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

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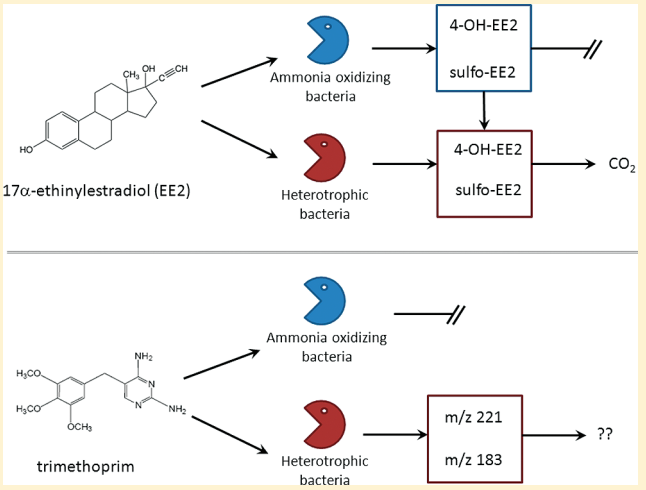
Supporting Information

ABSTRACT: The biological fate of 17α-ethinylestradiol (EE2; 500 ng/L to 1 mg/L) and trimethoprim (TMP; 1 μg/L to 1 mg/L) was evaluated with flow through reactors containing an ammonia oxidizing bacterial (AOB) culture, two enriched heterotrophic cultures devoid of nitrifier activity, and nitrifying activated sludge (NAS) cultures. AOBs biotransformed EE2 but not TMP, whereas heterotrophs mineralized EE2, biotransformed TMP, and mineralized EE2-derived metabolites generated by AOBs. Kinetic bioassays showed that AOBs biotransformed EE2 five times faster than heterotrophs. The basal expression of heterotrophic dioxygenase enzymes was sufficient to achieve the high degree of transformation observed at EE2 and TMP concentrations ≤ 1 mg/L, and enhanced enzyme expression was not necessary. The importance of AOBs in removing EE2 and TMP was evaluated further by performing NAS experiments at lower feed concentrations (500−1000 ng/L). EE2 removal slowed markedly after AOBs were inhibited, while TMP removal was not affected by AOB inhibition. Two key EE2 metabolites formed by AOB and heterotrophic laboratory-scale chemostats were also found in independent laboratory-scale mixed culture bioreactors; one of these, sulfo-EE2, was largely resistant to further biodegradation. AOBs and heterotrophs may cooperatively enhance the reliability of treatment systems where efficient removal of EE2 is desired.



INTRODUCTION

Researchers have observed that removal of pharmaceutically active trace organic contaminants during activated sludge treatment is enhanced in systems that are nitrifying.[1,2] Since nitrification is principally employed by wastewater treatment plants (WWTPs) to help meet nitrogen effluent guidelines, simultaneous removal of pharmaceuticals during nitrification is an unexpected bonus. 17α-Ethinylestradiol (EE2; a potent endocrine disruptor) and trimethoprim (TMP; an antibiotic) are examples of pharmaceuticals that may experience enhanced removal during nitrification[3−6] and are the focus of this study. EE2 is a potent synthetic estrogen present in wastewaters, and its adverse impact on aquatic species in receiving streams has been demonstrated.[7,8] TMP is typically used to treat urinary tract infections[9] and has been linked to the development of antibiotic resistance downstream of treatment plant effluents.[10]

EE2 (0.4−86 ng/L) and TMP (80−1300 ng/L) are commonly encountered in sewage.[11,12] TMP sorbs poorly to biomass,[13] but its loss has been demonstrated in nitrifying activated sludge (NAS) containing both active ammonia oxidizing bacteria (AOB) and heterotrophic bacteria.[3] Factors controlling EE2 fate remain controversial. Cirja et al.[14] proposed that sorption is the primary removal mechanism for EE2, whereas many other studies claim that biotransformation is primarily responsible; however, the nature of the biotransformation is unclear. Gaulke et al.[15] claim that AOB are incapable of EE2 biotransformation at low concentrations typically found in NAS and that heterotrophic bacteria are responsible instead.

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Several other studies conducted at concentrations higher than are typically found in NAS show that pure AOB cultures,[16,17] enriched ammonia oxidizing cultures,[5,6] and NAS cultures[4,18] are all capable of biotransforming EE2 and suggest their results may translate to full-scale NAS systems. To date, however, experiments designed to clarify the relative contribution of ammonia oxidizing and heterotrophic bacteria to EE2 or TMP biodegradation in NAS have not been clearly elucidated at ng/L concentrations. This study attempts to address the relative roles of these distinct functional microbial groups as it pertains to TMP and EE2.

Knowing the relevant functional microbial groups responsible for EE2 and TMP removal and the biodegradation kinetics for each can inform strategies for bioreactor design and operation that are geared toward enhancing pharmaceutical removal. We sought to apply our current understanding about aerobic catabolic pathways associated with substituted aromatic xenobiotic compounds and the importance of oxygenase enzymes in the biodegradation of these two pharmaceuticals, both which contain aromatic rings.[19−21] We hypothesized that both AOBs and heterotrophs contribute to the biotransformation of EE2 and TMP by NAS. To test this hypothesis, we evaluated the relative contribution of AOBs and heterotrophs in EE2 and TMP biotransformation using both single-culture and mixed-culture experiments. First, we separated the microbial groups (pure AOB or enriched heterotrophic cultures devoid of nitrifying activity) and evaluated the metabolic fate of EE2 and TMP removal in chemostat cultures operated alone or in series. Next, we evaluated the removal of EE2 + TMP in a mixed culture (nitrifiers + heterotrophs) chemostat. Despite using a range of reactor configurations and different feed concentrations of pharmaceuticals (from 500 ng/L to 1 mg/L), the results were quite consistent and provide an improved understanding of the relative role that AOBs and heterotrophs are likely to play in EE2 and TMP biodegradation in full-scale activated sludge systems.

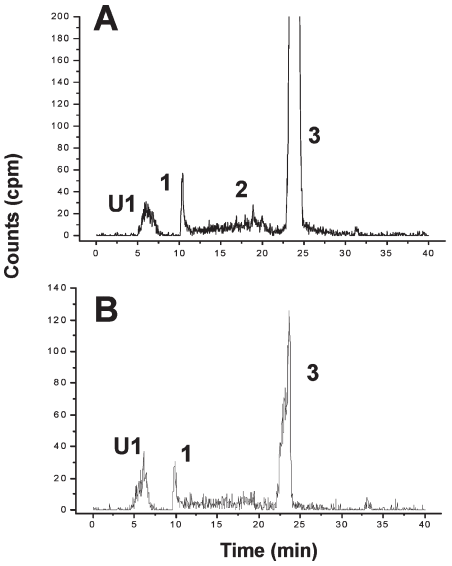
MATERIALS AND METHODS

Cultures. Four cultures were used for this study. *Nitrosomonas europaea* (ATCC strain 19718; designated AOB) was grown in batch prior to initiating chemostat growth conditions (dilution rate, $D = 0.14\ day^{-1}$), as described previously.[17] Master heterotrophic cultures were maintained in chemostats ($D = 0.14\ day^{-1}$) and fed either acetate to generate minimal oxygenase activity (cultures are designated Ox^-) or a mixture of benzoate, toluene, and acetate to generate enriched oxygenase activity (cultures are designated Ox^+). Nitrifying activity was inhibited in the Ox^- and Ox^+ cultures through continuous addition of 10 mg/L allylthiourea (ATU), a potent inhibitor of ammonia oxidizing bacteria. A nitrifying activated sludge culture was maintained in a chemostat ($D = 0.1\ day^{-1}$) and fed primary effluent from a local WWTP with minimal industrial input (designated CNAS). Full details about growth conditions and kinetic measurements for reactor experiments are described in the Supporting Information.

Single-Culture Biotransformation Experiments. Single-culture biotransformation experiments were performed with AOB (2 L), Ox^+ (1.5 L), or Ox^- (1.5 L) chemostat cultures ($D = 0.14\ day^{-1}$) that received the same synthetic feed used for the master chemostats, plus pharmaceuticals at a final concentration of 1 mg EE2/L and 10 μg ^{14}C-EE2/L (used as a tracer to assist in metabolite identification) or 1 mg TMP/L. Two NaOH traps (1 N) were connected in series to each EE2 chemostat to sequester $^{14}CO_2$ from the headspace. Duplicate chemostat cultures were maintained for at least 21 days during which grab samples were obtained and centrifuged at 10 000 × g for 30 min at room temperature to remove suspended solids. Aliquots were stored at −80 °C and eventually lyophilized (−80 °C) before being shipped to the University at Buffalo for EE2 analysis. Samples for TMP analysis were loaded onto solid-phase extraction cartridges and shipped to the University of Buffalo. The concentrations of EE2 and TMP were measured using liquid chromatography/mass spectrometry (LC/MS) under selected ion monitoring (SIM). Metabolites were characterized by LC with ion-trap mass spectrometry (LC-ITMS) and nuclear magnetic resonance spectroscopy (NMR). For solids and alkaline trap samples from ^{14}C-EE2-fed chemostats, total radioactivity was measured using liquid scintillation counting. Direct and indirect oxygenase assays (ammonia monooxygenase for AOB; toluene dioxygenase, catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O) for Ox^- and Ox^+) were performed before pharmaceuticals were introduced. Details of all methods are in the Supporting Information.

Serial Culture Biotransformation Experiments. To determine the heterotrophic fate of metabolites generated from AOB chemostats, effluent from the single culture AOB chemostats described above were supplemented with mineral salt media (MSM, recipe in the Supporting Information), the appropriate organic substrates used for Ox^- and Ox^+ growth, and 10 mg/L ATU before being fed into an Ox^- or Ox^+ chemostat ($D = 0.14\ day^{-1}$). Alkaline traps were used on all radiolabeled chemostats. Duplicate chemostat experiments were performed over 21 days during which grab samples (100 mL) were collected and processed as described previously for single-culture chemostat experiments.

NAS Culture Experiments. Experiments were performed using NAS grown under three different reactor configurations. Details regarding experiments with a completely mixed activated sludge lab system and a lab-scale membrane bioreactor are reported elsewhere;[22] however, metabolites found during those experiments are compared to those found with the single-population chemostat experiments. The CNAS experiments comprised the third NAS configuration. Four chemostats ($D = 0.1\ day^{-1}$) were fed primary effluent from a local domestic WWTP that was supplemented with 500 ng/L of each nonradiolabeled EE2 and TMP. A fifth chemostat was operated as a control and received primary effluent without pharmaceutical supplementation. Upon achieving steady state removal of soluble chemical oxygen demand (sCOD) and ammonia, two of the chemostats receiving pharmaceuticals were subjected to a continuous input of ATU (10 mg/L, known to inhibit the copper binding center of ammonia monooxygenase and to be selective for AOBs) for the duration of the experiment. Grab samples from all five chemostats were collected over the course of the experiment, centrifuged at 10 000 × g for 30 min at 4 °C to remove suspended solids, supplemented with deuterated EE2, and then loaded onto OASIS HLB cartridges that were preconditioned as described in the Supporting Information. EE2 and TMP concentrations were determined using LC/MS under SIM. Samples were screened for EE2 and TMP metabolites using full-scan LC/MS and LC-ITMS fragmentation of molecular ions from suspected metabolites. Biomass assays were performed to monitor AOB (specific nitrite generation rates, sNGR) and heterotrophic (endogenous specific oxygen update rates, sOUR) activities. Further details of all methods are in the Supporting Information.



Fraction	Identity	Ret. Time	$[M-H]^-$	% Contribution to ^{14}C activity added
1	sulfo-EE2	9.5	375	0.8 ± 0.3
2	4-hydroxy-EE2	19.1	311	0.6 ± 0.3
3	EE2	24	295	18.5 ± 7.6
U1	N/A	6	Unknown	0.8 ± 0.3
$^{14}CO2$	-	N/A	N/A	54.8 ± 14.6
Solids	-	N/A	N/A	9.8 ± 3.2
Avg ^{14}C Mass balance				85.2 ± 26.2

Fraction	Identity	Ret. Time	$[M-H]^-$	% Contribution to ^{14}C activity added
1	sulfo-EE2	9.5	375	9.9 ± 0.5
3	EE2	24	295	35.3 ± 1.8
U1	N/A	6	Unknown	4.5 ± 0.2
$^{14}CO2$	-	N/A	N/A	22.4 ± 23.9
Solids	-	N/A	N/A	16.1 ± 3.4
Avg ^{14}C Mass balance				88.2 ± 22.1

Figure 1. Selected radiochromatograms of EE2 and metabolites generated by chemostat cultures fed 1 mg/L EE2 and 10 μg/L ^{14}C-EE2. Results are from composite samples collected over 3 weeks in a single (A) chemostat Ox^- culture and (B) chemostat Ox^+ culture. Results are representative of analyses performed on replicated chemostats (triplicate Ox^- culture and duplicate Ox^+ culture). Data used to generate this figure are provided in Table S7, Supporting Information. For the complete ^{14}C mass balance on duplicate chemostats, see Table S8, Supporting Information. Structural characteristics of the transformation products are provided in Table S9, Supporting Information.

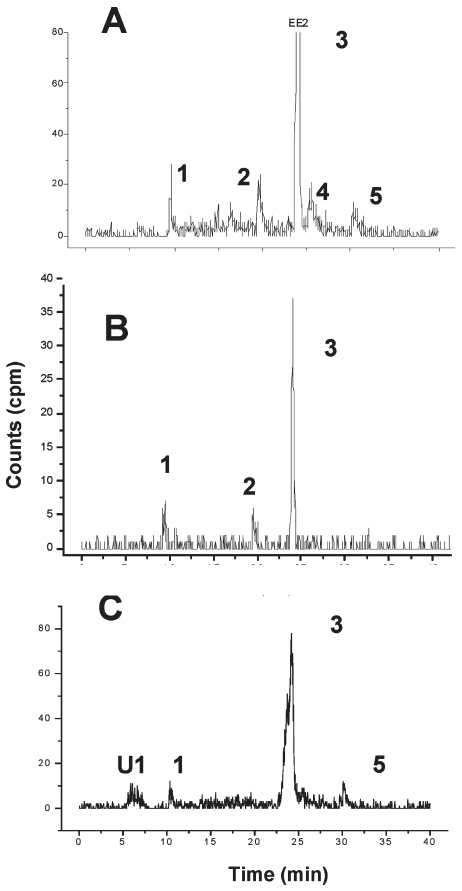
RESULTS AND DISCUSSION

Chemostat-Grown AOB Cultures Biotransform EE2. AOB chemostat cultures biotransformed EE2 (30% removal) and produced four metabolites but did not mineralize the compound. Detailed characterization of two of these metabolites (4-nitro-EE2 and 2-nitro-EE2) was previously reported by us.[17,23] The third metabolite, measured via negative mode LC/MS ($[M - H]^-$ = 311 m/z), was identified as 4-hydroxy-EE2 (4-OH-EE2) through ^{1}H NMR analyses (Figure S2, Supporting Information). This finding is consistent with the speculation that AOBs cometabolically biotransform EE2 by the ammonia monooxygenase (AMO) enzyme, which has been shown to cometabolize a range of organic compounds.[16,24−27] During these experiments, the high ammonia flux to the chemostat ensured strong and continuous AMO activity, as demonstrated by sNGR (Table S2, Supporting Information). Further characterization of the fourth metabolite ($[M - H]^-$ = 375 m/z) by LC-ITMS (Figure S1, Supporting Information) indicated that it was the product of a sulfonation reaction at ring A (sulfo-EE2).[28] This sulfonation is proposed to be catalyzed by sulfotransferase homologues coded within the genome of *N. europaea*.[29] Taken together, these results clearly show that AOBs can biotransform but not mineralize EE2 under ammonia-limited, AMO-rich growth conditions.

Biotransformation of EE2 by AOBs has been disputed by Gaulke et al.,[15] who suggested that EE2 removal in AOB experiments is strictly due to abiotic nitration reactions that form nitro-EE2 and that AOBs do not directly act on this compound when EE2 is present at low concentrations. However, they did not report an EE2 balance, so EE2 loss was not equated to the mass of nitro-EE2 produced. Without this data, it is not clear that all the EE2 that was transformed in their batch experiments is accounted for. Indeed, detection of 4-OH-EE2 and sulfo-EE2 in this study and metabolite M386 in our prior work[17] with *N. europaea* batch cultures indicate that AOBs directly metabolize EE2 at concentrations at or above 150 μg/L.

Ox^- and Ox^+ Chemostat Cultures Biotransformed EE2. EE2 was biotransformed (80% removal overall) and mineralized to similar extents by both Ox^- ($^{14}CO_2$ contributed 60−65% to total ^{14}C balance in triplicate experiments) and Ox^+ ($^{14}CO_2$ contributed 55−58% to total ^{14}C balance in duplicate experiments) cultures (Figure 1 and Table S8, Supporting Information). The EE2 metabolites formed in the Ox^- and Ox^+ cultures showed some differences. One stable metabolite (sulfo-EE2, 375 m/z) was found in both cultures. Both cultures also produced a peak (U1) that was not characterized further due to limited sample mass and an inability to chromatographically resolve what appears to be multiple compounds. The highly polar nature of U1 suggests that it is highly metabolized forms of ^{14}C-EE2 or anapleurotic compounds that are produced from central metabolic pathways. Finally, 4-OH-EE2 (311 m/z), where the hydroxylation occurred adjacent to the existing hydroxyl group on ring A to form a catechol, was found in Ox^- chemostats. The structure of 4-OH-EE2 was confirmed by NMR analysis of the isolated metabolite (Figure S2, Supporting Information). The 4-OH-EE2 is likely formed by nonspecific monooxygenase enzymes that exist in heterotrophic bacteria;[30] however, we did not measure monooxygenase activity in either heterotrophic culture. Interestingly, 4-OH-EE2 was not detected in the Ox^+ chemostats. Nonspecific dioxygenase activity in the Ox^+ culture was 23−63 times higher than that in the Ox^- culture (Table S4, Supporting Information) and can explain our inability to detect 4-OH-EE2 in Ox^+ if the intermediate was rapidly transformed via dioxygenase pathways. That the basal monooxygenase activity in Ox^- was presumably sufficient to allow for the formation of 4-OH-EE2 is consistent with what we found with basal dioxygenase activity, discussed next. Detection of catechol estrogens is significant because while they are less estrogenic than EE2;[17] they have been implicated as liver tumor promoters and weak hepatocarcinogens in rat, mice, and hamster models.[31,32]

Basal Catechol Oxygenase Activity Is Sufficient To Oxidize EE2 in Heterotrophs. Since EE2 possesses an aromatic ring,



Fraction	Identity	Ret. Time	[M-H]⁻	% Contribution to ^{14}C activity added
1	sulfo-EE2	9.5	375	3.6 ± 0.6
2	4-hydroxy-EE2	19.1	311	5.5 ± 0.9
3	EE2	24	295	70.1 ± 12
4	4-nitro-EE2	26	340	7.3 ± 1.2
5	2-nitro-EE2	31	340	4.5 ± 0.8
^{14}CO2	-	N/A	N/A	0
Solids	-	N/A	N/A	0
Avg ^{14}C Mass balance				91.0 ± 15.6

Fraction	Identity	Ret. Time	[M-H]⁻	% Contribution to ^{14}C activity added
1	sulfo-EE2	9.5	375	7.2 ± 0.3
2	4-hydroxy-EE2	19.1	Unknown	6.7 ± 0.3
3	EE2	24	295	37.4 ± 1.7
^{14}CO2	-	N/A	N/A	13.0 ± 13.2
Solids	-	N/A	N/A	15.5 ± 4.7
Avg ^{14}C Mass balance				79.8 ± 15.1

Fraction	Identity	Ret. Time	[M-H]⁻	% Contribution to ^{14}C activity added
1	sulfo-EE2	9.5	375	3.8 ± 1.9
3	EE2	24	295	48.7 ± 24.3
5	2-nitro-EE2	31	340	5.8 ± 2.9
U1	N/A	6	Unknown	5.8 ± 2.9
^{14}CO2	-	N/A	N/A	26.2 ± 7.4
Solids	-	N/A	N/A	14.3 ± 5.7
Avg ^{14}C Mass balance				104.5 ± 37.3

Figure 2. Selected radiochromatograms of EE2 and metabolites generated by chemostat cultures. Results are from composite samples collected over 3 weeks from the (A) effluent from an AOB chemostat fed 1 mg/L EE2 and 10 μg/L ^{14}C-EE2,[17] (B) effluent from an Ox$^-$ chemostat fed effluent from the AOB reactor, and (C) effluent from an Ox$^+$ chemostat fed effluent from the AOB reactor. Results are representative of analyses performed on duplicate chemostats (all cultures). Data used to generate this figure are provided in Table S7, Supporting Information. For the complete ^{14}C mass balance on duplicate reactors, see Table S8, Supporting Information. Structural characteristics of the transformation products are provided in Table S9, Supporting Information. N/A means not applicable.

aerobic mineralization requires ring cleavage catalyzed by catechol dioxygenases that are found in many heterotrophs. We theoretically determined whether the amount of EE2 applied to the Ox$^-$ and Ox$^+$ cultures could be oxidized through cleavage of the aromatic A ring in EE2 by the C12O and C23O activities measured in these cultures. If the rate of EE2 supplied to the system is greater than the theoretical rate of oxygenation that can be performed by ring cleaving catechol dioxygenases, ring cleavage reactions will limit EE2 biodegradation. In this study, combined theoretical rates of oxygenation were calculated by assuming a single ring-cleaving dioxygenation reaction equivalent to what happens when catechol is oxidized to *cis,cis*-muconate (C12O) or 2-hydroxy-*cis,cis*-muconic semialdehyde (C23O). The sum of C12O and C23O for Ox$^-$ (2.9 mg O_2 demand/d) and Ox$^+$ (95 mg O_2 demand/d; Table S5, Supporting Information) well exceeds the total oxygen demand required to mineralize the EE2 applied (0.78 mg EE2 as O_2 demand/day). Therefore, ring cleavage was not rate limiting. Furthermore, the basal level of nonspecific catechol dioxygenase expression in Ox$^-$ chemostats was sufficient to achieve unhindered EE2 mineralization and explain the lack of difference in the extent of EE2 mineralization in Ox$^-$ and Ox$^+$ chemostat cultures. These data indicate that EE2 can be biodegraded by heterotrophs present in NAS that are not specifically induced for enhanced dioxygenase activity.

Heterotrophs Degrade EE2-Associated Metabolites Generated by AOBs. Serial chemostat experiments (AOB effluent fed to Ox$^-$ or Ox$^+$) showed that EE2 was mineralized upon exposure to heterotrophs. In the AOB-Ox$^-$ series 25−37% of ^{14}C was recovered as $^{14}CO_2$, while the AOB-Ox$^+$ series showed 30−33% ^{14}C recovery as $^{14}CO_2$ (Table S8, Supporting Information). A theoretical evaluation of the oxygen demand imposed by EE2 was compared to the oxygen demand exerted by nonspecific dioxygenases in the downstream Ox$^-$ and Ox$^+$ cultures and again showed that EE2 mineralization was not limited by dioxygenase activity (Table S5, Supporting Information). Reduced mineralization in the heterotrophic chemostats fed AOB chemostat effluent may have been due in part to inhibition imposed by high nitrite concentrations passed on by the upstream AOB culture and proved to be inhibitory at or above 600 mg/L as N (Table S6, Supporting Information).

Radiochromatograms reveal that some of the AOB metabolites formed in the first chemostat were further degraded by the heterotrophic cultures (Figure 2). First, the 4-OH-EE2 generated by AOB is completely removed by Ox$^+$. This is consistent with our hypothesis that high dioxygenase activity rapidly cleaves the A ring of this catechol estrogen. The Ox$^-$ effluent still contained 4-OH-EE2; however, there are two sources of this metabolite (AOB[17] and Ox$^-$) and a possibility that Ox$^-$ is

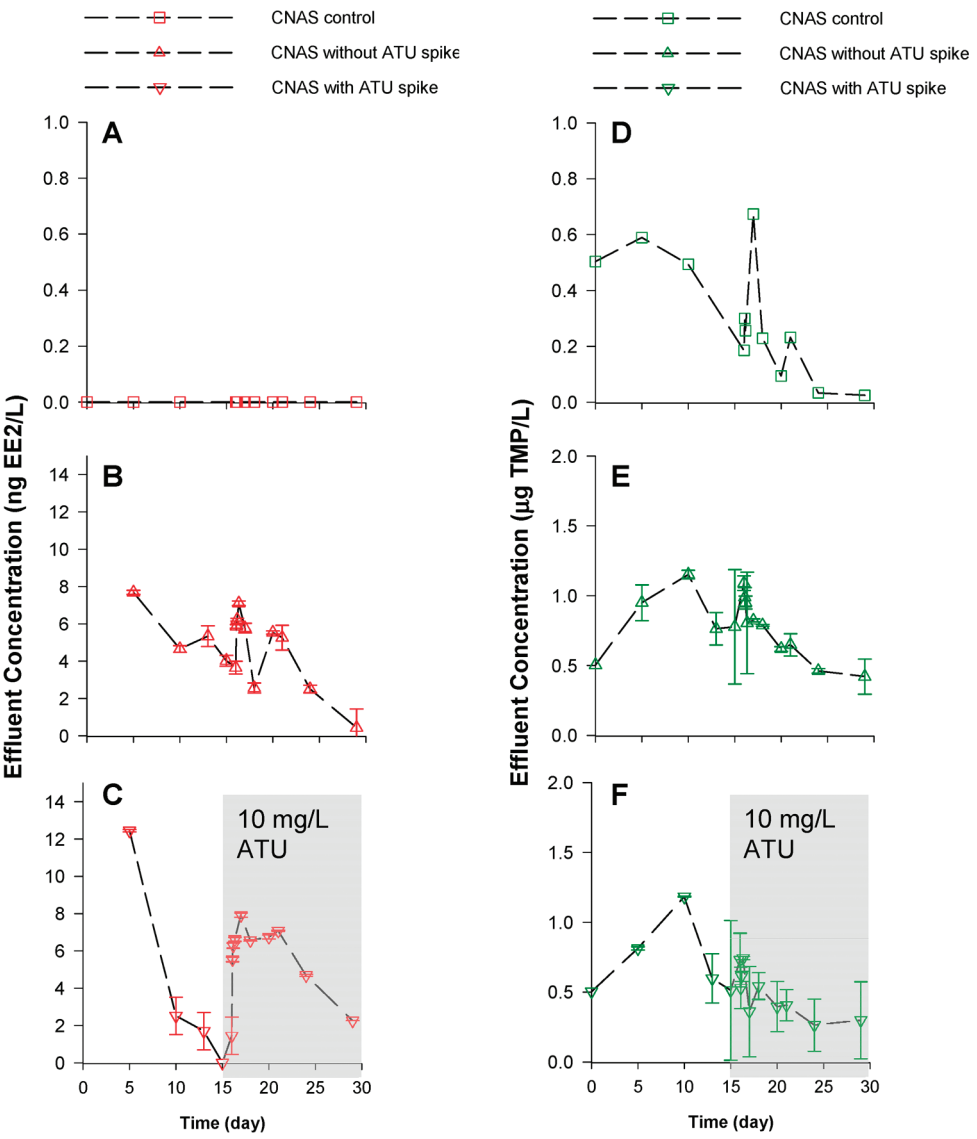


Figure 3. EE2 and TMP concentration profiles for CNAS experiments. (A and D) Control chemostats that were not supplemented with EE2 or TMP. (B and E) EE2 and TMP concentration profiles after receiving 500 ng/L of both EE2 and TMP. (C and F) EE2 and TMP concentration profiles after receving 500 ng/L of both EE2 and TMP and were subjected to continuous ATU addition from day 15 onward. The results are representative of duplicate chemostats except for the control reactor. Activity assay and chemical profile data are provided in the Supporting Information.

degrading it slowly, making interpretation of its relative fate complicated. Second, sulfo-EE2 was generated by all three cultures (noted above, and Figure 1). It is known that sulfonation of estrogens increases their solubility and may be a method of detoxification employed by prokaryotes.[28] The presence of residual sulfo-EE2 in the Ox^- and Ox^+ effluents (Figures 1 and 2) suggests that it is not deconjugated or deconjugates slowly under the conditions tested and may be recalcitrant. This result is supported by prior research where limited deconjugation of sulfo-EE2 was observed during activated sludge batch tests.[33] An important consequence of these findings is that sulfonation of EE2 may mask its detection in effluents, and the highly soluble sulfo-estrogen may deconjugate to the endocrine disrupting parent estrogen once in the environment. This deserves further study. A third set of AOB chemostat-derived compounds are 2-nitro-EE2 and 4-nitro-EE2, which are abiotically formed[15,17] due to the accumulation of nitrite and are an artifact of laboratory experimental protocols that are not representative of what would happen in a domestic WWTP where complete nitrification to nitrate is more common. Interestingly, both nitro-EE2 metabolites were completely removed by the Ox^- culture, but only 4-nitro-EE2 was completely removed by the Ox^+ culture. Gaulke et al.[34] showed that EE2 is biodegraded by activated sludge mixed liquor 3−4 times faster than 2-nitro-EE2, but the relative kinetics of 2-nitro-EE2 and 4-nitro-EE2 degradation were not evaluated. Our results lead us to speculate that 2-nitro-EE2 is more recalcitrant to overall heterotrophic biodegradation than 4-nitro-EE2 and can be explained because the unsubstituted meta position of 4-nitro EE2 is more accessible relative to the meta position of 2-nitro EE2 because of the adjacent ring structure.

AOBs Transform EE2 Faster than Ox^- or Ox^+. Extant kinetic assays performed with washed aliquots of chemostat-grown AOB, Ox^-, and Ox^+ cultures show that AOBs transform EE2

approximately 5 times faster than Ox^- or Ox^+ cultures. The average biotransformation rate for AOB, Ox^-, and Ox^+ from duplicate experiments (range of values) was 13.6 (12.9, 14.3), 2.1 (1.1, 3.1), and 2.7 (2.3, 3.1) L/g biomass as COD-day. Nitrite concentrations were <5 mg/L as N during these experiments. We believe that this rate difference occurs, in part, because EE2 biotransformation by AOBs involves a single monohydroxylation or sulfonation reaction whereas EE2 biotransformation by heterotrophs can involve multiple enzymatic reactions, including ring cleavage and mineralization. Ring cleavage is often the rate-limiting step for substituted aromatic ring biodegradation.[21]

TMP Was Biotransformed by Ox^- and Ox^+ Cultures but Not AOB Cultures. Considerably less work has been dedicated to evaluating TMP fate in NAS than has been done with EE2. Previously, we postulated that TMP removal by NAS is driven by AOB activity only,[3] but this was due in part to a misunderstanding of the microbial ecology present in the bioreactor studied. Here, we showed that TMP was not removed by AOB chemostat cultures (data not shown) and that TMP was actively removed by Ox^- (49% removal) and Ox^+ (27% removal) chemostat cultures (Figure S3, Supporting Information). The first-order rates of TMP transformation by Ox^- and Ox^+ cultures were measured in triplicate and are 1.2 ± 0.13 and 0.58 ± 0.09 L/g biomass as COD-day, respectively. During heterotrophic biotransformation, two metabolites were formed in both the Ox^- and the Ox^+ chemostats (Figure S4, Supporting Information). These novel metabolites, M220 (221 *m/z*) and M182 (183 *m/z*), were detected in full scan + ESI LC/MS but were not quantified due to the lack of reference standards. Since we could not obtain ^{14}C-TMP, it was impossible to determine the extent of TMP mineralization. Nevertheless, the presence of these intermediates implies that M220 and M182 may be resistant to further degradation under the conditions tested in this study. It is possible, as with EE2, that heterotrophic dioxygenase enzymes were involved with forming the metabolites because the oxygen demand mass balance implies that dioxygenase activities measured for both Ox^- and Ox^+ were not limiting (Table S5, Supporting Information).

These findings contradict previous assertions by Batt et al.[3] and imply that TMP losses in NAS are due to heterotrophs only. Serial reactor experiments in which effluent from an AOB chemostat fed TMP was fed to Ox^- and Ox^+ chemostats confirmed that heterotrophs were indeed solely responsible for TMP removal under the conditions tested. Since linking the heterotrophic reactors downstream of the AOB chemostat resulted in an increase in TMP in the heterotrophic chemostat effluents over time, biodegradation was inferred by comparing the measured TMP profile over time to theoretical concentration profiles for TMP if it was not biodegradable (a theoretical step feed of inert tracer into a completely mixed chemostat). The inert tracer curve greatly overpredicted the measured TMP concentration in the system (Figure S5, Supporting Information). This provides additional evidence that TMP can be biotransformed by heterotrophic chemostat cultures. Note that the downstream TMP heterotrophic chemostats received high concentrations of nitrite and may have had slower biotransformation rates than would be observed in the field where nitrite levels are often very low.

CNAS Experiment Shows That AOBs Degrade EE2 but Not TMP at ng/L Concentrations. The experiments discussed to this point were all conducted with EE2 and TMP concentrations that are much higher than is typically found in domestic wastewater treatment plants. Therefore, we designed the CNAS experiment to evaluate the biological fate of EE2 and TMP when (a) they are present together, (b) they are present at ng/L concentrations in primary effluent, (c) they are present in the absence of high residual nitrite and ammonia concentrations, and (d) they are added to a culture containing a mixture of AOBs and heterotrophs grown in a chemostat. Part way through the experiment, ATU was added to two of the five chemostats to stop the activity of AOBs in order to observe the dynamic impact on EE2 biotransformation. Prior to adding the inhibitor, effluent ammonia and nitrate concentrations across all five reactors averaged 0.55 ± 0.18 and 23.1 ± 2.7 mg/L as N, respectively (Figure S6, Supporting Information), while sNGRs (indicating AOB activity) averaged 114 ± 35 mg N/g protein-day (Figure S7, Supporting Information). Nitrite concentrations were 0.5 mg/L as N or less throughout the experiment. Upon adding ATU, EE2 removal slowed significantly while TMP removal was not affected (Figure 3). This result for EE2 is in contrast to that found by Zhou and Oleszkiewicz,[35] who reported that EE2 losses did not change when 5 mg/L ATU was added to a culture grown on synthetic feed in sequencing batch reactors and exposed to EE2 concentrations approaching 5 mg/L. The mechanistic reason for the discrepancy is unknown; however, the growth conditions between the experiments were dramatically different, and the current study used growth conditions much closer to what one might find in a full-scale WWTP. TMP concentrations were much higher than intended (500−1000 ng/L was present in the primary effluent, while 500 ng/L was added in the lab). EE2 accumulation occurred over 5 days, after which EE2 concentrations started to decrease and approach concentrations observed in the control chemostats not supplemented with ATU. Ammonia levels increased up to 15 ± 0.1 mg/L as N, and both nitrate concentrations and nitrite generation rates decreased to zero for the remaining 15 days of the experiment in the inhibited reactors (Figures S6 and S7, Supporting Information), indicating that AOBs remained inhibited for the duration of the experiment. Metabolites identified during the ^{14}C experiments were not detected in any samples from this experiment (data not shown), due to the low concentrations present and our inability to perform this experiment with ^{14}C-EE2. However, an independent experiment performed with a lab-scale completely mixed activated sludge reactor and lab-scale MBR that each received 1 mg/L EE2 with a radioactive ^{14}C-EE2 tracer showed that both *m/z* 311 (monohydroxylated EE2) and *m/z* 375 (sulfonated EE2) were detected.[22] It is not clear if the metabolites occur only during high EE2 concentrations or if they are also present at very low EE2 concentrations, but their presence across such a broad range of reactor configurations and in both pure and mixed cultures is notable.

Taken in concert with the other experiments performed during this study, we postulate the following scenario for how AOBs and heterotrophs interact in degrading EE2 and TMP. We believe that EE2 is preferentially biotransformed by AOBs to metabolites, probably hydroxyl-EE2 and sulfo-EE2; our data suggest that the former is further biotransformed by surrounding heterotrophs but not the latter. Upon inhibiting the AOBs in two of the reactors, EE2 accumulated because the culture that was primarily responsible for the first step in the primary transformation pathway was not available. Although the heterotrophs may have been biotransforming some of the EE2 before the ATU was added, they seemed to adapt over time to biotransform EE2 in the absence of AOB activity. These results suggest that there are

multiple pathways for EE2 biotransformation in nitrifying activated sludge systems and that the pathway that starts with AOB transformation is preferred as long as AOBs are active. In contrast to what was observed with EE2, we postulate that AOBs are not involved with TMP degradation and that heterotrophs are the major contributors to TMP removal in NAS.

This study shows that both AOBs and heterotrophs participate in EE2 biodegradation and heterotrophs in TMP biodegradation under conditions approximating those experienced in activated sludge systems. AOBs biotransformed EE2 more rapidly than heterotrophic organisms; however, the heterotrophs mineralized EE2 independent of AOB activity and also degraded some EE2-associated metabolites generated by AOBs. The results imply that relative contributions to EE2 biodegradation by AOBs and heterotrophs can vary as conditions in reactors vary, the cultures can adapt fairly rapidly (within a few days), and multiple pathways are possible. Overall, these results suggest that nitrification may be beneficial for the biodegradation of some trace organic compounds of emerging concern, and AOBs and heterotrophs may function cooperatively to enhance the reliability of treatment systems where efficient removal of these compounds is desired. This kind of knowledge is important for WWTP designers and operators to know when making decisions about plant design and operation. Finally, the methods deployed in this study show that great care is needed in elucidating the roles of different ecological groups and that additional pharmaceuticals should be screened in a similar manner to enhance our database on their fate during wastewater treatment.

■ ASSOCIATED CONTENT

Supporting Information. More detailed information. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ DISCLOSURE

Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

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NOTE ADDED AFTER ASAP PUBLICATION

There were errors in the abstract artwork in the version of this paper published March 23, 2011. The correct version published March 28, 2011.