Elucidating the Relative Roles of Ammonia Oxidizing and Heterotrophic Bacteria during the Biotransformation of 17α-Ethinylestradiol and Trimethoprim

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SUPPLEMENTARY INFORMATION

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MATERIALS AND METHODS

Reagents

Deuterium (d4)-labeled 17α-ethinylestradiol-2,4,16,16-d_4 (EE2-d4) was purchased from C/D/N Isotopes, Inc. (Pointe-Claire, Quebec, Canada) and radioabeled EE2 (^{14}C-EE2) was a gift from Schering AG, Germany. Non-labeled EE2 was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Deuterium (d9)-labeled trimethoprim (TMP) was purchased from Toronto Research Chemicals Inc, (North York, Ontario, Canada). Non-labeled TMP was obtained from MP Biomedicals (Solon, OH, USA) (radiolabeled TMP could not be obtained for this study). The purity of all pharmaceutical chemicals exceeded 99%. Stock solutions of non-radiolabeled EE2 (20 g EE2/L) and ^{14}C-EE2 were prepared in acetone. Stocks of non-radiolabeled TMP (400 mg/L) was prepared in Nanopure™ water. Acetonitrile, sodium bicarbonate, methanol, and ammonium acetate were obtained from J.T. Baker (Phillipsburg, NJ, USA) and Thermo Fisher Scientific Inc. (Pittsburg, PA). Acetone, formic acid, and toluene were purchased from EM Science (Gibbstown, NJ. USA). Dansyl chloride was purchased from MP Biomedicals (Solon, OH, USA). Catalase (from *Aspergillus niger*) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used in this study were reagent grade and purchased from Fisher/Thermo Scientific (Pittsburgh, PA).

Cultivation of *Nitrosomonas europaea* (ATCC strain 19718)

The ammonia oxidizing bacterium (AOB) *Nitrosomonas europaea* (ATCC 19718) was cultured in 2 L of medium in chemostat reactors using autotrophic media containing (mg/L): $MgSO_4 \cdot 7H_2O$ (200), $CaCl_2 \cdot 2H_2O$ (20), K_2HPO_4 (87), KH_2PO_4 (405), $Na_2MoO_4 \cdot 2H_2O$ (0.01), $MnSO_4 \cdot H_2O$ (0.017), $CoCl_2 \cdot 7H_20$ (0.0004), $CuCl_2 \cdot 2H_2O$ (0.17), $ZnSO_4 \cdot 7H_2O$ (0.01), chelated iron (1), and $(NH_4)_2SO_4$ (3,330). Sterile aeration was provided via stirring for batch reactors or air pumps outfitted with 0.22 μm HEPA® filters for chemostats. pH was controlled by manual (batch; pH 7.5 ± 0.5) addition or automated (chemostat; pH 7.5 ± 0.01) addition of $NaHCO_3$ (50 g/L). Undiluted aliquots (100 μL) from all reactors were periodically plated on Luria Bertani (LB) solid microbiological media and incubated at room temperature for 7 days to determine if heterotrophic contamination was present.

Cultivation of Ox^- and Ox^+ cultures

A 50:50 blend of nitrifying mixed liquors from the Blacksburg-VPI Sanitation Authority WWTP (BVPISA) and Peppers Ferry Regional Wastewater Treatment Facility (PFRWWTF) were used to seed a heterotrophic enrichment culture that was acclimated to toluene and benzoate (Ox^+), and a heterotrophic enrichment culture that was grown on acetate and no known aromatic organics (Ox^-). Each enrichment culture was maintained in a well-mixed, chemostat reactor. The Ox^+ reactor (V = 15.5 L) was fed an organic feed mixture of toluene (333 mg COD/L), sodium benzoate (333 mg COD/L), and acetic acid (333 mg COD/L). The Ox^- reactor (V = 4 L) was fed 1,000 mg/L acetic acid as COD. Both reactors also received a mineral salts media (MSM) comprised of (mg/L): NH_3-N/L (100), $CaCl_2 \cdot 2H_2O$ (5.3), $FeCl_3 \cdot 6H_2O$ (3), $CoCl_2 \cdot 6H_2O$ (0.3), $ZnCl_2$ (0.31), $CuCl_2 \cdot 2H_2O$ (0.09), H_3BO_3 (0.03), $MgSO_4 \cdot 7H_2O$ (30), $MnSO_4 \cdot 7H_2O$ (0.85), $Na_2MoO_4 \cdot 2H_2O$ (0.12), KH_2PO_4 (54), mg/L K_2HPO_4 (136) and allythiourea (ATU; 10), an inhibitor of AOBs. Dissolved oxygen was provided to the Ox^+ reactor by blending H_2O_2 (9%) and catalase (16 U/mL; 7 times the stoichiometric requirement) in a drip tube upstream of the reactor to form dissolved O_2 without degassing volatile feed constituents [1]. Oxygen uptake rate measurements indicated that the biomass activity was not inhibited by the addition of chemically-produced O_2 (data not shown). Overall, this aeration method was sufficient for our purposes and an adequate level of dissolved oxygen was maintained in the reactor (DO_{avg} = 4 to 5 mg/L). The Ox^- culture was aerated using air pumps to maintain a dissolved oxygen concentration of 7 to 8 mg/L O_2. Experiments were performed once the mixed liquor suspended solids (MLVSS) of the reactors had stabilized around 200 mg/L and achieved 85 to 95% chemical oxygen demand (COD) removal efficiency (typically within 3 SRTs, or 21 days).

Cultivation of CNAS cultures

Nitrifying mixed liquors from two local wastewater treatment plants were used to seed a mixed-culture, nitrifying activated sludge chemostat reactor (20 L) (CNAS). The pH of the CNAS culture was controlled at 7.5 ± 0.2 using automated addition of $NaHCO_3$ (50 g/L) or H_2SO_4 (1 N). The culture was fed primary effluent from a local wastewater treatment plant that received minimal input of industrial wastes over the course of this study. Biotransformation experiments were performed in five, 2 L chemostats using the master CNAS culture as seed,

once stable removal of chemical oxygen demand (COD; 85 to 95% removal) and ammonia (> 99%) was achieved (> 3 HRT operation).

Whole cell biotransformation kinetics

The rates of EE2 and TMP removal by AOB, Ox^- and Ox^+ chemostat cultures were determined assuming pseudo-first order kinetics. Biomass samples were harvested, washed and resuspended into MSM supplemented with chloramphenicol (100 mg/L) to inhibit new protein synthesis. EE2 (200 µg/L) or TMP (200 µg/L) and NH_3 (15 mg N/L for AOB experiments) were added to start kinetic assays. Aliquots were collected every 30 minutes for up to 3 hours, processed as described in SI**,** and monitored for nitrite (AOB experiments), total protein, and EE2 or TMP concentrations. The linearized initial rate of EE2 or TMP loss was used to estimate the kinetic constant . Rates are normalized to measured biomass protein which was converted to COD units (1.5 g COD/g biomass protein). Abiotic (EE2 or TMP in sterile MSM) and sorption (EE2 or TMP with NaN_3 inactivated biomass) controls were also performed. The effect of nitrite on heterotrophic EE2 degradation rates was investigated by supplementing chloramphenicol-treated cells with various concentrations of nitrite (10, 200, 600 mg NO_2^--N/L) and 200 µg/L EE2, and monitoring the specific oxygen uptake rate over time. All experiments were performed in duplicate as a minimum.

Specific Nitrite Generation Rate (sNGR) Assay

Ammonia monooxygenase (AMO) activity was measured indirectly by a specific whole cell nitrite generation rates (NGR) assay as described previously [2] with modifications as follows. Cells from AOB and CNAS chemostat cultures were immediately supplemented with 100 mg/L chloramphenicol to minimize new protein synthesis [3], centrifuged at 13,000×g for 10 min at room temperature and resuspended into autotrophic media minus ammonium and amended with chloramphenicol (100 mg/L). For CNAS experiments only, an optimized dose of sodium azide (75 µM) was added to selectively inhibit nitrite oxidizers [4]. The reaction was then initiated by adding 15 mg NH_3-N/L, and samples were collected every 30 minutes over a period of 3 hours at room temperature. Samples were analyzed for nitrite and total protein concentrations as described below. Plots of nitrite versus time were generated, and the slope of the line that gave the lowest residual square error was determined and called the nitrite

generation rate (NGR, mg N/L-min). Specific NGR (sNGR, mg N/mg protein-day) values were calculated by dividing NGRs by the protein concentration of the assay mixture. Experiments confirmed that minimal cell growth occurred over the 3 hour incubation period (data not shown). All AMO assays were performed in duplicate.

Toluene dioxygenase assay

Toluene dioxygenase (Tod) activity was measured by monitoring the conversion of indole to indigo by whole cell suspensions as described previously [5]. Briefly, cells from Ox^- and Ox^+ reactors were centrifuged at 12,000×g for 10 min at room temperature and resuspended in 1 X phosphate buffered saline (PBS) solution (7.6 g/L NaCl, 0.38 g/L NaH_2PO_4, 0.97 g/L Na_2HPO_4, pH 7.5). This procedure was repeated three (3) times to ensure complete removal of electron donor. The assay was performed at 37^0C for 60 min and was initiated by the addition of indole (100 μM). Cell associated indigo was extracted by resuspending cells in dimethyl-formamide (DMF) and centrifuging at 12,000×g for 5 min. The rate of indigo production was determined by measuring the optical density of the supernatant at 610 nm over time and converting to a molar basis using the extinction coefficient for indigo (ε = 15900 L/mol-cm) [6]. Specific rates of indigo production were obtained by normalizing rates using the protein concentration of the cell mixture. Experiments confirmed that minimal cell growth occurred over the 1 hour incubation period (data not shown). All Tod assays were performed in duplicate.

Catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O) assays

Intradiol and extradiol catechol dioxygenase activities were measured as described previously [7, 8]. Briefly, cells from Ox^- and Ox^+ reactors were prepared as per the Tod assay described above. The cell suspension was then subjected to six rounds of sonication (30 sec bursts at 30% output with 30 sec rest period on ice) using a Branson Sonifier 250 (Branson Ultrasonics Corp., Danbury, CT). Cell free extracts (CFE) were then obtained by centrifuging the lysed cell mixture at 12,000×g for 60 min at 4^0C. C12O activity was determined by adding catechol (4 mM) to the CFE and monitoring the rate of cis,cis muconate formation at OD_{260nm} over a 5 min period at room temperature. The conversion of 1 μmol of catechol to cis, cis

muconate results in a change of absorbance of 5.6 units at 260 nm [9]. C23O activity was determined by measuring the rate of 2-hydoxy-cis,cis muconic semialdehyde formation at OD_{375nm}. The conversion of 1 μmol of catechol to 2-hydoxy-cis,cis muconic semialdehyde results in a change of absorbance of 14.7 units at 375 nm [9]. Specific C12O and C23O activities were determined by normalizing to CFE protein content. All C12O and C23O assays were performed in duplicate.

EE2 biotransformation experiments with MBR and CBR biomass

Biomass (400 mL) from the MBR and the CBR was seeded into triplicate semi-continuous bioreactors (i.e. the bioreactors were fed continuously, but reactor volume was discharged only during sampling periods) and radiolabeled EE2 (24.5 μg/L) was applied to the system over a 48 hour period. Samples were obtained at 1, 24, and 48 hr intervals, passed through a 45 μm filter and lypholized at -50 ^{0}C prior to analysis. Alkaline traps (1 N KOH) were used to monitor mineralization of EE2 and subsequent metabolites. Samples (liquid, solids and alkaline trap) were analyzed as described for chemostat samples.

Specific oxygen uptake rate assay for heterotrophic inhibition

Forty mL of cells from Ox^-, Ox^+ and CNAS chemostats were harvested and aerated for 10 minutes prior to testing. Heterotrophic biomass (Ox^- and Ox^+) were supplemented with various concentrations of EE2 or TMP (0.01, 1, 10 mg/L) and acetate (0, 50 mg as COD/L) while CNAS biomass was tested without further substrate additions. Oxygen uptake rates (OURs) were determined in duplicate using Orion model 97-08 oxygen electrodes (Orion Research, Inc., Beverly, MA) connected to an Accumet dual channel pH/Ion meter (model AR25) such that DO readings were recorded every 6 seconds using an automated data acquisition system (Labview 6.0). The OUR is the slope of the line representing the least residual square error fit to the dissolved oxygen concentration versus time. Endogenous and solvent controls were also performed for heterotrophic cultures. Specific OUR (sOUR) values were determined by dividing the OUR values by the protein content of samples obtained at the end of each test. Percent inhibition was calculated as the difference between rates in the absence (control) and presence of EE2 or TMP divided by the control rate (x 100%):

$$\text{Percent Inhibition (\%) sOUR} = \frac{\text{sOUR}_{\text{control}} - \text{sOUR}_{\text{EE2 or TMP}}}{\text{sOUR}_{\text{control}}} \times 100\% \quad \text{(S-1)}$$

All experiments were performed in duplicate or quadruplicate and results were tested for statistical differences against control cultures (no pharmaceutical or nitrite) using the two-tailed student's t-test ($\alpha = 0.025$) using MS Excel®.

EE2 and TMP analyses by high performance liquid chromatography and UV-VIS diode array (LC-UV)

Non-radiolabeled EE2 and TMP were analyzed with a Hewlett Packard 1090 HPLC equipped with UV–VIS diode array detector. Separation was achieved using a Thermo Scientific Betabasic C_{18} column (100 x 2.1 mm i.d., 3.0 µm particle size) with a Thermo Scientific Betabasic C_{18} guard column (10 x 2.1 mm i.d., 3 µm particle size). The mobile phases are described in Table S1 and were delivered at a constant flow rate of 0.2 mL/min. Quantification was performed by constructing external standard curves (2 mg EE2/L to 200 ng EE2/L; 2 mg TMP/L to 0.01 mg TMP/L) with use of internal standards. Recovery of spiked additions exceeded 85% for all pharmaceuticals tested.

TMP analysis by liquid chromatography/mass spectrometry (LC/MS)

Solid phase extraction (SPE) was performed by loading pre-conditioned (with 6 mL of acetonitrile followed by 6 mL of pure water) Oasis® HLB cartridges with samples, followed by elution with 4 mL acetonitrile (2x) into a glass tube. Extracts were evaporated under nitrogen to 0.2 mL and then diluted with nanopure™ water to a final sample volume of 1 mL. The samples were analyzed using a LCQ Advantage™ ion trap mass spectrometer (LC-ITMS) with an electrospray ionization (ESI) source in positive ion mode (Thermo Finnigan, San Jose, CA, USA). Separation was achieved using a Thermo Scientific Betabasic-18 C_{18} column, (100 X 2.1 mm i.d, 3 µm particle size), equipped with a Thermo Scientific guard cartridge (10 X 2.1 mm i.d., 3 µm particle size), using a gradient mobile phase consisting of acetonitrile (A) and water with 0.3% formic acid (B). The initial conditions were 5% A and 95% B and after one minute A was increased to 50% over 4 minutes and then A was further increased to 70% over 6 minutes.

The initial mobile phase condition was then restored within one minute and run for 8 more minutes to maintain the condition. The flow rate was set at 250 µL/min, the injection volume was 10 µL, and the column oven temperature was held at 30°C. The total run time was 20 minutes including post-run protocols.

For additional verification of the proposed M220 metabolite in Figure S4, H/D exchange experiments were performed. Samples prepared above were diluted with 200 mL of pure water then loaded to pre-conditioned Oasis® HLB cartridges. The loaded samples were eluted twice with 4 mL of acetonitrile into a glass tube, evaporated under nitrogen to 0.2 mL and then diluted with deuterium oxide (Cambridge Isotope Laboratories, Inc., Andover, MA) to a final sample volume of 1 mL. Separation was achieved using the same mobile phases described above for TMP except that water was replaced with deuterium oxide with 0.3% d4-acetic acid buffer (Cambridge Isotope Laboratories, Inc., Andover, MA). During H/D exchange, 4 exchangeable protons were replaced in TMP forming [d4-TMP]+D^+ with 296 *m/z,* while no exchangeable deuterium was observed for M220 metabolite forming [M220]+D^+ with 222 *m/z*.

Analysis by liquid chromatography/ mass spectrometry (LC/MS) and LC/ radiochromatographic detection

Lyophilized samples were reconstituted in 45 mL of HPLC-grade methanol (Honeywell B & J, Muskegon, MI) and extracted for analysis by LC/radiochromatographic detection and LC/MS. Since all samples contained ^{14}C-labelled EE2 analysis was performed using an Agilent 1100 HPLC equipped with an on-line radiochromatographic detector (IN/US Systems, Inc., Tampa, FL) as described previously [10].

Once the retention times of the radioactive peaks were identified an aliquot of the sample was re-injected into the LC column with the eluate being split between the radioactive detector and a triple quadrupole mass spectrometer (Agilent 6410 MSD). The splitter was put in place to ensure that the LC/MS data corresponded with the radioactive peaks. All LC conditions were the same. LC/MS analysis was carried out under full scan monitoring of ions from *m/z* 100 to 600 in electrospray ionization (ESI) negative mode. The triple quadrupole mass spectrometer was equipped with nitrogen as a drying gas at a temperature of 350^oC and flow rate of 12 L/min. The capillary spray voltage used was 4000 V and the nebulizer pressure was set at 35 psi. Use of LC/MS in conjunction with a radioactive detector allows for identification of the *m/z* ratios for specific EE2-related metabolites.

Liquid chromatography/ ion-trap mass spectrometry (LC-ITMS) analysis of EE2 and M376 metabolite

Previously extracted and reconstituted samples bioreactors were characterized by LC ion-trap mass spectrometer (LC-ITMS, Thermo Finnigan LCQ Advantage LC with ion-trap MS). Separation was obtained using a reversed phase BetaBasic-18 column (100 X 2.1 mm 3 um; Thermo Scientific, CA, USA). The starting mobile phase, 20:80 (v:v) MeOH/H_2O, was held constant for 1 min followed by an increase to 100% MeOH over 25 minutes at a flow rate of 0.2 mL/min. MS^n fragmentation of the M376 metabolite and EE2 standard was obtained by in-source collision-induced dissociation in negative ESI mode.

The parent ion, *m/z* 375, was selected from the full scan method described beforehand and in LC-ITMS subjected to MS^n fragmentation. LC-ITMS characterization of M376 shown in Figure S1 provides additional verification for the sulfate-EE2 structure proposed by Yi and Harper, 2007. The MS^2 fragmentation suggests M376 contains a sulfate group by loss of 80 (SO_3; Figure S1A) leaving EE2 *m/z* 295 confirmed by MS^3 of *m/z* 375→295 (Figure S1B). The MS transitions of *m/z* 295 from the MS^3 (*m/z* 375→295) of selected bioreactor samples can be matched to those of a standard EE2 (Figure S1C) validating the sulfate-EE2 structure (Table S9).

Nuclear Magnetic Resonance (NMR) Spectroscopy Analysis of EE2 Metabolite

EE2 and metabolites identified through mass spectrometry were eluted from OASIS® HLB cartridges (500 mg) using acetone and subsequently evaporated to dryness. Samples were reconstituted in H_2O/Acetonitrile (ACN) (90%/10%) and initially fractionated via HPLC (Agilent 1100, CA, USA). Separation was achieved with a reversed phase C-18 column (Agilent ZORBAX C8 5µm, 4.6 x 250mm) using a gradient elution starting with methanol and water (40/60%) to 100% methanol over a 15 minute period at a constant flow rate of 0.4 mL/min. Fractions were collected every 30 seconds and further analyzed using an LC ion trap mass spectrometer system (Thermo Finnigan, CA, USA) to verify presence and purity of the M312 metabolite. Each fraction was directly injected with an isocratic gradient of methanol and water (80%/20%). Mass spectrometry was performed in negative mode using full scan monitoring of *m/z* from 100-600. Fractions containing metabolites were evaporated to dryness, reconstituted in

D_6-DMSO and transferred to NMR tubes. ^1H NMR data was performed with Inova-500 MHz NMR Spectrometer (Varian, CA, USA). Results are given in Figure S2.

Wet chemistry analytical methods

Total and volatile suspended solids (TSS and VSS) were performed as described previously [11]. Cell counts for *N. europaea w*ere performed using a Helber bacteria single round cell counting chamber manufactured by Weber Scientific International (Middlesex, UK). Total protein content was measured using the micro-bicinchoninic acid (BCA) method modified with an alkaline digestion step [12]. Briefly, cells were resuspended to a final concentration of 1 M NaOH and incubated at 105^0C for 15 min prior to addition of microBCA reagents. All standards were prepared in 1N NaOH and supplemented with chloramphenicol or sodium azide as necessary per experimental conditions. Total protein content was used to monitor biomass concentrations in reactors receiving radiolabeled substrates.

Samples for nitrogen analyses were filtered through 0.45 μm filters and frozen (nitrite and nitrate) or acidified with 5N H_2SO_4 to pH 2 and stored at 4^0C for up to 1 month. Nitrite and ammonia concentrations were analyzed colorimetrically in duplicate using the Griess reagent and phenate methods, respectively [11]. Nitrate was determined via ultraviolet spectrophotometric detection at 229 nm [13]. To account for nitrite interference, samples were acidified with concentrated H_2SO_4 and total oxidized nitrogen was measured. Nitrate concentrations were determined by subtracting nitrite concentrations obtained from Greiss assays from the total oxidized nitrogen concentration. Controls were performed using spiked additions to determine the percent recovery of standards in the matrix of the samples; recoveries always exceeded 85%.

Soluble chemical oxygen demand was measured via the closed reflux method as described previously [11] with minor modifications. Briefly, samples were filtered through 0.45 μm filters and acidified with 5N H_2SO_4 to pH 2 and stored at 4^0C for up to 1 month. Upon addition of reagents (17 mM $K_2Cr_2O_7$, 5.6 mM $HgSO_4$, 32 mM $AgSO_4$), samples were digested at 150^0C for 2 hours and then cooled to room temperature. At this point, samples were analyzed via spectrophotometry at 600 nm. External standard curves were constructed using potassium hydrogen phthalate (0 – 500 mg as COD/L) and sample concentrations were determined by direct comparison to this standard curve. Spiked additions were also performed during each digestion and used to determine the percent recovery, which always exceeded 90%.

Liquid scintillation counts for radiolabeled experiments were performed on an LS1800 Beckman Coulter system (maximum counting time of 5 minutes) by adding 0.1 mL of sample and 5 mL of Scintiverse^{TM} BD scintillation cocktail (Fisher Scientific, USA). Quench was accounted for by using external quench standards and sample to channel ratio. This protocol was adopted after extensive testing of alternative sample to cocktail ratios.

FIGURES

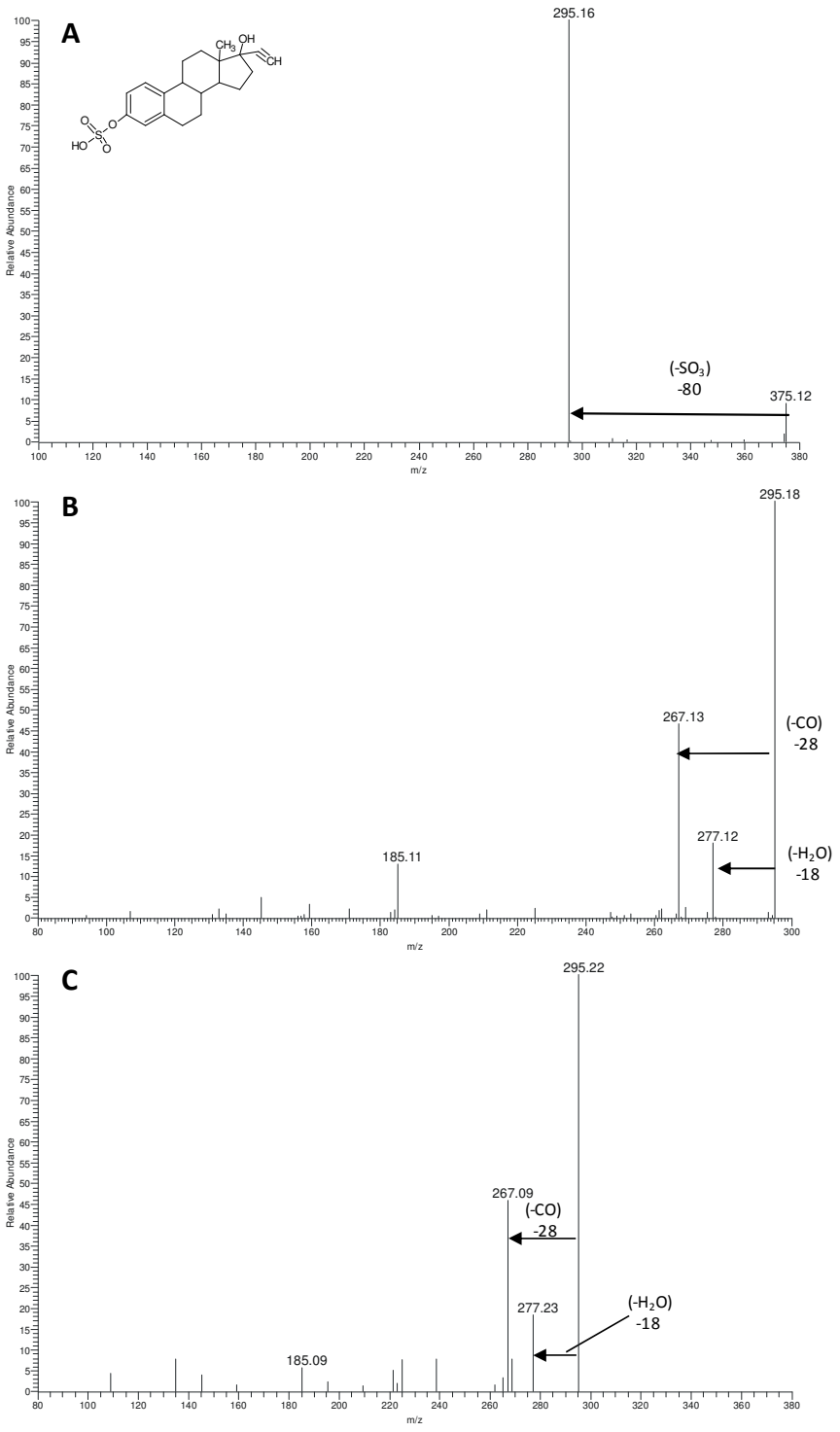


Figure S1. MS^n spectra of M376 metabolite. A: *m/z* 375, B: *m/z* 375→295 and C: *m/z* 295 from EE2 standard.

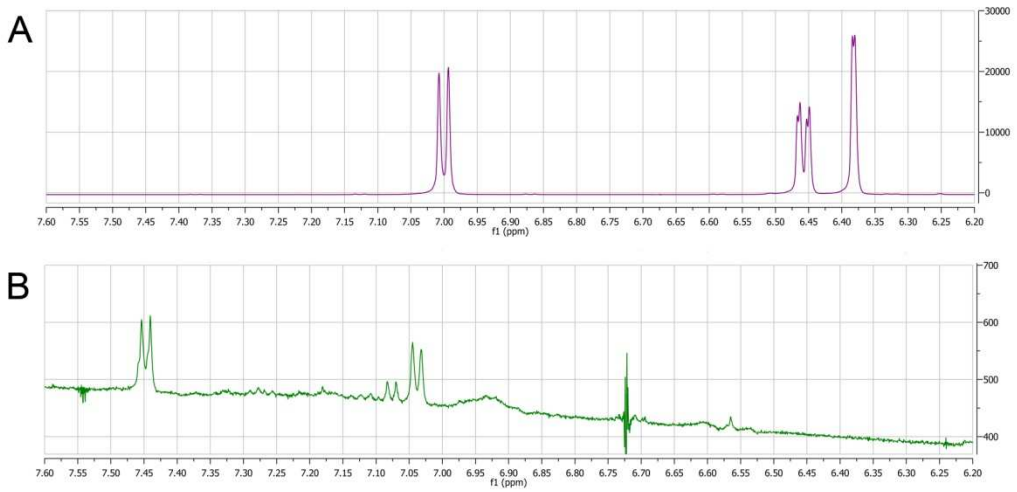


Figure S2. ^{1}H-NMR spectra of A: EE2 (m/z 295) and B: 4-OH-EE2 (*m/z* 311). Note doublet in B means that the aromatic protons are adjacent to each other.

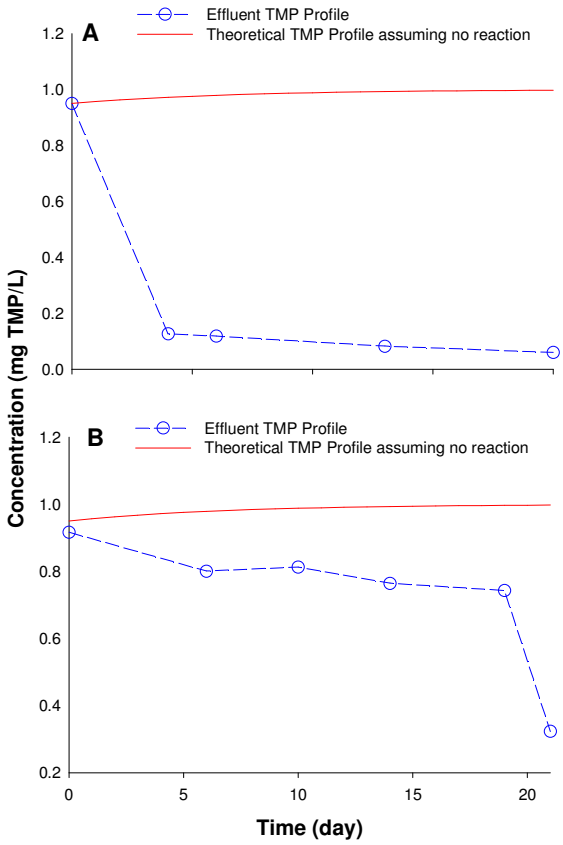


Figure S3. TMP profile for heterotrophic chemostat reactors. A: Ox^- reactor fed 1000 mg sCOD/L and 1 mg TMP/L, B: Ox^+ reactor fed 1000 mg sCOD/L and 1 mg TMP/L. Results are representative of profiles observed in duplicate reactors.

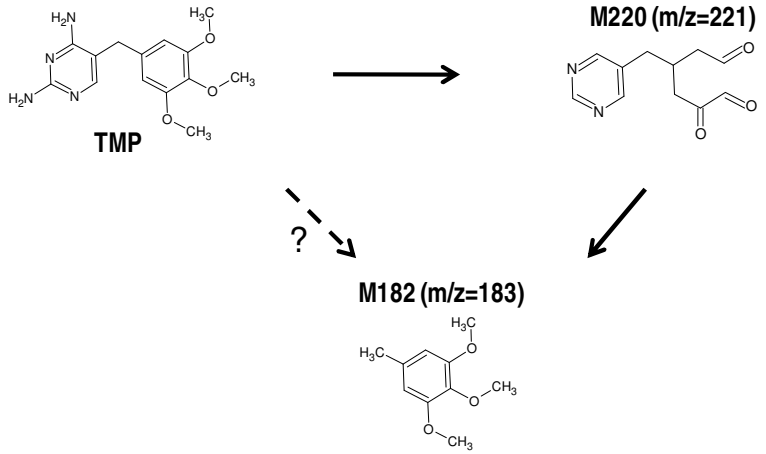


Figure S4. Transformation products of TMP generated in heterotrophic reactors. M220: 2-oxo-4-(pyrimidin-5-methyl)hexanedial, M182: 1,2,3-trimethoxy-5-methylbenzene.

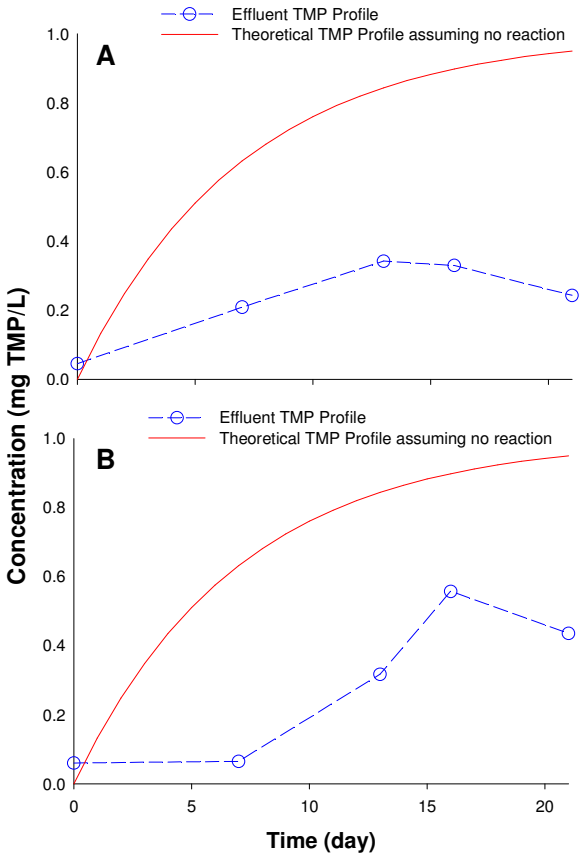


Figure S5. TMP profile for heterotrophic chemostat reactors fed effluent from AOB chemostat. A: Ox^- reactor fed effluent from AOB reactor, B: Ox^+ reactor fed effluent from AOB reactor. Results are representative of profiles observed in duplicate reactors.

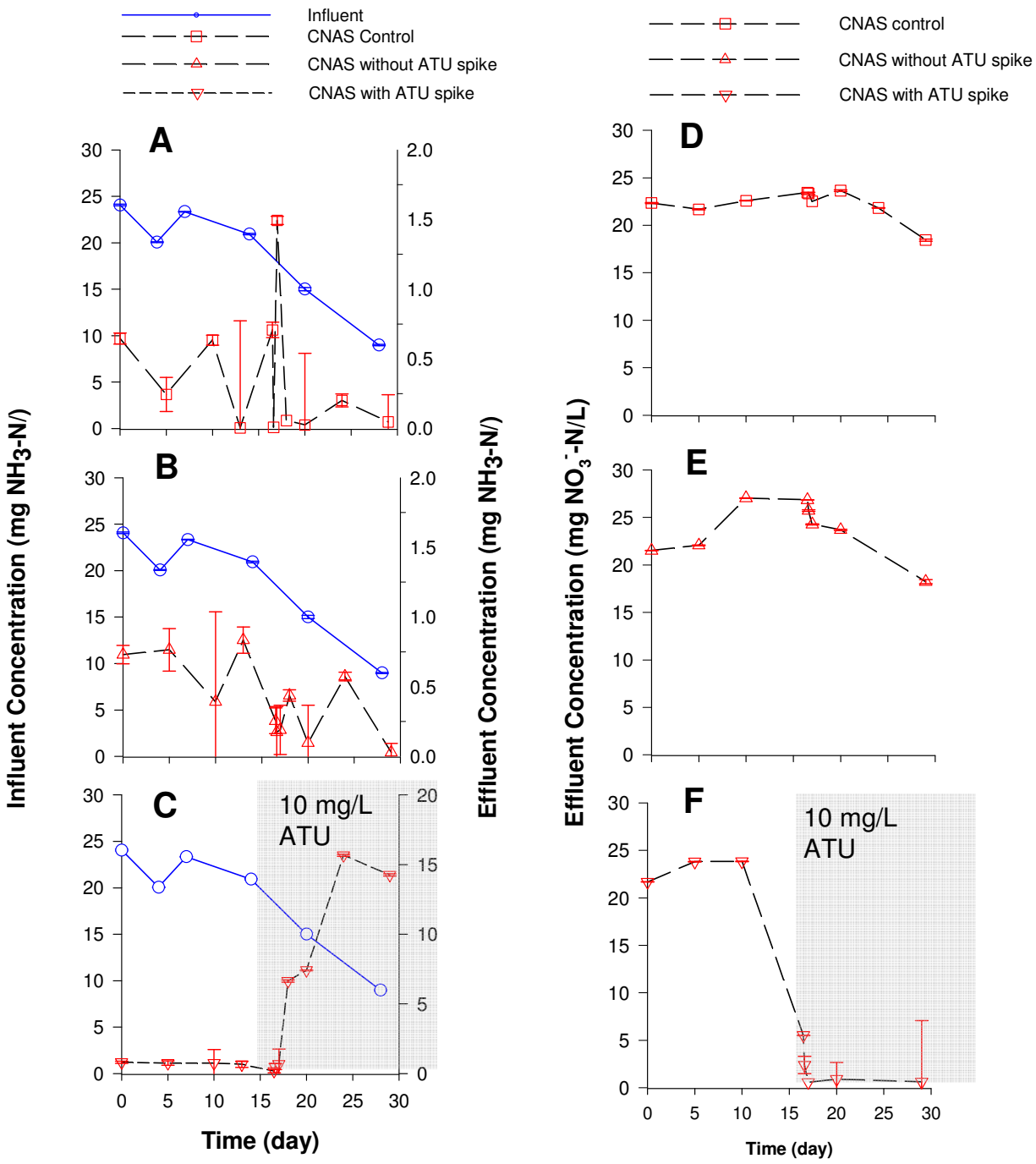


Figure S6. Ammonia and nitrate concentration for CNAS cultures subjected to AOB inhibition experiments. A and D: CNAS control receiving no EE2 or TMP supplementation, B and E: CNAS receiving 500 ng EE2/L and 500 ng TMP/L, C and F: CNAS receiving 500 ng EE2/L and 500 ng TMP/L and subject to ATU continuous addition from day 15 onwards. Results are representative of duplicate chemostats except for control reactor, which was not replicated.

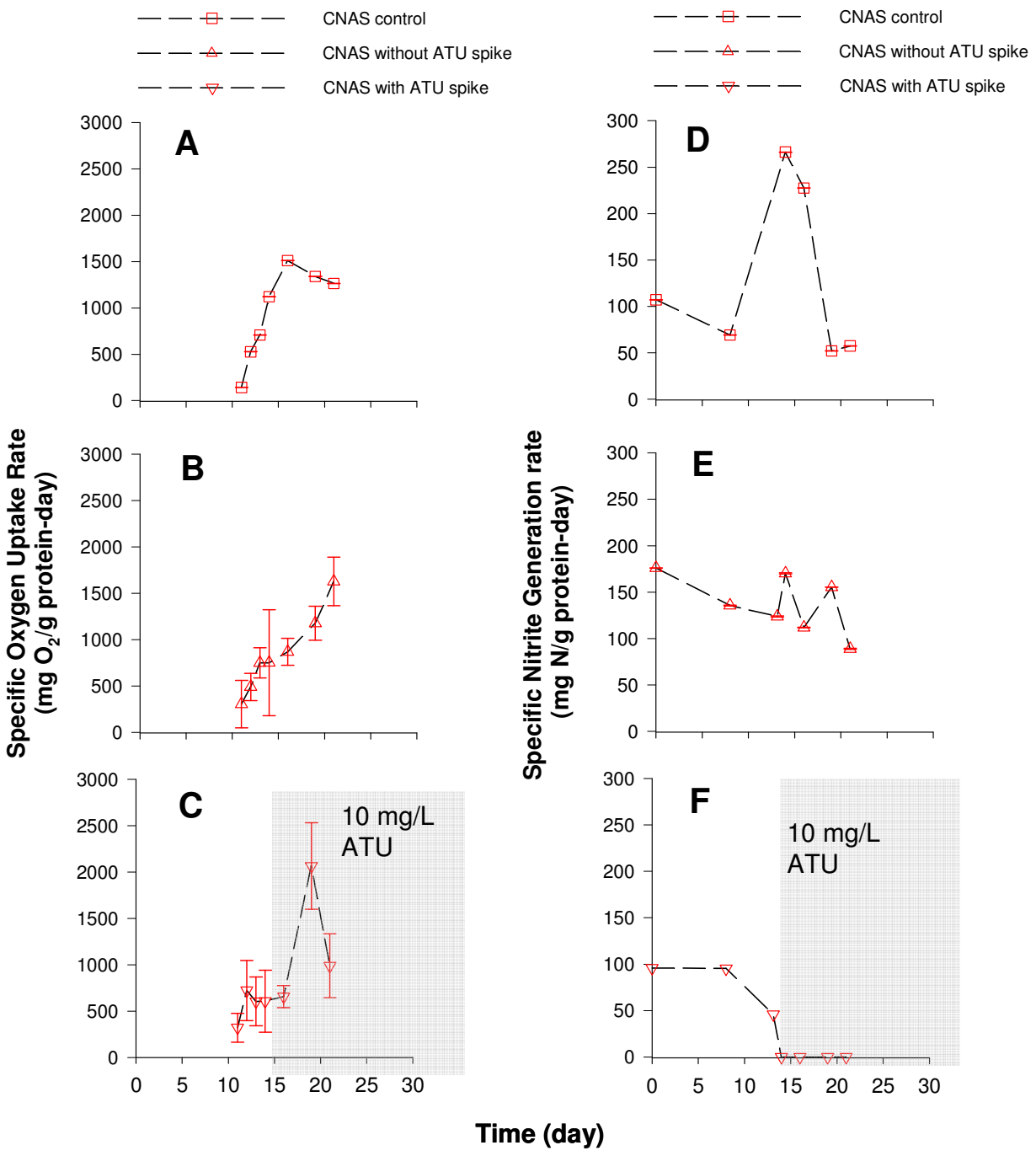


Figure S7. sOUR and sNGR activity measurements for CNAS cultures subjected to AOB inhibition experiments. A and D: CNAS control receiving no EE2 or TMP supplementation, B and E: CNAS receiving 500 ng EE2/L and 500 ng TMP/L, C and F: CNAS receiving 500 ng EE2/L and 500 ng TMP/L and subject to ATU continuous addition from day 15 onwards. Results are representative of duplicate chemostats except for the control reactor, which was not replicated.

TABLES

Table S1. Conditions used for HPLC-UV detection of pharmaceuticals. .

Compound	Internal Standard	Mobile Phase	Runtime (min)	UV Detection Wavelength (nm)	% Recoveries
EE2	17β-estradiol; 0.1 mg/L	Acetonitrile (35%)/NANOpure™ water (65%)	15	254, 281	95-102
TMP	Allythiourea; 10 mg/L	Methanol (11%) / 20 mM NaH_2PO_4 (89%)	45	231, 254	85-104

Table S2. Specific nitrite generation rates as indication of ammonia monooxygenase (AMO activity in AOB cultures when exposed to EE2 or TMP.

Concentration (μg/L)	sNGR (mg N/g protein.day)			
	EE2		TMP	
	Exponential Phase cells	Chemostat cells	Exponential Phase cells	Chemostat cells
0	1140 ± 370	630 ± 140	2380 ± 980	980 ± 150
0.01	930 ± 290	490 ± 60	1930 ± 0.00	670 ± 40
10	1250 ± 120	570 ± 60	1560 ± 490	880 ± 210
1000	1060 ± 70	470 ± 10	1450 ± 690	780 ± 100
Acetone Control	1220 ± 320		N/A	

N/A – TMP stock solution was prepared in Nanopure™ water.

Rates were calculated from duplicate reactor vessels and are reported as $\bar{x} \pm RMD$ where RMD is the relative mean difference.

Table S3. Specific oxygen uptake rates for heterotrophic chemostat cultures in the presence of EE2 or TMP.

Concentration (mg EE2 or TMP/L)	sOUR (mg O_2/g protein.day)			
	EE2		TMP	
	Ox^- culture	Ox^+ culture	Ox^- culture	Ox^+ culture
0	283 ± 61	274 ± 34	68 ± 9	84 ± 28
0.01	309 ± 123	440 ± 31	96 ± 16	68 ± 14
1	348 ± 161	443 ± 112	98 ± 11	99 ± 28
10	265 ± 88	472 ± 45	79 ± 19	71 ± 16
Acetone (46 mg/L)	300 ± 68	503 ± 47	N/A	

N/A – TMP stock solution was prepared in Nanopure™ water.

Rates were calculated from triplicate or quadruplicate reactor vessels and are reported as $\bar{x} \pm s_x$ where s_x is the standard deviation.

Table S4. Summary of oxygenase activity in Ox^- and Ox^+ chemostat cultures.

		Ox^- culture[1]	Ox^+ culture[2]	AOB-Ox^- culture[3]	AOB-Ox^+ culture[4]
Catechol 1,2 Oxygenase (Ortho) activity (μM catechol consumed/g protein-hr)	Avg. from EE2 experiments	1.48 ± 1.06	93.7 ± 17.2	2.63 ± 0.43	54.4 ± 54.5
	Avg. from TMP experiments	1.59 ± 0.23	86.3 ± 95.7	2.50 ± 1.18	90.4 ± 91.5
Catechol 2,3 Oxygenase (Meta) activity (μM catechol consumed/g protein-hr)	Avg. from EE2 experiments	5.77 ± 2.93	136 ± 53.6	0.10 ± 0.11	4.42 ± 3.47
	Avg. from TMP experiments	3.18 ± 2.64	73.3 ± 5.58	0.09 ± 0.08	24.5 ± 2.08
Toluene Dioxygenase activity (μmol indigo/g protein-hr)	Average from EE2 and TMP experiments	Below detection	0.09 ± 0.05	Below detection	N/D

[1] Ox^- culture fed 1000 mg acetate as COD/L.
[2] Ox^+ culture fed 333 mg acetate as COD/L, 333 mg benzoate as COD/L and 333 mg toluene as COD/L.
[3] Ox^- culture fed AOB reactor effluent supplemented with 1000 mg acetate as COD/L.
[4] Ox^+ culture fed AOB reactor effluent supplemented with 333 mg acetate as COD/L, 333 mg benzoate as COD/L and 333 mg toluene as COD/L.
N/D – Not determined.
Rates were calculated from duplicate reactor vessels and are reported as $\bar{x} \pm RMD$ where RMD is the relative mean difference.

Table S5. Comparison of the rate of EE2 and TMP applied to heterotrophic chemostats versus rate of catechol dioxygenation.

	Ox^-	Ox^+	AOB-Ox^-	AOB-Ox^+
	EE2 Experiments			
Rate of EE2 applied to system (mg EE2 as COD/day)	0.78			
Rate of Catechol 1,2 Dioxygenation (mg O_2/day)	0.68	42.92	1.20	24.92
Rate of Catechol 2,3 Dioxygenation (mg O_2/day)	2.20	51.91	0.04	1.69
	TMP Experiments			
Rate of TMP applied to system (mg TMP as COD/day)	0.44			
Rate of Catechol 1,2 Dioxygenation (mg O_2/day)	0.73	39.53	1.15	41.41
Rate of Catechol 2,3 Dioxygenation (mg O_2/day)	1.21	27.98	0.03	9.35

Table S6. Specific oxygen uptake rates for Ox^- and Ox^+ cultures exposed to various concentrations of nitrite.

Concentration (mg NO_2^--N/L)	**sOUR (mg O_2/g protein.day)[a]**	
	Ox^- culture	**Ox^+ culture**
0	527 ± 67	335 ± 42
10	506 ± 74	278 ± 27
200	375 ± 92	274 ± 98
600	359 ± 36	281 ± 85

[a]Rates were calculated from triplicate or quadruplicate reactor vessels and are reported as $\bar{x} \pm s_x$ where s_x is the standard deviation.

Table S7. Fractionation of ^{14}C constituents at three operational time-points in representative chemostats[1].

		Activity in Effluent fractions (μCi)								Total Activity in Trap (μCi)	Total Activity in Solids (μCi)	% Recovered	% Contribution to Overall ^{14}C activity									Avg % Recovery
		1	2	3	4	5	U1	U2	Total					1	2	3	4	5	U1	U2	CO_2	Solids	
Ox⁻ (Data for Figure 1A)	t_0	0.01	0.01	0.29	0.00	0.00	0.01	0.00	0.3	0.96	0.15	61.64		0.56	0.42	12.50	0.00	0.00	0.56	0.00	41.29	6.46	85.2
	t_1	0.04	0.03	0.85	0.00	0.00	0.04	0.00	0.9	2.81	0.53	80.59		0.71	0.53	15.97	0.00	0.00	0.71	0.00	52.86	9.98	26.2
	t_2	0.08	0.06	1.89	0.00	0.00	0.08	0.00	2.1	4.90	0.90	113.37		1.21	0.90	27.12	0.00	0.00	1.21	0.00	70.32	12.92	
													Avg	***0.82***	***0.62***	***18.53***	***0.00***	***0.00***	***0.82***	***0.00***	***54.82***	***9.79***	
													Stdev	***0.34***	***0.25***	***7.64***	***0.00***	***0.00***	***0.34***	***0.00***	***14.61***	***3.23***	
Ox⁺ (Data for Figure 1A)	t_0	0.19	0.67	0.00	0.00	0.00	0.00	0.08	0.9	0.11	0.25	70.55		10.19	36.16	0.00	0.00	0.00	0.00	4.58	6.10	13.52	88.2
	t_1	0.46	1.62	0.00	0.00	0.00	0.00	0.21	2.3	0.50	0.89	82.59		10.26	36.44	0.00	0.00	0.00	0.00	4.62	11.22	20.05	21.1
	t_2	0.69	2.46	0.00	0.00	0.00	0.00	0.31	3.5	3.69	1.10	111.52		9.35	33.20	0.00	0.00	0.00	0.00	4.21	49.89	14.87	
													Avg	***9.93***	***35.27***	***0.00***	***0.00***	***0.00***	***0.00***	***4.47***	***22.40***	***16.14***	
													Stdev	***0.51***	***1.80***	***0.00***	***0.00***	***0.00***	***0.00***	***0.23***	***23.94***	***3.45***	
AOB (Data for Figure 2A)	t_0	0.04	0.06	0.72	0.07	0.00	0.00	0.00	0.9	0.0	0.0	90.0		3.60	5.40	69.28	7.20	4.50	0.00	0.00	0.00	0.00	91.0
	t_1	0.08	0.11	1.47	0.15	0.00	0.00	0.00	1.9	0.0	0.0	75.9		3.04	4.56	58.47	6.08	3.80	0.00	0.00	0.00	0.00	15.6
	t_2	0.13	0.20	2.57	0.27	0.00	0.00	0.00	3.3	0.0	0.0	107.1		4.28	6.42	82.45	8.57	5.35	0.00	0.00	0.00	0.00	
													Avg	***3.64***	***5.46***	***70.07***	***7.28***	***4.55***	***0.00***	***0.00***	***0.00***	***0.00***	
													Stdev	***0.62***	***0.94***	***12.01***	***1.25***	***0.78***	***0.00***	***0.00***	***0.00***	***0.00***	
Ox⁻ (Data for Figure 2B)	t_0	0.01	0.01	0.04	0.0	0.00	0.0	0.00	0.1	0.009	0.020	83.7		7.53	6.99	39.24	0.00	0.00	0.00	0.00	9.15	20.82	79.8
	t_1	0.02	0.02	0.12	0.0	0.00	0.0	0.00	0.2	0.007	0.040	63.1		6.87	6.38	35.84	0.00	0.00	0.00	0.00	2.15	11.90	15.1
	t_2	0.03	0.03	0.16	0.0	0.00	0.0	0.00	0.2	0.120	0.060	92.5		7.12	6.62	37.15	0.00	0.00	0.00	0.00	27.76	13.88	
													Avg	***7.17***	***6.66***	***37.41***	***0.00***	***0.00***	***0.00***	***0.00***	***13.02***	***15.53***	
													Stdev	***0.33***	***0.31***	***1.72***	***0.00***	***0.00***	***0.00***	***0.00***	***13.24***	***4.69***	
Ox⁺ (Data for Figure 2C)	t_0	0.01	0.0	0.07	0.0	0.01	0.01	0.0	0.1	0.0	0.0	140.9		5.87	0.00	74.37	0.00	8.81	8.81	0.00	22.22	20.82	104.5
	t_1	0.01	0.0	0.10	0.0	0.01	0.01	0.0	0.1	0.1	0.0	66.3		2.06	0.00	26.09	0.00	3.09	3.09	0.00	21.55	10.41	37.3
	t_2	0.02	0.0	0.20	0.0	0.02	0.02	0.0	0.3	0.2	0.1	106.4		3.61	0.00	45.71	0.00	5.41	5.41	0.00	34.70	11.57	
													Avg	***3.85***	***0.00***	***48.72***	***0.00***	***5.77***	***5.77***	***0.00***	***26.16***	***14.27***	
													Stdev	***1.92***	***0.00***	***24.28***	***0.00***	***2.88***	***2.88***	***0.00***	***7.41***	***5.71***	

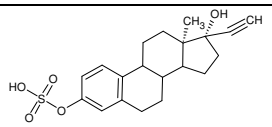
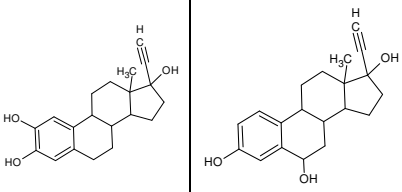
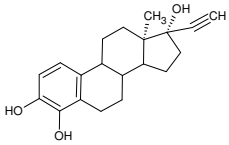
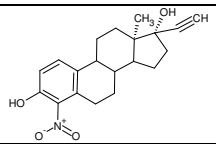
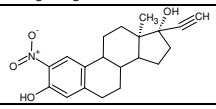
[1]This data is calculated from three individual timepoints (1, 2 and 2.5 weeks into operation) and does not represent the cumulative ^{14}C mass balance. See Table S8 for the cumulative mass balance.

Table S8. Cumulative ^{14}C mass balances on all reactors in this study[1].

	Standalone experiments					Sequential experiments					
	Ox^-			Ox^+		AOB	Ox^-	Ox^+	AOB	Ox^-	Ox^+
Total activity in experiment (μCi)	7.58	11.25	8.68	10.51	7.19	2.24	0.48	0.48	2.96	0.38	1.38
Total activity in traps (μCi)	4.92	6.73	5.57	6.08	3.95	0.00	0.12	0.16	0.00	0.14	0.41
Total activity in effluent/reactor (μCi)	2.11	3.41	1.34	2.84	2.11	2.01	0.22	0.18	2.42	0.26	0.54
Activity in solids (μCI)	0.93	1.79	1.60	1.10	1.07	0.00	0.06	0.07	0.00	0.05	0.19
Total recovered activity (μCi)	7.96	11.93	8.51	10.02	7.13	2.01	0.40	0.41	2.42	0.45	1.14
Percent recovery with solids (%)	**105.00**	**106.00**	**98.04**	**95.34**	**99.12**	**89.73**	**83.33**	**84.79**	**81.76**	**118.42**	**82.61**
Percent contribution to total ^{14}C added to experiments: Mineralization (%)	**64.91**	**59.82**	**64.17**	**57.85**	**54.91**	**0.00**	**25.00**	**33.33**	**0.00**	**36.84**	**29.71**
Percent contribution to total ^{14}C added to experiments: Solids (%)	**12.29**	**15.87**	**18.43**	**10.47**	**14.88**	**0.00**	**12.50**	**13.96**	**0.00**	**13.16**	**13.77**
Percent contribution to total ^{14}C added to experiments: Aqueous (%)	**27.80**	**30.31**	**15.44**	**27.02**	**29.33**	**89.73**	**45.83**	**37.50**	**81.76**	**68.42**	**39.13**

[1]-Data in this table represents the cumulative mass balance on ^{14}C in each system,

Table S9. Description of EE2 transformation products generated in this study.

Product	$[M-H]^-$	Proposed structure	IUPAC name		Ref.
sulfo-EE2 ($C_{20}H_{24}O_5S$)	375		(17-ethynyl-17-hydroxy-13-methyl-7,8,9,11,12,14,15,16-octahydro-6H-cyclopenta[a]phenanthren-3-yl) hydrogen sulfate		[14]
Mono-OH-EE2 ($C_{20}H_{24}O_3$)	311		17-ethynyl-13-methyl-7,8,9,11,12,14,15,16-octahydro-6H-cyclopenta[a]phenanthrene-2,3,17-triol	17-ethynyl-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthrene-2,3,17-triol	[10, 14, 15]
4-OH-EE2 ($C_{20}H_{24}O_3$)	311		17-ethynyl-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthrene-3,4,17-triol		This study
4-nitro-EE2 ($C_{20}H_{23}NO_4$)	340		17-ethynyl-13-methyl-4-nitro-7,8,9,11,12,14,15,16-octahydro-6H-cyclopenta[a]phenanthrene-3,17-diol		[10, 16]
2-nitro-EE2 ($C_{20}H_{23}NO_4$)	340		17-ethynyl-13-methyl-2-nitro-7,8,9,11,12,14,15,16-octahydro-6H-cyclopenta[a]phenanthrene-3,17-diol		[10, 16]

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