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Degradation of EE_2 by Different Consortium of Enriched Nitrifying Activated Sludge

Pantip Kayee

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***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.

P. Kayee is with the Department of Environmental Science, Suan Sunandha Rajabhat University, Bangkok 10300, Thailand (e-mail: pantip.ka@ssru.ac.th).

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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 7-8mg·O_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 $MnCl_2 \cdot 6H_2O$, 40g of $MnSO_4 \cdot 7H_2O$, 24mg of $NiCl_2$, 36mg of $CuCl_2 \cdot 2H_2O$, 2.1g of $FeSO_4 \cdot 7H_2O$, 30mg of H_3BO_3, 190mg of $CoCl_2 \cdot 6H_2O$, 144mg of $ZnSO_4 \cdot 7H_2O$, 36mg of $Na_2MoO_4 \cdot 2H_2O$ in 1L of deionised water), 1ml of selenite-tungstate solution (400mg of NaOH and 6mg of $Na_2SeO_3 \cdot 5H_2O$ in 1L of deionised water), 1ml of vitamin mixture (10Mm of sodium phosphate buffer at pH 7.1, 40mg of 4-aminobensoic acid, 10mg of D-biotin, 100mg of nicotinic acid, 50mg of Calcium D-patothenate, and 15mg of pyridoxide dihydrochloride in 1l of deionised 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 (QBiogenes, 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 *amoA* genes were enumerated using primers Arch-*amoA*F and Arch-*amo* [7]. Standard DNA for AOA *amoA* genes was the PCR product of *Candidatus nitrosopumilus maritimus* SCM1 ranging from 5.98×10^1 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 *amoA* genes was performed using primers amoA1F and amoA2R [8]. Standard DNA for AOB *amoA* genes was generated from the PCR product of *Nitrosomonas europaea*. A standard curve was prepared in a range of 6.3×10^1 to 6.3×10^7 copies (PCR efficiencies = 1.82 and $R^2 = 0.99$). A PCR condition was the same as for AOA *amoA* genes.

Primers amoNo550D2f and amoNo754r [9] were used to quantify the *amoA* genes of *Nitrosomonas oligotropha* cluster. The PCR product with *Nitrosomonas oligotropha amoA* gene in a range of 6.2×10^1 to 6.2×10^7 copies (PCR efficiencies = 1.85 and $R^2 = 0.98$) 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 5.6×10^1 to 5.6×10^7 copies of the PCR product of the 16S rRNA genes of *Nitrosomoanas 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 monooxygenase [11] while shaking at 250rpm. Visible light is an inhibitor of ammonia monooxygenase [12], [13] which is known as a photosensitive enzyme.

E. Chloramphenicol Inhibition of New Ammonia Monooxygenase 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 enriched nitrifying activated sludge to inhibit the new ammonia monooxygenase 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 Ee_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 EE_2 degradation of enriched nitrifying activated sludge of two

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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 EE_2 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 monooxygenase on ammonia oxidation and EE_2 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 monooxygenase [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. EE_2 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 λ= 210nm. 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 EE_2 is 11.52min.

TABLE I
PRIMERS USED IN THIS STUDY

Primer	Sequence 5'-3'	Target genes	References
Arch-amoAF	STAATGGTCTGGCTTAGACG	AOA *amoA* genes	[7]
Arch-amoAR	GCGGCCATCCATCTGTATGT		
amoA1F	GGGGTTTCTACTGGTGGT	AOB *amoA* genes	[8]
amoA2R	CCCCTCKGSAAAGCCTTCTTC		
amoNo550D2f	TCAGTAGCYGACTACACMGG	*amoA* genes of *N.oligotropha cluster*	[9]
amoNo754r	CTTTAACATAGTAGAAAGCGG		
NSMeur-828F	GTTGT CGGAT CTAAT TAAG	16S rRNA genes of *N.europaea cluster*	[10]
NSMeur-1028R	TGTCT TGGCT CCCTT TC		

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 10^6 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

Reactors	AOA		AOB		*N.oligotropha*		*N.europaea*	
	copies/mg·MLSS	SD	copies/mg·MLSS	SD	copies/mg·MLSS	SD	copies/mg·MLSS	SD
2 mM	9.60 x 10^5	1.12 x 10^5	3.50 x 10^6	2.24 x 10^5	8.21 x 10^6	4.25 x 10^6	<LOD	
5 mM	8.15 x 10^5	3.15 x 10^5	1.22 x 10^7	3.17 x 10^6	8.97 x 10^7	5.75 x 10^7	<LOD	

LOD = Limit of detection

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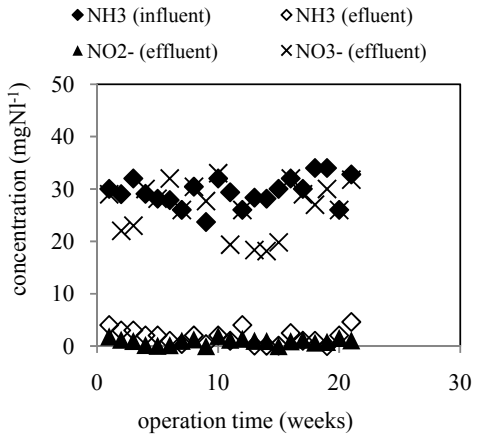
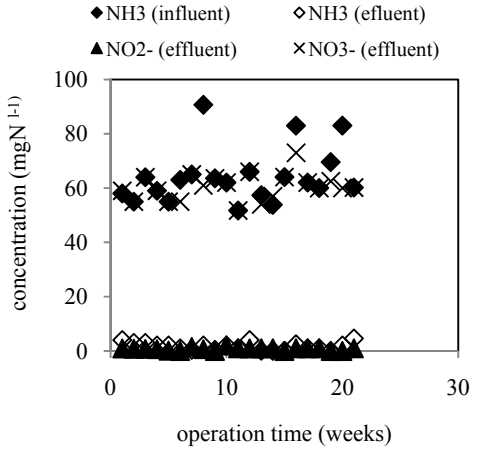


(a) 2 mM reactor



(b) 5 mM reactor

Fig. 1 Concentration of nitrogen in NAS reactor

C. Ammonia Oxidation and EE_2 Degradation by Different Consortium of Enriched Nitrifying Activated Sludge

Ammonia oxidation did not occurred in every set of abiotic test indicating that the decreasing in ammonia concentration would be caused biodegradation. For all inhibition test with all initial ammonia concentrations (2, 10 and 30mM), no ammonia oxidation occurred confirming that ammonia was not degraded by heterotrophs (Figs. 2 and 3).

For NAS from 2 mM reactor (containing AOA *amoA* genes $1.44x10^6$ copies and AOB *amoA* genes $5.25x10^6$ copies; *N.oligotropha amoA* genes $1.2x10^7$ copies), initial concentration of ammonia 2mM was completely degraded within 4 days in the degradation test treated with light and without any treatment of both light and chloramphenicol (no treatment) while it was completely degrade within 11 days in degradation test with only chloramphenicol. On the other hand, ammonia did not degrade in degradation test treated with both light and chloramphenicol. At initial ammonia concentration of 10mM, NAS from 2mM can degrade ammonia completely within 20 days in degradation test treated with light and without any treatment while treating with only chloramphenicol, ammonia could be degraded only by 35%. 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 $3.50x10^5$ 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.

EE_2 was not degraded in all abiotic tests (initial ammonia concentration of 2, 10 and 30mM) which mean that EE_2 was degrade by biodegradation. No EE_2 degradation occurred in all inhibition tests used NAS from 2 and 5mM reactor showing that EE_2 did not degraded 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, EE_2 was degraded by 66%, 42%, and 31% in degradation test with no treatment, with light and with chloramphenicol only, respectively, whereas no EE_2 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 EE_2 by 64%, 51% and 21%, respectively. Conversely, EE_2 was not degraded in degradation test with light and chloramphenicol. At initial ammonia concentration of 30mM, NAS from 2 mM reactor can remove EE_2 only by 38%, 24% and 13%.

For NAS from 5mM, at initial ammonia concentration 2 mM, EE_2 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 EE_2 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, EE_2 was degraded by 49%, 27%, and 25%, respectively. On the other hand, no EE_2 degradation occurred in degradation test with light and chloramphenicol. At initial ammonia concentration 30mM, EE_2 was not degraded in all tests.

In degradation tests with chloramphenicol, only existing AMO enzyme respond to ammonia oxidation and EE_2

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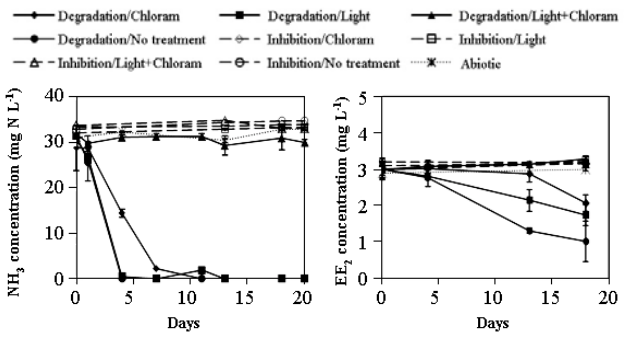
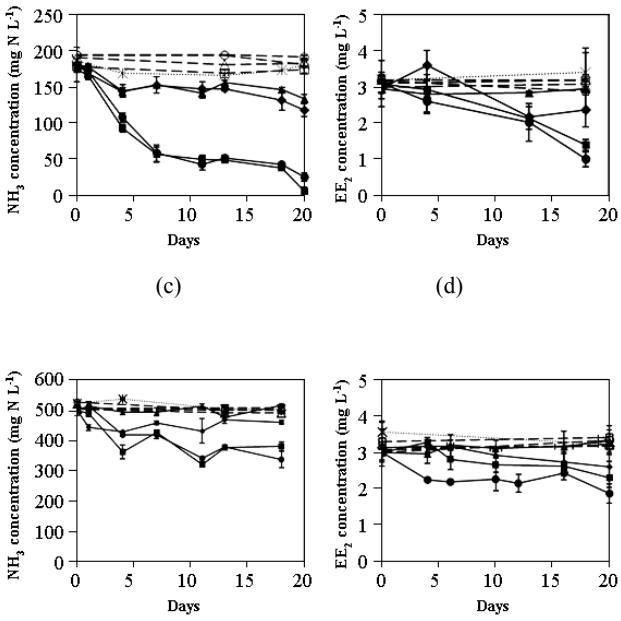
degradation while ammonia and EE_2 was degraded by the newly synthesized AMO enzyme in degradation tests with light. For degradation tests with no treatment, both existing and newly produced AMO enzyme contribute to ammonia oxidation and EE_2 degradation. In contrast, no existing and newly synthesized 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 EE_2 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 EE_2 degradation, the highest EE_2 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 EE_2 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 EE_2 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 EE_2 degradation occurred. This result can confirm that co-metabolism could not happen without metabolizing of ammonia occurred. Therefore, it was verified that EE_2 was degraded via co-metabolism by autotrophs [4], [15]-[17], however, this disagreed with the result of Gaulke et al. [19] which suggested that EE_2 decreased due to the abiotic transformation.



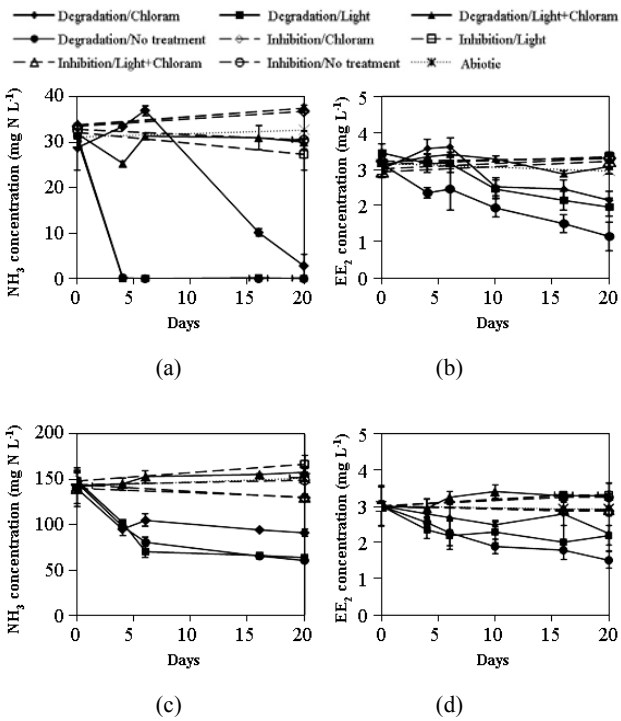
(a) (b)



(c) (d)

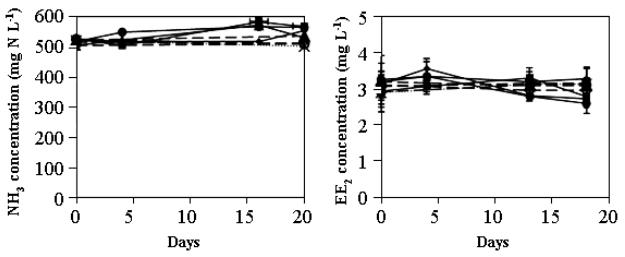
(e) (f)

Fig. 2 Ammonia oxidation and EE_2 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



(a) (b)

(c) (d)



(e) (f)

Fig. 3 Ammonia oxidation and EE_2 degradation in batch experiment under various initial ammonia concentrations in NAS from 5mM reactor (a) and (b) initial ammonia concentration 2mM, (c) and (d) initial ammonia concentration 5mM, and (e) and (f) initial ammonia concentration 30mM

IV. Conclusion

The result from this study may verify that AOA or AOB of *N. oligotropha* cluster in NAS from 2mM and 5mM, commonly found in municipal WWTPs, could degrade EE_2 in wastewater. However, this finding need further study to clarify this co-metabolism because no studies, available so far, have been confirmed that *N. oligotropha* and AOA can degrade EE_2.

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