy and DHy enrichment cultures, respectively, whereas no significant degradation of EE2 was observed in these two cultures.

TABLE 2 Percentage of ED degradation by the enrichment cultures

Enrichment culture and ED	% (SD) of ED degradation at transfer[a]:						
	13	14	15	18	19	20	23
AHyYE							
E1	99.1 (0.5)	99.8 (0.0)	99.6 (0.1)	ND	ND	ND	ND
E2	99.6 (0.1)	99.9 (0.1)	99.8 (0.1)	ND	ND	ND	ND
E3	91.6 (1.0)	97.1 (1.0)	90.5 (0.0)	ND	ND	ND	ND
EE2	0.0 (7.2)	0.8 (5.5)	7.9 (0.1)	ND	ND	ND	ND
AHy							
E1	ND	99.8 (0.0)	98.8 (0.0)	99.7 (0.1)	99.5 (0.0)	99.7 (0.0)	−67.3 (37.9)
E2	ND	99.9 (0.0)	98.8 (0.1)	99.8 (0.1)	99.8 (0.1)	100.0 (0.0)	88.2 (1.4)
E3	ND	97.1 (0.4)	94.3 (1.4)	97.2 (0.7)	97.7 (0.2)	97.9 (1.7)	50.8 (5.7)
EE2	ND	0.3 (13.3)	−1.2 (4.1)	0.9 (5.7)	12.1 (5.7)	50.1 (2.8)	17.9 (6.9)
BHyYE							
BPA	0.0 (4.2)	−2.5 (1.7)	2.3 (0.8)	ND	ND	ND	ND
NP	99.9 (0.1)	34.2 (3.2)	44.0 (1.9)	ND	ND	ND	ND
BHy							
BPA	ND	−3.0 (5.8)	−42.8 (4.3)	99.9 (0.1)	98.9 (0.1)	99.9 (0.0)	68.2 (2.3)
NP	ND	35.7 (1.8)	−3.7 (0.8)	80.5 (1.3)	50.2 (4.2)	95.5 (1.0)	31.5 (3.0)
DHyYE							
E1	99.8 (0.0)	99.8 (0.1)	99.8 (0.1)	ND	ND	ND	ND
E2	99.8 (0.0)	99.8 (0.1)	99.4 (0.1)	ND	ND	ND	ND
E3	95.1 (0.3)	95.9 (0.6)	96.2 (1.9)	ND	ND	ND	ND
EE2	15.3 (2.5)	−0.6 (3.8)	−7.1 (3.0)	ND	ND	ND	ND
BPA	99.8 (0.1)	99.7 (0.1)	99.8 (0.1)	ND	ND	ND	ND
NP	99.9 (0.0)	50.2 (1.8)	47.1 (2.4)	ND	ND	ND	ND
DHy							
E1	ND	99.6 (0.2)	99.8 (0.0)	99.7 (0.1)	98.4 (0.8)	99.8 (0.1)	−47.1 (9.4)
E2	ND	99.8 (0.1)	98.9 (0.0)	99.8 (0.2)	99.6 (0.1)	99.9 (0.0)	90.3 (1.0)
E3	ND	95.3 (0.7)	94.6 (0.4)	97.2 (0.8)	97.1 (0.2)	98.3 (0.2)	61.1 (13.4)
EE2	ND	1.2 (1.3)	−9.1 (3.5)	−10.8 (9.7)	14.9 (14.7)	54.2 (2.8)	8.3 (6.6)
BPA	ND	99.6 (0.1)	99.8 (0.1)	99.7 (0.1)	98.5 (1.2)	99.8 (0.2)	42.9 (5.2)
NP	ND	39.5 (6.2)	45.7 (0.7)	66.4 (3.7)	54.2 (18.6)	88.2 (1.8)	34.1 (3.9)
Abiotic control CtAHy							
E1	ND	ND	ND	ND	ND	48.6 (1.8)	4.8 (13.8)
E2	ND	ND	ND	ND	ND	38.7 (1.7)	9.1 (15.8)
E3	ND	ND	ND	ND	ND	34.3 (1.8)	9.4 (9.1)
EE2	ND	ND	ND	ND	ND	42.3 (2.4)	9.4 (20.2)
Abiotic control CtBHy							
BPA	ND	ND	ND	ND	ND	57.4 (6.3)	4.0 (17.1)
NP	ND	ND	ND	ND	ND	90.5 (1.9)	2.5 (6.8)
Abiotic control CtDHy							
E1	ND	ND	ND	ND	ND	43.1 (37.9)	−23.0 (6.8)
E2	ND	ND	ND	ND	ND	64.7 (28.3)	−12.8 (4.5)
E3	ND	ND	ND	ND	ND	59.3 (29.9)	−2.9 (6.9)
EE2	ND	ND	ND	ND	ND	51.7 (3.5)	−24.7 (9.8)
BPA	ND	ND	ND	ND	ND	49.5 (15.3)	−60.6 (23.6)
NP	ND	ND	ND	ND	ND	62.3 (19.7)	−15.3 (9.2)

[a] The ED concentrations in Hytrel were measured at the beginning and 4 weeks later, except for the 23rd transfer, for which they were determined 1 week later. A negative value means no degradation or transformation of E2 into E1. Values in parentheses are standard deviations (SD) of triplicate values. ND, not determined.

BPA was 40% and 66% degraded in the BHy and DHy TPP enrichment cultures, respectively, compared with a 30% degradation of NP in these two cultures. Taken together, these results demonstrate that although some of the ED disappearance might reflect irreversible adsorption onto Hytrel, considerable microbial degradation of the EDs also occurred.

Bacterial diversity profiles of the enrichment cultures. Total DNA was extracted for all enrichment cultures, and a region of the

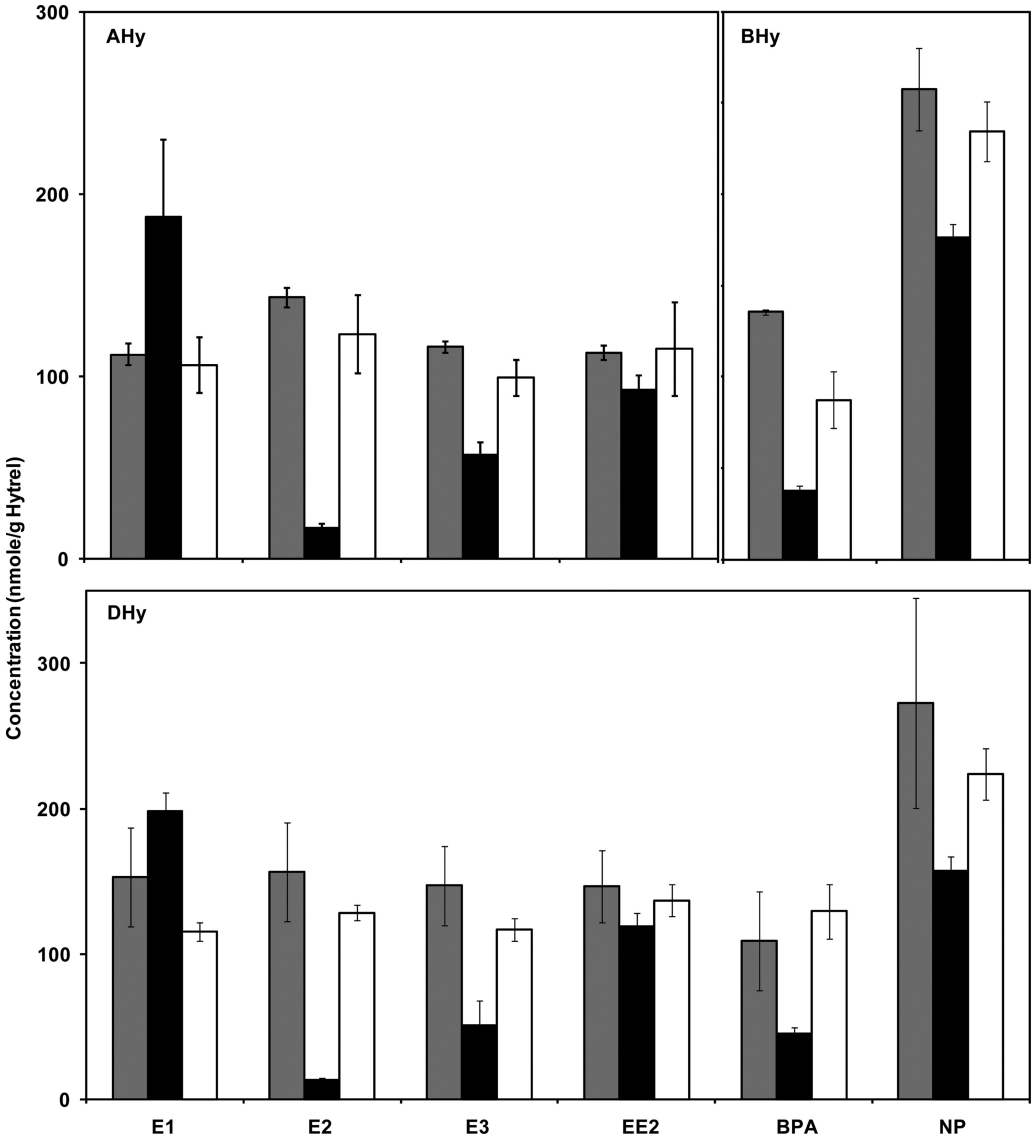

FIG 2 Degradation of the EDs using 1-week enrichment cultures. ED concentrations in the respective enrichment cultures immediately after (gray bars) and 1 week after (black bars) the 23rd transfer. White bars, abiotic control 1 week after the 23rd transfer. The error bars represent the standard deviations from triplicate flasks.

16S rRNA gene sequence was PCR amplified and segregated using DGGE. Respective to each type of enrichment culture, the DGGE migration profiles were similar after the 14th transfer (see Fig. S1A in the supplemental material), suggesting the stabilization of the bacterial populations. DNA-extracted enrichment culture at the 19th transfer showed identical DGGE migration profiles in the respective triplicates (see Fig. S1A in the supplemental material). The analysis of the migrating band profiles (see Fig. S1B in the supplemental material) revealed a clear distinction between the composition of the bacterial populations of the MHy enrichment cultures and the three ED-charged enrichment cultures. The similarity analysis also showed that the bacterial populations of AHy and DHy enrichment cultures were more related to each other than to that of the BHy enrichment cultures. DNA from the 19th transfer was used to determine the composition of the bacterial populations by pyrosequencing and 16S rRNA gene libraries.

Bacterial composition of the enrichment cultures. A region of the 16S rRNA gene sequence was PCR amplified from total DNA extracted from each enrichment culture, and the amplicons were subjected to pyrosequencing (see Table S1 in the supplemental material). The most probable affiliation at the genus level of the bacterial populations was possible with the resulting sequences (Table 3). As predicted from the DGGE profiles, the Fast UniFrac analysis (see Fig. S2 in the supplemental material) showed no significant differences in the phylogenic affiliations between AHy and DHy ($P > 0.1$). Similarly, a clear distinction was observed between the phylogenic affiliation of MHy and those of AHy, BHy, and DHy (see Fig. S2 in the supplemental material).

Table 3 shows the affiliation with the bacterial genera present above a 1% proportion in at least one enrichment culture. Except for MHy, all the enrichment cultures were dominated by alphaproteobacteria, particularly *Rhizobiales* and *Sphingomonadales*

TABLE 3 Most probable affiliation of the 16S rRNA gene sequences and proportion of the affiliated reads in each genus[a]

Affiliation	Proportion (%) in each enrichment culture			
	AHy	BHy	DHy	MHy
Alphaproteobacteria				
Rhizobiales				
Hyphomicrobium	38.2	27.1	34.5	6.0
Bosea	2.8	0.74	1.0	0.00
Nitrobacter	2.7	3.3	3.4	5.8
Mesorhizobium	0.05	0.44	3.8	0.84
Pseudaminobacter	1.4	1.3	1.6	0.14
Aminobacter	1.2	0.23	0.00	0.08
Ochrobactrum	0.00	15.7	0.05	0.92
Sphingomonadales				
Sphingomonas	3.1	13.3	6.9	0.73
Porphyrobacter	26.8	0.00	34.1	0.00
Novosphingobium	0.02	2.6	2.1	0.07
Betaproteobacteria				
Burkholderiales				
Pigmentiphaga	0.81	1.1	0.69	5.1
Pandoraea	2.8	9.0	0.13	0.43
Gammaproteobacteria				
Xanthomonadales				
Rhodanobacter	4.0	3.6	0.02	22.4
Dokdonella	0.35	0.52	0.33	2.4
Actinobacteria				
Actinomycetales				
Leifsonia	10.6	14.4	0.53	0.55
Microbacterium	1.7	2.7	5.1	53.3
Others[b]	3.1	3.9	5.6	1.3

[a] 16S rRNA gene sequences were obtained using the pyrosequencing approach.
[b] Includes sequences affiliated with *Pseudomonas*, *Planctomycetes*, and *Bacteroidetes*/*Sphingobacteriales*.

TABLE 4 Most probable affiliation of 16S rRNA sequences from clone libraries[a]

Affiliation	% identity	Clone name(s)
Hyphomicrobium denitrificans	99.5	AHy44
Hyphomicrobium sp. MC1	>99	DHy61, MHy88
Sphingomonas sp. JEM-1	97–98	AHy13, AHy33, DHy15, DHy25, DHy97
Sphingomonas sp.	93–96	BHy42, BHy54
Rhodanobacter lindaniclasticus	99	AHy52, MHy106
Pseudomonas citronellolis	99.4	BHy79
Ochrobactrum anthropi	99.7	BHy124
Mesorhizobium sp.	98–99	DHy65, DHy91
Microbacterium esteraromaticum	>99	MHy35, MHy37, MHy52
Pigmentiphaga sp.	99	MHy109
Planctomyces sp.	95–98	AHy34
Sphingobacteriales	91–92	BHy3, BHy102

[a] 16S rRNA gene sequences (at least 1,400 nt) were searched for the most closely related sequences using BLASTN in the gene databases. The sequence affiliations at the species level showed nearly 100% identity. GenBank accession numbers for the sequences are KC502947 to KC502969.

(see Fig. S3 in the supplemental material), among which high proportions of sequences related to the genus *Hyphomicrobium* (ranging from 27 to 38%) (Table 3) were identified in the three ED-charged enrichment cultures. In the MHy enrichment cultures, more than half of the reads were affiliated with *Actinomycetales* (see Fig. S3 in the supplemental material), particularly with the genus *Microbacterium* (Table 3). *Rhodanobacter* spp. were also present in high proportions (23%) in these cultures.

16S rRNA gene libraries. 16S rRNA gene libraries were derived for each enrichment culture using almost-complete PCR-amplified 16S rRNA gene sequences. This approach was used to confirm results of the pyrosequencing and obtain sequences with sufficient length for affiliation at the species level. Twenty-three representative clones of these libraries were sequenced (Table 4). Sequences related to the genus *Hyphomicrobium* were derived from all enrichment cultures. All the other affiliations were also observed in the pyrosequencing approach.

Residual methanol in Hytrel. Despite 16 h of drying of Hytrel granules at 70°C, residual methanol still left after loading the EDs might explain the high proportion of *Hyphomicrobium* spp. in the AHy, BHy, and DHy enrichment cultures. To evaluate this parameter, Hytrel granules were mixed with ED-free methanol and treated under the same conditions as applied when EDs were loaded into Hytrel. We found residual methanol concentrations of 11.07 ± 0.05 μg/g Hytrel.

Isolation of ED-degrading bacterial isolates from the AHy enrichment cultures. The microbial biomass from the AHy enrichment cultures was cultured on four solid media, R2A, TSA, and mineral medium containing EDs with or without vitamins. Twenty isolates with distinct colony morphologies were obtained. These isolates were affiliated (16S rRNA sequences) with *Rhodococcus* (11 isolates), *Rhodanobacter* (4 isolates), *Bacillus* (3 isolates), *Microbacterium* (1 isolate), and *Pusillimonas* (1 isolates). Six representative isolates were examined for their capacity to degrade E1, E2, E3, and EE2 (1 mg/liter each) in TSB medium for 4 days. None of the isolates degraded EE2. The ESM-V1A, R2A-4, R2A-5, and TSA-1 isolates performed only the oxidation of E2 into E1, with no significant degradation of E3. The TSA-7 isolate achieved the opposite reaction, the reduction of the ketone group of E1 to produce E2. No significant degradation of E3 was observed with this isolate. Only the EMS-1 isolate, affiliated with *Rhodococcus* spp., was able to significantly degrade E1, E2, and E3 in 4 days, with 54% and 72% degradation of E1 and E3, respectively. A 26% increase of the E2 concentrations occurred from the reduction of E1 into E2. Taken together, the residual E1 and E2 concentrations decreased by 26% with the EMS-1 isolate.

DISCUSSION

In a previous study (29), we demonstrated the potential of using Hytrel in a TPP system to extract and concentrate E1, E2, E3, EE2, BPA, and NP from water but also from treated wastewater that was spiked with these EDs. Hytrel 8206 is a copolymer of poly(butylene teraphthalate) and butylene ether glycol teraphthalate and is becoming one of the most used solid polymers in TPPBs for the degradation of pollutants and also for the transformation of molecules of interest (16, 18–21, 28, 38, 39, 54, 55). It is a polymer of choice because of its biocompatibility, nonbiodegradability, and high affinity of target molecules tested so far but also because of its low cost and reusability (16, 25). In the development of ED-degrading enrichment cultures, our strategy involving the enrich-

ment cultures in a solid-liquid TPP system with ED-charged Hytrel was chosen for four reasons. First, the low solubility of the EDs in the aqueous phase cannot sustain substantial bacterial growth. Second, there is possible selection of microorganisms with better surface adherence and biofilm formation to the solid phase. Third, Hytrel granules provide a better surface for microorganism attachment and ED diffusion. Finally, the acclimated enrichment cultures for the solid phase were able to be more readily used in the development of solid-liquid TPPBs. To our knowledge, this is the first time that enrichment cultures have been performed in a solid-liquid TPP system instead of using a traditional approach performed only in an aqueous phase.

A small concentration of yeast extract was included during the first 15 transfers to stimulate growth in case some microorganisms used cometabolism for ED degradation. In the MHy enrichment cultures, yeast extract was the only added source of carbon. However, when it was no longer added to the cultures, growth continued, showing that Hytrel can, unexpectedly, serve as a carbon source. The MHy enrichment cultures were dominated by *Microbacterium* spp. and *Rhodanobacter* spp. (75% of pyrosequencing reads), which apparently feed on Hytrel alone. However, we never observed the disaggregation of the Hytrel granules in our TPP systems. Degradation of acrylic polymers and polyvinyl alcohol involving *Microbacterium* spp. has been reported (56, 57).

Although Hytrel was able to serve as a carbon source in all our TPP systems, the composition of the bacterial populations in the three ED-charged TPP systems was clearly distinct from that in the MHy enrichment cultures (Table 3; see also Fig. S1 and S2 in the supplemental material). This suggests that the EDs were more readily available sources of carbon than Hytrel. Interestingly, growth was higher in the MHy enrichment cultures (Fig. 1) than in the others. The bacterial populations of the ED-loaded enrichment cultures may have had lower specific growth rates, which would have affected the biomass yield. Alternatively, the EDs by themselves or their degradation intermediates, such as phenol, may have induced some growth inhibition.

Nearly 100% degradation of E1, E2, E3, and BPA and more than 80% degradation of NP were achieved by the ED-loaded TPP systems after 4 weeks. However, abiotic controls showed a significant loss of all the EDs after 4 weeks, which may have been indicative of abiotic degradation in our TPP systems. Based on the 1-week degradation assays, the reduction in the ED concentrations in the TPP systems clearly resulted from microbial degradation, with a minimal reduction in the ED concentrations observed in the abiotic controls. These results rule out the possibility of abiotic degradation in the TPP systems. A time-induced irreversible absorption of the EDs to Hytrel more likely occurred in the abiotic controls, which consequently can no longer be extracted with methanol. Most of the E2 was oxidized into E1 within the first week, as expected as a first step in the degradation of this compound. In addition, more than 50% degradation of E3 and BPA and approximately 30% degradation of NP occurred.

None of the enrichment cultures were capable of degrading EE2, suggesting that efficient EE2-degrading bacteria were not present in the biomass sources used to constitute the original inoculum. Among the three sources, only the activated sludge obtained from the municipal wastewater treatment plant exhibited the potential to contain EE2-degrading microorganisms. However, this particular treatment plant is operated for biological oxygen demand removal only, with a low solid retention time (SRT; 2 to 3 days). These conditions do not favor the establishment of ED-metabolizing populations, as the efficient removal of EDs is generally observed in plants operated for nitrification or nitrification-denitrification with a long SRT (11, 12, 58).

There are many reports that used activated sludge, sediments, soils, and consortia for the degradation of EDs (see a review in Cajthaml et al. [59]). However, very limited information was provided on the composition of the bacterial populations in these systems. In our study, the 16S rRNA gene sequences derived from pyrosequencing (Table 3), gene libraries (Table 4), and the cultured strains provided a good indication of the bacterial composition of the enrichment cultures and the presence of ED-degrading bacteria. There are multiple reports on the isolation of ED-degrading bacterial strains showing affiliations with *Sphingomonas*, *Novosphingobium*, *Ochrobactrum*, *Microbacterium*, *Pusillimonas*, *Rhodococcus*, *Planctomyces*, and *Bacteroidetes*/*Sphingobacteriales* (41, 60–68), among the genera detected in our enrichments.

High prevalence of *Hyphomicrobium* spp. was shown in the AHy, BHy, and DHy enrichment cultures (between 27 and 38% of pyrosequencing reads). No ED-degrading *Hyphomicrobium* strain has previously been reported. *Hyphomicrobium* spp. are methylotrophic bacteria that typically use C-1 carbon sources, such as methanol, but can grow (suboptimally) on variety of complex carbons (69). The presence of these bacteria in high proportions in our enrichments may be the result of residual methanol from loading EDs into Hytrel granules, favoring *Hyphomicrobium* growth. It is also possible that the ED-degrading microorganisms generated C-1 metabolites, such as formate, which can be used by *Hyphomicrobium* spp. Alternatively, Hytrel might have promoted *Hyphomicrobium* growth. Indeed, *Hyphomicrobium* spp. were also present in relatively high proportions (6%) in the MHy enrichment cultures, in which the Hytrel was never treated with methanol. Hytrel 8206 is produced through the polycondensation of dimethyl terephthalate and 1,4-butanediol, accompanied by the production of significant amounts of tetrahydrofuran as a side product (70). Some *Hyphomicrobium* spp. use *n*-butyrate as a carbon source (69), suggesting that 1,4-butanediol or tetrahydrofuran might have been used as a carbon source.

Sequences related to *Sphingomonas* spp. were present in high proportions in the three ED-charged enrichment cultures. Several *Sphingomonas* sp. strains capable of degrading EDs have been isolated from soil, wastewater, or activated sludge (63, 64, 66–68, 71). For instance, in *Sphingomonas* sp. ED8, which can degrade E1 and E2, the 4-hydroxyestrone and 4-hydroxyestradiol were showed to be the degradation intermediates before the metacleavage of the aromatic ring (71). The five clones retrieved from the AHy and DHy 16S rRNA gene libraries were closely related to *Sphingomonas* sp. strain JEM1. This strain was originally isolated from biological wastewater treatment plants containing estrogenic substances (GenBank accession number AB219359) (72). Mattieu et al. (73) screened a wide variety of bacteria isolated from soil or wastewater treatment plant activated sludge to use 7-ketocholesterol as the sole carbon and energy source. One isolate, highly related to strain JEM1, was able to degrade 7-ketocholesterol. The 16S rRNA gene sequence of strain JEM-1 is also closely related to *Novosphingobium tardaugens* strain ARI-1. This strain was isolated from a sewage treatment plant in Tokyo and was able to degrade 17β-estradiol (60, 74).

The high proportion of 16S rRNA pyrosequences affiliated with *Ochrobactrum* spp. were retrieved from the BHy enrichment

cultures, and the 16S clone BHy124 was almost identical to the *Ochrobactrum anthropi* 16S rRNA sequence. BHy124 is also nearly identical to the 16S rRNA sequence of the M16.1B isolate, which was isolated from the biomass of the bioreactor used to treat swine wastes (41), one of the sources of the original inoculum. This isolate was shown to oxidize E2 into E1, which lowers the molecule estrogenic activity. The capacity to degrade BPA and NP was, however, not tested. 16S rRNA sequences related to *Ochrobactrum* spp. were not present in the AHy enrichment cultures and were present in very low proportions in the DHy enrichment cultures. *Ochrobactrum* sp. T was shown to mineralize tetrabromobisphenol-A (75), and *Ochrobactrum* spp. were found dominant during aerobic degradation of nonylphenol in soil (76).

High proportions of 16S rRNA pyrosequences affiliated with *Pandoraea* spp., *Porphyrobacter* spp., and *Leifsonia* spp. were obtained in ED-degrading enrichment cultures. These genera have never been reported to specifically degrade the six studied EDs. However, some members can attack aromatic compounds (e.g., PAHs, polychlorinated biphenyl) (77, 78) and therefore may be involved in the aromatic ring cleavage during ED degradation (71).

Among the bacterial isolates derived from the AHy enrichment cultures, only *Rhodococcus* sp. strain EMS-1 was able to significantly degrade E1 (6 μg in 4 days), E2, and E3 (9 μg in 4 days) under the conditions tested. Curiously, pyrosequencing reads affiliated with *Rhodococcus* spp. were not retrieved from the AHy enrichment cultures. Since *Rhodococcus* spp. are high-GC-content bacteria, their 16S rRNA gene sequences may have been missed by pyrosequencing because of GC bias, as reported by Jaenicke et al. (79). *Rhodococcus* sp. EMS-1 reduced E1 into E2, which is an unusual reaction under aerobic conditions. This transformation was also observed with strain TSA-7 and has been reported once by Isabelle et al. (41) for *Methylobacterium* sp. MI6.1R. Bacterial strains affiliated with *Rhodococcus* sp., *Rhodococcus equi*, *Rhodococcus erythropolis*, *Rhodococcus rhodochrous*, and *Rhodococcus zopfii* were isolated for their high performance of E1, E2, E3, and EE2 degradation (68, 71, 80, 81). For instance, Yoshimoto et al. (81) isolated several *Rhodococcus* strains (affiliated with *R. zopfii* and *R. equi*) from activated sludge of a wastewater treatment plant which performed high levels of degradation of E1, E2, E3, and EE2 (1,000 μg within 24 h). Kurisu et al. (71) showed >90% degradation of E1 and E2 (720 μg in 5 days) for 3 *Rhodococcus* strains, isolated from soil. However, none of these studies reported transformation of E1 into E2, as strain EMS-1 is doing.

In conclusion, we succeeded in establishing enrichment cultures for the degradation of five out of the six EDs in solid-liquid TPP systems. Failure to have EE2-degrading microorganisms in our enrichment cultures could be compensated for by the addition of known EE2-degrading strains, such as *Rhodococcus* sp., in the enrichment cultures. Our results also provide evidence that Hytrel can serve as a carbon source. As Hytrel granules are intended to be reused in TPPBs, long-term experiments should be performed to assess the impact of the microorganisms on the granules' integrity. Still, this did not preclude the use of Hytrel in the development of an ED-degrading TPPB with our enrichment cultures for wastewater treatments. This TPPB could be included just before the treated water was released into the environment. This configuration will not affect the primary and secondary treatment.

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