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The Link between Nitrification and Biotransformation of 17α-Ethinylestradiol

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Biological treatment processes are probably important for preventing the proliferation of steroidal compounds in the environment, and a growing number of reports suggest that nitrification may play a role in removing these chemicals from wastewater. The link between nitrification and biotransformation of 17α-ethinylestradiol (EE_2) was investigated using enriched cultures of autotrophic ammonia-oxidizers. Batch experiments showed that ring A of EE_2 is the site of electrophilic initiating reactions, including conjugation and hydroxylation. Ring A was also cleaved before any of the other rings were broken, which is likely because the frontier electron density of the ring A carbon units is higher than those of rings B, C, or D. EE_2 and NH_3 were degraded in the presence of an ammonium monooxygenase (AMO) containing protein extract, and the reaction stoichiometry was consistent with a conceptual model involving a binuclear copper site located at the AMO active site. Continuous tests showed a linear relationship between nitrification and EE_2 removal in enriched nitrifying cultures. Taken together, these results support the notion that EE_2 biotransformation can be cometabolically mediated under operating conditions that allow for enrichment of nitrifiers.

Introduction

There is continuing interest in removing micropollutants from wastewaters. This interest was borne in part from concerns about discharging these chemicals into water bodies, and potentially causing genetic and developmental abnormalities in microorganisms, aquatic life, and possibly even humans. Surveys of treated and untreated wastewaters have shown that there are a wide range of micropollutants present, and that in some cases a significant fraction of these chemicals pass through the wastewater treatment process. Recent applied research has demonstrated that micropollutant removal efficiencies depend on a number of factors including the chemical characteristics of the compound(s) (e.g., log K_{ow}), the operating solids retention time, and the biomass particle characteristics (*1*−*4*).

One class of micropollutants that has attracted considerable attention in the literature is the steroidal compounds, including natural estrogens such as 17β-estradiol (E_2) and estrone (E_1), and the synthetic steroid 17α-ethinylestradiol (EE_2). These compounds tend to adsorb strongly onto activated sludge particles, and much of the previous work has determined equilibrium partitioning coefficients (K_d). The values available in literature generally show good agreement. Clara et al. found that the log(K_d) for steroid estrogens E_2 and EE_2 was 2.84 (2.64−2.97) and 2.84 (2.71−3.00), respectively (*5*). In the work by Ternes et al. the log(K_d) for EE_2 was determined to be 2.54 (2.49−2.58) (*6*). Yi et al. found that the log(K_d) for EE_2 was 2.7 for membrane bioreactor sludge and 2.3 when the sludge was taken from a sequencing batch reactor. Andersen et al. determined distribution coefficients (K_d) with activated sludge biomass for the steroid estrogens E_1, E_2, and EE_2 in batch experiments, and they determined log(K_d) values for those steroid estrogens of 2.6, 2.7, and 2.8, respectively (*7*). Taken together, these partitioning coefficients enable practitioners to model sorption in activated sludge processes, and numerically evaluate the importance of sorption as a removal mechanism.

Biotransformation of steroidal compounds is an area where the consensus is still developing. Biotransformation is likely due to cometabolic activity because steroidal compounds (like other micropollutants) are not present in high enough concentration to support substantial biomass growth. Clear evidence of cometabolism is still needed, but progress is being made, as there is a growing body of reports suggesting that EE_2 can be biotransformed in enriched autotrophic nitrifying cultures. Vader et al. degraded EE_2 using nitrifying activated sludge, and they noted the presence of unidentified hydrophilic daughter products (*8*). Yi et al., Shi et al., and Dytczak et al. also biologically degraded EE_2 using nitrifying mixed cultures, and in each case, the simultaneous disappearance of EE_2 and ammonia was reported (*3, 9, 10*). Cometabolic activity requires a catalyst, and Yi et al. investigated the possibility that ammonium monooxygenase (AMO) can mediate EE_2/NH_3 cometabolism. Their data suggested that AMO can remove EE_2 and ammonia simultaneously (*3*), but their results were in conflict with those of others that suggested AMO may be inhibited by acetylene (an analogue of the C17 EE_2 function group) (*11*−*13*); since EE_2 contains an acetylene group (at C17), the possibility of EE_2 inhibiting AMO must be considered.

These results have provided useful information about the removal of EE_2 in nitrifying cultures, but there remain important and unanswered questions, and this manuscript addresses three of them. The first unanswered question concerns the reaction mechanisms (e.g., ring cleavage, hydroxylation) which are at work when EE_2 is initially biotransformed. Most previous experiments were not conducted in a manner that allowed for the identification of metabolites, leaving the nature of the chemical reactions unclear. There has also been little effort dedicated toward the development of a better fundamental and conceptual understanding of EE_2/NH_3 cometabolism. This need raises the second unanswered question, which concerns identifying conceptual models that can be used to understand the stoichiometry of EE_2/NH_3 cometabolism. There is also not enough information in the literature to analytically characterize the relationship between nitrification and EE_2 biotransformation, which are presumably linked cometabolically. The third question concerns the relationship between EE_2 and NH_3 biotransformation rates. The objectives of this study are to address these questions using laboratory-scale experimentation.

Materials and Methods

Experimental Overview. A nitrifying completely mixed stirred tank reactor (NCSTR) with sludge recycle was operated to cultivate an enriched nitrifying microbial community. This bioreactor was originally seeded with mixed liquor from the

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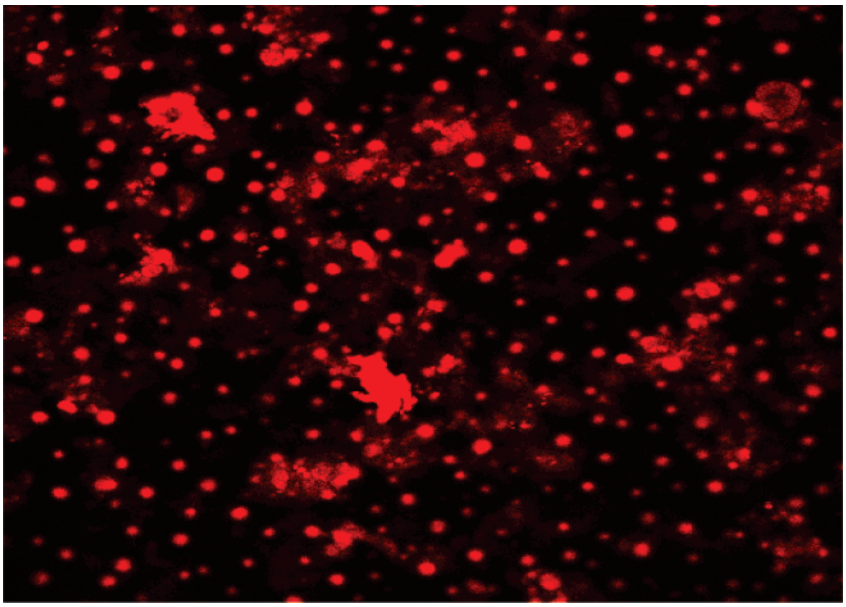


FIGURE 1. Fluorescent in-situ hybridization of *Nitrosomonas* sp., *Nitrosococcus* sp., and *Nitrosospira* sp. cells from nitrifying sludge reactor. Cell hybridization was done using Nitri-VIT (Vermicon AG).

H.C. Morgan Water Pollution Control Facility in Auburn, AL. The experimental strategy was to use the waste activated sludge for a series of batch experiments. The batch experiments involved extracting the AMO enzyme from the biomass and incubating the protein extract with EE_2, E_2, and NH_3. These experiments were done to investigate reaction stoichiometry and to determine whether the EE_2 acetylene group at the C17 inhibited nitrification. These batch tests were done three times, and samples were collected and analyzed in triplicate. Another series of batch tests involved incubating whole cells with EE_2, NH_3, and sometimes allylthiourea to confirm the link between nitrification and EE2 removal. After the batch tests were completed, the NCSTR was used for continuous experiments in which EE_2 was included in the influent and the effluent was collected for metabolite detection. During the continuous experiments, the influent NH_4^+ concentration was varied in order to change the nitrification rate and to evaluate the ensuing effects on the rate of EE_2 biodegradation. Finally, computational experiments were carried out to determine with electron density of the EE_2 compound, and to investigate whether the electron density of EE_2 is related to the sites where the initial biotransformation steps take place.

Nitrifying Bioreactor. The NCSTR (with sludge recycle) was operated at a HRT of 0.75 d and an SRT of 20 d. Peristaltic pumps were used to control the flow of influent and effluent, and the pH was controlled between 7.5 and 8.5. The SRT was maintained by manual wasting of solids directly from the bioreactor, and in most cases the waste sludge was used for analytical purposes. The influent feed consisted of the following (as mg/L total influent concentration): $(NH_4)_2SO_4$ (660), $MgSO_4$ (40), KH_2PO_4 (83.3), $CaCl_2$ (34), $CuSO_4$ (0.2), $NaHCO_3$ (1500), and $FeCl_3$ (0.4). The influent EE2 concentration was 300 μg/L and influent ammonium concentrations were between 100 and 400 mg/L as NH_4^+-N. Ammonia was used as the primary substrate to select for an autotrophic microbial community, and fluorescence in situ hybridization (FISH) was done at each operating condition to confirm the structure of the bioreactor population. FISH was performed using the hybridization and washing buffers provided by Vermicon AG (Munich, Germany) and as described by Tarre and Green (*14*). The hybridized samples were analyzed with a BioRad Laser Scanning Confocal Microscope, and the results confirmed the presence of autotrophic nitrifiers (Figure 1).

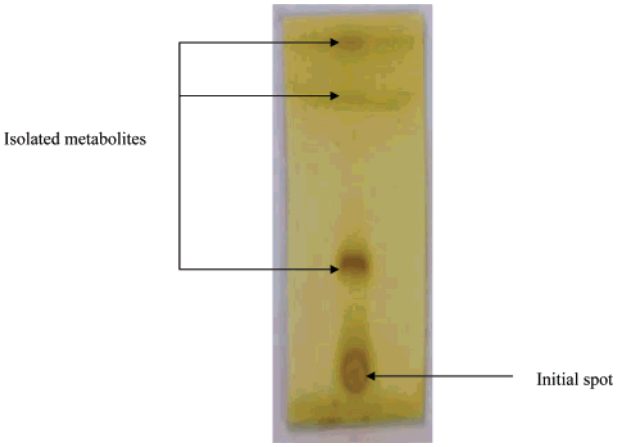


FIGURE 2. Typical thin layer chromatography plate.

Standard Analytical Methods. EE_2 and E_2 were detected by HPLC (Hewlett-Packard, HP 1100). The system consisted of a degasser (G1322A), a quaternary pump (G1311A), an ALS auto-sampler (G1313A), a colcomp column oven (G1316A), and a variable wavelength UV−vis detector (G1314A). A Hypersil ODS C18 (125 × 46 mm, 5 um) column was used. HPLC operating conditions were as follows: UV detector wavelength, 197 nm, and mobile phase, acetonitrile and water (40:60), with solvent delivered at a constant flow rate of 1 mL/min. The total runtime of the HPLC analysis was 10 min. Total suspended solids (TSS), volatile suspended solids (VSS), and ammonia-N were analyzed according to Standard Methods (APHA 1992) (*15*). NADH was measured colorimetrically as described by Hage and Hartmans (*16*).

Thin-Layer Chromatography. The effluent from the nitrifying sludge reactor was collected and metabolites were extracted using solid-phase extraction (SPE). Extracted samples were dried in a Savant Speed Vac System (GMI, USA). Solid samples were dissolved in methanol. Chromatography was conducted using TLC plates of silica gel (Whatman no. 4745-010, Florham Park, NJ) with a solvent system of hexane/ethyl acetate (3:1 v/v). The metabolites on the TLC plate were visualized by exposure to iodine vapors (see Figure 2).

Column Chromatography. SPE was carried out as described above and metabolites were eluted using methanol.

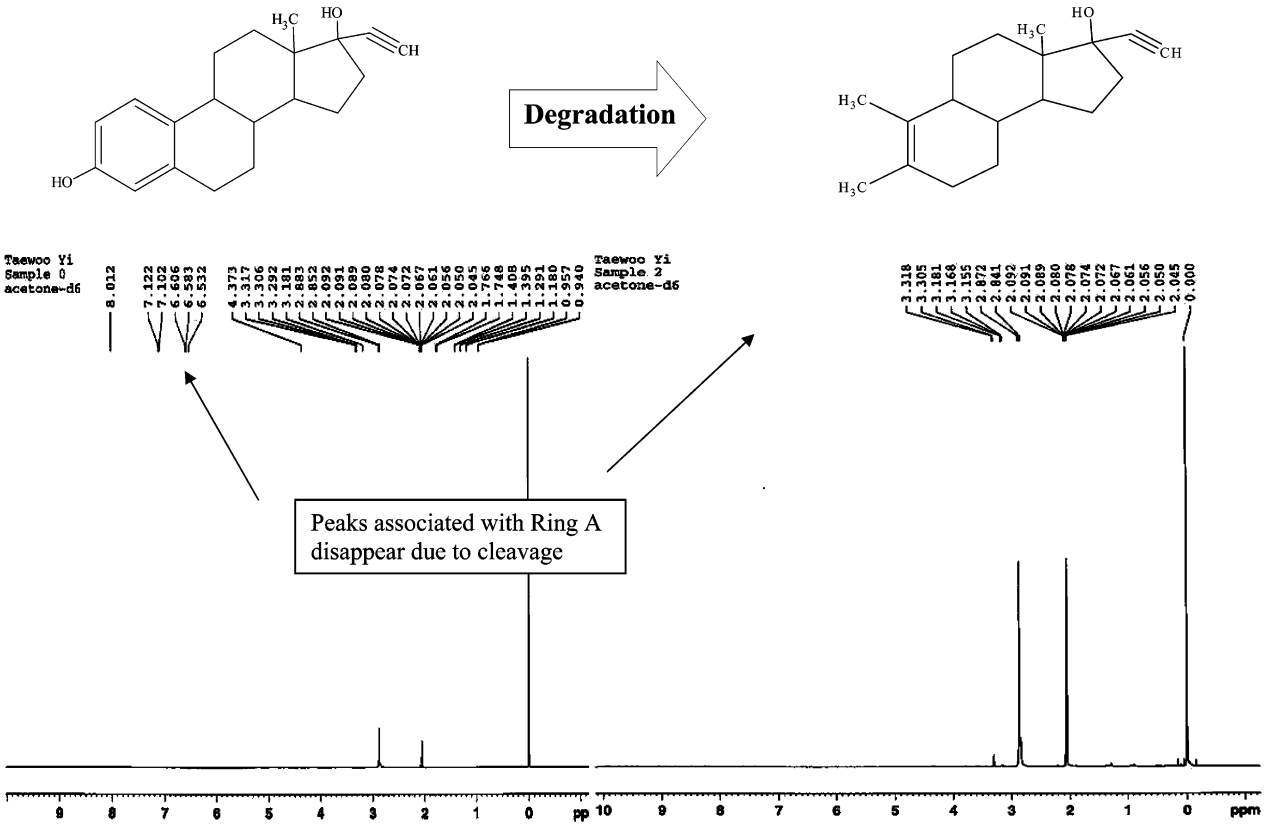
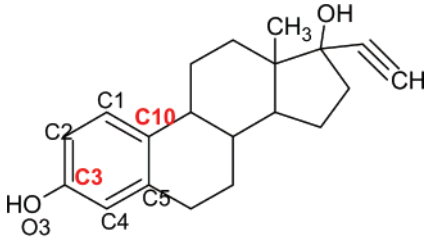


FIGURE 3. Example of an observed NMR spectrumshowing that Ring A of ethinylestradiol was cleaved.

The eluted samples were mixed with fresh silica gel (Whatman 4745-010, Kent, UK) and then dried in a vacuum evaporator. A glass column was packed with silica gel. Silica gel containing dried metabolites was loaded on the top of the silica gel column. The column was eluted with solvent mixture of hexane/ethyl acetate (3:1 v/v) by gravity and the eluting solvent was collected at the bottom of the silica gel column in each 50 mL fraction. Total volume of eluting solvent was 1 L. Each fraction of solvent was dried in the vacuum evaporator and then the sample was dissolved in acetone-D6 for NMR tests.

Nuclear Magnetic Resonance Spectroscopy (NMR). The ^{1}H NMR of purified metabolites was obtained using a Bruker 400 MHz model (Bruker-Oxford Imaging Comp, Oxford, UK). The samples were dissolved in acetone-D6 (Aldrich Chemical, no. 29621-0, Milwaukee, WI). Samples (600 μL) were placed in a new NMR tube. Figure 3 shows an example of an NMR spectrum that was observed.

AMO Extraction and Activity. AMO enzyme extraction was explained previously (*3, 17*). Briefly, biomass from the NCSTR was harvested by centrifugation at 5000*g* at 4 °C for 30 min and resuspended in 10 mM Tri-HCl (pH 8.0). The resuspended pellet was sonicated for 10 min at 40% amplitude in an ice bath using a Fisher Scientific Sonic Dismembrator (model 550, maximum power of 500 W at a frequency of 20 kHz). The particulate fraction of the sonicated product was separated by centrifugation at 5000*g* and the pellet was reconstituted in 10 mM Tris-HCl (pH 8.0) supplemented with 1% dodecyl-β-D-maltoside. The resuspended pellet, which contained the membrane-bound AMO protein, was incubated for 1 h, and then separated by centrifugation at 5000*g* at 4 °C for 1 h. Chromatography was performed on the supernatants and the resuspended pellet in a glass column (1 cm × 10 cm) packed with DEAE Sepharose CL6B (weak anion exchange resin). The column was eluted with 140 mM NaCl in 10 mM Tris-HCl (pH 9.0) + 0.02% dodecyl-β-D-maltoside. The removal of EE_2 and E_2 was investigated in 10 mM Tris-HCl using eluted enzyme, electron donors (0.5 mM NADH, 0.6 units diaphorase and 0.5 mM duroquinone), and ammonia. Control tests were always performed in the same way without the enzyme extract; these controls confirmed the absence of abiotic transformation of EE_2 or E_2.



Electron density values for 17α-ethinylestradiol	
Atom No.	Electron density, *fr*
C3	**0.26**
C4	0.10
C5	0.09
C1	0.09
C2	0.15
C10	**0.38**
O3	0.18
All other electron density values < 0.01.	

FIGURE 4. 17α-Ethinylestradiol structure with electron density shown for the carbon units with the highest FED values.

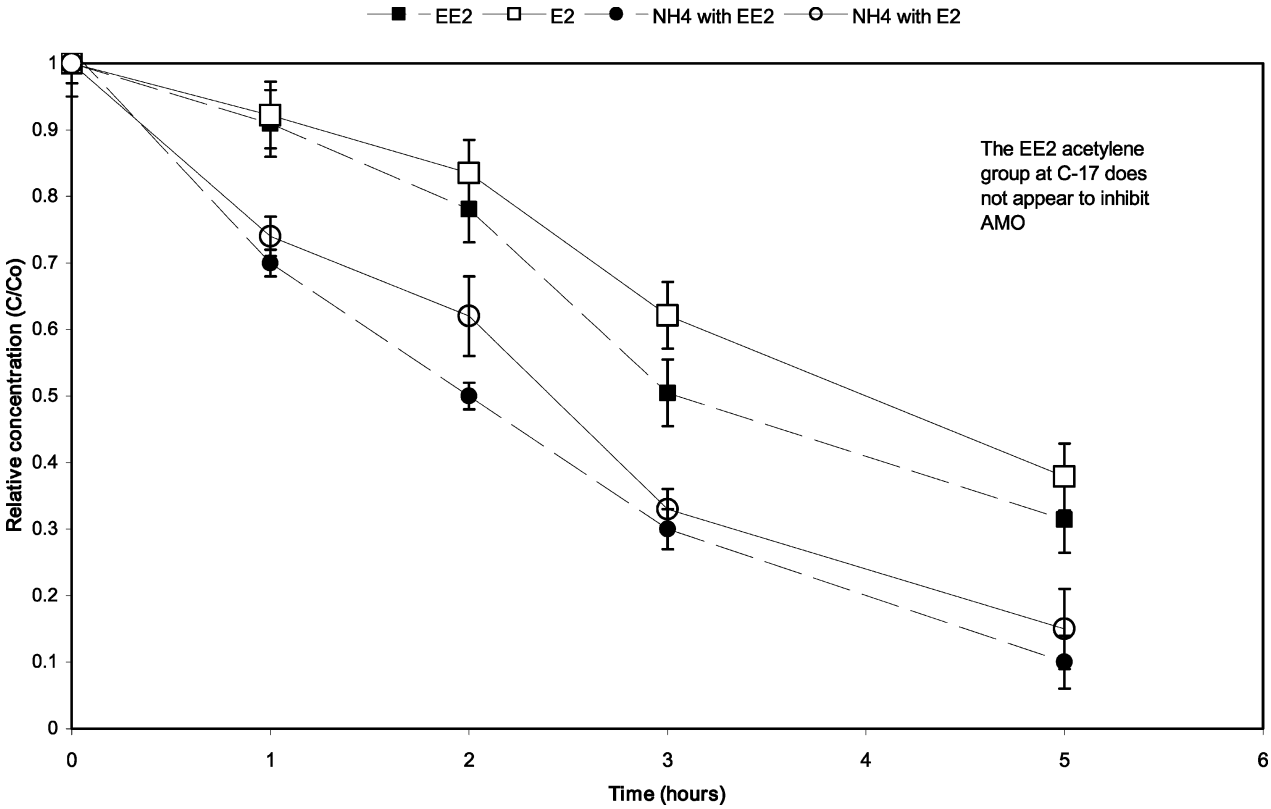
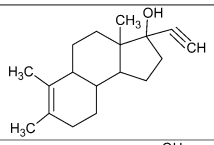
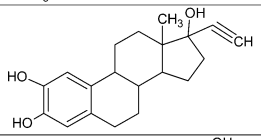
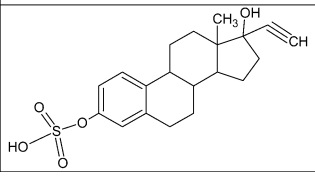


FIGURE 5. EE2 and E2 removal in the presence of an AMO-containing extract.

TABLE 1. EE2 Biotransformation Byproducts Detected by NMR

Name	Structure	Reaction mechanism
ETDC (3-ethynyl-3a,6,7-trimethyl-2,3,3a,4,5,5a,8,9,9a,9b-decahydro-1*H*-cyclopenta[a]naphthalen-3-ol) (IUPAC)		Ring A cleavage
OH-EE2		Hydroxylation
Sulfate-EE2		Conjugation

FED Analysis. Frontier electron density (FED) values were obtained by using Gaussian 03 program on the supercomputer in Alabama Supercomputer Center. Optimization of the EE_2 structure was carried out with the STO-3G basis set at level of Unrestricted Hartree−Fock (UHF). Based on optimized structure, highest occupied molecular orbital (HOMO) was calculated using the same method and basis set. The frontier electron density $\times c4r$ can be calculated as

$$\times c4r = [2^* \sum (C_{ri}\mathrm{HOMO})^2]$$

where r is the number of carbon atoms in i: 2s, 2px, 2py, and 2pz.

Results and Discussion

Electrophilic Initiating Reactions and Ring A Cleavage. EE_2 is polycyclic with a single aromatic ring A, and it includes

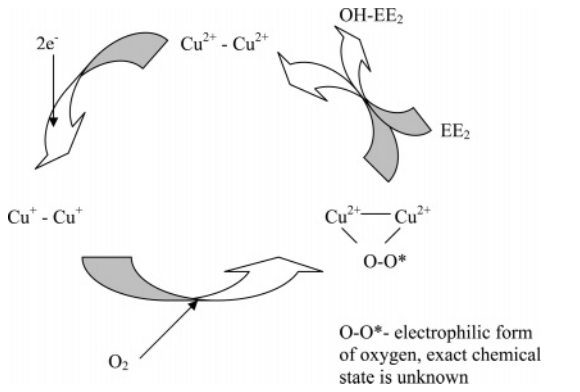


FIGURE 6. Conceptual model for AMO role in cometabolic transformation: catalytic reaction cycle involving a binuclear copper site.

polycyclic rings B, C, and D; ring D carries a hydroxyl group and acetylene group at C17. The electron density associated with ring A is significantly higher than that in other rings of the compound (Figure 4). The pi electrons associated with this ring are sterically unhindered (i.e., accessible to attacking reagents because of their location in the circular clouds above and below the plane of ring A). This leaves ring A vulnerable to electrophilic substitutions that may serve as initiating reactions. Therefore, we expected to identify daughter products that showed electrophilic substitution, and we further expected to identify daughter products that demonstrated ring A cleavage. Using NMR, three primary daughter products were identified: ETDC (3-ethynyl-3a,6,7-trimethyl-2,3,3a,4,5,5a,8,9,9a,9b-decahydro-1H-cyclopenta[a]naphthalen-3-ol), EE_2-OH, and EE_2-SO_4 (Table 1). The first, ETDC, shows that ring A was removed, which was expected since the electron density around the EE_2 ring A was relatively high. The second, EE_2-OH, is hydroxylated at the C-2, and EE_2-SO_4 is conjugated at C3, and C3 and C2 are also high FED carbon units. These results show that the high FED

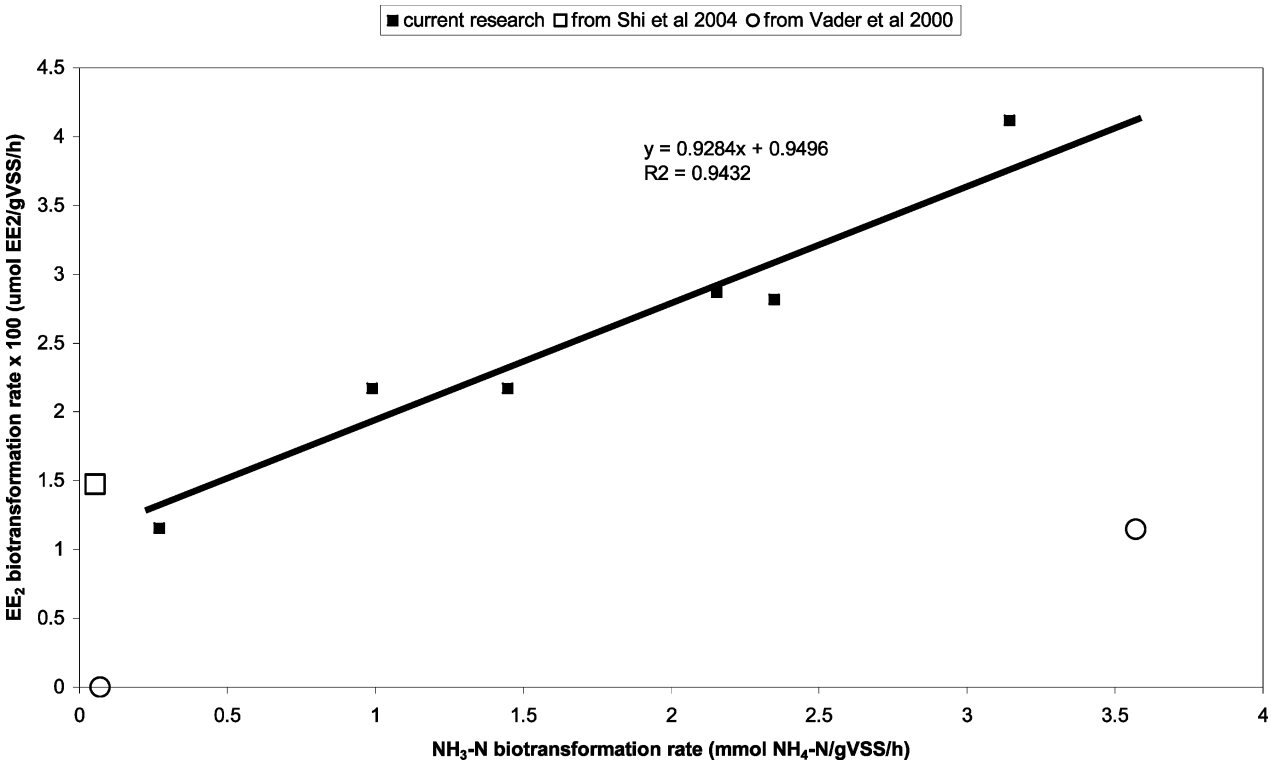


FIGURE 7. Relationship between NH_3-N and EE_2 biotransformation rate.

regions of the EE_2 structure are involved in initiating reactions. These results also show ring A cleavage can occur before modification of ring B or C; this latter finding is different from what was found by Haiyan et al. (*18*). They used a *Sphingobacterium* sp. JCR5 to degrade EE_2, and based on the daughter products they detected, they proposed that EE_2 is initially oxidized to E_1, and that the pathway continues with ring opening oxidation reactions on ring B, leaving ring A initially intact. These current results offer another view of these initiating reactions by whole cells because they show that ring A cleavage can occur before ring B is broken.

Acetylene Group Inhibition. Previous results have shown that acetylene (an analogue of EE_2) irreversibly inactivates AMO (*11*−*13*); since EE_2 contains an acetylene group (at C17), the possibility of EE_2 inhibiting AMO must be considered. EE_2 and E_2 were both incubated in the presence of an AMO-containing extract and with NH_4^+. Figure 5 shows that the rate of ammonia removal for both incubations is similar. When EE_2 is present, the ammonia removal efficiency is 90% and with E_2 the ammonia removal efficiency is 85%. The EE_2 removal efficiency was 70% and that of E_2 was 63%. Since the only difference between EE_2 and E_2 is the C-17 acetylene group of EE_2, and the action of AMO does not appear to be inhibited by the C-17 acetylene group. This result makes sense in light of the previously shown evidence (from Table 1) that initial transformations occur at ring A of the EE_2 structure and not at C17. Whole cell incubations with E_2 and EE_2 also did not show any inhibition of NH_3 removal rates (data not shown). This result shows that the C-17 group does not affect AMO activity or nitrification.

Cometabolic Action. Figure 6 is a conceptual picture showing a detailed example of how EE_2 and nitrification may be connected. AMO converts NH_3 to NH_2OH in the presence of oxygen. This step requires reducing power that is regenerated as NH_2OH is oxidized to NO_2^- by hydroxylamine oxidoreductase. Electrons then enter a catalytic cycle involving a binuclear copper site located at the AMO active site. Oxygen reacts to convert the Cu(I) to Cu(II), but the oxygen remains bound as an electrophilic radical. This oxygenated form of the enzyme then reacts with organic substrates to produce the Cu(II) form.

This conceptual model is based on monooxygenase activity, but dioxygenase enzymes must be considered also, and for two reasons in particular. First, dioxygenase enzymes are capable of mediating cometabolic biotransformation of polyaromatic compounds (*19*). Second, the microorganisms in this study were harvested from an *enriched* (not pure) culture of nitrifiers, so it is possible that heterotrophic activity may be present, which would increase the possibility that dioxygenase activity may be occurring. Fortunately, cometabolic dioxygenase-mediated biotransformation of EE_2 can be distinguished from monooxygenase-mediated activity because the NADH/EE_2 molar ratio of the former is 1:1 (as opposed to 2:1). Therefore, in order to investigate whether the biotransformation of EE_2 was monoxygenase or dioxygenase mediated, the ratio of EE_2/NADH removed was determined by incubating EE_2 and NADH in the presence of an AMO-containing enzyme extract. The molar ratio of NADH/EE_2 determined during the incubation was 2.2, which is consistent with the action of monooxygenase-mediated biotransformation. This result demonstrates the potential for monooxygenase-mediated EE2 biotransformation *in vitro* and it also provides a conceptual model which could be useful for the design of future experimental efforts.

Nitrification and EE2 Biotransformation. The aforementioned whole cell cultures were used as a resource for further evaluating links between nitrification and EE_2 biotransformation. One of the objectives of this work is to characterize the relationship between nitrification rate and EE_2 biotransformation rate. Figure 7 shows the relationship between the measured NH_3 removal rate and the measured EE_2 biotransformation rate. Current data, as well as data taken from Shi et al. and Vader et al., are shown. The continuous experiments showed a linear relationship between nitrification and EE_2 biodegradation rates over the range of NH_3 and EE_2 biotransformation rates tested. The EE_2 biotransformation rate increased from 1.1 to 4.1 μmol EE_2/g VSS/h, while the NH_3 biotransformation rate increased from 0.3 to 3.1 mmol NH_3/g

VSS/h. The current data agree well with the results of Shi et al. (*9*), who measured an EE_2 biotransformation rate of 1.5 μmol/g VSS/h at a nitrification rate of 0.1 mmol/g VSS/h. The EE_2 biotransformation rates observed by Vader et al. (*8*) where less than those reported by Shi et al. and by the current work, but the Vader et al. work showed a trend that is consistent with the current data. These data taken together strongly show a linear link between nitrification and EE_2 removal in enriched nitrifying cultures, and therefore supports the notion that EE_2 biotransformation can be cometabolically mediated in bioreactors that are enriched for autotrophic nitrifiers.

Results from the whole cell experiments provide evidence for AMO involvement in EE_2 biotransformation. The appearance of EE_2-OH as a metabolite (from Table 1) is consistent with monooxygenase activity, and the correlation between the EE_2 and NH_3 biotransformation rate (from Figure 7) are both strongly suggestive. Also, nitrifying whole cells were incubated with NH_4^+-N and EE_2, both with and without allylthiourea (a nitrification inhibitor). Nitrification and EE_2 removal were observed without the inhibitor, but in the presence of the inhibitor, EE2 was not removed and nitrification did not occur (data not shown). This result also supports the idea that AMO is involved because allylthiourea inhibits nitrification by reacting with AMO (*20*). There are also other reports presenting similar evidence for AMO oxidation of organic compounds (e.g., *21*). Taken together these observations offer a considerable body of evidence.

These conclusions make it tempting to assert that autotrophic cultures are key to controlling the passage of micropollutants through full-scale biological wastewater treatment plants, but the role of heterotrophic organisms must be considered. There are many fast-growing heterotrophic microorganisms in the activated sludge processes that have a variety of mono- and di-oxygenase enzymes; these cultures may contribute to or even dominate micropollutant biotransformations. The current results show that nitrifiers can initially degrade EE_2 into intermediates; these compounds may serve as a substrate for heterotrophic organisms. The work of Shi et al. also supports this idea. They conducted EE_2 biodegradation experiments with a nitrifying pure culture and a mixed culture of nitrifiers and heterotrophs. They detected daughter products in the pure culture experiments but not in the mixed culture experiments, perhaps because the heterotrophs completely degraded the daughter products. It is not clear that autotrophic nitrifiers can play a significant role in transforming micropollutants in systems that support significant heterotrophic populations. Exocellular enzymes and other scavenging, biodegradative mechanisms are also present in bioreactors. The relative importance of nitrifiers and heterotrophs remains open for future investigations.

Acknowledgments

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