DEGRADATION OF 17α-ETHYNYLESTRADIOL (EE2) BY NITRIFYING ACTIVATED SLUDGE CONTAINING DIFFERENT AMMONIA-OXIDIZING BACTERIAL COMMUNITIES

Miss Panida Sermwaraphan

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Environmental Management
(Interdisciplinary Program)
Graduate School
Chulalongkorn University
Academic Year 2006

การย่อยสลาย 17α-เอทินิวเอสตระไดออล (EE2) ด้วยไนตริไฟอิงแอกทิเวเต็ดสลัดจ์ที่มีกลุ่มประชากรแอมโมเนียออกซิไดซ์ซิงค์แบคทีเรียแตกต่างกัน

นางสาวพนิดา เสริมวราพันธ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาการจัดการสิ่งแวดล้อม (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2549

Thesis Title	DEGRADATION OF 17α-ETHYNYLESTRADIOL (EE2) BY NITRIFYING ACTIVATED SLUDGE CONTAINING DIFFERENT AMMONIA-OXIDIZING BACTERIAL COMMUNITIES
By	Miss Panida Sermwaraphan
Field of Study	Environmental Management
Thesis Advisor	Tawan Limpiyakorn, Ph.D.
Thesis Co-advisor	Assistant Professor Futoshi Kurisu, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Graduate School
(Assistant Professor M.R. Kalaya Tingsabadh, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Manaskorn Rachakornkij, Ph.D.)

.....Thesis Advisor
(Tawan Limpiyakorn, Ph.D.)

.....Thesis Co-advisor
(Assistant Professor Futoshi Kurisu, Ph.D.)

.....Member
(Ekawan Luepromchai, Ph.D.)

.....Member
(Assistant Professor Wiboonluk Pungrasmi, Ph.D.)

พนิดา เสริมวราพันธ์ : การย่อยสลาย 17α-เอทินิวเอสตระไดออล (EE2) ด้วยไนตริไฟอิงแอกทิเวเต็ดสลัดจ์ที่มีกลุ่มประชากรแอมโมเนียออกซิไดซ์ซิงค์แบคทีเรียแตกต่างกัน (DEGRADATION OF 17α-ETHYNYLESTRADIOL (EE2) BY NITRIFYING ACTIVATED SLUDGE CONTAINING DIFFERENT AMMONIA-OXIDIZING BACTERIAL COMMUNITIES) อ. ที่ปรึกษา: อาจารย์ ดร.ตะวัน ลิมปิยากร , อ. ที่ปรึกษาร่วม: Assistant Professor Futoshi Kurisu, Ph.D., 99 หน้า.

17α-เอทินิวเอสตระไดออล (EE2) เป็นฮอร์โมนเอสโตรเจนสังเคราะห์ซึ่งเป็นส่วนประกอบหลักของยาเม็ดคุมกำเนิด EE2 จัดเป็นสารที่รบกวนการทำงานของระบบต่อมไร้ท่อ จากการศึกษาเมื่อไม่นานมานี้พบการตกค้างของฮอ รโมนดังกล่าวในแหล่งน้ำธรรมชาติ ทั้งนี้ถึงแม้ว่า EE2 จะมีความคงทนในระบบบำบัดน้ำเสียแบบแอกทิเวเต็ดสลัดจ์ การศึกษาล่าสุดพบว่า EE2 ถูกย่อยสลายได้ด้วยกลุ่มประชากรแอมโมเนียออกซิไดซ์ซิงค์แบคทีเรีย (AOB) ผ่านกระบวนการโคเมทตาบอลิซึม อย่างไรก็ตาม การศึกษาที่ผ่านมาได้ทำการศึกษาเฉพาะกลุ่มประชากร AOB จากระบบที่มีภาระแอมโมเนียสูง จึงเกิดข้อสงสัยที่ว่า AOB ในระบบบำบัดน้ำเสียชุมชนซึ่งเป็นแหล่งรองรับฮอร์โมนเอสโตรเจน สามารถย่อยสลาย EE2 ได้หรือไม่ เนื่องจากระบบดังกล่าวรับภาระแอมโมเนียต่ำกว่าระบบที่มีการศึกษามาก่อนหน้านี้ ด้วยเหตุผลดังกล่าวงานวิจัยชิ้นนี้จึงมุ่งเน้นที่การศึกษาการย่อยสลาย EE2 ด้วยไนตริไฟอิงแอกทิเวเต็ดสลัดจ์ (NAS) ที่มีกลุ่มประชากร AOB ที่แตกต่างกัน โดยได้ทำการศึกษาปัจจัยที่มีผลต่อการย่อยสลาย EE2 (กลุ่มประชากร AOB ปริมาณแอมโมเนียที่ถูกย่อยสลาย และสารอินทรีย์ตัวอื่นๆ) โดยในขั้นต้นได้ทำการพัฒนา NAS ให้มีกลุ่มประชากร AOB ที่แตกต่างกัน โดยการนำสลัดจ์จากระบบบำบัดน้ำเสียชุมชนมาทำการเพาะเลี้ยงในถังปฏิกรณ์3 ถัง ด้วยอาหารเลี้ยงเชื้อที่มีปริมาณแอมโมเนียที่แตกต่างกัน (2 mM 10 mM และ 30 mM) หลังจากนั้นได้ทำการวิเคราะห์กลุ่มประชากร AOB ในถังปฏิกรณ์แต่ละถัง โดยการถอดรหัสพันธุกรรมของยีนส์ 16srRNA และ *amoA* ซึ่งผลการศึกษาพบว่า กลุ่มประชากร AOB ในถังปฏิกรณ์แต่ละถังแตกต่างกัน โดยในสลัดจ์เริ่มต้นพบกลุ่ม *Nitrosomonas communis* และ กลุ่ม *Nitrosomonas oligotropha* เป็นกลุ่มเด่น ขณะที่ NAS จากถังปฏิกรณ์ 2 mM พบกลุ่ม *Nitrosomonas communis* เป็นกลุ่มเด่น และใน NAS จากถังปฏิกรณ์ 10 mM พบกลุ่ม unknown *Nitrosomonas* เป็นกลุ่มเด่น ซึ่ง AOB กลุ่มนี้มีลักษณะคล้ายกับ *Nitrosomonas sp.* Is343 ซึ่งพบในระบบบำบัดน้ำเสียจากชุมชน โรงงานน้ำมัน และโรงงานเครื่องดื่ม ขณะที่ใน NAS จากถังปฏิกรณ์ 30 mM พบกลุ่ม *Nitrosomonas europaea –Nitrosococcus mobilis* เป็นกลุ่มเด่น จากการศึกษาการย่อยสลาย EE2 พบว่า EE2 ถูกย่อยสลายได้ด้วย NAS จากทุกถังปฏิกรณ์ที่ทุกระดับความเข้มข้นแอมโมเนียเริ่มต้น (2 mM 10 mM และ 30 mM) อย่างไรก็ตามรูปแบบการย่อยสลายมีความแตกต่างกันโดยจะขึ้นอยู่กับกลุ่มประชากร AOB ในถังปฏิกรณ์ ซึ่งผลการศึกษานี้ แสดงให้เห็นว่าการผลิตเอนไซม์ การแสดงออกของเอนไซม์ และกิจกรรมของเอนไซม์ในกลุ่มประชากร AOBแต่ละกลุ่มมีความแตกต่างกัน นอกจากนี้ยังพบว่าความสามารถในการย่อยสลาย EE2 จะเพิ่มขึ้นเมื่อระดับความเข้มข้นเริ่มต้นของแอมโมเนียสูงขึ้น อย่างไรก็ตามการย่อยสลาย EE2 ไม่ได้เป็นสัดส่วนโดยตรงต่อการย่อยสลายแอมโมเนีย การศึกษาผลกระทบของสารอินทรีย์ตัวอื่นต่อการย่อยสลาย EE2 พบว่า เอสตระไดออล (E2) ซึ่งเป็นสารที่มีโครงสร้างคล้ายกับ EE2 ทำให้การย่อยสลายของ EE2 ลดลง อย่างไรก็ตาม สารอินทรีย์ในน้ำเสียโรงอาหารกลับไม่มีผลกระทบต่อการย่อยสลายของ EE2 ผลการทดลองจากงานวิจัยนี้ แสดงให้เห็นว่า AOB ในระบบบำบัดน้ำเสียชุมชนสามารถย่อยสลาย EE2 ได้ ซึ่งจะนำไปสู่การพัฒนากระบวนการกำจัด EE2 และสารอินทรีย์ย่อยสลายยากตัวอื่นๆในน้ำเสียด้วยแบคทีเรียกลุ่ม AOB ต่อไป

สาขาวิชาการจัดการสิ่งแวดล้อม
ปีการศึกษา 2549

ลายมือชื่อนิสิต พนิดา เสริมวราพันธ์
ลายมือชื่ออาจารย์ที่ปรึกษา
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม

488 94664 20: MAJOR ENVIRONMENTAL MANAGEMENT
KEY WORD: AMMONIA-OXIDIZING BACTERIAL/ DEGRADATION/ EE2/ MICROBIAL COMMUNITY/ NITRIFYING ACTIVATED SLUDGE/ 17α-ETHYNYLESTRADIOL

PANIDA SERMWARAPHAN: DEGRADATION OF 17α-ETHYNYLESTRADIOL (EE2) BY NITRIFYING ACTIVATED SLUDGE CONTAINING DIFFERENT AMMONIA-OXIDIZING BACTERIAL COMMUNITIES. THESIS ADVISOR: TAWAN LIMPIYAKORN, Ph.D., THESIS COADVISOR: ASSISTANT PROFESSOR FUTOSHI KURISU, Ph.D., 99 pp.

17α-ethynylestradiol (EE2), a synthetic estrogen, is a key ingredient in oral contraceptives pill. EE2 is reported as an endocrine disruptor, high in estrogenicity. Recent studies on the occurrence of pharmaceutical compounds in environments suggested the existence of EE2 in receiving water. Although previous studies suggested that EE2 persist in contact with activated sludge, more recent studies showed that EE2 can be degraded by ammonia-oxidizing bacteria (AOB) via co-metabolism. Nevertheless, all of the studies so far involved in only AOB enriched under high ammonium loads. The question still arises about whether AOB in municipal wastewater treatment systems (WWTS), as a potential reservoir for estrogens, which receive much lower ammonium loads than in the previous studies can degrade EE2. As a result, this study aimed to investigate the degradation of EE2 by nitrifying activated sludge (NAS) containing different AOB communities and factors affecting the degradation of EE2 by NAS (AOB communities, ammonia oxidation, and other organic matters). To develop NAS containing different AOB communities, sludge taken from a municipal wastewater treatment system was enriched in three reactors receiving inorganic medium containing different ammonium concentrations of 2, 10 and 30 mM. Community of AOB in each NAS was analyzed using specific Polymersase Chain Reaction amplification followed by Denaturing Gel Gradient Elecrophoresis and sequencing of 16S rRNA genes or *amoA* genes. The results showed that AOB community in each reactor differed depending on the ammonium load supplied. Predominant AOB species in the seed sludge related to *Nitrosomonas communis* cluster and *Nitrosomonas oligotropha* cluster, while that of NAS from 2 mM reactor related to *Nitrosomonas communis* cluster and that from 10 mM reactor related to unknown *Nitrosomonas* cluster, which was related closely to the strain *Nitrosomonas sp.* Is343 previously found in municipal, oil industry, and brewery WWTS. Whereas, that from 30 mM reactor related to *Nitrosomonas europaea –Nitrosococcus mobilis* cluster. Degradation studies suggested that EE2 can be degraded by all NAS under all different initial ammonium concentrations of 2, 10, and 30 mM. However, the degradation patterns varied among NAS. This result suggested that enzyme induction, enzyme expression, and enzyme activity may differ among AOB communities, and thus among distinct AOB species. Initial ammonium concentrations also affected the degradation of EE2. The results showed that the higher the initial ammonium concentration, more EE2 can be degraded. However, the amount of ammonia oxidized was not proportional to the amount of EE2 degraded. Study on the competition effect of other organic compounds on EE2 degradation showed that estradiol (E2), that have similar structure to EE2, competed the degradation of EE2, whereas organic compounds in canteen wastewater did not. The major finding of this study is that AOB found in municipal WWTS can degrade EE2. This will lead to the new means of treatment technology in removing EE2 and also other persistent organic compounds in wastewater using AOB.

Department: Environmental Management Student's signature พนิดา เสริมมาพันธุ์

Academic year 2006 Advisor's signature ตวัน ~

Co-advisor's signature 栗拓太

ACKNOWLDGEMENTS

I do wish to express my graceful appreciation to Dr. Tawan Limpiyakorn who is my thesis advisor and Assistant Professor Dr. Futoshi Kurisu who is my thesis co-advisor for their guidance and support during the entire of my study and for their time, patience and suggestions and comments, I am deeply indebted.

Moreover, I would like to express my appreciation and sincere gratitude to Dr. Manaskorn Rachakornkij who is a chairman of my thesis committee, Dr. Ekawan Luepromchai, and Assistant Professor Wiboonluk Pungrasmi who are committee members for their suggestions and recommendations throughout my research work.

In addition, I would like to thank the National Center of Excellence for Environmental and Hazardous Waste Management (NCE-EHWM) for the full scholarship, funding and supporting facilities for my study.

Special thanks to Miss Ramnaree Netvichien who is a Laboratory supervisor, Miss Chantana Intim who is a Laboratory coordinator and all staffs at NCE-EHWM program for their helps especially in the analytical works.

I thank to Miss Supreeda Homklin, Miss Maliwan Kutoko, and Miss Sainatee Champirat for helping me at any time, suggestion for this research work, and listening to my complaints and frustrations.

Thank also to my fellow all students at NCE-EHWM for making the time enjoyable in the lab.

Last but definite not least, I would like to express my deep appreciation to my family. Without their constant support, encouragement, I would not have been able to arrive this far.

CONTENTS

Page

Page

Page

LIST OF FIGURES

Figure **Page**

Figure **Page**

Figure **Page**

LIST OF TABLES

Table **Page**

ABBREVIATIONS

AMO	=	ammonia monooxygenase
AOB	=	ammonia-oxidizing bacteria
C18	=	octadecyl
Conc.	=	concentration
DAD	=	diode array detectors
DGGE	=	denaturing gradient gel electrophoreses
DW	=	dry weight
E. coli	=	*Escherichia coli*
E1	=	Estrone
E2	=	Estradiol
E3	=	Estriol
EDs	=	endocrine disruptors
EE2	=	17α-ethynylestradiol
EPA	=	environmental protection agency
g	=	gram
GC	=	gas chromatography
HAO	=	hydroxylamine oxidoreductase
HPLC	=	high performance liquid chromatography
Kow	=	octanol-water partitioning coefficient
LC	=	liquid chromatography
LOD	=	limit of detection
MeEE2	=	Mestranol
mg/l	=	milligram per liter
MW	=	molecular weight
NAS	=	nitrifying activated sludge
ND	=	no data.
ng/l	=	nanogram per liter
NOB	=	nitrite-oxidizing bacteria
PCR	=	polymerase chain reaction
SPE	=	solid phase extraction
WWTS	=	wastewater treatment system

CHAPTER I

INTRODUCTION

1.1 Background and motivation

Estrogens are predominantly female hormones, which are important for maintaining health of reproductive tissues, breasts, skin and brain. Human and animals release of estrogens is widespread concern for endocrine disruptor which interfere the endocrine and reproductive function in human and living organisms. For example, as low as nanogram per litter of EE2 can cause decreasing in sperm count and increasing in incident to testicular cancer and male fertilizer disorder (Perdom et al., 1994). In addition, estrogens can induce vitellogenin synthesis in some fish (Routledge et al., 1998). Estrogens can be divided into two groups: natural estrogens and synthetic estrogens. Natural estrogens consisting of estrone (E1), 17α-estradiol (E2), and estriol (E3) are produced by living organism body and synthetic estrogen, 17α-ethynylestradiol (EE2), is a key ingredient in oral contraceptives pill (Ying et al., 2002). Estrogens can be released to the environments by excretion of humans and animals through their urine and feces, most of which flows into wastewater treatment systems (WWTS). In humans and animals estrogens undergo various transformations mainly in the liver and are excreted to the final conjugation with glucuronic acid or sulphates. Municipal WWTS are an important facility that markedly reduces the concentrations of estrogens. Removal efficiencies of estrogens by activated sludge treatment range from 61% to 89% for E1, 87 % and 99% for E2 and 80% to 85% for EE2 (Ternes et al., 1999a; Baronti et al., 2000). These results correspond to the study of degradation of natural estrogens in batch experiment but are in contrast to that of synthetic estrogen. In batch experiment with activated sludge, 1 μg/l of E2 was oxidized to E1, and then E1 was eliminated; whereas, 1μg/l of EE2 appeared to be mainly stable in contact with activated sludge (Ternes et al., 1999a). Regarding EE2, nitrifying activated sludge (NAS) in high ammonium concentration could degrade EE2 at an initial concentration of 50 μg/l within 6 days (Vader et al., 2000). These results allow the conclusion that NAS, consisting of heterotrophic and ammonia-oxidizing bacteria (AOB), is able to degrade EE2. By using inhibitor for ammonia monooxygenase, which is the key enzyme for ammonia oxidation by AOB more

recent publication suggested that in NAS, natural estrogens (E1, E2, and E3) were degraded by heterotrophic bacteria whereas EE2 was degraded by AOB (Shi et al., 2004). In general, physiological properties of AOB differ among distinct AOB species. This reflects the distribution pattern of AOB in the environments. For example, *N. europaea-Nc. mobilis* cluster, as the AOB with low affinity to ammonia, predominate in the environments high in ammonia, whereas *N. communis* cluster as the AOB with moderate affinity to free ammonia (K_s, 14 to 43 μM) and *N. oligotropha* cluster and *Nitrosospira* cluster, as the AOB with high affinity to ammonia, dominate in the environments low in ammonia (Limpiyakorn et al., 2005). Regarding EE2, although AOB in NAS have been proven for their ability to eliminate EE2, the question still arise about whether AOB in municipal WWTS, as a potential reservoir for estrogens, can degrade EE2 or not, since all studies so far concerning to the degradation of EE2 by NAS dealed with high ammonia enriching cultures which possible possess only *N. europaea-Nc. mobilis* cluster instead of *N. communis* cluster, *N. oligotropha* cluster and *Nitrosospira* cluster, the dominant AOB found in municipal WWTS. As ammonia in wastewater is the most important factor in the inclusion of AOB in WWTS, in order to apply NAS to remove EE2 from wastewater, it is necessary to understand how different AOB in WWTS degrade EE2. Consequently, this study investigated the degradation of EE2 by NAS containing different AOB communities and clarified factors, which consists of species of AOB, ammonia oxidation, and organic matter, affecting the degradation of EE2 by NAS.

1.2 Objectives

This study focuses mainly on degradation of EE2 by NAS containing different AOB communities. The objectives of this study are as follows:

1. To analyze community of AOB in NAS.
2. To study degradation of EE2 by NAS containing different AOB communities.
3. To investigate effect of ammonia oxidation on degradation of EE2 by NAS containing different AOB communities.
4. To observe competition effect of other organic compounds on degradation of EE2 by NAS.

1.3 Hypotheses

1. Different AOB degrade EE2 differently.
2. Degradation of EE2 depends on ammonia oxidation.
3. Other organic matters can deteriorate degradation of EE2 by AOB.
4. AOB belonging to *N. communis* cluster, *N. oligotropha* cluster and *Nitrosospira* cluster degrades EE2 in municipal wastewater treatment system**.**

1.4 Scope of the study

1. The study was focused on EE2 which is a synthetic estrogen.
2. Seed sludge was taken from an aeration tank of a municipal wastewater treatment system.
3. NAS was enriched in laboratory-scale continuous-flow reactor.
4. Degradation study was carried out in laboratory-scale batch test.

CHAPTER II

LITERATURE REVIEW

Estrogens are steroid hormone that produces from ovary grand in female of living organism. In mammal body, estrogens are very important because they control the second sex characteristic and reproductive system. When estrogens are excreted out of the living organism they become high potential compound or so called “endocrine disruptor”. Although, they can be degraded by microorganisms, the estrogenic potential of them are in the range of nanogram level. The characterization of estrogens and related literature are described as following.

2.1 Steroid hormone

A steroid is a lipid characterized by a carbon skeleton with four combined rings. All steroids are derived from the cholesterol or the acetyl CoA biosynthetic pathway. Different steroids vary in the functional groups attached to these rings. Some of the common categories of steroids include:

a. Anabolic steroids are a class of steroids that interact with androgen receptors to increase muscle and bone synthesis. There are natural and synthetic anabolic steroids. There are the steroids used by athletes to increase performance.

b. Corticosteroids include glucocorticoid and mineralocorticoids

- Glucocorticoids regulate many aspects of metabolism and immune function, and are often prescribed by doctors to reduce inflammatory condition like asthma and arthritis.

- Mineralocorticoids are corticosteroids that help maintain blood volume and control renal excretion of electrolytes.

c. Sex steroids are a subset of sex hormones that produce sex differences or support reproduction. They include androgens, estrogens and progestagens

d. Phytosterols are steroids naturally occurring in plants

e. Ergosterols are steroids occurring in fungi. These includes some vitamin D supplements

2.2 Estrogen Hormones

2.2.1 Type of estrogens

Estrogens are female hormone that controls the second sex characteristic of female. Estrogens can be divided into two groups which are natural estrogens and synthetic estrogens. Natural estrogens are naturally produced in living organism body including human and animals. Natural estrogens consist of estrone (E1), estradiol (E2) and estriol (E3). The systematic IUPAC name of E1, E2, E3 and EE2 are 3-hydroxyestra-1,3,5[10]-trien-17-one, 1,3,5[10]-estratriene-3,17β-diol, 1,3,5[10]-estratriene-3,16α,17β-triol, and 17α-ethnyl-1,3,5[10]-oestratriene-3,17β-diol respectively. Synthetic estrogens are normally used as ingredient of contraceptive pill for birth control. Moreover, synthetic estrogens used to treat menopausal woman who suffer from lack of hormone as hormone therapy. Synthetic estrogens comprise 17α-ethynylestradiol (EE2) and mestranol (MeEE2).

2.2.2 Structures of estrogens

Natural and synthetic estrogens have a similar main structure. Estrogens consist of 1 aromatic ring at A ring, 2 hexacyclic rings at B and C ring and 1 pentacyclic at D ring (Figure 2.1).

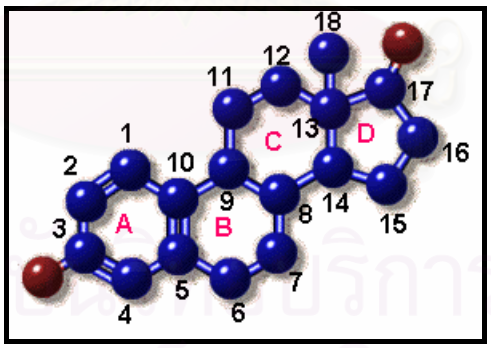
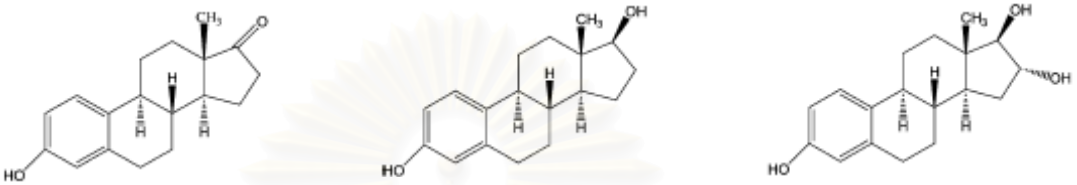


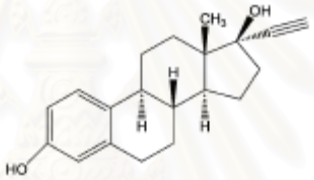
Figure 2.1 Structure of estrogen

The distinguish parts between them are functional group at C3, C16 and C17 positions. E1 has hydroxyl (OH) and ketone (C=O) groups at C3 and C17. E2 has hydroxyl (OH) groups at both C3 and C17. Moreover, E2 has two patterns that depend on the position of hydroxyl (OH) group at C17. If a hydroxyl group is downward from the molecule, it is α configuration. If a hydroxyl group is upward from the molecule, it is β configuration (Hanselman, Graetz, and Wilkie, 2003). E3

has hydroxyl groups as C3, C16 and C17. EE2 has structure as same as structure of E2 except triple bond at C17. Main structure of conjugated estrogens is similar to the free form except the functional group at C3 and C17. The former functional groups at C3 and C17 are replaced by glucoronide and/or sulfate group. However, conjugated form is less concern because the potential of conjugated form is less than the potential of free form (Figure 2.2).



Estrone (E1) Estradiol (E2) Estriol (E3)



Ethynylestradiol (EE2)

Figure 2.2 Structure of estrogens

2.2.3 Physicochemical properties of estrogens

The physicochemical properties of natural and synthetic estrogens in free form are shown in Table 2.1. From table, natural estrogens have water solubility about 13 mg/l at 20 ^{0}C while synthetic estrogen has lower water solubility than natural estrogen which is about 4.8 mg/l at 20 ^{0}C. Moreover, Log Kow of E1, E2, E3 and EE2 is 3.43, 3.94, 2.81 and 4.15, respectively. According to water solubility and Log Kow values, it can be indicated that estrogens are easily to be captured in soil or sediment more than to be dissolved in water, especially for EE2. Vapor pressure of E1, E2, E3 and EE2 is $2x10^{-10}$, $2.3x10^{-10}$, $6.7x10^{-15}$ and $4.5x10^{-11}$ mmHg, respectively. Vapor pressure of both natural and synthetic estrogens is significantly low. It indicated that they are hardly to vaporize (Lai et al., 2000). There is no information about the conjugated estrogens physicochemical properties. However, Hanselman et al. (2003) suggested that conjugated estrogens can be dissolved in water more than free form because of

the high polarity of functional group as glucuronide and sulfate. However, conjugated estrogens have been less concerned because they are less estrogenic potential than free forms.

Table 2.1 Structures and properties of estrogens

Substance	MW [a]	Water solubility (mg/l at 20°)	Vapor pressure (mmHg)	Log K_{ow} [b]
E1	270.4	13	$2.3x10^{-10}$	3.43
E2	272.4	13	$2.3x10^{-10}$	3.94
E3	288.4	13	$6.7x10^{-15}$	2.81
EE2	296.4	4.8	$4.5x10^{-11}$	4.15

Symbols and Abbreviations: [a] Molecular weight, [b] Octanol-water partitioning coefficient

Available from: Lai et al. (2000)

2.2.4 Forms of estrogens

In humans and animals, estrogens undergo various transformations mainly in the liver and are excreted through their urine principally as inactive polar conjugate such as glucuronides and sulphates. Inactive polar conjugate can re-activate to active from (Figure 2.3). This re-formation or de-conjugation of estrogens depends on the acid-base properties of the environment and on the possibility of bacterial process. Conjugation of E2 and EE2 can occur in the C_3 position, in the C_{17} position and in both the C_3 and C_{17} position. E3 conjugate occurs in all the previous positions and can occur in the C_{18} position, as well. Sulphatation can also be expected in all the previously cited positions on the molecule. Conjugates possessing both Glucuronidation and Sulphatation also exist because the estrogen receptor is an unspecific receptor, a response will depend only on de-conjugation in the C_3 position (Flemming and Bent, 2003).

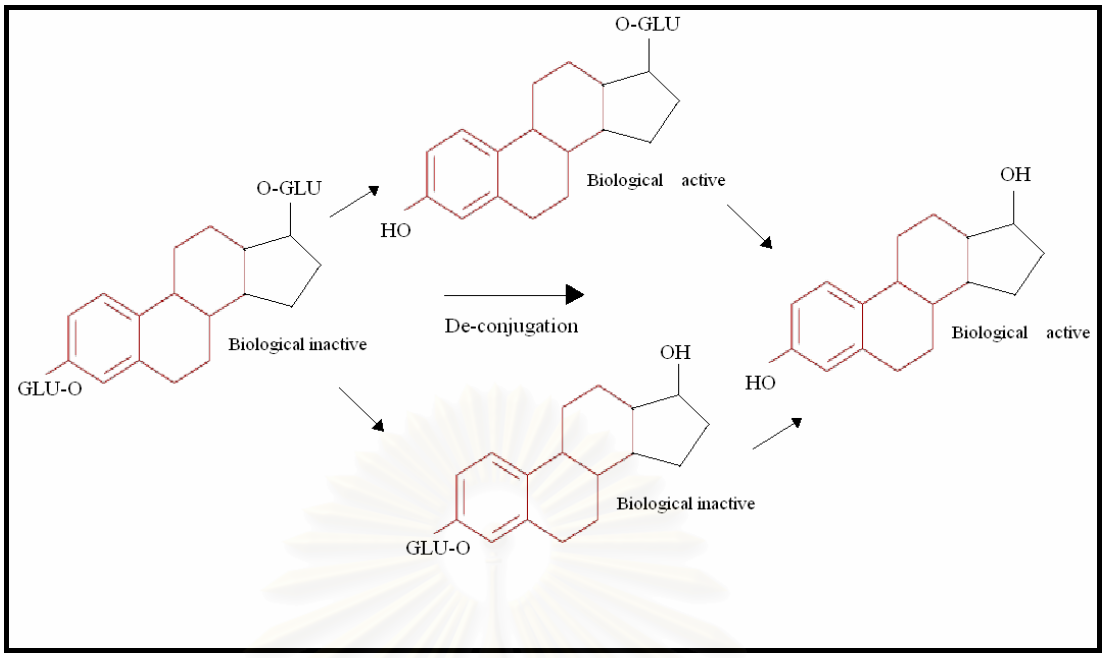


Figure 2.3 De-conjugation of 17β-estradiol (E2) into biological active compounds Available from: Flemming and Bent (2003)

2.2.5 Fate of estrogens in environments

Conjugate and de-conjugate estrogens are forms of estrogens that are found in excretion of human and animal through municipal wastewater treatment systems. Estrogens excreted in urine or feces are in glucuronides or sulfate conjugated forms (Orme, Back, and Breckenridge, 1983; Baronti et al., 2000). The structure of conjugated estrogens are similar to those of de-conjugation ones, except for a sulfate and/or glucuronides group which is instead of the C_3 and /or C_{17} positions of the parent compound (Hanselman et al., 2003). However, the occurrence of free estrogens in MTSs effluents and rivers (Baronti et al., 2000; Belfroid et al., 1999; Desbrow et al., 1998; Johnson, Belfroid, and Di Corcia, 2000; Ternes et al., 1999) indicate that estrogen metabolites are converted back into active forms somewhere between houses and municipal wastewater treatment systems outlets. Conjugated estrogens can be cleaved to de-conjugated ones by bacteria in the collection system. *Escherichia coli* (*E. coli*), which is eliminated in large quantities in the feces, is able to synthesize large amounts of the β-glucuronidase enzyme. A laboratory biodegradation test confirmed that conjugation with glucuronic is readily de-conjugated in unmodified domestic wastewater, due to the large amounts of the β-glucuronidase enzyme (D'Ascenzo et al., 2003).

2.3 Adverse effects of estrogens

2.3.1 Endocrine disruptors

Endocrine disruptors are compound that have negative impact to human and animal. They can interfere with the normal function of endocrine and reproductive system of human and animal. The US Environmental Protection Agency (EPA) defines endocrine disruptors as: "*An exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior*". Estrogenic endocrine disruptor compounds consist of natural hormones and pharmaceutical estrogens, phytoestrogens, surfactants, pesticides and industrial compounds. Although, surfactants, pesticide and industrial compounds are not estrogen hormones, they can affect to living organism the same as estrogens. They can also interfere with endocrine and reproductive system of human and animal. This research only concerns to estrogenic endocrine disruptor compound that are natural hormones and pharmaceutical estrogens (Institute of Population Health, 2007).

2.3.2 Effects of estrogens on living organisms

2.3.2.1 Effect of estrogens on human being

The intake of estrogens via food or drinking water may be caused decreasing of sperm count, increasing of incident of testicular cancer and male fertility disorder in human (Sharp and Skakkeback, 1993).

2.3.2.2 Effect of estrogens on aquatic organisms

When endocrine disruptors enter into environment, they affect on living organisms that live near the contaminated environment. Especially, aquatic organisms are directly affected by endocrine disruptor because water from wastewater treatment plant is discharged into water resources such as river, reservoir, lake, and ocean. Aquatic organisms can exposure to endocrine disruptor compounds especially natural hormone and pharmaceutical estrogens that remain in effluent of wastewater. Estrogen contamination of waterways is concerned because low concentrations (10-100 ng/l) of estrogens in water can adversely affect the reproductive biology of vertebrate species such as fish, turtles, and frogs by disrupting the normal function of their endocrine systems (Hanselman et al., 2004). For example 1 ng/l of E2 can lead

to the induction of vitellogenin (an egg yolk precursor protein that is normally produced only by adult females) in male trout (Desbrow et al., 1998; Jobling et al., 1998). A laboratory study on the endocrine disrupting potency of EE2 demonstrated that EE2 at low concentrations of 1-10 ng/l caused estrogenic response in caged fish (Purdom et al., 1994) and these changes may be expressed later in the life cycle or even in future generations.

2.3.2.3 Effect of estrogens on terrestrial organisms

Estrogens may interfere with the normal functioning of endocrine systems and affect reproduction and development in wildlife (Jobling et al., 1998). Hormone steroids in the environment may affect not only wildlife but also plants. Shore, Correll, and Charkraborty (1995) reported that Alfalfa irrigated with municipal effluent, which contained hormone steroids, was observed to have elevated levels of phytoestrogens.

2.4 Sources of estrogens

2.4.1 Estrogens from humans

Generally the endogenous excretion of hormones by healthy pre-menopausal women is reported to range from 10 to 100 μg/day (Table 2.2). Menstruating women excrete 8 μg/day of E1, 3.5 μg/day of E2 and 4.8 μg/day of E3. After menopause, women only excrete 4 μg/day of E1, 2.3 μg/day of E2 and 1 μg/day of E3. Pregnant women excrete 600 μg/day of E1, 259 μg/day of E2 and 600 μg/day of E3. Women using contraception pill are assumed to excrete the whole daily dose of 35 μg. The average values for normal men are 3.9 μg/day of E1, 1.6 μg/day of E2 and 1.5 μg/day of E3 in their urine (Johnson et al., 2000).

Table 2.2 Daily excretions (μg) of estrogens in humans

Category	Concentration (μg/day)			
	E1	E2	E3	EE2
Pre-menopausal females	100	10	10	-
Menstruating females	8	3.5	4.8	-
Menopausal females	4	2.3	1	-
Pregnant women	600	259	600	-
Women using contraception pill	-	-	-	35
Males	3.9	1.6	1.5	-

Available from: Johnson et al. (2000)

2.4.2 Estrogens from animals

Possible exposure to estrogens may come from animal manures that are applied to agricultural fields. The animal manures are from sheep, cattle, pigs and poultry, as well as other animals. Steroid drugs are frequently used in cattle as well as other livestock, which control the estrus cycle, treat reproductive disorders and induce abortion (Refsdal, 2000). This could greatly increase the generation of hormone steroids in urine of livestock. In poultry waste, a concentration ranging from 14 to 533 ng/g dry waste with an average of 44 ng/g for E2 was reported by Shemesh and Shore (1994). The E2 concentration in urine of cattle was found to be 13 ng/l on average by Erb, Chew, and killer (1977).

2.5 Level of estrogens in the environments

2.5.1 Level of estrogens in surface water

The concentrations of estrogens in surface water ranged from 0.4 to 1.5 ng/l for E1, from 0.11 to 2.1 ng/l for E2 and from less than 0.1 to 0.4 ng/l for EE2 (Table 2.3). From table 2.3, it can be seen that E1 was detected in 7 of 11 Netherlands coastal/estuarine and freshwater samples with a median concentration of 0.3 ng/l, while E2 and EE2 were only detected in 4 and 3 of 11 samples, with the concentrations less than 0.3 for E2 and less than 0.1 for EE2 (Belfroid et al., 1999). The measurements in Italy resemble the situation in the Netherlands. E1 was found in Tiber River in Italy with a highest concentration of 1.5 ng/l, while E2, E3 and EE2 were found to be 0.11, 0.33 and 0.04 ng/l, respectively (Baronti et al., 2000). The concentration of E2 found in 109 Japanese rivers is higher in summer more than in autumn (Tabata, 2001). Moreover, Estrogen, E1 E2 and EE2, were also detected in some water samples from southern Germany with an average concentration of 0.4, 0.3 and 0.4 ng/l, respectively (Kuch and Ballschmiter, 2001).

Table 2.3 Mean concentrations of estrogens in surface water

Location	Concentration (ng/l)				Reference
	E1	E2	E3	EE2	
Netherlands coastal/estuarine/fresh water	0.3	<0.3	-	<0.1	Belfroid et al. (1999)
Italian river	1.5	0.11	0.33	0.04	Baronti et al. (2000)
Japanese rivers	-	2.1[a] 1.8[b]	-	-	Tabata (2001)
Germany river	0.4	0.3	-	0.4	Kuch and Ballschmiter (2001)

Symbols and Abbreviations: [a] Summer, [b] Autumn

2.5.2 Level of estrogens in municipal wastewater treatment systems

2.5.2.1 Level of estrogens in influents of municipal wastewater treatment systems

The concentrations of estrogens in influents of municipal wastewater treatment systems ranged from 11 to 140 ng/l for E1, from less than limit of detection (LOD) to 90 ng/l for E2 and from less than 0.2 to 8.8 ng/l for EE2 (Table 2.4). From table 2.4, in the raw sewage of the Brazilian MTSs (municipal wastewater treatment systems), E1, E2 and EE2 were detected with average concentrations of 40, 21 and 6 ng/l, respectively (Ternes et al., 1999). Moreover, estrogens were detected in three Netherlands MTSs with concentrations ranged from 11 to 140 ng/l for E1, from below LOD to 48 ng/l for E2 and from less than 0.2 to 8.8 ng/l for EE_2 (Johnson et al., 2000). For a median concentration of E1, E2, E3 and EE2 in influents of six Italian activated sludge municipal wastewater treatment systems were 52, 12, 80 and 3 ng/l, respectively (Baronti et al., 2000). In addition, the concentrations of E_2 in influents of Japanese MTSs ranged from 20 to 94 ng/l in summer and from 30 to 90 ng/l in autumn (Nasu, 2000).

Table 2.4 Concentrations of estrogens in influents of municipal wastewater treatment systems

Location	Concentration (ng/l)				Reference
	E1	E2	E3	EE2	
Brazilian	40	21	-	6	Ternes et al. (1999)
Netherlands	11-140	< LOD -48	-	<0.2-8.8	Johnson et al. (2000)
Italian	52	12	80	3	Baronti et al. (2000)
Japanese	-	20-94[a] 30-90[b]	-	-	Nasu et al. (2000)

Symbols and Abbreviations: [a] Summer, [b] Autumn

2.5.2.2 Level of estrogens in effluents of municipal wastewater treatment systems

The concentrations of estrogens in the effluents ranged from below LOD to 64 ng/l for E2, from below LOD to 82 ng/l for E1, from 0.43 to 18ng/l for E3 and from less than LOD to 42 ng/l for EE2 (Table 2.5). From the table 2.5, it can be seen that E2 was present at higher concentrations in the effluents from MTSs in Canada, UK and Japan than those from other countries. In British MTSs, the concentrations of E1 in the effluents varied widely from 1.4 to 76 ng/l, while E2 concentrations from 2.7 to 4.8 ng/l (Desbrow et al., 1998). However, EE2 was only found in 7 of 21 effluent samples from domestic MTSs in British, with concentrations ranging from below LOD to 7 ng/l. In Canadian MTSs, E1 and E2 were determined with maximum concentrations of 48 and 64 ng/l, respectively. EE2 was detected in 9 of 10 effluent samples with a maximum concentration of 42 ng/l (Ternes et al., 1999). The levels of estrone in the effluents from different countries are quite comparable. Estriol (E3) was only reported in Italian MTSs and Baronti et al. (2000) reported maximum concentrations are 82 ng/l for E1 and 18 ng/l for E3. E2 was detected in Japanese MTSs effluent samples with concentrations ranged from 3.2 to 55 ng/l in summer and from 2.8 to 30 ng/l in autumn (Tabata, 2001). In addition, Spengler, Korner, and Metzger (2001) recently reported a maximum concentration of 15 ng/l for E2 in effluents of MTSs in Germany.

Table 2.5 Concentration of estrogens in effluents of municipal wastewater treatment systems

Location	Concentration (ng/l)				Reference
	E1	E2	E3	EE2	
British	1.4-76	2.7-4.8	-	< LOD-7	Desbrow et al. (1998)
Canadian	<LOD-48	<LOD-64	-	<LOD-42	Ternes et al. (1999)
Italy	2.5-82	-	0.43-18	-	Baronti et al. (2000)
Japanese	-	3.2-55[a] 2.8-30[b]	-	-	Tabata (2001)
Germany	-	<LOD-15	-	-	Spengler et al. (2001)

Symbols and Abbreviations: [a] Summer, [b] Autumn

2.6 Biotransformation of estrogens

2.6.1 Biotransformation by metabolisms

2.6.1.1 Biotransformation by metabolisms in mixed culture

Weber et al. (2005) used mixed culture consisting of two strains, which were *Achromobacter xylosoxidans* and *Ralstonia picketii* to transform E2 with transformation rate 0.013-0.015mg/hr. Moreover, 1μg/l of E2 was oxidized to E1, and then E1 was eliminated with activated sludge (Ternes et al., 1999a).

2.6.1.2 Biotransformation by metabolisms in pure culture

Shi et al. (2004) isolated EE2-degrading microorganism, *Fusarium proliferatum* strain HNS-1, which degrade EE2 at an initial concentration of 25 mg/l in 6 day. Moreover, Gram-negative bacteria were isolated from activated sludge, *Novo-sphingobium sp*., which degrades E2 within 44 days (Fujii et al., 2002), but long time was required for degradation. In contrast, *R.zopfii* Y 50158 and *R.equi* Y 50155, Y 50156, and Y 50157 degraded E2 and E1 at an initial concentration of 100 mg/l completely in 24 hr and EE2 was degraded by about 80% in 24 hr (Yoshimoto et al., 2004).

2.6.2 Biotransformation by co-metabolisms

2.6.2.1 Biotransformation by co-metabolisms in mixed culture

In batch experiments with nitrifying activated sludge (NAS), 0.050 mg/l of EE2 was degraded completely within 6 days by oxidizing ammonium at rate of 50 mg NH_4^+/gDW/ hr and degrading EE2 at maximum rate of 1 μg/gDW/hr (Vader et al., 2000). Furthermore, in initial concentration of 1 mgL^{-1} of estrogen were degraded with NAS by the degradation rate of 0.056 hr^{-1} for E1, 1.3 hr^{-1} for E2, 0.030 hr^{-1} for E3, and 0.035 hr^{-1} for EE2. By using inhibitor for ammonia monooxygenase, the key enzyme for ammonia oxidation by AOB confirmed that NAS significantly degrade E1, E2, E3 and EE2. In NAS, E1, E2 and E3 were degraded by heterotrophic bacteria whereas EE2 was degraded by AOB (Shi et al., 2004).

2.6.2.2 Biotransformation by co-metabolisms in pure culture

Ammonia-oxidizing bacteria (AOB), *Nitrosomonas europaea*, degraded 0.4 mg/l estrogens with constant biodegradation rates of 0.0022 mg/l/hr for E1, 0.0020 mg/l/hr for E2, 0.0016 mg/l/hr for E3 and 0.0019 mg/l/hr for EE2. Corresponding ammonia consumption rates were 1.5 $mgNH4^+$-N/l/hr for E1, 1.45 $mgNH4^+$-N/l/hr for E2, 1.35 $mgNH4^+$-N/l/hr for E3 and 1.55 $mgNH4^+$-N/l/hr for EE2 (Shi et al., 2004).

2.7 Measurement of estrogens in environments

2.7.1 Sample storage

Samples in form of liquid and solid must be stored in refrigerator at 4 °C. Samples from river and wastewater should be collected in glass bottles that prior are rinsed by samples. 1% formaldehyde should be added into sample to reduce the estrogen degradation by microorganism. Sample should be analyzed within 72 hrs. Baronti et al., (2000) studied the recovery of estrogen in the bottle in different time storage and preservation stage. The result expressed that estrogens that was not preserved with 1% formaldehyde and was kept more than 7 days were severally lost more than the preserved sample except for EE2. They found that the storage time for more than 60 days can cause 40-50 % loss in all types of estrogens except for E1. They believed that the increase in amount of E1 came from the oxidation of E2 to E1 since formaldehyde is affected on the slow degradation of bacteria while activity is not completely inhibited.

2.7.2 Sample preparation

2.7.2.1 Filtration method

Because wastewater usually contains a high load of organic material and suspended particles, filtration is usually the first step of sample preparation. The filtration step is particularly necessary when subsequent extraction of the sample is based on the use of solid-phase extraction (SPE), because suspended solids could easily clog the absorbent bed. The most filtration step use glass filters with a pore size between 0.22-1.2 μm (Desbrow et al., 1998). Analysts often wash the filtration system with methanol after filtration of the wastewater samples to remove any analyze adsorbed on the particles in the filter. A few studies also use centrifugation of samples in addition to filtration for removing suspended matter.

2.7.2.2 Extraction method

Extraction of estrogen is usually performed by solid-phase extraction (SPE). Both disks and cartridges have been employed for the SPE of estrogens. Both disks and cartridges have advantages and disadvantages. Disks are not clogged by suspended matter present in the sample as easily as cartridges. Disks also have a comparatively larger surface area for adsorbent-matrix contact, which results in the higher extraction rates, and finally disk samples are free of contamination, whereas

cartridge samples can be contaminated by plasticizers leached from the cartridge support material during elution. Cartridge have the advantage of being amenable to system automation, because devices are available for automated washing, conditioning, sample loading, drying and elution of a large number of sample. SPE has many absorbent such as octadecyl (C_{18}) boned silica, graphitized carbon black, and styrenedivinylbenzene. Sample loading flow rates varied greatly among applications but were usually between 0.5-70 ml/min. Subsequent drying of the cartridge with either nitrogen or air. Elution of the compounds retained by C_{18} is usually performed with pure or aqueous (80-85%) methanol, in two steps with total elution volumes varying between 10 and 20 ml for cartridges and between 15 and 60 ml for disks. Graphitized carbon black adsorbents which are also often used for the extraction of estrogens behave both as non-specific adsorbents and anionic exchangers (D'Ascenzo et al., 2003).

2.7.2.3 Evaporation method

Volume reductions techniques can be used in the different means, for example, rotary evaporation and nitrogen evaporation. The choice depended mainly on the volume of extract to be concentrated.

2.7.3 Measurement of estrogens by gas chromatography (GC)

The analytical determination of estrogens in environmental has been dominated by the use of GC-MS and GC-MS-MS. The detection limits achieved with the different methods employing GC-MS or GC-MS-MS as final analytical techniques were in the range of 0.5-7.4 ng/l and 0.1-24 ng/l. The analysis is conducted after sample derivatization. Several derivatization agents such as bis - (trimethylisilyl) - triflouroacetamide, N – methyl - N-(tert.) – Butyl – dimethylsilyl - triflouroacetamide (MTBSTFA) and heptaflouro – butyric anhydride, have been used depending on the choice of ionization technique (Kelly 2000). The analytic are usually derivatized in the –OH groups of the steroid ring.

2.7.4 Measurement of estrogens by high performance liquid chromatography (HPLC)

The main advantage of applying the liquid chromatography based methods for environmental analysis of estrogens is that glucuronic and sulphuric metabolites can be detected while the derivatisation of the analytic needed in the GC-systems is un-necessary. The usual means of achieving separation is in columns with octadecyl silica based stationary phases. The mobile phases consist of water: acetonitrile or water: methanol mixtures with gradient elution from 20-50% to 100 % organic phases. Synder (1999) used fluorescence detection of E2 and EE2. Ying, Kookana, and Ru (2002) recently presented a similar method with similar limit of detection. The sensitivity of the fluorescence methods is low. This technique is rarely used because of severe problems with interference from the matrix and is obviously not recommended. The used of spectrophotometric techniques including diode array detectors (DAD) is common in HPLC systems. This technique is also widely used (Shimada, Mitamura, and Higashi, 2001).

2.7.5 Measurement of estrogens by Immunoassays

Immunoassays were the first methods applied for detection of environmental estrogens (Shore et al., 1993). The analytical validity of these and other early works are generally considered insufficient when compared to the level of more recent publications. This may explain why the immunoassays are less used than classical analytical techniques for detection of steroid estrogens. This method provides very sensitive methods, especially for wastewater and MTSs effluent, but the selectivity is poor.

2.8 Ammonia-oxidizing bacteria

2.8.1 Nitrification

Nitrification is the two step process by which ammonia is oxidized to nitrite and subsequently to nitrate. Ammonia is first oxidized to nitrite by ammonia-oxidizing bacteria (AOB), and subsequently nitrite is oxidized to nitrate by nitrite-oxidizing bacteria (NOB).

2.8.2 Phylogeny of ammonia-oxidizing bacteria

The current understanding of evolutionary relationships and the natural diversity of AOB is based on comparative sequence analyses of their genes encoding the 16S rRNA genes and *amoA* genes the gene that encode enzyme ammonia monooxygenase (AMO). Comparative 16S rRNA gene sequence analyses of cultured AOB found that members of physiological group are limited to two monophyletic lineages within the *Proteobacteria: Gammaproteobacteria* and *Betaproteobacteria. Nitrosococcus oceani* is member in the *Gammaproteobacteria*, despite members of the genera *Nitrosomonas* (including *Nitrosococcus mobilis*), *Nitrosospira, Nitrosolobus* and *Nitrosovibrio* from a closely related grouping within the *Betaproteobacteria* (Perkhold et al., 2000). Figure 2.4 shows a phylogenetic 16S rRNA based tree of those AOB demonstrated to represent different genospies (DNA-DNA similarity less than 60% and/or 16 rRNA sequence similarity less than 97.5%). Recently, the *amoA* gene, coding for the active site polypeptide of the ammonia monooxygenase has been used as an additional phylogenetic marker molecule for AOB. Phylogeny inference based on the deduced amino acid sequence of the *amoA* gene fragment is overall consistent with the 16S rRNA phylogeny of AOB (Figure 2.5) (Koops et al., 2003).

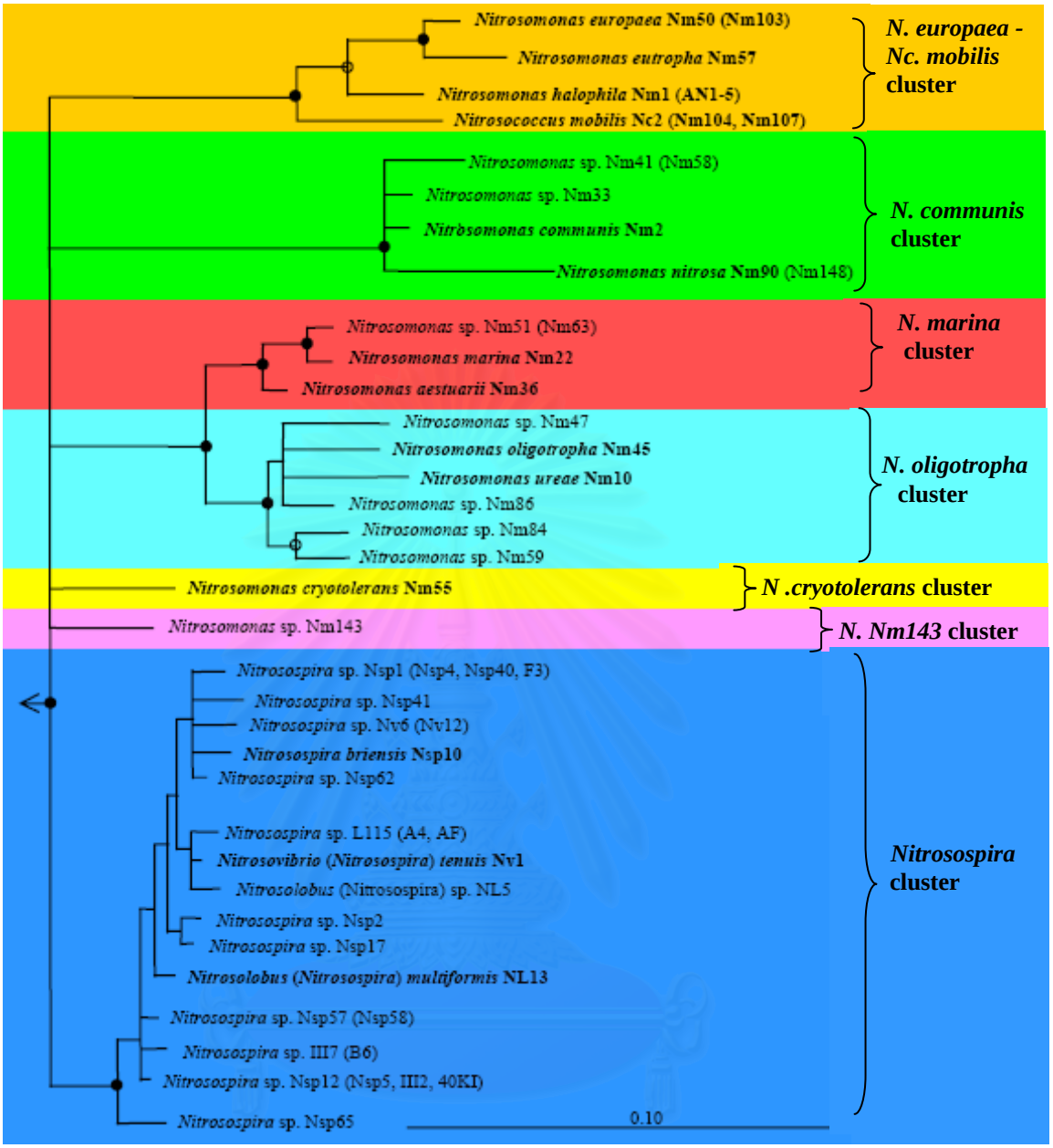


Figure 2.4 16S rRNA-based phylogenetic tree of the *Betaproteobacterial* AOB. Described species are depicted in bold. Maximum likelihood, maximum parsimony, and neighbor-joining trees were calculated and merged. Multifurcations connect branches for which a relative order cannot be unambiguously determined by applying different treeing methods. Filled and empty dots indicate parsimony bootstrap values (100 resamplings) above 90% and 70%, respectively. Scale bar represents 10% estimated sequence divergence

Available from: Koops et al. (2003)

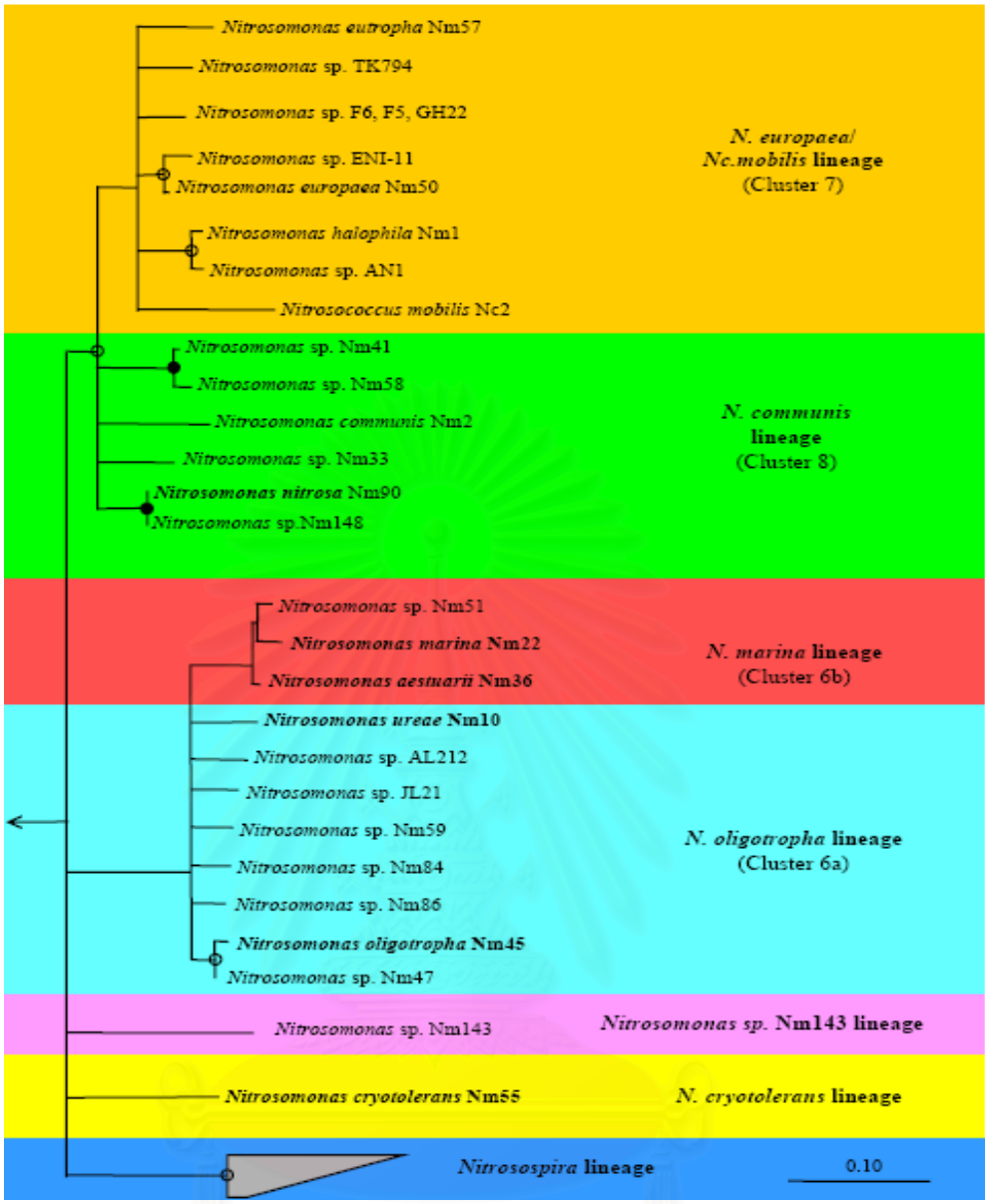


Figure 2.5 AmoA-based phylogenetic tree of the *Betaproteobacterial* AOB. Described species are depicted in bold. The 453-bp gene fragment obtainable with the most commonly used *amoA* PCR primers was used for phylogeny inference. AmoA sequences shorter than 414 nucleotides were excluded from the analysis. Protein maximum likelihood, protein maximum parsimony, neighbor-joining, and Fitch trees were calculated and merged. Multifurcations connect branches for which a relative order cannot be unambiguously determined by applying different treeing methods. Filled and empty dots indicate parsimony bootstrap values (100 resamplings) above 90% and 70%, respectively. Scale bar represents 10% estimated sequence divergence.
Available from: Koops et al. (2003)

2.8.3 Physiological properties of ammonia-oxidizing bacteria

All AOB use ammonia as a sole energy source but the characterization of AOB differ significantly among species and various distribution patterns of distinct species in different habitats (Table 2.6; Koops et al., 2003).

Table 2.6 Physiological properties and preferred habitats of described AOB species

Species	G+C (mol %)	Substrate (NH_3) affinity (K_s in μM)	Maximum ammonia tolerance NH_4Cl (in mM ; pH 8.0)	Salt Requirement	Maximum salt tolerance (in mM)	Preferred habitats
N .europaea	50.6–51.4	30–61	400	–	400	Sewage disposal plants, freshwater and brackish water
N. eutropha	47.9–48.5		600	–	400	
N. halophila	53.8		400	+	900	
Nc. mobilis	49.3		250	+	500	
N. communis	45.6–46.0	14–43	250	–	250	Soils (not acid) and eutrophic freshwater Oligotrophic freshwater and natural soils
N. nitrosa	47.9	19–46	100	–	300	
N. ureae	45.6–46.0	1.9–4.2	200	–	200	
N. oligotropha	49.4–50.0		50	–	150	
N. marina	47.4–48.0	50–52	200	+	800	Marine environments
N. aestuarii	45.7–46.3		400	+	600	
N. cryotolerans	45.5–46.1	42–59	400	+	550	
Ns. multiformis	53.5	ND	50	–	200	Soils (not acid)
Ns. tenuis	53.9	ND	100	–	100	Soils, rocks and freshwater
Ns. briensis	54	ND	200	–	250	

Symbols and Abbreviations: +, present; –, not present; +/–, present in some strains; and ND, no data.

Available from: Koops et al. (2003)

2.8.4 Co-metabolism of organic compound by ammonia-oxidizing bacteria

AOB, which is obligate chemolithotrophic aerobe using ammonia as a sole energy source, is widely for the oxidation of hydrocarbon substrates through the action of ammonia monooxgenase (AMO) (Arciero, Vannelli, and Hooper, 1989).

During oxidation of ammonia to nitrite, AMO catalyzes the oxidation of ammonia to hydroxylamine. Subsequently, hydroxylamine is oxidized to nitrite by hydroxylamine oxidoreductase (HAO). During the last process four electrons are released. Two of four electrons transfer to AMO in order to activate oxygen and maintain steady-state rate of ammonia oxidation. The rest two electrons are used in another oxidation reaction which is called co-metabolism (Arciero et al., 1989; William and Daniel, 1993). Currently, many hydrocarbons and halogenated hydrocarbons which are able to be degraded by co-metabolism of AOB such as in Figure 2.6 show ethylene is degraded by co-metabolism of AOB.

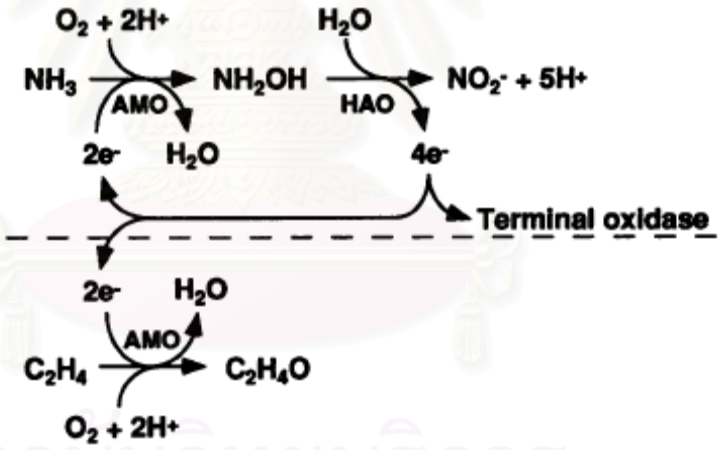


Figure 2.6 Co-metabolism of ethylene by AOB
Available from: William and Daniel (1993)

CHAPTER III

METHODOLOGY AND MATERIALS

3.1 Experimental framework

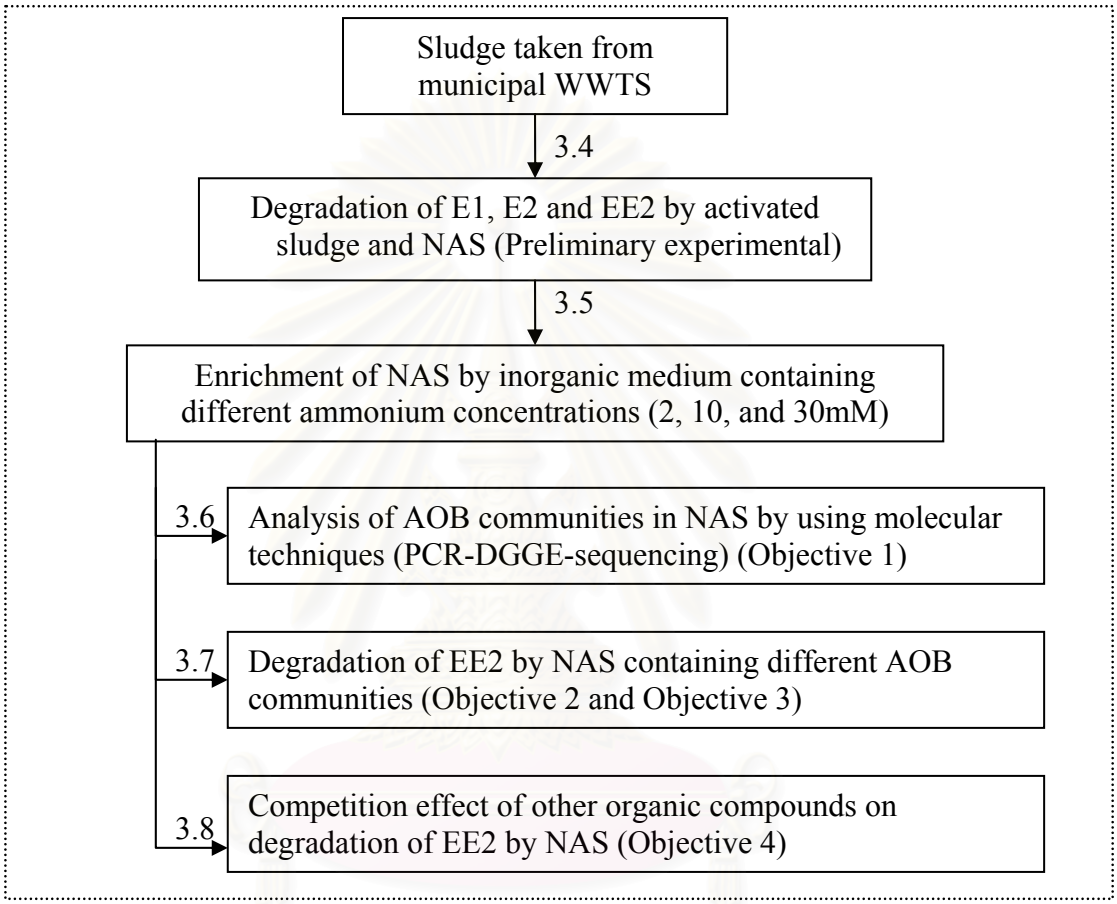


Figure 3.1 Experimental framework

The main part of this study concerns to the degradation EE2 by NAS containing different AOB communities. Experiment is divided into 5 parts (Figure 3.1): 1) Preliminary experimental using activated sludge and NAS to degrade E1, E2 and EE2, 2) Enrichment of NAS by inorganic medium containing different ammonium concentrations (2, 10, and 30 mM), 3) Analysis of AOB communities in NAS by using molecular techniques (PCR-DGGE-sequencing), 4) Degradation of EE2 by NAS containing different AOB communities, and 5) Competition effect of other organic compounds on degradation of EE2 by NAS.

3.2 Materials and apparatus

3.2.1 Chemicals

E1, E2 (>99%pure), and EE2 (>98% pure) were purchased from Sigma (St.Louis, MO, USA). Stock solutions of estrogens were prepared to 40mg/l in methanol.

3.2.2 Media

3.2.2.1 Medium for degradation of estrogens by activated sludge

The inorganic medium for degradation of estrogens by activated sludge contained 100 mg of NH_4Cl, 1 g of $NaNO_3$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of EDTA-Fe, 0.05 g of $CaCl_2 \cdot 2H_2O$, 0.05 g of K_2HPO_4, 4 g of HEPES, 0.6 mg of $MnCl_2 \cdot 4H_2O$, 0.5 mg of H_3BO_3, 0.1 mg of $ZnCl_2$, 0.1 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.6 mg of $CoCl_2 \cdot 6H_2O$, 0.12 mg of $NiCl_2 \cdot 6H_2O$, 0.12 mg of $CuSO_4 \cdot 5H_2O$ per liter, pH was adjusted to 7.5-8.0 using 40 g/l $NaHCO_3$ (modified from Chao et al., 2004). For degradation test, estrogens in methanol solution were added to test tubes to achieve a final concentration of 10 mg/l. Nitrogen gas flow was purged to remove methanol and then 5 ml of inorganic medium described above was added.

3.2.2.2 Medium for enriching nitrifying activated sludge

The inorganic medium for enriching NAS contained $(NH_4)_2SO_4$, 40 mg of $MgSO_4 \cdot 7H_2O$, 40 mg of $CaCl_2 \cdot 2H_2O$, 200 mg of KH_2PO_4, 1 mg of $FeSO_4 \cdot 7H_2O$, 0.1 mg of $Na_2Mo_4O_4 \cdot 2H_2O$, 0.2 mg of $MnCl_2 \cdot 4H_2O$, 0.02 mg of $CuSO_4 \cdot 5H_2O$, 0.1 mg of $ZnSO_4 \cdot 7H_2O$, and 0.002 mg of $CoCl_2 \cdot 6H_2O$ per liter (Limpiyakorn et al., 2007). $NaHCO_3$ was added to achieve 2 mg bicarbonate (HCO_3^-) per 1 mg of ammonium added. pH was adjusted to around 7.5-8.0 using 40 g/l $NaHCO_3$.

3.2.2.3 Medium for degradation of EE2 by nitrifying activated sludge

The inorganic medium for degradation of EE2 by NAS contained $(NH_4)_2SO_4$, $NaHCO_3$, 40 mg of $MgSO_4 \cdot 7H_2O$, 40 mg of $CaCl_2 \cdot 2H_2O$, 200 mg of KH_2PO_4, 1 mg of $FeSO_4 \cdot 7H_2O$, 0.1 mg of $Na_2Mo_4O_4 \cdot 2H_2O$, 0.2 mg of $MnCl_2 \cdot 4H_2O$, 0.02 mg of $CuSO_4 \cdot 5H_2O$, 0.1 mg of $ZnSO_4 \cdot 7H_2O$, and 0.002 mg of $CoCl_2 \cdot 6H_2O$, 5 g of $CaCO_3$ and 0.5% phenol 10 mg/l (modified from Limpiyakorn et al., 2007). Nitrogen gas flow was purged to remove methanol and then 5 ml of inorganic medium described above was added.

3.2.3 Seed sludge

Seed sludge was taken from a sludge buffer tank of a Chong Nonsi municipal wastewater treatment plant in September 2006. This system is Cyclic Activated Sludge System (CASS) which is modified from Sequencing Batch Reactor (SBR) and can receive up to 200,000 m^3/day. On the day of sampling, biological oxygen demand (BOD) in the influent was 40 mg/ l, whereas ammonium concentration was 13 mg N/l. BOD and ammonium removal efficiencies of this system were 92.5 % and 84.6 %, respectively. Nitrite concentration in the aeration tank was 0.01 mg N/l, and pH was controlled around 6-7. Mixed-liquor suspended solids (MLSS) concentration on the day of sampling was 9385 mg/l.

3.3 Sample preparation and analytical method

3.3.1 Sample preparation

1 ml of liquid medium was taken from test tubes to analyze for nitrogen concentrations. Equal volume of methanol (4 ml) was added into test tube containing remaining liquid medium (4 ml). Test tube was then vortexed to allow completely dissolving estrogens.

3.3.2 Measurement of ammonium

Inorganic medium added with methanol was diluted with deionized water to achieve a final concentration of ammonium ranging from 0 to 0.5 mg/l. 5 ml of dilution sample and 0.2 mL of phenol solution (Mix 11.1 mL liquefied phenol ($\geq$89%) with 95% v/v ethyl alcohol to a final volume of 100 mL) were added and then mixed. 0.2 mL of sodium nitroprusside solution (0.5% w/v: dissolve 0.5 g of sodium nitropusside in 100 mL of deionized water), and 0.5 mL of oxidizing solution (Mix 100 mL alkaline citrate solution: dissolve 200 g of trisodium citrate and 10 g of sodium hydroxide in 1000 mL of deionized water with 25 mL of sodium hypochloride) were added into the tube. Sample was covered with plastic wrap or paraffin wrapper film and kept at room temperature in subdued light for at least 1 hr to develop color. Sample was measured for absorbance at 640 nm with UV visible spectrophotometers (Thermo Electron Corporation, Hexious α, Cambridge, UK) (Strickland and Parson, 1972).

3.3.3 Measurement of nitrite

Inorganic medium added with methanol was diluted with deionized water to achieve a final concentration of nitrite ranging from 0 to 0.3 mg/l. 5ml of diluted sample and 0.1mL of Sulphanilamide solution (dissolve 5 g of Sulphanilamide and 50 mL of hydrochloric in 500 mL) was added, and allowed to react 5 min, then 0.1 mL of NNED solution (dissolve 1 g of (N-(1-Naphthyl)-Ethylenediamine Dihydrochloride in 1000mL of de-ionized water) was added and allowed at room temperature in subdued light for at least 1 hr to develop color . Sample was measured for absorbance at 543 nm with UV visible spectrophotometers (Thermo Electron Corporation, Hexious α, Cambridge, UK) (Strickland and Parson, 1972).

3.3.4 Measurement of nitrate

Inorganic medium added with methanol was diluted with deionized water to achieve a final concentration of nitrate ranging from 0 to 0.5 mg/l. 5 mL of diluted sample was filtered and measured for absorbance at 220 nm to obtain NO_3^- reading and absorbance at 275 nm to determine interference due to dissolved organic matter with UV visible spectrophotometers (Thermo Electron Corporation, Hexious α, Cambridge, UK) (Strickland and Parson, 1972).

3.3.5 Measurement of estrogens

1 ml of inorganic medium added with methanol was filtered through 0.45 μm filter. Estrogens were analyzed using High Performance Liquid Chromatography (HPLC; Agilent 1100 Series LC, Germany) with UV diode array detector (Agilent 1100 Series LC, Germany) at λ= 210 nm. Elution was carried out by using 40 % v/v acetonitrile/water at a flow rate of 1 ml/min with retention time of 15 min (Weber et al., 2005). Retention time of E1, E2 and EE2 were 13.153 min, 9.257 min and 12.094 min, respectively.

3.3.6 Measurement of COD using closed reflux method

2.5 ml of sample, 1.5 ml of standard potassium dichromate solution (12.259 g of Potassium dichromate dissolved in 1 l of deionized water), and 3.5 ml of silver sulfate (22 g of diluted silver sulfate in 4 kg of sulfuric acid) was added in digestion vessel and allowed to react for 2 hr in block digester. Solution were titrated with standard ferrous ammonium sulfate until color of ferroin indicator (1.485 g of 1,10 phenanthroline monohydrate and 0.695 g of Ferrous sulfate dissolved in 100 ml of deionized water) changed from green to red (American Public Health Association, 1992).

3.3.7 Analysis of ammonia-oxidizing bacterial communities

3.3.7.1 Preparation of sample

Sludge of approximately 2 mg of MLSS was transferred into a 1.7 ml eppendorf tube and centrifuged at 14,000 rpm for 10 min. The supernatant was removed, and the pellet was kept at -20 ^{0}C until analysis.

3.3.7.2 DNA extraction

DNA was extracted from samples using Fast-DNA SPIN kits for soil (QBiogene, Solon, Ohio, USA) with a small modification at the initial step: 1 ml of sodium phosphate buffer solution was added to and mixed with the sample, and then the tube was sonicated for 30 s on ice. The remaining steps followed the manufacturer's instructions. The product from DNA extraction was verified by electrophoresis in 2% agarose (Bio-Rad, Spain).

3.3.7.3 Polymerase chain reaction (PCR)

Primers CTO189f and CTO654r (Kowalchuck et al., 1997) were used to amplify 465-bp of 16S rRNA gene fragment and primers amoA 1F and amoA 2R were used to amplify 500-bp of *amoA* gene fragment of *Betaproteobacteria*. The oligonucleotide sequences of the primers are shown in Table 3.1. Extracted DNA was PCR-amplified using the primer sets (the forward primer had a GC clamp) for 35 cycles in a 25 μl reaction volume. DNA eluted from bands excised from DGGE gels were amplified for 20–25 cycles using the primer set lacking the GC clamp in a 25 μl reaction volume. The PCR mixture was prepared using AmpliTaq Gold DNA polymerase (PE Applied Biosystems, CA, USA) following the manufacturer's

instructions. PCR amplification was performed in an Authorized thermal cycler (Biorad, USA) under the conditions of 3 min at 94 ^{0}C followed by 35 cycles of 1min at 94 ^{0}C, 1min at 57 ^{0}C, and 1min at 72 ^{0}C, followed by 10min final extension at 72 ^{0}C **(**Limpiyakorn et al., 2007).

Table 3.1 Primers used in this study

Primer	Nucleotide sequence (5′–3′)
CTO 189A/Bf	GGAGRAAAGCAGGGGATCG
CTO189A/Bf-GC	CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAGRAA AGCAGGGGATCG
CTO 189Cf	GGAGGAAAGTAGGGGATCG
CTO 189Cf-GC	CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAGGAA AGTAGGGGATCG
CTO 654r	CTAGCYTTGTAGTTTCAAACGC
amoA 1F	GGGGTTTCTACTGGTGGT
amoA 1F-GC	CGCCGCGCGGCGGGCGGGGCGGGGGCGGGGTTTCTACTGGTGGT
amoA 2R	CCCCTCTGCAAAGCCTTCTTC

Available from: Limpiyakorn et al. (2005)

3.3.7.4 Denaturing gradient gel electrophoreses

Denaturing gradient gel electrophoreses was performed according to the modification of a described method (Kurisu et al., 2002). We use 8% polyacrylamide gels, and the urea–formamide denaturant gradient was 35–50%. Gels were run on the D Code system (Bio-Rad Laboratories, Hercules, CA, USA) for 16 h at 60 ^{0}C and 75 V. After electrophoresis, the gels are stained with Ethidium bromide (Amersham Biosciences, Munich, Germany) and analyzed by gel documentation (Dolphin-DOC Plus, NV, USA). Prominent bands were excised and dissolved in 30µl sterilized water. DNA was recovered from the gel by freeze–thawing three times.

3.3.7.5 Homology Search

Homology search of the analyzed sequences by using Blast program and aligned with those of closely related and reference AOB using ClustalW**.**

3.4 Degradation of E1, E2 and EE2 by activated sludge and nitrifying activated sludge (Preliminary experimental)

This experiment aimed to investigate the potential of activated sludge from a municipal wastewater treatment system in Thailand in degrading natural estrogens (E1 and E2) and synthetic estrogen (EE2). Two parallel batch tests comprising of degradation test and control test were carried out in triplicate. Activated sludge was added into inorganic medium containing 10 mg/l of E1, E2 or EE2 to obtain final MLSS concentration of 150 mg/l. The control test was prepared in the same manner as the degradation test except that for the control test, no NAS was added. The cultivations were at 25 ^{0}C with a rotating speed of 250 rpm. Samples were taken at time 24, 48, 72, 96, 120, 144 and 168 hr for analysis of estrogen concentrations.

3.5 Enrichment of nitrifying activated sludge by inorganic medium containing different ammonium concentrations (2, 10, and 30 mM)

This experiment aimed to develop NAS containing different AOB communities. Sludge taken from the municipal wastewater treatment system was enriched in three laboratory-scale continuous flow reactors without sludge recycling introduced with inorganic medium containing three different ammonium concentrations: 2, 10 and 30 mM NH_4^+-N (28, 140, and 420 mg N/l, respectively). Total volume of each reactor was 4 l, with an effective volume of 2 l. To obtain the optimum condition for AOB growth, temperature was kept at 30 ^{0}C, DO concentration was controlled at around 2 mgl^{-1}, pH was maintained in a range of 7.5-8.0 using 1 N HCl and 1 N NaOH, and mixing was provided at rotating speed of 300rpm. Inorganic medium was introduced into all reactors at a fixed dilution rate of 0.01 hr^{-1} **(**Limpiyakorn et al., 2007).

3.6 Analysis of ammonia-oxidizing bacterial communities in nitrifying activated sludge by using molecular techniques

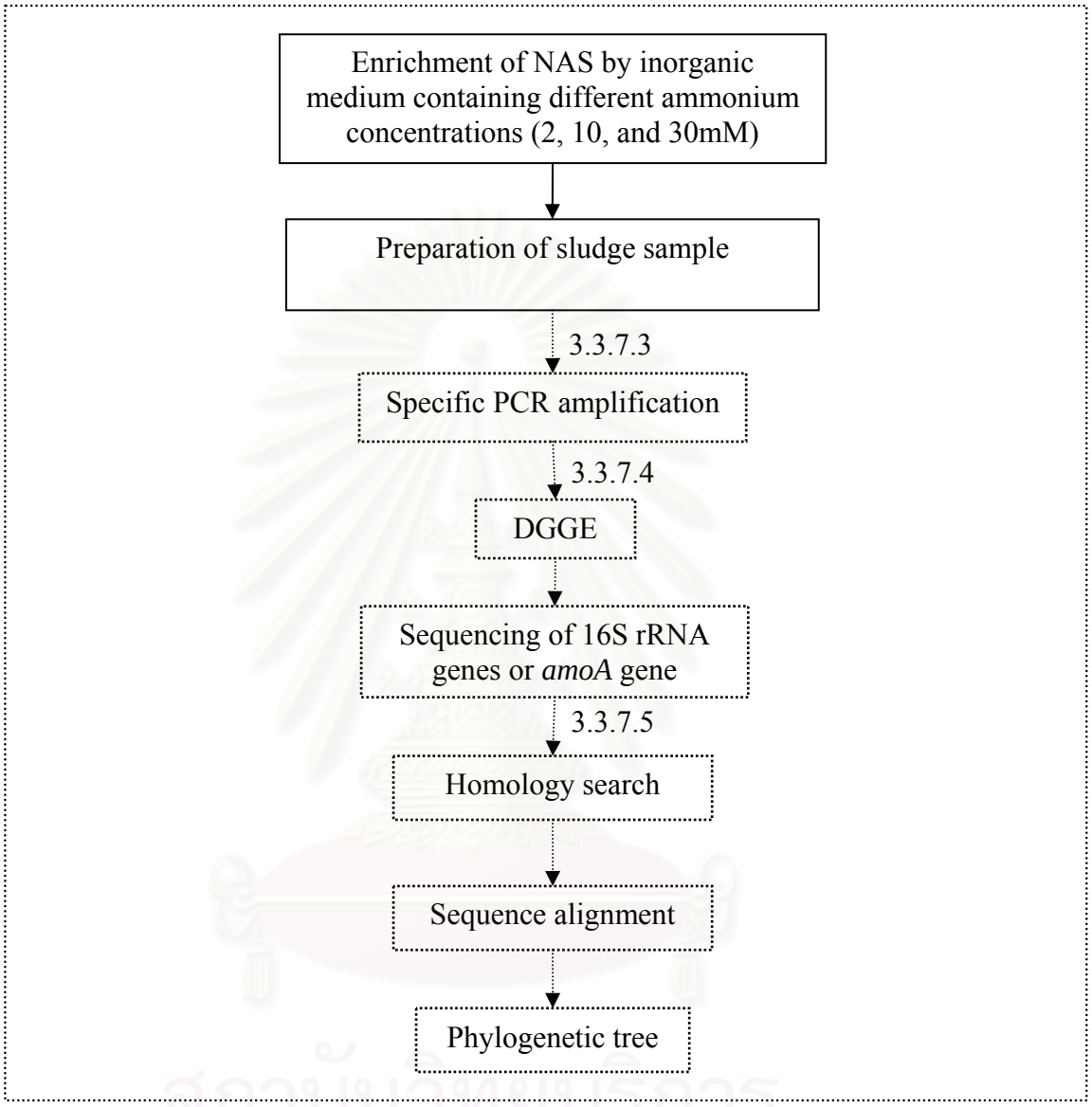


Figure 3.2 Experimental framework for analysis of ammonia-oxidizing bacterial communities in nitrifying activated sludge

This experiment aimed to investigate AOB communities in NAS enriched with inorganic medium containing different ammonium concentrations. Communities of AOB in NAS were analyzed by specific PCR application followed by DGGE, and sequencing of 16S rRNA genes or *amoA* genes of AOB belonging to *Betaproteobacteria* (Figure 3.2).

3.7 Degradation of EE2 by nitrifying activated sludge containing different ammonia-oxidizing bacterial communities

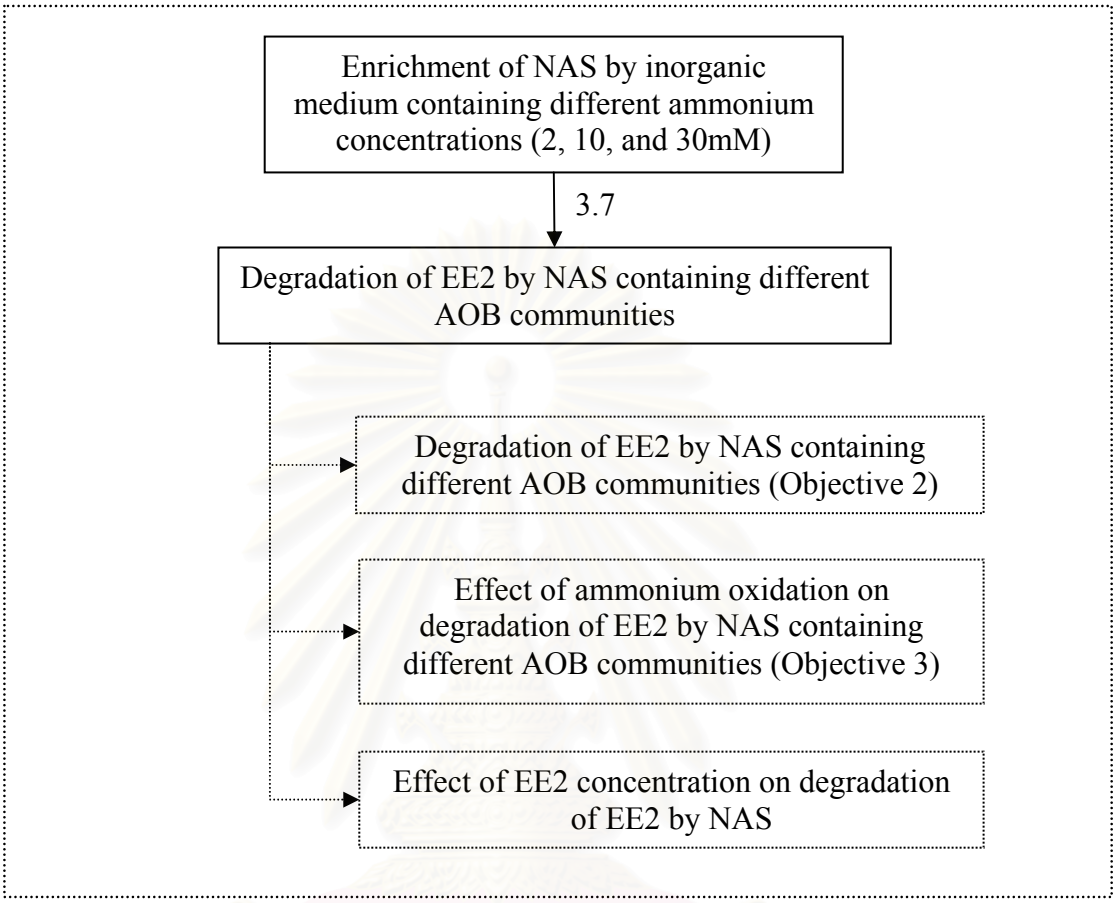


Figure 3.3 Experimental framework for degradation of EE2 by nitrifying activated sludge with different ammonia-oxidizing bacteria communities

This experiment aimed to study degradation of EE2 by NAS containing different AOB communities (Objective 2), effect of ammonia oxidation on degradation of EE2 by NAS containing different AOB communities (Objective 3) and effect of EE2 concentration on degradation of EE2 by NAS.

NAS from 3 reactors containing different AOB communities were tested for their ability to degrade 10 mg/l of EE2 under varying concentrations of ammonium (2, 10 and 30 mM NH_4^+-N). Three parallel batch tests comprising of degradation test, inhibition test and control test were performed in triplicate. In degradation test NAS was added into 5 ml of inorganic medium containing EE2 (10 mg/l) and varying concentration of ammonium (2, 10 and 30 mM NH_4^+-N) to obtain final MLSS concentration of 150 mg/l. Inhibition test and control test were prepared in the same

manner as the degradation test except that for inhibition test, allythiourea (10 mg/l) (Shi et al., 2004) was added to inhibit ammonia oxidation by AOB and for control test, no NAS was added. The cultivations were at 25 ^{0}C with rotating speed of 250 rpm. Samples in each tube were taken at time 24, 48, 72, 96, 120, 144 and 168 hr. Concentrations of ammonium, nitrite, nitrate and EE2 were analyzed as described previously.

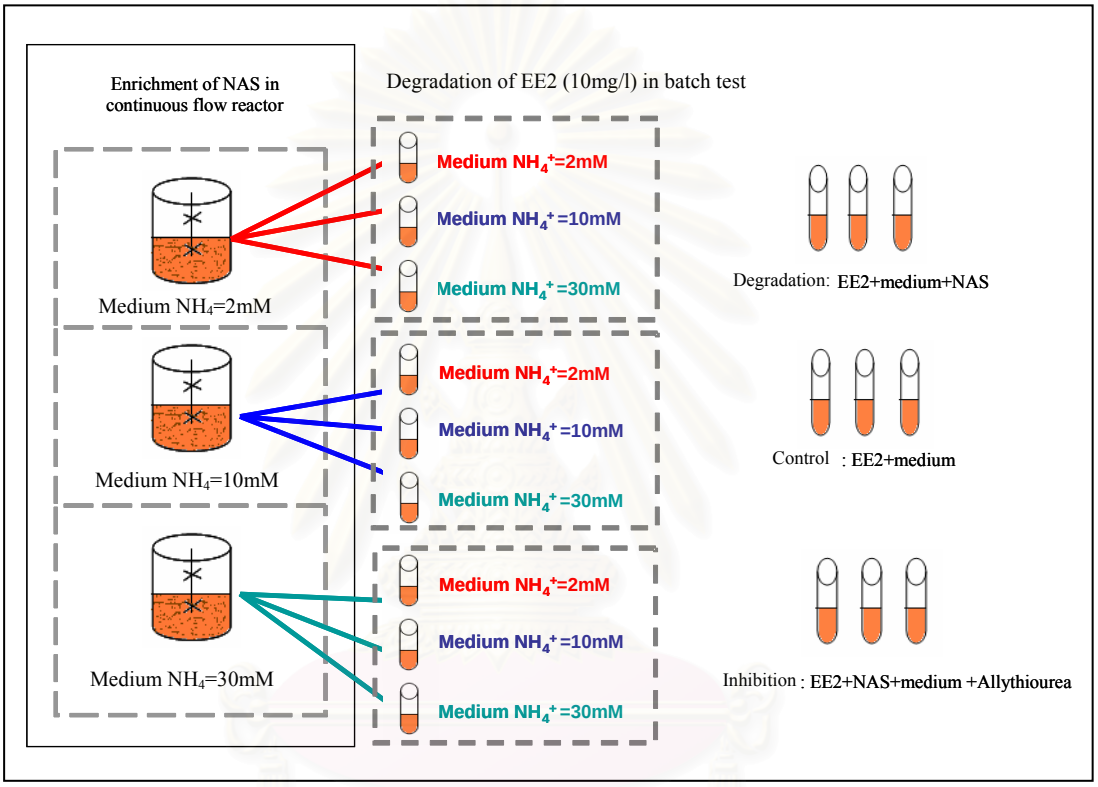


Figure 3.4 Degradation of EE2 by nitrifying activated sludge containing different ammonia-oxidizing bacteria communities

3.8 Competition effect of other organic compounds on degradation of EE2 by nitrifying activated sludge

This experiment aimed to observe competition effect of other organic compounds on degradation of EE2 by NAS (Objective 4). E2 and canteen wastewater were selected as model compounds (separated study). E2 were selected as to represent organic compounds that have similar structure to EE2. Canteen wastewater was selected to observe the actual phenomena in municipal wastewater treatment systems. Six parallel batch tests (Table 3.2 and Table 3.3) comprising of four degradation tests, degradation test with additional E2 or wastewater, control test, and inhibition test were performed in triplicate for each study. In degradation test, NAS was added into 5 ml inorganic medium containing EE2 (10 mg/l), ammonium (30 mM), and E2 (10 mg/l) or wastewater (COD=217 mg/l) to obtain MLSS concentration of 150 mg/l. Control test and inhibition test were prepared in the same manner as degradation test except that for control test, no NAS was added, and for inhibition test, allythiourea was added to inhibit ammonia oxidation by AOB. The cultivations were at 25 ^{0}C with rotating speed of 250 rpm. Samples were taken at 24, 48, 72, 96, 120, 144 and 168 hr. Concentrations of ammonium, nitrite, nitrate, EE2, E2 and COD were analyzed as described above.

Table 3.2 Competition effect of E2 on degradation of EE2 by nitrifying activated sludge

Test	NAS (2mM reactor)	Ammonium (30mM)	E2	EE2	Allythiourea
EE2+E2	+	+	+	+	-
EE2+E2+Inhibitor	+	+	+	+	+
EE2+E2+Control	-	+	+	+	-
EE2	+	+	-	+	-
E2	+	+	+	-	-
Medium	+	+	-	-	-

Symbols and Abbreviations: +, added; –, not added

Table 3.3 Competition effect of canteen wastewater on degradation of EE2 by nitrifying activated sludge

Test	NAS (2mM reactor)	Ammonium (30mM)	Wastewater	EE2	Allythiourea
EE2+Wastewater	+	+	+	+	-
EE2+Wastewater+Inhibitor	+	+	+	+	+
EE2+Wastewater+Control	-	+	+	+	-
EE2	+	+	-	+	-
Wastewater	+	+	+	-	-
Medium	+	+	-	-	-

Symbols and Abbreviations: +, added; –, not added

CHAPTER IV

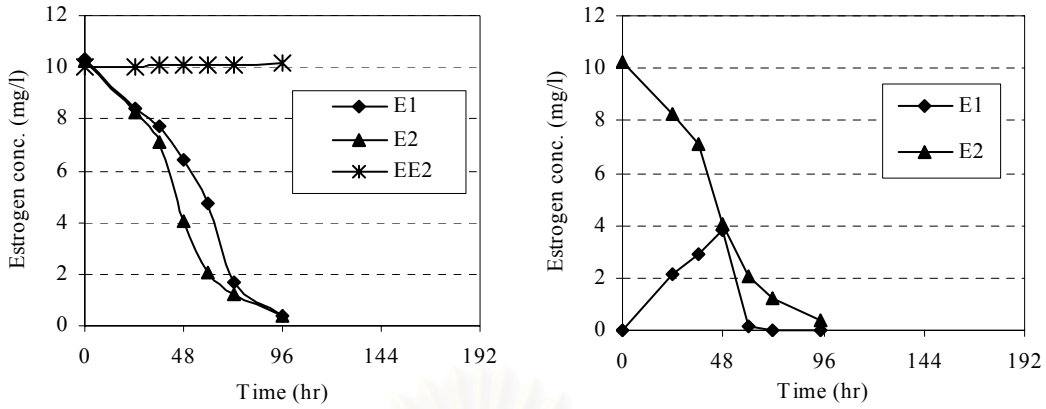
RESULTS AND DISCUSSION

4.1 Degradation of E1, E2 and EE2 by activated sludge and nitrifying activated sludge (Preliminary experiment)

Estrogens can be released into the environments by excretion of humans and animals through their urine and feces. Municipal wastewater treatment systems are an important facility that markedly reduced the concentrations of estrogens. This experiment was conducted to investigate the potential of activated sludge from municipal wastewater treatment system in Thailand (Chong Nonsi municipal wastewater treatment plant) in degrading natural estrogens (E1 and E2) and synthetic estrogen (EE2). The results suggested that activated sludge was able to degrade 10 mg/l of E1 and E2 (Figure 4.1a). 96.25% and 96.62% of E1 and E2 can be degraded within 4 days.

Previously, similar data was illustrated by Ternes et al. (1999a) and Shi et al. (2004). They found that E2 with initial concentration of 1 mg/l could be degraded by using activated sludge with in 2 hr (Shi et al., 2004) and 3 hr (Ternes et al., 1999a). Here, activated sludge degraded 10 mg/l of E1 and E2 within 4 days of which the degradation rates and the degradation rate constants were 0.103 mg/hr and 0.0334 1/hr, respectively for E1 and 0.103 mg/hr and 0.0369 1/hr, respectively for E2. It was noted that E1 was formed during the degradation of E2. This compound was accumulated until hour 48 and then eliminated (Figure 4.1b). No other metabolite was found using HPLC analysis.

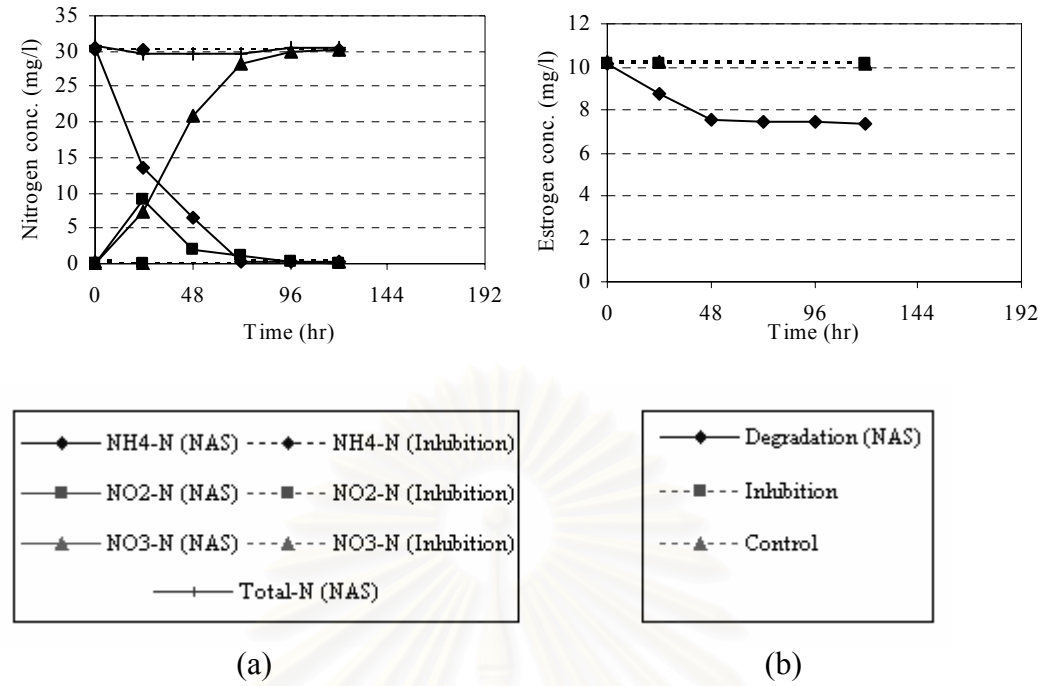
In contrast to natural estrogens, EE2 was not degraded by activated sludge. These results confirmed that E1 and E2 were degradable, whereas EE2 was persistent in contact with activated sludge.



(a) (b)

Figure 4.1 Degradation of E1, E2 and EE2 by activated sludge (a) concentration of estrogens during degradation (b) formation of E1 during degradation of E2

Although EE2 was not degraded by activated sludge, recent publication suggested that AOB in NAS can degrade several persistence organic compounds via co-metabolism. Vader et al., (2000) suggested that 0.05 mg/l of EE2 was degraded completely within 6 days by NAS enriched with high ammonium concentration. Moreover, Shi et al., (2004) suggested that 1 mg/l of EE2 was degraded completely within 4 days. In this experiment, NAS from 30 mM reactor was used to degrade 10mg/l of EE2 under an initial ammonium concentration of 2 mM. Three parallel batch tests comprising of degradation test, inhibition test and control test were performed. In the inhibition test, allythiourea was used as an inhibitor of ammonia oxidation by AOB (Figure 4.2a). The result suggested that ammonium concentration in degradation test decreased, nitrite temporarily increased and then decreased, nitrate increased, while total nitrogen were nearly stable. In contrast to the inhibition test, no reduction of ammonium concentrations was observed, which indicated that allythiourea completely inhibited ammonia oxidation of AOB. EE2 concentration in the degradation test decreased 25% within 48hr, whereas that in the inhibition tests remained the same throughout the experiment. These results indicated that EE2 concentrations were decreased by co-metabolism of AOB.



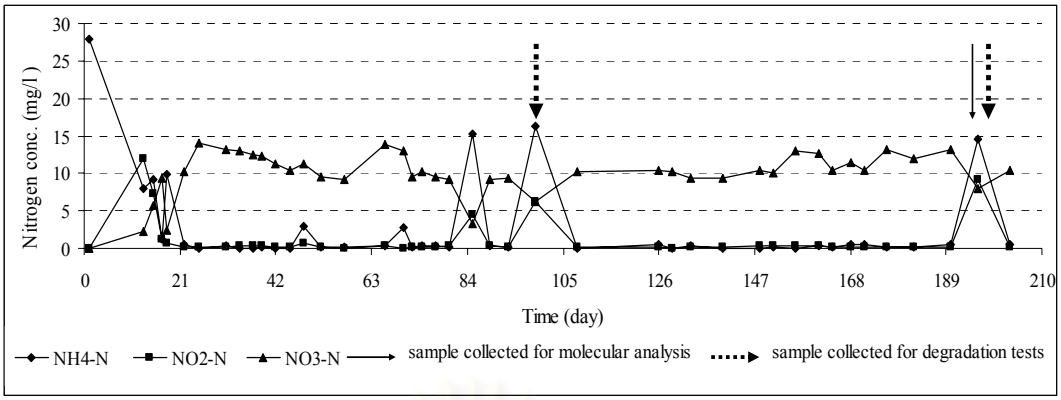
(a) (b)

Figure 4.2 Degradation of EE2 by nitrifying activated sludge (a) concentrations of nitrogen during degradation (b) concentration of EE2 during degradation

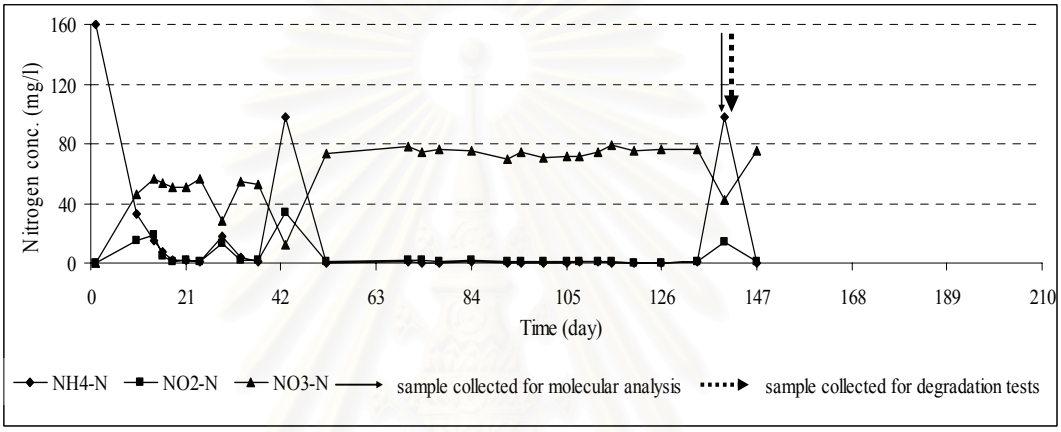
4.2 Enrichment of nitrifying activated sludge by inorganic medium containing different ammonium concentrations (2, 10, and 30 mM)

This experiment aimed to develop NAS containing different AOB communities. Sludge taken from the municipal wastewater treatment system was enriched in three reactors receiving inorganic medium containing different ammonium concentration of 2, 10 and 30 mM. 2, 10, and 30 mM reactor were operated for 203, 147, and 203 days, respectively.

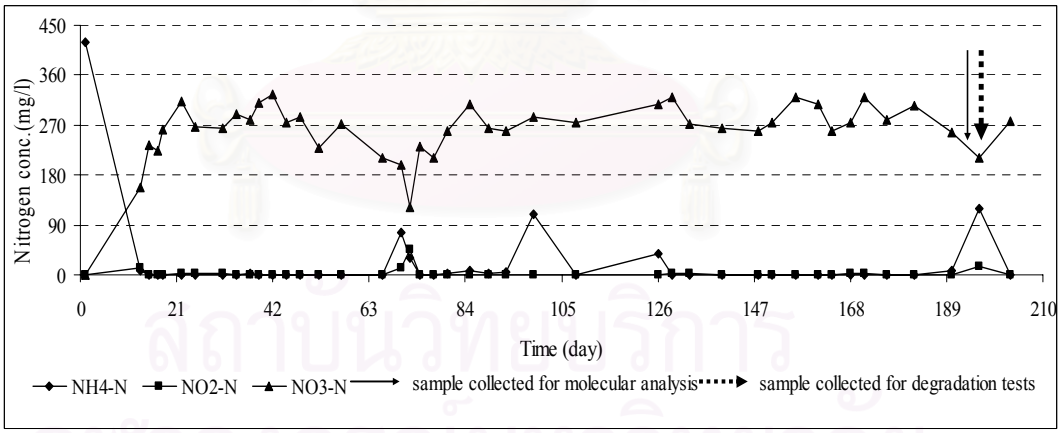
In all three reactors, the tendencies of nitrogen concentrations were very similar (Figure 4.3). Ammonium concentration reached the steady state condition at zero after around 21 days of operation. This indicated that ammonium was completely oxidized. However, it must be noted that few ammonium peaks were found when sludge samples were taken due to adding medium to obtain volume of 2 l. Nitrite temporarily increased at the early operation period and then decreased. Nitrate increased and reached the steady state condition. Total nitrogen concentrations were nearly stable through at the experiment suggesting that no nitrate was reduced to nitrogen gas and thus no anoxic condition arose in the reactors. DO concentrations in each reactor were >2 mg/l confirmed again that the reactors were absolutely in aerobic condition. After reaching the steady state conditions of nitrogen, sludge samples were taken for molecular analysis and degradation tests.



(a)



(b)

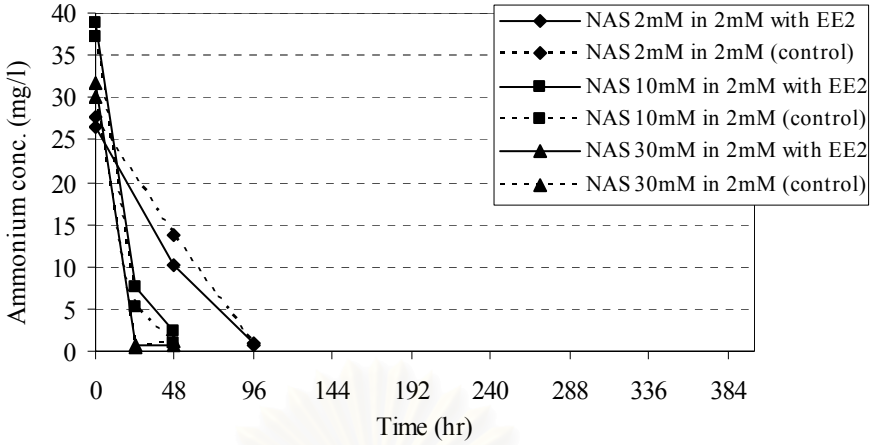


(c)

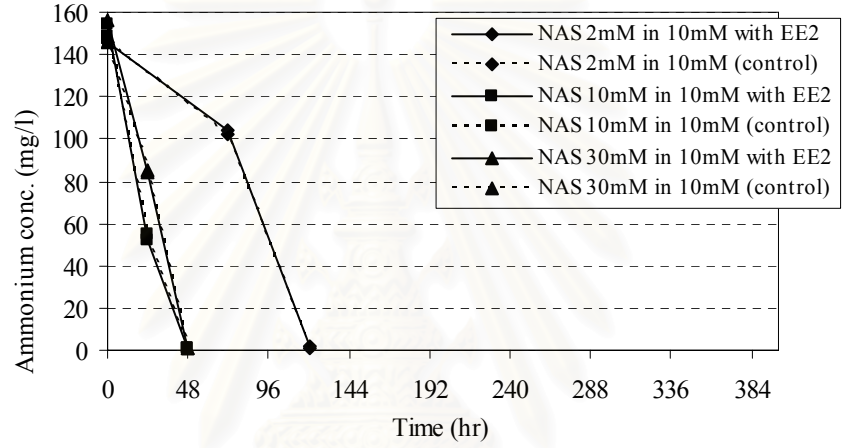
Figure 4.3 Concentrations of nitrogens during enrichment of nitrifying activated sludge by inorganic medium containing different ammonium concentrations of (a) 2 mM reactor, (b) 10 mM reactor, and (c) 30 mM reactor

4.3 Effect of EE2 on ammonia oxidation

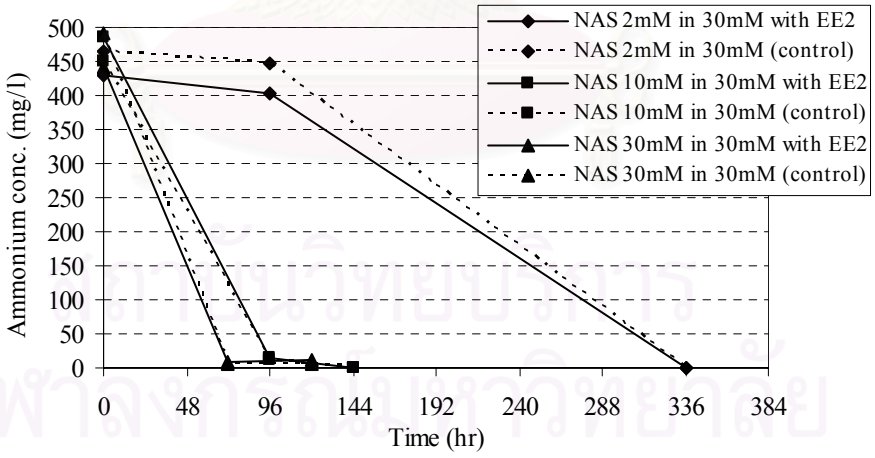
This experiment aimed to observe the inhibitory effect of EE2 on ammonia oxidation. Degradation tests and control tests were prepared in the same manner except that in the control test, no EE2 was added. All of the cases (Figure 4.4) suggested no significant difference in ammonium oxidations between the degradation tests (with EE2) and the control tests (without EE2). These confirmed that EE2 did not affect ammonia oxidation of AOB.



(a)



(b)



(c)

Figure 4.4 Concentrations of ammonium during the study of effect of EE2 on ammonia oxidation with NAS from 2, 10, and 30 mM reactors with initial ammonium concentrations of (a) 2 mM, (b) 10 mM, and (c) 30 mM

4.4 Analysis of ammonia-oxidizing bacterial communities in nitrifying activated sludge (Objective 1)

Specific PCR amplification followed by DGGE, cloning, and sequencing of 16S rRNA gene or *amoA* gene were used to analyze AOB communities in seed sludge and NAS from different reactors. Analysis based on sequence of 16s rRNA gene was performed for all samples. However, after several attempts, amplification of 16s rRNA gene of AOB in sample from 2 mM reactor never have been succeeded. To solve this problem, sequences of *amoA* gene were analyzed for this sample instead. After the PCR-amplified products of all samples had been run on DGGE gels (Figure 4.5), a total of 11 bands were selected for sequence analysis.

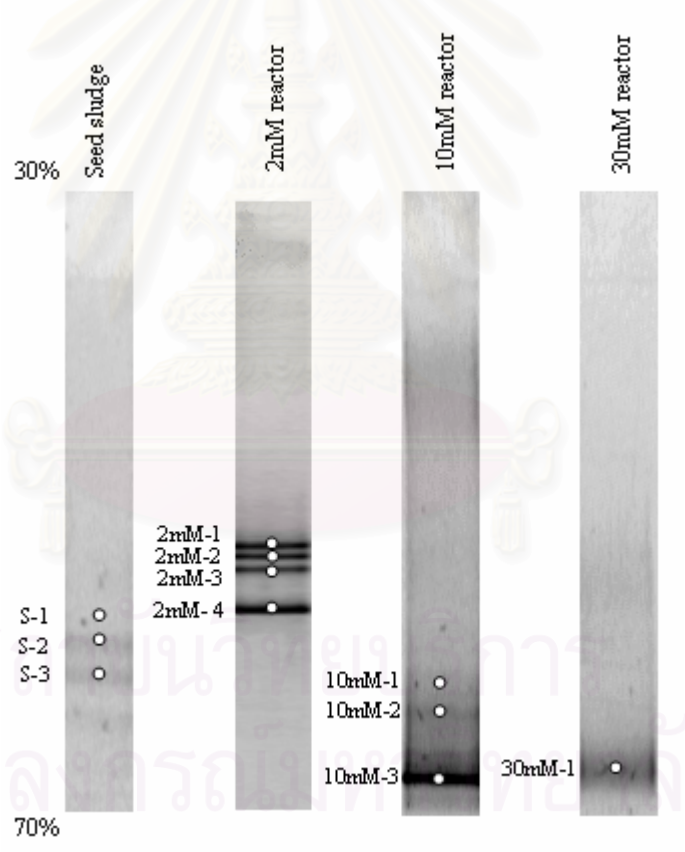


Figure 4.5 DGGE images of the PCR-amplified products of seed sludge (16S rRNA gene), and nitrifying activated sludge from 2 mM (*amoA* gene), 10 mM (16S rRNA gene), and 30 mM (16S rRNA gene) reactors

Homology search of the analyzed sequences using Blast program (Table 4.1) suggested that most of the sequences analyzed showed 97% to 100% identity to the AOB sequences available in the database. Sequences of 16s rRNA genes from this study were aligned with those of closely related and reference AOB using ClustalW, then phylogenetic tree was drawn (Figure 4.6).

Table 4.1 Closely related neighbor of analyzed sequences from DGGE bands

Reactor	**Band**	**Score**	**Percent identity**	**Gap**	**Accession No. of closely related sequence**	**Closely related sequence**
Seed sludge	S-1	819	98(436/443)	2/443	AB222811	DGGE 0NO2c-3
	S-2	821	98(441/446)	3/446	AJ297415	clone GaN50304
	S-3	868	99(441/442)	0/442	AJ297415	clone GaN50304
2mM	2mM-1	918	99(470/471)	1/471	DQ437762	clone Y41
	2mM-2	872	99(446/448)	0/448	AY352918	clone CB1-21
	2mM-3	848	100(428/428)	0/428	AF202649	clone S6
	2mM-4	821	100(414/414)	0/414	EF105354	clone 3GN01a07
10mM	10mM-1	848	99(445/448)	2/448	CP000450	*Nitrosomonas eutropha* C91
	10mM-2	745	97(434/446)	6/446	EF175894	clone S_1
	10mM-3	789	99(408/410)	1/410	EF175894	clone S_1
30mM	30mM-1	833	99(437/440)	2/440	AF210051	clone HB3

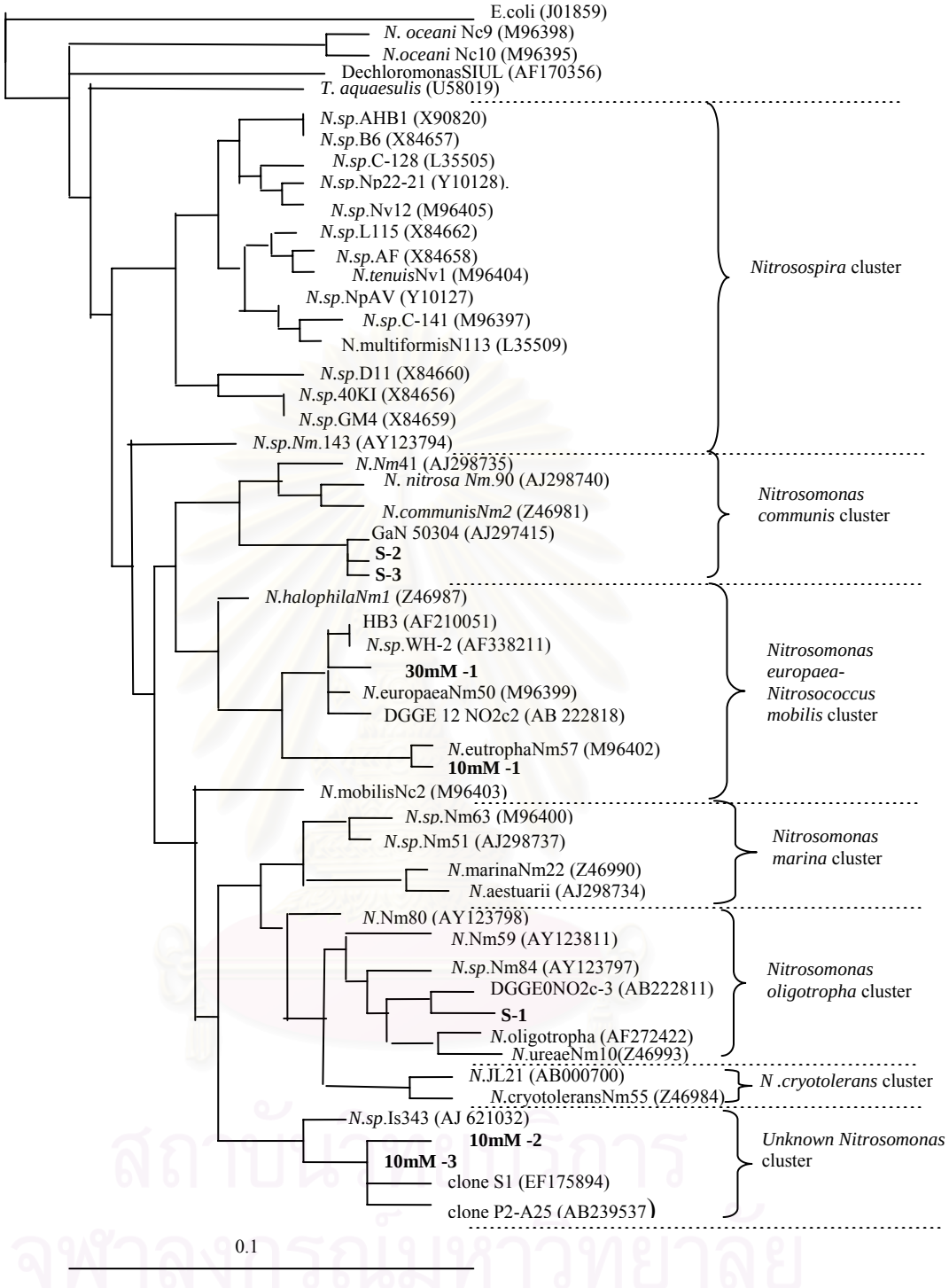


Figure 4.6 16S rRNA-based phylogenetic tree based on partial 400-bp sequences of 16S rRNA gene of ammonia-oxidizing bacteria of β-Proteobacteria. AOB genus abbreviations are *N.* for *Nitrosomonas*, *Nc.* for *Nitrosococcus*, and *Ns*. for *Nitrosospira*

Table 4.2 lists and summarizes the AOB found in each sample. All of the bands analyzed were related to *Nitrosomonas spp* which were always found in several studies of wastewater treatment systems (Limpiyakorn et al., 2005; Purkhold et al., 2000).

Table 4.2 Summary of ammonia-oxidizing bacteria found in samples

AOB cluster	Seed sludge	2mM reactor	10mM reactor	30mM reactor
Nitrosospira cluster				
Nitrosomonas communis cluster	+	+		
Nitrosomonas europaea- Nitrosococcus mobilis cluster		+	+	+
Nitrosomonas marina cluster				
Nitrosomonas oligotropha cluster	+			
Nitrosomonas cryotolerans cluster				
Unknown Nitrosomonas cluster			+	

Symbols and Abbreviations: +, present

Bands analyzed from seed sludge related closely to *N. communis* cluster and *N. oligotropha* cluster. The sequences of *N. oligotropha* cluster was reported for their high affinity to free ammonium (K_s=1.9 to 4.2 μM; Koops et al., 2003). Sequence of this AOB cluster often are recovered from oligotrophic environments, including freshwater sediment (Bollmann and Laanbroek, 2001), wastewater treatment systems receiving low-ammonium influents (Gieseke et al., 2001). Although *N. communis* was reported to have moderate affinity to free ammonium (K_s=14 to 43 μM; Koops et al., 2003). They were often recovered from wastewater treatment systems (WWTS) receiving influents low in ammonium load (Gieseke et al., 2001; Koops et al., 2003; Limpiyakorn et al., 2005). Recently (Limpiyakorn et al., 2005), the numbers of *N. communis* cluster and *N. oligotropha* cluster were first time revealed in 12 sewage treatment systems in Tokyo. This study suggested an importance of these two AOB in the systems.

Bands analyzed from 2 mM reactor related closely to *N. communis* cluster and *N. europaea – Nc. mobilis* cluster. The sequences of *N. communis* cluster found in this

reactor were also found in the seed sludge. In contrast to *N. communis* that were often found in WWTS receiving low ammonium loads effluents , *N. europaea – Nc. mobilis* cluster were reported to have low affinity to free ammonium (K_s > 30 μM; Koops et al., 2003). This AOB were commonly found in eutrophic environments. However, previous study on effect of ammonium (Limpiyakorn et al., 2007) showed clearly that this AOB were not the predominant AOB in the systems with this ammonium load. The numbers of this AOB found at this ammonium load were lower that the detection limits of real time PCR quantification. In addition, the homology search (Table 4.1) suggested that the sequence 2 mM-3 has 100% identity to clone S6 which was a member *N. europaea – Nc. mobilis* cluster. However, this clone was recovered from anoxic biofilm. As discussed previously, 2 mM reactor was strictly aerobic condition (DO >2 mg/l) and the intensity of this band was weak, this AOB should be present in the deep part of sludge floc and should not active. Therefore, in this reactor only *N. communis* cluster should be the dominant AOB.

Bands analyzed from 10 mM reactor related to unknown nitrosomonas cluster and *N. europaea – Nc. mobilis* cluster. The unknown *Nitrosomonas* cluster previously were found in WWTP (municipal, oil industry, brewery), which in general low in ammonium concentration. In this reactor, this band was strong compared to others. This implied that this AOB should be the dominant AOB in this reactor. Band related to *N. europaea – Nc. mobilis* cluster was also found as very smear and weak. This result suggested that this AOB may occur as minority in this reactor. Previous study using the similar operational condition to this study (Limpiyakorn et al., 2007) found that *N. europaea – Nc. mobilis* cluster were not an important AOB in 10 mM reactor.

Band analyzed from 30 mM reactor related to only *N. europaea – Nc. mobilis* cluster which was reported for their low affinity to free ammonium (K_s > 30 μM; Koops et al., 2003). This AOB were common for eutrophic environments, including in high ammonium concentration reactors (Limpiyakorn et al., 2007). Previously (Limpiyakorn et al., 2007), this AOB comprised the majority of AOB population in 30 mM reactor. Therefore, it can be implied that this AOB was also predominate in this reactor.

4.5 Degradation of EE2 by nitrifying activated sludge containing different ammonia-oxidizing bacteria communities (Objective2 and Objective3)

This experiment aimed to study effect of AOB communities (Objective2) and ammonia oxidation (Objective3) on degradation of EE2 by NAS containing different AOB communities. Different AOB communities in NAS (2, 10, 30 mM reactor) were tested to degrade 10mg/l of EE2 under different initial ammonium concentrations of 2, 10, 30mM in three parallel batch tests comprising of degradation test, inhibition test and control test which were performed in triplicate. Inhibition test were prepared in the same manner as the degradation test except that allythiourea was added to inhibit ammonia oxidation by AOB. Figure 4.7-4.9 suggested that during the test, ammonium concentrations in degradation tests decreased, nitrite concentrations temporarily increased and then decreased, nitrate concentrations increased, while the total nitrogen concentrations were nearly stable. In contrast, no change in ammonium concentrations was observed in the inhibition tests. This indicated that allythiourea completely inhibited ammonia oxidation of AOB. EE2 concentrations in the degradation tests also decreased whereas EE2 concentrations in the inhibition tests were not changed. This suggested that EE2 were degraded by AOB in NAS.

In order to observe the activity of ammonia oxidation during the test, ammonia oxidation rate were calculated for each test base on the steady state conditions of nitrogen (Table 4.3). The degradation of ammonium obeyed first-order reaction kinetic. The results suggested that AOB in 2 mM reactor had lowest activity (0.0299, 0.0287, and 0.0131 1/hr) whereas 10mM and 30 mM reactor had similar activities (0.0673, 0.0675, and 0.0417 1/hr for 10mM reactor, and 0.0595, 0.0510, and 0.0204 1/hr for 30 mM reactor).

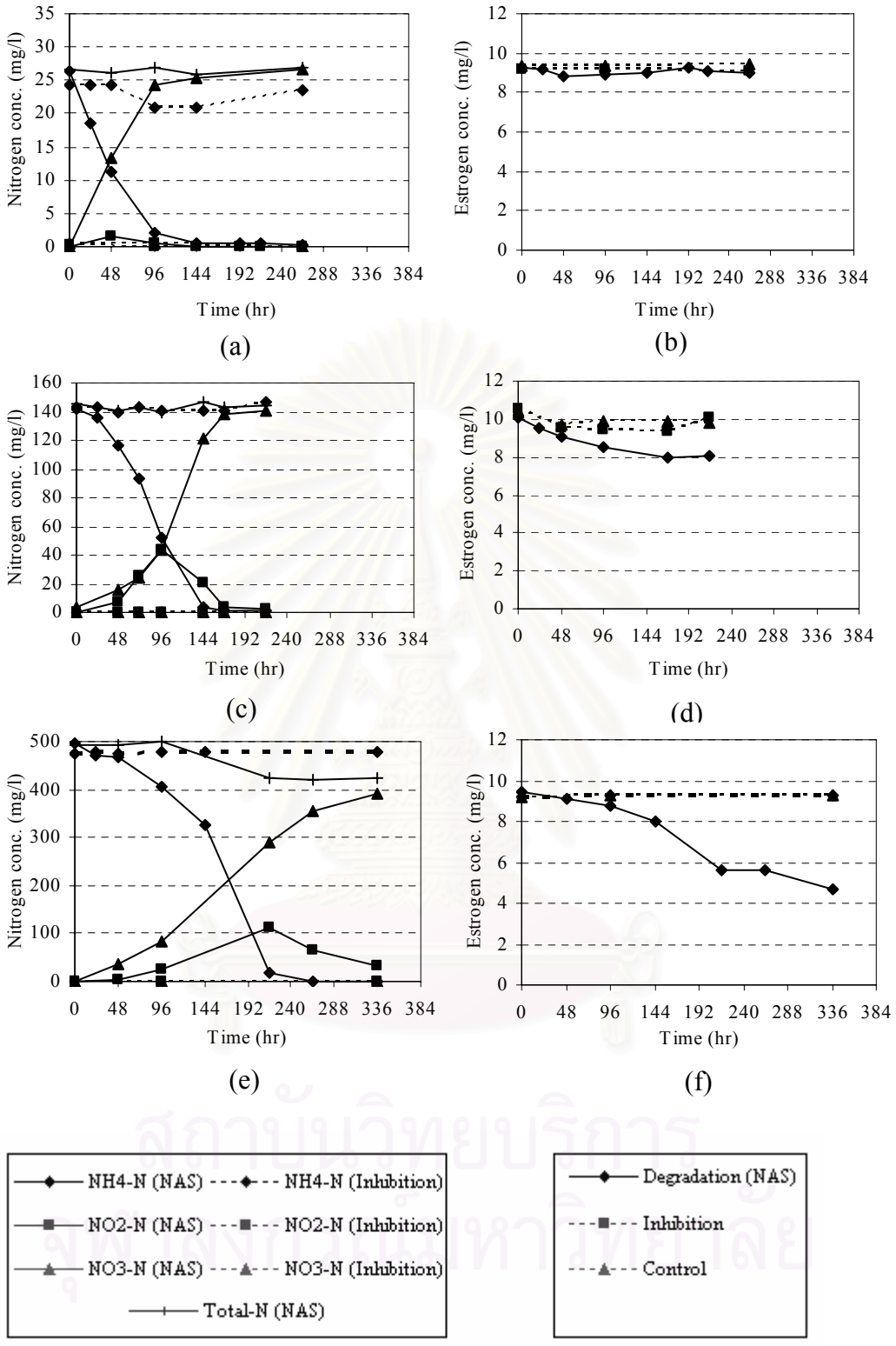


Figure 4.7 Degradation of EE2 by nitrifying activated sludge from 2 mM reactor with initial ammonium concentrations of 2 mM (a and b), 10 mM (c and d), and 30 mM (e and f)

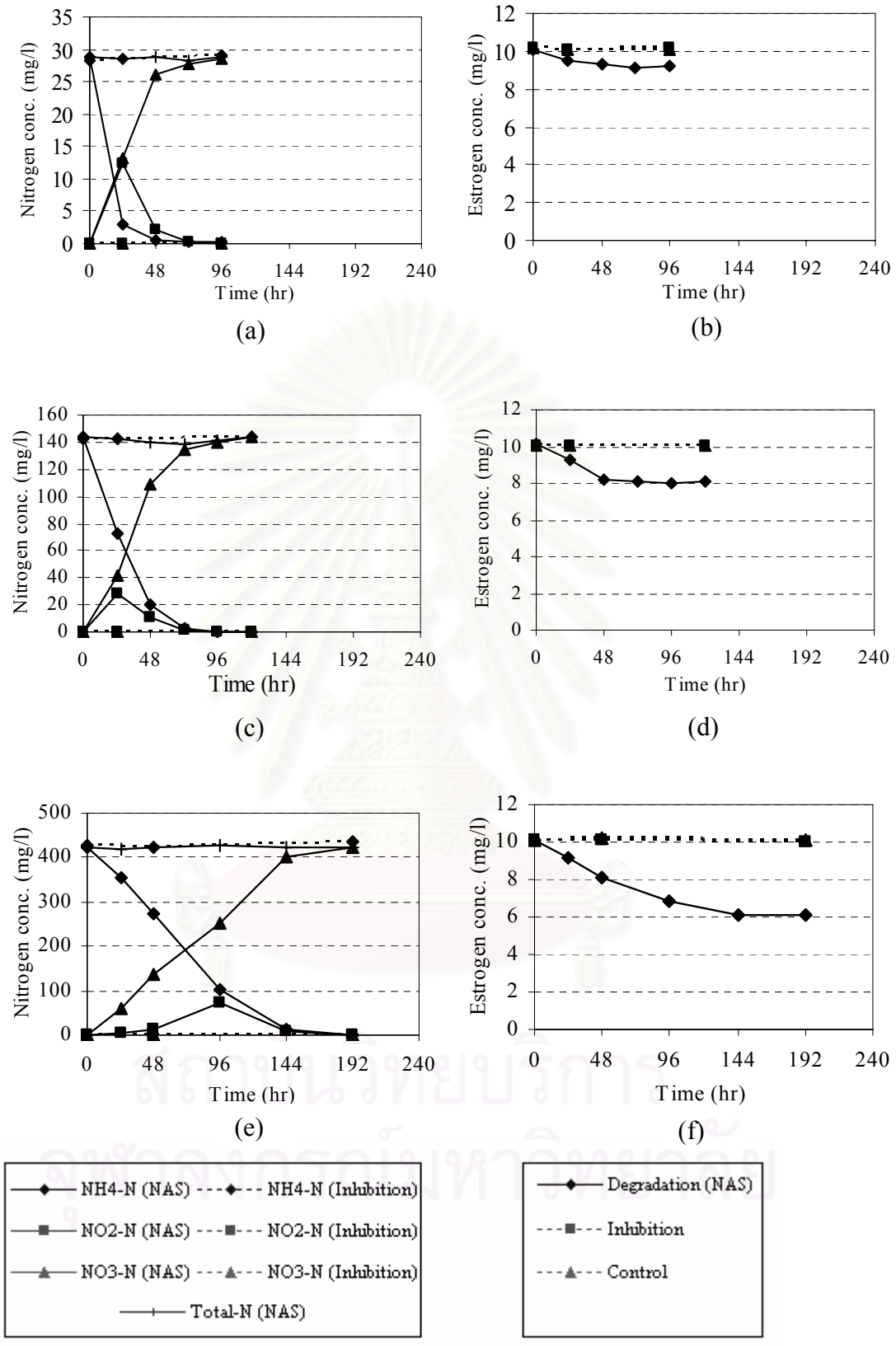


Figure 4.8 Degradation of EE2 by nitrifying activated sludge from 10 mM reactor with initial ammonium concentrations of 2 mM (a and b), 10 mM (c and d), and 30 mM (e and f)

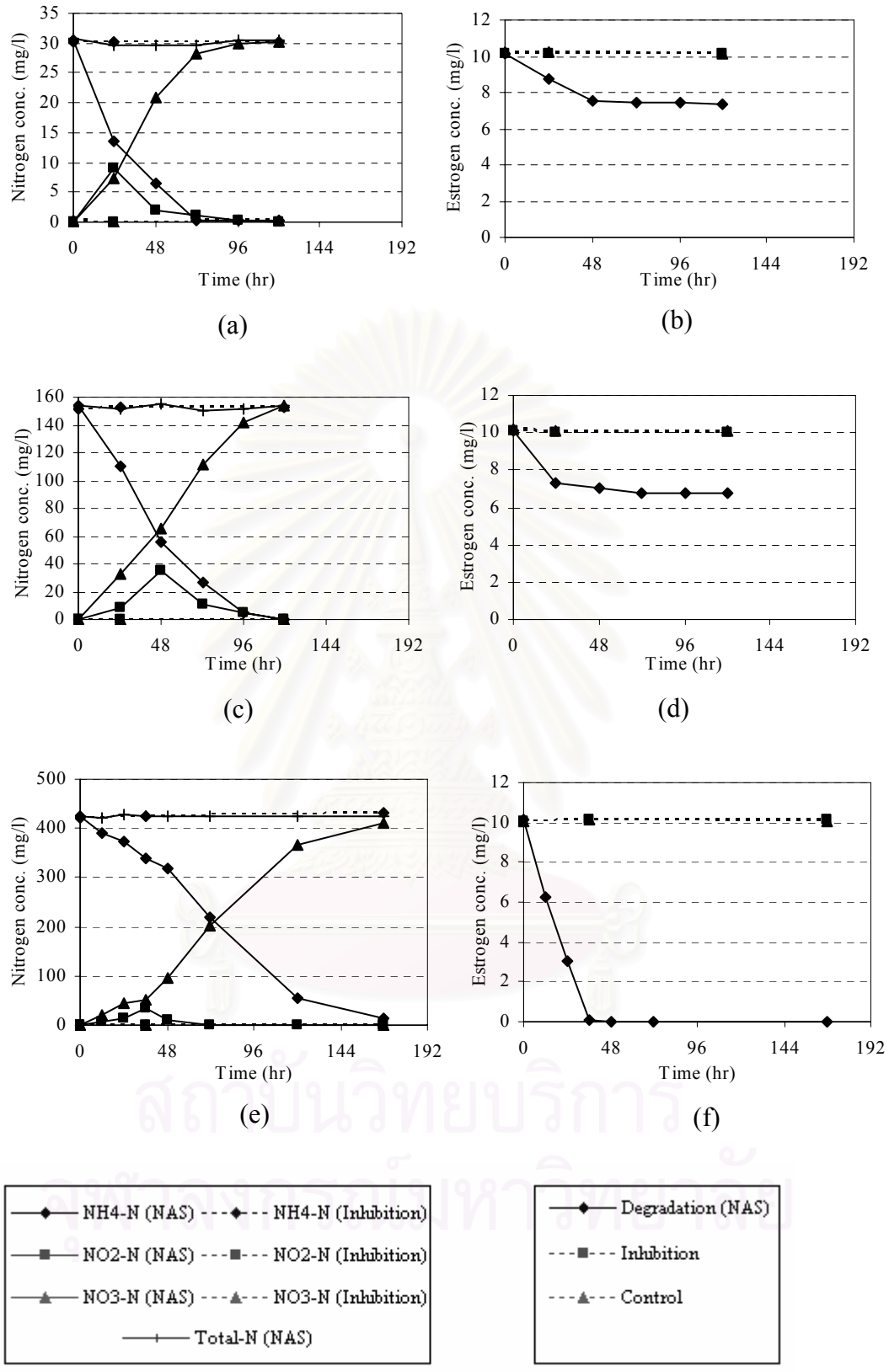


Figure 4.9 Degradation of EE2 by nitrifying activated sludge from 30 mM reactor with initial ammonium concentrations of 2 mM (a and b), 10 mM (c and d), and 30 mM (e and f)

Table 4.3 Degradation rate of 10mg/l EE2 until ammonia disappearance

Reactor	Initial NH4 conc. (mM)	ammonia (mg)			time	ammonia rate	
		initial	end	loss		(gNH4/hr)	K (1/hr)
2	2	26.40	0.40	26.00	144	180.56	0.0299
	10	142.05	1.45	140.60	168	836.90	0.0287
	30	491.50	0.02	491.48	264	1861.67	0.0131
10	2	28.73	0.23	28.50	72	395.83	0.0673
	10	146.50	0.23	146.27	96	1523.65	0.0675
	30	422.50	0.08	422.42	192	2200.10	0.0417
30	2	30.60	0.34	30.26	72	420.28	0.0595
	10	154.20	0.23	153.97	120	1283.08	0.0510
	30	424.50	14.65	409.85	168	2439.58	0.0204

4.5.1 Effect of ammonia-oxidizing bacterial communities on degradation of EE2 by nitrifying activated sludge containing different ammonia-oxidizing bacterial communities (Objective2)

EE2 can be degraded by all NAS under all different ammonium concentrations via co-metabolism (Table 4.4). However, the level of EE2 degradation varied depending on several factors which will be discussed further. Previously, municipal wastewater treatment systems were found to remove EE2. However, in batch experiment, Ternes et al. (1999a) found that 1 μg/l of EE2 appeared to be mainly stable in contact with activated sludge. Recently, Vader et al. (2000) and Shi et al. (2004) found that EE2 was degraded by NAS enriched with high ammonium concentration expecting *N.europaea* cluster as a predominant AOB species. So far, the ability of other AOB cluster has never been observed. The question still appears about how EE2 degrade in municipal WWTS and whether AOB in municipal WWTS can degrade EE2. The major finding of this study over other studies is that AOB community found in municipal WWTS can degrade EE2. This study will lead to the new means of treatment technology in removing persistence organic compounds in municipal WWTS using AOB.

In this part, we consider effect of ammonia-oxidizing bacterial community on EE2 degradation at each initial ammonium concentration (Table 4.4). By comparing among different NAS, the amounts of EE2 degraded (loss) for NAS from 2mM reactor were < those from 10mM reactor < those from 30 mM reactor at every initial ammonium containing (initial ammonium concentration of 2 mM: 0.47, 0.84, and 2.57 mg; initial ammonium concentration of 10 mM: 2.04, 1.91, and 2.81 mg; initial ammonium concentration of 30 mM: 3.83, 4.03, and 10.10 mg, for NAS from 2, 10, and 30 mM reactors, respectively).

In addition, the decrease in EE2 concentrations obeyed first-order reaction kinetic (Table 4.4). The degradation rates and the degradation rate constants for NAS from 2 mM reactor were < that from 10 mM reactor < that from 30 mM reactor (initial concentration of 2 mM: the degradation rate and the degradation rate constants were 0.0098 mg/hr and 0.0011 1/hr for NAS from 2 mM reactor, 0.0175 mg/hr and 0.0018 1/hr for NAS from 10 mM reactor, and 0.0535 mg/hr and 0.0061 1/hr for NAS from 30 mM reactor; initial concentration of 10 mM: the degradation rate and the degradation rate constants were 0.0142 mg/hr and 0.0016 1/hr for NAS from 2 mM

reactor, 0.0398 mg/hr and 0.0044 1/hr for NAS from 10 mM reactor, and 0.1171 mg/hr and 0.0136 1/hr for NAS from 30 mM reactor; initial concentration of 30 mM: the degradation rate and the degradation rate constants were 0.0177 mg/hr and 0.0023 1/hr for NAS from 2 mM reactor, 0.0280 mg/hr and 0.0035 1/hr for NAS from 10 mM reactor, and 0.2806 mg/hr and 0.1324 1/hr for NAS from 30 mM reactor).

Although, the amounts of EE2 degraded, the rates of EE2 degradation, and the rate constants of EE2 degradation for NAS from 2 mM reactor were < that from 10 mM reactor < that in 30 mM reactor (Table 4.4). The amounts of degraded EE2 per the amounts of ammonia oxidized for NAS from 2 mM reactor were as same as those of 10 mM reactor, and lower than those of 30 mM reactor**.**

The amounts of EE2 degraded may depend on the amount of enzyme produced. Our results showed the similar EE2 degradation patterns ($mgEE2/gNH_4^+$) for NAS from 2 mM and 10 mM reactors which were different from these of 30 mM. This result suggested the difference in enzyme expression between NAS from 2 mM and 10 mM reactors and that from 30 mM reactor. So far, there was no report on the difference of enzyme expression among distinct AOB species. This needs further study to clarify since this aspect is very important for taking advantage of AOB co-metabolism in degrading persistence organic compound in environments.

4.5.2 Effect of ammonia oxidation on degradation of EE2 by nitrifying activated sludge containing different ammonia-oxidizing bacterial communities (Objective 3)

In this part, we consider effect of ammonia oxidation of degradation of EE2 by each NAS (Table 4.4). By comparing among different initial ammonium concentrations, the amounts of degraded EE2 (loss) under an initial ammonium concentration of 2 mM were < those of 10 mM < those of 30 mM. (2 mM reactor: 0.47, 2.04, and 3.83 mg; 10 mM reactor: 0.84, 1.91, and 4.03 mg; initial 30 mM reactor: 2.57, 2.81, and 10.10 mg, for initial ammonium concentrations of 2, 10, and 30 mM, respectively).

In addition, the decrease in EE2 concentrations obeyed first-order reaction kinetic (Table 4.4). The degradation rates and the degradation rate constants with an initial ammonium concentrations of 2 mM were < those of 10 mM < those of 30 mM for each NAS. (2 mM reactor: the degradation rate and the degradation rate constant

were 0.0098 mg/hr and 0.0011 1/hr for an initial ammonium concentration of 2 mM, 0.0142 mg/hr and 0.0016 1/hr for an initial ammonium concentration of 10 mM, and 0.0177 mg/hr and 0.0023 1/hr for an initial ammonium concentration of 30 mM; 10 mM reactor: the degradation rate and the degradation rate constant were 0.0175 mg/hr and 0.0018 1/hr for an initial ammonium concentration of 2 mM, 0.0398 mg/hr and 0.0044 1/hr for an initial ammonium concentration of 10 mM, and 0.0280 mg/hr and 0.0035 1/hr for an initial ammonium concentration of 30 mM; 30 mM reactor: the degradation rate and the degradation rate constant were 0.0535 mg/hr and 0.0061 1/hr for an initial ammonium concentration of 2 mM, 0.1171 mg/hr and 0.0136 1/hr for an initial ammonium concentration of 10 mM, and 0.2806 mg/hr and 0.1324 1/hr for an initial ammonium concentration of 30 mM).

In general, initial substrate concentrations affect the induction of enzyme in metabolism and co-metabolism (Michael and Oliver, 1998). In this study, higher initial ammonium concentration may induce more genes to produce more enzyme causing increasing in EE2 degradation.

However, the ratio of the amounts of EE2 degraded per the amounts of ammonia oxidized decrease with increasing initial ammonium concentrations for NAS from 2 and 10 mM reactors (except for those of in 30 mM reactor). The question occurs about why when increasing in initial ammonium concentration EE2 degradation increased (more enzymes produced), the ration of EE2:NH_4^+ was in contrast. One possible explanation is that the level of enzyme induction and enzyme expression may not be related proportionally. For example, in case of NAS from 2 mM reactor, although the amounts of enzyme produced were less when exposed to initial ammonium of 2 mM than that of 30 mM. One unit of enzyme in the first case may degrade more ammonium than in the second case.

However, this phenomenon did not occur for the case of NAS from 30 mM reactor. This may be caused by the fact that the enzyme of this AOB expressed differently. The results from the analysis of AOB communities suggested that the predominant AOB in the NAS from 2 and 10 mM reactors were previously found in the systems with lower ammonium load whereas, that of 30 mM were found in eutrophic environments. AOB in NAS from 2 and 10 mM reactors may express enzyme differently from those from 30 mM reactor. So far, there was no report on the difference of enzyme expression among distinct AOB species. This needs further study to clarify.

Table 4.4 Degradation of EE2 by nitrifying activated sludge from 2, 10, and 30 mM reactor with initial ammonium concentrations of 2, 10, and 30 mM

Reactor	Initial NH_4 conc. (mM)	estrogen (mg)			ammonia (g)			time	EE2 rate		Ammonia rate		EE2:ammonia	
		initial	end	loss	initial	end	loss		mg/hr	K (1/hr)	mg/hr	K (1/hr)	mgEE2/gNH4	mgEE2/gNH4/hr
2	2	9.25	8.78	0.47	26.40	11.25	15.15	48	0.0098	0.0011	0.32	0.0178	31.02	0.646
	10	10.04	8.00	2.04	142.05	3.72	138.33	144	0.0142	0.0016	0.96	0.0239	14.75	0.102
	30	9.44	5.61	3.83	495.00	19.65	475.35	216	0.0177	0.0023	2.20	0.0131	8.06	0.037
10	2	10.11	9.27	0.84	28.73	0.54	28.19	48	0.0175	0.0018	0.59	0.0828	29.80	0.621
	10	10.12	8.21	1.91	143.50	20.76	122.74	48	0.0398	0.0044	2.56	0.0403	15.56	0.324
	30	10.15	6.12	4.03	422.50	11.15	411.35	144	0.0280	0.0035	2.86	0.0248	9.80	0.068
30	2	10.10	7.53	2.57	30.60	6.50	24.10	48	0.0535	0.0061	0.50	0.0323	106.64	2.222
	10	10.12	7.31	2.81	154.20	110.82	43.38	24	0.1171	0.0136	1.81	0.0318	64.78	2.699
	30	10.17	0.07	10.10	424.50	339.00	85.50	36	0.2806	0.1324	2.38	0.0061	118.13	3.281

4.6 Degradation of EE2 by nitrifying activated sludge from 2mM reactor with lower initial EE2 concentration (2mg/l)

This experiment aimed to observe the ability of AOB to degrade EE2 at low concentration. NAS from 2 mM reactor were selected as a model for the test as the community of AOB in this NAS was similar to those in full-scale municipal wastewater treatment systems. Two initial ammonium concentrations of 2 mM and 30 mM were selected for the test. 2 mM was selected to represent the actual ammonium concentration found in municipal wastewater treatment systems and 30 mM was selected to show the clear effect. 2 mg/l of EE2 was selected as the solubility of EE2 is around this value and also to ensure the detectability of EE2 during the degradation.

By comparing among different initial EE2 concentration (Table 4.5), the amount EE2 degraded for 2 mg/l was < 10 mg/l for both initial ammonium concentrations (in initial ammonium concentration of 2 mM: 0.23 mg and 0.47 mg; in initial ammonium concentration of 30 mM: 1.54 mg and 3.83 mg, for initial EE2 concentrations of 2 mg/l and 10 mg/l, respectively).

In addition, the decrease in EE2 concentrations obeyed first-order reaction kinetic, the degradation rates and the degradation rate constants for initial EE2 concentration of 2 mg/l was <10 mg/l (in initial ammonium concentration of 2mM: 0.0024 mg/hr and 0.0013 1/hr for initial EE2 concentration of 2 mg/l, and 0.0098 mg/hr and 0.0011 1/hr for initial EE2 concentration of 10 mg/l; in initial ammonium concentration of 30 mM: 0.0071 mg/hr and 0.0071 1/hr for initial EE2 concentration of 2 mg/l, and 0.0177 mg/hr and 0.0023 1/hr for initial EE2 concentration of 10 mg/l), while those of ammonia oxidation did not differ significantly. These results suggested that initial EE2 concentration affect the degradation of EE2.

Table 4.5 Degradation of EE2 by nitrifying activated sludge from 2mM reactor with different EE2 concentrations

Reactor	Initial NH4 conc. (mM)	initial EE2 conc. (mM)	estrogen (mg)			ammonia (mg)			time	EE2 rate		Ammonia rate		EE2:ammonia	
			initial	end	loss	initial	end	loss		mg/hr	K (1/hr)	mg/hr	K (1/hr)	mgEE2/gNH4	mgEE2/gNH4/hr
2	2	2	2.00	1.77	0.23	30.40	0.48	29.92	96	0.0024	0.0013	0.31	0.0427	7.69	0.080
		10	9.25	8.78	0.47	26.40	11.25	15.15	48	0.0098	0.0011	0.32	0.0178	31.02	0.646
	30	2	2.03	0.49	1.54	426.12	1.17	424.95	216	0.0071	0.0071	1.97	0.0276	3.62	0.017
		10	9.44	5.61	3.83	495	19.65	475.35	216	0.0177	0.0023	2.2	0.0131	8.06	0.037

4.7 Competition effects of other organic compounds on degradation of EE2 by nitrifying activated sludge from 2 mM reactor (Objective 4)

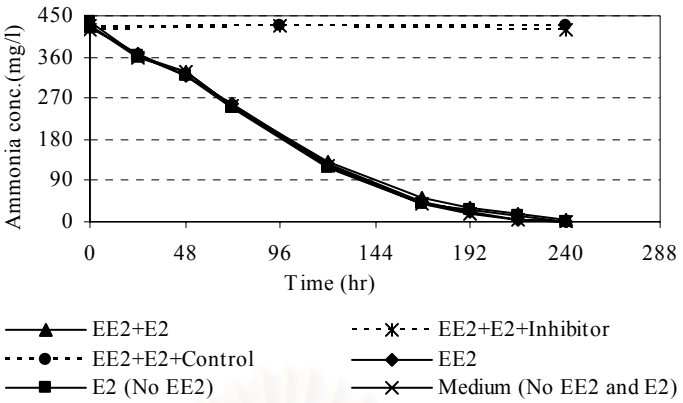
AOB in NAS is capable of co-metabolism several organic compounds. So far, no research mentions on the competitions effect of other organic compounds on co-metabolism of AOB. This experiment therefore aimed to observe the competition effect of other organic compounds on the degradation of EE2 by NAS. We used NAS from 2 mM reactor as the model NAS because the AOB communities in this NAS represent that of full-scale municipal wastewater treatment systems. The initial ammonium concentration of 30 mM was selected for the test as this concentration will show the clear effect. E2 and canteen wastewater were selected as model organic compounds (separated study). 10 mg/l of E2 was selected to represent the organic compounds that have similar structure to EE2 and wastewater from canteen was selected to observe the actual phenomena in municipal wastewater treatment systems. Six parallel batch tests comprising of four degradation tests, degradation test with additional organic compound (E2 or wastewater), inhibition test, and control test were performed in triplicate.

4.7.1 Competitions effect of E2 on degradation of EE2 by nitrifying activated sludge from 2mM reactor

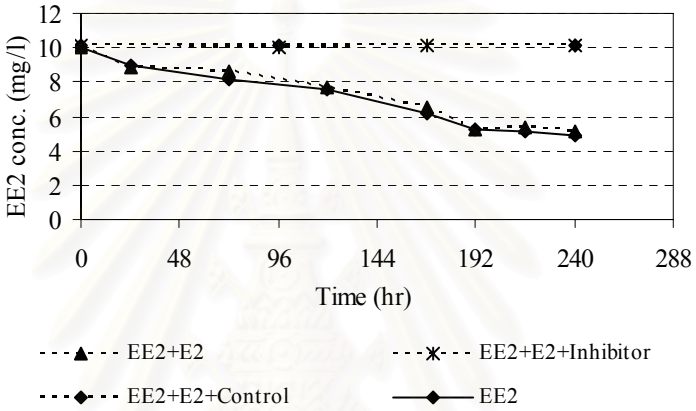
Ammonium concentrations in EE2+E2, EE2, E2, and medium (no EE2 and E2) decreased during the test (Figure 4.10a). No remarkable difference was found among these four batch tests. These results suggested that EE2 and E2 did not inhibit ammonium oxidation. In contrast, ammonium concentration in the inhibition tests showed no change, indicating that allythiourea completely inhibited ammonia oxidation of AOB.

Concentrations of EE2 in EE2+E2 and EE2 (Figure 4.10b) decreased whereas EE2 concentrations in the inhibition tests remained the same throughout the experiment. These results suggested that EE2 were degraded by AOB in NAS. In addition, the decrement of EE2 concentration in EE2 was > EE2+E2 indicating that E2 which represent organic compounds that have similar structure to EE2, competed the degradation of EE2 during co-metabolism of AOB.

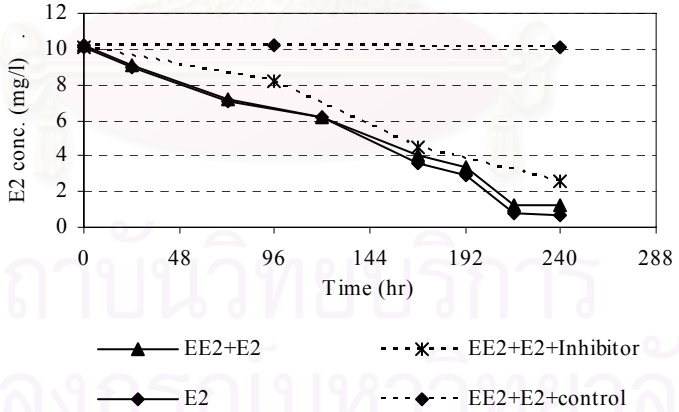
Concentrations of E2 in EE2+E2, E2, and even in EE2+E2+Inhibitor decreased (EE2+E2+inhibit < EE2+E2 < E2) (Figure 4.10c). These results suggested that most of E2 were decreased by heterotrophic bacteria in NAS. Some parts of E2 in EE2+E2 and E2 were decreased by co-metabolism of AOB. When compare between E2 concentration in EE2+E2 and E2, E2, increased more in the absence of EE2, suggest the competition effect of these two estrogens on co-metabolism.



(a)



(b)



(c)

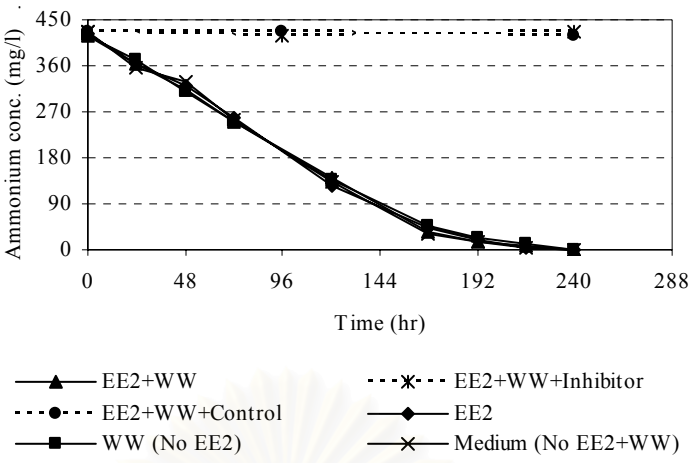
Fiugure 4.10 Competition effect of E2 (10 mg/l) on degradation of EE2 (10 mg/l) by nitrifying activated sludge from 2 mM reactor (a) ammonium concentrations (b) EE2 concentrations (c) E2 concentrations

4.7.2 Competitions effect of canteen wastewater on degradation of EE2 by nitrifying activated sludge from 2 mM reactor

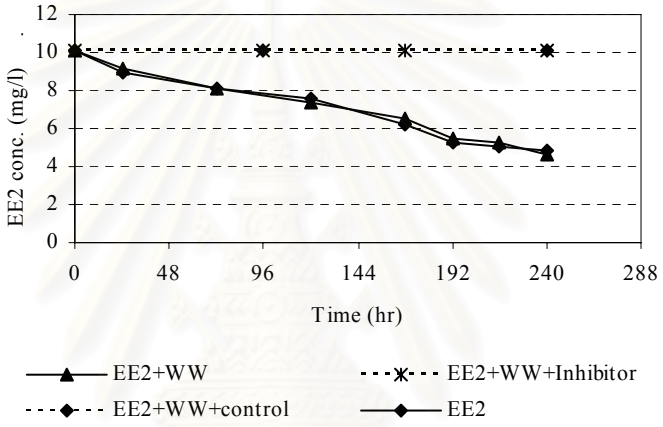
Ammonium concentrations in EE2+wastewater (WW), EE2, WW, and medium (no EE2 and E2) decreased during the test. No remarkable difference was found among these four batch tests. These results suggested that EE2 and organics in wastewater did not inhibit ammonium oxidation. In contrast, ammonium concentration in the inhibition test showed no change, which indicated that allythiourea completely inhibited ammonia oxidation of AOB.

Concentrations of EE2 in EE2+WW and EE2 decrease whereas EE2 concentrations in the inhibition tests remained the same throughout the experiment. These results suggested that EE2 were degraded by AOB in NAS. No remarkable difference was observed between EE2+WW and EE2 indicating that organic compounds in wastewater did not compete the degradation of EE2 by NAS.

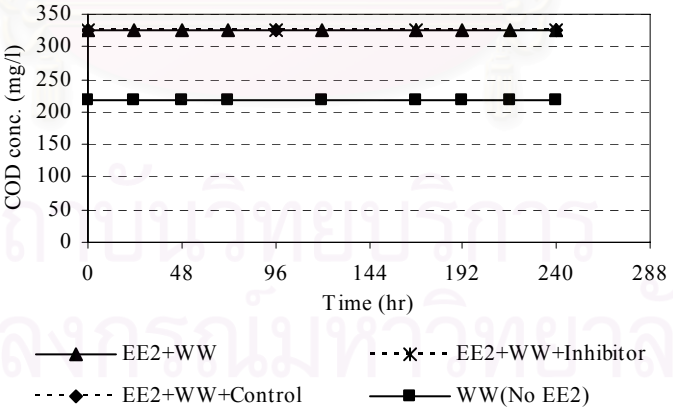
Concentrations of COD in EE2+WW, EE2+WW+Inhibitor, and EE2+WW+ control were more than that in wastewater alone suggesting that added EE2 can be account for 109 mg/l of COD. However, the concentrations of COD in all tests were stable through out the experiment. This suggested that heterotrophic bacteria in wastewater and NAS did not degrade organic compounds in wastewater and also organic compound in wastewater did not degrade via co-metabolism of AOB.



(a)



(b)



(c)

Fiugure 4.11 Competition effect of canteen wastewater on degradation of EE2 (10 mg/l) by nitrifying activated sludge from 2 mM reactor (a) ammonium concentrations (b) EE2 concentrations (c) COD concentrations

CHAPTER V

CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORKS

5.1 Conclusions

This study investigated the degradation of EE2 by NAS containing different AOB communities and factors affecting EE2 degradation by NAS. The findings of this study fulfill all the objectives. Significant details of the findings can be summarized as follow.

1. E1 and E2 were easily degraded by activated sludge, whereas EE2 was persist in contact with activated sludge. During the degradation of E2, formation of E1 occurred and then was eliminated. However, EE2 can be degraded by AOB in NAS via co-metabolism EE2 did not significantly affect ammonia oxidation of AOB.

2. Community of AOB in each NAS differed depending on the levels of ammonium for enrichment. Predominant AOB in seed sludge were *N. communis* cluster and *N. oligotropha* cluster whereas that of NAS from 2mM reactor related to *N. communis* cluster, that of NAS from 10mM reactor were unknown *Nitrosomonas* cluster which was closely to related strain *Nitrosomonas* sp. Is343 found in WWTP (municipal, oil industry, and brewery). Whereas that of 30mM reactor related to *N. europaea- Nc. mobilis* cluster.

3. EE2 can be degraded by all NAS under all different initial ammonium concentrations via co-metabolism. These results also indicated that AOB in municipal wastewater treatment systems can degrade EE2 which may be main reason for the reduction of EE2 in full-scale municipal WWTS.

4. AOB communities in NAS affect the degradation of EE2. The degradation patterns varied among NAS. This result suggested that enzyme induction, enzyme expression, and enzyme activity may differ among AOB communities, and thus among distinct AOB species.

5. Initial ammonium concentrations also affected the degradation of EE2. The results showed that the higher the initial ammonium concentration, more EE2 can be degraded. However, the amount of ammonia oxidized was not proportional to the amount of EE2 degraded. One possible explanation is that the level of substrate induction and enzyme expression may not be related proportionally.

6. Initial concentrations of EE2 (2 and 10 mg/l) also affected the degradation of EE2. The amount degraded EE2 for 2 mg/l was < 10 mg/l.

7. Study on the competition effects of other organic compounds on EE2 degradation showed that E2, that have similar structure to EE2, competed the degradation of EE2, whereas organic compounds in canteen wastewater did not.

8. This was the first study investigating the degradation of persistent organic compounds by NAS containing different AOB communities. The major finding is that AOB community found in municipal WWTS can degraded EE2. This will lead to the new means of treatment technology in removing EE2 and also other persistent organic compounds in wastewater using AOB.

5.2 Suggestions for future works

As this is one of the pioneer studies on this area. Several findings should be studied in more detail as listed below:

1. Mechanism of different AOB species in substrate induction, enzyme expression, and enzyme activity.

2. Mechanism of co-metabolism in distinct AOB species.

3. Degradation of other persistent organic compounds (pharmaceutical compounds and endocrine disruption compounds) via co-metabolism of AOB.

4. Detailed competition effects of other organic compounds on EE2 degradation.

REFFERENCES

American Public Health Association. Standard methods for the examination of water and wastewater. 18th edition, Maryland, USA, 1992

Arciero, D. T., Vannelli, M. L., and Hooper, A. B. Degradation of trichloroethylene by the ammonia-oxidizing bacterium Nitrosomonas europaea. Archives of Biochemistry and Biophysics 159 (1989): 640-643.

Ballmann, A., and Laanbroek, E. L. Continuous culture enrichments of ammonia-oxidizing bacteria at low ammonium concentrations. FEMS Microbiology Ecology 37 (2001): 211-221.

Baronti, C., Curini, R., D'A scenzo, G., Corcia, A., Gentili, A., and Samperi, R. Monitoring natural and synthetic estrogens at activated treatment plants and in receiving river water. Environmental Science and Technology 34 (2000): 5059-5066.

Belfroid, A. C., Van, D. H., Vethaak, A. D., Schafer, A. J., Rijs. G. B. J., Wegener, J., and Cofino, W. P. Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in the Netherlands. Sciene of the Total Environment 225 (1999): 101-108.

Chao, Y., Kurisu, F., Saitoh, S., and Yagi, O. Degradation of Estrdiol by Sphingomonas sp. Strain D12 Isolated from soil. Journal of Environmental Biotecnology 3(2) (2004):89-94.

D'Ascenzo, G., Coria, A., Gentili, A., Mancini, R., Mastropasqua, R., Nazzari, M., and Samperi, R. Fate of natural estrogen conjugates in municipal sewage transport and treatment facilities. The Science of the Total Environment 302 (2003): 199–209.

Desbrow, C., Routledge, E. J., Brighty, G., Sumpter, J. P., and Waldock, M. Identification of estrogenic chemicals in STW effluent-I. Chemical fractionation and in vitro biological screening. Environmental Science and Technology 32 (1998): 1549-1558.

Erb, R. E., Chew, B. P., and Keller, H. F. Relative concentrations of estrogen and progesterone in milk and bold, and excretion of estrogen in urine. Journal of Animal Science 46 (1977): 617-626.

Flemming, l., and Bent, H. Evaluation of analytical chemical methods for detection of estrogens in the environment. Working report 44, 2003.

Fujii, K., Kikuchi, S., Satomi, M., Ushio, S. N., and Morita, N. Degradation of 17 - Estradiol by a Gram-Negative Bacterium Isolated from Activated Sludge in a Sewage Treatment Plant in Tokyo, Japan. Applied and Environment Microbiology 68 (2002): 2057–2060.

Gieseke, A., Purkhold, U., Wagner, M., Amann, R., and Schramm, A. Community structure and activity dynamics of nitrifying bacteria in a phosphate-removing biofilm. Applied and Environment Microbiology 67 (2001): 1351–1362.

Hanselman, T. A., Graetz, D. A., and Wilkie, A. C. Review of Manure-Borne Estrogens as Potential Environmental Contaminants. Soil and Water Science Department 33 (2003): 5471-5478.

Institute of Population Health. Endocrine disruption[online]. University of Ottawa, R. Samuel McLaughlin Centre for Population Health Risk Assessmen. 2007. Available from: http://www.emcom.ca/EM/index.shtml[2007, Jan 09]

Jamal, D., and Michel, J. P. Nitrification and autotrophic nitrifying bacteria in a hydrocarbon polluted soil. Applied and Environment Microbiology 65 (1999): 4008-4013.

Jobling, S., Sheaham, D. A., Osborne, J. A., Matthiessen, P., and Sumpter, J. P. Widespread sexual disruption in wild fish. Environmental Science and Technology 32 (1998): 2498-2506.

Johnson, A. C., Belfroid, A., and Corcia, Di. A. Estimating steroid estrogen inputs into activated sludge treatment works and observations on their removal from the effluent. Sciene of the Total Environment 256 (2000): 163-173.

Kelly, K. Analysis of steroids in environment water samples using solids-phase extraction and ion-trap gas chromatography-mass spectrometry and gas chromatography-tandem mass spectrometry. Journal of Chromatography A 879 (2000): 97-112.

Koops, H. P., Purkhold, U., Pommeren, R. A., Timmermann, G., and Wagner, M. The Lithoautotrophic Ammonia-Oxidizing Bacteria. In M. Dworkin et al (eds.), The Prokaryotes: An Evolving Electronic Resource For the Microbiological Community, pp.1-22. New York, 2003.

Kowalchuck, G. A., Stephen, J. R., Boer, W., Prosser, J. I., Embley, T. M., and Woldendorp, J. W. Analysis of ammonia-oxidizing bacteria of the b subdivision of the class Proteobacteria in costal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Applied and Environment Microbiology 63(4) (1997): 1489–1497.

Kuch, H. M., and Ballschmitter, K. Determination of endocrine-disrupting phenolic compounds and estrogens in surface and drinking water by HRGC-(NCI)-MS in the picogram per liter range. Environmental Science and Technology 35 (2001): 3201-3206.

Kurisu, F., Satoh, H., Mino, T., and Matsuo, T. Microbial community analysis of thermophilic contact oxidation process by using ribosomal RNA approaches and the quinone profile method. Water Research 36 (2002): 429–438.

Lai, K. M., Scrimshaw, K. M., and Lester, J. N. Binding of waterborne steroid estrogens to solid phases in river and estuarine systems. Environmental Science and Technology 34 (2000): 3890-3894.

Limpiyakorn, T., Futoshi, K., Yoriko, S., and Osami, Y. Communities of ammonia-oxidizing bacteria in activated sludge of various sewage treatment plants in Tokyo. FEMS Microbiology Ecology 54 (2005): 205-217.

Limpiyakorn, T., Futoshi, K., Yoriko, S., and Osami, Y. Effects of ammonia and nitrite on communities and populations of ammonia-oxidizing bacteria in laboratory-scale continuous-flow reactors. FEMS Microbiology Ecology (DOI: 10.1111/j.1574-6941.2007.00307.x)

Michael, H. K., and Oliver, J. H. Cometabolic degradation of chlorophenols by acinetobacter species. Water Research 33 (1999): 562-574.

Nasu, M. Study on endocrine disrupting chemicals in wastewater treatment plants. Water Science and Technology 43(2) (2000): 101-108.

Orme, M., Back, D., and Breckenridge, A. Clinical pharmacokinetics of oral contraceptive steroids. Clin Pharmacokinet 8 (1983): 95–136.

Purdom, C. E., Hardiman, P. A., Bye, V. J., Eno, N. C., Tyler, C. R., and Sumpter, J. P. Estrogenic affects of effluents from sewage treatment works. Journal Of Chemical Ecology 8 (1994): 275-285.

Purkhold, U., Wagner, M., Timmermann, G., Pommerening. R., and Koops, H. P. Phylogeny of all Recognized Species of Ammonia oxidiziers Based on Comparative 16S rRNA and amoA Sequence Analysis: Implication for Molecular Diversity Surveys. Applied and Environment Microbiology 66 (2000):5368-5382.

Refsdal, A. O. To treat or not to treat: a proper use of hormones and antibiotics. Animal Reproduction Science 60/61 (2000): 109- 119.

Routledge, E. J., Sheahan, D., Desbrow, C., Brighty, G. C., Waldock, M., and Sumpter, J. P. Identification of estrogenic chemicals in STW effluent-II. In vivo responses in trout and roach. Environmental Science and Technology 32 (1998): 1559-1565.

Sharpe, R. M., and Skakkebaek, N. E. Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? Lancet 341 (1993): 1392-1395.

Shemesh, M., and Shore, L. S. Effect of hormones in the environment on reproduction in cattle. In Fields, M. J.; and Sand, R. S (eds.), Factors affecting calf crop, pp 287-297. Florida: CRC Press, 1994.

Shi, J., Fujisawa, S., Nakai, S., and Hosomi, M. Biodegradation of natural and synthetic estrogens by nitrifying activated sludge and ammonia-oxidizing bacterium Nitrosomonas europaea. Water Research 38 (2004): 2323-2330.

Shimada, K., Mitamura, K., and Higashi, T. Gas chromatography and high performance liquid chromatography of natural steroids. Journal of Chromatography A 935 (2001): 141-172.

Shore, L. S., Correll, D. L., and Chakraborty, P. K. Relationship of fertilization with chick manure and concentrations of estrogens in small streams. In Steele, K (ed.), Animal waste and the land -water interface, pp.155-162. Florida: CRC Press, 1995a.

Spengler, P., Korner, W., and Metzger, J. W. Substances with estrogenic activity in effluents of sewage treatment plants in south-western Germany: 1.Chemical analysis. Environmental Toxicology and Chemistry 20 (2001): 2133-2141.

Strickland, J. D., and Parson, T. R. A practical handbook of seawater analysis. Fishery research board of Canada. Otawa (1972), pp 310.

Synder, SA. Analytical methods for detection of selected estrogenic compounds in aqueous mixtures. Enviromental Science and Technlogy 33 (1999): 2814-2820.

Tabata, A. Estrogenic influence of estradiol-17h, p-nonylphenol and bisphenol A on Japanese Medaka (Oryzias iatipes) at detected environmental concentrations. Water Science and Technology 43(2) (2001): 109-116.

Ternes, T. A., Stumpf, M., Mueller, J., Haberer, K., Willken, R. D., and Servos, M. Behaviour and occurrence of estrogens in municipal sewage treatment plants-I. Investigations in Germany, Canada and Brazil. Science of the Total Environment 225 (1999a): 81- 90.

Vader, J. S., Ginkel, C., Stokman, F. M., Sperling, G. M., Jong, J., Boer, W., Graaf, J., Most, M., and Stokman, P. Degradation of ethinyl estradiol by nitrifying activated sludge. Chemosphere 41 (2000): 1239-1243.

Weber, S., Leuschner, P., Peter, K., Wolfgang, D., and Juliane, H. Degradation of Estradiol and Ethinyl estradiol by activated sludge and by a defined mixed culture. Applied Microbioligy and Biotechnology 67 (2005): 106-112.

William, K. K., and Daniel, J. A. Kinetic studies of ammonia monooxygenase inhibition in Nitrosomonas europaea by hydrocarbons and halogenated hydrocarbons in an optimized whole-cell assay. Applied and Environment Microbiology 59 (1993): 501-510.

Ying, G., Kookana, R. S., and Ru, Y. J. Occurrence and fate of hormone steroids in the environment. Environment International 28 (2002): 545– 551.

Yoshimoto, T., Nagai, F., Fujimoto, J., Watanabe, K., Mizukoshi, H., Makino, T., Kimura, K., Saino, H., and Omura, H. Degradation of Estrogens by Rhodococcus zopfii and Rhodococcus equi Isolates from Activated Sludge in Wastewater Treatment Plants. Applied and Environment Microbiology 70 (2004): 5283–5289.

APPENDICES A

Sequences of DGGE bands

>S-1
GGGATCGAAAGACCTTATGCTTTTGGAGCGGCCGATGTCTGATTAGCTAG
TTGGTAGGGTAATGGCCTACCAAGGCGACGATCAGTAGTTGGTCTGAGAG
GATGgCCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
CAGCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCAGCAATGCC
GCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGTTGAGAGGA
AAAGNTTGTGACTAATAATCACAATTCATGACAGTATCAGACAGAAGAAG
CACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCG
TTAATCGGAATTATTGGGCGTAAAGGGTGCGCAGGCGGTTCTGTAAGTCA
GATGTGAAATCCCCGGGCTTAACCTGGGAATTGCGTTTGAAACT

>S-2
GGGATCGAAAGACCTCGTGCTTTGAGGGTGGCCGATGTCTGATTAGCTAG
TTGGTAGGGTAAAGGCCTACCAAGGCGACGATCAGTAGCTGGTCTGAGAG
GACGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAG
GCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCCATGC
CGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCGGTCGGAAAG
AAATATCTATAAAAAATATTTATAGAGGATGACGGTANCCGACATAAGAA
GCCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAG
CGTTAATCGGAATTATTGGGCGTAAAGGGTGCGCAGGCGGTGTTGTAAGT
CAGATGTGAAATCCCCCGGGCTTAACCTGGGAATTGCGTTTGAAACTAC

>S-3
GGGATCGAAAGACCTCGTGCTTTGAGGGTGGCCGATGTCTGATTAGCTAG
TTGGTAGGGTAAAGGCCTACCAAGGCGACGATCAGTAGCTGGTCTGAGAG
GACGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAG
GCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCCATGC
CGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCGGTCGGAAAG
AAATATCTATAAAAAATATTTATAGAGGATGACGGTACCGACATAAGAAG
CACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCG
TTAATCGGAATTATTGGGCGTAAAGGGTGCGCAGGCGGTGCTGTAAGTCA
GATGTGAAATCCCCGGGCTTAACCTGGGAATTGCGTTTGAAAC

>2mM-1
CCAATCAACTTTGTTACCCCATCGATCATGATTCCGGGTGCATTGATGTTG
GATATCACGTTGTACTTGACCCGTAACTGGATTGGTAACGGCGCTGATCG
GAGGCGGATTCTTTGGTCTGTTATTCTATCCAGGCAACTGGCCAATTTTTG
GACCGACTCACTTGCCTGTCGTTGCAGAAGGCGTATTGCTTTCAATGGCAG
ACTACATGGGACACCTTTATATCCGTACAGGTACACCTGAATACGTACGC
CTGATTGAGCAGGGTTCACTGCGTACCTTTGGCGGCCATACCACGGTGATT
GCTGCGTTCTTTGCGGCGTTTGTATCGATGTTGATGTTTGTTGTTTGGTGGT
TCCTCGGTAAAGTTTACTGTACAGCATTCTTTTACGTTAAAGGTAAAAGAG
GCCGTATT

>2mM-2
CCAATCAACTTTGTTACCCCATCGATCATGATTCCGGGTGCATTGATGTTG
GATATCACGTTGTACTTGACTCGTAACTGGTTGGTAACGGCGCTGATCGGA
GGCGGATTCTTTGGTCTGTTATTCTATCCGGGCAACTGGCCAATTTTTGGA
CCGACGCACTTGCCTGTCGTTGCAGAAGGCGTATTGCTTTCAATGGCAGAC
TACATGGGTCACCTCTACATCCGTACAGGTACACCTGAGTATGTGCGTTTG
ATTGAACAAGGATCGTTGCGTACCTTTGGTGGCCATACCACGGTGATTGCT
GCGTTCTTTGCGGCGTTTGTATCGATGTTGATGTTTGTTGTTTGGTGGTTCC
TCGGTAAAGTTTACTGTACCGCATTCTTTTACGTTAAAGGTAAAAGAGGCC
GTATT

>2mM-3
CCCATCAACTTCGTGACACCGGGCATTATGCTTCCGGGTGCGCTGATGCTG
GACTTCACGCTGTATCTGACGCGCAACTGGCTGGTAACAGCTCTGGTTGG
AGGTGGATTCTTCGGTCTGCTGTTCTACCCAGGTAACTGGCCGATCTTTGG
TCCAACGCATCTGCCAATCGTTGTAGAAGGAACACTGTTGTCGATGGCTG
ACTACATGGGCCATATGTATGTTCGTACGGGTACACCCGAGTATGTTCGTC
ATATTGAGCAAGGTTCACTGCGTACCTTTGGTGGTCATACtACCGTTATTGC
AGCATTCTTCTCTGCGTTCGTATCAATGTTGATGTTCACTGTATGGTGGTAT
CTCGGAAAAGTTTACTGTACAGCCTTTTTCTACGTTAAAGGTAAAAGAGGT
CGTAT

>2mM-4
CCAATCAACTTTGTAACCCCATCGATCATGATTCCGGGTGCATTGATGTTG
GATATCACGCTGTACTTGACCCGTAGCTGGTTGGTAACGGCACTGATTGGC
GGTGGCTTCTTTGGTCTGTTATTCTATCCAGGCAACTGGCCAATTTTTGGA
CCGACTCACTTGCCTGTCGTTGCAGAAGGCGTATTGCTTTCAATGGCAGAC
TACATGGGGCACCTTTATATCCGTACAGGTACACCTGAGTATGTGCGTTTG
ATTGAACAAGGATCGTTGCGTACCTTTGGTGGTCATACCACGGTGATTGCT
GCGTTCTTCTCAGCGTTTGTATCGATGCTGATGTTTGTTGTTTGGTGGTACC
TCGGTAAAGTCTATTGCACAGCCTTCTTCTACGTTAAAGGTAAAAGAGGC
CGTATT

>10mM-1
GGGATCGAAAGACCTTGCGGCTAAAGGAGCGGCTGATGTCTGATTAGCTA
GTTGGTGGGGTAAgGGCTTACCAAGGCAACGATCAGTAGCTGGTCTGAGA
GGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA
GGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCCATG
CCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTAGTCGGAAA
GAAAGAGTCATAGTAAATAGCTATGATTTATGACGGTACCGACAGAAAAA
GCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGC
GTTAATCGGAATTACTGGGCGTAAAGGGTGCGCAGGCGGCCTTGTAAGTC
AGATGTGAAAGCCCCTGGGCTTAACCTGGGAATTGCGTTTGAAAC

>10mM-2
GGGATCGNAAGACCTTGCGTTTTTGGGAGCGGGCCGATGTCTGATTAGCT
AGTTGGTGGGGTAAGGGCCTACCAAGGNCgACGATCAGTAGTTGGTCTGA
GAGGACGACCAgCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCaAT
GCCGCGTGAGtGAAGAAGGCCTTCGGGTTGTAAAGCCTCTTTCACTCGAgA
AGAAAAGGTGCAgTGAATAACTGTAgTTTATGACGGTATCGACAGAANAA
GCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGC
GTTAATCGGAATTACTGGGCGTAAAGGGTGCGCAGGCGGTTTTGTAAGTC
AGATGTGAAATCCCCCGGGCTTAACCTGGGAATTGCGTTTGAAA

>10mM-3
CGATGTCTGATTAGCTAGTTGGTGGGGTAAGGGGCCTACCAAGGCGACGA
TCAGTAGTTGGTCTGAGAGGACGACCAGCCACACTGGGACTGAGACACGG
CCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGCA
AGCCTGATCCAGCAATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAA
GCTCTTTCAGTCGAGAAGAAAAGGCTGCAGTGAATAACTGTAGTTTATGA
CGGTATCGACAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGGGTGCG
CAGGCGGTTTTGTAAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAAT
TGCGTTTGAAA

>30mM-1
GGGTATCGCAAGACCTTGCGCTAAAGGAGCGGGCCGATGTCTGATTAGCT
AGTTGGTGGGGTAAAGGGCTTACCAAGGCAACGATCAGTAGTTGGTCTGA
GAGGACGGCCAACCACACTGGGACTGAGACACGGCCCAGACTCCTACGG
GAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCC
ATGCCGCGTGAATGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTAGTCGG
AAAGAAAGAGTTGCAATGAATAATTGTGATTTATGACGGTACCGACAGAA
AAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCG
AGCGTTAATCGGAATTACTGGGCGTAAAGGGTGCGCAGGCGGTCTTGCAA
GTCAGATGTGAAAGCCCCGGGCTTAACCTGGGAATTGCGTTTGAAA

APPENDICES B

Table B-1 Concentration of estrogens during degradation of E1, E2 and EE2 by activated sludge

Hr	Estrogen concentrations (mg/l)					
	E1	E1-control	E2	E2-control	EE2	EE2-control
0	10.30	10.09	10.25	10.09	10.00	10.17
24	8.43	10.17	8.28	10.19	10.02	10.07
36	7.69	10.13	7.13	10.24	10.07	10.15
48	6.40	10.14	4.07	10.23	10.11	10.07
60	4.73	10.16	2.05	10.30	10.07	10.01
72	1.69	10.14	1.23	10.23	10.09	10.13
96	0.39	10.13	0.35	10.24	10.13	10.06

Table B-2 Formation of E1 during degradation of E2 by activated sludge

Hr	Estrogen concentrations (mg/l)		
	E2	E2-control	E1
0	10.25	10.09	0.00
24	8.28	10.14	2.11
36	7.13	10.49	2.88
48	4.07	10.48	3.84
60	2.05	10.50	0.13
72	1.23	10.43	0.00
96	0.35	10.24	0.00

78

APPENDICES C

Table C-1 Degradation of EE2 by nitrifying activated sludge from 2 mM reactor with initial ammonium concentrations of 2 mM

Hr	Ammonia concentrations (mg/l)			Nitrite concentrations (mg/l)			Nitrate concentrations (mg/l)			Estrogen concentrations (mg/l)		
	Degradation	Inhibition	Control	Degradation	Inhibition	Control	Degradation	Inhibition	Control	Degradation	Inhibition	Control
0	26.40	24.20	25.20	0.11	0.14	0.10	0.02	0.40	7.00	9.25	9.17	9.38
24	18.55	24.20	27.00									
48	11.25	24.20	27.00	1.60			13.26			8.78		
96	2.21	21.00	27.80	0.40	0.08	0.12	24.24	0.64	7.44	8.91	9.16	9.35
144	0.40	21.00	27.80	0.12			25.24			9.01		
192	0.48			0.12						9.22		
216	0.55			0.10						9.07		
264	0.20	23.43	26.50	0.11	0.10	0.10	26.62	0.04	6.54	8.99	9.20	9.46

Table C-2 Degradation of EE2 by nitrifying activated sludge from 2 mM reactor with initial ammonium concentrations of 10 mM

Hr	Ammonia concentrations (mg/l)			Nitrite concentrations (mg/l)			Nitrate concentrations (mg/l)			Estrogen concentrations (mg/l)		
	Degradation	Inhibition	Control	Degradation	Inhibition	Control	Degradation	Inhibition	Control	Degradation	Inhibition	Control
0	142.05	143.20	144.00	0.26	0.04	0.03	3.17	0.11	2.58	10.04	10.51	10.40
24	135.51	143.20	144.00							9.53		
48	116.50	139.60	140.00	7.70	0.03	0.03	16.07	0.09	2.58	9.07	9.52	9.75
72	93.05	143.20	140.20	25.50	0.03	0.03	24.07	0.10	2.68	9.05		
96	52.20	140.20	142.00	44.10	0.03	0.03	43.55	0.10	2.98	8.32	9.47	9.86
144	3.72	141.20	142.00	20.90	0.03	0.04	121.53	0.10	3.05	8.00		
168	1.45	141.20	143.00	3.21	0.01	0.03	138.71	0.10	3.02	7.98	9.35	9.87
216	1.04	147.25	144.40	2.54	0.03	0.04	140.99	0.10	3.05	8.08	10.12	9.82

Table C-3 Degradation of EE2 by nitrifying activated sludge from 2 mM reactor with initial ammonium concentrations of 30 mM

Hr	Ammonia concentrations (mg/l)			Nitrite concentrations (mg/l)			Nitrate concentrations (mg/l)			Estrogen concentrations (mg/l)		
	Degradation	Inhibition	Control	Degradation	Inhibition	Control	Degradation	Inhibition	Control	Degradation	Inhibition	Control
0	426.50	421.00	426.00	0.10	0.10	0.10	0.30	0.01	0.02	10.11	10.13	10.18
24	371.50			7.80			44.30			9.43		
48	298.50			15.00			112.40			9.26		
96	154.00	429.00	432.00	74.80	0.08	0.10	195.00	0.05	0.01	8.84	10.05	10.11
144	60.50			13.50			348.00			8.38		
192	9.25			3.00			409.45			8.07		
240	1.45	426.00	427.00	0.10	0.20	0.20	424.50	0.02	0.01	7.97	10.12	10.21

Table C-4 Degradation of EE2 by nitrifying activated sludge from 10 mM reactor with initial ammonium concentrations of 2 mM

Hr	Ammonia concentrations (mg/l)			Nitrite concentrations (mg/l)			Nitrate concentrations (mg/l)			Estrogen concentrations (mg/l)		
	Degradation	Inhibition	Control	Degradation	Inhibition	Control	Degradation	Inhibition	Control	Degradation	Inhibition	Control
0	28.73	28.20	28.40	0.02	0.02	0.01	0.01	0.01	0.01	10.11	10.16	10.15
24	2.86	28.50	28.23	12.36	0.02	0.02	13.22	0.02	0.02	9.49	10.10	10.08
48	0.54			2.14			26.12			9.27		
72	0.23			0.32			27.81			9.13		
96	0.24	29.20	29.31	0.09	0.02	0.02	28.43	0.01	0.01	9.17	10.15	10.10

Table C-5 Degradation of EE2 by nitrifying activated sludge from 10 mM reactor with initial ammonium concentrations of 10 mM

Hr	Ammonia concentrations (mg/l)			Nitrite concentrations (mg/l)			Nitrate concentrations (mg/l)			Estrogen concentrations (mg/l)		
	Degradation	Inhibition	Control	Degradation	Inhibition	Control	Degradation	Inhibition	Control	Degradation	Inhibition	Control
0	143.50	142.00	148.00	0.30	0.20	0.30	0.01	0.01	0.02	10.12	10.05	10.07
24	72.75	143.00	144.00	28.00	0.10	0.10	41.50	0.03	0.03	9.28	10.06	10.05
48	20.76			11.00			108.60			8.21		
72	2.60			1.90			134.50			8.11		
96	0.23			0.50			140.05			8.02		
120	0.13	144.00	145.00	0.20	0.20	0.20	143.23	0.02	0.01	8.13	10.08	10.03

Table C-6 Degradation of EE2 by nitrifying activated sludge from 10 mM reactor with initial ammonium concentrations of 30 mM

Hr	Ammonia concentrations (mg/l)			Nitrite concentrations (mg/l)			Nitrate concentrations (mg/l)			Estrogen concentrations (mg/l)		
	Degradation	Inhibition	Control	Degradation	Inhibition	Control	Degradation	Inhibition	Control	Degradation	Inhibition	Control
0	422.50	426.00	424.00	0.02	0.01	0.01	0.01	0.10	0.10	10.15	10.08	10.14
24	353.50			6.00			61.00			9.13		
48	275.50	421.00	425.00	12.50	0.02	0.01	134.76	0.20	0.10	8.07	10.15	10.22
96	102.50			72.00			251.00			6.87		
144	11.15			10.50			401.09			6.12		
192	0.08	435.00	424.00	0.58	0.02	0.01	421.13	0.20	0.10	6.14	10.05	10.12

Table C-7 Degradation of EE2 by nitrifying activated sludge from 30 mM reactor with initial ammonium concentrations of 2 mM

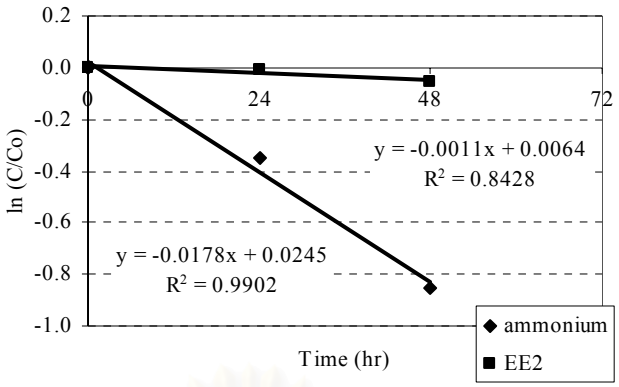
Hr	Ammonia concentrations (mg/l)			Nitrite concentrations (mg/l)			Nitrate concentrations (mg/l)			Estrogen concentrations (mg/l)		
	Degradation	Inhibition	Control	Degradation	Inhibition	Control	Degradation	Inhibition	Control	Degradation	Inhibition	Control
0	30.60	30.16	31.45	0.02	0.01	0.01	0.02	0.20	0.10	10.10	10.16	10.19
24	13.50	30.23	31.13	9.00	0.01	0.02	7.20	0.10	0.20	8.78	10.11	10.23
48	6.50			2.10			21.00			7.53		
72	0.34			1.10			28.23			7.43		
96	0.32			0.20			29.83			7.41		
120	0.19	30.12	31.25	0.10	0.01	0.02	30.10	0.20	0.10	7.37	10.12	10.17

Table C-8 Degradation of EE2 by nitrifying activated sludge from 30 mM reactor with initial ammonium concentrations of 10 mM

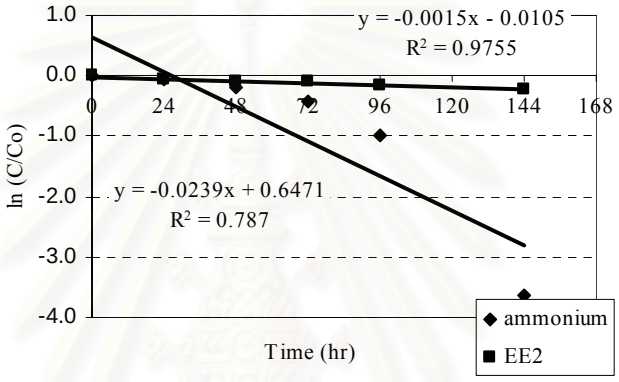
Hr	Ammonia concentrations (mg/l)			Nitrite concentrations (mg/l)			Nitrate concentrations (mg/l)			Estrogen concentrations (mg/l)		
	Degradation	Inhibition	Control	Degradation	Inhibition	Control	Degradation	Inhibition	Control	Degradation	Inhibition	Control
0	154.20	152.00	153.20	0.01	0.02	0.02	0.10	0.10	0.10	10.12	10.08	10.19
24	110.82	153.12	155.21	8.00	0.03	0.01	33.00	0.10	0.20	7.31	10.05	10.07
48	55.50			35.00			65.00			7.03		
72	27.00			11.00			112.00			6.80		
96	4.50			4.70			142.00			6.76		
120	0.23	153.00	151.00	0.30	0.01	0.02	153.60	0.20	0.10	6.75	10.03	10.12

Table C-9 Degradation of EE2 by nitrifying activated sludge from 30 mM reactor with initial ammonium concentrations of 30 mM

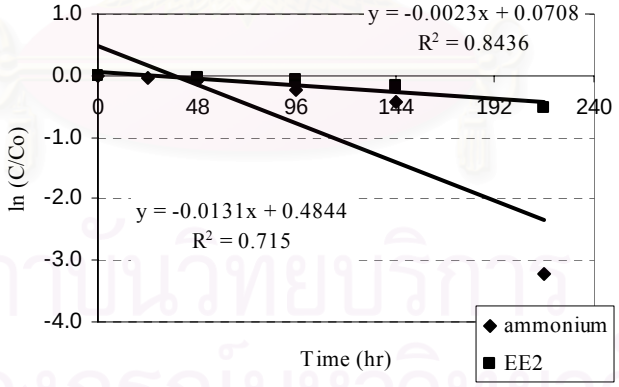
Hr	Ammonia concentrations (mg/l)			Nitrite concentrations (mg/l)			Nitrate concentrations (mg/l)			Estrogen concentrations (mg/l)		
	Degradation	Inhibition	Control	Degradation	Inhibition	Control	Degradation	Inhibition	Control	Degradation	Inhibition	Control
0	424.50	421.00	424.00	0.19	0.12	0.16	0.01	0.01	0.03	10.17	10.03	10.08
12	392.00			8.00			21.20			6.23		
24	372.00			14.00			42.92			3.02		
36	339.00	423.00	428.00	33.00	0.10	0.10	52.00	0.02	0.02	0.07	10.12	10.14
48	318.50			9.00			96.00			0.00		
72	220.50			1.20			202.00			0.00		
120	55.29			1.02			367.76			0.00		
168	14.65	431.00	428.00	0.24	0.14	0.13	409.31	0.01	0.02	0.00	10.17	10.08



(a)

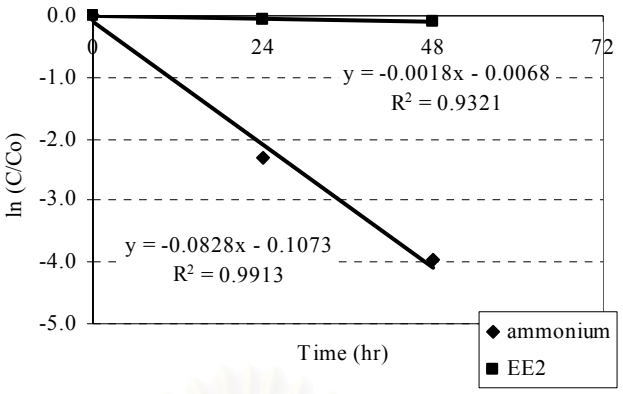


(b)

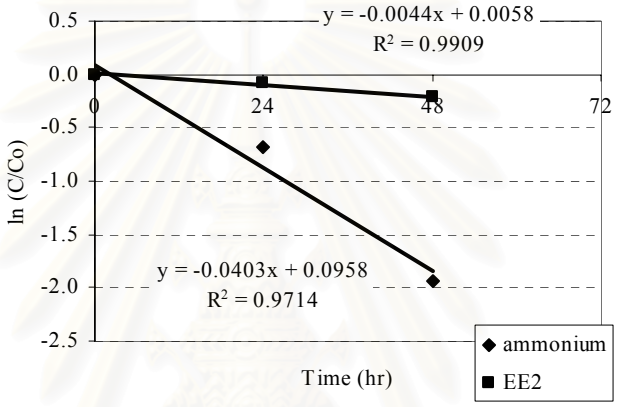


(c)

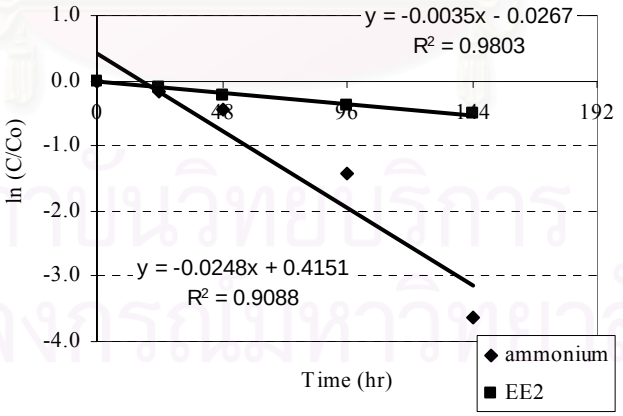
Figure C-1 First-order reaction kinetic of ammonium and estrogens during degradation of EE2 by nitrifying activated sludge from 2 mM reactor with initial ammonium concentrations of 2 mM (a), 10 mM (b), and 30 mM (c)



(a)

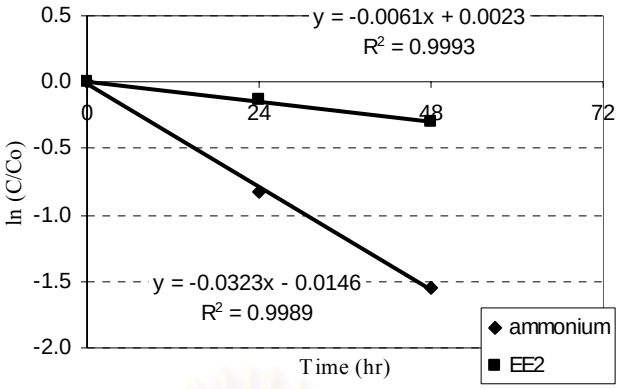


(b)

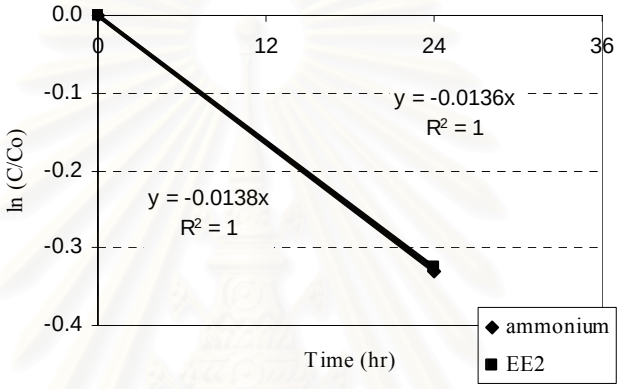


(c)

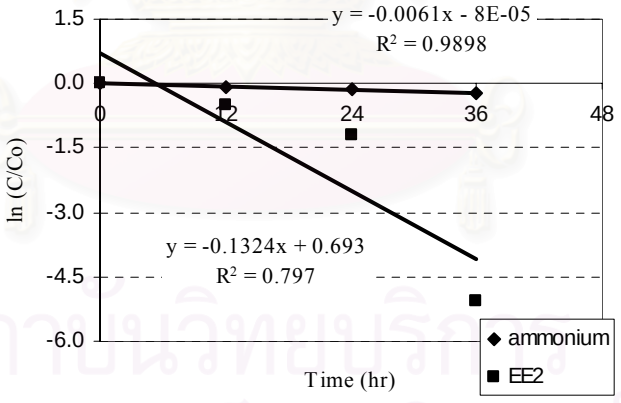
Figure C-2 First-order reaction kinetic of ammonium and estrogens during degradation of EE2 by nitrifying activated sludge from 10 mM reactor with initial ammonium concentrations of 2 mM (a), 10 mM (b), and 30 mM (c)



(a)

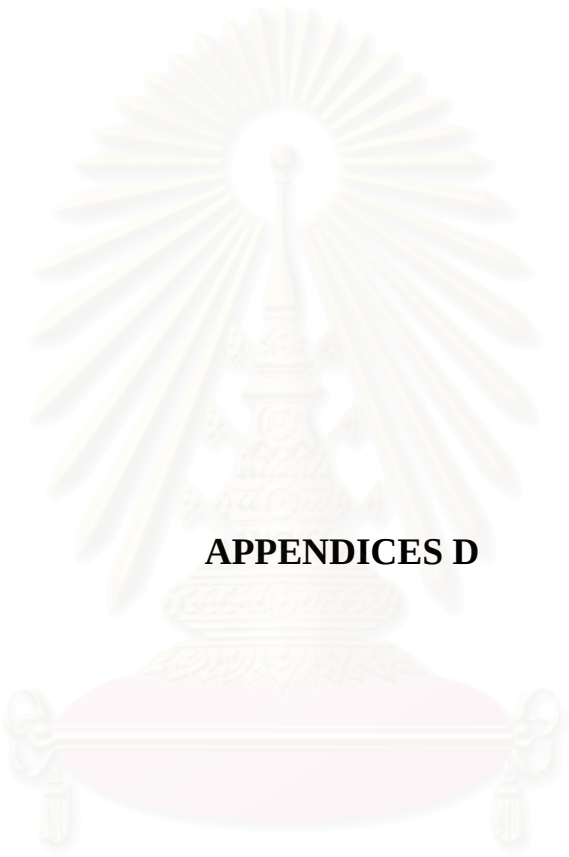


(b)



(c)

Figure C-3 First-order reaction kinetic of ammonium and estrogens during degradation of EE2 by nitrifying activated sludge from 30 mM reactor with initial ammonium concentrations of 2 mM (a), 10 mM (b), and 30 mM (c)



APPENDICES D

Table D-1 Ammonium concentrations during competitive effect of E2 on degradation of EE2 by nitrifying activated sludge from 2mM reactor

Time (hr)	Ammonium concentrations (mg/l)					
	EE2+E2	EE2+E2+Inhibitor	EE2+E2+Control	EE2	E2	Medium
0	424.50	420.00	424.00	424.00	435.00	426.00
24	363.00			365.00	361.00	357.00
48	326.50			319.00	321.00	329.00
72	252.50			257.00	250.00	253.00
96		428.00	430.00			
120	130.50			127.00	119.00	121.00
168	52.00			42.00	41.50	39.00
192	31.50			21.00	27.13	17.00
216	16.65			4.14	13.20	3.00
240	2.26	419.00	426.00	0.21	1.40	0.11

Table D-2 Nitrite concentrations during competitive effect of E2 on degradation of EE2 by nitrifying activated sludge from 2mM reactor

Time (hr)	Nitrite concentrations (mg/l)					
	EE2+E2	EE2+E2+Inhibitor	EE2+E2+Control	EE2	E2	Medium
0	0.20	0.20	0.01	0.01	0.10	0.10
24	2.80			2.70	3.10	2.30
48	12.00			14.60	15.10	12.30
72	32.00			25.00	22.00	17.00
96		0.02	0.01			
120	54.00			57.00	74.50	65.80
168	56.80			77.60	81.30	80.30
192	48.00			56.00	41.00	51.80
216	12.30			14.00	9.00	15.00
240	3.29	0.01	0.02	0.12	0.02	3.50

Table D-3 Nitrate concentrations during competitive effect of E2 on degradation of EE2 by nitrifying activated sludge from 2mM reactor

Time (hr)	Nitrate concentrations (mg/l)					
	EE2+E2	EE2+E2+Inhibitor	EE2+E2+Control	EE2	E2	Medium
0	0.20	0.20	0.01	0.10	0.03	0.01
24	62.00			62.00	65.00	64.00
48	97.00			101.00	102.00	94.00
72	145.00			147.00	142.00	158.00
96		0.02	0.01			
120	234.00			236.00	228.00	232.00
168	312.00			301.00	298.00	306.00
192	352.00			342.00	351.00	353.00
216	397.00			402.00	398.00	406.00
240	419.00	0.01	0.02	421.00	422.00	416.00

Table D-4 Estrogens concentrations during competitive effect of E2 on degradation of EE2 by nitrifying activated sludge from 2mM reactor

Time (hr)	EE2 concentrations (mg/l)				E2 concentrations (mg/l)			
	EE2+E2	EE2+E2+Inhibitor	EE2+E2+Control	EE2	EE2+E2	EE2+E2+Inhibitor	EE2+E2+Control	E2
0	10.05	10.14	10.18	10.06	10.17	10.14	10.15	10.05
24	8.80			8.98	9.13			8.95
48								
72	8.60			8.13	7.15			7.10
96		10.06	10.17			8.23	10.20	
120	7.66			7.59	6.18			6.17
168	6.57	10.13		6.16	4.02	4.46		3.54
192	5.28			5.28	3.35			2.88
216	5.34			5.09	1.23			0.74
240	5.09	10.09	10.15	4.86	1.18	2.61	10.14	0.73

Table D-5 Ammonium concentrations during competitive effect of wastewater (WW) on degradation of EE2 by nitrifying activated sludge from 2mM reactor

Time (hr)	Ammonium concentrations (mg/l)					
	EE2+WW	EE2+WW+Inhibitor	EE2+W+Control	EE2	WW	Medium
0	420.00	428.00	426.00	424.00	420.00	426.00
24	362.00			365.00	371.00	357.00
48	315.00			319.00	311.00	329.00
72	251.00			257.00	250.00	253.00
96		418.00	428.00			
120	141.00			127.00	137.00	132.80
168	36.50			42.00	47.00	31.00
192	16.30			21.00	25.00	15.20
216	7.56			4.14	12.00	4.00
240	0.02	426.00	417.00	0.21	1.30	0.21

Table D-6 Nitrite concentrations during competitive effect of wastewater (WW) on degradation of EE2 by nitrifying activated sludge from 2mM reactor

Time (hr)	Nitrite concentrations (mg/l)					
	EE2+WW	EE2+WW+Inhibitor	EE2+WW+Control	EE2	WW	Medium
0	0.20	0.10	0.20	0.10	0.10	0.10
24	2.80			2.70	2.90	2.30
48	12.00			14.60	14.00	12.30
72	27.00			25.00	22.00	17.00
96		0.10	0.10			
120	61.70			57.00	42.00	38.80
168	42.80			77.60	65.00	53.00
192	54.20			56.00	50.00	42.10
216	8.04			14.00	9.50	3.43
240	4.68	0.02	0.01	0.12	0.67	0.01

Table D-7 Nitrate concentrations during competitive effect of wastewater (WW) on degradation of EE2 by nitrifying activated sludge from 2mM reactor

Time (hr)	Nitrate concentrations (mg/l)					
	EE2+WW	EE2+WW+Inhibitor	EE2+W+Control	EE2	WW	Medium
0	0.20	0.01	0.01	0.10	0.03	0.01
24	61.00			62.00	53.00	64.00
48	97.00			101.00	101.00	94.00
72	144.00			147.00	150.00	158.00
96		0.02	0.01			
120	223.00			236.00	243.00	251.00
168	342.00			301.00	312.00	336.00
192	352.00			342.00	337.00	362.00
216	407.00			402.00	398.00	413.00
240	416.00	0.01	0.02	421.00	418.00	422.00

Table D-8 Estrogen and COD concentrations during competitive effect of wastewater (WW) on degradation of EE2 by nitrifying activated sludge from 2mM reactor

Time	EE2 concentrations (mg/l)				COD concentrations (mg/l)			
(hr)	EE2+WW	EE2+WW+Inhibitor	EE2+WW+Control	EE2	EE2+WW	EE2+WW+Inhibitor	EE2+WW+Control	WW
0	10.11	10.09	10.07	10.06	326.4	326.4	326.4	217.6
24	9.13			8.98	326.4			217.6
48					326.4			217.6
72	8.16			8.13	326.4			217.6
96		10.11	10.1			326.4	326.4	
120	7.35			7.59	326.4			217.6
168	6.51	10.07		6.16	326.4	326.4	326.4	217.6
192	5.43			5.28	326.4			217.6
216	5.24			5.09	326.4			217.6
240	4.62	10.08	10.08	4.86	326.4	326.4	326.4	217.6

BIOGRAPHY

Miss Panida Sermwaraphan was born on January 8, 1983 in Suratthani province, Thailand. She obtained her B.Sc. Degree in Environmental Technology from the Faculty of Environmental and Resource Studies of Mahidol University in 2004. She pursued her Master Degree at The International Postgraduate Programs in Environmental Management, Inter-Department of Environment Management, Chulalongkorn University, Bangkok, Thailand since May 2005. She finished her Master of Science Degree in Environmental Management in May 2007.