Importance of culture history on 17 α -ethinylestradiol cometabolism by nitrifying sludge

Angkana Jantanaprasartporn^{1,2}, Suppasil Maneerat³, Chaiwat Rongsayamanont^{2,4*}

¹International Postgraduate Programs in Environmental Management, Graduate School, Chulalongkorn University, Bangkok 10330, Thailand

²Center of Excellence on Hazardous Substance Management (HSM), Bangkok 10330, Thailand

³Biotechnology for Bioresource Utilization Laboratory, Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Songkhla 90110, Thailand

⁴Environmental Assessment and Technology for Hazardous Waste Management Research Center, Faculty of Environmental Management, Prince of Songkla University, Songkhla 90110, Thailand

ABSTRACT

17 α -ethinylestradiol (EE2), a synthetic estrogen which interfere the endocrine and reproductive function in living organisms, has been found extensively to be deposited into municipal wastewater treatment plants and the environment via human excretion. EE2 has long been known to be efficiently cometabolized by ammonia-oxidizing bacteria (AOB) during ammonia (NH₃) oxidation. Current study aims to investigate the effect of culture history on the biotransformation of EE2 by nitrifying sludge which was enriched under different ammonia loading rates in continuous flow reactors. Result showed that past growth condition largely affected not only the metabolic rate of NH₃ oxidation but also EE2 cometabolism. Sludge previously acclimated with higher NH₃ loads as well as sludge dominated with AOB belong to high growth cluster (*Nitrosomonas europaea*-*Nitrosococcus mobilis*) showed higher rate of EE2 biotransformation than those one being acclimated with lower NH₃ loads because of its ability to provide more reducing power from NH₃ oxidation. Moreover, the correlation between the degradation rates of NH₃ and EE2 was higher in sludge being acclimated with higher load of NH₃ in comparison with other sludge. Implication of the findings emphasized the role of volumetric NH₃ loading rate in determining EE2 removal in wastewater treatment system.

Keywords: Ammonia-oxidizing bacteria, Cometabolism, Culture history, Nitrifying sludge, 17 α -ethinylestradiol

1. Introduction

Estrogens are essential group of hormones that regulate the growth and development of female sexual characteristics in humans and animals. Estrogens strongly interfere with endocrine and reproductive functions in living organisms including disturbance on immunological system and fertility, reproductive failure due to thinning of eggshells and reduction in egg production [1], feminization and masculinization, and altered sexual development [2]. 17 α -ethinylestradiol (EE2), a synthetic estrogen, is generally used as the oral contraceptive pill to control the birth rate and hormone therapy. EE2 prompt to be released to the environment via human excretion which is later discharged into municipal wastewater treatment plants (WWTS) as hotspots for the release of estrogens in the environment [3].

Some estrogens are able to be degraded in the WWTS. The estrogens removal efficiencies in activated sludge process range from 61% to 98% for estrone (E1), 67% and 99% for estradiol (E2) and 85% to 90% for EE2 [4]. Natural estrogens (E1 and E2) are readily metabolized by heterotrophic microorganisms in WWTS, while the biodegradation of more recalcitrant hormone like EE2 is known mainly through cometabolism of ammonia monooxygenase enzyme (AMO) by ammonia-oxidizing bacteria (AOB) [5, 6]. Some essential factors like the availability of ammonia (NH₃) as the growth substrate of AOB [5] have been investigated for its roles on EE2 cometabolism by AMO. However, microorganism generally produce enzyme and other macromolecules at a rate which is unique to their previous growth condition [7, 8], therefore, the culture history of AOB may be another factor determining not only the rate of NH₃ utilization [9] but also the rate of EE2 cometabolism.

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Copyright © 2018 Korean Society of Environmental Engineers

Received April 8, 2017 Accepted July 18, 2017

* Corresponding author

Email: milesgodfather@yahoo.com

Tel: +66-7428-6839 Fax: +66-7442-9758

For all of the reasons above, this study aims to determine effect of culture history on EE2 cometabolism by autotrophic AOB. AOB was enriched in mixed culture at different growth conditions. Effect of growth substrate concentration on bacterial cometabolism was also investigated. Although the past growth condition could potentially affect the bacterial cometabolism, but influence of the culture history of AOB on its EE2 cometabolism has never been investigated.

2. Materials and Methods

2.1. Chemicals

EE2 (17 α -ethinylestradiol) was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). The stock solution of EE2 was prepared to 500 mg/L in methanol. Other chemicals were purchased from Carlo Erba Reagents (Val de Reuil, France). All chemicals used in this study are analytical reagent grade.

2.2. Enrichment Media for AOB

Enrichment media, which consist of synthetic wastewater plus NH_3 at various concentrations, was used for enrichment of AOB. Synthetic wastewater A was prepared by dissolving NaCl (1 g), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.4 g), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.1 g), KH_2PO_4 (0.2 g), and KCl (0.5 g) into one liter of de-ionized (DI) water and then adding one milliliter (mL) of each mineral solutions including the solution of non-chelated trace element, selenite-tungstate, vitamin mixture, thiamine, vitamin B12 [10]. Synthetic wastewater A was added with 0.34 g of $(\text{NH}_4)_2\text{SO}_4$ per liter to achieve final NH_3 concentration of 70 mg N/L. Synthetic wastewater B, which contained Na_2HPO_4 (4.05 g), K_2HPO_4 (2.1 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05 g), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.01 g), and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.09 g) in one liter of DI water [11], was added with 0.68 and 2.02 g of $(\text{NH}_4)_2\text{SO}_4$ per liter to make up final NH_3 concentrations of 140 and 420 mg N/L, respectively. Synthetic wastewater A and B were further used to prepare medium for batch degradation test.

2.3. Enriched Reactors Setup and Operation

Three vessels with an effective volume of 6 liters were identically set up as continuous flow enriched reactors without cell recycle. For each reactor, air was supplied from air blower connecting with a small stone diffuser to provide oxygen and mix the mixed liquor completely. The concentration of dissolved oxygen (DO) in bulk solution was maintained between 7-8 mgO_2/L by adjusting air flow rate with a small needle valve. pH in mixed liquor was controlled at 7.5 ± 0.2 by a pH controller (Liquitron DP 5000, LMI Milton Roy, USA) equipped with a pH probe (Orion 9156DJWP, Thermo scientific, UK) as well as a solution of 0.5 N HCl and 0.5 N NaOH. Reactors were operated at room temperature between 23-25°C. Mixed liquor taken from full scale activated sludge process was used as inoculum to start up the reactors. Enriched reactors were operated differently in order to enrich AOB which belongs to different growth characteristics. Enrichment medium containing NH_3 was continuously fed into each one of three reactors at different NH_3 concentrations to enrich the AOB population which belongs

to different rates of NH_3 utilization. The reactors named '70NS', '140NS' and '420NS' were fed with NH_3 of 70, 140 and 420 mg N/L and controlled at sludge age of 4, 3, and 4 d, respectively. For each gram of added NH_3 , 0.068 grams of HCO_3^- was put into an enrichment medium to provide carbon source for AOB growth.

2.4. Batch Degradation Test

2.4.1. Reactor setup and operation

Several 25 mL small Plexiglas® vessels, which were initially filled with synthetic wastewater, were used as batch reactors. Sludge taken from enriched reactors during steady operation were washed and inoculated into the batch reactors. Synthetic wastewater was later filled into batch reactors until final sludge concentration of 300 mg mixed liquor suspended solid (MLSS)/L was reached. Then, a predetermined volume of the stock EE2 solution was added and fully mixed in order to achieve final EE2 concentration of 3 mg/L in the reactors. NH_3 was later added into the reactors in a similar manner with that of EE2. Synthetic wastewater A was used particularly for tests with having initial NH_3 levels of 28 and 70 mg N/L while for tests belongs to the initial NH_3 concentration of 140 and 420 mg N/L, wastewater B was used instead. Air and mixing was provided to mixed liquor by shaking in an orbital shaker at 250 rpm. The reactor was incubated at room temperature. While running the tests, pH in bulk solution was maintained within a range of 8.0 ± 0.2 by adding HEPES buffer solution to a final concentration of 30 g/L.

2.4.2. Test procedure

Sludge containing the AOB population which belongs to different the NH_3 utilization rates was taken from each one of three enriched reactors during steady operation and then, brought into batch degradation tests. Various initial concentrations of NH_3 were used in the tests in order to determine effect of growth substrate concentration on the rates of NH_3 oxidation and EE2 degradation. Loss due to abiotic sorption and volatilization of NH_3 and EE2 were observed through batch tests with heat-killed cell and without cell. EE2 biodegradability by heterotrophic microorganism in enriched sludge was investigated in the presence of 10 mg/L allylthiourea (ATU) for the suppression of the AMO usage by AOB [12]. Furthermore, 1,000 mg/L chloramphenicol was also added into the reactors in order to evaluate the NH_3 and EE2 biodegradability remained in the harvested sludge under the suppression of AMO-induced de novo synthesis [13]. All tests were carried out in triplicate except the test with heat-treated cells and with no cells were done in duplicate. For all tests, samples were periodically collected and analyzed for the concentration of EE2 and inorganic nitrogen species in bulk solution for the whole two weeks of the test period.

2.5. Chemical Analysis

Mixed liquor samples were collected from enriched and batch reactors and then, filtered through 0.45 μm Polytetrafluoroethylene membrane filter. Filtrate were analyzed for EE2 and inorganic nitrogen species (NH_3 , NO_2^- , NO_3^-) while filtrated were measured for MLSS and mixed liquor volatile suspended solid by gravimetric method. EE2 was analyzed by a C18 reversed-phase high-performance liquid chromatography with ultraviolet detector (Shimadzu

Series LC-10ADvp) using a 60:40 acetonitrile:H₂O as mobile phase. NH₃ was measured by gas-sensitive combination electrode (Cole Palmer, Co.) and salicylated-colorimetric method with UV visible spectrophotometer (Thermo Electron Corporation, Hexious α , Cambridge, UK) at 640 nm [14]. NO₂⁻ was colorimetrically analyzed with UV visible spectrophotometer at 543 nm while NO₃⁻ was analyzed by UV screening method at 220 and 275 nm [14]. pH, DO concentration and temperature in mixed liquor were monitored during sludge enrichment and degradation test. pH value was determined by pH probe (250A+, Thermo Orion Inc., Germany). The concentration of DO was determined by membrane sensing electrode (DO 083005D probe with 850A meter, Thermo Orion Inc., Germany). Temperature was measured by a laboratory glass thermometer.

2.6. Analysis of Ammonia-oxidizing Bacterial Communities

2.6.1. Sample preparation and DNA extraction

Sludge samples (2 mg dry sludge/L) from two enriched reactors including '70NS' and '420NS' were concentrated via centrifugation at 14,000 rpm for 10 min and stored at -20°C. Genomic DNA was extracted from each samples (2 mg dry sludge) using Fast-DNA SPIN kits for soil (QBiogenes, USA) following to the manufacturer's instruction. The extracted DNA was verified using 1.5% agarose gel electrophoresis (Bio-Rad, Spain).

2.6.2. Polymerase chain reaction (PCR), cloning and sequencing

Primers amoA 1F and 2R [15] (forward 5-GGGGTTCCTACTGGTGGT-3; reverse 5-CCCCCTCKGSAAGCCTTCTTC-3) were used for PCR amplification. Bacterial amoA gene fragments were amplified using a Takara polymerase (Takara Bio Inc., Japan) in a thermal cycler (Biorad Laboratories, USA) for 10 min at 95°C, followed by 35 cycles of 60 s at 95°C, 60 s at 56°C, and 30 s at 72°C, followed by 15 min of final extension at 78°C. Later, the amplified PCR products were purified by gel electrophoresis using a NucleoSpin Extract II Kit (Clontech Laboratories Inc., USA). Then, the purified PCR products were cloned using the pGEM-T Easy vector system (Promega, USA). Later, five and four clones of '70NS' and '420NS' samples were randomly chosen for sequencing at Macrogen Inc., Korea, respectively.

3. Results and Discussion

3.1. Enriched Reactors Performance

Table 1 and Fig. 1 showed long-term performance of all three enriched reactors. All reactors showed steady performance in terms of NH₃, NO₂⁻ and NO₃⁻ effluent concentrations after five weeks of operation. At steady state condition, NH₃ removal efficiencies

Fig. 1. Concentration profiles of inorganic nitrogen species during sludge enrichment with NH₃ concentration of a) 70 mg N/L, b) 140 mg N/L and c) 420 mg N/L.

Table 1. System Performance of Enriched Reactors

Parameter	Treatment performance		
	A	B	C
	70 mg N/L NH ₃ -fed reactor	140 mg N/L NH ₃ -fed reactor	420 mg N/L NH ₃ -fed reactor
Influent NH ₃ (mg N/L)	69.14 ± 1.44	138.05 ± 3.79	409.37 ± 17.55
NH ₃ loading rate (mg N/(L-d))	16.59 ± 0.35	33.13 ± 0.91	98.25 ± 4.21
Effluent NH ₃ (mg N/L)	0.49 ± 0.15	1.95 ± 0.58	11.00 ± 10.76
NH ₃ oxidation rate (mg N/(gVSS-day))	219.7	275.0	637.4
Effluent NO ₂ ⁻ (mg N/L)	0.12 ± 0.08	0.84 ± 0.69	12.35 ± 13.16
Effluent NO ₃ ⁻ (mg N/L)	66.47 ± 3.38	125.88 ± 3.20	353.55 ± 18.40
Total N	67.09 ± 3.01	128.67 ± 3.51	376.90 ± 27.03
Dominated AOB cluster	<i>Nitrosomonas oligotropha</i> cluster	-	<i>Nitrosomonas europaea</i> - <i>Nitrosococcus mobilis</i> cluster

for reactors fed with NH_3 of 70 mg N/L and 140 mg N/L were more than 99%. But for 420 mg N/L NH_3 -fed reactor, a slight less efficiency for NH_3 removal (96.23%) was observed due to receiving higher NH_3 load compared to other reactors. In addition, an increase in NH_3 load led to stimulate specific NH_3 oxidation rate. No NO_2^- accumulated in any reactors indicating NH_3 oxidation is the rate determining step for nitrification.

3.2. Ammonia-oxidizing Bacterial Communities in Enriched Sludge

Sludge '70NS' and '420NS' were brought to further analyze for ammonia-oxidizing bacterial communities using PCR-cloning-sequencing technique. Results showed that AOB belongs to *Nitrosomonas oligotropha* (*N. oligotropha*) cluster dominated (4 of 5 clones) in reactor fed with NH_3 of 70 mg N/L at which the NH_3 concentration were less than 1 mg N/L in effluent (Table 1). AOB belongs to *N. oligotropha* cluster is known as a high NH_3 affinity cluster as indicated by low Monod half-saturated coefficient (K_s : 0.32-3.16 mg N/L [16, 17]). However, reactor fed with 420 mg N/L NH_3 , which its effluent contained 15.82 mg N/L NH_3 , was dominated (all 4 clones) by AOB belongs to *Nitrosomonas europaea*-*Nitrosococcus mobilis* (*N. europaea*-*N. mobilis*) cluster. AOB belongs to *N. europaea*-*N. mobilis* cluster has much lower NH_3 affinity than that belongs to *N. oligotropha* cluster as indicated by much higher K_s (1-100 mg N/L, [16-19]). These results corresponded with the finding reported in Limpiyakorn et al. [20]. Limpiyakorn et al. [20] showed that *N. europaea*-*N. mobilis* cluster dominated in chemostats fed with > 140 mg N/L NH_3 while for the reactor fed with ≤ 140 mg N/L NH_3 , the dominant AOB belonged to *N. oligotropha* cluster.

3.3. Batch NH_3 Oxidation

3.3.1. Effect of culture history

In order to investigate effect of continuous culture history on batch substrate utilization, three enriched sludge being acclimated with various loads of the growth substrate ('70NS', '140NS', and '420NS')

were brought to determine the rate of substrate utilization under batch condition. Results showed that continuous culture history of enriched sludge strongly influence on batch NH_3 oxidation rate (Table 2). For any given values of initial NH_3 concentration, higher NH_3 oxidation rate was found in sludge previously being acclimated with higher load of NH_3 .

This is likely because continuous cultivation of mixed sludge at higher NH_3 load leading to the selection for a particular AOB having higher NH_3 utilization rate. Generally, the dominant bacterial population mainly determined the rate of substrate utilization and bacterial growth of mixed culture. Sludge dominated by AOB with *N. europaea*-*N. mobilis* cluster (420NS) showed higher batch NH_3 utilization rate as compared with that of dominated by *N. oligotropha* AOB cluster (70NS). For all results shown above, an intrinsic NH_3 oxidation rate clearly depended on the physiology of dominant AOB population in enriched nitrifying sludge.

3.3.2. Effect of initial NH_3 concentration

As a result, NH_3 oxidation was limited by substrate availability. Larger specific NH_3 oxidation rate was observed at higher initial NH_3 (Fig. 2). However, maximum batch NH_3 oxidation rate of enriched sludge was attained under initial NH_3 concentration which had been previously used for feeding during sludge enrichment. Enriched sludge in reactors fed with 70 mg N/L NH_3 (70NS) achieved its maximum NH_3 oxidation rate at 70 mg N/L while at least 140 mg N/L was needed by sludge taken from the 140 and 420 mg N/L NH_3 -fed reactors (140NS and 420NS). In other words, *N. oligotropha*-dominating sludge (70NS) achieved its maximum NH_3 oxidation rate at lower NH_3 concentration than that of sludge dominated with *N. europaea*-*N. mobilis* AOB cluster (420NS). Higher NH_3 affinity of the 70NS as compared to that of the 420NS because it was dominated by the *N. oligotropha* cluster which has higher NH_3 affinity than the *N. europaea*-*N. mobilis* cluster.

Moreover, at initial NH_3 concentration of 420 mg N/L, slight lower NH_3 was oxidized as a result of substrate inhibition. However, sludge dominated with *N. oligotropha* AOB cluster (70NS) were

Table 2. Comparison of the Rates of EE2 Degradation by Nitrifying Activated Sludge and Enriched Nitrifying Sludge in Aerated Batch Reactor

Culture	EE2 degradation rate (mg/(gVSS-day))	NH_3 oxidation rate (mg N/(gVSS-day))	Initial concentration of EE2 & NH_3	Ref
Nitrifying activated sludge	0.0936	1,320	1.0 mg/L EE2; 100 mg N/L NH_3	[12]
AMO-containing extract from enriched nitrifying sludge	7.83-29.17	129.6-1,339.2	-	[23]
Enriched nitrifying sludge	0.096	1,260	0.75 mg/L EE2; 52.5 mg N/L NH_3	[26]
Nitrifying activated sludge	0.024	933.6	0.05 mg/L EE2; 50 mg N/L NH_3	[28]
Enriched nitrifying sludge				
^a 70NS (<i>N. oligotropha</i> -dominating)	^a 0.28-0.52	^a 51.1-225.4	3.0 mg/L EE2;	This study
^b 140NS	^b 0.31-0.66	^b 67.5-312.7	28-420 mg N/L	
^c 420NS (<i>N. europaea</i> -dominating)	^c 0.44-0.88	^c 150.9-630.7	NH_3	

Fig. 2. Profiles of NH_3 and NO_3^- concentration during incubation test with and without adding ATU at initial NH_3 concentration of 28 mg N/L (a-c), 70 mg N/L (d-f), 140 mg N/L (g-i) and 420 mg N/L (j-l) using sludge 70NS (a,d,g,j), 140NS (b,e,h,k) and 420NS (c,f,i,l).

apparently affected far less by the substrate inhibition than *N. europaea*-*N. mobilis*-dominating sludge indicating higher tolerability to adverse condition in slow-growing AOB. This emphasized an importance of past cultivation condition of sludge on its rate of substrate utilization in batch reactor.

3.4. Batch EE2 Degradation

EE2 biodegradation by sludge enriched with different loads of NH_3 was investigated to observe effect of culture history on the AMO cometabolism. As shown in Fig. 2 and 3, NH_3 and EE2 degradation was observed only in the absence of ATU. Furthermore, there was not any EE2 degradation in batch with heat-treated cells or no cell (data not shown) indicating the loss of EE2 due to abiotic processes (ie. sorption and volatilization) was negligible in our study. This indicates that AMO cometabolism by AOB is main degradation pathway of EE2 rather than heterotrophic and abiotic degradation that is correspondence to several studies [12, 21-25]. Moreover, EE2 and NH_3 degradation in the presence of AMO-induced inhibitor was not found (data not shown) indicating residual AMO utilization by harvested cells in order to complete both substrate degradation was negligible.

EE2 biotransformation clearly depended on NH_3 oxidation. During EE2 cometabolism, EE2 was degraded by using a reducing power from NH_3 oxidation. Therefore, more NH_3 was oxidized,

higher EE2 was degraded via the cometabolism of AMO. Moreover, not only initial NH_3 concentration largely affected on NH_3 oxidation rate but on EE2 biotransformation rate as well. Higher available NH_3 improved both the rates of NH_3 and EE2 removals indicating a reductant-limiting condition for AMO metabolism and cometabolism. While a reductant-inhibiting condition decreased NH_3 oxidation and EE2 degradation at initial NH_3 of 420 mg N/L. The results shown here is in good agreement with previous studies [22-24] which found strong positive correlation between initial concentration of NH_3 and batch EE2 biotransformation rate by nitrifying sludge.

In addition, after 5 d of sludge incubation with initial NH_3 of 28-140 mg N/L, all NH_3 were utilized, but substantial biotransformation of EE2 was still occurring even in the absence of NH_3 . This was consistent with the results obtained by a number of studies [22, 26-28] which revealed the capability of nitrifying sludge to cometabolize EE2 in NH_3 -free environment. This phenomenon is likely to be a result of utilizing AMO accumulated within the AOB cell for degrading EE2 during NH_3 -limiting condition.

Moreover, EE2 degradation of enriched sludge was affected by not only initial NH_3 level but also the enrichment condition. Higher EE2 biotransformation rate was achieved by sludge previously being enriched with higher NH_3 load (Fig. 4). As mentioned earlier, the enrichment of nitrifying bacteria at higher NH_3 load may pro

Fig. 3. EE2 degradation using different sludge at initial NH_3 concentration a) 28 mg N/L b) 70 mg N/L c) 140 mg N/L d) 420 mg N/L.

Fig. 4. The degradation rate of a) NH_3 and b) EE2 under different sludge and initial NH_3 concentrations.

mote specific AOB having higher utilization rate of NH_3 over those having the lower one. The results show that AOB population in enriched nitrifying sludge affected on the EE2 degradation in the same manner as NH_3 oxidation. Higher EE2 degradation was found in sludge dominated by higher-growth-AOB than lower-growth-AOB-dominating sludge. EE2 cometabolic rate of sludge dominated with the *N. europaea*-*N. mobilis* AOB cluster were approximately 1.6-1.7 (420NS) times higher than that belongs to

Fig. 5. The relationship between NH_3 and EE2 degradation rates for a) 70NS b) 140NS c) 420NS.

the *N. oligotropha* AOB cluster (70NS). Greater EE2 cometabolic rate of higher-growth-AOB may be caused by more electron available for cometabolism.

Furthermore, the correlation between NH_3 and EE2 degradation rates of sludge was found strongest in the sludge being previously cultivated with highest NH_3 load (Fig. 5). It can be implied that more electron provided by NH_3 oxidation is needed by sludge being previously cultivated with the highest load of NH_3 to cometabolize a certain amount of EE2. Our finding firstly reveals the importance of past AOB growth condition regarding its determination on simultaneous N and EE2 removals. The rates of EE2 biotransformation found in this study were compared with those reported in previous studies (Table 2). Higher rates of EE2 degradation obtained in this study as compared to other studies is likely because much higher initial concentration of EE2 was used.

4. Conclusions

This research firstly determined the importance of culture history of nitrifying sludge on simultaneous NH_3 and EE2 removals in wastewater. Higher available NH_3 improved both the rates of NH_3 and EE2 removals indicating a reductant-limiting condition for AMO metabolism and cometabolism. Sludge being acclimated with higher NH_3 load as well as the one dominated with higher-growing-AOB showed higher rate of EE2 cometabolism due to its ability to provide more reducing power from NH_3 oxidation. The NH_3 and EE2 degradation rates were found having a stronger correlation particularly in sludge being previously cultivated with higher NH_3 load.

Acknowledgments

The research was supported by a grant from Prince of Songkla University, contract no. ENV550371S.

References

- Scholz S, Gutzeit HO. 17α -ethinylestradiol affects reproduction, sexual differentiation and aromatase gene expression of the medaka (*Oryzias latipes*). *Aquat. Toxicol.* 2000;50:363-373.
- Hester RE, Harrison RM, Phillips B, Harrison P. Overview of the endocrine disruptors issue. In: Harrison RM and Hester RE, eds. Endocrine disrupting chemicals. London: Royal Society of Chemistry; 1999. p. 1-26.
- Lust MJ, Ziels RM, Strand SE, Gough HL, Stensel HD. Biodegradation kinetics of 17α -ethinylestradiol in activated sludge treatment processes. *Environ. Eng. Sci.* 2015;32:637-646.
- Johnson AC, Williams RJ. A model to estimate influent and effluent concentrations of estradiol, estrone, and ethinylestradiol at sewage treatment works. *Environ. Sci. Technol.* 2004;38:3649-3658.
- Tran NH, Urase T, Ngo HH, Hu J, Ong SL. Insight into metabolic and cometabolic activities of autotrophic and heterotrophic microorganisms in the biodegradation of emerging trace organic contaminants. *Bioresour. Technol.* 2013;146:721-731.
- Khunjar WO, Mackintosh SA, Skotnicka-Pitak J, Baik S, Aga DS, Love NG. Elucidating the relative roles of ammonia oxidizing and heterotrophic bacteria during the biotransformation of 17 α -ethinylestradiol and trimethoprim. *Environ. Sci. Technol.* 2011;45:3605-3612.
- Ozkok IP, Rehman A, Yagci N, Cokgor EU, Jonas D, Orhon D. Characteristics of mixed microbial culture at different sludge ages: Effect on variable kinetics for substrate utilization. *Bioresour. Technol.* 2012;126:274-282.
- Grady CPL, Smets BF, Barbeau DS. Variability in kinetic parameter estimates: A review of possible causes and a proposed terminology. *Water Res.* 1996;30:742-748.
- Munz G, Szoke N, Oleszkiewicz JA. Effect of ammonia oxidizing bacteria (AOB) kinetics on bioaugmentation. *Bioresour. Technol.* 2012;125:88-96.
- Widdel F, Bak F. Gram-negative mesophilic sulfate-reducing bacteria. In: Balows A, Truper HG, Dworkin M, Harder W, Schleifer KH, eds. The prokaryotes: A handbook on the biology of bacteria: Ecophysiology, isolation, identification, applications. New York: Springer-Verlag; 1992. p. 3352-3378.
- Rongsayamanont C, Limpiyakorn T, Law B, Khan E. Relationship between respirometric activity and community of entrapped nitrifying bacteria: Implication for partial nitrification. *Enzyme Microbial Technol.* 2010;46:229-236.
- Shi J, Fujisawa S, Nakai S, Hosomi M. Biodegradation of natural and synthetic estrogens by nitrifying activated sludge and ammonia-oxidizing bacterium *Nitrosomonas europaea*. *Water Res.* 2004;38:2323-2330.
- Gerards S, Duyts H, Laanbroek HJ. Ammonium-induced inhibition of ammonium-starved *Nitrosomonas europaea* cells in soil and sand slurries. *FEMS. Microbiol. Ecol.* 1998;26: 269-280.
- APHA-AWWA-WEF. Standard methods for the examination of water and wastewater. 20th ed. Washington D.C.: American Public Health Association, American Water Works Association, Water Environment Federation; 1998. p. 4-91-4-143.
- Rotthauwe JH, Witzel KP, Liesack W. The ammonia mono-oxygenase structural gene *amoA* as a functional marker: Molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl. Environ. Microbiol.* 1997;63:4704-4712.
- Martens-Habbena W, Berube PM, Urakawa H, De la Torre JR, Stahl DA. Ammonia oxidation kinetics determine niche separation of nitrifying archaea and bacteria. *Nature* 2009;461: 976-979.
- Koops HP, Purkhold U, Pommerening-Röser A, Timmermann G, Wagner M. The lithoautotrophic ammonia-oxidizing bacteria. In: Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E, eds. The prokaryotes: An evolving electronic resource for the microbiological community. New York: Springer-Verlag; 2003. p. 778-811.
- Wood PM. Nitrification as a bacterial energy source. In: Prosser JL, ed. Nitrification. Oxford: IRL Press; 1986. p. 39-62.
- Suzuki I, Dular U, Kwok SC. Ammonia or ammonium ion as substrate for oxidation by *Nitrosomonas europaea* cells and

- extracts. *J. Bacteriol.* 1974;120:556-558.
20. Limpiyakorn T, Kurisu F, Sakamoto Y, Yagi O. Effects of ammonium and nitrite on communities and populations of ammonia-oxidizing bacteria in laboratory-scale continuous-flow reactors. *FEMS. Microbiol. Ecol.* 2007;60:501-512.
21. Forrez I, Carballa M, Noppe H, De Brabander H, Boon N, Verstraete W. Influence of manganese and ammonium oxidation on the removal of 17 α -ethinylestradiol (EE2). *Water Res.* 2009;43:77-86.
22. Ren YX, Nakano K, Nomura M, Chiba N, Nishimura O. Effects of bacterial activity on estrogen removal in nitrifying activated sludge. *Water Res.* 2007;41:3089-3096.
23. Yi T, Harper WF. The link between nitrification and biotransformation of 17 α -ethinylestradiol. *Environ. Sci. Technol.* 2007;41:4311-4316.
24. Yi T, Harper WF, Holbrook RD, Love NG. Role of particle size and ammonium oxidation in removal of 17 α -ethinylestradiol in bioreactors. *J. Environ. Eng.* 2006;132:1527-1529.
25. Andersen H, Siegrist H, Halling-Sorensen B, Ternes TA. Fate of estrogens in a municipal sewage treatment plant. *Environ. Sci. Technol.* 2003;37:4021-4026.
26. De Gussemme B, Pycke B, Hennebel T, et al. Biological removal of 17 α -ethinylestradiol by a nitrifier enrichment culture in a membrane bioreactor. *Water Res.* 2009;43:2493-2503.
27. Forrez I, Carballa M, Boon N, Verstraete W. Biological removal of 17 α -ethinylestradiol (EE2) in an aerated nitrifying fixed bed reactor during ammonium starvation. *J. Chem. Technol. Biotechnol.* 2008;84:119-125.
28. Vader JS, Van Ginkel CG, Sperling FM. Degradation of ethinylestradiol by nitrifying activated sludge. *Chemosphere* 2000;41:1239-1243.