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17α-ethinylestradiol Transformation via Abiotic Nitration in the Presence of Ammonia Oxidizing Bacteria

LINDA S. GAULKE,*,† STUART E. STRAND,† THOMAS F. KALHORN,‡ AND H. DAVID STENSEL†

Department of Civil and Environmental Engineering University of Washington, Box 352700 Seattle, Washington 98195-2700, and Department of Medicinal Chemistry, University of Washington, Box 357610, Seattle, Washington 98195-7610

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Impacts of trace concentrations of estrogens on aquatic ecosystems are a serious environmental concern, with their primary source being wastewater treatment facility effluents. Increased removal of 17α-ethinylestradiol (EE2) has been reported for activated sludge treatment with long enough solids retention time for nitrification. Previous work based on batch tests with *Nitrosomonas europaea* and nitrifying activated sludge at high EE2 concentrations (>300 000 ng/L) and high NH_4—N concentrations (>200 mg/L) has led to the hypothesis that ammonia oxidizing bacteria cometabolically degrade EE2. This work investigated EE2 transformation with *N. europaea* and *Nitrosospira multiformis* at environmentally relevant EE2 concentrations and LC-MS-MS to observe transformation products. Degradation of EE2 was not observed in batch tests with no NH_4—N addition or with 10 mg/L NH_4—N fed daily. At increased NH_4—N concentrations (200−500 mg/L) EE2 transformation was observed, but the only detected products were nitrated EE2. Abiotic assays with growth medium confirmed EE2 removal by nitration, which is enhanced at low pH and high NO_2—N concentrations. These results suggest that EE2 removal at low concentrations found in municipal treatment activated sludge systems is not due to cometabolic degradation by ammonia oxidizing bacteria, or to abiotic nitration, but most likely due to heterotrophic bacteria.

Introduction

Anthropogenic estrogens in the environment have the ability to alter sexual characteristics of aquatic species at trace concentrations as low as 1 ng/L (*1*). Wastewater treatment facility (WWTF) effluents have been identified as a primary source for endocrine disrupting compounds in the environment, with the bulk of their endocrine disrupting activity resulting from natural and synthetic estrogen compounds (*2, 3*). The synthetic estrogen, 17α-ethinylestradiol (EE2), and the natural estrogens, estrone (E1) and 17β-estradiol (E2), are the greatest contributors to endocrine disrupting activity in WWTF effluent (*4*) with EE2 showing the greatest recalcitrance in WWTFs (*5*). Influent concentrations range from below detection to 70 ng/L for EE2, 670 ng/L for E1, and 150 ng/L for E2 (*6, 7*).

Biodegradation has been suggested as the most important estrogen removal mechanism in activated sludge wastewater treatment (*8*), for which 60−95% removal of influent estrogen compounds has been reported (*9, 10*). Heterotrophic bacterial isolates have been identified that can grow on exceptionally high concentrations of estrogens (25 000 000−100 000 000 ng/L) as sole sources of carbon and energy (*11, 12*), but the biodegradation mechanism(s) or organism(s) responsible for removal at the very low concentrations typical of WWTFs is not known. In general, estrogen removal efficiency in activated sludge processes increases as solids retention time (SRT) increases (*13*). However, at lower SRTs with no nitrification, EE2 degradation is not significant (*8*), from which it has been inferred that the ammonia oxidizing bacteria play an important role in estrogen degradation due to their cometabolic capabilities.

The importance of nitrification was reinforced in recent bench-scale tests showing that E1, E2, estrone (E3), and EE2 are removed by nitrifying activated sludge and pure cultures of the ammonia oxidizing bacteria *Nitrosomonas europaea* at initial estrogen concentrations of 300 000−1 000 000 ng/L (*14*−*16*). When allythiourea (ATU), an inhibitor of the enzyme ammonia monooxygenase (AMO), was added to the nitrifying activated sludge enrichment, EE2 degradation was reduced by 40% over 56 h (*15*). When it was added to a pure culture of *N. europaea*, E2 degradation was completely inhibited (*15*). This evidence, together with previous work showing the nonspecific AMO catalytic diversity (*17, 18*), has led to the conclusion that estrogen degradation by ammonia oxidizing bacteria is due to the enzymatic action of AMO. However, the estrogen concentrations in previous bench-scale studies were 3000−10 000 times higher than those in WWTF influents, and the initial NH_4—N concentration was 10−20 times higher. The aim of this study was to determine if EE2 degradation by ammonia oxidizing bacteria could occur at estrogen concentrations near those typical of WWTFs and to determine the effects of NH_4—N concentration on EE2 degradation.

Materials and Methods

Experimental Overview. A series of batch tests evaluating EE2 transformation with *N. europaea* ATCC 19718 and *Nitrosospira (Ns.) multiformis* ATCC 25197 as representative ammonia oxidizing bacteria were carried out. Cells were harvested by centrifugation, rinsed with phosphate buffer solution, and then brought to volume in growth medium with no NH_4—N. Cells were kept on ice for a maximum of five hours during harvesting and experimental setup. 50 mL of culture, with a biomass of 15−41 mg/L as volatile suspended solids (VSS), were incubated in 250 mL Erlenmeyer flasks in the dark at 20 °C on a shaker. At time 0 in batch tests, EE2 and NH_4—N were spiked into the flasks from stock solutions of 1 mg/L EE2 in Milli-q water and 50 g/L NH_4SO_4. Samples for EE2 and NO_2—N or NH_4—N were taken daily over the period of the assay. NH_4—N was added and the pH was adjusted to 7.8 daily by addition of 10N NaOH based on NH_4—N consumption. Controls for EE2 degradation included bacteria killed with 100 mg/L sodium azide and EE2 with no biomass. Biological assays were conducted in triplicate and abiotic assays in duplicate.

N. europaea and *Ns. multiformis* were maintained as batch-fed pure cultures at 20 °C. The following mineral salt growth medium was used: $(NH_4)_2SO_4$ (25 mM), HEPES buffer (50 mM), $NaHCO_3$ (1.5 mM), K_2HPO_4 (250 μM), $CaCl_2$ (150

* Corresponding author phone: (206) 883-8571; fax: (206) 685-9185; e-mail: lsg@u.washington.edu.
† Department of Civil and Environmental Engineering.
‡ Department of Medicinal Chemistry.

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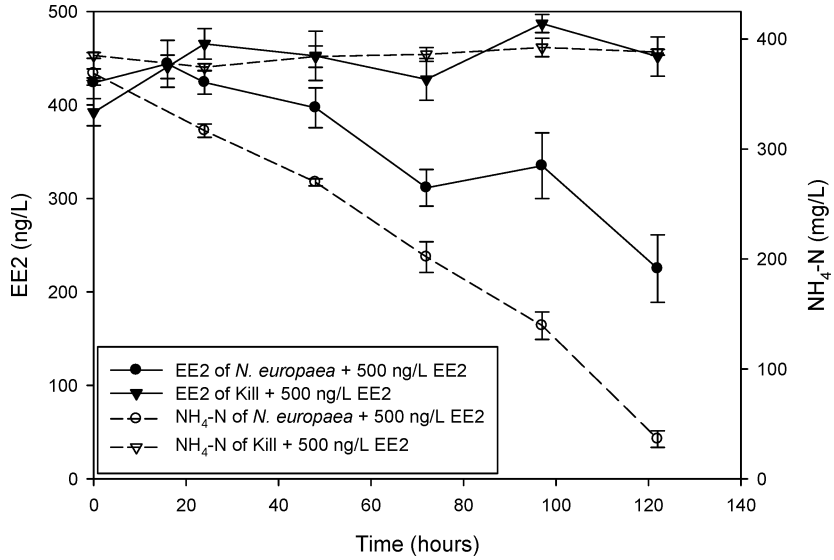

FIGURE 1. Decrease in EE2 and NH_4—N concentration versus time in batch tests inoculated with *N. europaea* with initial biomass, EE2 and NH_4—N concentrations of 41 mg/L VSS, 500 ng/L and 400 mg/L, respectively.

μM), $MgSO_4$ (150 μM), FeNaEDTA (5 μM), $NaMoO_4 \times 2H_2O$ (46 nM), $MnCl_2 \times 2H_2O$ (124 nM), $CoCl \times 6H_2O$ (1nM), $ZnSO_4 \times 7H_2O$ (35 nM), and $CuSO_4 \times 5H_2O$ (8nM).

All glassware was washed with detergent and then rinsed with tap water (all rinses three times), followed by 10% dilute sulfuric acid, Milli-q water, acetone and methylene chloride. Samples for nitrogen (NH_4—N, NO_2—N, NO_3—N) were analyzed with Hach chemical kits and spectrophotometer. Total suspended solids (TSS) and VSS were analyzed by Standard Methods (*19*). The pH was measured with 100 μL samples using a MI-410 pH electrode (Microelectrodes, Inc.).

Estrogen Analyses. EE2 was detected with a Waters Acuity HPLC and Micromass Quattro Premier XE MS-MS tandem quadrupole mass spectrometer (LC-MS-MS). Sample aliquots (0.5 mL) were transferred to 13 × 100 mm glass tubes and 125 pg internal standard (d4EE2) added. Samples were then extracted with 3 mL ethyl acetate for 5 min, after which the organic phase was transferred to a clean tube and evaporated to dryness under nitrogen. Phenolic hydroxyl groups were dansylated as follows: the residue was reconstituted in 100 μL $NaHCO_3$ buffer (pH 10.5), vortexed 1 min, and 100 μL 1 mg/mL dansyl chloride in acetonitrile added. The samples were sealed, vortexed 1 min, and heated at 60 °C for 5 min (*20*). A Waters Acuity BEH phenyl 2.2 × 50 mm 1.7 μm dp column was used for separation with a flow rate of 0.4 mL/min acetonitrile and 0.1% formic acid. The gradient was initially 25% acetonitrile, adjusted linearly to 100% at 3−4.5 min, and back to 25% at 4.6 min. The MS-MS was operated in electrospray positive mode with a cone voltage of 40 V and collision energy of 45 eV. The transitions for EE2 and d4EE2 were monitored at m/z 530 > 171 and 534 > 171 respectively. The method level of detection was 1 ng/L and the method lower level of quantification was 2.5 ng/L.

For analysis of EE2, nitro-EE2, dinitro-EE2, A-ring hydroxylated-EE2 and nonaromatic hydroxylated-EE2, the same extraction and derivatization procedure was used with modification of 0.1% w/v ascorbic acid added to samples prior to extraction with methylene chloride (*21*). The catechol form of E1 was used as a positive control to verify formation of the didansyl derivative. A Waters Acuity C8 2.2 × 50 mm 1.7 μm dp column was used with a flow rate of 0.4 mL/min acetonitrile and 0.1% formic acid. The gradient was initially 30% acetonitrile, adjusted linearly to 87% at 2.5 min, 100% at 3−4.5 min and back to 30% at 4.6 min. The MS-MS was operated in electrospray positive mode with a cone voltage of 40 V and collision energy of 45 eV. The transitions for EE2, d4EE2, nonaromatic hydroxylated-EE2, nitro-EE2, dinitro-EE2, and A-ring hydroxylated-EE2 were monitored at 530 > 171, 534 > 171, 546 > 171, 575 > 171, 620 > 171, and 779 > 170, respectively.

Nuclear Magnetic Resonance Spectroscopy (NMR). The 1HNMR of the purified nitro-EE2 in deuterated chloroform ($CDCl_3$) was obtained using a Bruker 500 MHz model (Bruker-Oxford Imaging Comp, Oxford, UK).

Results and Discussion

batch tests with Trace Concentrations of EE2 and Low NH_4—N Concentrations. To evaluate whether cometabolic EE2 degradation occurs at environmentally relevant concentrations, batch tests were conducted with pure cultures of ammonia oxidizing bacteria from the *Nitrosomonas* and *Nitrosospira* clusters of the β-subdivision of the proteobacteria. The initial EE2 concentration was 500 ng/L in separate tests with *N. europaea* biomass at 16 mg/L VSS and *Ns. multiformis* biomass at 22 mg/L VSS. Tests were conducted at pH 7.8 with either 0 or 10 mg/L NH_4—N added daily. No EE2 degradation by either *N. europaea* or *Ns. multiformis* occurred over 5 days, even though the NH_4—N was fully oxidized to NO_2—N (10 mg/L/day) in the fed bottles. The lack of cometabolic degradation of other aromatic substrates by ammonia oxidizing bacteria with no NH_4—N feed has been reported (*18*), but the absence of EE2 degradation in the cultures with NH_4—N oxidation was not expected.

Transformation of Trace Concentrations of EE2 with *N. europaea* at High NH_4—N Concentrations. To investigate how EE2 removals occurred in previous studies, a series of batch tests were conducted with *N. europaea* at initial NH_4—N concentrations of 200−500 mg/L to be in the range of previous work (50−1400 mg/L NH_4—N) and to ensure a continuous supply of NH_4—N for cell growth. EE2 was transformed in these assays, with results from a representative experiment shown in Figure 1. Results confirmed that EE2 transformation seen previously with high EE2 concentrations could be replicated at environmentally relevant EE2 concentrations of 500 ng/L. The EE2 removal rate was independent of NH_4—N concentration in the presence of 200−500 mg/L NH_4—N, and the range of specific EE2 degradation rates were 0.46−0.96 ng EE2/mg VSS-day.

To evaluate whether rates were also independent of EE2 concentration, batch tests with *N. europaea* were conducted with initial EE2 concentrations of 500 and 10 000 ng/L. EE2 removal rates were proportional to the initial EE2 concen-

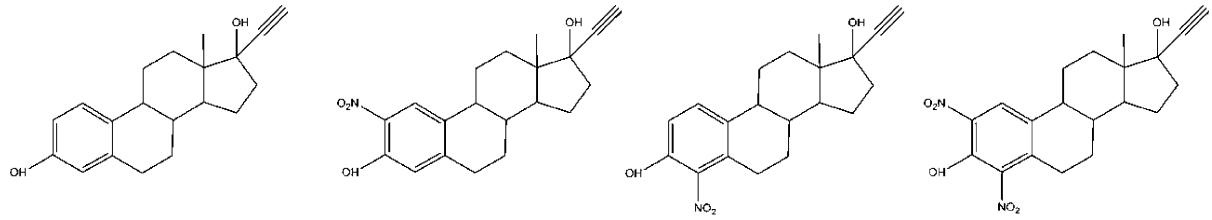

FIGURE 2. Chemical structures of 17α-ethinylestradiol (EE2) and nitrated forms of EE2.

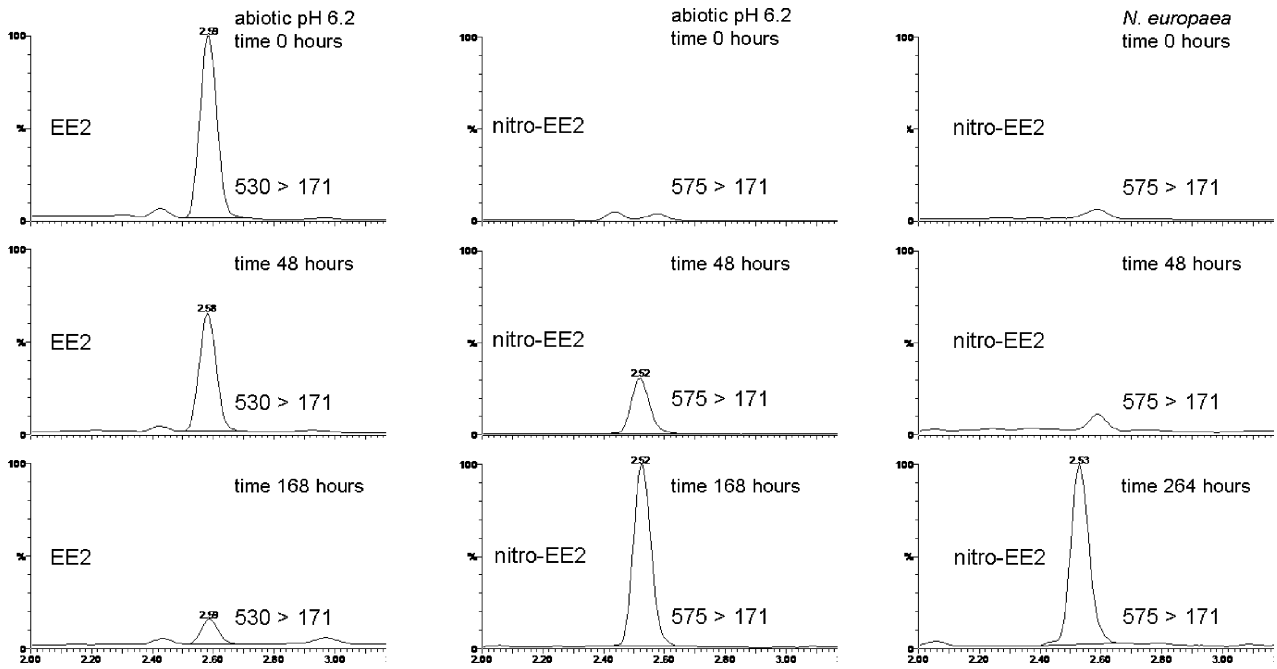

FIGURE 3. Chromatograms show EE2 depletion and nitro-EE2 production with time in the abiotic pH 6.2 treatment and nitro-EE2 production with time in the *N. europaea* treatment. Initial EE2 concentration was 500 ng/L.

tration for both concentrations. The first order rate constant was 0.0012−0.0016/hr, which is similar to the 0.0019/hr rate constant reported by Shi et al. (*15*) with *N. europaea* at initial EE2 concentrations of 400 000 ng/L and NH_4−N of 280 mg/L.

Product Formation in EE2 Bioassays. To gain an understanding of the reactions involved in EE2 transformation, MS-MS parent ion scans were conducted to identify products of EE2 transformation following complete EE2 removal in 10 000 ng/L EE2 assays. Only two products were detected by MS-MS scans and had *m*/*z* ratios of 575 (dansylated EE2 + 45, eluting at 2.52 min) and 620 (dansylated EE2 + 90, eluting at 2.48 min). Based on the *m*/*z* of the parent ions, the products were tentatively identified to be nitro- and dinitro-EE2 (Figure 2). Predicted end-products of cometabolic degradation based on hydroxylation by the AMO include hydroxylation of the aromatic A-ring (779 > 170) and/or hydroxylation of the nonaromatic rings (546 > 171). They were not detected, even though catechol-EE2 has been reported from a nitrifying activated sludge enrichment (*16*). Results of the parent ion scans provided evidence that EE2 transformation in the bioassays was not due to enzymatic attack by the *N. europaea* AMO on EE2, but rather by nitration of EE2 with NO_2−N produced by *N. europaea*.

Comparison of Bioassay versus Abiotic EE2 Nitration. To test the hypothesis that EE2 transformation was not a result of ammonia oxidizing bacteria, abiotic assays without *N. europaea* were conducted at 500 mg/L NO_2−N at pH 6.2, 7, and 8, representative of a range of conditions in *N. europaea* batch tests in this work and others. Parallel batch tests were also done with *N. europaea* at initial EE2 of 500 and 1000 ng/L. Samples were analyzed for dansylated derivatives of the following compounds: EE2 (530 > 171), d4EE2 (534 > 171), nonaromatic hydroxylated-EE2 (546 > 171), nitro-EE2 (575 > 171), dinitro-EE2 (620 > 171), and A-ring hydroxylated-EE2 (779 > 170). Over a period of 7 days, the only metabolite ions observed in any of the treatments was nitro-EE2, chromatograms are shown in Figure 3. Abiotic cultures were sampled again after 17 days, at which time dinitro-EE2 was detected in the abiotic treatment at pH 6.2.

TABLE 1. First Order Rate Constants for the Abiotic Nitration of 17α-Ethinylestradiol[a]

abiotic nitration of EE2					
k (hour^{-1})	R^2	HEPES buffer (50 mM)	phosphate buffer (10 mM)	NO_2−N (mg/L)	pH
0.0192	0.98	yes	no	464	6.2
0.0071	0.99	yes	no	475	6.5
0.0018	0.95	yes	no	460	7.0
0.0006	0.69	yes	no	471	8.0
0.0278	0.99	no	yes	482	6.4
0.0072	0.99	no	yes	44	6.5
0.0022	0.72	no	yes	9	6.5

[a] Rate constants and R^2 values obtained by linear regression of natural log-transformed data.

The EE2 transformation rate was pH dependent, with much higher rates at pH 6.2 than 7 or 8. (Table 1). EE2 transformation with time in abiotic assays and EE2 transformation, NO_2−N and pH of the *N. europaea* bioassays are

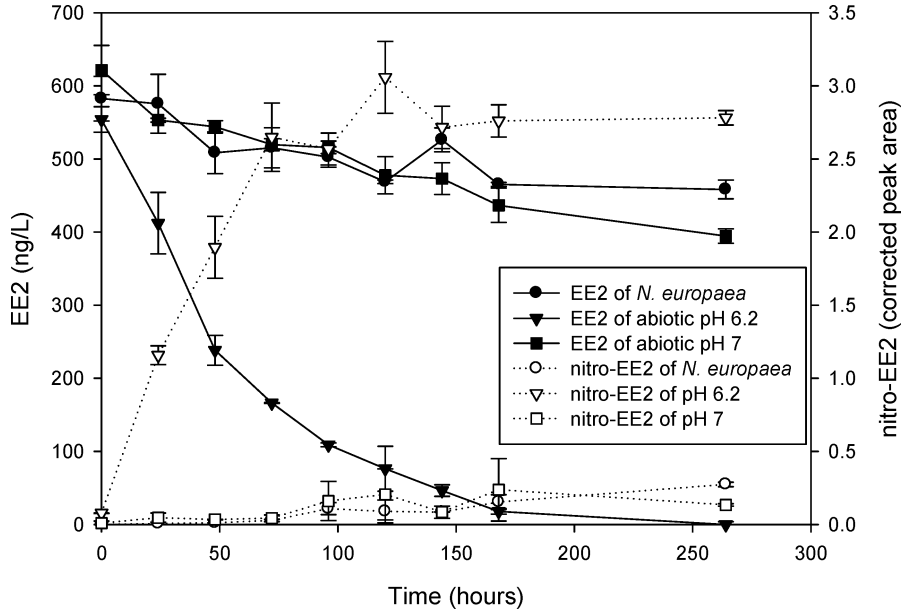

FIGURE 4. EE2 depletion and nitro-EE2 formation with time. Batch tests with *N. europaea* were maintained at 200 mg/L NH_4—N, and abiotic batch tests 500 mg/L NO_2—N. Nitro-EE2 reported on the second axis as peak area normalized to deuterated EE2 internal standard.

provided in the Supporting Information. During days 0−7 of the *N. europaea* batch test, the pH varied between 7.2 and 7.8 due to NH_4—N oxidizing activity and the daily adjustment of pH. One more sample point was taken at day 11, at which time the pH was 6.8. NO_2—N was continually produced and by day 11 had reached 400−550 mg/L. As shown in Figure 4, the EE2 transformation rate in the *N. europaea* test was similar to the abiotic EE2 nitration rates at similar pH conditions, being between that of the abiotic pH 8 and abiotic pH 7 treatments. The increase in nitro-EE2 concentration was proportional to the amount of EE2 transformed. These results provided further evidence that EE2 transformation in the *N. europaea* incubation is an abiotic reaction with NO_2—N, not biological transformation of EE2 by the AMO.

Characteristics of EE2 Nitration. To characterize rates of abiotic nitration, effects of pH, buffer and NO_2—N concentration were evaluated. EE2 nitration was pH dependent and appeared to be first order with respect to EE2, as demonstrated by linearity of the log-transformed data. The nitration rate increased with decreasing pH and increasing concentrations of free nitrous acid (HNO_2). Buffer composition was also found to greatly affect the reaction (Figure 5). At the same pH and NO_2—N concentration, with HEPES buffer in the growth medium the first order rate constant was 0.0071/hour. In contrast, with phosphate buffer the first order rate constant was 0.0278/hour, four times higher. A threshold NO_2—N concentration was investigated because no EE2 transformation occurred in pure cultures with 10 mg/L NH_4—N added daily (oxidized to NO_2—N) together with no transformation of EE2 in abiotic experiments at pH 7 and 1 mg/L NO_2—N (data not shown). In batch tests at pH 6.4 without HEPES, at four days 93% of EE2 was transformed in the presence of 500 mg/L NO_2—N, 50% with 50 mg/L NO_2—N and 18% with 10 mg/L NO_2—N. First order rate constants are presented in Table 1. At the same pH, the EE2 removal rate with 50 mg/L NO_2—N without HEPES buffer was the same as with 500 mg/L NO_2—N with HEPES buffer.

Identification of 2-Nitro-EE2 Product. To confirm the product of this reaction, 2-nitro-EE2 was synthesized by the action of excess sodium nitrite on 100 mg EE2 in 50 mL of 1:1 acetonitrile and acetate buffer (pH 4) at room temperature for 48 h. The acetonitrile was then removed in vacuum and the steroids were extracted into three 50 mL aliquots of ethyl acetate. Following isolation, the crude product was purified

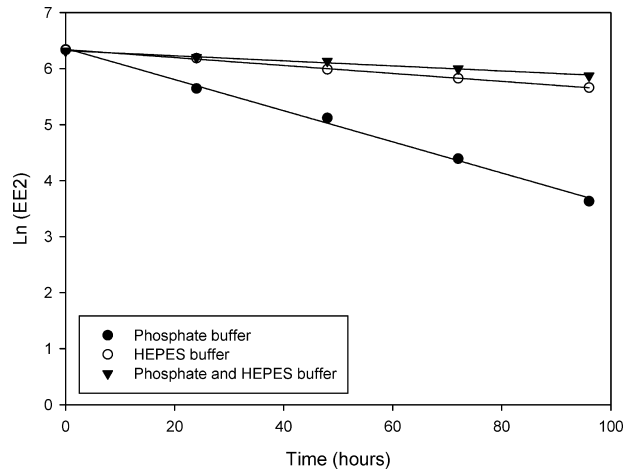

FIGURE 5. Transformation of EE2 versus time in milli-q water with 500 mg/L NO_2—N and 5 μM FeEDTA at pH 6.4. Initial EE2 concentration of 500 ng/L.

by column chromatography with a 1:4 ratio of ethyl acetate and hexane. The formula was confirmed by high resolution mass spectrometry and 500 MHz proton NMR. The MS daughter ion scan of the molecular ion shows several dominant ions that are the same as EE2 + 45, which is consistent with the proposed structure. The NMR shifts for the aromatic protons in the isolated 2-nitro-EE2 are consistent with those described for 2-nitro-E2, and differ radically in shift and splitting pattern from those of 4-nitro-E2 (*22*). MS fragmentation patterns and NMR shifts are presented in the Supporting Information. The nitro-EE2 generated during assays eluted as only one peak, which occurred at the same time as the confirmed 2-nitro-EE2.

This is the first reported work investigating the ability of ammonia oxidizing bacteria to degrade EE2 at concentrations close to those observed in WWTFs, attempting to gain an understanding of increased EE2 removals at higher SRT systems that include nitrification. Previous studies, with much higher concentrations of EE2, have concluded that ammonia oxidizing bacteria degrade EE2 by cometabolic biodegradation (*14*−*16*). In contrast, this study found that pure cultures of *N. europaea* and *Ns. multiformis* did not biodegrade EE2.

Instead, as the high NH_4—N concentrations in the batch tests were oxidized, they produced high NO_2—N concentrations and reduced the pH creating conditions favorable for EE2 removal by nitration. Thus, the apparent EE2 biodegradation was actually abiotic nitration and was an artifact of the batch test conditions. These findings illustrate the importance of taking into account the artificial environment created in batch tests, and of understanding the complex chemical and biological changes that compounds undergo, as opposed to just monitoring for their disappearance.

Though pH was not reported, similar conditions for the nitration of EE2 would have been present in *N. europaea* batch tests reported by Shi et al. (*15*). EE2 removal in batch tests was concurrent with the oxidation of 280 mg/L NH_4—N by *N. europaea,* and EE2 was almost entirely transformed over 160 h. This occurred at rates similar to the abiotic EE2 transformation rates in this study. Shi et al. (*15*) also reported inhibition of EE2 degradation in the presence of the AMO inhibitor, ATU. However, in this case ATU would have also prevented the production of NO_2—N that is needed for the abiotic nitration of EE2, as opposed to preventing the cometabolic degradation of EE2.

Cometabolic degradation of EE2 by ammonia oxidizing bacteria has also been claimed by researchers working with nitrifying activated sludge enrichments (*14, 16*), but they did not consider the possibility of heterotrophic EE2 degradation, or the abiotic nitration reaction observed in this study. Vader et al. (*14*) reported on batch tests with nitrifying activated sludge enrichments from a continuous flow reactor seeded with municipal wastewater activated sludge, operated with a 20-day SRT and fed 4000 mg/L NH_4—N. EE2 degradation in batch tests was first order with a rate constant of 0.026/hr, an order of magnitude higher than that reported by Shi et al. (*15*), indicating more significant degradation in the presence of heterotrophic bacteria. Vader et al. also reported that in batch tests their nitrifying enrichments were able to degrade EE2 in the absence of NH_4—N. In contrast, this study found that neither *N. europaea* nor *Ns. multiformis* were able to degrade EE2 in the absence of NH_4—N, or in the presence of 10 mg/L NH_4—N. If this lack of degradation by *N. europaea* and *Ns. multiformis* is indicative of other ammonia oxidizing bacteria, then degradation in the absence of NH_4—N may well be due to another type of bacteria. EE2 degradation could have been a result of heterotrophic bacteria in the mixed community developed during the 20-day SRT, nitration due to high NO_2—N concentrations in batch tests from incomplete oxidation of the 4000 mg/L NH_4—N fed to the enrichment reactor, or potentially even due to a different species of ammonia oxidizing bacteria that is able to degrade EE2. However, there was not sufficient experimental information provided for interpretation of their results based on the results of this study.

Yi and Harper (*16*) based their conclusion of EE2 removal by ammonia oxidizing bacteria on product formation, EE2 conversion with extracted AMO, batch tests with and without ATU, and increasing specific EE2 degradation rates with increasing specific ammonia oxidation rates. They reported product formation of catechol-EE2 and cleavage of the EE2 A-ring. However, a possible alternative is that the catechol-EE2 resulted from heterotrophic oxygenase activity in the mixed culture. They also reported transformation of EE2 with extracted enzymes containing AMO, but again, since it was obtained from a mixed culture the extracted enzymes could have contained oxygenases other than AMO. In addition, the experimental conditions that generated a correlation between higher specific EE2 degradation with higher specific NH_4—N oxidation were not given. If increased specific NH_4—N oxidation rates were achieved by making stepwise reductions of the reactor SRT, it would have resulted in the increase of all specific biodegradation rates with the resulting decrease in reactor biomass. Hence, specific rates of EE2 degradation would increase along with rates of NH_4—N oxidation, regardless of which bacteria in the mixed culture were responsible for EE2 degradation. The lack of EE2 degradation in batch tests with ATU addition is also not conclusive evidence of EE2 degradation by ammonia oxidizing bacteria, as ATU is able to inhibit oxygenase activity of bacteria other than ammonia oxidizing bacteria (*23, 24*).

Shi et al. (*15*) also reported first order EE2 removal with a nitrifying activated sludge enrichment with a first order rate constant of 0.035/hr, similar to the rate of Vader et al. (*14*). EE2 transformation was most likely not due to nitration as their maximum NO_2—N concentration (30 mg/L) was too low and their pH range (7.5—8.0) too high for significant nitration to occur. However, heterotrophic bacteria would have been present in these mixed community tests and cannot be ruled out as the mechanism for EE2 transformation. This possibility is also supported by the incomplete inhibition of EE2 transformation by ATU in their nitrifying activated sludge enrichments (*15*).

This research provides evidence suggesting that cometabolic degradation of EE2 by ammonia oxidizing bacteria at relevant EE2 concentrations is not an important removal mechanism in WWTFs. The significant EE2 removals observed in WWTFs are also probably not due to the nitration of EE2 from NO_2—N produced by ammonia oxidizing bacteria as the NO_2—N concentration in WWTF aeration tanks is much lower (typically <1 mg NO_2—N/L) than the threshold NO_2—N concentration for EE2 nitration found in this study. Thus, the primary mechanism for EE2 degradation in WWTFs is more likely due to the activity of heterotrophic bacteria.

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Supporting Information Available

Details pertaining to rates of EE2 removal in *N. europaea* batch tests, and the MS fragmentation pattern and proton NMR shifts for the synthesized 2-nitro-EE2. This material is available free of charge via the Internet at http://pubs.acs.org.

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