Degradation of EE$_2$ by Different Consortium of Enriched Nitrifying Activated Sludge

Pantip Kayee

Abstract—17α-ethinylestradiol (EE$_2$) is a recalcitrant micropollutant which is found in small amounts in municipal wastewater. But these small amounts still adversely affect for the reproductive function of aquatic organisms. Evidence in the past suggested that full-scale WWTPs equipped with nitrification process enhanced the removal of EE$_2$ in the municipal wastewater. EE$_2$ has been proven to be able to be transformed by ammonia oxidizing bacteria (AOB) via co-metabolism. This research aims to clarify the EE$_2$ degradation pattern by different consortium of ammonia oxidizing microorganism (AOM) including AOA (ammonia oxidizing archaea) and investigate contribution between the existing ammonia monooxygenase (AMO) and new synthesized AOM. The result showed that AOA or AOB of $N$. oligotropha cluster in enriched nitrifying activated sludge (NAS) from 2mM and 5mM, commonly found in municipal WWTPs, could degrade EE$_2$ in wastewater via co-metabolism. Moreover, the investigation of the contribution between the existing ammonia monooxygenase (AMO) and new synthesized AOM demonstrated that the new synthesized AMO enzyme may perform ammonia oxidation rather than the existing AMO enzyme or the existing AMO enzyme may has a small amount to oxidize ammonia.

Keywords—17α-ethinylestradiol, nitrification, ammonia oxidizing bacteria, ammonia oxidizing archaea.

I. INTRODUCTION

Nitriﬁcation-denitrification is the important biological process for nitrogenous substance removal in wastewater. Along with this process, the ammonia oxidation, the first step of a nitrification process, is believed to be the rate-limiting step of biological nitrogen removal in wastewater treatment plants. Before the discovery ammonia oxidizing archaea (AOA), ammonia oxidizing bacteria (AOB), chemolithoautotrophic bacteria, was long thought to be the only microorganisms capable for the ammonia oxidation. Thus far, the study of the contribution of AOA to ammonia oxidation in WWTPs is still lacking.

Ammonia monooxygenase (AMO) is the key enzyme for ammonia oxidation which has been known to have broad substrates. Consequently, beside ammonia, AOB are capable of degrading a few organic pollutants such as aliphatic hydrocarbons, chlorinated hydrocarbons [1]-[3] and endocrine disrupting compounds [4]. This can be done by co-metabolic mechanism during the ammonia oxidation. Recent idea has arisen to use AOB to remove recalcitrant micropollutants which remain and are non-degradable by heterotrophic microorganisms in WWTPs.

A pharmaceutical compound, namely 17α-ethinylestradiol (EE$_2$) is a synthetic estrogen which commonly uses as a main ingredient in oral contraceptive pill. EE$_2$ is typically released by excretion of human and then contaminated to municipal wastewater. The municipal wastewater treatment plants (WWTPs) is the reservoir for receiving and treating the municipal wastewater. Generally, EE$_2$ was treated during nitrification which performed by ammonia oxidizing bacteria (AOB) via co-metabolism.

However, the completely EE$_2$ removal was not achieved by WWTP and was therefore released and accumulated in environment. EE$_2$ is reported as an endocrine disrupting compound having a potential to adversely affect for the reproductive function of aquatic organisms [5].

Due to the increasing of environmental concern about endocrine disrupting compounds, the EE$_2$ removal efficiency should be enhanced to reduce the contamination of EE$_2$ in environment. Moreover, the degradation product in effluent should be test for the estrogenic activity to observe the toxicity for aquatic organisms. However, the enhancement of EE$_2$ removal efficiency is required the understanding of metabolic (ammonia oxidation) and co-metabolic (EE$_2$ degradation) behavior of ammonia oxidizing microorganism. Previously, the degradation of EE$_2$ was studied only by pure culture of AOB with low affinity to ammonia ($Nitrosomonas europaea$) and nitrifying activated sludge containing AOB with low affinity to ammonia. The degradation patterns of ammonia and EE$_2$ have never been studied in sludge containing different consortium of ammonia oxidizing microorganism (AOM) including AOA. So far, no information verified that whether AOA can degrade EE$_2$ or other substances, although, AOA were known that might involve in ammonia oxidation. Therefore, in order to apply AOM to remove EE$_2$ from wastewater, it is necessary to understand how the different in consortium of AOM in WWTPs affect to the degradation of EE$_2$. Consequently, this study investigated the degradation patterns of EE$_2$ by enriched nitrifying activated sludge (NAS) containing different consortium of AOM. Moreover, the contribution of AMO between new synthesized enzyme and existing enzyme on ammonia oxidation and EE$_2$ biodegradation was investigated. In doing so, we will understand clearly the microbiology, activity (metabolic and co-metabolic behavior) and enzymology of AOM. This study would be very useful to improve the removal efficiency of ammonia and recalcitrant micropollutants in WWTPs.

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II. METHODS

A. Enrichment of Nitrifying Activated Sludge

Activated sludge of municipal full-scale wastewater treatment plants were enriched in two chemostat reactors fed with medium containing essential supplements for growth of ammonia oxidizing archaea and bacteria either at 2mM or 5mM to obtain different compositions of AOM. In nitrifying activated sludge (NAS) reactor fed with 2mM ammonia, AOA and Nitrosomonas oligotropha were the predominant microorganisms while Nitrosomonas oligotroph was the major AOM in NAS reactor fed with 5mM ammonia. Both reactors were operated at dilution rate of 0.01hr\(^{-1}\) and pH was adjusted in range 7.5±0.1 by pH controllers (Liquitron DP 5000, LMI Milton Roy, USA). The DO was maintained to 7-8mgO\(_2\)/l using aerator.

B. Inorganic Medium for Nitrifying Activated Sludge Enrichment and Degradation Test

Inorganic medium used for this study containing vitamin and trace elements to promote ammonia oxidizing archaea [6]. The medium solution 1L comprised of 1ml of steriled non-chelated trace element (12.5ml of 25 % of HCl, 5g of MnCl\(_2\cdot6\)H\(_2\)O, 40g of MnSO\(_4\cdot7\)H\(_2\)O, 24mg of NiCl\(_2\), 36mg of chelated trace element (12.5ml of 25 % of HCl, 5g of CoCl\(_2\cdot6\)H\(_2\)O, 144mg of ZnSO\(_4\cdot7\)H\(_2\)O, 36mg of CuCl\(_2\cdot2\)H\(_2\)O, 2.1g of FeSO\(_4\cdot7\)H\(_2\)O, 30mg of H\(_3\)BO\(_3\), 190mg of CoCl\(_2\cdot6\)H\(_2\)O, 144mg of ZnSO\(_4\cdot7\)H\(_2\)O, 36mg of Na\(_2\)SeO\(_3\cdot5\)H\(_2\)O in 1L of deionised water), 1ml of vitamin B1 (10mg of thiamine chloride water), 1ml of vitamin B12 (5mg of cyanocobalamin in 100ml of deionised water), 1ml of vitamin B1 (10mg of thiamine chloride dihydrochloride in 100ml of 25mM sodium phosphate buffer at pH 3.4).

C. Quantitative Polymerase Chain Reaction

DNA was extracted from a sample (2mg dry weight) using Fast-DNA SPIN kits for soil (QBiogene, USA) according to the manufacturer’s instruction. An extract was verified on 1.5% agarose gel electrophoresis (Bio-Rad, Spain). For each sample, duplicate sets of extracts were prepared. In each set of extracts, two extracts were pooled to minimize bias. Quantitative polymerase chain reaction (PCR) was performed separately for duplicate sets of extracts using a Light Cycler 480 instrument (Roche, Germany) with a Maxima SYBR Green/ROX QPCR Master Mix (Fermentas, USA). For each set of extracts, qPCR was carried out with four 10 dilution series, each in duplicates.

AOA amo\(_A\) genes were enumerated using primers Arch-am04F and Arch-am07 [7]. Standard DNA for AOA amo\(_A\) genes was the PCR product of Candidatus nitrosopumilus maritimus SCM1 ranging from 5.98 × 10\(^5\) to 5.98 × 10\(^7\) copies (PCR efficiencies = 1.82 and R\(^2\) = 0.99). PCR reaction was carried out with 95°C for 10min, followed by 40 cycles of 60s at 95°C, 60s at 56°C, and 30s at 72°C, and data capture for each cycle at 78°C for 15s.

Quantification of AOB amo\(_A\) genes was performed using primers amoA1F and amoA2R [8]. Standard DNA for AOB amo\(_A\) genes was generated from the PCR product of Nitrosomonas europaea. A standard curve was prepared in a range of 6.3 × 10\(^7\) to 6.3 × 10\(^9\) copies (PCR efficiencies = 1.82 and R\(^2\) = 0.99). A PCR condition was the same as for AOA amo\(_A\) genes.

Primers amoNo550D2F and amoNo754r [9] were used to quantify the amo\(_A\) genes of Nitrosomonas oligotropha cluster. The PCR product with Nitrosomonas oligotropha amo\(_A\) gene in a range of 6.2 × 10\(^7\) to 6.2 × 10\(^9\) copies (PCR efficiencies = 1.85 and R\(^2\) = 0.99) was used for standard DNA. A PCR condition was 95°C for 10min, followed by 40 cycles of 30s at 95°C, 60s at 56°C, and 60s at 72°C, with data capture for each cycle at 78°C for 15s.

Nitrosomonas europaea 16S rRNA genes were quantified using primers NSMeur-828F and NSMeur-1028R [10]. A range of 5.6 × 10\(^7\) to 5.6 × 10\(^8\) copies of the PCR product of the 16S rRNA genes of Nitrosomonas europaea was used to construct a standard curve (PCR efficiencies = 1.8 and R\(^2\) = 0.99). A condition for PCR amplification was 95°C for 10min, followed by 40 cycles of 10s at 94°C, 30s at 60°C, and 60s at 72°C, with data capture for each cycle at 78°C for 15s.

D. Light Inactivation of Ammonia Monooxygenase

Enriched NAS was harvested from a reactor and was washed with the medium without ammonia for 2-3 times to eliminate existing ammonia. Before adding ammonia, enriched NAS were exposed with light intensity 500 watts, at 25cm distance from light source, for one hour to inactivate ammonia monoxygenase [11] while shaking at 250rpm. Visible light is an inhibitor of ammonia monoxygenase [12], [13] which is known as a photosensitive enzyme.

E. Chloramphenicol Inhibition of New Ammonia Monoxygenase Synthesis

Enriched nitrifying activated sludge was prepared the same as in Light inactivation experiment. Before applying ammonia, chloramphenicol (Chloram) at the final concentration 1,000 mg/l [14] was added to enrich nitrifying activated sludge to inhibit the new ammonia monoxygenase synthesis. Chloramphenicol is an effective inhibitor for gram negative and positive bacteria. It was proven to inhibit de novo protein synthesis but did not inhibit existing enzyme [15]. Chloramphenicol inhibit protein elongation step by preventing the peptidyl transferase activity of ribosome. It binds to A2451 and A2452 residues in the 23S rRNA of the 50S ribosomal subunit which is the preventing of peptide bond formation.

F. Ammonia Oxidation and E\(_2\) Degradation under Various Ammonia Concentrations by Different Consortium of Enriched Nitrifying Activated Sludge

Three parallel batch tests, control test (abiotic test), degradation test (biotic test) and inhibition test were performed to investigate ammonia oxidation and E\(_2\) degradation of enriched nitrifying activated sludge of two
reactors under various ammonia concentrations (2, 10 and 30mM). All batch tests were done in test tube as triplicate. Inorganic medium (as described in section B) added with EE2 at the final concentration 3mg/l were used to conduct in all batch tests. The number of AOB amoA genes 5.25 x 10^6 cells were applied for degradation test (biotic test) and inhibition test while no cells was applied in control (abiotic test). The degradation test (biotic test) was conducted to investigate the enzymology of ammonia monoxygenase on ammonia oxidation and EE2 degradation.

The degradation test consist of four tests which were (1) the degradation test with no treatment (no treatment on cells), (2) the degradation with light (cells were exposed with light as described in section D) [12], (3) the degradation test with chloramphenicol (cells were treated with chloramphenicol 1000 mg/l as described in section E) and (4) the degradation test with light and chloramphenicol (Cells were exposed with light and were treated with chloramphenicol as describe in section D and E, respectively). For inhibition test, allythiourea (ATU) at the final concentration 10 mg/l were added to inhibit ammonia monoxygenase [4] and carried out in four set as same as the degradation test.

The pH of medium in all tests was adjusted to obtain pH rang of 7.5±0.1 by initially adding 40mg/l of HEPES. All tests were shaking at 250rpm, 25°C. Samples were taken at time course. The ammonia, nitrite and nitrate concentration were measured by ion chromatography (IC; DIONEX) with ED50 Electrochemical Detector and CS12A column. EE2 concentration were analyzes by high performance liquid chromatography (HPLC; Agilent 1100 Series LC, Germany) with UV diode array detector (Agilent 1100 Series LC, Germany) at \(\lambda=210\)nm. Elution was be carried out by using 40% v/v acetonitrile/water at a flow rate of 1ml/min with retention time of 15 min. Retention time of EE2 is 11.52min.

### TABLE I

**PRIMERS USED IN THIS STUDY**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’-3’</th>
<th>Target genes</th>
<th>References</th>
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<tbody>
<tr>
<td>Arch-amoAF</td>
<td>STAATGGTCTGGCTTAGACG</td>
<td>AOA amoA genes</td>
<td>[7]</td>
</tr>
<tr>
<td>Arch-amoAR</td>
<td>GCGGCCATCCATCTGTATGT</td>
<td>AOB amoA genes</td>
<td>[8]</td>
</tr>
<tr>
<td>amoA1F</td>
<td>GGGGTTTCTACTGGTGGTG</td>
<td>amoA genes of AOA</td>
<td>[7]</td>
</tr>
<tr>
<td>amoA2R</td>
<td>CCCCTCAGGAAAGCTTTTCT</td>
<td>amoA genes of AOB</td>
<td>[9]</td>
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<tr>
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<td>[10]</td>
</tr>
<tr>
<td>amoNo754e</td>
<td>CTTTAAAGTAGGAAAGGG</td>
<td>N.europaea cluster</td>
<td>[11]</td>
</tr>
<tr>
<td>NSMeur-828F</td>
<td>GTTGT CGGAT CTAAT TAAG</td>
<td>16S rRNA genes of N.europaea</td>
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<tr>
<td>NSMeur-1028R</td>
<td>TGCTT TGGCT CCGTT TC</td>
<td>16S rRNA genes of N.europaea</td>
<td>[13]</td>
</tr>
</tbody>
</table>

### III. RESULTS AND DISCUSSIONS

#### A. Operational Performance of Enriched Nitrifying Activated Sludge Reactors under Various Ammonia Concentrations

To observe the performance of NAS reactors during operation, influent and effluent were analyzed every week for the concentrations of ammonia, nitrite and nitrate. The performance of ammonia oxidation of two reactors was shown in Fig. 1. It was found that nitrite did not accumulated in both reactors and converted to nitrate which confirmed that nitrification process occurred in the reactors. In addition, nitrate production gradually increased until ammonium was completely oxidized and reached the steady state within two week of operation.

#### B. Abundance of Consortium of Ammonia Oxidizing Microorganism in Enriched Nitrifying Activated Sludge

Before conducting the experiment, sample were taken from reactor of 2mM and 5mM ammonia treated to observe and quantify the abundance of ammonia oxidizing microorganism in reactors. In NAS from reactor 2mM, the number of AOA amoA genes was 9.60 x 10^5 ± 1.12 x 10^5 copies/mg.MLSS and the abundance of AOB amoA genes was 3.50 x 10^6 ± 2.24 x 10^5 copies/mg.MLSS. The number of AOA and AOB amoA gene found in NAS from 5mM reactor were 8.15 x 10^5 ± 3.15 x 10^5 copies/mg.MLSS and 1.22 x 10^7 ± 3.17 x 10^6 copies/mg.MLSS, respectively.

The majority AOB cluster in both of reactor was *Nitrosomonas oligotropha* cluster (8.21 x 10^6 ± 4.25 x 10^5 copies/mg.MLSS in NAS from 2mM and 8.97 x 10^7 ± 5.75 x 10^7 copies/mg.MLSS in NAS from 5 reactors, respectively).

### TABLE II

**ABUNDANCE OF AMMONIA OXIDIZING MICROORGANISM OF ENRICHED NITRIFYING ACTIVATED SLUDGE**

<table>
<thead>
<tr>
<th>Reactors</th>
<th>AOA copies/mg MLSS ± SD</th>
<th>AOB copies/mg MLSS ± SD</th>
<th>N.oligotropha copies/mg MLSS ± SD</th>
<th>N.europaea copies/mg MLSS ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mM</td>
<td>9.60 x 10^5 ± 1.12 x 10^5</td>
<td>3.50 x 10^6 ± 2.24 x 10^5</td>
<td>8.21 x 10^6 ± 4.25 x 10^5</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>5 mM</td>
<td>8.15 x 10^5 ± 3.15 x 10^5</td>
<td>1.22 x 10^7 ± 3.17 x 10^6</td>
<td>8.97 x 10^7 ± 5.75 x 10^7</td>
<td>&lt;LOD</td>
</tr>
</tbody>
</table>

LOD = Limit of detection
In addition, the degradation of ammonia was not observed in treatment with both light and chloramphenicol. In case of initial ammonia concentration of 30mM, NAS from 2mM reactor can degrade ammonia only by 33%, 24%, and 8% in degradation test with no treatment, with light only, and with chloramphenicol only, respectively. However, no ammonia degradation occurred in degradation test with both light and chloramphenicol.

For NAS from 5mM reactor (containing AOA amoA gene 3.50x10^7 copies and AOB amoA gene 5.25x10^6 copies; N.oligotropha amoA genes 4.0x10^7 copies) at initial ammonia concentration 2mM, ammonia was degraded completely within 4 days in degradation test with no treatment and with light while ammonia was degraded completely within 20 days in degradation test with chloramphenicol only. However ammonia was not degraded in degradation test with both light and chloramphenicol. At initial ammonia concentration 10mM, NAS from 5mM reactor can degrade ammonia by 59%, 57% and 35% in degradation test with no treatment, with light, and with chloramphenicol, respectively. However, no ammonia oxidation occurred in degradation test treated with both light and chloramphenicol. For initial ammonia concentration 30mM, NAS from 5mM reactor did not degrade ammonia in all degradation tests.

EE2 was not degraded in all abiotic tests (initial ammonia concentration of 2, 10 and 30mM) which mean that EE2 was degrade by biodegradation. No EE2 degradation occurred in all inhibition tests used NAS from 2 and 5mM reactor showing that EE2 did not degrade by heterotrophs but by autotrophs (Figs. 2 and 3). This result agreed with the several previous studies [1], [16], [17] but contrast to some study [18]. For NAS from 2mM, at initial ammonia concentration 2mM, EE2 was degraded by 66%, 42%, and 31% in degradation test with no treatment, with light and with chloramphenicol, respectively whereas no EE2 degradation occurred in degradation test treated with both light and chloramphenicol.

For initial ammonia concentration of 10 mM in degradation test with no treatment, with light, and with chloramphenicol, NAS from 2mM reactor can degrade EE2 by 64%, 51% and 21%, respectively. Conversely, EE2 was not degraded in degradation test with light and chloramphenicol. At initial ammonia concentration of 30mM, NAS from 2 mM reactor can remove EE2 only by 38%, 24% and 13%.

For NAS from 5mM, at initial ammonia concentration 2 mM, EE2 was degraded by 66%, 42%, and 31% in degradation test with no treatment, degradation test with light and degradation test with chloramphenicol, respectively whereas no EE2 degradation occurred in degradation test with light and chloramphenicol. In case of initial ammonia concentration 10 mM in degradation test with no treatment, degradation test with light and degradation test with chloramphenicol, EE2 was degraded by 49%, 27%, and 25%, respectively. On the other hand, no EE2 degradation occurred in degradation test with light and chloramphenicol. At initial ammonia concentration 30mM, EE2 was not degraded in all tests.

In degradation tests with chloramphenicol, only existing AMO enzyme respond to ammonia oxidation and EE2.
degradation while ammonia and EE₂ was degraded by the newly synthesized AMO enzyme in degradation tests with light. For degradation tests with no treatment, both existing and newly produced AMO enzyme contribute to ammonia oxidation and EE₂ degradation. In contrast, no existing and newly synthesized enzymes were presented when treating with both light and chloramphenicol.

The comparison of ammonia oxidation between the degradation tests with light and the degradation tests with chloramphenicol in NAS from 2mM and 5mM reactors suggested that the existing AMO enzyme performed ammonia oxidation less than the new synthesized AMO enzyme. Moreover, the comparison of ammonia oxidation in the degradation tests with no treatment and the degradation tests with light in NAS from 2mM and 5mM reactors showed the similar results which demonstrated that the newly synthesized AMO enzyme may contributed to ammonia oxidation rather than the existing AMO enzyme or the existing AMO enzyme may has a small amount to perform ammonia oxidation and EE₂ degradation. At the initial ammonia concentration of 30 mM, NAS from 5mM reactor did not degrade ammonia which can be imply that AOM in NAS from 5mM may not be able to tolerate high concentration of ammonia; however, detailed explanation must further be clarified.

In case of EE₂ degradation, the highest EE₂ degradation happened in the degradation tests with no treatment and followed by the degradation tests with light while the degradation test with chloramphenicol gave the lowest EE₂ degradation in both of NAS from 2mM and 5mM reactors. This result suggested that the existing AMO enzyme and the new synthesized AOM show the greatest EE₂ degradation due to the highest amount of AMO enzyme. In case of NAS from 5mM and initial ammonia concentration 30mM, no ammonia oxidation and no EE₂ degradation occurred. This result can confirm that co-metabolism could not happen without metabolizing of ammonia occurred. Therefore, it was verified that EE₂ was degraded via co-metabolism by autotrophs [4], [15]-[17], however, this disagree with the result of Gaulke et al. [19] which suggested that EE₂ decreased due to the abiotic transformation.

Fig. 2 Ammonia oxidation and EE₂ degradation in batch experiment under various initial ammonia concentrations in NAS from 2mM reactor (a) and (b) initial ammonia concentration 2mM, (c) and (d) initial ammonia concentration 5mM, and (e) and (f) initial ammonia concentration 30mM.
Fig. 3 Ammonia oxidation and EE2 degradation in batch experiment under various initial ammonia concentrations in NAS from 5 mM reactor (a) and (b) initial ammonia concentration 2 mM, (c) and (d) initial ammonia concentration 5 mM, and (e) and (f) initial ammonia concentration 30 mM

IV. CONCLUSION

The result from this study may verify that AOA or AOB of \textit{N. oligotropha} cluster in NAS from 2 mM and 5 mM, commonly found in municipal WWTPs, could degrade EE2 in wastewater. However, this finding need further study to clarify this co-metabolism because no studies, available so far, have been confirmed that \textit{N. oligotropha} and AOA can degrade EE2.

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