

# Effect of Real Wastewater on Biotransformation of 17 $\alpha$ -ethynylestradiol by Ammonia-Oxidizing Bacteria in Nitrifying Activated Sludge

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**Abstract**—17 $\alpha$ -ethynylestradiol (EE2) is a synthetic estrogen used as a key ingredient in an oral contraceptives pill. EE2 is an endocrine disrupting compound, high in estrogenic potency. Although EE2 exhibits low degree of biodegradability with common microorganisms in wastewater treatment plants (WWTPs), this compound can be biotransformed by ammonia-oxidizing bacteria (AOB) via a co-metabolism mechanism in WWTPs. This study aimed to investigate the effect of real wastewater on biotransformation of EE2 by AOB. A preliminary experiment on the effect of nitrite and pH levels on abiotic transformation of EE2 suggested that the abiotic transformation occurred at only pH <6.8. Biotransformation of EE2 under the presence of municipal or industrial wastewater demonstrated that different types of wastewater affect EE2 biotransformation differently. Organic matters in wastewater were believed to deteriorate EE2 biotransformation via the competition effect. At a lower initial ammonium concentration, EE2 biotransformation can be retarded and the extent of the deterioration was COD-concentration dependent. However, when an initial ammonium concentration was elevated, this phenomena disappeared. This is because when increasing the amount of the primary substrate, more AMO enzymes can be produced resulting in unlimited transformation of all compounds in the tests reducing the competitive effect of organic matters on EE2.

**Keywords**—17 $\alpha$ -ethynylestradiol, ammonia-oxidizing bacteria, nitrifying activated sludge, wastewater.

## I. INTRODUCTION

THE release of steroid estrogens by human activities into urban environment is widespread concern due to their renowned endocrine disrupting properties. Among steroid estrogens, 17  $\alpha$ -ethynylestradiol (EE2), a synthetic estrogen is used as a key ingredient in an oral contraceptives pill, exhibits the highest estrogenic potency [4]. Although wastewater treatment plants (WWTPs) are capable of removing this compound, the extent of the removal is inadequate to reduce the released amounts of EE2 to the safe levels [4].

Although EE2 is a questionable recalcitrant organic pollutant and batch experiments showed no transformation of this compound by activated sludge [9], [11], nitrifying activated sludge (NAS) was found to capable of degrading

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EE2 during ammonia oxidation [10]. By using an inhibitor for ammonia monooxygenase (AOM), the key enzyme for ammonia oxidation, EE2 was found to be degraded mainly by ammonia-oxidizing bacteria (AOB) via cometabolism [8]. AMO has the extraordinary broad range of substrates [12]. Thus, many organic compounds, such as hydrocarbons, halogenated hydrocarbons and steroid hormones, can be biotransformed by this enzyme [1].

A number of studies demonstrated the potential of AOB to remove EE2 in wastewater. However, all studies thus far limited the experiments with EE2 as a sole organic compound. In fact, several types of organic matters are present in wastewater and these compounds can deteriorate EE2 biotransformation by competing EE2 for the active site of AMO. Prior to the application, fundamental knowledge of how AMO interact with alternative substrates is required. As the result, this study aimed to investigate the effect of real wastewater on biotransformation of EE2 by AOB in NAS.

## II. MATERIALS AND METHODS

### A. Effect of Nitrite and pH Levels on Abiotic Transformation of EE2

An earlier study demonstrated that EE2 can be transformed via an abiotic process at high nitrite and low pH levels [2]. To clarify from what levels of nitrite and pH the abiotic transformation take place, experiments were conducted without NAS at the EE2 concentration of 10mg/l with various ammonia, nitrite and pH levels: Test 1 at 30mM NH<sub>4</sub><sup>+</sup>-N, 70 mg/l nitrite and pH of 6.0, 6.2, 6.4, 6.8, 7.0, and 8.0; Test 2 at 10mM NH<sub>4</sub><sup>+</sup>-N, 45 mg/l nitrite and pH of 6.0, 6.2, 6.4, 6.8, and 7.0; Test 3 at 2mM NH<sub>4</sub><sup>+</sup>-N, 12mg/l nitrite and pH of 6.0, 6.2, 6.4, 6.8, and 7.0. Each condition was tested in triplicate at 25 °C with a rotating speed of 250rpm. Samples were taken at 24, 48, 72, 96, 120, 144 and 168hr. for chemical analysis.

### B. Enrichment of Nitrifying Activated Sludge

To produce NAS with a similar community composition of AOB to what are found in municipal WWTPs [3], [6], [7], sludge from a municipal WWTP was enriched in a laboratory-scale continuous flow reactor without sludge recycling. The reactor was introduced with an inorganic medium containing the ammonium concentration of 2mM NH<sub>4</sub><sup>+</sup>-N (28 mg N/l) which is the concentration close to the influent of municipal WWTPs. A total volume of the reactor was 5l with an effective volume of 2l. To obtain the optimum condition for AOB growth, temperature was kept at 30°C, DO concentration

was controlled at  $> 2\text{mg/l}$ , pH was maintained in a range of 7.5-8.0, and mixing was provided at a rotating speed of 300 rpm. The inorganic medium for NAS enrichment contained  $(\text{NH}_4)_2\text{SO}_4$ , 40mg of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 40mg of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 200 mg of  $\text{KH}_2\text{PO}_4$ , 1mg of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1mg of  $\text{Na}_2\text{Mo}_4\text{O}_4 \cdot 2\text{H}_2\text{O}$ , 0.2mg of  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.02mg of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.1mg of  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.002mg of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  per liter [5].  $\text{NaHCO}_3$  was added to achieve 2 mg bicarbonate ( $\text{HCO}_3^-$ ) per 1mg of ammonium added. pH was adjusted to around 7.5-8.0 using 40g/l  $\text{NaHCO}_3$ .

#### C. Effect of Real Wastewater on Biotransformation of EE2 by Ammonia-Oxidizing Bacteria in Nitrifying Activated Sludge

Three separated sets of experiments were carried out with two types of wastewater and two initial concentrations of ammonium. The first set was prepared with municipal wastewater and the initial ammonium concentration of 2mM. In the second set, the ammonium concentration was 30mM and this set was carried out with municipal wastewater. With the last set, industrial wastewater was used and the initial ammonia concentration was fixed at 2mM. The EE2 concentration of 3.5mg/l was used for all sets. It is noted that the results from set 1 and 3 clarify the effect of types of wastewater and the effect of initial ammonium concentration can be found from the results of set 1 and 2.

For each set, six parallel batch tests (Table I) comprising of four transformation tests, one inhibition test, and one control test were performed, each batch test in triplicate. For the transformation tests, NAS was added into 5ml inorganic medium containing EE2 (3.5mg/l), ammonium (2mM or 30mM  $\text{NH}_4^+\text{-N}$ ) and wastewater (municipal or industrial wastewater) at different COD concentrations of 0, 70, and 140 mg/l for municipal wastewater and 0, 70, 140, 1000, and 2000 mg/l for industrial wastewater. The final MLSS concentration in all batch tests was 150mg/l. The inhibition test and the control test were prepared in the same manner as for the transformation tests except that for the inhibition test, allythiourea (10mg/l) was added to inhibit ammonia oxidation of AOB and for the control test, no NAS was added. The cultivations were at  $25^\circ\text{C}$  with a rotating speed of 250rpm. Samples were taken at 0, 1, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, and 18 days of operation. Concentrations of ammonium, nitrite, nitrate, EE2, and COD were analyzed.

TABLE I  
SIX PARALLEL BATCH TESTS

Test	NAS	Ammonium	EE2	Wastewater	Allythiourea
Transformation 1	+	+	-	-	-
Transformation 2 (EE2)	+	+	+	-	-
Transformation 3 (wastewater)	+	+	-	+	-
Transformation 4 (EE2+wastewater)	+	+	+	+	-
Inhibition	+	+	+	+	+
Control	-	+	+	+	-

+, added; -, not added

#### D. Measurement of EE2 Concentration

An equal volume of methanol was added into a test tube containing 5 ml of inorganic medium, then vortexed to allow EE2 to be completely dissolved. EE2 concentration was analyzed using High Performance Liquid Chromatography (HPLC; Agilent 1100 Series LC, Germany) equipped with UV diode array detector (Agilent 1100 Series LC, Germany) at  $\lambda = 210\text{nm}$ . Elution was carried out by using 40%v/v acetonitrile/water at a flow rate of 1ml/min with retention time of 15min [11].

### III. RESULTS AND DISCUSSION

#### A. Effect of Nitrite and pH Levels on Abiotic Transformation of EE2

Abiotic transformation can be divided into three groups exhibiting minimum (Group A), medium (Group B), and maximum (Group C) transformation (data not shown). In overall, the results demonstrated that EE2 can be abiotically transformed and the reaction was pH dependent. Abiotic EE2 transformation occurred with all ammonium and nitrite levels tested when pH was below 6.8. Above this pH level, abiotic EE2 transformation was not found. In the later parts of the work, pH was monitored along experimental periods of all tests to ensure that abiotic transformation of EE2 was excluded from EE2 biotransformation.

#### B. Effect of Types of Wastewater on Biotransformation of EE2 by Ammonia-Oxidizing Bacteria in Nitrifying Activated Sludge

Municipal and industrial wastewater has different patterns of organic constituents. Fig. 1 shows the transformation of EE2 by AOB in NAS in the presence of municipal and industrial wastewater. Figs. 1 (a1-a2) suggest that during the tests, ammonia and nitrite oxidation took place in the transformation tests. No change in nitrogen concentrations was found in the inhibition tests indicating that allythiourea completely inhibited ammonia oxidation of AOB. Figs. 1 (b1-b2) show that EE2 concentrations reduced only in the transformation tests but not in the inhibition tests. In addition, pH levels along transformation time courses were  $>7$ . This indicated that the removal of EE2 was driven via co-metabolism during ammonia oxidation of AOB, not heterotrophic microorganisms or abiotic transformation.

Fig. 1 (b1) shows that for municipal wastewater, complete EE2 transformation occurred after day 12, 13, and 14 of operation for the tests with different diluted wastewater (COD concentrations of 0, 70, and 140mg/l of COD, respectively). This resulted in the difference in EE2 transformation rates of -0.0719, -0.0746, and -0.068mg/day, respectively. The results suggested that organic matters in municipal wastewater may deteriorate EE2 transformation and the EE2 transformation was COD concentration dependent. For industrial wastewater, Fig. 1 (b2) shows that in most tests, incomplete EE2 transformation was found along 18 days of operation.

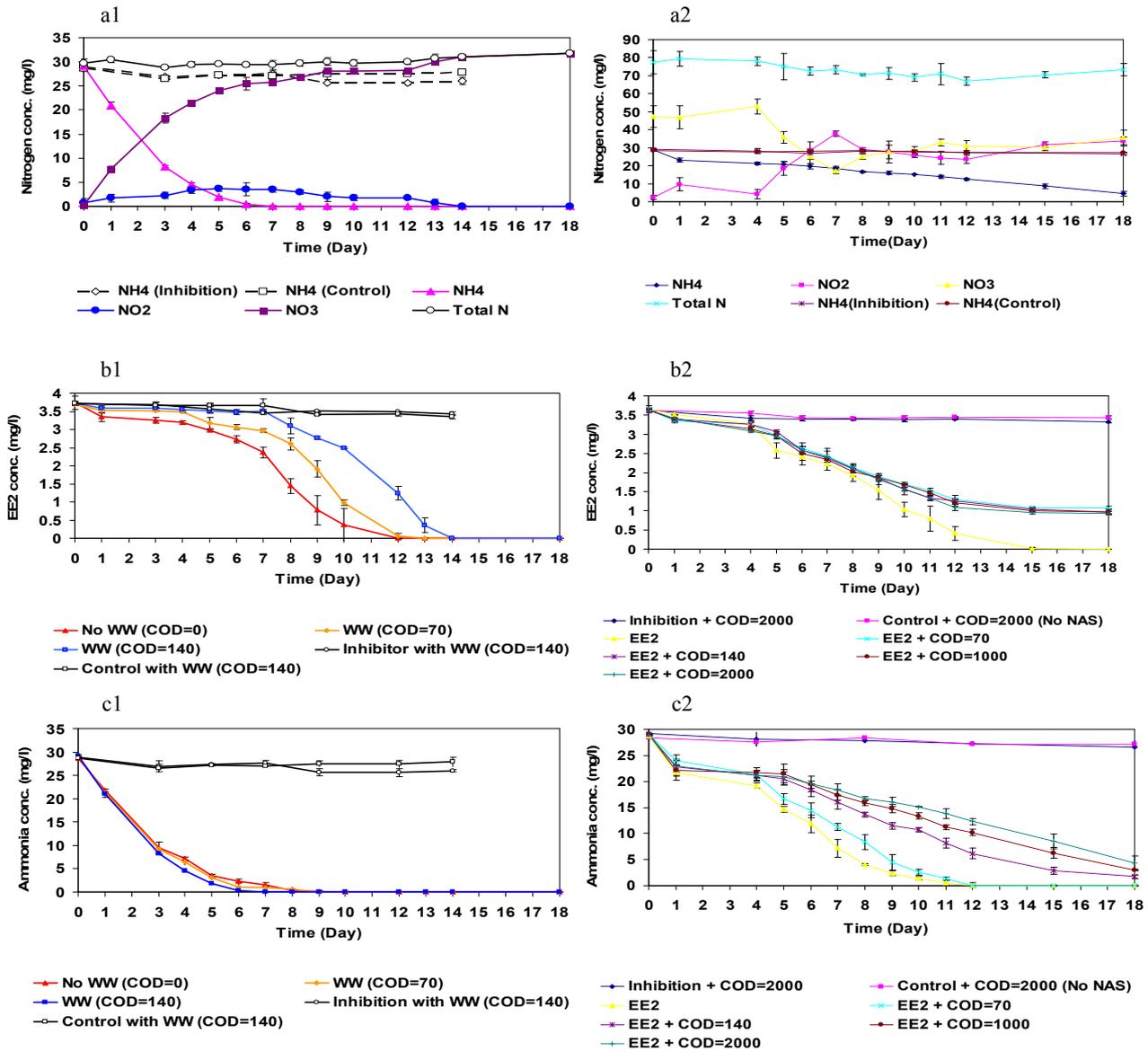


Fig. 1 Transformation of EE2 by AOB in NAS in the presence of municipal wastewater (1) and industrial wastewater (2): a) nitrogen concentrations in selected tests, b) EE2 concentrations, and c) ammonia concentrations

Complete EE2 degradation occurred only in the case with no wastewater added. For COD concentrations of 0, 70, 140, 1000 and 2000mg/l, the transformation rates were -0.066, -0.0453, -0.0479, -0.0467, and -0.0484mg.day<sup>-1</sup>, respectively. These results showed that organic matters in industrial wastewater may retard EE2 transformation but EE2 transformation was independent from COD concentrations.

In overall, different types of wastewater influence distinctly patterns of EE2 transformation. This may be caused by the difference in organic constituents in wastewater resulting in different inhibition types to ammonia oxidation and its co-substrate.

*C. Effect of Ammonia Concentrations on Biotransformation of EE2 by Ammonia-Oxidizing Bacteria in Nitrifying Activated Sludge*

Fig. 2 shows that at the lower initial ammonium concentration (2mM), EE2 transformation can be deteriorated and this is COD concentration dependent. In contrast, with the higher ammonium level (30mM), the phenomena disappeared, as the results showed the same transformation patterns with various COD concentrations. This suggested that initial ammonia concentrations influence EE2 transformation. When increasing the amounts of the primary substrate, more AMO enzymes can be produced resulting in unlimited transformation of all compounds in the medium via co-

metabolism mechanism. This reduced the effect of organic

matters in wastewater to compete to EE2 degradation.

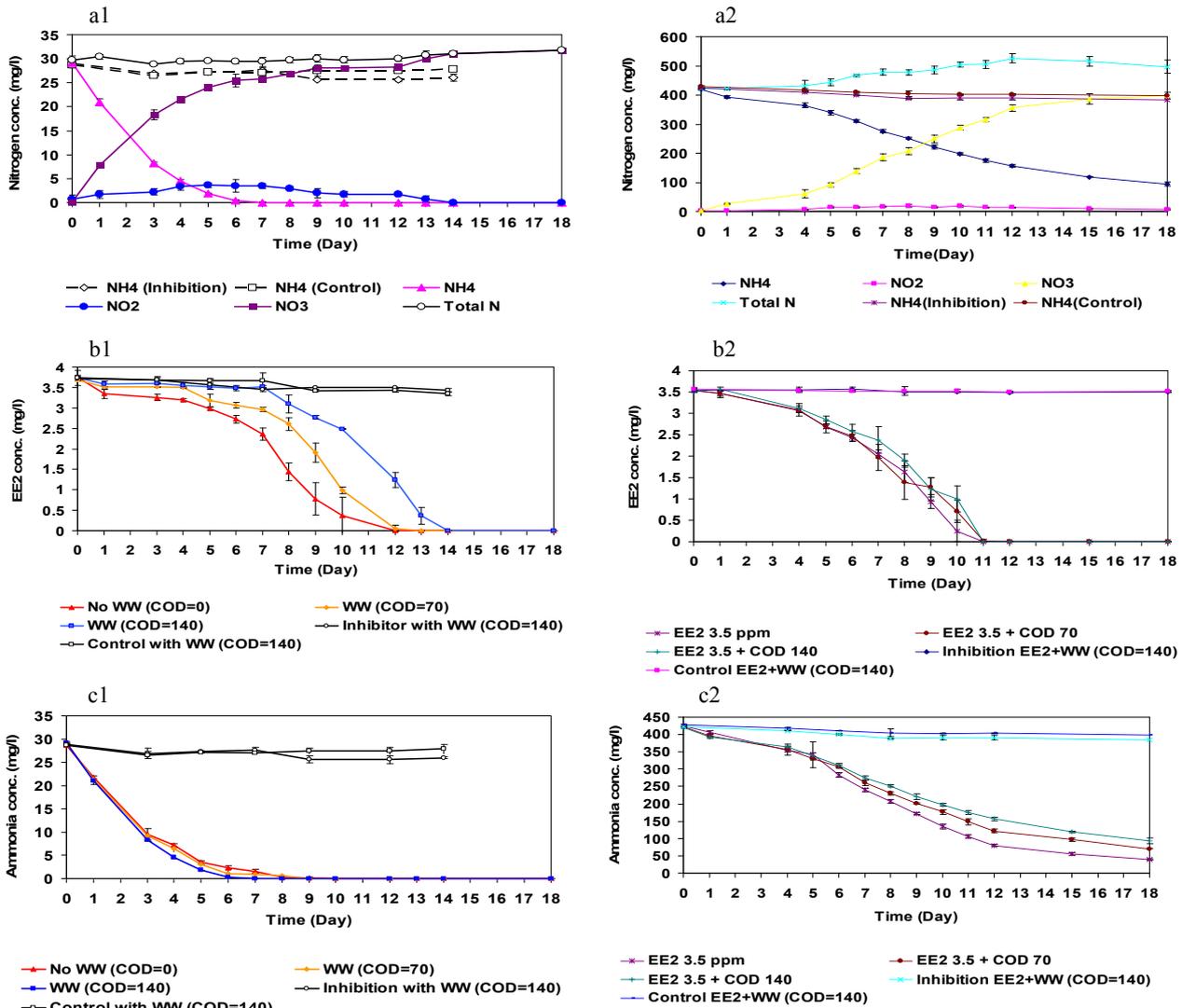


Fig. 2 Transformation of EE2 by AOB in NAS in the presence of municipal wastewater at the initial ammonia concentrations of 2mM (1) and 30 mM (2): a) nitrogen concentrations in selected tests, b) EE2 concentrations, and c) ammonia concentrations

#### IV. CONCLUSIONS

The results of this study suggested that it is necessary to consider organic matters in wastewater prior to developing AOB to degrade recalcitrant organic pollutants in wastewater. One example is to promote pre-denitrification process rather than post-denitrification process. With pre-denitrification process, nitrification tanks are placed behind denitrification tanks. Therefore, the major parts of organic matters can be removed earlier by heterotrophic microorganisms in the denitrification tanks. With this approach, reduced amounts and types of organic matters enter the nitrification tank to compete EE2.

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