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BIOREMEDIATION AND BIODEGRADATION

Reversible Reduction of Estrone to 17β-Estradiol by *Rhizobium*, *Sphingopyxis*, and *Pseudomonas* Isolates from the Las Vegas Wash

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Abstract

Environmental endocrine-disrupting compounds (EDCs) are a growing concern as studies reveal their persistence and detrimental effects on wildlife. Microorganisms are known to affect the transformation of steroid EDCs; however, the diversity of estrogen-degrading microorganisms and the range of transformations they mediate remain relatively little studied. In mesocosms, low concentrations of added estrone (E1) and 17β-estradiol (E2) were removed by indigenous microorganisms from Las Vegas Wash water within 2 wk. Three bacterial isolates, *Rhizobium* sp. strain LVW-9, *Sphingopyxis* sp. strain LVW-12, and *Pseudomonas* sp. strain LVW-PC, were enriched from Las Vegas Wash water on E1 and E2 and used for EDC transformation studies. In the presence of alternative carbon sources, LVW-9 and LVW-12 catalyzed near-stoichiometric reduction of E1 to E2 but subsequently reoxidized E2 back to E1; whereas LVW-PC minimally reduced E1 to E2 but effectively oxidized E2 to E1 after a 20-d lag. In the absence of alternative carbon sources, LVW-12 and LVW-PC oxidized E2 to E1. This report documents the rapid and sometimes reversible microbial transformation of E1 and E2 and the slow degradation of 17α-ethinylestradiol in urban stream water and extends the list of known estrogen-transforming bacteria to the genera *Rhizobium* and *Sphingopyxis*. These results suggest that discharge of steroid estrogens via wastewater could be reduced through tighter control of redox conditions and may assist in future risk assessments detailing the environmental fate of estrogens through evidence that microbial estrogen transformations may be affected by environmental conditions or growth status.

Core Ideas

- Estrone and 17β-estradiol were rapidly degraded in Las Vegas Wash water.
- Microbial genera involved included *Rhizobium*, *Sphingopyxis*, and *Pseudomonas*.
- Microbial isolates catalyzed the reversible reduction of estrone to 17β-estradiol.
- Oxidation-reduction activities on estrogens may be controlled by redox status.

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Environmental endocrine-disrupting compounds (EDCs) are a growing concern as studies reveal their persistence and detrimental effects on wildlife. Endocrine-disrupting chemicals are defined as "exogenous chemical substances or mixtures that alter the structure or function(s) of the endocrine system and cause adverse effects at the level of the organism, its progeny, populations, or subpopulations" (Crisp et al., 1998). Although a wide variety of anthropogenic compounds are estrogenic, some of the most potent are the steroid hormones estrone (E1), 17β-estradiol (E2), and 17α-ethinylestradiol (EE2) (Routledge and Sumpter, 1996), with predicted no-effect concentrations for fish of only 6, 2, and 0.1 ng L^{-1}, respectively (Caldwell et al., 2012). Although individual studies and alternative methods targeting different organisms yield different effective concentrations, EE2 is generally considered tens of times more potent than E2, which in turn is generally reported as approximately two to three times more potent than E1 (Thorpe et al., 2003; Van den Belt et al., 2004).

Steroid hormones are excreted through human waste, and although 90% or more may be removed during wastewater treatment (Joss et al., 2004), residuals make their way into waterways through wastewater effluent, posing potential risks to wildlife downstream (Routledge et al., 1998). To assess the fate of the remaining steroid hormones in wastewater entering the environment, more detailed knowledge of the types of organisms that transform these compounds, the conversions they perform, and the environmental conditions (redox, carbon sources, etc.) required for these processes to work is needed. If microorganisms that are effective in transforming steroid hormones can be isolated, they may be useful to engineer treatment processes.

A number of studies have investigated estrogen biodegradation by mixed cultures of microorganisms in sewage sludge and natural aquatic ecosystems under both aerobic (Jürgens et al., 2002; Lee and Liu 2002; Weber et al., 2005; Yi and Harper, 2007) and anaerobic (Czajka and Londry, 2006) conditions. In

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Abbreviations: EDCs, endocrine-disrupting compound; E1, estrone; E2, 17β-estradiol; EE2, 17α-ethinylestradiol.

most of these studies, E2 is oxidized to E1, followed by degradation of E1 to estriol and then to nonsteroidal products (Casey et al., 2003; Das et al., 2004; Lee and Liu, 2002). 17α-ethinylestradiol is generally more refractory but is degraded by a combination of biological activity (Zhang et al., 2016) and photodegradation (Jürgens et al., 2002). The reversible interconversion of E2 and E1 is known to occur under anaerobic conditions, although EE2 biodegradation has only been observed under iron-reducing conditions (Ivanov et al., 2010). Two recent studies of systems treating manure waste streams, for example, have reported the near-stoichiometric reductive transformation of E1 to E2, followed by complete reoxidation of E2 to E1 (Prater et al., 2015; Zheng et al., 2012). One of these studies showed that the reduction of E1 to E2 was sensitive to oxygen (Prater et al., 2015). Other studies have shown that the loss of E2 can be associated with an accumulation of E1 (Lee and Liu, 2002; Ying and Kookana, 2003) or that E1 can be reduced back to E2, with the steady-state concentration of E2 being dependent on the dominant electron-accepting reaction (Czajka and Londry, 2006). Thus, the tendency of estrogens to reversibly shift between redox states rather than undergo complete degradation suggests that they would accumulate in anoxic environments.

A diversity of bacteria is known to transform estrogens, including members of the phyla Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria (reviewed in Yu et al. [2013] and Zhang et al. [2016]). Many of these microorganisms can cometabolically oxidize E2 to E1, and some can degrade estrogens to small, nontoxic products, by using estrogens as sole carbon sources and electron donors for heterotrophic growth, with examples including *Novosphingobium* strain ARI-1 (Fujii et al., 2002), *Sphingomonas* sp. KC8 (Yu et al., 2007), and several *Rhodococcus* strains (Yoshimoto et al., 2004). However, very few microorganisms have been shown to reduce E1 to E2. One study described the reversible reduction of E1 to E2 by pure cultures identified as *Alcaligenes fecalis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* and suggested that these activities are likely to be responsible for the high concentration of reduced estrogens in feces from humans and other animals (Järvenpää et al., 1980); however, few details were provided, such as the source or identity of the strains, the details of the cultivation conditions, the concentration of estrogens used, or whether the transformations were coupled with growth. In a more recent study, Isabelle et al. (2011) showed that *Methylobacterium* strain MI6.1R, isolated from an activated sludge bioreactor, is capable of the reduction of E1 to E2 under aerobic conditions.

In this study, we examined the role of microorganisms in estrogen transformations in water collected from the Las Vegas Wash in Nevada. The Las Vegas Wash is a tributary that enters the Boulder Basin of Lake Mead through Las Vegas Bay and is primarily supplied by tertiary-treated wastewater from four plants (Benotti et al., 2010; Rosen et al., 2006). Significant amounts of organic contaminants also enter the Las Vegas Wash through historic industrial land uses adjacent to the wash and through urban runoff (Bevans et al., 1996; Boyd and Furlong, 2002; Covay and Leiker, 1998; Rosen et al., 2006, 2010; Rosen and Van Metre, 2010). Various EDCs have been found in the Las Vegas Wash and Las Vegas Bay, including E2, which has been detected at concentrations as high as 2.7 ng L^{-1} (Snyder et al., 1999). Toxicity tests of water collected in Las Vegas Bay where wastewater and urban runoff enters Lake Mead show the estimated estrogenicity was fivefold to ninefold greater at depths of 3.0 and 4.7 m as compared with surface water, which correlates with the greater occurrence of many synthetic organic chemicals related to wastewater effluents and urban runoff at these depths and indicates that wastewater and urban runoff is the likely cause of this toxicity (Alvarez et al., 2012). Carp and largemouth bass collected from the Las Vegas Wash and Las Vegas Bay have been found to contain high concentrations of synthetic organic chemicals and biomarkers of endocrine disruption, such as vitellogenin, an egg yolk precursor (Bevans et al., 1996; Goodbred et al., 2007, 2015; Patiño et al., 2003, 2015). Understanding contaminant fate and transport is important, particularly in arid regions such as the American Southwest, as population growth and climate change pit an ever-increasing demand for water against dwindling supplies. As a result, these communities are engaging in beneficial water reuse practices, thus raising the stakes for strategies to remediate EDCs and other wastewater-derived pollutants. Here we show that a microcosm containing Las Vegas Wash water was capable of rapid degradation of E1 and E2 and slow degradation of EE2. Three representative microbial isolates obtained from the Las Vegas Wash, identified as members of the genera *Rhizobium*, *Sphingopyxis*, and *Pseudomonas*, were shown to catalyze the reversible co-metabolic reduction of E1 to E2. These findings may be useful in developing methods for the effective treatment of wastewater before it enters the environment or may allow an assessment of natural attenuation and/or degradation occurring downstream of effluent outfalls.

Materials and Methods

Sample Collection, Microcosm Experiment, and Microbial Isolation

Water samples were collected in sterile containers from the Las Vegas Wash (36.092° N, 114.969° W), transported to the laboratory on ice, and used to create a microcosm to examine estrogen degradation. The microcosm consisted of 18.9 L (5 gallons) of Las Vegas Wash water, which was gently stirred in a glass bioreactor in the dark at room temperature. An aqueous stock solution containing estrone (99% pure), 17β-estradiol (98% pure), and 17α-ethinylestradiol (98% pure) (Sigma-Aldrich, Inc.) was prepared by adding neat compounds to deionized water rather than a solvent such as methanol. This was done to avoid introducing an alternative carbon and potential energy source for the microbes. Because the stock solution was a mixture of several compounds, the entire stock did not completely dissolve before the microcosm was spiked, resulting in lower-than-expected initial concentrations. Initial measured concentrations for E1, E2, and EE2 in the microcosm were 22.1, 60, and 148 ng L^{-1} (0.08, 0.22, and 0.5 nM), respectively. Water samples (1 L) were aseptically collected and analyzed to measure attenuation of E1, E2, and EE2 at 0, 1, 2, 4, 7, 14, 29, 56, and 120 d.

Fresh Las Vegas Wash samples were serially diluted into 16-mm slip-cap culture tubes containing M1 mineral medium (Myers and Nealson, 1988) supplemented with E1, E2, and EE2 (2000 μg mL^{-1}) as the sole sources of carbon and energy for growth (1:10 dilution of sample to media) and incubated at room temperature in the dark. The most dilute enrichments showing visible turbidity were subsequently used as sources for

microbial isolation by streaking onto the same medium solidified with 1.5% (w/v) high-purity agar, followed by incubation in the dark at room temperature. For the solid medium, 100× stock solutions of E1, E2, and EE2 in methanol were incorporated by spreading with a sterile glass rod onto agar plates containing M1 minimal medium lacking any other added carbon source (for a final concentration of 10 mg L^{-1} each of E1, E2, and EE2). To minimize the possibility that the added methanol might serve as a potential carbon and energy source for microorganisms, methanol was allowed to evaporate from plates in a laminar flow hood overnight, leaving only the steroid estrogens behind. Colonies with distinct morphologies were subjected to three rounds of streak plate isolations. Culture purity was verified through microscopic observation and sequencing of the 16S rRNA gene.

16S rRNA Gene Polymerase Chain Reaction, Sequencing, and Microbial Identification

DNA was extracted from cell pellets using the MoBio Ultraclean soil kit (MoBio), and 16S rRNA genes were amplified by PCR using primers 9bF (Eder et al., 1999) and 1512uR (Eder et al., 2001) and sequenced using Sanger technology as previously described (Costa et al., 2009). Reads were trimmed to remove bases with quality scores of less than 20, and sequence contigs were assembled using Sequencher V4.9 (Gene Codes). The near full-length 16S rRNA gene sequences were used as queries to the NCBI 16S rRNA gene sequence database using BLASTN and to EZTaxon (Kim et al., 2012).

Microbial Growth and Estrogen Degradation Characteristics

The capacity for growth on, and degradation of, EDCs was assessed in the defined medium using two different culture conditions. In the first condition, a mixture of E1, E2, and EE2 (2000 μg mL^{-1} each) was provided as the sole carbon and energy source. In the second condition, the same medium was used with, in addition to the estrogens, an equimolar mixture of simple organics (2.5 mM each of sodium formate, sodium lactate, sodium acetate, and D-glucose). These organic compounds were chosen to represent organics that are common in natural systems as products of biomass depolymerization (glucose) and fermentation (organic acids) and are used as electron donors for a wide variety of microorganisms. For both culture conditions, the inoculum consisted of a 1:50 dilution of early stationary-phase cultures, which were grown in M1 liquid with the equimolar mixture of EDCs and simple organics, centrifuged, rinsed twice, and resuspended in fresh M1 without amendments. All experiments were run in triplicate.

In the microcosm experiment, steroid analysis used solid-phase extraction followed by LC-MS/MS (Vanderford et al., 2003). Water samples were spiked with isotopically labeled standards and extracted using Oasis HLB cartridges (Waters Corp.) using an Autotrace automated solid-phase extraction system (Zymark Corp.). Cartridges were preconditioned with 5 mL each of dichloromethane, tert-butyl methyl ether, methanol, and reagent water. Water samples were filtered through the cartridges at 15 mL min^{-1}, rinsed with 5 mL reagent water, and dried with nitrogen gas for 60 min. Samples were eluted with methanol, evaporated to 250 μL, and stored at −20°C until analysis. The LC-MS/MS was performed using an Agilent G1312A binary pump with a binary mobile phase of 0.1% formic acid in water and 100% methanol at a flow rate of 0.7 mL min^{-1} and an injection volume of 10 μL followed by an Applied Biosystems API 4000 triple quadrupole mass spectrometer, using multiple reaction monitoring with electrospray ionization in positive and negative modes.

For the growth experiment, estrogen concentrations were determined by extraction of culture broth with acetonitrile (1:1 v/v) and filtration using 0.2-μm polytetrafluoroethylene filters. Extracts were quantified using high-performance liquid chromatography (Agilent 1200 Series) with an Agilent Zorbax ODS C18 column (4.6 mm ID × 250 mm; particle size, 5 μm; pore size, 70 Å) and a UV detector (200 nm). The mobile phase was acetonitrile/water (45/55, v/v), with a flow rate of 600 μL min^{-1}, column temperature of 35°C, and an injection volume of 100 μL. Microbial growth was assessed by using the FASTEST Total Viable Organisms Kit by Flow cytometry (MicroPro, Becton Dickinson).

Nucleotide Accession Numbers

Near full-length 16S rRNA gene sequences have been deposited in GenBank with the accession numbers KU291420, KU291421, and KU291422.

Results

Estrogen Transformations: Microcosm Study

A microcosm was established from the Las Vegas Wash by adding low concentrations of the estrogens E1, E2, and EE2 (Table 1) to Las Vegas Wash water (Fig. 1). In this experiment, E2 was rapidly removed without lag, reaching the detection limit within 1 wk. The concentration of E1 increased transiently, consistent with the commonly observed pattern of oxidation of E2 to E1 under aerobic conditions, but then was completely removed within 2 wk. The concentration of EE2 decreased slowly and steadily over the course of the incubation, such that roughly one-third of the original 150 ng μL^{-1} remained at the end of the 120-d incubation period. Half-lives for E1, E2, and EE2 were calculated as 27, 2.9, and 150 d, respectively.

Estrogen Transformations by Pure Cultures

A number of putative estrogen-degrading bacteria were isolated from the Las Vegas Wash based on their apparent ability to grow on agar plates with estrogens as sole carbon and energy sources. However, subsequent screening failed to confirm

Table 1. 16S rRNA gene identity to closest cultivated relatives.

Organism	Closest cultivated relative	% Identity	Accession no.
Rhizobium sp. LVW-9	*Rhizobium radiobacter* ATCC 19358^T	99.7%	KU291420
Sphingopyxis sp. LVW-12	*Sphingopyxis alaskensis* RB2256^T	99.1%	KU291421
Pseudomonas sp. LVW-PC	*Pseudomonas taiwanensis* BCRC 17751^T	99.8%	KU291422

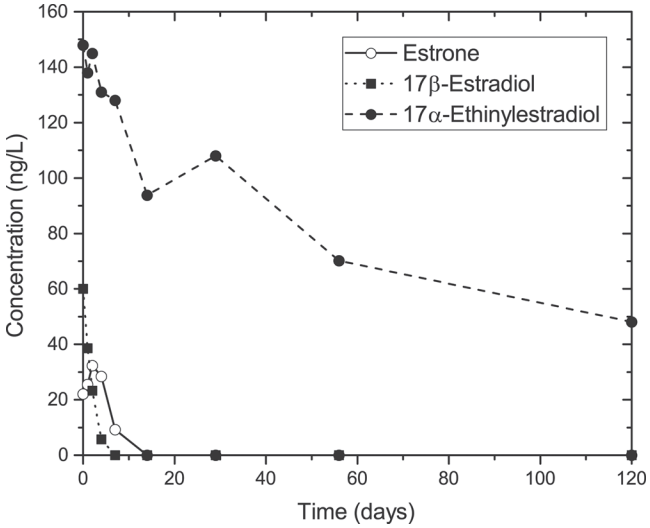

Fig. 1. Transformation of estrogens by a Las Vegas Wash microcosm. A mixture of estrone, 17β-estradiol, and 17α-ethinylestradiol was added (22.1, 60, and 148 ng L^{-1}, respectively) to 18.9 L of Las Vegas Wash water. The microcosm was gently stirred in the dark at room temperature and periodically sampled for estrogens by LC-MS-MS analysis. (See Supplemental Table S1 for data used in this figure.)

estrogen degradation by many of the pure cultures. We speculate that these potential nontarget microorganisms may have maintained slow growth by using impurities in agar or residual methanol used in our estrogen stock solutions as carbon and energy sources. Regardless, three isolates from this collection, designated LVW-9, LVW-12, and LVW-PC, were confirmed to transform estrogens. These three isolates were identified as strains of *Rhizobium* (LVW-9), *Sphingopyxis* (LVW-12), and *Pseudomonas* (LVW-PC) based on analysis of near-complete 16S rRNA gene sequences (Table 1).

In pure culture experiments, all three strains co-metabolically catalyzed the near-stoichiometric reduction of relatively high concentrations of E1 to E2 in the presence of simple organics (2.5 mM each of sodium formate, sodium lactate, sodium acetate, and D-glucose) and later reoxidized E2 to E1. However, these degradation patterns differed in several details (Fig. 2a–c). Strain LVW-9 reduced approximately half of the 2000 $\mu g\,\mu L^{-1}$ of E1 to E2 slowly over a 10-d period, during which time minimal growth was observed. Then, between 10 and 20 d of incubation, two-thirds of the E2 was reoxidized back to E1, concomitant with a marked increase in cell density (i.e., from $\sim 1.0 \times 10^8$ to 1.0×10^9 cells mL^{-1}). Under the same conditions, strain LVW-12 grew without lag to a cell density of $\sim 6.0 \times 10^8$ cells mL^{-1} and reduced a similar amount of E1 to E2 within 5 d of incubation. However, longer incubation led to the near-complete reoxidation of E2 to E1 during stationary and death phases of growth. In contrast, strain LVW-PC reduced E1 to E2 only slightly during growth on simple organics over a 20-d time period, during which cell counts reached $\sim 6.0 \times 10^8$ cells mL^{-1}, followed by a slow decline in cell numbers and oxidation of E2 to E1.

In all three cultures, EE2 was not significantly transformed, and all estrogens were stable in parallel abiotic incubations. Although there was no evidence that any of the strains degraded estrogens to smaller compounds or for the coupling of estrogen transformation to growth, strains LVW-12 and LVW-PC were both able to oxidize E2 to E1 during incubation under nongrowing conditions without supplemental organics; in contrast, strain LVW-9 did not transform any estrogens in the absence of co-metabolic substrates (Fig. 2d–f).

Discussion

This study resulted in the characterization of estrogen transformation by environmental isolates of two genera not previously known to transform estrogens, *Rhizobium* and *Sphingopyxis*, both belonging to the class Alphaproteobacteria in the phylum Proteobacteria. Various members of the class Alphaproteobacteria are well known for their abilities to degrade estrogens (reviewed in Yu et al. [2013] and Zhang et al. [2016]); however, these two genera have never previously been implicated in estrogen transformations. *Rhizobium* is a member of the family Rhizobiaceae, which includes known estrogen-transforming bacteria in the genera *Aminobacter* (Yu et al., 2007) and *Phyllobacterium* (Pauwels et al., 2008). Members of these genera are able to couple degradation of E1 and E2 to nonsteroidal products and growth, but neither is known to reduce E1 to E2, as was shown here for *Rhizobium*. *Sphingopyxis* is a member of the family Sphingomonadaceae, which includes estrogen-transforming bacteria in the genera *Sphingomonas* (Ke et al., 2007; Kurisu et al., 2010; Yu et al., 2007) and *Novosphingobium* (Fujii et al., 2002; Hashimoto et al., 2010). These strains carry out a variety of transformations of E1, E2, estriol, and EE2, but none is known to reduce E1 to E2. Thus, the current study expands the list of *Alphaproteobacteria* genera known to transform estrogen and adds to the known estrogen transformation repertoire of this group.

The genus *Pseudomonas* has previously been observed to participate in estrogen transformations in a variety of capacities. Järvenpää et al. (1980) described the reversible reduction of E1 to E2 in *P. aeruginosa* cultures over 35 yr ago. An isolate of *P. aeruginosa* isolated from activated sludge has also been described as being capable of growth on E2 as a sole carbon and energy source (Zeng et al., 2009). Finally, several *Pseudomonas putida* isolates have been shown to participate in EE2 degradation indirectly through the production of reactive Mn oxides (Sabirova et al., 2008).

The physiological basis for the reversible reduction–oxidation of estrogens observed here is not currently understood and could be a basis for further study. In this study, a range of strain-specific estrogen-transforming behaviors was documented. Strain LVW-9 was observed to transform estrogens only in the presence of co-metabolic substrates. Even with co-metabolic substrates, E1 was only reduced to E2 slowly and with a long lag phase, after which E2 was reoxidized during and possibly after exponential growth. In contrast, LVW-12 and LVW-PC transformed estrogens under the tested conditions both with and without co-metabolic substrates. In the presence of co-metabolic substrates, LVW-12 rapidly reduced E1 to E2 during exponential growth phase and stationary phase. However, prolonged incubation resulted in the reoxidation of E2 to E1. A similar pattern was observed in LVW-PC; however, the reduction of E1 was minimal in the presence of the co-metabolites. LVW-12 and LVW-PC were also able to oxidize E2 to E1 during nongrowth conditions in the absence of a carbon source. For these two strains, we speculate that estrogen oxidation–reduction activities are controlled by oxygen

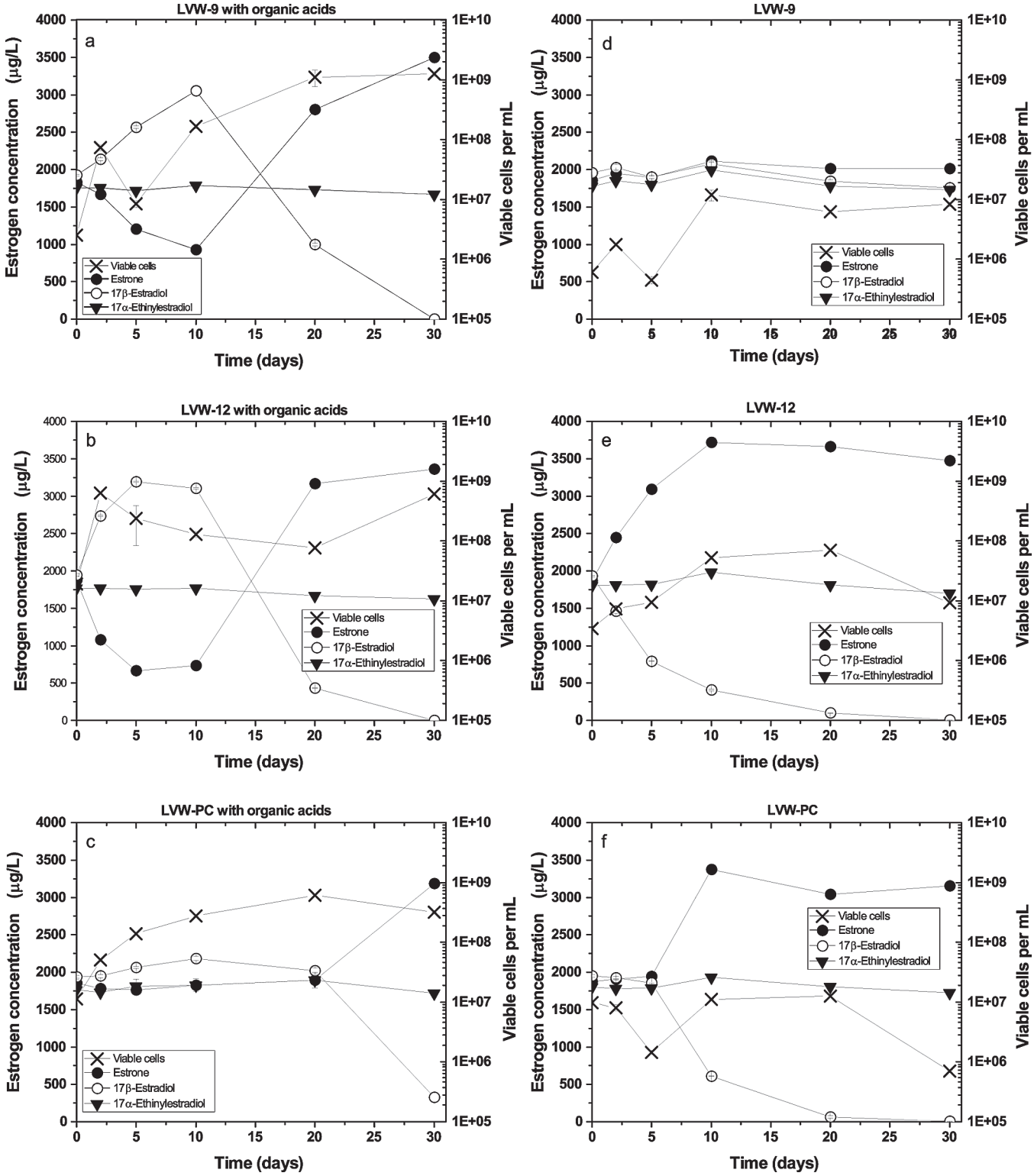

Fig. 2. Transformations of estrogens by pure cultures. Co-metabolic estrogen transformation experiments containing estrone, 17β-estradiol, and 17α-ethinylestradiol (2000 μg L^{-1} each) and an equimolar mixture of simple organics (formate, lactate, acetate, and glucose, 2.5 mM each) with strain (a) LVW-9, (b) LVW-12, and (c) LVW-PC. Similar estrogen transformation experiments without co-metabolic substrates with strain (d) LVW-9, (e) LVW-12, and (f) LVW-PC. (See Supplemental Table S2 for data used in this figure.)

concentration or redox status, as has been observed for bioreactor studies with swine manure (Prater et al., 2015). In our study, because these cultures were only shaken slowly, we suspect that rapid growth on organic acids and glucose likely led to suboxic or hypoxic conditions, which may have favored the transfer of electrons to E1 as a mechanism to establish redox homeostasis. In any case, redox reactions involving estrogens are important for the fate of estrogens in wastewater streams and other environments. The reduction of E1 to E2 observed here may be significant to understanding the environmental dynamics of estrogen transformations because E2 is much more estrogenic than E1 (Thorpe et al., 2003), but it is also degraded more quickly in the

environment than E1. Conversely, because E1 tends to be present at higher concentrations than E2 in at least some environments (Furuichi et al., 2004; Salste et al., 2007), E1 may be the more impactful of the two. The results presented here may represent a first step toward optimization of treatment processes for the elimination of estrogens from waste streams through redox manipulation and the assessment of risks posed by these compounds in receiving waters.

Conclusions

This study is the first to describe estrogen transformations in the genera *Rhizobium* and *Sphingopyxis* and adds to a body of literature on estrogen transformations by *Pseudomonas* species. The transformations that these (and likely other) microorganisms perform on estrogens are affected by the presence of co-metabolic substrates, which is possibly an indirect effect of the redox status of the medium. Continued study of the microbial transformation of EDCs is increasingly important to reduce or eliminate estrogen from effluent wastewater that is directed toward natural wetlands, rivers, or lakes. This is particularly important in arid regions where there is increasing societal pressure on water resources and increasing water reuse and a limited supply of natural freshwater where effluent can be directed.

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