Title: Enhancement and evaluation of microbial degradation of endocrine disrupting compounds

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Report to TWRI

Introduction

The presence of steroid hormones in the environment has been linked at surprisingly low concentrations (ng/L) to potential adverse effects on humans and ecosystem. Estrogens of the steroid hormones are referred to as endocrine disrupting compounds (EDCs) with higher potency. Their sources are many; some examples are discharges from wastewater treatment plants (WWTPs) as a point source and animal litter application to land as a nonpoint source. Many researchers have been detecting the steroid hormones in the various sources and matrix through advanced analytical tools, indicating that the major mechanism of the estrogen loss in water and soil system is biodegradation [12, 13, 14, 15]. Estrogen removal in WWTPs is associated with the sorption on the activated sludge and the biodegradation. Anderson et al. demonstrated that biodegradation is more important than sorption for the fate of estrogen in WWTPs [16].

Biodegradation can remove estrogen in WWTPs and in soils from agricultural practices. Estrogen biodegradation has been documented in activated sludge, nitrifying bacteria, or other pure cultures [1, 2, 3, 11]. However, pure cultures isolated from the mixed consortia are limited in their estrogen degradation ability because most of the cultures have low rate constant of estrogen degradation [1, 2, 3]. Several studies report that estrogens are biologically degraded by autotrophic or heterotrophic bacteria [1]. Vader et al. report biodegradation of 17α-ethynylestradiol (EE2) by nitrifying activated sludge [1]. Shi et al. also demonstrated that four estrogens were degraded with a pure culture, *Nitrosomonas europaea* [2]. The results from both studies indicate that estrogen can be degraded via cometabolism. Moreover, *Rhodococcus zopfii* and *Rhodococcus equi* isolated from activated sludge by Yoshimoto et al. degraded

estrone (E1), 17β-estradiol (E2), estriol (E3), and EE2 as their sole carbon and energy source under heterotrophic role with much better degradation capacity [3]. Generally, the nitrifying bacteria transform ammonia (NH_3), which more commonly occurs as in ionic form (NH_4^+), to nitrate (NO_3^-). Ammonia oxidizing bacteria (AOB) play a key role in the oxidation of NH_3 to NO_2^- as a first step. Ammonia is initially oxidized to hydroxylamine by ammonia monooxygenase (AMO) expressed by *Nitrosomonas europaea* and the hydroxylamine is then further oxidized to NO_2^- with four electrons by hydroxylamine oxidoreductase (HAO) [4]. *N. europaea* is the most widely used AOB bacterium for cometabolism research [5]. Several studies indicate that estrogens can be biodegraded through cometabolic oxidation using *N. europaea* and mixed cultures, nitrifying activated sludge of WWTPs [1, 2, 11].

Research Objectives

The objective of the research was to study the biodegradation kinetics of estrogen compounds. Four potential kinetic models were best fit to the experimental data. The rate coefficient ($k_{1,Es}$) of estrogens was estimated for cometabolic degradation of estrogens with the best fit model.

Materials and Methods

Chemicals and Analysis: Estrone (E1), 17β-estradiol (E2), Estriol (E3), 17α-ethynylestradiol (EE2), and 17α-estradiol were purchased from Sigma Chemical Co. Deuterated estrone (d4-E1) and deuterated 17β-estradiol (d4-E2) were purchased from CDN ISOTOPES. All chemicals were ACS certified. Estrogens were dissolved with acetone to make a stock solution of approximately 100 g/L of each estrogen. The stock solutions were preserved at -20 °C.

Cell Growth: The pour culture of *N. europaea* was purchased from ATCC. The culture was grown in 2-L Erlenmeyer flasks with aluminum foils on 1-L of media including 50 mM $(NH_4)_2SO_4$, 43mM KH_2PO_4, 0.73 mM $MgSO_4$, 0.2 mM CaCl, 0.01mM $FeSO_4$, 0.017mM EDTA, 0.007mM $CuSO_4$, 4.4mM NaH_2PO_4,

and 0.04% (wt/vol) Na_2CO_3 [4]. An inoculum of approximately 10% (v/v) was used for cell transfer every 10 days. The flasks were placed on rotary shakers in a dark room at 30 ^oC. The suspension in the flasks was turbid in 3 or 4 days after cell transfer and before the kinetic experiment.

Batch Kinetic Assay: For kinetic experiments with a pure culture, pre-grown N. europaea organisms were harvested from Erlenmeyer flasks by centrifugation, washing, centrifugation, and resuspension in fresh buffer medium (8 mM phosphate and 10 mM carbonate, pH 8). The initial concentration of substrate was adjusted with 100 ml of growth medium after evaporation of 250 µl of 2 g/L stock solution in 300-ml amber bottles to obtain approximately 0.5 mg/L of estrogen. The required volume of the suspension of concentrated cells was added to each reactor to obtain an OD_{600} of 0.4. The biomass concentration was quantified at the beginning of each test by a protein assay using BSA as a standard. 5-ml samples from each reactor was added to 20-ml amber vials including 5 ml ethyl ether for extraction at predetermined sampling times. 1 ml samples from each reactor will be filtered with 0.2 µm membrane filters to 10-ml centrifuge tube for ammonia, nitrite, and nitrate analysis using HACH kit and ion chromatography. After sample extraction and derivatization, the samples were analyzed with GC/MS.

Estimation of Kinetic Coefficients: For estrogens, if the substrate concentrations used in the experiment are low or less than K_s, the use of nonlinear regression techniques is widely accepted [6] as an accurate method of analysis [7]. A kinetic model will be developed with NH_3 as the only species linked to the active site of AMO [8]. NH_3 concentration in the models will be calculated as the product of the ionization fraction and the experimentally measured total concentration of ammonia and ammonium ($TOTNH_3$). An alternative means of estimating the Monod parameters is by solving the integrated form of the Monod equation in computer code or simple spreadsheet [6, 9]. The numerical integration of the differential equations is commonly achieved through a fourth-order Runge-Kutta numerical approximation. The approximated value was fit to the data by minimizing the normalized residual sum of squares (NRSS) between the model predicted values and experimental values. This value is based on an

iterative search by the Solver spreadsheet function (Microsoft Excel 2007). For estrogens, four different kinetics models were evaluated by fitting them to data on ammonia removal. Half saturation constants (Ks) from the kinetic experiment of ammonia degradation were used to determine the kinetic parameters for estrogen degradation. Finally, the estrogen rate constant and the initial concentration of each estrogen was determined. Kinetic models and their major assumptions are provided in Table 2. The assumptions for estrogen degradation by a cometabolic model were based on a model for cometabolic degradation of trihalomethanes (THMs) by Wahman et al. [5]. The nonlinear regression analysis yields estimates of the estrogen rate constant ($K_{1,Es}$), the ammonia maximum specific rate of degradation (k_{TOTNH3}), and the ammonia half-saturation constant ($K_{sNH3\text{-}N}$), as well as the initial concentration (S_0) for ammonia and each estrogen.

Table 1 Estrogen and ammonia kinetic model equation summery (Wahman et al. [5])

Substrate	Chemical	Model Name	Fitting Parameter	Rate equation and Assumption(s)
Primary Substrate	Ammonia	Saturation Kinetics	3	$r_{TOTNH3} = \frac{k_{TOTNH3} X S_{TOTNH3} \alpha_1}{K_{S_{NH3-N}} + S_{TOTNH3} \alpha_1}$ 1. Estrogens do not compete with ammonia
Secondary Substrate	E1, E2, E3,17α	First-order	2	$r_{estrogen} = -k_{1,estrogen} X S_{estrogen}$ 1. Ammonia does not compete with estrogens. 2. Estrogens do not compete with ammonia. 3. Estrogens do not compete with each other. 4. One limiting reactant (estrogen)
Secondary substrate	E1, E2, E3,17α	Competition	2	$r_{estrogen} = \frac{k_{1,estrogen} X S_{estrogen}}{1 + \frac{S_{TOTNH3} \alpha_1}{K_{S_{NH3-N}}}}$ 1. Ammonia competes with estrogens 2. Estrogens do not compete with ammonia 3. Estrogens do not compete with each other 4. One limiting reactants (estrogen)
Secondary substrate	E1, E2, E3,17α	Reductant	2	$r_{estrogen} = \frac{k_{1,estrogen} X S_{estrogen}}{1 + \frac{K_{S_{NH3-N}}}{S_{TOTNH3} \alpha_1}}$ 1. Ammonia does not compete with estrogens. 2. Estrogens do not compete with ammonia. 3. Estrogens do not compete with each other. 4. Two limiting reactants (estrogen and reductant).
Secondary substrate	E1, E2, E3,17α	Combined	2	$r_{estrogen} = \frac{k_{1,estrogen} X S_{estrogen}}{\left(1 + \frac{S_{TOTNH3} \alpha_1}{K_{S_{NH3-N}}}\right)\left(\frac{K_{I_{NH3-N}}}{S_{TOTNH3} \alpha_1} + 1\right)}$ 1. Ammonia competes with estrogens. 2. Estrogens do not compete with ammonia. 3. Estrogens do not compete with each other. 4. Two limiting reactants (estrogen and reductant).

Result

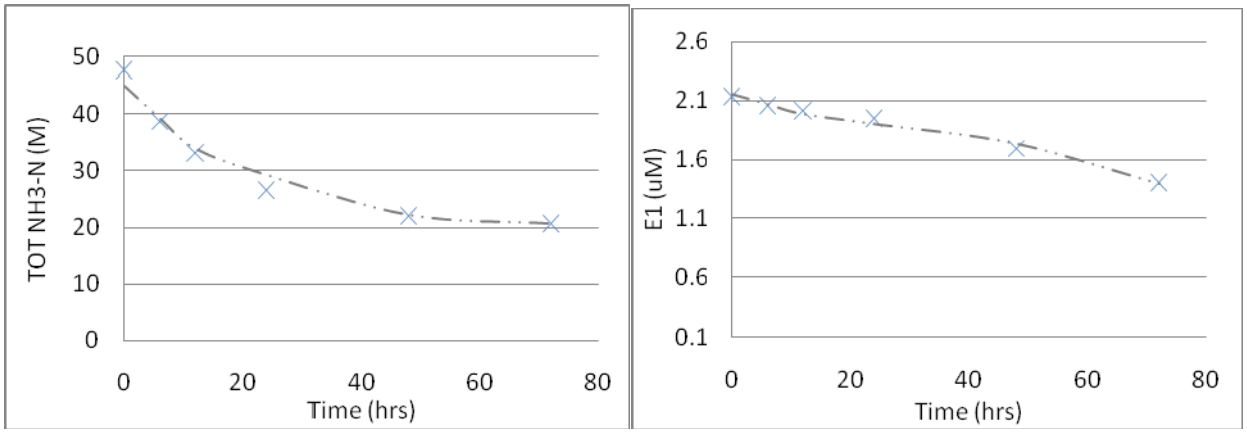

Figure 1 Kinetics of Ammonia & Estrone (E1) degradation (Combined model)

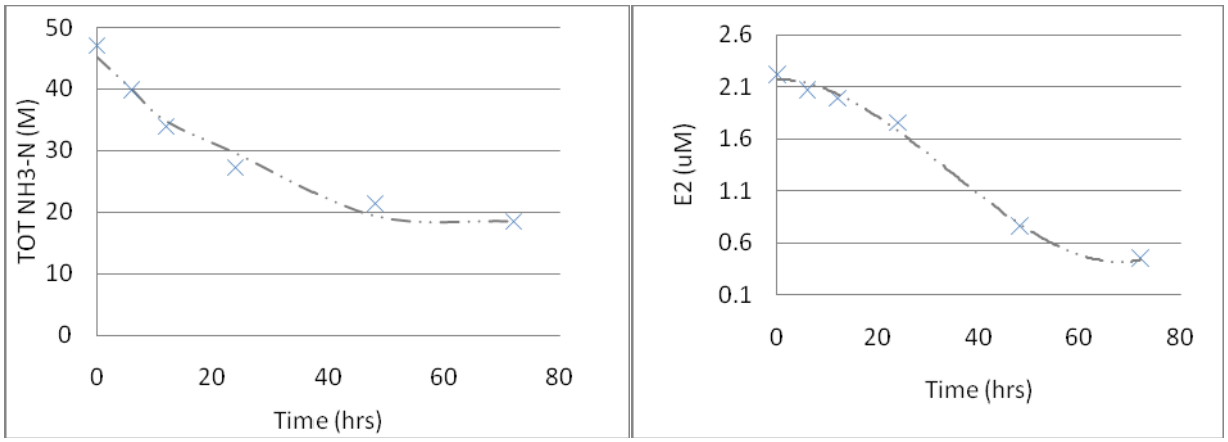

Figure 2 Kinetics of ammonia & 17β-estradiol (E2) degradation (Combined model)

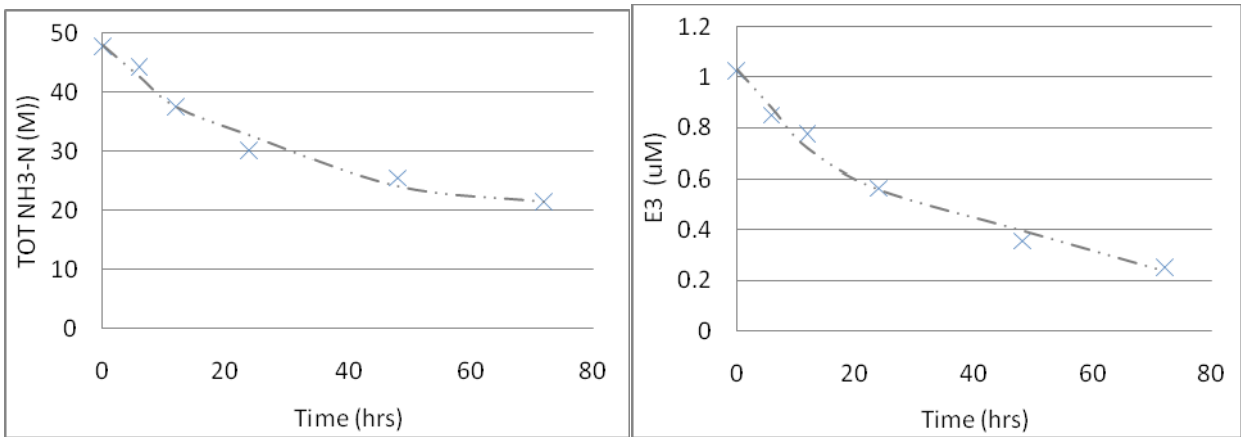

Figure 3 Kinetics of Ammonia & Estriol (E3) degradation (Reductant model)

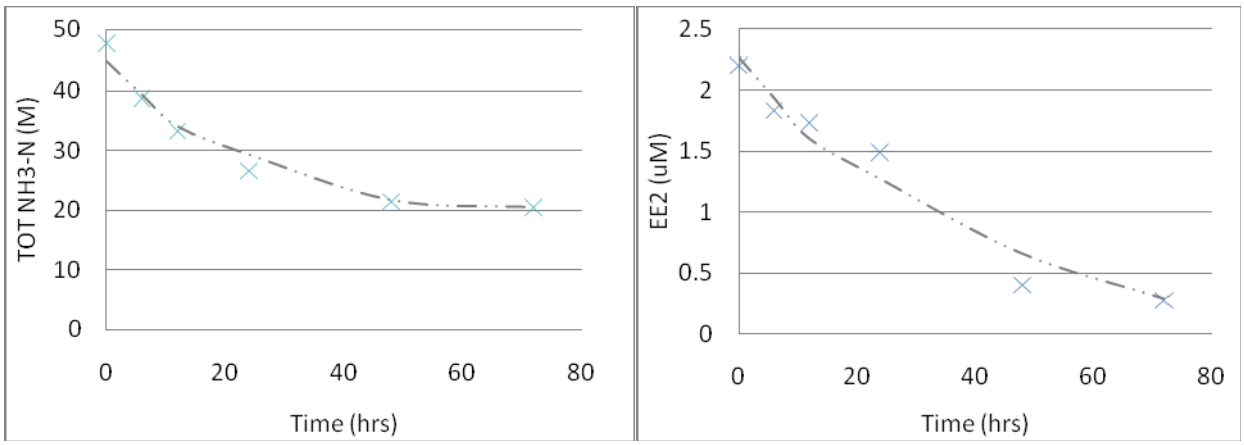

Figure 4 Kinetics of Ammonia & 17α-ethynylestradiol (EE2) degradation (Reductant model)

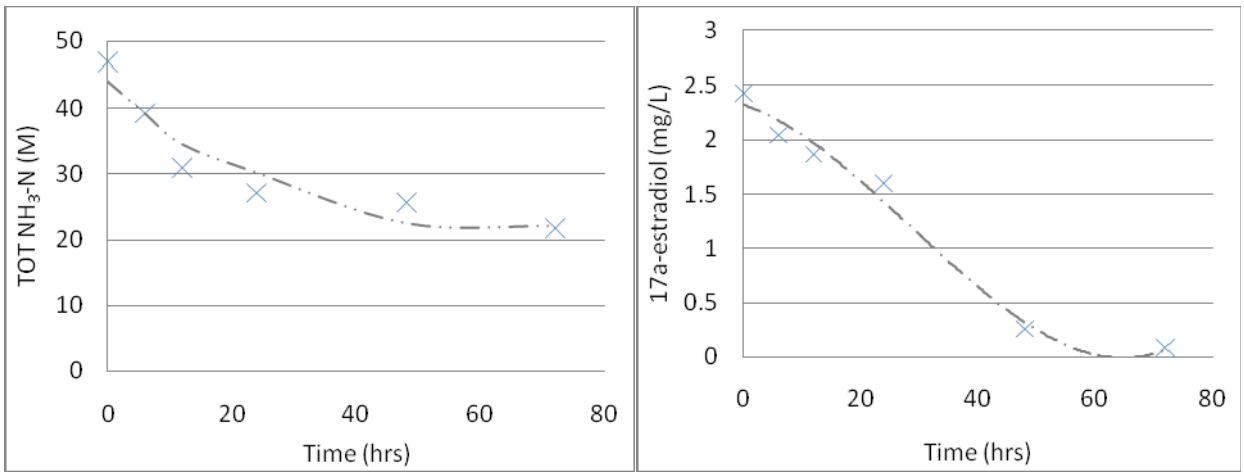

Figure 5 Kinetics of Ammonia & 17α-estradiol degradation (Combined model)

Table 2 NRSS (Normalized residual sum of squares) of five estrogens, **bold numbers** indicate the best fit model with an observed value

Kinetic model	Estrogen (10^{-3})			
	E1 (Estrone)	E2 (17β-estradiol)	E3 (Estriol)	EE2 (17α-ethynylestradiol)
1st order	0.48	19.7	1.08	30.4
Competition	0.42	17.6	0.46	24.0
Reductant	0.39	17.9	**0.45**	**13.0**
Combined	**0.38**	**13.5**	1.60	35.0

Table 3 Kinetic coefficients of estrogens with the best fit model using NRSS value

Parameters	E1 (Estrone)	E2 (17b-estradiol)	E3 (Estroil)	EE2 (17a-ethynylestradiol)	17α-Estradiol
Model	Combined	Combined	Reductant	Reductant	Combined
X (mg BSA/L)	68.80±7.63	48.50±11.80	52.90±10.47	89.20±15.98	69.90 ± 1.63
K_{es} (L/mg BSA-hr^{-1})	$1.32x10^{-4}$±0.0001	$3.21x10^{-4}$±0.0002	$1.72x10^{-4}$±0.0002	$2.82x10^{-4}$±0.00001	$2.16x10^{-4}$±0.0001
$K_{s,NH3-N}$ (mg/L)	21.15±1.47	40.84±15.84	18.04±0.74	18.35±2.04	16.99±2.4

Each estrogen was evaluated using Normalized Residual Sum of Square values that were approximated by non-linear regression (Table 2). E1, E2, E3, EE2, and 17α-estradiol were best fit with combined, combined, reductant, reductant, and combined model respectively. Observed and predicted results for each of the estrogens were compared by using the best fit model of the four candidate models for estrogen kinetic parameter determination (Figures 1-6). A summary of the ammonia and estrogen kinetic parameters determined from these experiments is presented in Table 3. The half saturation coefficient ($K_{s,NH3}$) for ammonia degradation ranged from 16.99 to 40.84 mg/L NH_3-N, values higher than typically reported for nitrifiers because probably both the ammonia concentration and microorganism density were high. The estrogen rate constants ($k_{1,es}$) ranged from $3.21x10^{-4}$ to $0.39x10^{-4}$ (L/mg BSA-hr). All estrogens have very similar rate constant, indicating E2 was most easily degraded, which was similar with Shi et al research [2].

The kinetic models examined proved to be effective in fitting the experimental data. The cometabolic assumptions resulted in different degradation rate constants and suggest mechanisms of estrogen

degradation in the presence of high ammonia concentrations, emphasizing the importance of AOB role in WWTPs. E3 and EE2 might be governed as mostly biodegraded by a reductant (electrons) generated by ammonia oxidation. However, E1, E2, and 17α-estradiol might be governed by both a reductant and competition by either ammonia or its oxidative metabolites (NO_2^- or NO_3^-). Recently there has been debating in key bacteria responsible for hormone degradation in WWTPs. Finally, AOB may be effective for hormone removal in WWTPs, indicating that each estrogen can be biologically degraded with low rate constants and mechanisms. Higher estrogen removal (>95%) seems to be partly attributed from high AOB population in WWTPs, explaining difference between in the absence (>85%) or in the presence (>95%) of a nitrifying step in terms of estrogen removal efficiency. A study demonstrated that high estrogen removal appeared to be related, not statistically, with higher sludge retention time (SRT, 15-20 day) resulting in higher population of AOB in WWTPs [17, 18]. Further investigation of relationship between AOB population and solid retention time (SRT) in WWTPs is needed to understand the potential for cometabolic degradation in environmental relative concentration as an application study in WWTPs.

Reference

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