ผลของสารอินทรีย์ในน้ำเสียต่อการย่อยสลาย 17แอลฟา-เอทินิวเอสตระไดออล (EE2) ด้วยไนตริไฟอิงแอกทิเวเต็ดสลัดจ์ที่มีแอมโมเนียออกซิไดซ์ซิงค์แบคทีเรีย

นางสาว นัทวรรณ ลิขิตมงคลสกุล

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EFFECT OF ORGANIC MATTERS IN WASTEWATERS ON DEGRADATION OF 17α-ETHYNYLESTRADIOL BY NITRIFYING ACTIVATED SLUDGE CONTAINING AMMONIA-OXIDIZING BACTERIA

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for the Degree of Master of Science Program in Environmental Management
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นัทวรรณ ลิขิตมงคลสกุล : ผลของสารอินทรีย์ในน้ำเสียต่อการย่อยสลาย 17แอลฟา-เอทินิวเอสตระไดออล (EE2) ด้วยไนตริไฟอิงแอกทิเวเต็ดสลัดจ์ที่มีแอมโมเนียออกซิไดซ์ซิงค์แบคทีเรีย. (EFFECT OF ORGANIC MATTERS IN WASTEWATERS ON DEGRADATION OF 17α-ETHYNYLESTRADIOL BY NITRIFYING ACTIVATED SLUDGE CONTAINING AMMONIA-OXIDIZING BACTERIA) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ดร.ตะวัน ลิมปิยากร 140 หน้า.

17α-เอทินิลเอสตระไดออล (EE2)เป็นฮอร์โมนเอสโตรเจนสังเคราะห์ซึ่งเป็นส่วนประกอบหลักของยาเม็ดคุมกำเนิด จัดเป็นสารที่รบกวนการทำงานของระบบต่อมไร้ท่อ จากการศึกษาเมื่อไม่นานมานี้พบการตกค้างของฮอร์โมนดังกล่าวในแหล่งน้ำธรรมชาติ ซึ่ง EE2 จะถูกขับออกมาทางระบบขับถ่ายของคนและสัตว์ โดยจะพบเป็นส่วนใหญ่ในน้ำเสียชุมชน ทั้งนี้ถึงแม้ว่าโรงบำบัดน้ำเสียจะสามารถลดปริมาณ EE2 ได้ แต่ก็ไม่สามารถลดปริมาณให้อยู่ในระดับที่ปลอดภัยได้ เนื่องจาก EE2 จะมีความคงทนในระบบบำบัดน้ำเสียแบบแอกทิเวเต็ดสลัดจ์ (logKow = 4.15) การศึกษาล่าสุดพบว่า EE2 ถูกย่อยสลายได้ด้วยกลุ่มประชากรแอมโมเนียออกซิไดซ์ซิงค์แบคทีเรีย (AOB) ผ่านกระบวนการโคเมทาบอลิซึม อย่างไรก็ตามจากการศึกษาทั้งหมดที่ผ่านมาพบว่าเป็นการศึกษาการย่อยสลายของ EE2 เพียงตัวเดียวในระบบ ซึ่งในความเป็นจริงในน้ำเสียนั้นยังประกอบไปด้วยสารประกอบสารอินทรีย์อื่นๆหลายชนิด ที่มีผลไปรบกวนการย่อยสลาย EE2 โดยไปแข่งขันทำปฏิกิริยาที่บริเวณ active site ของเอนไซม์แอมโมเนียโมโนออกซิจิเนส ดังนั้นในทางปฏิบัติจริงของการใช้ AOB ในการย่อยสลาย EE2 ในโรงบำบัดน้ำเสียต้องอาศัยความรู้พื้นฐานเกี่ยวกับปฏิกิริยาของเอนไซม์ AMO กับสารต่างๆ ด้วยเหตุผลดังกล่าวงานวิจัยชิ้นนี้จึงมุ่งเน้นที่การศึกษาผลของสารอินทรีย์ในน้ำเสียต่อการย่อยสลาย EE2 ด้วยไนตริไฟอิงแอกทิเวเต็ดสลัดจ์ (NAS) ที่มี AOB โดยมีจุดประสงค์หลักเพื่อศึกษาผลของชนิดของน้ำเสีย (น้ำเสียจากแหล่งชุมชนและน้ำเสียจากโรงงานอุสาหกรรม), ผลของความเข้มข้นของแอมโมเนียเริ่มต้น (2 และ 10 mM), และผลของความเข้มข้นเริ่มต้นของ EE2 เริ่มต้น (3.5 และ 10 mg/l) ในขั้นต้นได้ทำการพัฒนา NAS ให้มีกลุ่มประชากร AOB คล้ายกับสภาวะจริง โดยการนำสลัดจ์จากระบบบำบัดน้ำเสียชุมชนมาทำการเพาะเลี้ยงในถังปฏิกรณ์ ด้วยอาหารเลี้ยงเชื้อที่มีปริมาณแอมโมเนีย 28 มิลลิกรัมต่อลิตร ในแต่ละการทดลองน้ำเสียตัวอย่างจะถูกทำให้เจือจาง เพื่อให้ได้ค่าความเข้มข้น Chemical Oxygen Demand (COD) ที่ระดับต่างๆ โดยในการศึกษาขั้นต้นได้ทำการศึกษาถึงผลของระดับ pH และความเข้มข้นของไนไตรท์ต่อปฏิกิริยาเปลี่ยนรูปแบบไร้เชื้อของ EE2 จากผลการศึกษาพบว่าการเปลี่ยนรูปของ EE2 แบบไร้เชื้อจะเกิดขึ้นที่ระดับ pH น้อยกว่า 6.8 เท่านั้นและความเข้มข้นเริ่มต้นของไนไตรท์ไม่มีผลต่อการเปลี่ยนรูปแบบไร้เชื่อของ EE2 ผลการศึกษาการย่อยสลาย EE2 แสดงให้เห็นว่าน้ำเสียทั้งสองชนิดจะประกอบไปด้วยสารอินทรีย์ที่แตกต่างกัน ซึ่งจะมีคุณสมบัติการยั้บยั้งการย่อยสลาย EE2 แตกต่างกันด้วย โดยในการทดลองกับน้ำเสียชุมชนพบว่า สารอินทรีย์ส่วนใหญ่มีคุณสมบัติเป็น competitive inhibitor ต่อแอมโมเนีย ซึ่งจะทำปฏิกิริยากับเอนไซม์ที่บริเวณเดียวกับ EE2 ทำให้ไม่มีผลต่อปฏิกิริยา ammonia oxidation แต่จะมีผลไปรบกวนการย่อยสลายของ EE2 ในทางกลับกันในการทดลองกับน้ำเสียจากโรงงานอุตสาหกรรมพบว่า สารอินทรีย์ส่วนใหญ่มีคุณสมบัติเป็น noncompetitive inhibitor ต่อแอมโมเนียซึ่งจะมีผลไปรบกวนปฏิกิริยา ammonia oxidation ในการทดลองที่ความเข้มข้นแอมโมเนียต่ำ, การย่อยสลายของ EE2 จะถูกรบกวนด้วยระดับความเข้มข้นของ COD แต่เมื่อระดับความเข้มข้นเริ่มต้นของแอมโมเนียเพิ่มมากขึ้นระดับความเข้มข้นของ COD จะไม่มีผลต่อการย่อยสลายของ EE2 เนื่องจากความเข้มข้นที่มากเกินพอของสารตั้งต้นแอมโมเนียจะมีผลทำปริมาณเอนไซม์ AMO เพิ่มมากขึ้น ทำให้การย่อยสลาย EE2 ไม่ถูกจำกัดและสารอินทรีย์ในน้ำเสียไม่มีผลต่อปฏิกิริยาเม็ทาบอลิซึ่มร่วมของ EE2 อย่างไรก็ตามถึงแม้ว่าสารอินทรีย์ในน้ำเสียชุมชนส่วนใหญ่จะมีคุณสมบัติเป็น noncompetitive inhibitor ต่อแอมโมเนีย ระดับความเข้มข้นของ COD ยังผลไปรบกวนปฏิกิริยา ammonia oxidation ซึ่งอาจเกิดมาจากความเป็นพิษของผลิตภัณฑ์ที่ได้จากการย่อยสลายสารอินทรีย์ ค่าความเข้มข้นเริ่มต้นของ EE2 ไม่มีผลต่อปฏิกิริยาการย่อยสลาย EE2

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5087524320: MAJOR ENVIRONMENTAL MANAGEMENT
KEYWORDS : 17α-ETHYNYLESTRADIOL / AMMONIA-OXIDIZING BACTERIA / COMETABOLISM / NITRIFYING ACTIVATED SLUDGE / WASTEWATER

NATTHAWAN LIKITMONGKONSAKUN: EFFECT OF ORGANIC MATTERS IN WASTEWATERS ON DEGRADATION OF 17α-ETHYNYLESTRADIOL BY NITRIFYING ACTIVATED SLUDGE VIA COMETABOLISM OF AMMONIA-OXIDIZING BACTERIA. ADVISOR : TAWAN LIMPIYAKORN, Ph.D., 140 PP.

17α-ethynylestradiol (EE2), a synthetic estrogen, is a key ingredient in oral contraceptive pill. This recalcitrant organic pollutant is reported as an endocrine disruptor, very high in estrogenicity. Previous studies on the occurrence of pharmaceutical compounds in environments suggested the existence of EE2 in several receiving waters (logKow = 4.15). Municipal wastewater is a potential source of EE2 since EE2 is released mainly to the environments by excretion of humans and animals through their urine and feces. Although wastewater treatment plants (WWTPs) are capable of removing EE2 from wastewater, the potential removals of EE2 by WWTPs are not enough to reduce the released amounts of EE2 to the safe levels. In batch experiment, EE2 appeared to be mainly stable in contact with activated sludge, while nitrifying activated sludge (NAS) could completely degrade EE2. In NAS, EE2 is proven to be degraded by ammonia-oxidizing bacteria (AOB) via co-metabolism. However, all the studies so far have provided only the information obtained from the study with single EE2 compound. In fact, other several types of organic matters are present in wastewater. Such organic compounds in wastewater can result in retarding EE2 degradation by competing EE2 for active site of ammonia monooxynase (AMO) enzyme. Therefore, applications of AOB in degrading EE2 in WWTPs require fundamental knowledge of AMO and its interaction with alternate substrates. This study aimed to investigate effect of organic matters in wastewaters on cometabolism of EE2 by AOB in NAS. Specific objectives included effect of types of wastewaters (municipal and industrial wastewaters), effect of initial ammonium concentration (2 and 10 mM), and effect of initial EE2 concentration (3.5 and 10 mg/l). To develop NAS, sludge taken from a municipal WWTP was enriched in a reactor receiving inorganic medium containing 2mM (28 mg-N/l) ammonium concentration. Each experiment was carried out with diluted wastewater to obtain various final chemical oxygen demand (COD) concentrations. Preliminary experiment on effect of pH and nitrite concentration (nitration) on abiotic transformation of EE2 suggested that abiotic EE2 transformation occurred at only pH < 6.8 and initial nitrite concentrations showed no effect on abiotic EE2 transformation. Degradation of EE2 under the presence of municipal or industrial wastewater showed that different types of wastewaters that may contain district compositions of organic matters exhibited inhibition behaviors differently. In the case of municipal wastewater, most amounts of organic matters may be noncompetitive inhibitors to ammonia which have the same binding site to EE2 causing no effect on ammonia oxidation but deceleration of EE2 degradation. In contrast, in the case of industrial wastewater, the major portions of organic matters may be competitive inhibitors to ammonia causing deceleration of ammonia oxidation. At low initial ammonium concentration, EE2 degradation can be deteriorated by COD concentrations. But when initial ammonium concentration increased, these phenomena disappeared. This is because when increasing the amount of the primary substrate, more AMO enzymes were produced resulting in unlimited degradation of all compounds in the medium reducing the effect of organic matters on cometabolism of EE2. However, although organic matters in municipal wastewater were more in noncompetitive forms to ammonia, COD concentrations were found to deteriorate ammonia oxidation at high initial ammonium concentration. This may cause by product toxicity when organic matters were more degraded. Initial EE2 concentration did not affect cometabolism of EE2.

Field of Study : Environmental Management Student's Signature : ณัทธวรรธน์ ลิขิตมงคลกุล

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ABBREVIATIONS

AMO	=	ammonia monooxygenase
AOB	=	ammonia-oxidizing bacteria
Conc.	=	concentration
E1	=	Estrone
E2	=	Estradiol
E3	=	Estriol
EDs	=	endocrine disruptors
EE2	=	17α-ethynylestradiol
g	=	gram
GC	=	gas chromatography
HAO	=	hydroxylamine oxidoreductase
HPLC	=	high performance liquid chromatography
IWW	=	Industrial wastewater
Kow	=	octanol-water partitioning coefficient
LC	=	liquid chromatography
LOD	=	limit of detection
mg/l	=	milligram per liter
mM	=	milimolar
MW	=	molecular weight
MWW	=	Municipal wastewater
NAS	=	nitrifying activated sludge
ND	=	no data.
ng/l	=	nanogram per liter
WW	=	wastewater
WWTP	=	wastewater treatment plant
WWTS	=	wastewater treatment system
X	=	Found heavy metals in major industries
+	=	present
–	=	not present
+/–	=	present in some strains

CHAPTER I

INTRODUCTION

1.1 Background and motivation

Wastewater is any water that has been adversely affected in quality by anthropogenic influence. It comprises of liquid waste discharged by domestic residences, commercial properties, industry, and agriculture that can encompass a wide range of potential contaminants. There are various characteristics of wastewater discharged because the number of raw materials, processes and types of products involved are complicated as well as the content of wastewater, its concentration, and the volume of wastewater. In the past several decades, many techniques have been developed to find an economic and efficient way to reclaim the wastewater (Overcash and Pal, 1979; Gao, 1986; Jin, 1993; Raisin and Mitchell, 1994; Zhao and Wang, 1994). However, the physical and chemical treatments are more difficult and costly (Tchobanoglous et al., 2003). High capital construction costs and high operation costs limited their application to a great extent, especially in developing countries. As a result, most of wastewater treatments used a part of biological treatments that were effective and economic (Ou et al., 1992). Naturally, there are chemical substances which persist in the environment, bioaccumulate through the food web, and pose a risk of causing adverse effects to human health and the environment with the evidence of long-range transport. Although, they cannot be degraded by the normal bacteria, there is a common degradable ammonia-oxidizing bacteria (AOB) in natural can be used. The oxidation of ammonia to nitrite is initiated by ammonia mono-oxygenase (AMO). Nonetheless, considerable knowledge about AMO has been gained from

studies with intact cells and cell-free extracts. Most studies on AMO have focused on the obligate chemolithotroph, *Nitrosomonas europaea* (Wood, 1986). Because of the remarkable broad substrate range of AMO (Arp and Stein, 2003), attention has focused recently of the possibility of using nitrifiers such as *N. europaea* via cometabolism in the bioremediation of contaminated soils and aquifers and especially in the treatment of wastes. In 2005, the trihalomethanes (THMs) cometabolism was shown to occur with pure culture of *N. europaea* (Wahman et al.). AMO catalyzes the oxidation of NH_3 to hydroxylamine which is subsequently oxidation to NO^-_2 by hydroxylamine oxidoreductase (HAO) and releases four electrons. Two of them will be returned to AMO to activate O_2 and sustain ammonia oxidation rates. The remaining two electrons will be transferred to cellular metabolism for the cell's reductant needs including assimilation of inorganic nutrients and generation of the proton gradient (Arp and Stein, 2003). While ammonia is the only substrate for AMO that can support growth, there are many compounds that can be transformed by AMO such as hydrocarbons and halogenated hydrocarbons (Arp and Stein, 2003).

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). EE2 is known as a key ingredient in oral contraceptives pill (Ying et al., 2002) which 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). Due to its hydrophobic property (Log Kow = 3.67-4.15) (Lai et al., 2000) and tend to be accumulated in sediments, this predominantly female hormone interfere the endocrine and reproductive function in human and living

organisms. For example, as low as nanogram per liter of EE2 can cause decreasing in sperm count and increasing in incident to testicular cancer and male fertilizer disorder (Purdom et al., 1994). EE2 degraded slowly with an estimated half-life of 81 days in the aquifer material under aerobic conditions (Ying et. al, 2003) In batch experiment, EE2 appeared to be mainly stable in contact with activated sludge (Ternes et al., 1999a), while nitrifying activated sludge (NAS) in high ammonium concentration with enrichment culture of *N. europaea* could completely degrade EE2 at an initial concentration of 50 μg/l within 6 days (Vader et al., 2000). In the study of Shi et al. (2004), *N. europaea* degraded both natural and synthetic estrogens but did not degrade the intermediates. Current knowledge of catalytic activity of AMO in *N. europaea* is largely based on inhibitor and substrate studies (Ensign et al., 1993; Hooper et al., 1973; Suzuki et al., 1981). The varieties of inhibitors which are alternative hydrocarbon substrates of AMO have been identified (Keener and Arp, 1993). Although previous studies have provided information on degradation of EE2 and other hydrocarbons, but there is no study on degradation of EE2 in the presence of other organic matters. In the actual phenomena wastewater treatment, there are many organic matters in the systems. Single organic maters are competed to each other. The applications of these potential wastewater systems will require a fundamental knowledge of AMO and its interaction with alternate substrates. Consequently, this study investigated effect of organic matters in wastewaters on cometabolism of 17α-ethynylestradiol (EE2) by nitrifying activated sludge (NAS) containing ammonia-oxidizing bacterial (AOB) community.

1.2 Objectives

This study focuses mainly on effect of organic matters in wastewaters on cometabolism of 17α-ethynylestradiol (EE2) by nitrifying activated sludge (NAS) containing ammonia-oxidizing bacteria (AOB). The objectives of this study are as follows:

1. To investigate effect of pH levels and nitrite concentrations (nitration) on abiotic transformation of EE2.
2. To analyze competitive effect of wastewaters on degradation of EE2 by NAS.
    - Effect of organic matters in municipal and industrial wastewaters
    - Effect of initial ammonia concentration
    - Effect of initial EE2 concentration

1.3 Hypothesis

1. pH levels and nitrite concentrations (nitration) affect abiotic transformation of EE2.
2. Abiotic transformation of EE2 occurs at pH between 6.0-7.0.
3. Type of organic matters in wastewater competitively affects EE2 degradation by NAS differently.
4. Different source of wastewater, that contains district kinds of organic matters, affects degradation of EE2 by NAS differently.
5. Concentrations of organic matters affect degradation of EE2 (in wastewater) differently.
6. Initial ammonia concentration affect cometabolism of EE2.
7. Organic matters in wastewater are different in inhibition behavior.

1.4 Scope of study

1. NAS was enriched in laboratory-scale continuous-flow reactor receiving inorganic media containing 2mM ammonia concentration.
2. To avoid the abiotic transformation of EE2, degradation study was carried out in batch experiment at pH between 7.0 – 8.5.
3. Two different types of wastewaters (municipal and industrial wastewaters) were used in this study.
4. COD concentrations in wastewater were varied (0, 70, and 140 mg/l of COD for municipal wastewater and 0, 70, 140, 1000, and 2000 mg/l of COD for industrial wastewater).
5. Initial EE2 concentrations were varied, at 3.5 and 10 mg/l.

CHAPTER II

LITERATURE REVIEW

Characteristics of wastewater are varied in number of raw materials, types of products, and the volume of wastewater. It comprises of liquid waste discharged by domestic residences, commercial properties, industry, and agriculture that can encompass a wide range of potential contaminants and concentrations. Naturally, there are chemical substances which persist in the environment, bioaccumulate through the food web, and pose a risk of causing adverse effects to human health and the environment with the evidence of long-range transport. Although, they cannot be degraded by the normal bacterial, there is a common degradable ammonia-oxidizing bacteria in natural, *Nitrosomonas europaea*, can be used. The characterization of ammonia-oxidizing bacteria and related literature are described as following.

2.1 Ammonia-oxidizing bacteria

2.1.1 Nitrification

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

2.1.2 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 2.1 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 2.2) (Koops et al., 2003).

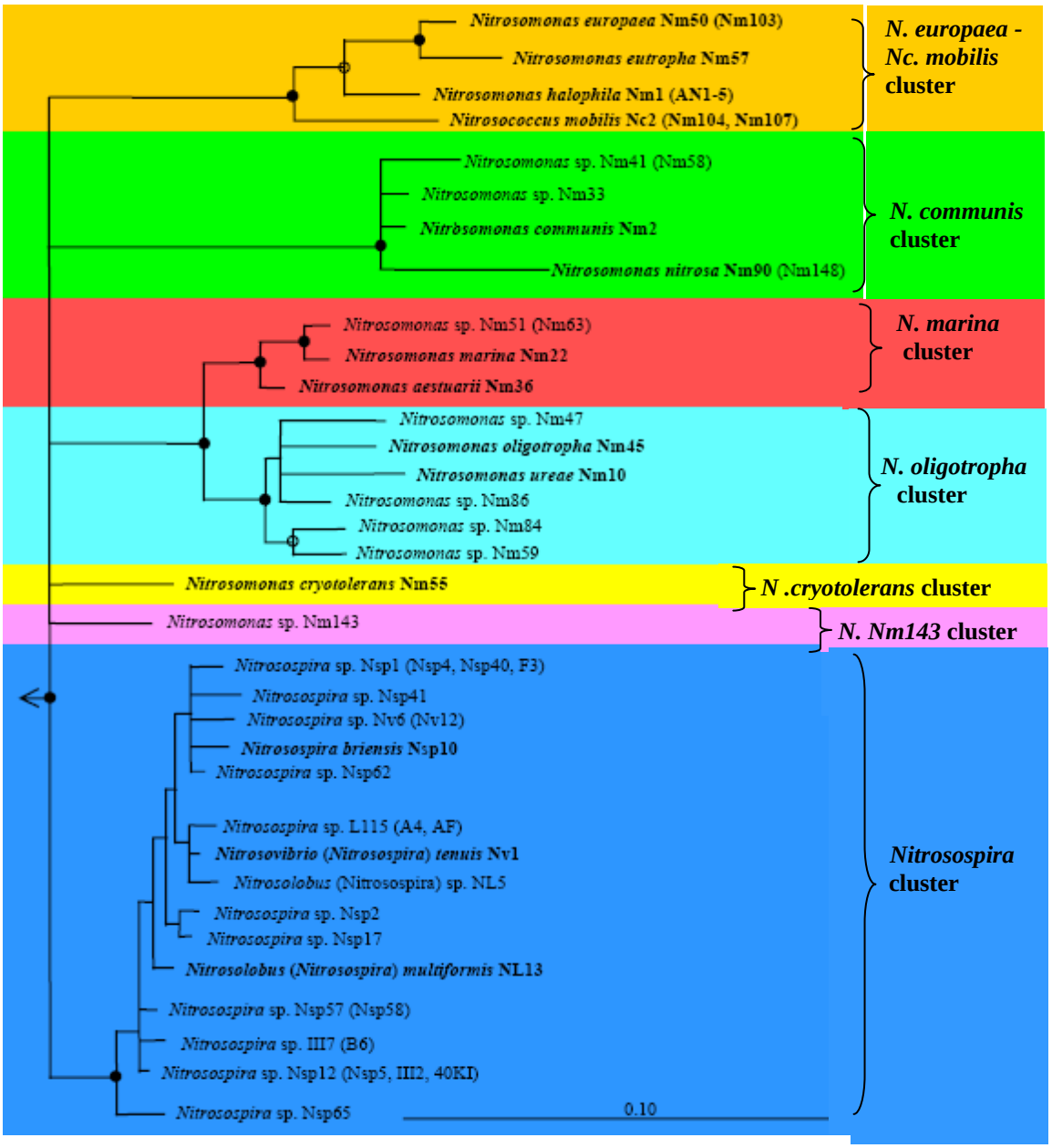


Figure 2.1 16S rRNA-based phylogenetic tree of the *Betaproteobacterial* AOB. Described species are depicted in bold. Maximum likelihood, maximum parsimony, and neighbor-joining trees were calculated and merged. Multifurcations connect branches for which a relative order cannot be unambiguously determined 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

(Source: Koops et al., 2003)

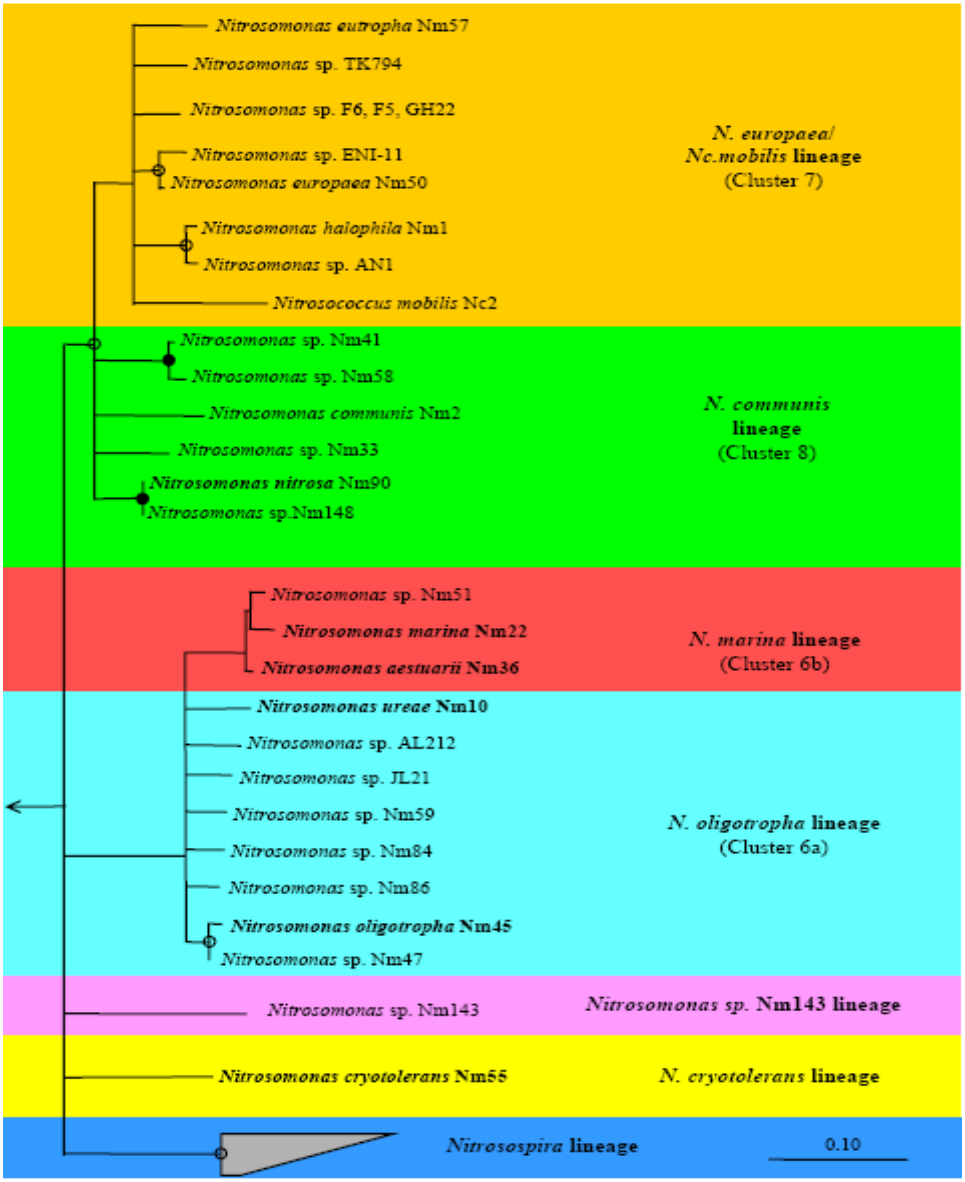


Figure 2.2 AmoA-based phylogenetic tree of the *Betaproteobacterial* AOB. Described species are depicted in bold. The 453-bp gene fragment obtainable with the most commonly used *amoA* PCR primers was used for phylogeny inference. AmoA sequences shorter than 414 nucleotides were excluded from the analysis. 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. (Source: Koops et al., 2003)

2.1.3 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 2.1; Koops et al., 2003).

Table 2.1 Physiological properties and preferred habitats of described AOB species (Source: Koops et al., 2003)

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.

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

AOB, which is obligate chemolithotrophic aerobe using ammonia as a sole energy source, is used 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 which is subsequently oxidation to nitrite by hydroxylamine oxidoreductase (HAO) and releases four electrons. Two of them will be transferred to cellular metabolism for the cell's reductant needs including assimilation of inorganic nutrients and generation of the proton gradient. The remaining two electrons will be returned to AMO to activate O_2 and sustain ammonia oxidation rates (Arp and Stein, 2003). In some cases, these last two electrons might be used in another oxidation reaction which is called co-metabolism (Arciero et al., 1989; William and Daniel, 1993).

While ammonia is the only substrate for AMO that can support growth, there are many compounds that can be transformed by cometabolism of AMO such as hydrocarbons and halogenated hydrocarbons in Figure 2.3 and Table 2.2.

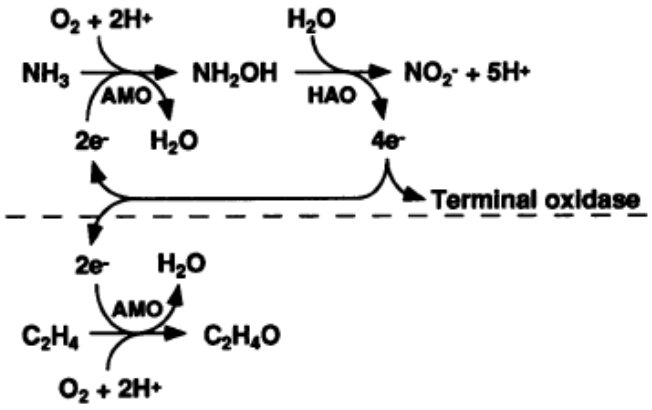


Figure 2.3 Co-metabolism of ethylene by AOB (Source: William and Daniel, 1993)

Table 2.2 Examples of substrates transformed by AMO and the resulting products (Source: Arp and Stein, 2003).

Substrates	Products	References
Ammonia	Hydroxylamine	Hofman and Lee, 1953
Alkanes	Alcohols	Hyman and Wood, 1983; Hyman et al., 1988
Methane	Methanol	
Butane	1-, 2-butanol	
Alkenes	Epoxides	Hyman and Wood, 1984; Hyman et al., 1988
Ethene	Ethylene oxide	
Peopene	Propylene oxide	
Aromatic hydrocarbons	Alcohols	Hyman et al., 1985; Vannelli and Hooper, 1995; Chang et al., 2002
Benzene	Benzyl alcohol	
Naphthalene	Naphthol	
Thioethers	Sulfoxides	Hyman et al., 1985; Juliette et al., 1993a; Vannelli and Hooper, 1995; Chang et al.,2002
Dimethylsulfide	Dimethylsulfoxide	
O-Ethers	Hydrolysis products	Hyman et al., 1994
Dimethyl ether	Methanol and formaldehyde	
Halogenated Compounds	Various compounds	Hyman and Wood,1984a; Rasche et al., 1990; Vannelli et al., 1990; William and Daniel, 1994
Bromoethane	Acetaldehyde and Br-	
Chlorobenzene	4-chlorophenol	
Tricholroethylene	TCE-epoxide	
Ethylbenzene	Styrene	William and Daniel, 1994
2-chloro-6-trichloromethyl-pyridine	2-cholro-6-dichloromethyl-pyridine	Vannelli and Hooper, 1993

2.2 Enzymes

Enzymes are biological catalysts involved in important pathways that allow chemical reactions to occur at higher rates (velocities) than would be possible without the enzyme. Enzymes are generally globular proteins that have one or more substrate binding sites. The kinetic behavior for many enzymes can be explained with a simple model as in figure 2.4.

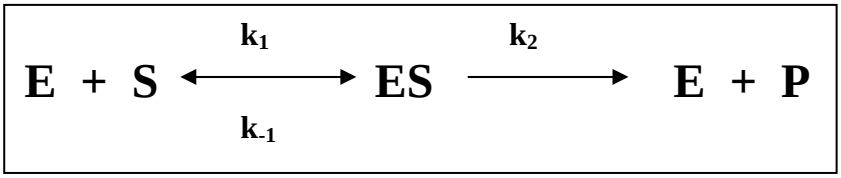


Figure 2.4 Enzymes kinetic

Where E is an enzyme, S is substrate and P is product(s). ES is an enzyme-substrate complex that is formed prior to the catalytic reaction. k1 is the rate constant for enzyme-substrate complex (ES) formation and k-1 is the dissociation rate of the ES complex. In this model, the overall rate-limiting step in the reaction is the breakdown of the ES complex to yield product, which can proceed with rate constant k2. The reverse reaction (E + P → ES) is generally assumed to be negligible.

Assuming rapid equilibrium between reactants (enzyme and substrate) and the enzyme-substrate complex resulted in mathematical descriptions for the kinetic behavior of enzymes based on the substrate concentration (Nelson and Cox, 2000). The most widely accepted equation (derived independently by Henri and subsequently by Michaelis and Menten) relates the velocity of the reaction to the substrate concentration as shown in the equation below, which is typically referred to as the Michaelis-Menten equation:

$$V = \frac{[S] \cdot Vmax}{[S] \cdot + Km}$$

Where

v = rate of reaction

Vmax = maximal reaction rate

[S] = substrate concentration

Km = Michaelis-Menten constant

2.3 Inhibitors

Inhibitors are compounds which interact with an enzyme to slow down its rates of reaction (Nelson and Cox, 2000). However, because the substrate and inhibitor are not identical the enzyme is unable to convert the inhibitor into product. There are three types of inhibitor which consist of:

2.3.1 Competitive Inhibitor

2.3.1.1 Competitive Inhibitor by active site binding

The competitive inhibitor is a compound which bears a close structural and chemical similarity to the substrate of the enzyme. Due to this similarity, the inhibitor binds to the active site in place of the substrate. The enzyme is unable to convert the inhibitor into product because the substrate and inhibitor are not identical. The inhibitor simply blocks the active site. While it's there the substrate can't enter and consequently the enzyme can't convert it to product. On the other hand, if the

substrate binds to the active site before the inhibitor, the inhibitor is incapable of binding. Both of them can't bind the active site at the same time.

2.3.1.2 Competitive inhibition by conformational change

The Competitive inhibitor binds not to the active site but to an inhibitor binding site which is remote from the active site. On binding, the inhibitor causes a conformation change in the enzyme. This has the effect of altering the active site that the substrate can no longer bind to it. Similarly, prior binding of the substrate to the active site causes a change in the inhibitor site which prevents the inhibitor from binding.

2.3.2 Non-competitive Inhibitor

A noncompetitive inhibitor binds to an inhibitor site on the enzyme which is remote from the active site and brings about a conformational change in the active site. It's similar to the competitive inhibitor types. The difference is that the change in the active site does not prevent substrate binding but only prevents the enzyme from converting the bound substrate to product.

A classical noncompetitive inhibitor has absolutely no effect on substrate binding. The change to the shape of the active site is almost certain to alter the ability of the substrate to bind. It doesn't stop it altogether but the affinity will be reduced. This Inhibitors are also called mixed inhibitors as they appear to have some of the properties of competitive and noncompetitive types. In natural environment, these classical noncompetitive inhibitors are very rare.

2.3.3 Uncompetitive Inhibitor

The Uncompetitive Inhibitor is incapable of binding to free enzyme. It can only bind to the enzyme-substrate complex. This could be because the substrate is itself directly involved in binding the inhibitor or because it brings about a conformational change in an inhibitor binding site which was previously incapable of binding the inhibitor. Once the inhibitor has bound it prevents the enzyme from turning the substrate into product. This also could be some kind of direct interaction, or due to a change in conformation of the active site. In natural environment, these uncompetitive inhibitors are very rare.

2.4 Inhibition of AMO

Since, AMO also catalyzes the oxidation of several alkanes, alkenes, aromatics (including benzene and several derivatives, several heterocycles, and several heteroatom ring compounds), ethers, thioethers and primary amines (Arciero et al., 1989; Rasche et al., 1991). Current knowledge of catalytic activity of AMO is largely based on inhibitor and substrate studies (Ensign et al., 1980; Hooper et al., 1973; Suzuki et al., 1981). A variety of inhibitors which are alternative substrates of AMO have been identified (William and Daniel, 1993). The kinetic analysis revealed that while some alternative substrates were competitive inhibitors, others were noncompetitive. For example, TCE (Ely et al., 1997 and Hyman et al., 1985), methane (CH_4) (Suzuki et al., 1976; William and Daniel, 1993), ethylene (C_2H_4), and etc. (William and Daniel, 1993) were reported to be a potent competitive inhibitor of ammonia oxidation by *N. europaea*., whereas alkanes (up to C4) and

monohalogenated (Cl, Br, I) alkanes were noncompetitive (William and Daniel, 1993).

Table 2.3 Inhibition patterns of substrates transformed by AMO (Source: William and Daniel, 1993).

Substrates	**Inhibition Patterns**	**Reference**
Methane (CH_4)	Competitive Inhibitor	Suzuki et al.,1976; William and Daniel, 1993
Ethylene (C_2H_4)	Competitive Inhibitor	William and Daniel, 1993
Trichloroethylene (TCE)	Competitive Inhibitor	Ely et al., 1997; Hyman et al., 1985;
Methanol (CH_3OH)	Competitive Inhibitor	William and Daniel, 1993
Carbon Monoxide (CO)	Competitive Inhibitor	William and Daniel, 1993
Napthalene	Competitive Inhibitor	William and Daniel, 1993
Ethane (C_2H_6)	Non-competitive Inhibitor	William and Daniel, 1993
Propane (C_3H_8)	Non-competitive Inhibitor	William and Daniel, 1993
Butane (C_4H_{10})	Non-competitive Inhibitor	William and Daniel, 1993
Bromomethane	Non-competitive Inhibitor	William and Daniel, 1993
Chloromethane (C_2Cl_4)	Non-competitive Inhibitor	William and Daniel, 1993
n-Chloropropane	Non-competitive Inhibitor	William and Daniel, 1993
Iodomethane (CH_3I)	Non-competitive Inhibitor	William and Daniel, 1993
Iodoethane (CH_3CH_2I)	Non-competitive Inhibitor	William and Daniel, 1993
Bromoethane (C_2H_5Br)	Non-competitive Inhibitor	William and Daniel, 1993
Benzene	Non-competitive Inhibitor	William and Daniel, 1993
Tetrachloroethylene (C_2Cl_4)	CD	William and Daniel, 1993
Carbon tetrachloride (CCl_4)	CD	William and Daniel, 1993
1,1,1,2-Tetrachloroethane (CCl_3CH_2Cl)	CD	William and Daniel, 1993

CD is concave-down curves. This pattern does not allow determination of K_{iE} and K_{iES} values.

2.5 Wastewater

Wastewater is any water that has been adversely affected in quality by anthropogenic influence. It comprises liquid waste discharged by domestic residences, commercial properties, industry, and agriculture and can encompass a wide range of potential contaminants and concentrations. In the most common usage, it refers to the wastewater that contains a broad spectrum of contaminants resulting from the mixing of wastewaters from different sources (Tchobanoglous et al., 2003)

2.5.1 Type and characteristics of wastewaters

The content of wastewater and its concentration as well as the volume of wastewater vary with the type of wastewater.

2.5.1.1 Municipal wastewater

Municipal wastewater is mainly comprised of water together with relatively small concentrations of suspended and dissolved organic and inorganic solids. Among the organic substances present in sewage are carbohydrates, lignin, fats, soaps, synthetic detergents, proteins and their decomposition products, as well as various natural and synthetic organic chemicals from the process industries. Organic chemicals usually exist in municipal wastewaters at very low concentrations and ingestion over prolonged periods would be necessary to produce detrimental effects on human health.

Metcalf and Eddy (1991) have analyzed the typical municipal wastewater characteristics for wastewater engineering treatment, disposal, and reuse (Table 2.4).

Table 2.4 Typical municipal wastewater characteristics (Source: Metcalf and Eddy, 1991)

	Weak (mg/l)	Medium (mg/l)	Strong (mg/l)
Biochemical oxygen demand (BOD)	110	220	400
Total suspended solid (TSS)	100	220	350
Dissolved solids (TDS)	250	500	850
Nitrogen (N)	20	4	85
Phosphorus (P)	4	8	15
Grease	50	100	200

Tieheng et. all.(1998) studied the influent belongs to the wastewater from toilet, bathroom and restaurant, without the involvement of any industrial wastewater, characterized by the bad smell and high COD, BOD5, TOC and NH4-N concentrations, as shown as Table 2.5. The ratio of BOD_5:COD was 0.50.

Table 2.5 Characteristics of wastewater (yearly means) (Source: Tieheng, et al., 1998)

Year	pH	COD (mg/1)	BOD_5 (mg/1)	NH_4^- (mg/1)	TOC (mg/1)	SS (mg/1)
1991	6.65	538	283	35.73	148	187
1992	7.22	585	318	24.00	161	221
1993	7.38	609	253	83.26	150	172
1995	6.77	395	217	51.10	-	188
Mean	7.21	588	294	44.87	157	202

2.5.1.2 Industrial wastewater

There are various characteristics of wastewater discharged from industry (Table 2.6) because the number of raw materials, processes and types of products involved in the industry are complicated as well as the content of wastewater, its concentration, and the volume of wastewater (Bond and Straub, 1974).

Table 2.6 Comparative strengths of wastewaters from industry

(Source: Bond and Straub, 1974)

Industry	BOD (mg/l)	COD (mg-O/l)	SS (mg/l)	pH
Slaughterhouse	1,500-2,500	200-400	800	7
Wool scouring	2,000-5,000	2,000-5,000	3,000-30,000	9-11
Potato processing	2,000	3,500	2,500	11-13
Farm	1,000-2,000	500-1,000	1,500-3,000	7.5-8.5
Brewery	850	1700	90	4-8
Beet sugar	450-2,000	600-3,000	800-1,500	7-8
Coke oven	780	1,650	70	7-11
Cotton	200-1,000	400-1,800	200	8-12
Tannery	1000-2,000	2,000-4,000	2,000-3,000	11-12
Laundry	1,600	2,700	250-500	8-9
Oil refining	100-500	150-800	130-600	2-6

In addition, industrial wastewater contains a variety of inorganic substances from domestic and industrial sources, including a number of potentially toxic elements such as aluminum, arsenic, cadmium, chromium, copper, lead, mercury, nigel, zinc, etc., as shown as Table 2.7 (Bond and Straub, 1974).

Table 2.7 Heavy Metals Found in Major Industries (Source: Bond and Straub, 1974)

Industry	Al	As	Cd	Cr	Cu	Hg	Pb	Ni	Zn
Pulp & paper mill				X	X	X	X	X	X
Organic chemicals	X	X	X	X		X	X		X
Alcalies, Chlorine	X	X	X	X		X	X		X
Fertilizers	X	X	X	X	X	X	X	X	X
Petroleum refines.	X	X	X	X	X		X	X	X
Steelworks		X	X	X	X	X	X	X	X
Aircraft plating, finishing	X		X	X	X	X		X	
Flat glass, cement				X					
Textile mills				X					
Tanning				X					
Power plants				X					

X = Found heavy metals in major industries

Even if toxic materials are not present in concentrations likely to affect humans, they might well be at phytotoxic levels, which would limit their agricultural use. However, from the point of view of health, a very important consideration in management of wastewater, the contaminants of greatest concern are the pathogenic micro- and macro-organisms.

2.5.1.2.1 Wastewater in food industry

Wastewater generated from agricultural and food operations has distinctive characteristics that set it apart from common industrial wastewater managed. it is biodegradable and nontoxic, but that has high concentrations of biochemical oxygen demand (BOD) and suspended solids (SS). The constituents of food and agriculture wastewater are often complex to predict due to the differences in BOD and pH in effluents from vegetable, fruit, and meat products and due to the seasonal nature of food processing and post harvesting.

Processing of food from raw materials requires large volumes of high grade water. Vegetable washing generates waters with high loads of particulate matter and some dissolved organics. It may also contain surfactants. Animal slaughter and processing produces very strong organic waste from body fluids, such as blood, and gut contents (Table 2.8). This wastewater is frequently contaminated by significant levels of antibiotics and growth hormones from the animals and by a variety of pesticides used to control external parasites. An insecticide residue in fleeces is a particular problem in treating waters generated in wool processing (Biljana, 2007).

Table 2.8 Average % of waste/by-product formation in food industrial processes (Source: Biljana, 2007).

Process	% waste or by-product	Waste/by-product
Fish canning	30 - 65	Rejected fish: heads, offal, tails, skins, bones, et al.
Beef slaughtering	40 - 52	Head, tail, udder, hooves, hides, et al.
Fresh, soft and coohed cheese production	85 – 90	Whey.
Fruit and vegetable juice production	30 – 50	Stem, stalks, rotten fruit, peels, seeds, et al.
Sugar production from sugar beet	86	Beet pulp, carbonation lime, molasses, et al.

Processing food for sale produces wastes generated from cooking which are often rich in plant organic material and may also contain salt, flavorings, coloring material and acids or alkali. Very significant quantities of oil or fats may also be present (Metcalf and Eddy, 1991).

2.5.2 Wastewater management

Wastewater reclamation and reuse has been globally accepted as a suitable solution to the serious water shortage around the world (Sun and Ou, 1994). In the past several decades, many techniques have been developed to find an economic and efficient way to reclaim the wastewater, including physical, chemical and biological treatment such as active sludge, trickling filtration system, lagoon, ozone oxidation, floatation, sedimentation, land treatment system and wetland system (Overcash and Pal, 1979; Gao, 1986; Jin, 1993; Raisin and Mitchell, 1994; Zhao and Wang, 1994). These technologies are usually highly efficient for wastewater containing special pollutants. Some of them are so successful as to have been widely used in the treatment of municipal and industrial wastewater in developed countries (Overcash and Pal, 1979; Jin, 1993). However, disposal of wastewaters from an industrial plant is a difficult and costly problem (Tchobanoglous et al., 2003). High capital construction costs and high operation costs limited their application to a great extent, especially in developing countries. As a result, natural and ecological processes that were effective and economic were sought and studies (Ou et al., 1992).

Many different types of organisms are particularly plentiful in wastewater and accomplish most of the treatment. Bacteria and other microorganisms live in wastewater and some are essential contributors to treatment. A variety of bacteria, protozoa, and worms work to break down certain carbon-based (organic) pollutants in wastewater by consuming them. Through this process, organisms turn wastes into carbon dioxide, water, or new cell growth. Most wastewater treatment systems are designed to rely in large part on biological processes. Aerobic and anaerobic processes are widely applied in the treatment of wastewaters and biological sludge.

Some wastewater may be highly treated and reused as reclaimed water. Ammonias and nitrates can be removed from wastewater by microbial nitrification and denitrification respectively (Baronti, et. all, 2000; Fujii, et. all., 2002; D'Ascenzo, et. all., 2003; Chao, et. all., 2004; Haiyan, et. all, 2007).

2.5.3 Wastewater quality indicators

Any oxidizable material present in a natural waterway or in wastewater will be oxidized both by biochemical (bacterial) or chemical processes. The result is that the oxygen content of the water will be decreased. Basically, the reaction for biochemical oxidation may be written as in Figure 2.5:

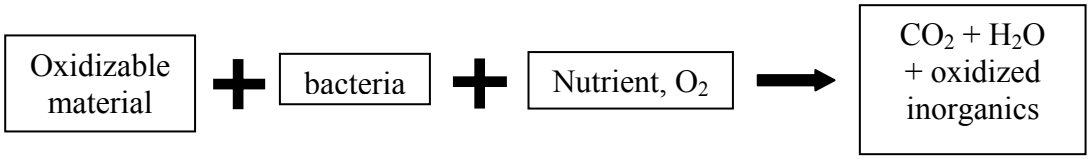


Figure 2.5 The reaction for biochemical oxidation

Since all wastewaters contain many materials, bacteria, O_2, and nutrients, it will be introduced into the biochemical reactions (Figure 2.9). Consequently, this biochemical reaction create what is measured in the laboratory as the Biochemical oxygen demand (BOD) and Chemical oxygen demand (COD) to determine the concentration of oxidizable organic compounds to indicate the quality of wastewater. Both the BOD and COD tests are a measure of the relative oxygen-depletion effect of a waste contaminant. Both have been widely adopted as a measure of pollution effect. The BOD test measures the oxygen demand of biodegradable pollutants whereas the

COD test measures the oxygen demand of biogradable pollutants plus the oxygen demand of non-biodegradable oxidizable pollutants (Bond, and Straub, 1974; Overcash and Pal, 1979; Gao, 1986; Metcalf, and Eddy, 1991; Ou et al., 1992; Jin, 1993; Raisin and Mitchell, 1994; Sun and Ou, 1994; Zhao and Wang, 1994; Tieheng, et. all., 1998; Tchobanoglous et al., 2003).

2.6 Estrogen Hormones

2.6.1 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).

2.6.2 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 2.6).

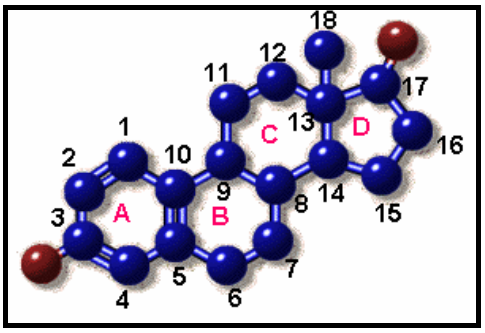
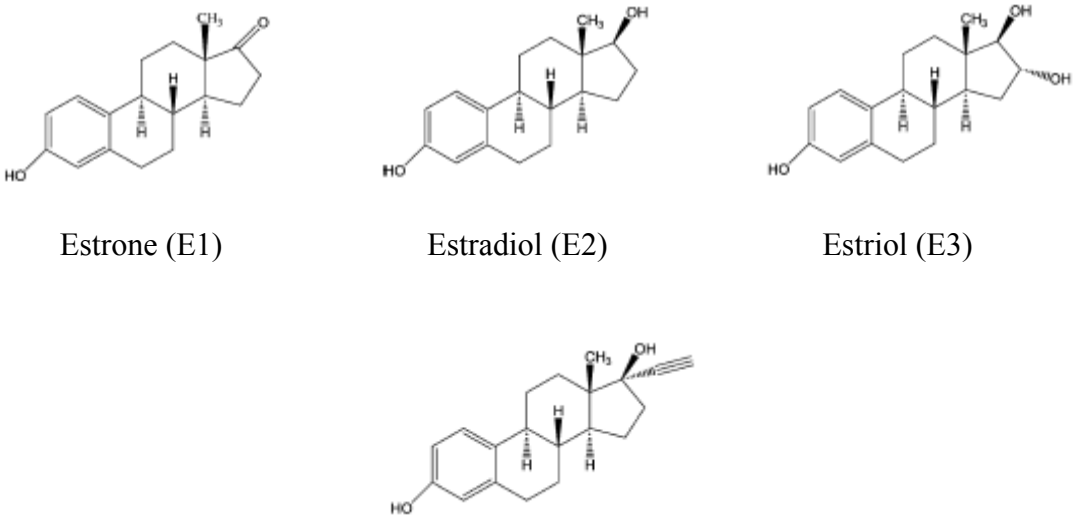


Figure 2.6 Structure of estrogen

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 2.7).



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

Ethynylestradiol (EE2)

Figure 2.7 Structure of estrogens

2.6.3 Physicochemical properties of estrogens

The physicochemical properties of natural and synthetic estrogens in free form are shown in Table 2.9. 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 2.9 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

(Source: Lai et al., 2000)

2.6.4 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 2.8). 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).

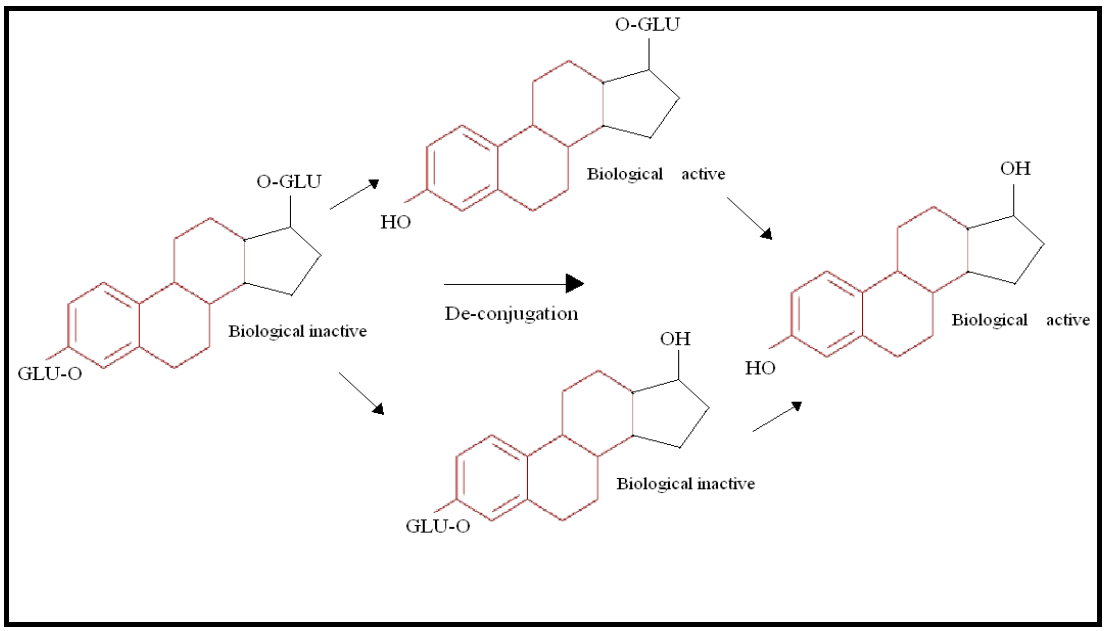


Figure 2.8 De-conjugation of 17β-estradiol (E2) into biological active compounds Available from: Flemming and Bent (2003)

2.6.5 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).

2.6.6 Adverse effects of estrogens

2.6.6.1 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).

2.6.6.2 Effects of estrogens on living organisms

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). 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. 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.

2.6.7 Sources of estrogens

2.6.7.1 Estrogens from humans

Generally the endogenous excretion of hormones by healthy pre-menopausal women is reported to range from 10 to 100 μg.day^{-1} (Table 2.10). Menstruating women excrete 8 μg.day^{-1} of E1, 3.5 μg.day^{-1} of E2 and 4.8 μg.day^{-1} of E3. After menopause, women only excrete 4 μg.day^{-1} of E1, 2.3 μg.day^{-1} of E2 and 1 μg.day^{-1} of E3. Pregnant women excrete 600 μg.day^{-1} of E1, 259 μg.day^{-1} of E2 and 600 μg.day^{-1} 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^{-1} of E1, 1.6 μg.day^{-1} of E2 and 1.5 μg.day^{-1} of E3 in their urine (Johnson et al., 2000).

Table 2.10 Daily excretions (μg) of estrogens in humans (Source: Johnson et al., 2000)

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	-

2.6.7.2 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).

2.6.8 Level of estrogens in the environments

2.6.8.1 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 2.11). 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 2.11 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

2.6.8.2 Level of estrogens in 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 2.12). 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 2.12 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]	-	-	Nasu et al. (2000)
		30-90[b]			

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

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 2.13). 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 2.13 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

2.6.9 Transformation of estrogens

2.6.9.1 Biotransformation by metabolisms

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).

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).

2.6.9.2 Biotransformation by co-metabolisms

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).

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).

2.6.9.3 Abiotic transformation

Previous work based on batch tests with AOB and nitrifying activated sludge at high EE2 concentrations (>300 mg/L) and high NH4sN concentrations (>200mg/L) has led to the hypothesis that ammonia oxidizing bacteria cometabolically degrade

EE2. However, the current study showed that abiotic assays with growth medium confirmed EE2 removal by nitration, which is enhanced at low pH (<7.0) and high NO2sN concentrations (Gaulke et al., 2008).

2.6.10 Measurement of estrogens in environments

2.6.10.1 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.

2.6.10.2 Sample preparation

2.6.10.2.1 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.

2.6.10.2.2 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).

2.6.10.2.3 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.

2.6.10.3 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.

2.6.10.4 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).

2.6.10.5 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.

CHAPTER III

METHODOLOGY

3.1 Experimental framework

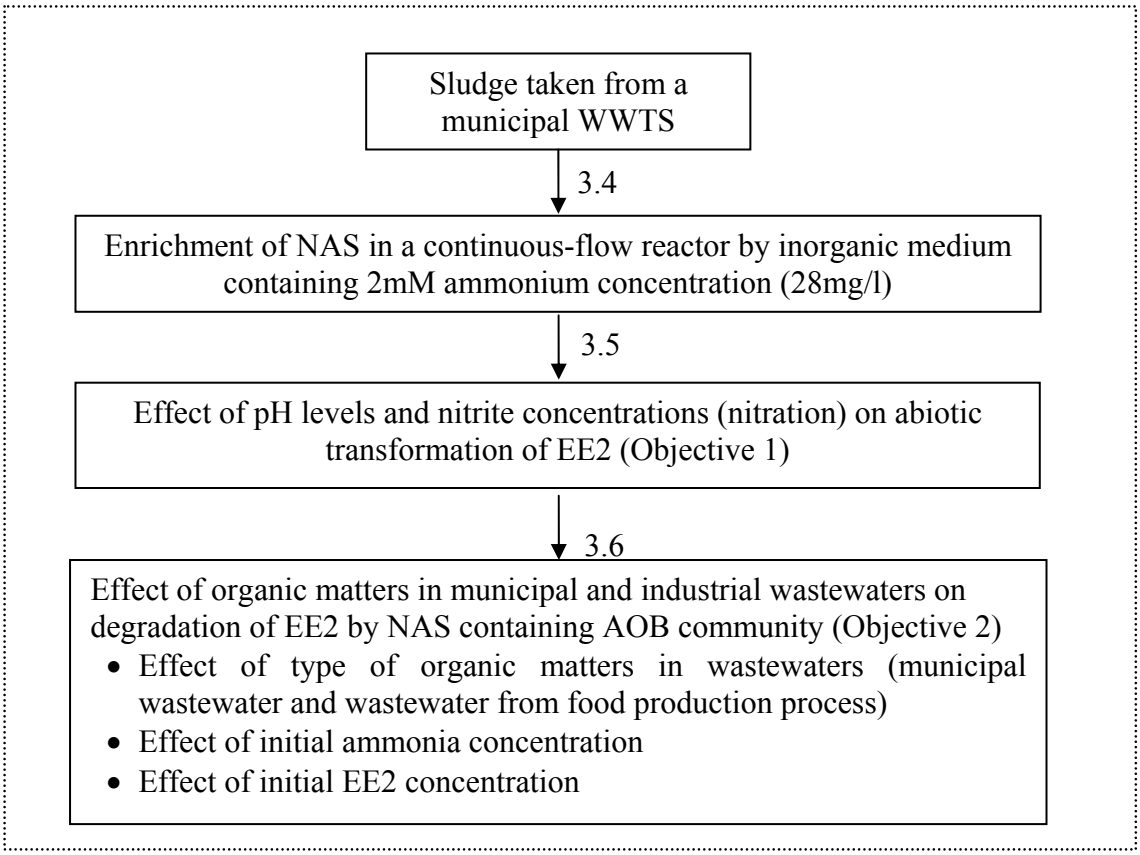


Figure 3.1 Experimental framework

The main part of this study concerns the effect of organic compounds on degradation of EE2 by NAS. Experiment is divided into 3 parts (Figure 3.1). The first part is enrichment of NAS in a continuous-flow reactor by using inorganic medium containing 2mM of ammonium concentration (28 mg/l). This part of experiment aims to enrich AOB under the ammonia level close to the actual municipal wastewater treatment systems.

The second part is to study the effect of pH levels and nitrite concentrations (nitration) on abiotic transformation of EE2. The current study with abiotic assays in growth medium confirmed the removal of EE2 by nitration, which is enhanced at low pH levels (< 7.0) and high NO_2^--N concentrations. The tests were conducted under three different ammonia concentrations (2, 10, and 30 mM) by varying pH levels and nitrite concentrations at fixed EE2 concentration of 10 mg/l. The cases of EE2 transformation with 30 mM of ammonia concentration and 70 mg of nitrite concentration represented the high concentration of nitrogen condition. The cases of EE2 transformation with 10mM of ammonia concentration and 45 mg of nitrite concentration represented the medium concentration of nitrogen condition. The cases of EE2 transformation with 2mM of ammonia concentration and 12 mg of nitrite concentration represented the low nitrogen condition. This part aim to confirm the range of pH levels on abiotic transformation occurs and applied to used for further step.

The last part is to study effect of organic matters in municipal and industrial wastewaters on degradation of EE2 by NAS containing AOB community. In the actual phenomena, there are many organic matters in the wastewaters. Municipal wastewater and wastewater from food production process were selected as model compounds (separated study). Municipal wastewater belonging to Bangkok Metropolitan Administration (BMA) was selected as a representative of municipal wastewater with low level of organic matters. Wastewater from food production process was selected as a representative of industrial wastewater with high level of organic matters. NAS containing AOB community were tested for their ability to degrade 3.5 and 10 mg/l of EE2 under different ammonium concentrations. NAS from

2 mM reactor was 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 plants. Degradation of EE2 (3.5 mg/l) by NAS under 2mM of ammonium concentrations represents the actual phenomena in wastewater treatment systems whereas degradation of EE2 (10 mg/l) by NAS under 2 mM of ammonium concentrations represents the effect of initial EE2 concentrations and degradation of EE2 (3.5 mg/l) by NAS under 30 mM of ammonium concentrations represents the effect of initial ammonium concentrations. Only EE2 (3.5 mg/l) was tested with both municipal and industrial wastewaters for ability of NAS under 2mM of ammonium concentrations to observe effect of wastewater from different source.

3.2 Materials and apparatus

3.2.1 Chemicals

EE2 (>98% pure) was purchased from Sigma (St.Louis, MO, USA). Stock solutions of EE2 were prepared to 50 mg/l in methanol.

3.2.2 Media

3.2.2.1 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 $NaHCO_3$.

3.2.2.2 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 $m^3.day^{-1}$. 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.

3.2.4 Wastewater

Municipal wastewater was taken from Huamark municipal wastewater treatment plant which belongs to Bangkok Metropolitan Administration (BMA). On the day of sampling, biological oxygen demand (BOD) and chemical oxygen demand

(COD) concentrations in wastewater were 48.50 mg/l and 164.47 mg/l, respectively, whereas ammonium concentration was 12.35 mg N/l. Wastewater from food production process was taken from a wastewater tank of a food factory wastewater treatment plant. On the day of sampling, biological oxygen demand (BOD) and chemical oxygen demand (COD) in wastewater was 3,463.95 mg/l and 4,216.33 mg/l, respectively, whereas ammonium concentration was 113.18 mg N/l. The characteristic of wastewater were as shown in Table 3.1. Wastewaters were autoclaved at 121^{o}C for 30 minutes and measured by measurement methods as described below.

Table 3.1 Characteristic of wastewaters.

Parameters	Municipal wastewater	Wastewater from food production process
COD concentration (mg/l)	164.47	4216.33
BOD concentration (mg/l)	48.50	3463.95
Total suspended solid (TSS) (mg/l)	184.02	2041.53
Dissolved solids (TDS) (mg/l)	213.04	1483.28
Ammonia concentration (mg/l)	12.35	113.18
Nitrite concentration (mg/l)	2.90	35.9252
Nitrate concentration (mg/l)	0.02	16.9526
Total inorganic nitrogen (mg/l)	15.27	166.0578

3.3 Sample preparation and analytical methods

3.3.1 Sample preparation

Equal volume of methanol (5 ml) was added into test tube containing remaining liquid medium (5 ml). Test tube was then vortexed to allow completely dissolving EE2.

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. 2 ml of dilution sample and 0.04 mL of phenol solution (Mix 11.1 mL liquefied phenol (≥89%) with 95 % v/v ethyl alcohol to a final volume of 100 mL) were added and then mixed. 0.04 mL of sodium nitroprusside solution (0.5% w/v: dissolve 0.5 g of sodium nitropusside in 100 mL of deionized water), and 0.25 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) (Phenate method, Standard Method for the Examination of Water and Wastewater 20^{th} edition).

3.3.3 Measurement of nitrite

Inorganic medium added with methanol was diluted with deionized water. 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 incubated 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) (Phenate method, Standard Method for the Examination of Water and Wastewater 20th edition).

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. 2 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) (Phenate method, Standard Method for the Examination of Water and Wastewater 20th edition).

3.3.5 Measurement of EE2

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 EE2 was 11.094 min.

3.3.6 Measurement of COD using closed reflux method

2.5 ml of sample, 1.5 ml of 0.0167M standard potassium dichromate digestion solution (4.913 g of potassium dichromate, 167 ml conc. Sulfuric acid, and 33.3 g of mercury sulfate dissolved in 1 l of deionized water), and 3.5 ml of silver sulfate (5.5 g

of silver sulfate in 1 kg of sulfuric acid) were added in digestion vessel and allowed to react for 2 hr in oven preheated to 150°c. After cool to room temperature, solutions were titrated with standard ferrous ammonium sulfate titrant (39.2 g of iron ammonium sulphate) 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 blue-green to reddish brown. In the same manner, a blank containing the reagents and volume of distilled water equal to that of the sample is refluxed and titrated (American Public Health Association, 1992).

3.4 Enrichment of nitrifying activated sludge by inorganic medium containing 2mM ammonium concentration

This experiment aimed to develop NAS containing AOB community. Sludge taken from a municipal wastewater treatment system was enriched in laboratory-scale continuous flow reactors without sludge recycling introduced with inorganic medium containing ammonium concentration: 2 mM NH_4^+-N (28 mg-N/l). Total volume of reactor was 5 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 between 7.5-8.0 using 1 N of HCl and 1 N of NaOH, and mixing was provided at rotating speed of 300 rpm. Inorganic medium was introduced into all reactors at a fixed dilution rate of 0.01 hr^{-1} **(**Limpiyakorn et al., 2007)

3.5 Effect of pH levels and nitrite concentrations (nitration) on abiotic transformation of EE2

This experiment aimed to study the effect of pH levels and nitrite concentrations (nitration) on abiotic transformation of EE2 (Objective 2).

Three parallel batch tests (Table 3.2) were performed in triplicate for each study with EE2 (10mg/l). In the first transformation test, EE2 was added into 5 ml inorganic medium containing ammonium (30 mM of NH_4^+-N), sodium nitrite (70mg of NO_2^--N) and HCl (40 mg/l) to obtain pH levels (6.0, 6.2, 6.4, 6.8, 7.0, and 8.0) (Test 1). The second transformation test, EE2 was added into 5 ml inorganic medium containing ammonium (10 mM of NH_4^+-N), sodium nitrite (45mg of NO_2^--N) and HCl (40 mg/l) to obtain pH levels (6.0, 6.2, 6.4, 6.8, and 7.0) (Test 2). The last transformation test, EE2 was added into 5 ml inorganic medium containing ammonium (2 mM of NH_4^+-N), sodium nitrite (12mg of NO_2^--N) and HCl (40 mg/l) to obtain pH levels (6.0, 6.2, 6.4, 6.8, and 7.0) (Test 3). 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 EE2 and nitrite were analyzed as described above.

Table 3.2 Effect of pH levels and nitrite concentrations on transformation of EE2 in batch tests

Test	Ammonia concentration (mM)	Nitrite concentration (mg)	pH level					
			6.0	6.2	6.4	6.8	7.0	8.0
1	30	70	+	+	+	+	+	+
2	10	45	+	+	+	+	+	-
3	2	12	+	+	+	+	+	-

Symbols and Abbreviations: +, with; –, without

3.6 Effect of organic matters in wastewaters on degradation of EE2 by AOB in NAS

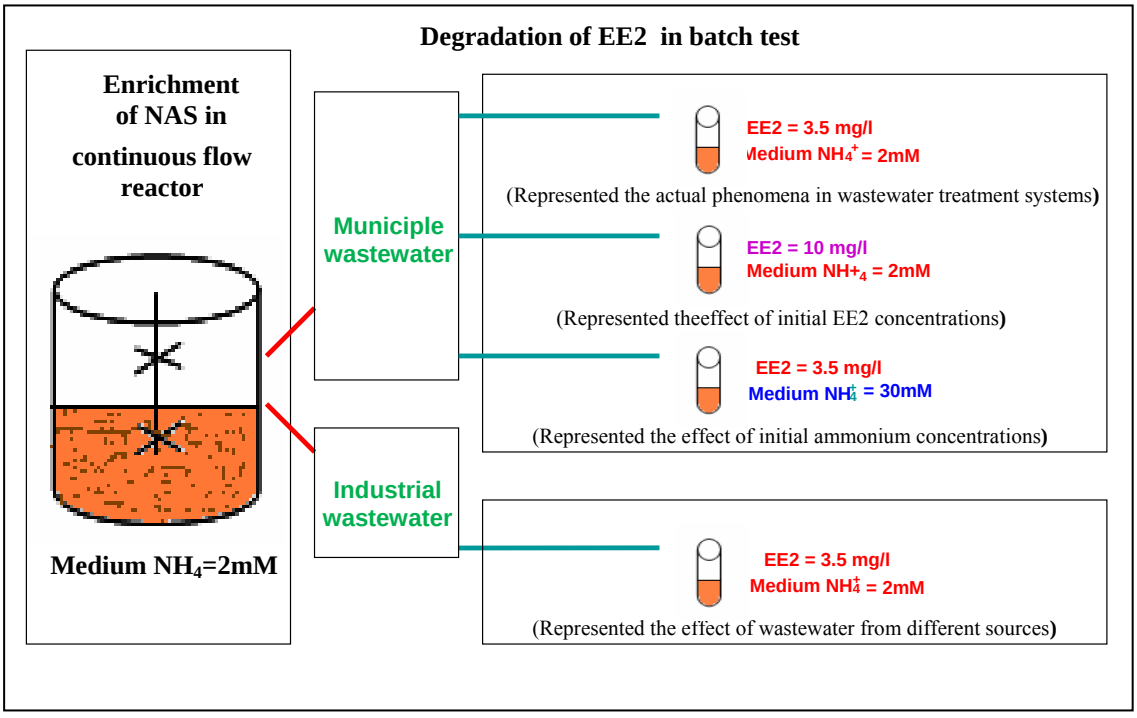


Figure 3.2 Effect of organic matters in wastewaters on degradation of EE2 by AOB in nitrifying-activated sludge NAS

This experiment aimed to analyze effect of organic matters in different wastewaters on degradation of EE2 by NAS containing AOB communities (2mM) (Objective 2).

Experiment was divided into 4 parts including the study with municipal wastewater under 2 mM of ammonia concentration and 3.5 mg/l of EE2 concentration (study 1), industrial wastewater under 2 mM of ammonia concentration and 3.5 mg/l of EE2 concentration (study 2), municipal wastewater under 30 mM of ammonia concentration and 3.5 mg/l of EE2 concentration (study 3), and municipal wastewater under 2 mM of ammonia concentration and 10 mg/l of EE2 concentration (study 4).

Each part comprised of six parallel batch tests (Table 3.3); four degradation tests, one control test, and one inhibition test were performed in triplicate for each study. In the degradation test, NAS (final MLSS concentration of 150 mg/l) were added into 5 ml of inorganic medium containing EE2 (3.5, or 10 mg/l), ammonium (2mM, or 30mM of NH_4^+-N), and wastewater (municipal or industrial) (0, 70, and 140 mg/l of COD for municipal wastewater and 0, 70, 140, 1000, and 2000 mg/l of COD for industrial wastewater). Inhibition test and control test were prepared in the same manner as the degradation test except that for the 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 were taken periodically. Concentrations of ammonium, nitrite, nitrate, EE2, and COD were analyzed as described previously.

Table 3.3 Six parallels batch tests in each study.

Test	Ammonium	NAS	EE2	Wastewater	Allythiourea
Degradation 1 (Medium)	+	+	-	-	-
Degradation 2 (EE2)	+	+	+	-	-
Degradation 3 (Wastewater)	+	+	-	+	-
Degradation 4 (EE2+Wastewater)	+	+	+	+	-
Inhibition 1 (EE2+Wastewater+Inhibitor)	+	+	+	+	+
Control 1 (EE2+Wastewater+Control)	+	-	+	+	-

Symbols and Abbreviations: +, with; –, without

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Effect of pH levels and nitrite concentrations (nitration) on abiotic transformation of EE2

Increased removal of EE2 has been reported for the treatment of wastewater by nitrifying activated sludge (NAS) processes where long enough solid retention time must be provided. Previous works based on batch tests with NAS and pure cultures of AOB at high EE2 concentrations (>300 mg/l) and high NH_4N concentrations (>200 mg/l) has led to the hypothesis that ammonia oxidizing bacteria cometabolically degraded EE2 (Clara et al., 2005; Shi et al., 2002; Shi et al., 2004; Vader et al., 2002; Yi and Harper, 2007; Yoshimoto et al., 2004). However, the current study with abiotic assays in growth medium confirmed the removal of EE2 by nitration, which is enhanced at low pH levels (< 7.0) and high NO_2^--N concentrations (Gaulke et al., 2008).

To confirm the hypothesis that abiotic transformation of EE2 occurs only at low pH levels (< 7.0), the abiotic tests were conducted. In this study, after the pH rang is defined EE2 degradation study will be done at above the effective pH level to avoid abiotic transformation of EE2 in our study. The abiotic tests were carried out with three initial ammonia concentrations of 2, 10, and 30 mM, the ammonium ranges that will be used in the later parts foe the EE2 degradation study. With each ammonium concentration, pH levels were varied in the range of 6.0 - 8.0 and nitrite concentrations were fixed (12 mg-N/l of NO_2^--N with 2 mM of NH_4^+-N, 40 mg of NO_2^--N with 10 mM of NH_4^+-N, and 70 mg of NO_2^--N with 30 mM of NH_4^+-N) at the

highest nitrite concentrations accumulated in the previous study (Sermwaraphan, 2006). As high as 10 mg/l of EE2, was selected to show the clear effect of EE2 transformation and also to ensure the detectability of EE2 during the transformation.

By comparing the amounts of EE2 transformation over the period of 240 hours, the results were divided in to three different groups; Group A, Group B, and Group C exhibiting lowest, medium, and highest EE2 transformation, respectively (Figure 4.1).

Group A; Figure 4.2-a showed that EE2 concentrations of group A remained the same throughout the experiment. No remarkable (around 10%) transformation of EE2 was observed. The initial pH levels found in the group A was in the range of initial pH 6.8 – 8.0 (30 mM of NH_4^+-N with 70 mg-N/l of NO_2^--N at pH 6.8, 7.0 and 8.0, 10 mM of NH_4^+-N with 45 mg-N/l of NO_2^--N at pH 6.8 and 7.0, 2 mM of NH_4^+-N with 12 mg-N/l of NO_2^--N at pH 6.8 and 7.0). However, in group A, all initial nitrite concentrations of 12, 45, and 70 mg were covered. These results suggested that nitrite concentration did not affect EE2 transformation.

Group B; EE2 concentrations dramatically decreased during the first 24 hours and remain stable after 24 hours. Incomplete EE2 transformations were found in all cases (30 mM of NH_4^+-N with 70 mg-N/l of NO_2^--N at pH 6.4; 10 mM of NH_4^+-N with 45 mg-N/l of NO_2^--N at pH 6.4; 2 mM of NH_4^+-N with 12 mg-N/l of NO_2^--N at pH 6.4). The pH levels found for group B were in a range of initial pH 6.4 – 6.8. Also this phenomenon occur at all initial nitrite concentrations (12, 45, and 70 mg).

Group C; EE2 concentrations dramatically decreased during the first 24 hours and gradually decreased after 24 hours. Complete EE2 degradations occurred after hour 216 and 240 in most cases (10 mM of NH_4^+-N with 45 mg-N/l of NO_2^--N at pH

6.0 and 2 mM of NH_4^+-N with 12 mg-N/l of NO_2^--N at pH 6.0 respectively), while in some cases incomplete EE2 degradations were found (30 mM of NH_4^+-N with 70 mg-N/l of NO_2^--N at pH 6.0 and 6.2, NH_4^+-N 10 mM with 45 mg-N/l of NO_2^--N at pH 6.0 and 6.2, 2 mM of NH_4^+-N with 12 mg-N/l of NO_2^--N at pH 6.0 and 6.2). pH levels in this case were between 6.0 - 6.2. These also happened with all initial nitrite concentrations (12, 45, and 70 mg).

In conclusion, Figure 4.1 confirmed that EE2 transformation can occur abiotically. EE2 transformation was pH dependent. The EE2 transformation rates at pH 6.0 were higher to those of 6.2 higher to those of 6.4. And at pH > 6.8, abiotic transformation of EE2 did not occur. In addition, initial nitrite concentrations showed no effect on abiotic EE2 transformation (Figure 4.2) Therefore, in later parts of the study, pH will be monitored along the time in all tests to confirm that abiotic transformation of EE2 do not involve in EE2 degradation.

pH played more important role than initial nitrite concentrations in abiotic transformation of EE2.

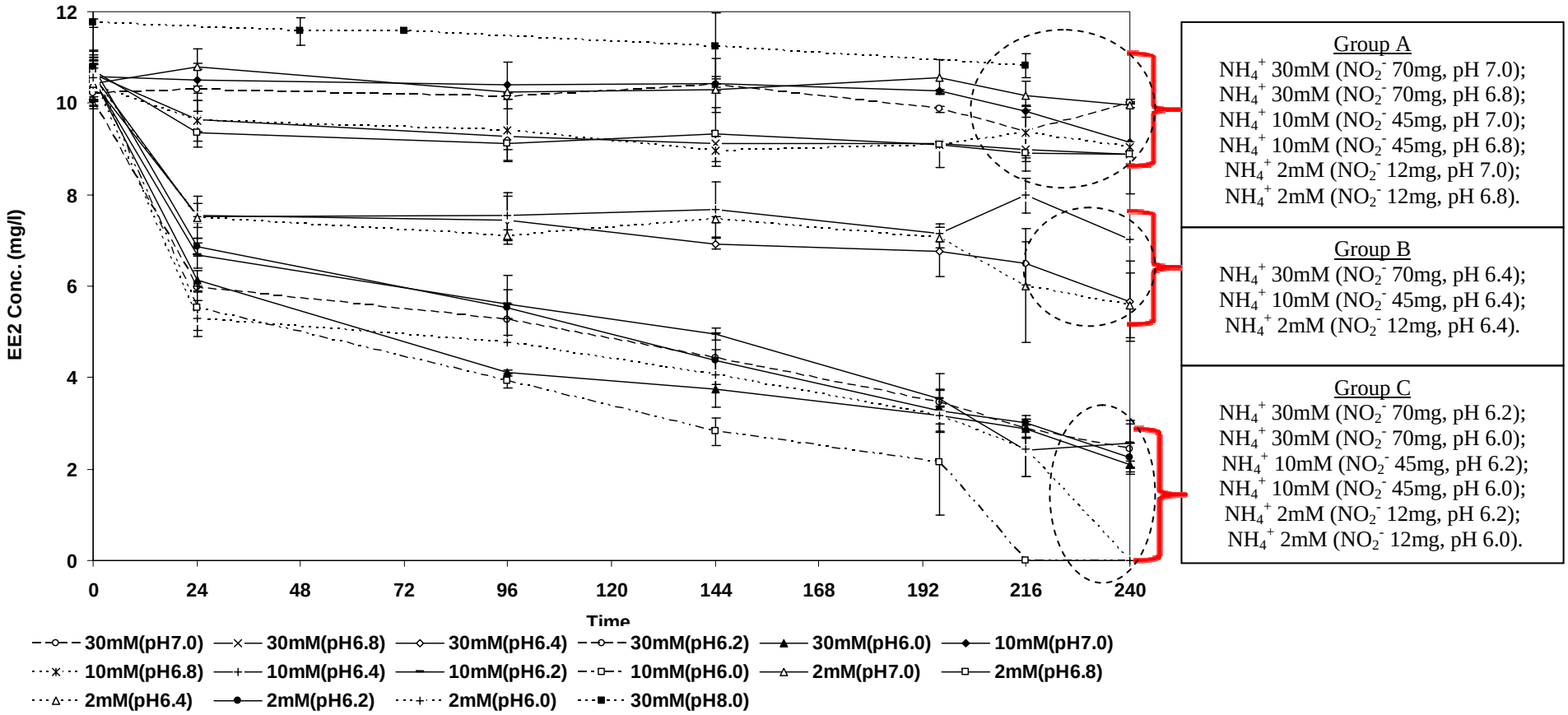


Figure 4.1: EE2 concentrations in abiotic assay.

Symbols and Abbreviations: amM(pHb); a = NH_4^+ concentration, b = pH level
For example; 30mM(pH7.0) = 30mM of NH_4^+ at pH 7.0, 10mM(pH6.8) = 10mM of NH_4^+ at pH 6.8, 2mM(pH6.4) = 2mM of NH_4^+ at pH 6.4.

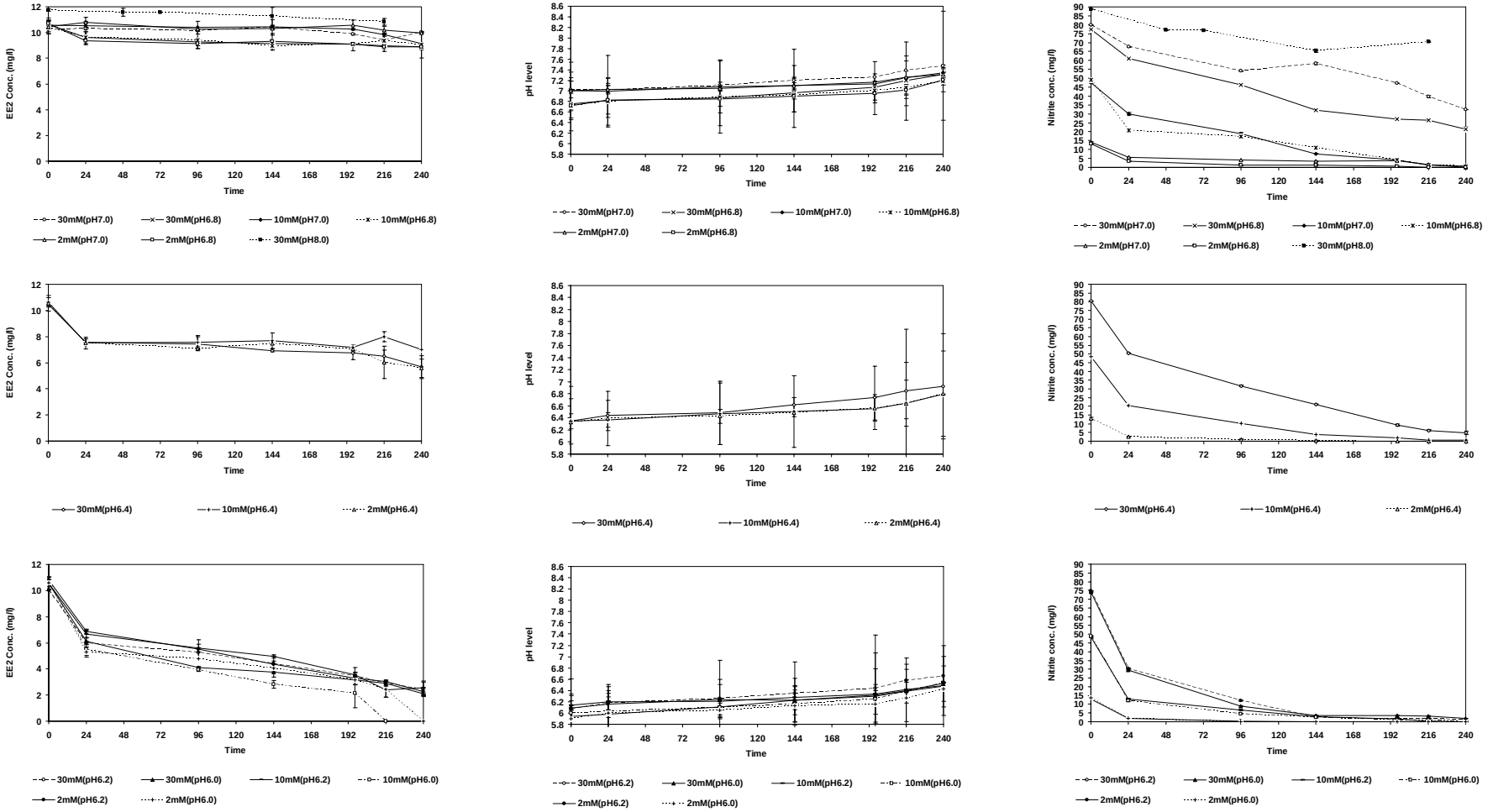


Figure 4.2: Effect of pH levels and nitrite concentrations on EE2 transformation; a) Group A, b) Group B, c) Group c.

Symbols and Abbreviations: a mM (pH b); a = NH_4^+ concentration, b = pH level
For example; 30 mM (pH7.0) = 30mM of NH_4^+ at pH 7.0, 10 mM (pH6.8) = 10mM of NH_4^+ at pH 6.8, 2 mM (pH6.4) = 2 mM of NH_4^+ at pH 6.4.

4.2 Competitive effect of organic matters (COD concentrations) in wastewaters on degradation of EE2 by nitrifying activated sludge

EE2 can be released into the environments by excretion of humans and animals through their urine and feces. Municipal wastewater treatment plant is an important facility that markedly reduced the concentrations of EE2 in municipal wastewater. In NAS, EE2 has been proven to be degraded by AOB via co-metabolism. AOB is capable of cometabolising several organic compounds. So far, no research mentions on the competitive effect of non target organic compounds on co-metabolism of target organic compounds by AOB. In fact, organic compounds in wastewater can result in retarding EE2 degradation by competing EE2 for active site of ammonia monooxynase (AMO) enzyme. This experiment was conducted to investigate the effect of organic matters (COD concentrations) in wastewaters on degradation of EE2 by NAS.

Experiment was divided into 4 parts including the study with municipal wastewater under 2 mM of ammonia concentration and 3.5 mg/l of EE2 concentration (study 1), industrial wastewater under 2 mM of ammonia concentration and 3.5 mg/l of EE2 concentration (study 2), municipal wastewater under 30 mM of ammonia concentration and 3.5 mg/l EE2 of concentration (study 3), and municipal wastewater under 2 mM of ammonia concentration and 10 mg/l EE2 of concentration (study 4). Each part comprised of six parallel batch tests; four degradation tests, one control test, and one inhibition test each of which was performed in triplicate.

- Combination of parts 1 and 2 will explain effect of type of wastewater.
- Combination of parts 1 and 3 will explain effect of initial ammonium concentration.

- Combination of parts 1 and 4 will explain effect of initial EE2 concentration.

4.2.1 Effect of type of wastewaters

Municipal and industrial wastewaters, which have different patterns of organic matters and thus inhibitor behaviors, were selected as model compounds (separated study). Municipal wastewater belonging to Bangkok Metropolitan Administration (BMA) was selected to represent wastewater that contained low strength in level of organic matters. Wastewater from food factory was selected as to represent wastewater with high strength level of organic matters. NAS from 2 mM reactor was selected for the test as the community of AOB in this NAS was similar to those in full-scale municipal wastewater treatment systems (Sonthiphand, 2008). Degradation of EE2 (3.5 mg/l) by NAS was tested under 2mM of ammonium concentration.

Wastewaters were autoclaved at 121^{o}C for 30 minutes and measured by measurement methods as described above (Table 4.1).

Table 4.1 Characteristic of autoclaved wastewaters.

Parameters	Municipal wastewater	Wastewater from food production process
COD concentration (mg/l)	177.50	2516.10
Ammonia concentration (mg/l)	11.7114	0.8201
Nitrite concentration (mg/l)	0.2795	0.4142
Nitrate concentration (mg/l)	5.4231	415.4667
Total inorganic nitrogen (mg/l)	17.414	416.701

Figure 4.3 shows degradation of EE2 (3.5 mg/l) by NAS in the presence of municipal and industrial wastewaters. Figure 4.3-a1 and 4.3-a2 show nitrogen concentrations in the selected test of 140 mg/l COD concentration of municipal

wastewater and 2000 mg/l COD concentration of industrial wastewater, respectively. Results 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 decreased whereas EE2 concentrations in the inhibition tests decreased in-significantly. In the case of inhibition tests, the highest amount of EE2 was 7.26 % in the case of no municipal wastewater (COD concentration of 0 mg/l). This suggested that EE2 were degraded mainly by AOB in NAS.

4.2.1.1 Effect of type of wastewaters on EE2 degradation

With the municipal wastewater, acclimation periods of more than 3 days were required for EE2 degradation with all COD concentrations (Figure 4.3-b1). Complete EE2 degradations occurred after day 12, 13, and 14 (with 0, 70, and 140 mg/l of COD concentrations respectively). For 0, 70, and 140 mg/l of COD concentrations, the degradation rates were -0.3495, -0.0574, and -0.0332 $mg.day^{-1}$ respectively (Table 4.2). These results suggested that organic matters in municipal wastewater deteriorated EE2 degradation and EE2 degradation was COD concentration dependent (Figure 4.4-a1).

In contrast, in the case of industrial wastewater, more than 3 days acclimation periods were acquired and incomplete EE2 degradations were found (Figure 4.3-b2). Complete EE2 degradations occurred after day 15 only in the case with no wastewater

(COD concentration = 0 mg/l). At day 18 the EE2 removals were 100 %, 70.41 %, 73 %, 73.05 %, and 74.14 % (with COD concentrations of 70, 140, 1000 and 2000 mg/l, respectively). For COD concentrations of 0, 70, 140, 1000 and 2000 mg/l, the degradation rates were -0.1487, -0.0932, -0.0981, -0.0965, and -0.0984 $mg.day^{-1}$, respectively (Table 4.2). These results showed organic matters in industrial wastewater retarded the degradation of EE2 but degradation of EE2 was independent from COD concentrations (Figure 4.4-a2).

Overall results show the different patterns of EE2 degradation in the present of municipal and industrial wastewater. This may caused by difference in composition of wastewater that came from different sources of wastewater. Organic matters in wastewaters exhibited inhibition behaviors differently. In this study, organic matters in industrial wastewater more highly deteriorated EE2 degradation than those in municipal wastewater as can be seen from the degradation rate (Table 4.2). In addition, in the case of municipal wastewater, the higher the organic matters (COD concentration), the less cometabolism of EE2 was found. But in the case of industrial wastewater, the present of COD concentration deteriorated EE2 degradation with no COD concentration dependence.

Table 4.2 Degradation of EE2 (3.5 mg/l) by NAS in the presence of municipal and industrial wastewaters**.**

Wastewater	COD concentration (mg/l)	EE2 loss (mg)	EE2 removal (%)	EE2 degradation rate	NH_4^+ loss (mg)	NH_4^+ oxidation (%)	NH_4^+ oxidation rate
Municipal wastewater	0	3.7479	100	-0.3495	28.6908	100	-0.5667
	70	3.6843	100	-0.0574	29.2102	100	-0.6167
	140	3.7299	100	-0.0332	29.1622	100	-0.4644
Industrial wastewater	0	3.6461	100	-0.1487	28.8146	100	-0.2961
	70	2.5755	70.41	-0.0932	28.9856	100	-0.2560
	140	2.6404	73	-0.0981	27.2827	94.06	-0.1152
	1000	2.6509	73.05	-0.0965	26.0328	89.70	-0.0976
	2000	2.6987	74.14	-0.0984	24.5268	84.91	-0.0701

4.2.1.2 Effect of type of wastewaters on ammonia oxidation

With municipal wastewater, complete ammonia oxidations occurred after day 12, 10, and 9 (COD concentrations of 0, 70, and 140 mg/l respectively) (Figure 4.3-c1). For COD concentrations of 0, 70, and 140 mg/l, the degradation rates were -0.5667, -0.6167, and -0.4644 $mg.day^{-1}$ respectively (Table 4.2). These suggested that organic matters in wastewater were did not deteriorate ammonia oxidation (Figure 4.4-b1).

In contrast, in the case of industrial wastewater, complete ammonia oxidations occurred after day 12 and 15 only in the cases of COD concentrations of 0 and 70 mg/l respectively (Figure 4.3-c2). In the cases of COD concentrations of 140, 1000 and 2000 mg/l, 94.06 %, 89.70 %, and 84.91 % of ammonia oxidation achieved in 18 days. For COD concentrations of 0, 70, 140, 1000 and 2000 mg/l, the degradation rates were -0.2961, -0.256, -0.1152, -0.0976, and -0.0701 $mg.day^{-1}$ respectively (Table 4.2). And these results showed clearly that organic matters in industrial wastewater decelerated the ammonia oxidation. The higher the initial COD

concentration of industrial wastewater, the more oxidation of ammonia was deteriorated (Figure 4.4-a2).

In summary, in the cases of municipal wastewater, no significant difference in ammonium oxidation between the degradation tests (COD concentration 0, 70, and 140 mg/l) was observed. These confirmed that organic matters in municipal wastewater did not affect ammonia oxidation of AOB. On the other hand, in the cases of industrial wastewater, organic matters in wastewater were found to inhibit ammonia oxidation and it was COD dependent. The more the COD concentration, the more ammonia oxidation deteriorated were observed (Figure 4.3).

4.2.1.3 pH levels during degradation tests

This experiment aimed to confirm that abiotic transformation of EE2 do not involve in EE2 degradation in this study. This can be observed by monitored pH level along the time in all tests.

In all cases (with municipal and industrial wastewater), pH levels during degradation tests remain at above 7.0 throughout the experiment (Figure 4.5). These results suggested that abiotic transformation of EE2 did not occur in EE2 degradation in this study.

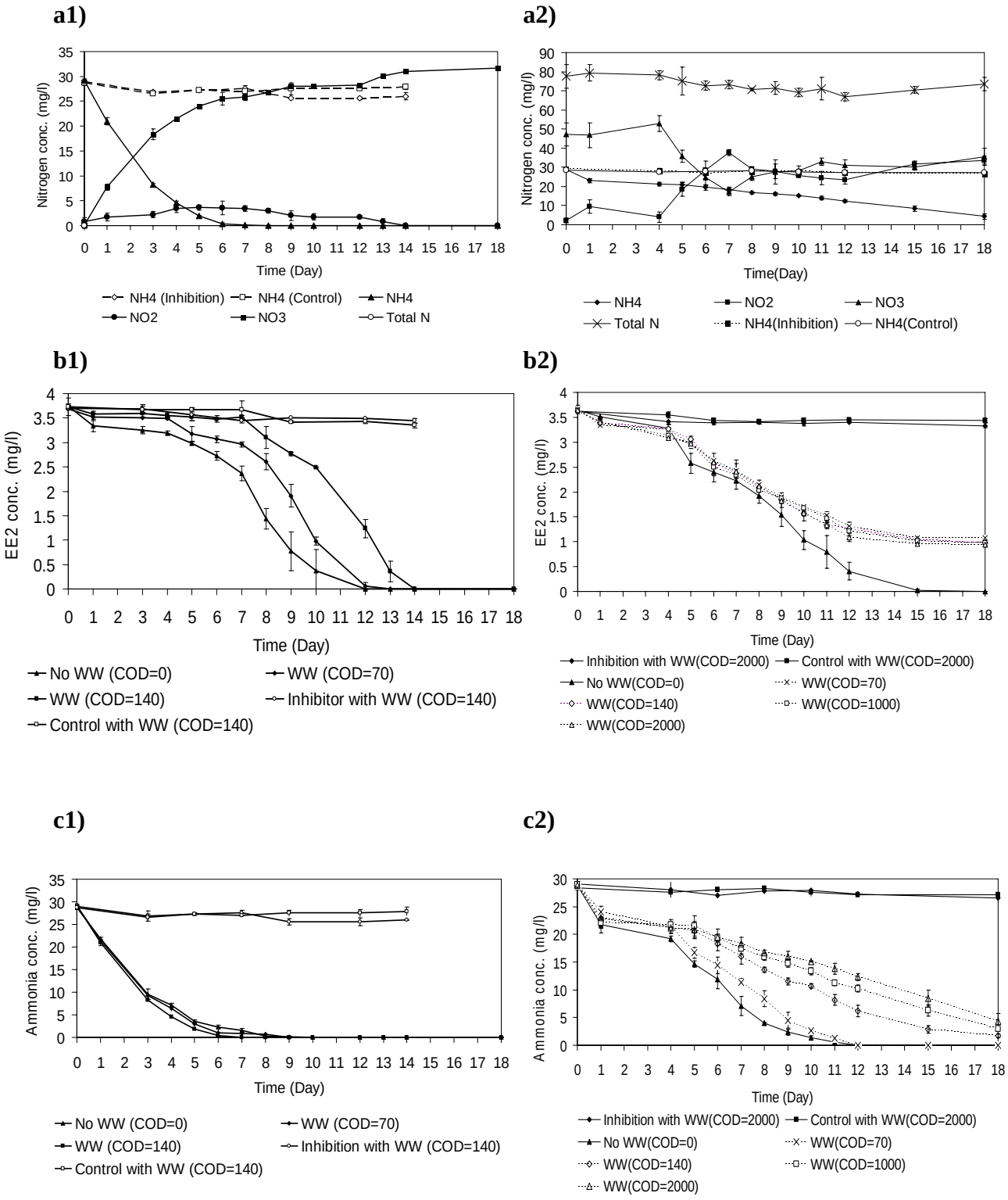


Figure 4.3: Degradation of EE2 (3.5 mg/l) by NAS in the presence of municipal wastewater (1) and industrial wastewater (2). a) Inorganic nitrogen concentration in the selected tests (a1;140 mg/l COD concentration of municipal wastewater, a2;2000 mg/l COD concentration of industrial wastewater), b) EE2 concentration, and c) Ammonia concentration

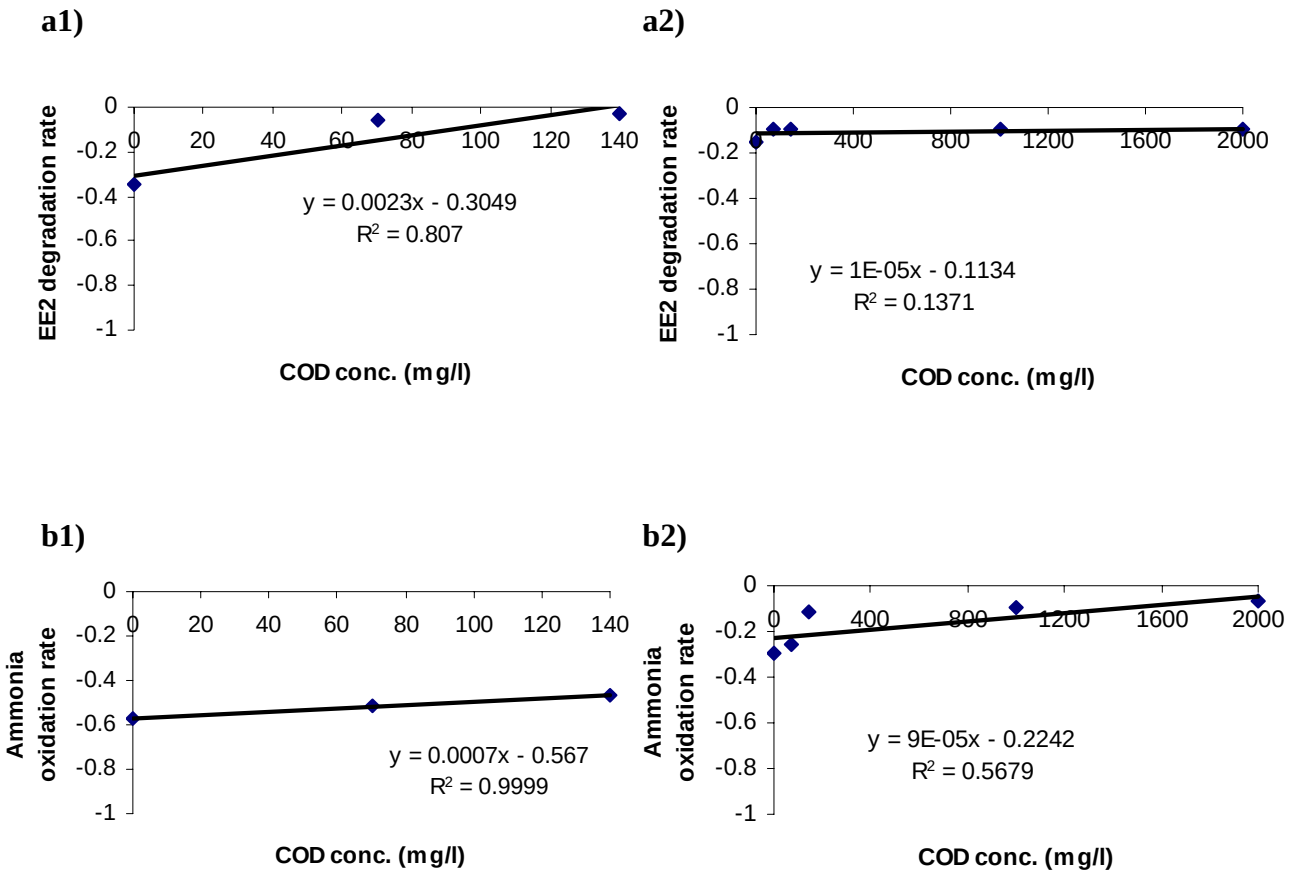
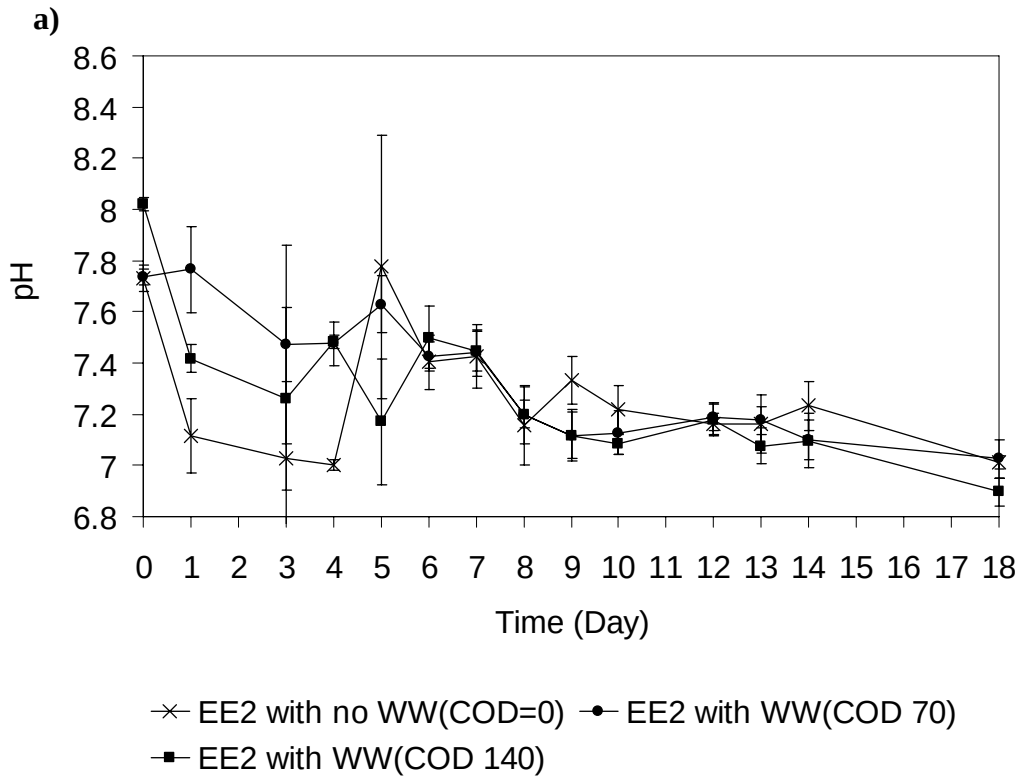


Figure 4.4: Degradation of EE2 (3.5 mg/l) by NAS in the presence of municipal wastewater (1) and industrial wastewater (2). a) EE2 degradation rate, b) Ammonia oxidation rate



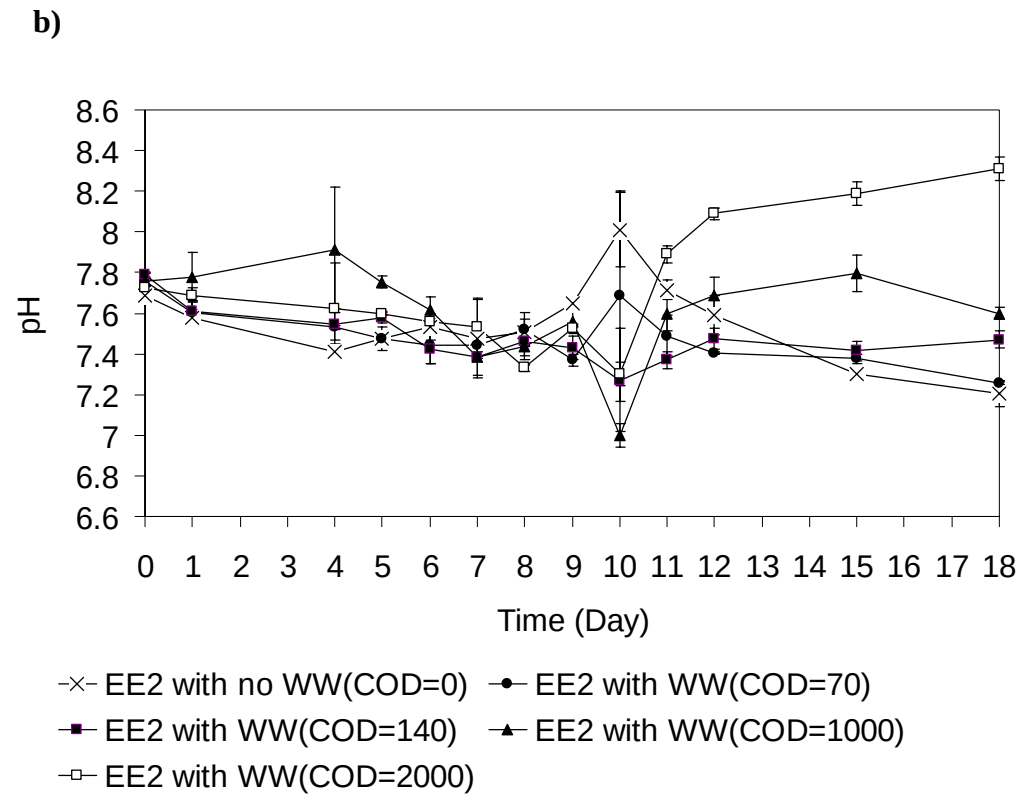


Figure 4.5: pH levels during degradation tests; a) With municipal wastewater; b) With industrial wastewater

4.2.1.4 COD removal during degradation tests

This experiment aimed to observe whether heterotrophs or AOB in NAS or heterotrophs existing in municipal or industrial wastewaters removal COD concentrations in wastewaters. This can be observed by the test with wastewater under various conditions under no NAS, NAS without ammonia in the medium and NAS with ammonia in the medium (Figure 4.6).

In all cases under the absence of NAS, COD concentrations remained the same throughout the experiment. These results suggested that the amount of heterotrophs in wastewater were not enough to significantly degrade organic matters in wastewater. In the cases of NAS without ammonia in the medium, COD concentrations decreased. These results suggested that organic matters in wastewater were degraded by heterotrophs in NAS. In the cases of NAS with ammonium in the medium, COD concentrations significantly decreased. These results suggested that some organic matters in wastewater were degraded by heterotrophs and some organic matters in wastewater were degraded by AOB in NAS via co-metabolism. These results confirmed that organic matters in wastewater associated with AMO enzyme (Figure 4.6).

Results suggested that parts of COD concentrations (25 % and 14.3%) in municipal and industrial wastewater were decreased by AOB via co-metabolism.

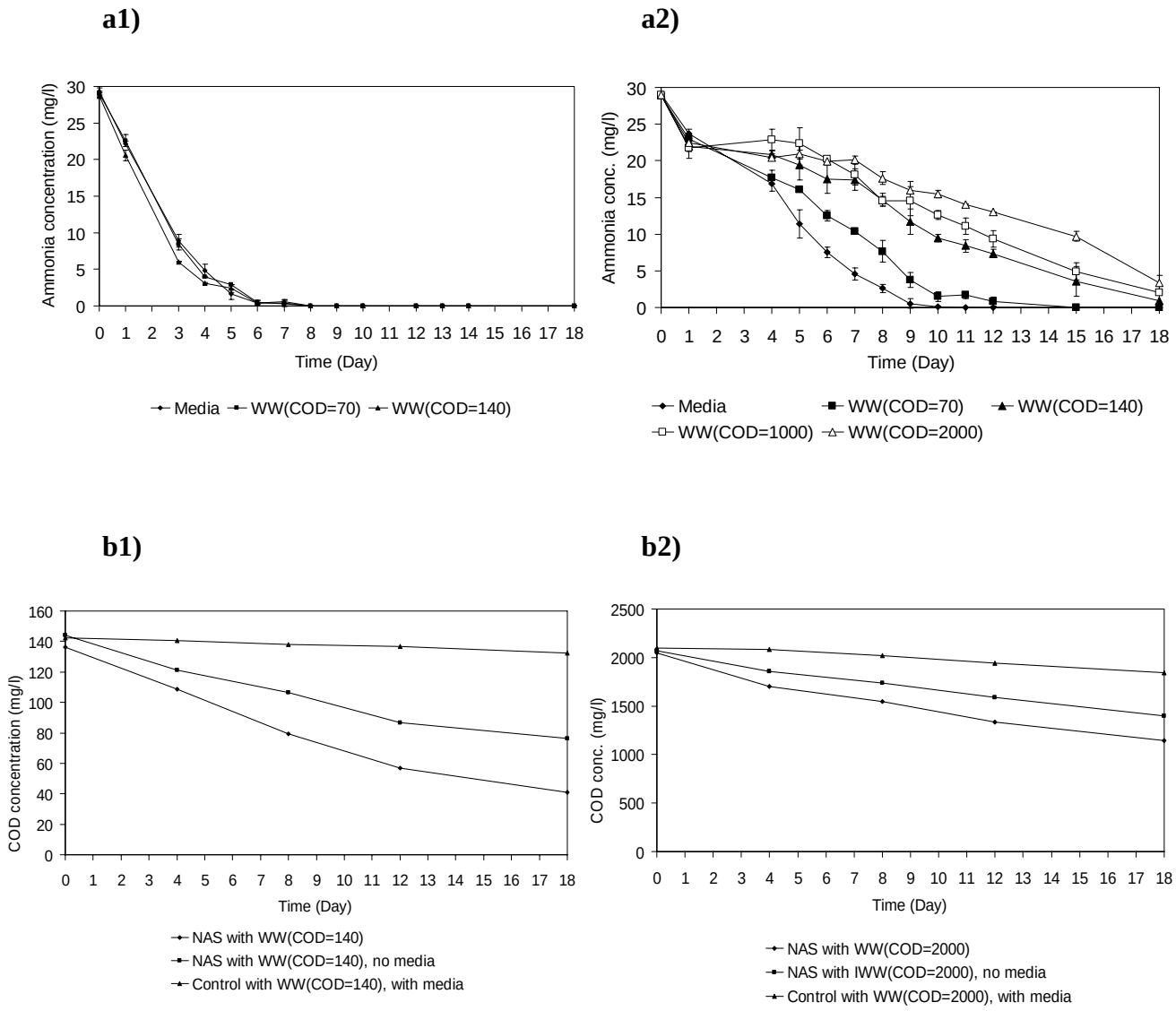


Figure 4.6: Type of organic matters in wastewater competitively affects ammonia degradation: a) Concentration of ammonia, b) Concentration of COD, 1) Municipal wastewater, 2) Industrial wastewater

4.2.1.5 Summary

In the case of municipal wastewater, organic matters deteriorated EE2 degradation and EE2 degradation was COD dependent while organic matters did not deteriorate ammonia oxidation.

In the cases of industrial wastewater, organic matters retarded the degradation of EE2 but degradation of EE2 was independent from COD concentration. In addition, organic matters in industrial wastewater decelerated the ammonia oxidation and ammonia oxidation was COD concentration dependent.

Generally, inhibitors are compounds which interact with an enzyme to slow down the rates of reaction. Competitive inhibitor is inhibitor that binds with enzyme and prevents enzyme-substrate binding whereas noncompetitive inhibitor is inhibitor that binds with enzyme and does not prevent enzyme-substrate binding (Nelson and Cox, 2000). And initial substrate concentrations affect the induction of enzyme in metabolism and cometabolism (Michael and Oliver. 1998). In this study, results suggested that different type of wastewaters contained different composition of inhibitors. In the cases of municipal, major parts of organic matters may be noncompetitive inhibitors to ammonia have the same binding site to EE2 causing no effect on ammonia oxidation but deceleration of EE2 degradation. In contrast, in the cases of industrial wastewater, major parts of organic matters in industrial wastewater may be competitive inhibitors to ammonia causing deceleration of ammonia oxidation. However, the minor parts of organic matters are noncompetitive inhibitors that have the same site to EE2. Therefore, they could deteriorate EE2. However this part may be high enough to deteriorate EE2 at all COD concentrations.

So far, there was no report on specialized organic matters in wastewaters and their inhibition behaviors to ammonia oxidation. This needs further study to clarify since this aspect is very important for taking advantage of AOB cometabolism in degrading other organic compounds in actual wastewater treatment plants.

4.2.2 Effect of initial ammonia concentrations

This experiment aimed to observe the inhibitory effect of municipal wastewater on EE2 degradation under different ammonia concentrations. The test was performed with municipal wastewater. Initial ammonium concentrations of 2 and 30 mM were selected for the tests. 2 mM was selected to represent the actual ammonium concentration found in municipal wastewater treatment plants and 30 mM was selected to provide unlimited primary substrate condition.

Figure 4.7 shows degradation of EE2 by NAS under initial ammonia concentrations of 2 and 30 mM. Figure 4.7-a shows nitrogen concentrations in the selected test of 140 mg/l COD concentration of municipal wastewater. Results 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 decreased whereas EE2 concentrations under 2 and 30 mM ammonia concentration in the inhibition tests decreased insignificantly. In the case of inhibition test, the highest amount of EE2 loss was 7.26 % in the case of 2 mM ammonia concentration with the absent of no wastewater

(COD concentration of 0 mg/l). This suggested that EE2 were degraded mainly by AOB in NAS.

4.2.2.1 Effect of initial ammonia concentrations on EE2 degradation

With the initial ammonia concentration of 2 mM, acclimation periods of more than 3 days were required for EE2 degradation with all COD concentrations (Figure 4.7-b1). Complete EE2 degradations occurred after day 12, 13, and 14 (with 0, 70, and 140 mg/l of COD concentrations respectively). For 0, 70, and 140 mg/l of COD concentrations, the degradation rates were -0.3495, -0.0574, and -0.0332 $mg.day^{-1}$, respectively (Table 4.3). These results suggested that in the cases of 2 mM ammonia concentration, initial ammonia concentration affected EE2 degradation and EE2 degradation was COD concentration dependent (Figure 4.8-a1).

In contrast, with the initial ammonia concentration of 30 mM, shorter acclimation periods were acquired (Figure 4.7-b2). For 0, 70, and 140 mg/l of COD concentrations, complete EE2 degradations occurred after day 11 with the degradation rates of -0.1234, -0.1138, and -0.1188 $mg.day^{-1}$, respectively (Table 4.3). These results suggested that in the cases of 30 mM ammonia concentration, initial ammonia concentration did not affect EE2 degradation and EE2 degradation was COD independent (Figure 4.8-a2).

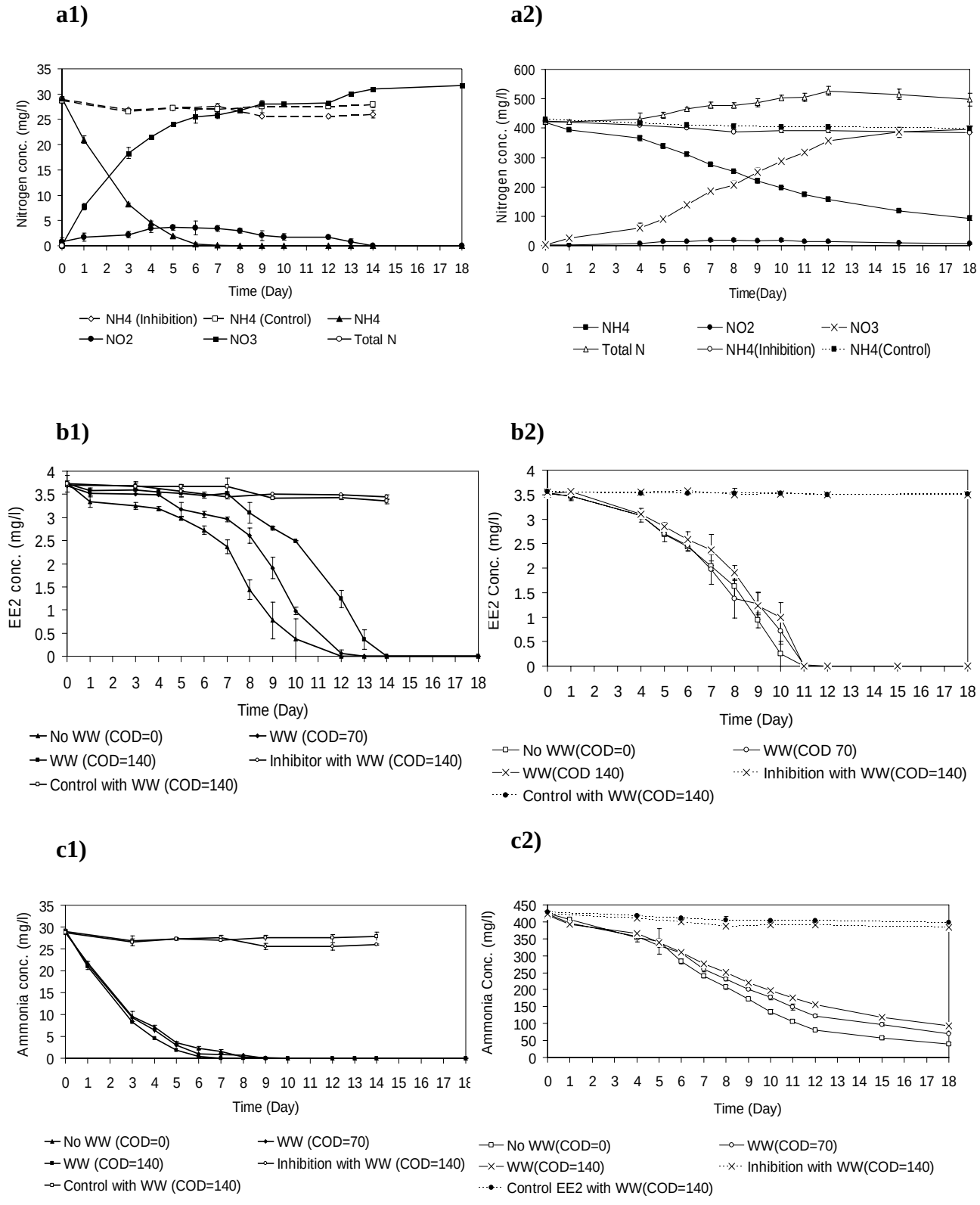


Figure 4.7: Degradation of EE2 (3.5 mg/l) by NAS under initial ammonia concentrations of 2mM (1) and 30 mM (2). a) Nitrogen concentration in the selected tests (a1;140 mg/l COD concentration under 2mM ammonia concentrations and a2;140 mg/l COD concentration under 30 mM ammonia concentrations; c2), b) EE2 concentration, and c) Ammonia concentration

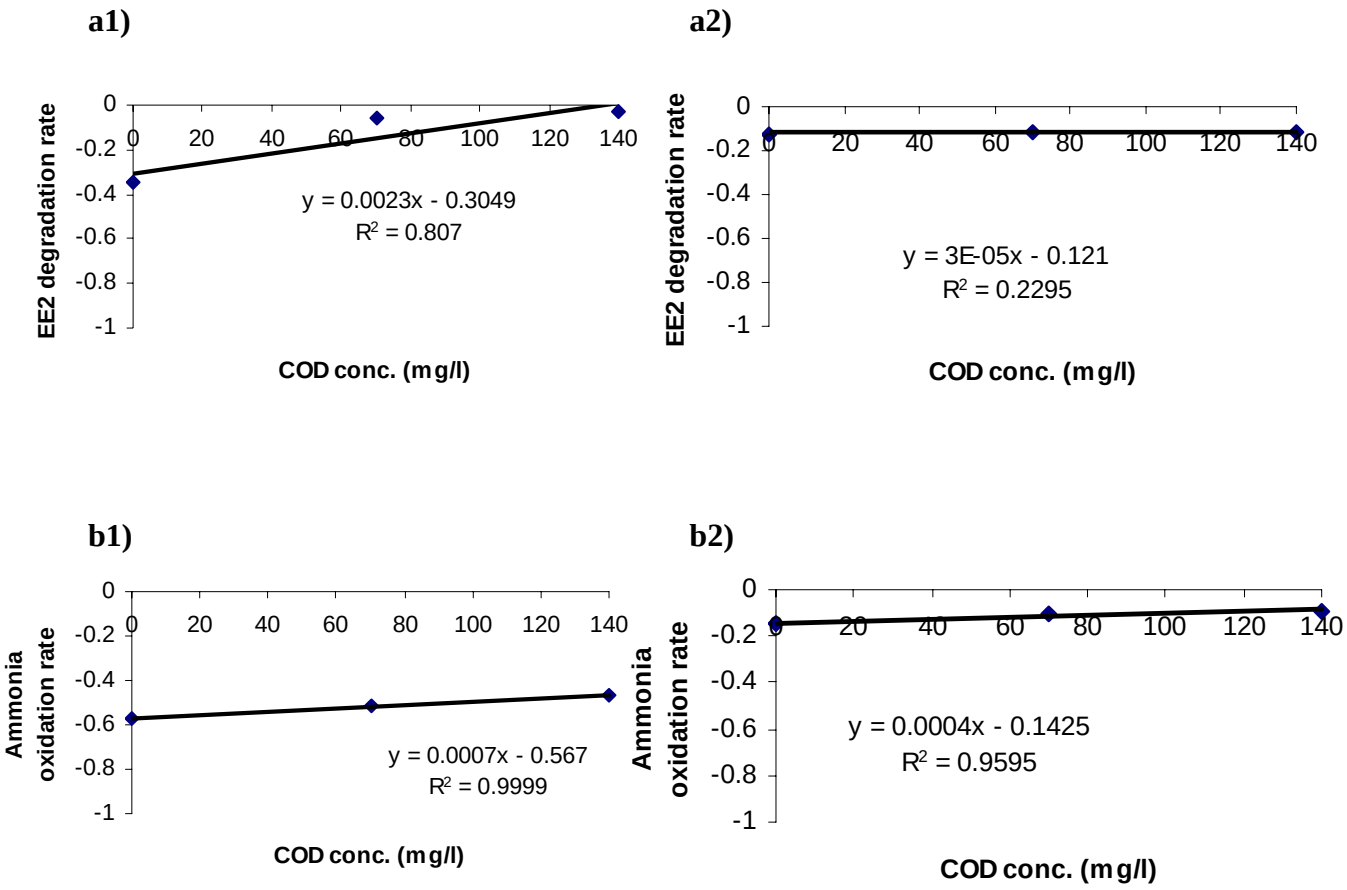


Figure 4.8 Degradation of EE2 (3.5 mg/l) by NAS under initial ammonia concentrations of 2mM (1) and 30 mM (2). a) EE2 degradation rate, b) Ammonia oxidation rate

In all cases of initial ammonia concentrations, EE2 concentrations completely degraded. Ammonia concentrations produced enough AMO enzymes for EE2 degradations. The higher initial ammonia concentration required less acclimation periods. Overall results showed similar patterns of EE2 degradation under different ammonia concentrations.

Table 4.3 Degradation of EE2 (3.5 mg/l) by NAS under initial ammonia concentrations of 2 and 30 mM.

Initial Ammonia Concentration (mM)	COD concentration (mg/l)	EE2 loss (mg)	EE2 removal (%)	EE2 degradation rate	NH_4^+ loss (mg)	NH_4^+ oxidation (%)	NH_4^+ oxidation rate
2	0	3.748	100	-0.3495	28.6908	100	-0.5667
	70	3.6844	100	-0.0574	29.2102	100	-0.6167
	140	3.7299	100	-0.0332	29.1622	100	-0.4644
30	0	3.5292	100	-0.1234	384.5358	90.56	-0.1458
	70	3.5279	100	-0.1138	353.193	83.60	-0.1075
	140	3.5151	100	-0.1188	325.8316	77.73	-0.0893

4.2.2.2 Effect of initial ammonia concentrations on ammonia oxidation

With the initial ammonia concentration of 2mM, no acclimation periods were required for ammonia oxidation with all COD concentrations (Figure 4.7-c1). Complete ammonia oxidation occurred after day 6, 7, and 8 (with 0, 70, and 140 mg/l of COD concentrations respectively). For 0, 70, and 140 mg/l of COD concentrations, the ammonia oxidation rates were -0.5667, -0.6167, and -0.4644 mg/day respectively (Table 4.3). These results suggested that in the cases of 2 mM ammonia concentration, initial ammonia concentration did not affect ammonia oxidation and ammonia oxidation was COD independent (Figure 4.8-b1).

In contrast, with the initial ammonia concentration of 30 mM, acclimation periods of more than 3 days were acquired and incomplete ammonia oxidations were

found (Figure 4.7-c2). For 0, 70, and 140 mg/l of COD concentrations, the ammonia oxidation rates at day 18 were 90.56 %, 83.60 %, and 77.73 % and the ammonia oxidation rates were -0.1458, -0.1075, and -0.0893 mg/day respectively (Table 4.3). These results suggested that in the cases of 30 mM ammonia concentration, initial ammonia concentration affected ammonia oxidation and ammonia oxidation was COD dependent (Figure 4.8-b2).

Overall results showed different patterns of ammonia degradation under initial ammonia concentrations. In cases of low initial ammonia concentration, the concentration of organic matters (COD concentration) did not involve in ammonia oxidation whereas, in cases of high initial ammonia concentration, the concentration of organic matters (COD concentration) deteriorated ammonia oxidation.

4.2.2.3 Effect of pH levels during degradation tests

This experiment aimed to confirm that abiotic transformation of EE2 do not involve in EE2 degradation in this study. This can be observed by monitored pH level along the time in all tests.

In all cases (under 2 and 30 mM ammonia concentrations), pH levels during degradation tests remain at above 6.8 throughout the experiment (Figure 4.9). These results suggested that abiotic transformation of EE2 did not occur in EE2 degradation in this study.

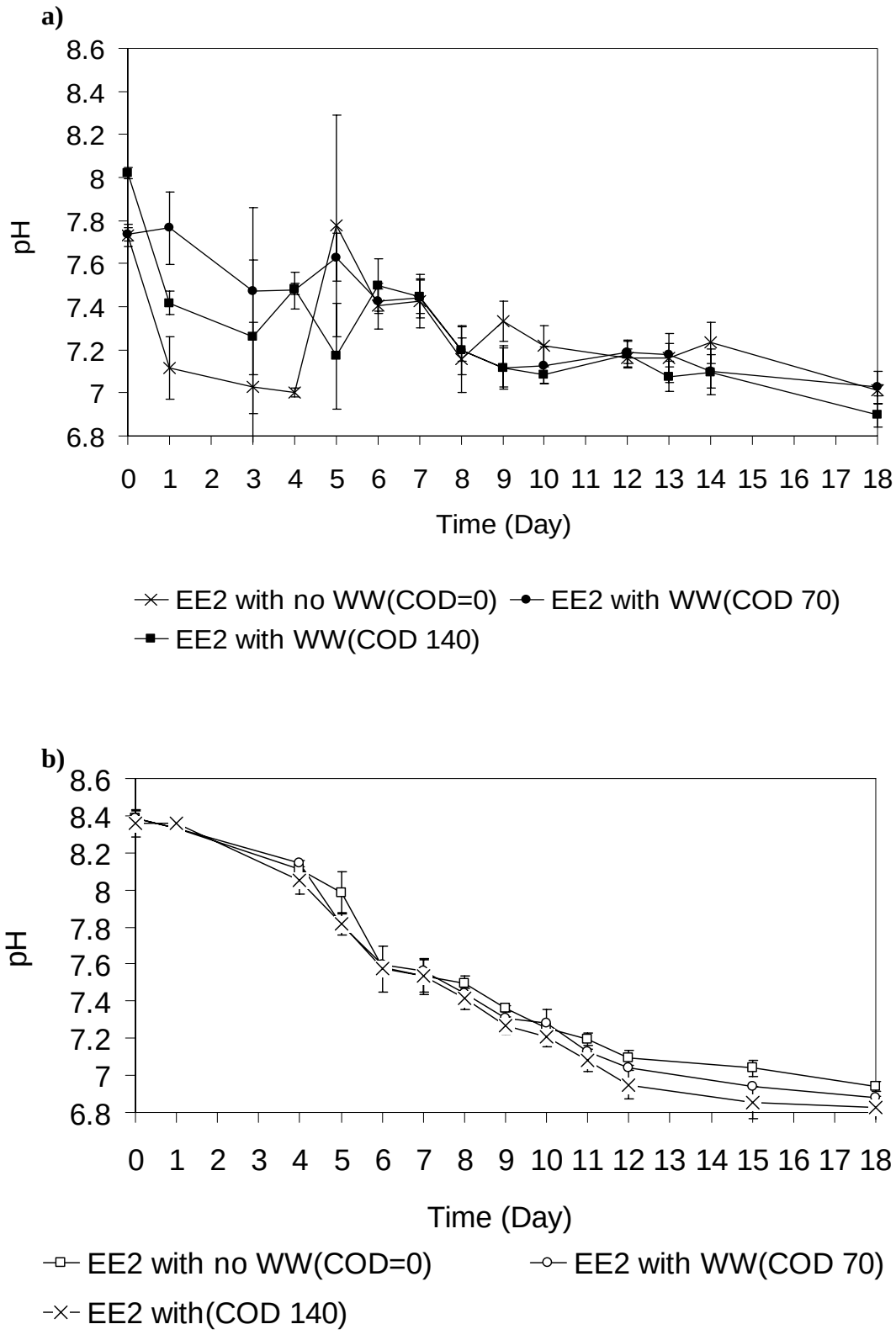


Figure 4.9: pH levels during degradation tests; a) Under initial 2 mM ammonia concentration; b) Under initial 30 mM ammonia concentration

4.2.2.4 COD removal during degradation tests

This experiment aimed to observe whether heterotrophs or AOB in NAS or heterotrophs existing in municipal wastewater under 2 and 30 mM of ammonia concentrations degraded COD concentrations in wastewater. This can be observed by the test with wastewater under various conditions under no NAS, NAS without ammonia in the medium and NAS with ammonia in the medium (Figure 4.10).

In all cases under the absence of NAS, COD concentrations remained the same throughout the experiment. These results suggested that the amount of heterotrophs in wastewater were not enough to significantly degrade organic matters in wastewater. In the cases of NAS without ammonia in the medium, COD concentrations decreased. These results suggested that organic matters in wastewater were degraded by heterotrophs in NAS. In the cases of NAS with ammonium in the medium, COD concentrations significantly decreased. These results suggested that some organic matters in wastewater were degraded by heterotrophs and some organic matters in wastewater were degraded by AOB in NAS via co-metabolism. These results confirmed that organic matters in wastewater associated with AMO enzyme.

Results suggested that parts of COD concentrations (25 and 17.86 %) under 2 and 30 mM of ammonia concentrations were decreased by AOB via co-metabolism.

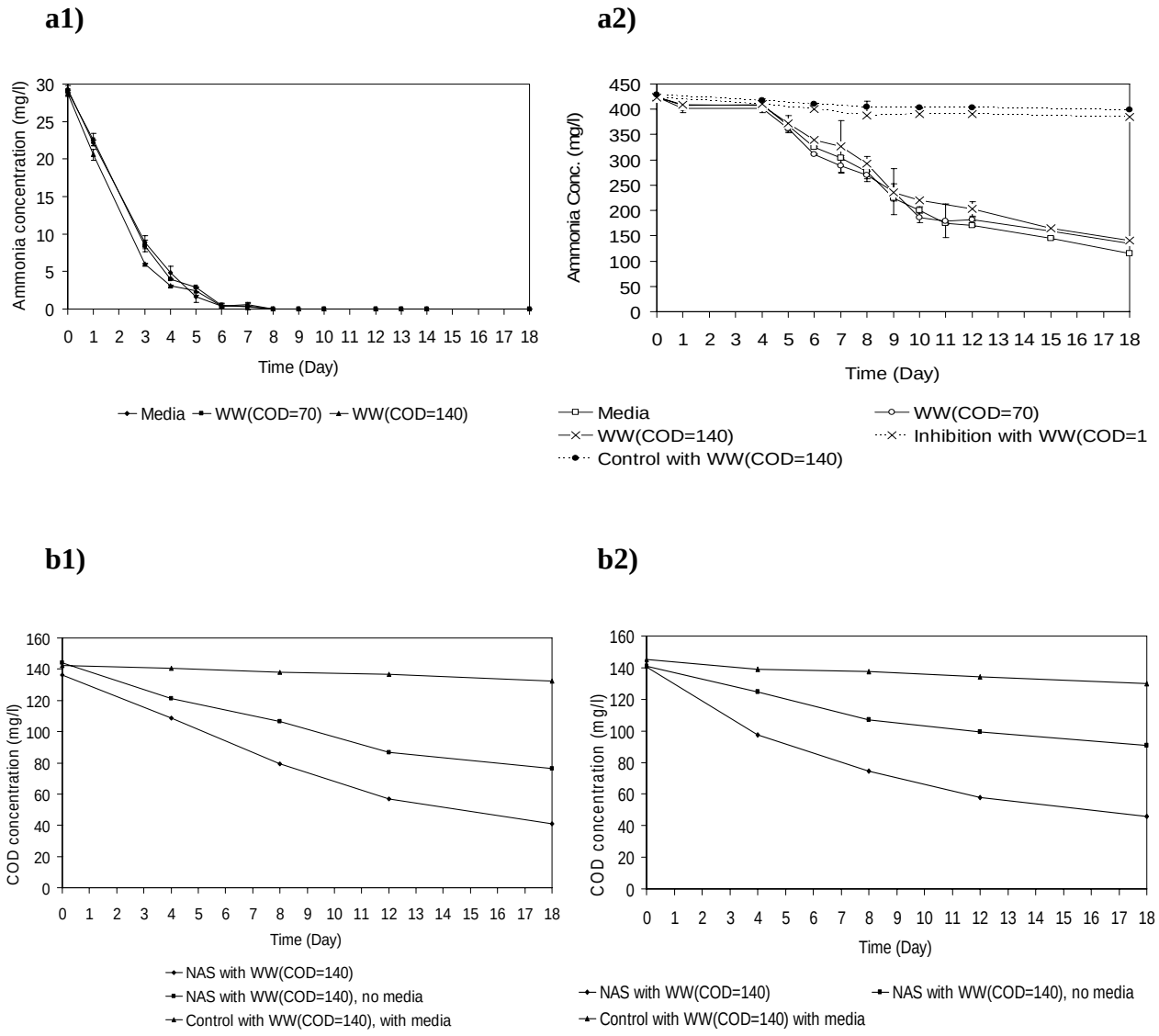


Figure 4.10: Type of organic matters in wastewater competitively affects ammonia degradation: a) Concentration of ammonia, b) Concentration of COD, (c1; 2 mM initial ammonia concentration, c2; 30 mM initial ammonia concentration)
Symbols and Abbreviations: WW = Wastewater

4.2.2.5 Summary

Initial ammonia concentration at a level of 2 mM of was found to affect EE2 degradation under different COD concentrations and the EE2 degradation was COD dependent while at this initial ammonia concentration no effect on ammonia oxidation under different COD concentrations was found.

In the cases of 30 mM of ammonia concentrations, no effect of EE2 degradation was observed. However, at this initial ammonia concentration, ammonia oxidation was found to retard dependently to COD concentration.

Competitive inhibitor binds with enzyme at the active site in place of the substrate blocking enzyme-substrate binding, or at an inhibitor binding site preventing enzyme-substrate binding. In contrast, noncompetitive inhibitor does not bind with enzyme at active site but binds with enzyme only at inhibitor binding site and does not prevent enzyme-substrate binding (Nelson and Cox, 2000). However, in some cases, the cometabolic oxidation of substances by a wide range of oxygenase enzymes can result in product toxicity. Although the specific products responsible for the observed product toxicity are not known, some previous works have been shown the toxic effects of product to the oxygenase enzymes (Fox et al. 1990; Ely et al. 1997) as well as to general cellular constituents (Wackett & Householder 1989; Alvarez-Cohen & McCarty 1991d; Oldenhuis et al. 1991; Rasche et al. 1991; Hyman et al. 1995; van Hylckama Vlieg et al.1997). In this study, at low initial ammonia concentration, EE2 degradation can be deteriorated by COD concentrations. However, when initial ammonia concentration increased, these phenomena disappeared. This can imply that when increasing the amount of primary substrate, more AMO enzymes had been produced resulting unlimited degradation at all compounds in the media reducing

effect of organic matters on cometabolism of EE2. However, the forms although as it was mentioned early that organic matters in wastewater are more in noncompetitive to ammonia, COD concentrations ere found to deteriorate ammonia oxidation at high initial ammonia concentration of 30 mM. This may cause by toxicity of products of organic matters degradation.

So far, there was no report on the inhibition behavior of EE2 or product toxicity of AMO enzyme to ammonia oxidation. This needs further study to clarify since this aspect is very important for supporting further application designs to improve treatment of wastewater in the actual wastewater treatment systems.

4.2.3 Effect of initial EE2 concentrations

This experiment aimed to observe the ability of AOB to degrade EE2 at different initial EE2 concentrations. Municipal wastewater was selected as a wastewater that mostly found contamination of EE2. NAS from 2 mM reactor was 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 plants. 2mM of ammonia concentration was selected as a model for the test as this ammonia concentration was similar to those in full-scale municipal wastewater treatment plants. Two initial EE2 concentrations (3.5 and 10 mg/l) were selected for the test. 3.5 mg/l was selected as the level is below the solubility of EE2 and 10 mg/l was selected to show the clear effect to ensure the detectability of EE2 during the degradation.

Figure 4.11 shows degradation of EE2 by NAS under initial EE2 concentrations of 3.5 and 10 mg/l. Figure 4.11-a shows nitrogen concentrations in the selected test of 140 mg/l COD concentration of municipal wastewater. Results are

shown in Figure 4.11-a1 and 4.11-a2 as examples, 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 decreased whereas EE2 concentrations in the inhibition tests decreased insignificantly. In the cases of inhibition tests, 10 mg of EE2 concentration, the highest amount of EE2 loss was 12.68 % in the absence of no wastewater (COD concentration of 0 mg/l). This suggested that EE2 were degraded by AOB in NAS.

4.2.3.1 Effect of initial EE2 concentrations on cometabolism of EE2

With the initial EE2 concentration of 3.5 mg/l, acclimation periods of more than 3 days were required for all COD concentrations (Figure 4.11-b1). Complete EE2 degradation occurred after day 12, 13, and 14, (with 0, 70, and 140 mg/l of COD concentrations, respectively) (Table 4.4). For 0, 70, and 140 mg/l of COD concentration, the degradation rates were -0.3495, -0.0574 and -0.0332 $mg.day^{-1}$ respectively. These results suggested that in the cases of 3.5 mg/l EE2 concentration, initial EE2 concentration did not affect EE2 degradation and EE2 degradation was COD concentration independent (Figure 4.12-a1).

In contrast, in the case of EE2 concentration of 10 mg/l, shorter acclimation periods were acquired and incomplete EE2 degradations were found (Figure 4.11-b2). The EE2 removals were 73.99 %, 71.90 %, and 70.70 % in 18 days and the degradation rates were -0.1234, -0.1058, and -0.0873 $mg.day^{-1}$ (with 0, 70, and 140

mg/l of COD concentrations respectively) (Table 4.4). These results suggested that in the cases of 10 mg/l EE2 concentration, initial EE2 concentration affected EE2 degradation and EE2 degradation was COD concentration independent (Figure 4.12-a2).

Overall results showed similar patterns of EE2 degradation of different initial EE2 concentrations.

Table 4.4 Degradation of EE2 by NAS under 3.5 and 10 mg/l under 2mM ammonia concentration with municipal wastewater.

Initial EE2 Concentration (mg/l)	COD concentration (mg/l)	EE2 loss (mg)	EE2 removal (%)	EE2 degradation rate	NH4+ loss (mg)	NH4+ oxidation (%)	NH4+ oxidation rate
3.5	0	3.748	100	-0.3495	28.6908	100	-0.5667
	70	3.6844	100	-0.0574	29.2102	100	-0.6167
	140	3.7299	100	-0.0332	29.1622	100	-0.4644
10	0	8.2686	73.99	-0.1234	28.933	100	-0.1352
	70	8.0612	71.90	-0.1058	29.0435	100	-0.564
	140	7.9334	70.70	-0.0873	29.141	100	-0.7638

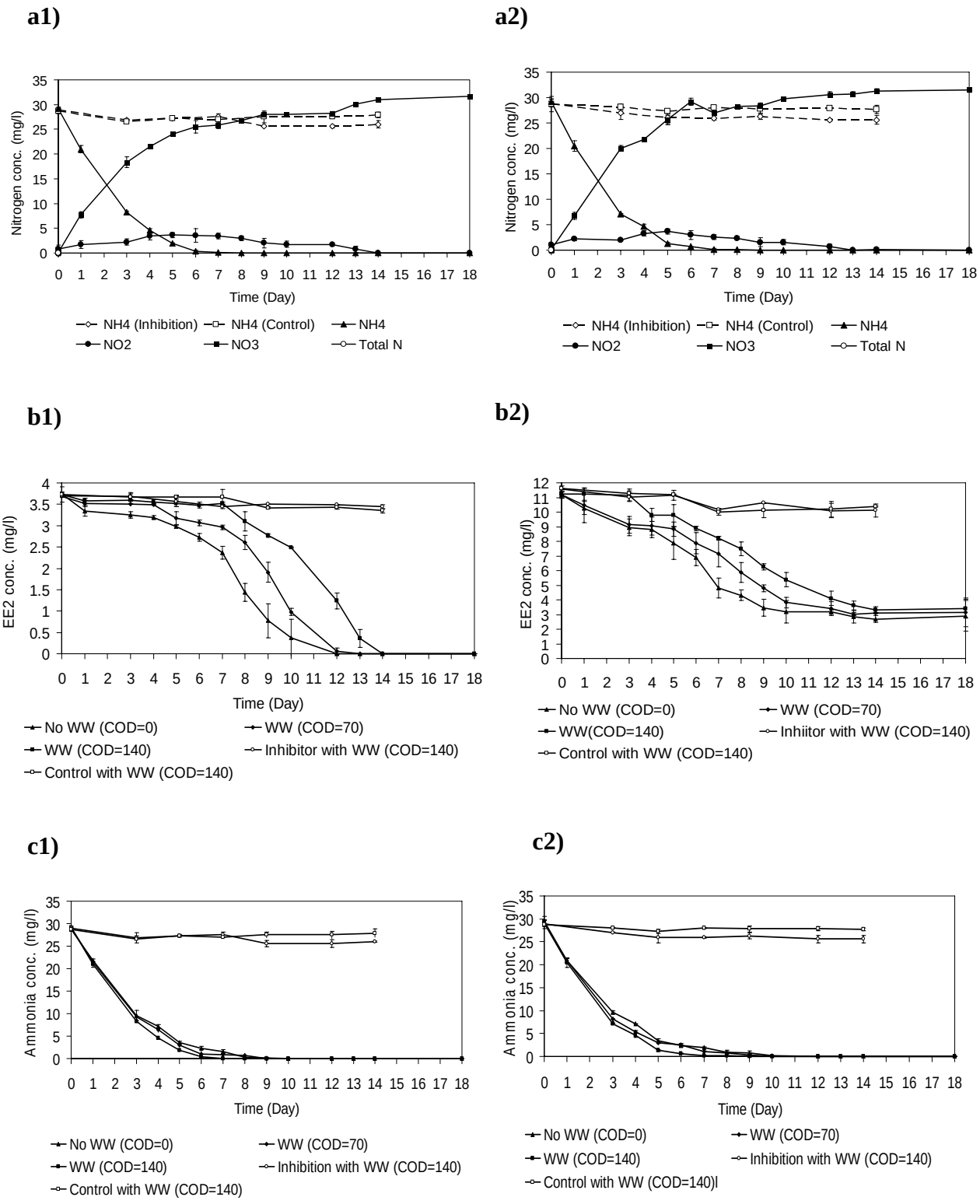


Figure 4.11: Degradation of EE2 by NAS under 3.5 mg/l (1) and 10 mg/l (2) EE2 concentrations. a) Nitrogen concentration in the selected tests (a1;140 mg/l COD concentration under 2mM ammonia concentrations and a2;140 mg/l COD concentration under 30 mM ammonia concentrations), b) EE2 concentration, and c) Ammonia concentration

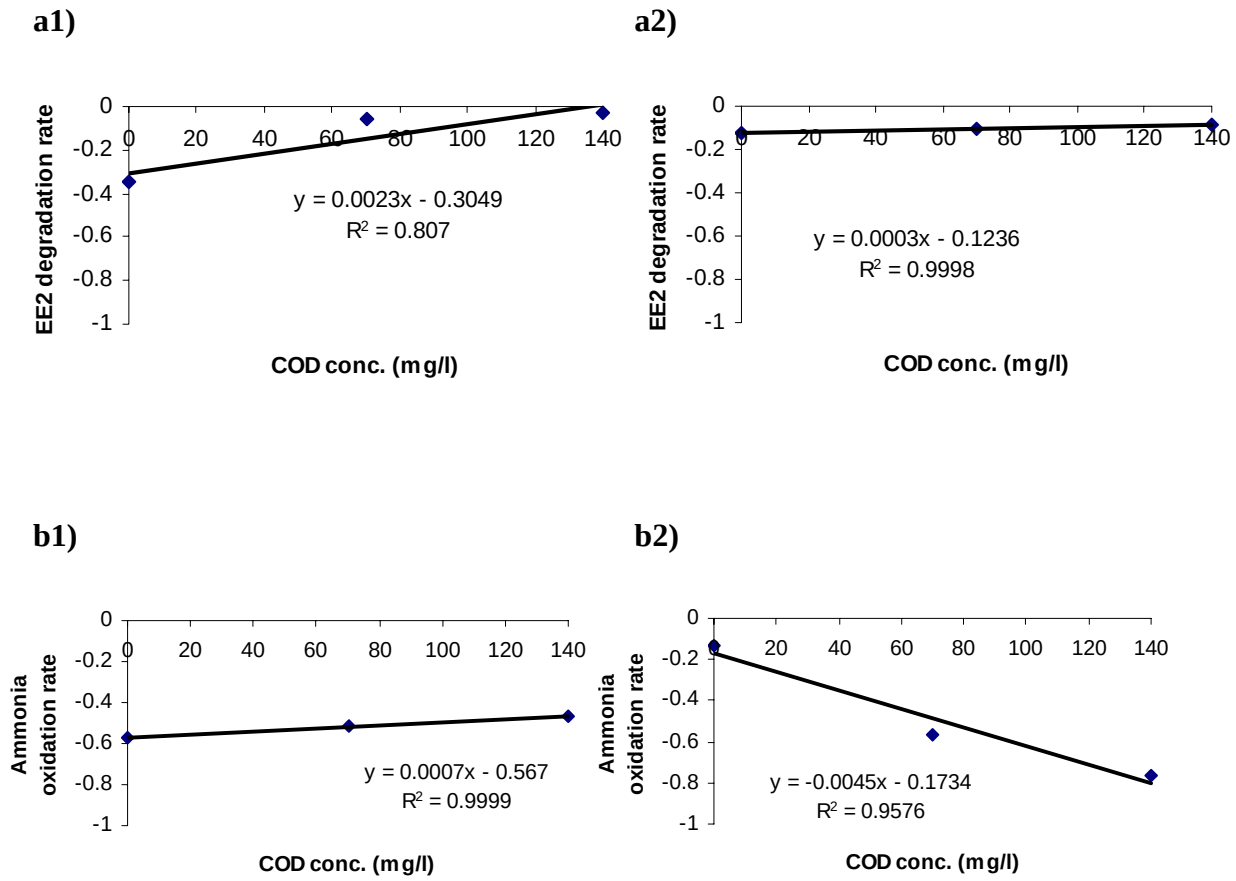


Figure 4.12 Degradation of EE2 by NAS under 3.5 mg/l (1) and 10 mg/l (2) EE2 concentrations. a) EE2 degradation rate, b) Ammonia oxidation rate

4.2.3.2 Effect of initial EE2 concentrations on ammonia oxidation

With two different initial EE2 concentrations, in all cases, ammonia was completely degraded within 12 days, without an acclimation period. There was no significant difference of ammonia oxidations. For 0, 70, and 140 mg/l of COD concentrations, the degradation rates were -0.5667, -0.6167, and -0.4644 $mg.day^{-1}$, in the cases of 3.5 mg/l of EE2 concentration and the degradation rates were -0.1352, -0.564, and -0.7638 $mg.day^{-1}$ in the cases of 10 mg/l of EE2 concentration (Table 4.4). The result suggested that initial EE2 concentration did not affect ammonia oxidation and ammonia oxidation was COD concentration independent (Figure 4.11).

Overall results showed similar patterns of ammonia oxidation with different initial EE2 concentrations (Figure 4.12).

4.2.3.3 pH levels during degradation tests

This experiment aimed to confirm that abiotic transformation of EE2 do not involve in EE2 degradation in this study. This can be observed by monitored pH level along the time in all tests.

In all cases (with 3.5 and 10 mg/l of EE2 concentrations), pH levels during degradation tests remain at above 7.0 throughout the experiment for (Figure 4.13). These results suggested that abiotic transformation of EE2 did not occur in EE2 degradation in this study.

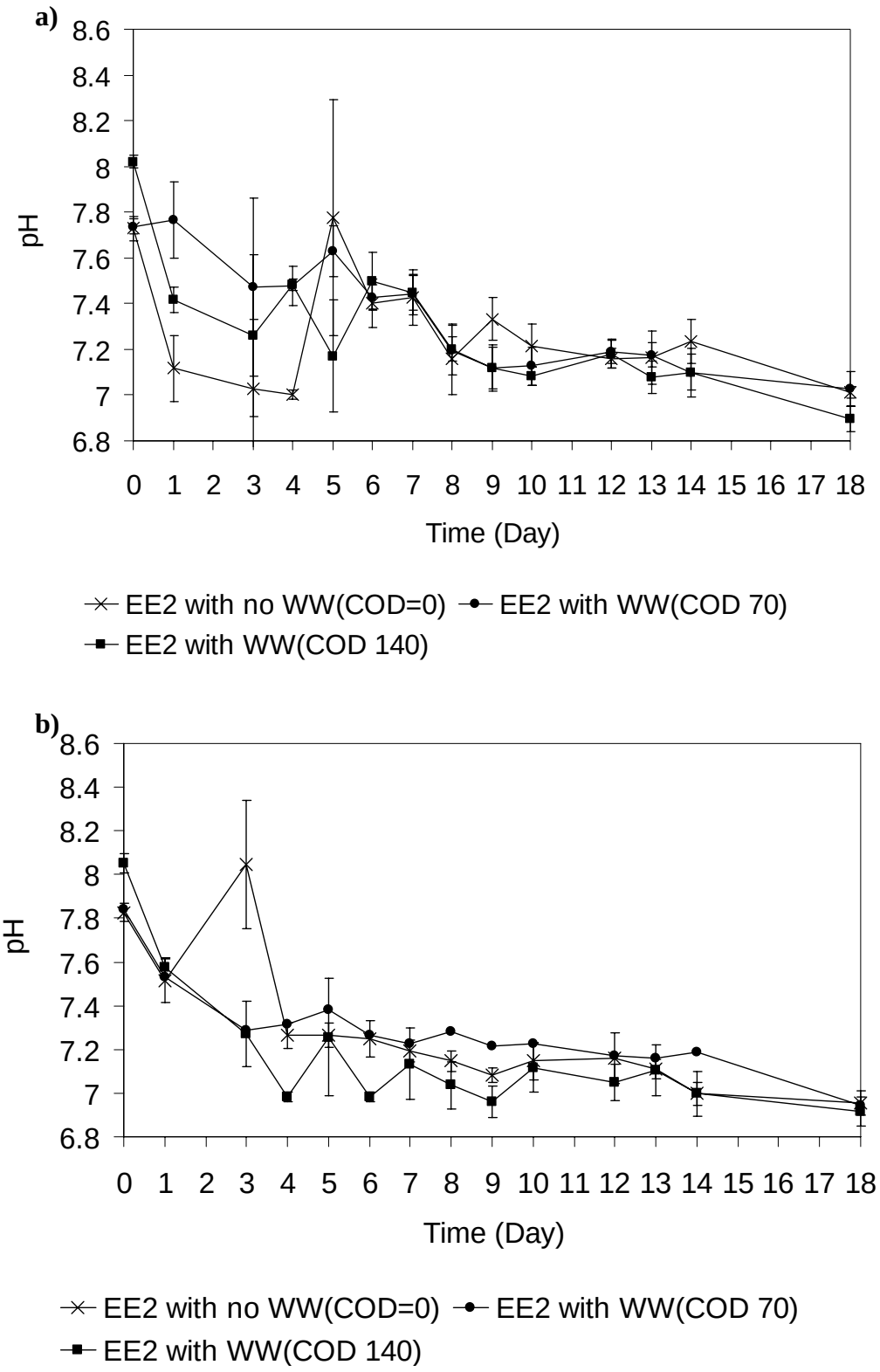


Figure 4.13: pH levels during degradation tests; a) 3.5 mg/l of EE2 degradation; b) 10 mg/l of EE2 degradation

4.2.3.5 Summary

In the case of 3.5 mg/l of EE2 concentrations, initial EE2 concentration did not affect EE2 degradation and EE2 degradation was COD independent whereas in the case of 10 mg/l of EE2 concentrations, initial EE2 concentration affected EE2 degradation and EE2 degradation was COD concentration independent. Initial EE2 concentration did not affect ammonia oxidation and ammonia oxidation was COD concentration independent.

In this study, results suggested that EE2 did not compete to ammonia to bind with enzyme whereas EE2 and organic matters in wastewater competitively binds with enzyme at the same site. Consequently, EE2 may be a noncompetitive inhibitor for AMO enzyme.

So far, there was no report on the mechanism of competitive inhibitors in cometabolism of EE2 by AOB. This needs further study to clarify.

CHAPTER V

CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORKS

5.1 Conclusions

This study investigated effect of organic matters in wastewaters on cometabolism of 17α-ethynylestradiol (EE2) by nitrifying activated sludge (NAS) containing ammonia-oxidizing bacteria (AOB).. The findings of this study fulfill all the objectives. Significant details of the findings can be summarized as follows.

1. EE2 transformation occurred abiotically AS pH dependent.
2. Abiotic EE2 transformation occurred at only pH < 6.8.
3. Initial nitrite concentrations showed no effect on abiotic EE2 transformation
4. Degradation of EE2 showed that different types of wastewater containing district compositions of organic matters exhibited inhibition behavior differently.
5. In the case of municipal wastewater, most amounts of organic matters may be noncompetitive inhibitors to ammonia. This inhibitors have the same binding sites to EE2 which causing no effect on ammonia oxidation but deceleration of EE2 degradation. In contrast, in the case of industrial wastewater, the major portions of organic matters may be competitive inhibitors to ammonia which causing deceleration of ammonia oxidation. However, the minor parts of organic matters in industrial wastewater may be noncompetitive inhibitors that have the same binding site to EE2. Therefore, they could deteriorate EE2

degradation and this part may be high enough to deteriorate EE2 at all COD concentrations.

6. At low initial ammonium concentration, EE2 degradation can be deteriorated by COD concentrations. When initial ammonium concentration increased, these phenomena disappeared. This is because when increasing the amount of the primary substrate, more AMO enzymes were produced resulting in unlimited degradation of all compounds in the medium reducing effect of organic matters on cometabolism of EE2. Although organic matters in municipal wastewater were more in noncompetitive forms to ammonia, COD concentrations were found to deteriorate ammonia oxidation at high initial ammonium concentration. This may cause by product toxicity when organic matters were more degraded causing decreasing in ammonia oxidation.
7. Initial EE2 concentrations did not affect cometabolism of EE2. The results of this study suggested that it is necessary to concern the effect of organic matters in wastewater before developing NASs to degrade recalcitrant organic pollutants in wastewater. This knowledge can support further application designs to improve treatment of wastewater in the actual wastewater treatment plants. One example is by promoting postnitrification process rather than prenitrification process. This is because in the prior case, nitrification tank is placed behind the denitrification tank. Major parts of organic matters will be removed earlier by heterotrophs in denitrification tank and front compartment of nitrification tank. This results in significant reducing the amounts of organic matters come to nitrifying compartment. Then, AOB in nitrifying compartment will be used to degrade EE2 more efficiently.

5.2 Suggestions for future works

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

1. Detailed competition effects of other types of wastewaters on EE2 degradation by AOB via cometabolism.
2. Competition effects of specialized non-target organic matters in wastewaters and their inhibition behaviors on EE2 degradation by AOB via cometabolism.
3. Product toxicity from organic matters in wastewaters on AMO enzyme that reduced ammonia oxidation rate.
4. Fundamental knowledge of AMO enzyme and its interaction to alternative substrates.

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Appendix

APPENDIX A
EFFECT OF pH LEVELS

Figure A-1 Average pH level of abiotic testes.

Ammonia	Nitrite	Initial pH	Time								
			0	24	48	72	96	144	196	216	240
30mM	70mg	8.0	8.295	-	8.33	8.34	-	8.475	-	8.425	-
		7.0	7.03	7.02	-	-	7.10	7.20	7.26	7.39	7.47
		6.8	6.76	6.81	-	-	6.87	6.96	7.07	7.19	7.31
		6.4	6.35	6.44	-	-	6.49	6.62	6.74	6.86	6.93
		6.2	6.07	6.18	-	-	6.26	6.35	6.44	6.58	6.66
		6.0	5.94	5.98	-	-	6.11	6.23	6.30	6.39	6.48
10mM	45mg	7.0	7.00	7.00	-	-	7.08	7.10	7.14	7.25	7.35
		6.8	6.72	6.80	-	-	6.88	6.92	7.00	7.06	7.19
		6.4	6.35	6.37	-	-	6.47	6.51	6.56	6.65	6.80
		6.2	6.14	6.20	-	-	6.21	6.28	6.34	6.42	6.49
		6.0	6.00	6.03	-	-	6.10	6.16	6.25	6.39	6.54
2mM	12mg	7.0	7.00	7.03	-	-	7.05	7.10	7.17	7.26	7.31
		6.8	6.72	6.82	-	-	6.85	6.90	6.95	7.02	7.20
		6.4	6.33	6.40	-	-	6.43	6.49	6.57	6.64	6.81
		6.2	6.09	6.16	-	-	6.24	6.23	6.32	6.40	6.53
		6.0	5.90	6.00	-	-	6.05	6.13	6.16	6.27	6.43

Figure A-2 Average EE2 concentration level of abiotic testes.

Ammonia	Nitrite	Initial pH	Time								
			0	24	48	72	96	144	196	216	240
30mM	70mg	8.0	11.7513	-	11.5757	11.5789	-	11.2495	-	10.826	-
		7.0	10.2095	10.3041	-	-	10.1432	10.3901	9.8733	9.3926	10.0057
		6.8	10.6285	9.6365	-	-	9.2821	9.1285	9.1065	8.9848	8.8829
		6.4	10.4476	7.5484	-	-	7.4397	6.9283	6.7472	6.4967	5.6695
		6.2	10.0566	5.9868	-	-	5.2667	4.4183	3.4569	2.8879	2.4334
		6.0	10.5568	6.1192	-	-	4.1069	3.7537	3.1663	2.8850	2.1015
10mM	45mg	7.0	10.5746	10.5075	-	-	10.3918	10.4217	10.2603	9.8209	9.1341
		6.8	10.4118	9.6081	-	-	9.4182	8.9492	9.0920	9.3428	9.0485
		6.4	10.6219	7.5150	-	-	7.5422	7.6823	7.1608	7.9813	7.0247
		6.2	10.5405	6.6791	-	-	5.6079	4.9503	3.5449	2.4038	2.5557
		6.0	10.7492	5.5265	-	-	3.9407	2.8175	2.1429	0.0000	0.0000
2mM	12mg	7.0	10.4199	10.8045	-	-	10.2491	10.2923	10.5700	10.1592	9.9654
		6.8	10.7040	9.3626	-	-	9.1256	9.3200	9.0962	8.8954	8.8877
		6.4	10.5573	7.5055	-	-	7.1061	7.4614	7.0605	6.0103	5.5835
		6.2	10.7979	6.8563	-	-	5.5224	4.3665	3.2691	3.0119	2.2606
		6.0	10.5719	5.2959	-	-	4.7800	4.0682	3.1768	2.4335	0.0000

Figure A-3 Average nitrite concentration level of abiotic testes.

Ammonia	Nitrite	Initial pH	Time								
			0	24	48	72	96	144	196	216	240
30mM	70mg	8.0	88.7323	-	76.9428	76.8758	-	65.37	-	70.4634	-
		7.0	79.8342	67.5762	-	-	54.0058	58.1317	47.1371	39.6994	32.5136
		6.8	77.4803	61.1463	-	-	46.2142	32.0257	27.1058	26.5616	21.3576
		6.4	80.3898	50.5317	-	-	31.6837	21.1468	9.2571	6.0868	4.7756
		6.2	74.8294	30.5094	-	-	11.9667	2.6299	1.5249	1.4348	1.3883
		6.0	74.1075	29.5421	-	-	9.0199	3.5422	3.4660	3.2114	1.9150
10mM	45mg	7.0	47.5513	30.0199	-	-	18.9714	7.5821	4.1314	1.2561	0.6056
		6.8	49.0475	20.6571	-	-	17.3259	11.0160	4.0557	1.4159	0.5440
		6.4	48.4002	20.2898	-	-	10.2263	3.7429	1.8175	0.7447	0.5490
		6.2	48.5085	12.9847	-	-	6.5272	2.9263	1.5624	0.2375	0.1186
		6.0	48.9126	12.0314	-	-	4.5269	2.5140	1.0093	1.0148	0.0702
2mM	12mg	7.0	14.0571	5.5766	-	-	3.9744	3.5380	3.8016	1.5428	0.0000
		6.8	13.0933	3.6039	-	-	1.1046	1.3648	0.4733	0.0408	0.0000
		6.4	13.1984	2.5587	-	-	1.0408	0.4208	0.0177	0.0000	0.0000
		6.2	12.9661	1.8027	-	-	0.2611	0.0000	0.0000	0.0000	0.0000
		6.0	13.5323	1.8580	-	-	0.1434	0.0000	0.0000	0.0000	0.0000

APPENDIX B

EFFECT OF ORGANIC MATTERS IN WASTEWATERS

Figure B-1 COD concentration of municipal and industrial wastewaters in EE2 degradation by AOB in NAS under varied ammonia concentrations (2 and 30 mM).

Day	NAS with IWW Media 2mM COD=2000	NAS with IWW No Media COD=2000	Control with IWW Media 2mM COD=2000	NAS with MWW Media 2mM COD=140	NAS with MWW No Media COD=140	Control with MWW Media 2mM COD=140	NAS with MWW Media 30mM COD=140	NAS with MWW No Media COD=140	Control with MWW Media 30mM COD=140
0	2035.14	2042.00	2104.05	139.93	146.20	138.57	140.52	145.05	143.04
	2005.86	2104.23	2086.36	132.05	142.01	143.01	142.06	140.04	144.93
	2101.50	2059.11	2104.78	136.81	143.47	145.00	138.02	138.05	148.02
4	1684.25	1902.46	2053.84	108.87	120.95	142.35	104.02	124.90	139.25
	1684.82	1838.83	2085.89	111.61	119.21	140.71	92.06	120.11	140.01
	1742.60	1835.53	2104.58	106.05	122.88	138.15	96.04	129.45	138.13
8	1504.28	1726.42	1982.05	79.86	102.96	136.25	78.25	110.01	135.06
	1642.07	1773.59	2008.59	80.01	109.01	141.53	76.02	107.53	143.64
	1496.48	1703.50	2068.65	77.60	107.01	135.75	69.13	102.70	133.60
12	1386.05	1585.63	1995.09	56.23	84.48	132.06	55.20	99.21	136.30
	1325.09	1663.05	1902.98	54.43	88.61	142.05	57.86	97.44	135.07
	1293.14	1513.58	1932.03	60.08	87.14	136.09	59.90	101.26	130.58
18	1123.34	1411.08	1892.82	40.10	75.21	131.76	45.15	98.68	129.61
	1092.04	1409.17	1827.17	41.01	74.47	130.96	40.93	87.51	131.88
	1213.26	1385.01	1805.58	41.77	79.05	133.86	51.05	85.53	128.07

Symbols and Abbreviations: MWW = Municipal wastewater, IWW = Industrial wastewater

Figure B-2.1 Average concentration of EE2 in EE2 degradation (3.5 mg/l) by AOB in NAS with municipal wastewater under 2 mM ammonia concentrations.

Day	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70	COD 140	COD 140	COD 140
0	3.7480	3.7075	3.7365	3.6844	3.7078	3.7351	3.7299	3.7501	3.7308
1	3.3483	-	-	3.5216	-	-	3.5816	-	-
3	3.2478	3.6781	3.7859	3.5089	3.7480	3.6760	3.5964	3.6675	3.6737
4	3.1908	-	-	3.4977	-	-	3.5555	-	-
5	2.9768	3.5707	3.6199	3.1769	3.6453	3.6965	3.5143	3.7006	3.6679
6	2.7246	-	-	3.0661	-	-	3.4804	-	-
7	2.3643	3.4532	3.5508	2.9656	3.3141	3.5884	3.5149	3.4308	3.6725
8	1.4421	-	-	2.6030	-	-	3.0977	-	-
9	0.7770	3.5026	3.5508	1.9079	3.4738	3.5501	2.7676	3.4981	3.5207
10	0.3700	-	-	0.9804	-	-	2.4850	-	-
12	0.0000	3.4897	3.5178	0.0544	3.4221	3.5455	1.2402	3.4104	3.5426
13	0.0000	-	-	0.0000	-	-	0.3612	-	-
14	0.0000	3.4384	3.5437	0.0000	3.4879	3.5678	0.0000	3.5198	3.5497
18	0.0000	-	-	0.0000	-	-	0.0000	-	-

Figure B-2.2 Average concentration of ammonia in EE2 degradation (3.5 mg/l) by AOB in NAS with municipal wastewater under 2 mM ammonia concentrations.

Day	Media	WW	WW	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 140	COD 70	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70	COD 140	COD 140	COD 140
0	29.3459	28.7399	29.0259	28.6908	28.9914	28.9373	29.2102	28.7282	29.1180	29.1622	28.9308	28.6965
1	22.1898	20.5782	22.5903	21.8141	-	-	21.3967	-	-	20.9665	-	-
3	8.8696	6.0414	8.4355	9.5459	27.2054	28.8461	9.2332	27.6904	28.5095	8.2402	26.8776	26.5680
4	4.8103	3.0710	3.9642	7.1577	-	-	6.3949	-	-	4.5631	-	-
5	1.6151	2.3862	2.8887	3.5048	27.2609	28.3722	3.0607	27.1013	28.0280	1.9081	27.2589	27.2172
6	0.3573	0.3840	0.4588	2.3497	-	-	1.0358	-	-	0.3780	-	-
7	0.3798	0.6102	0.3332	1.5609	25.4927	28.3989	0.8747	26.6830	28.3853	0.0675	27.5686	27.0229
8	0.0000	0.0000	0.0000	0.2924	-	-	0.6444	-	-	0.0507	-	-
9	0.0000	0.0000	0.0000	0.1523	26.0015	28.8639	0.0572	25.9404	27.5887	0.0000	25.5911	27.5276
10	0.0000	0.0000	0.0000	0.0274	-	-	0.0000	-	-	0.0000	-	-
12	0.0000	0.0000	0.0000	0.0000	26.3095	28.4179	0.0000	26.7320	28.6223	0.0000	25.5680	27.5465
13	0.0000	0.0000	0.0000	0.0000	-	-	0.0000	-	-	0.0000	-	-
14	0.0000	0.0000	0.0000	0.0000	27.5186	26.5161	0.0000	26.7337	28.9163	0.0000	26.0181	27.8895
18	0.0000	0.0000	0.0000	0.0000	-	-	0.0000	-	-	0.0000	-	-

Symbols and Abbrivations: WW = Wastewater.

Figure B-2.3 Average concentration of nitrite in EE2 degradation (3.5 mg/l) by AOB in NAS with municipal wastewater under 2 mM ammonia concentrations.

Day	Media	WW	WW	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 140	COD 70	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70	COD 140	COD 140	COD 140
0	0.0000	0.0318	0.0400	0.0000	0.0000	0.0000	0.3484	0.1871	0.2431	0.7597	0.9190	1.2754
1	1.8889	1.8189	0.6571	0.9326	-	-	1.7771	-	-	1.7329	-	-
3	2.2861	2.3179	1.5528	1.1908	0.1091	0.0099	2.4430	0.1082	0.3408	2.2259	0.6110	0.5171
4	1.2485	4.1447	2.6011	1.1976	-	-	2.6417	-	-	3.3899	-	-
5	2.6209	1.5782	2.2186	1.5903	0.6994	0.0798	2.9595	0.0315	0.0028	3.6429	0.9619	0.9680
6	1.1154	1.8811	1.4868	2.2780	-	-	3.2366	-	-	3.5009	-	-
7	0.4849	1.4801	0.8855	2.3552	0.4664	0.6404	3.0217	0.5339	0.5859	3.4676	0.3968	0.5367
8	0.0604	0.5634	0.5373	3.6081	-	-	3.0367	-	-	2.9875	-	-
9	0.0325	0.4200	0.0895	2.2899	0.0798	0.4206	2.6415	1.3839	0.6030	2.0025	1.0288	1.1792
10	0.0485	0.0362	0.2160	2.2822	-	-	3.3026	-	-	1.7544	-	-
12	0.0000	0.0000	0.0000	0.7133	0.3349	0.0137	2.0362	1.0806	0.3909	1.6998	0.7707	-
13	0.0000	0.0000	0.0000	0.5823	-	-	1.0455	-	-	0.7848	-	-
14	0.0000	0.0000	0.0000	0.0000	0.6541	0.1587	0.0000	0.5940	1.3233	0.0000	0.7569	0.7734
18	0.0000	0.0000	0.0000	0.0000	-	-	0.0000	-	-	0.0000	-	-

Symbols and Abbrivations: WW = Wastewater.

Figure B-2.4 Average concentration of nitrate in EE2 degradation (3.5 mg/l) by AOB in NAS with municipal wastewater under 2 mM ammonia concentrations.

Day	Media	WW	WW	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 140	COD 70	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70	COD 140	COD 140	COD 140
0	0.0000	0.1478	0.0284	0.0000	0.0000	0.0000	0.0185	0.0375	0.0247	0.1330	0.0850	0.1298
1	4.1564	5.8578	5.0999	6.8888	-	-	6.6991	-	-	7.7304	-	-
3	16.7843	20.5704	18.5845	17.4782	0.5596	0.4025	16.7704	0.9766	0.3373	18.3440	1.3529	1.0585
4	22.9713	21.8096	21.1221	20.7027	-	-	20.6011	-	-	21.5076	-	-
5	26.1791	25.0205	23.0791	24.3513	1.4871	1.2379	23.2897	2.7088	0.7722	24.0120	1.4841	0.5952
6	28.8011	26.5330	26.0646	25.0606	-	-	25.4162	-	-	25.5010	-	-
7	28.9902	27.2327	26.5648	25.6850	2.5366	0.6585	25.7397	1.6321	0.7622	25.8289	1.5302	0.9338
8	29.0681	29.0875	28.2315	25.4292	-	-	26.0718	-	-	26.7442	-	-
9	30.2411	30.0767	29.0800	26.6922	2.0435	0.1210	27.1044	1.7759	1.0656	28.0558	2.1039	0.7751
10	29.8880	30.5822	30.4212	27.1295	-	-	27.1780	-	-	28.0467	-	-
12	30.1329	30.5498	30.2770	28.9822	1.8100	0.7930	28.1342	1.5052	0.2901	28.2588	2.0214	0.7810
13	30.6557	30.8998	30.1517	29.4821	-	-	29.4475	-	-	30.0287	-	-
14	30.0731	30.6126	30.5335	30.9576	2.0044	0.1496	30.9532	2.7711	0.1210	31.0437	1.6144	0.0507
18	30.5790	31.3553	30.6062	31.0814	-	-	31.7052	-	-	31.7315	-	-

Symbols and Abbrivations: WW = Wastewater.

Figure B-2.5 Average concentration of total nitrogen in EE2 degradation (3.5 mg/l) by AOB in NAS with municipal wastewater under 2 mM ammonia concentrations.

Day	Media	WW	WW	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 140	COD 70	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70	COD 140	COD 140	COD 140
0	29.3459	28.9195	29.0942	28.6908	28.9914	28.9373	29.4548	28.9528	29.3859	29.7573	29.9348	30.1017
1	28.2351	28.2549	28.3472	29.6355	-	-	29.8730	-	-	30.4298	-	-
3	28.3920	28.9296	28.5727	28.2149	27.8740	29.2585	28.4467	28.7751	29.1876	28.8100	28.8415	28.1435
4	29.0301	29.0252	27.6874	29.0580	-	-	29.6378	-	-	29.4605	-	-
5	30.4150	28.9848	28.1863	29.4464	29.4474	29.6898	29.3100	29.8415	28.8030	29.5630	29.7049	28.7804
6	30.2738	28.7981	28.0101	29.6883	-	-	29.6886	-	-	29.3799	-	-
7	29.8548	29.3229	27.7835	29.6011	28.4956	29.6977	29.6361	28.8490	29.7334	29.3639	29.4956	28.4934
8	29.1284	29.6509	28.7687	29.3297	-	-	29.7528	-	-	29.7824	-	-
9	30.2736	30.4967	29.1695	29.1344	28.1247	29.4055	29.8031	29.1001	29.2572	30.0583	28.7237	29.4819
10	29.9364	30.6184	30.6371	29.4390	-	-	30.4806	-	-	29.8011	-	-
12	30.1329	30.5498	30.2770	29.6954	28.4543	29.2246	30.1705	29.3177	29.3032	29.9587	28.3921	28.4475
13	30.6557	30.8998	30.1517	30.0645	-	-	30.4930	-	-	30.8134	-	-
14	30.0731	30.6126	30.5335	30.9576	2.6585	0.3083	30.9532	30.0971	30.0665	31.0437	27.9712	28.4905
18	30.5790	31.3553	30.6062	31.0814	-	-	31.7052	-	-	31.7315	-	-

Symbols and Abbreviations: WW = Wastewater.

Figure B-2.6 Average pH of EE2 degradation (3.5 mg/l) by AOB in NAS with municipal wastewater under 2 mM ammonia concentration.

Day	Media	WW	WW	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 140	COD 70	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70	COD 140	COD 140	COD 140
0	7.5067	8.5067	8.3033	7.7300	7.7100	7.8650	7.7367	8.3600	8.4650	8.0200	8.2050	8.3000
1	7.0767	7.0100	7.6333	7.1167	-	-	7.7667	-	-	7.4167	-	-
3	7.7500	7.0400	7.1833	7.0300	8.1150	8.5000	7.4733	8.4450	8.8500	7.2600	8.3650	8.8150
4	6.9467	6.7700	7.0900	7.0033	-	-	7.4767	-	-	7.4833	-	-
5	7.4667	7.1933	7.2133	7.7767	7.6150	7.8300	7.6300	7.7900	8.5450	7.1700	8.0200	8.5650
6	7.0367	7.2067	7.1800	7.4033	-	-	7.4267	-	-	7.5000	-	-
7	6.9133	6.7700	7.0100	7.4267	7.7500	7.8250	7.4400	7.7950	8.5500	7.4467	8.1250	8.6600
8	6.9167	6.8333	7.0267	7.1567	-	-	7.1967	-	-	7.2000	-	-
9	6.8800	6.8767	7.0633	7.3333	7.7050	7.8750	7.1167	7.6950	8.5050	7.1167	7.8950	8.4850
10	7.0733	6.8533	6.9867	7.2167	-	-	7.1267	-	-	7.0833	-	-
12	6.9433	6.8933	6.8567	7.1600	7.7000	7.7700	7.1900	7.6200	8.4450	7.1800	7.6000	8.5000
13	6.7267	6.7267	6.8867	7.1633	-	-	7.1767	-	-	7.0767	-	-
14	6.8100	6.8967	6.7833	7.2333	7.5550	7.8400	7.1000	7.6400	8.2950	7.0967	7.6900	8.3400
18	6.6667	6.6667	6.7033	7.0133	-	-	7.0267	-	-	6.8967	-	-

Symbols and Abbreviations: WW = Wastewater.

Figure B-3.1.1 Average concentration of EE2 in EE2 degradation (3.5 mg/l) by AOB in NAS with industrial wastewater under 2 mM ammonia concentrations.

Day	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70	COD 140	COD 140	COD 140
0	3.6461	3.5849	3.6055	3.6577	3.4911	3.7061	3.6169	3.5248	3.5160
1	3.5095	-	-	3.3422	-	-	3.3887	-	-
4	3.2793	3.5770	3.5760	3.2532	3.5343	3.5215	3.2618	3.5379	3.6398
5	2.5762	-	-	2.9565	-	-	3.0625	-	-
6	2.4002	3.4110	3.4132	2.6348	3.4303	3.4595	2.5747	3.3958	3.4707
7	2.2205	-	-	2.4196	-	-	2.3694	-	-
8	1.9260	3.3801	3.4136	2.1373	3.3856	3.4437	2.0961	3.4041	3.4263
9	1.5416	-	-	1.8990	-	-	1.8482	-	-
10	1.0366	3.4094	3.4411	1.6948	3.3908	3.4357	1.5657	3.3717	3.4338
11	0.7946	-	-	1.5303	-	-	1.3336	-	-
12	0.4088	3.4069	3.5274	1.3035	3.3872	3.4369	1.2598	3.4006	3.4377
15	0.0191	-	-	1.0718	-	-	1.0366	-	-
18	0.0000	3.3963	3.4459	1.0822	3.3481	3.4144	0.9765	3.3872	3.4197

Figure B-3.1.2 Average concentration of EE2 in EE2 degradation (3.5 mg/l) by AOB in NAS with industrial wastewater under 2 mM ammonia concentrations.

Day	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 1000	COD 1000	COD 1000	COD 2000	COD 2000	COD 2000
0	3.6287	3.6236	3.6506	3.6399	3.6237	3.6172
1	3.4017	-	-	3.4044	-	-
4	3.1282	3.4324	3.4858	3.0849	3.4183	3.5455
5	2.9621	-	-	2.9666	-	-
6	2.4938	3.4775	3.4411	2.5887	3.3876	3.4329
7	2.3354	-	-	2.4087	-	-
8	2.0344	3.4067	3.4457	2.1180	3.3975	3.4079
9	1.8741	-	-	1.8174	-	-
10	1.6740	3.4044	3.4209	1.5802	3.3795	3.4365
11	1.4716	-	-	1.3304	-	-
12	1.2122	3.3935	3.4492	1.0849	3.4013	3.4533
15	1.0109	-	-	0.9524	-	-
18	0.9778	3.3996	3.4160	0.9412	3.3304	3.4325

Figure B-3.2.1 Average concentration of ammonia in EE2 degradation (3.5 mg/l) by AOB in NAS with industrial wastewater under 2 mM ammonia concentrations.

Day	Media	WW	WW	WW	WW	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 2000	COD 1000	COD 140	COD 70	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70
0	29.0468	28.9353	28.9888	28.9820	28.8585	28.8146	28.9535	28.9987	28.9856	28.9696	28.6333
1	23.6768	22.5006	21.7919	21.9894	22.9383	21.7689	-	-	24.0940	-	-
4	16.8664	20.4750	22.8835	20.8703	17.7420	19.1961	28.2788	29.3484	21.3626	27.6032	28.5985
5	11.3703	20.9805	22.3761	19.4187	16.0625	14.6317	-	-	16.6667	-	-
6	7.5421	19.9298	20.1904	17.4982	12.5195	11.9365	28.6238	28.0910	14.4458	27.6274	28.0033
7	4.6182	20.1389	18.1235	17.4318	10.3430	7.1296	-	-	11.3243	-	-
8	2.5948	17.6233	14.5227	14.6832	7.6658	4.0500	28.0287	28.6055	8.3874	27.1204	27.6582
9	0.5389	15.9406	14.5039	11.7113	3.7408	2.3559	-	-	4.5139	-	-
10	0.0715	15.4784	12.6366	9.4715	1.4958	1.3671	28.2310	28.7007	2.6190	27.5987	28.2846
11	0.0047	13.9898	11.0814	8.4148	1.6808	0.5202	-	-	1.2909	-	-
12	0.0000	13.0095	9.3667	7.3622	0.8281	0.0000	27.0781	28.4490	0.0492	27.2525	27.6927
15	0.0000	9.7114	4.9024	3.5570	0.0000	0.0000	-	-	0.0000	-	-
18	0.0000	3.3565	2.0831	0.9070	0.0000	0.0000	27.6093	27.7135	0.0000	27.0551	27.2833

Symbols and Abbrivations: WW = Wastewater.

Figure B-3.2.2 Average concentration of ammonia in EE2 degradation (3.5 mg/l) by AOB in NAS with industrial wastewater under 2 mM ammonia concentrations.

Day	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 140	COD 140	COD 140	COD 1000	COD 1000	COD 1000	COD 2000	COD 2000	COD 2000
0	29.0055	28.3030	29.0485	29.0206	28.9715	29.2578	28.8866	29.1198	28.4233
1	22.7525	-	-	22.1084	-	-	22.9924	-	-
4	21.1349	27.5794	28.7689	21.6989	27.5853	28.5537	21.1689	28.0573	27.6209
5	20.5611	-	-	21.4929	-	-	20.8615	-	-
6	18.2916	26.9344	27.5694	19.3492	27.5315	28.4007	19.5069	27.0089	28.0393
7	16.0355	-	-	17.4186	-	-	18.3629	-	-
8	13.6435	27.0249	28.6744	15.8747	27.5095	28.9541	16.7322	27.8174	28.3115
9	11.5208	-	-	14.8184	-	-	16.0663	-	-
10	10.6961	28.0172	27.7061	13.3602	27.8413	28.4854	15.1347	27.9523	27.5858
11	8.1570	-	-	11.2329	-	-	13.8054	-	-
12	6.1576	27.0637	28.1897	10.2354	27.5014	27.5701	12.3260	27.3013	27.0985
15	2.8893	-	-	6.3069	-	-	8.5175	-	-
18	1.7228	27.5800	27.3342	2.9878	27.0934	27.5563	4.3598	26.5391	27.1279

Figure B-3.3.1 Average concentration of nitrite in EE2 degradation (3.5 mg/l) by AOB in NAS with industrial wastewater under 2 mM ammonia concentrations.

Day	Media	WW	WW	WW	WW	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 2000	COD 1000	COD 140	COD 70	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70
0	0.0000	2.0318	0.5400	0.5318	1.0400	0.0000	0.0000	0.0000	3.8484	4.6871	2.2431
1	1.3429	19.1439	18.9612	6.5618	1.5170	0.9146	-	-	11.3812	-	-
4	2.1937	19.4386	14.5214	2.6291	17.4512	1.4682	0.4954	0.1189	9.7000	7.1143	7.9581
5	3.4152	17.4902	12.9657	0.5190	9.0502	2.3739	-	-	10.9515	-	-
6	3.5922	18.0692	16.5585	0.9946	1.5671	2.3533	0.4574	0.9200	12.3854	4.8541	4.9020
7	3.1641	24.5339	8.6012	1.2212	1.9383	3.7289	-	-	14.0728	-	-
8	1.4887	28.4522	11.5905	12.5263	0.0165	3.3443	1.3491	0.9566	1.0277	4.5890	6.5196
9	1.0811	31.5804	15.3448	18.5283	1.4845	2.9700	-	-	3.0738	-	-
10	0.5122	30.5301	23.7872	19.5308	6.0567	1.5500	0.5052	0.9560	0.2851	16.5435	7.1940
11	0.0703	35.7463	15.8612	3.5500	1.4586	0.7165	-	-	0.9811	-	-
12	0.0000	28.5589	23.5574	1.7843	12.5512	0.4930	0.6534	0.5533	0.0000	16.4788	10.3120
15	0.0000	26.0729	13.8887	0.5289	28.9532	0.0245	-	-	0.0000	-	-
18	0.0000	24.5517	12.1518	0.0635	0.2514	0.0000	0.9694	0.6633	0.0000	15.4163	14.4087

Symbols and Abbrivations: WW = Wastewater.

Figure B-3.3.2 Average concentration of nitrite in EE2 degradation (3.5 mg/l) by AOB in NAS with industrial wastewater under 2 mM ammonia concentrations.

Day	EE2 COD 140	Inhibition COD 140	Control COD 140	EE2 COD 1000	Inhibition COD 1000	Control COD 1000	EE2 COD 2000	Inhibition COD 2000	Control COD 2000
0	0.9855	1.5250	0.9971	3.8484	1.1871	0.7431	2.2597	2.9190	1.2754
1	5.3659	-	-	9.3430	-	-	9.5858	-	-
4	8.8671	3.6129	1.1597	14.9699	14.5382	14.7682	4.0380	5.8345	3.1278
5	7.1267	-	-	16.5768	-	-	18.4656	-	-
6	14.3758	8.5456	9.5491	20.7122	3.5180	1.9793	28.3665	5.1704	6.0711
7	16.6233	-	-	26.4226	-	-	37.8100	-	-
8	6.0816	11.5334	5.0192	28.0819	1.5193	0.5162	29.0626	0.5596	0.5467
9	5.3507	-	-	27.0528	-	-	27.7599	-	-
10	2.0227	14.9822	9.6005	20.6340	1.0708	0.8327	25.6108	1.5531	0.4752
11	3.4064	-	-	10.1160	-	-	24.3659	-	-
12	2.0773	14.0181	8.4482	11.4017	10.5641	4.5250	23.4406	0.5245	1.9865
15	2.3525	-	-	24.2775	-	-	31.7637	-	-
18	0.0000	15.5840	5.0546	18.4329	0.5254	1.0360	33.6862	1.6945	0.7093

Figure B-3.4.1 Average concentration of nitrate in EE2 degradation (3.5 mg/l) by AOB in NAS with industrial wastewater under 2 mM ammonia concentrations.

Day	Media	WW	WW	WW	WW	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 2000	COD 1000	COD 140	COD 70	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70
0	0.0039	48.6991	32.5526	26.1478	15.0284	0.0029	0.0000	0.0000	14.0475	13.2164	14.7608
1	10.7563	30.3046	19.4601	21.7591	23.1730	12.4697	-	-	14.8798	-	-
4	14.2498	33.5991	29.0476	27.5556	18.5925	22.3076	0.8593	0.5202	19.5712	14.6447	13.7636
5	22.0916	33.6374	26.7008	35.6174	29.5941	26.2610	-	-	22.2543	-	-
6	22.6216	31.0922	28.7868	40.5593	34.5125	28.4403	2.6004	1.4290	24.7106	16.1592	15.8606
7	26.8899	28.1183	46.9394	67.5583	53.6269	32.2666	-	-	28.0346	-	-
8	31.4448	22.5568	35.0468	26.4630	53.4167	31.2565	2.9016	1.7658	44.7391	15.6734	13.3091
9	32.9453	21.3805	33.0642	25.1157	49.5789	27.9351	-	-	47.2995	-	-
10	31.8998	22.6319	27.8640	27.8640	44.3894	31.0271	1.9111	0.9739	54.1350	11.4583	14.7158
11	34.3918	19.2955	37.0921	42.3920	57.3330	30.7052	-	-	63.9950	-	-
12	32.6399	22.1193	21.3805	49.0595	46.5787	31.8691	1.0470	0.0800	62.1197	13.3616	13.7003
15	32.0465	26.7248	40.0307	54.4892	27.1068	31.1196	-	-	63.0655	-	-
18	33.3790	34.2646	45.9124	58.6059	63.0283	30.8420	2.6284	1.2619	67.9880	16.5604	12.6506

Symbols and Abbrivations: WW = Wastewater.

Figure B-3.4.2 Average concentration of nitrate in EE2 degradation (3.5 mg/l) by AOB in NAS with industrial wastewater under 2 mM ammonia concentrations.

Day	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 140	COD 140	COD 140	COD 1000	COD 1000	COD 1000	COD 2000	COD 2000	COD 2000
0	25.7739	26.5226	20.6367	35.0897	33.5375	29.5247	47.1104	43.0850	38.1298
1	22.6240	-	-	35.8253	-	-	46.8000	-	-
4	23.0006	20.0878	20.5325	27.9487	20.0320	21.2941	52.9187	27.5631	30.0192
5	24.7514	-	-	25.3513	-	-	35.7624	-	-
6	24.1039	23.1108	23.9189	24.2841	32.5300	30.0306	24.7796	39.0524	30.8825
7	24.6529	-	-	23.9882	-	-	17.1160	-	-
8	37.6656	24.5812	19.8003	12.5281	33.0642	30.7319	24.8907	47.1097	40.4946
9	41.2614	-	-	26.3707	-	-	27.6069	-	-
10	47.3579	19.4876	22.7537	35.6512	29.6263	29.6071	28.2502	41.1775	40.5862
11	51.9425	-	-	48.0141	-	-	32.8946	-	-
12	54.7841	21.2965	21.1634	45.8770	26.5456	27.8562	31.1385	42.6985	35.5814
15	61.5243	-	-	38.2428	-	-	30.2261	-	-
18	73.5663	23.6073	25.0770	50.4052	40.0981	28.9339	35.4217	43.1410	39.7278

Figure B-3.5.1 Average concentration of total nitrogen in EE2 degradation (3.5 mg/l) by AOB in NAS with industrial wastewater under 2 mM ammonia concentrations.

Day	Media	WW	WW	WW	WW	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 2000	COD 1000	COD 140	COD 70	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70
0	29.0507	79.6661	62.0814	55.6615	44.9268	28.8175	28.9535	28.9987	45.5986	46.8730	45.6372
1	35.7760	71.9490	60.2131	50.3103	47.6283	35.1532	-	-	50.3550	-	-
4	33.3099	73.5126	66.4525	51.0550	53.7856	42.9719	29.6335	29.9875	50.6338	49.3621	50.3202
5	36.8770	72.1081	62.0426	55.5551	54.7068	43.2665	-	-	49.8725	-	-
6	33.7558	69.0912	65.5357	59.0521	48.5991	42.7300	31.6815	30.4399	51.5419	48.6407	48.7659
7	34.6722	72.7911	73.6641	86.2113	65.9082	43.1251	-	-	53.4317	-	-
8	35.5283	68.6323	61.1599	53.6725	61.0990	38.6508	32.2794	31.3278	54.1542	47.3827	47.4869
9	34.5653	68.9015	62.9129	55.3553	54.8041	33.2610	-	-	53.5662	-	-
10	32.4834	68.6405	64.2878	56.8663	51.9418	33.9442	30.9557	30.9402	57.0391	55.0604	50.6236
11	34.4668	69.0316	64.0347	54.3568	60.4724	31.9419	-	-	66.2670	-	-
12	32.6399	63.6878	54.3047	58.2060	58.9944	32.3622	28.7786	29.0823	61.1457	57.0929	51.7050
15	32.0465	62.5091	58.8217	58.5751	56.0600	31.1441	-	-	63.0655	-	-
18	33.3790	62.1729	60.1473	59.5764	63.2797	30.8420	31.2070	29.6387	67.9880	59.0319	54.3425

Symbols and Abbrivations: WW = Wastewater.

Figure B-3.5.2 Average concentration of total nitrogen in EE2 degradation (3.5 mg/l) by AOB in NAS with industrial wastewater under 2 mM ammonia concentrations.

Day	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 140	COD 140	COD 140	COD 1000	COD 1000	COD 1000	COD 2000	COD 2000	COD 2000
0	55.4364	56.3505	50.6823	66.6758	63.6961	59.5256	77.5035	75.1238	67.8284
1	50.7424	-	-	67.2766	-	-	79.3782	-	-
4	53.0026	51.2800	50.4611	64.6175	62.1554	64.6159	78.1255	61.4549	60.7678
5	52.4392	-	-	63.4210	-	-	75.0895	-	-
6	56.7714	58.5907	61.0374	64.3455	63.5795	60.4106	72.6530	71.2317	64.9928
7	57.3117	-	-	67.8295	-	-	73.2889	-	-
8	57.3907	63.1394	53.4939	56.4846	62.0930	60.2022	70.6855	75.4866	69.3528
9	58.1330	-	-	68.2419	-	-	71.4332	-	-
10	59.8590	62.3948	60.6597	69.8261	57.7895	58.5582	69.0662	70.6350	69.1166
11	63.5060	-	-	69.3630	-	-	71.0659	-	-
12	63.0190	62.3783	57.8013	67.5141	64.6110	59.9513	66.9050	70.5243	64.6664
15	66.7661	-	-	68.8271	-	-	70.5073	-	-
18	75.2890	66.7714	57.4658	72.8722	67.7169	57.5261	73.4677	71.3745	67.5649

Figure B-3.6.1 Average pH of EE2 degradation (3.5 mg/l) by AOB in NAS with industrial wastewater under 2 mM ammonia concentration.

Day	Media	WW	WW	WW	WW	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 2000	COD 1000	COD 140	COD 70	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70
0	7.8350	7.6700	7.5750	7.5550	7.5150	7.6900	7.7300	7.6050	7.7567	7.6450	7.6300
1	7.6350	7.6200	7.6150	8.0350	7.5700	7.5767	-	-	7.6033	-	-
4	7.4300	7.4700	7.6700	7.9450	7.6850	7.4100	7.6050	7.5400	7.5300	7.6200	7.5600
5	7.3050	7.8550	7.7900	7.7900	7.5050	7.4767	-	-	7.4767	-	-
6	7.3950	7.6100	7.6550	7.6950	7.3950	7.5300	7.5300	7.7900	7.4400	7.9200	8.1750
7	7.3300	7.4850	7.5300	7.7050	7.2350	7.4750	-	-	7.4450	-	-
8	7.2750	7.3000	7.4400	7.6200	7.1150	7.5050	8.0650	7.9750	7.5200	8.3050	8.3950
9	7.1150	7.1000	7.2950	7.6550	7.2950	7.6500	-	-	7.3700	-	-
10	7.1650	6.8600	7.1450	7.5800	7.6650	8.0100	8.0500	8.0600	7.6850	8.2100	8.1900
11	6.9350	7.0700	7.4550	7.2350	7.1400	7.7150	-	-	7.4900	-	-
12	6.8700	7.9100	7.6250	7.2800	7.2450	7.5900	8.0350	8.1300	7.4050	8.1600	8.1150
15	6.8100	8.3100	7.7950	7.4050	7.3000	7.3000	-	-	7.3750	-	-
18	6.7350	8.1900	7.6000	7.4700	7.2050	7.2050	8.0850	8.0850	7.2550	8.2100	8.1250

Symbols and Abbrivations: WW = Wastewater.

Figure B-3.6.2 Average pH of EE2 degradation (3.5 mg/l) by AOB in NAS with industrial wastewater under 2 mM ammonia concentration.

Day	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 140	COD 140	COD 140	COD 1000	COD 1000	COD 1000	COD 2000	COD 2000	COD 2000
0	7.65	7.63	7.79	7.65	7.70	7.76	7.73	7.69	7.72
1	-	-	7.61	-	-	7.78	-	-	7.69
4	7.62	7.56	7.54	7.51	7.64	7.91	7.58	7.57	7.62
5	-	-	7.58	-	-	7.75	-	-	7.60
6	7.92	8.18	7.43	7.76	7.90	7.62	7.61	7.73	7.56
7	-	-	7.39	-	-	7.39	-	-	7.53
8	8.31	8.40	7.46	8.28	8.33	7.44	8.31	8.38	7.33
9	-	-	7.43	-	-	7.56	-	-	7.53
10	8.21	8.19	7.27	8.23	8.26	7.00	8.35	8.35	7.30
11	-	-	7.37	-	-	7.60	-	-	7.89
12	8.16	8.12	7.48	8.20	8.16	7.69	8.31	8.25	8.09
15	-	-	7.42	-	-	7.80	-	-	8.19
18	8.21	8.13	7.47	8.13	8.14	7.60	8.21	8.26	8.31

Symbols and Abbrivations: WW = Wastewater.

Figure A-4.1 Average concentration of EE2 in EE2 degradation (3.5 mg/l) by AOB in NAS with municipal wastewater under 30 mM ammonia concentrations.

Day	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70	COD 140	COD 140	COD 140
0	3.5292	3.51045	3.538	3.5279	3.5211	3.5505	3.51513	3.5432	3.56175
1	3.46797	-	-	3.46557	-	-	3.5664	-	-
4	3.07913	3.53395	3.5365	3.0694	3.50755	3.58555	3.10503	3.54865	3.52465
5	2.68354	-	-	2.6963	-	-	2.84712	-	-
6	2.43347	3.5093	3.55085	2.4687	3.559	3.51185	2.58571	3.5699	3.5209
7	2.04523	-	-	1.97015	-	-	2.3731	-	-
8	1.63367	3.52555	3.53615	1.3792	3.56145	3.58135	1.9024	3.49885	3.52195
9	0.93389	-	-	1.27687	-	-	1.23013	-	-
10	0.24878	3.5315	3.49355	0.709	3.54595	3.49985	0.99345	3.508	3.52265
11	0.00567	-	-	0.0221	-	-	0.0000	-	-
12	0.0000	3.49605	3.5078	0.0000	3.49135	3.52395	0.0000	3.4915	3.49955
15	0.0000	-	-	0.0000	-	-	0.0000	-	-
18	0.0000	3.4876	3.51435	0.0000	3.4937	3.4967	0.0000	3.49945	3.50905

Figure A-4.2 Average concentration of ammonia in EE2 degradation (3.5 mg/l) by AOB in NAS with municipal wastewater under 30 mM ammonia concentrations.

Day	Media	WW	WW	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 140	COD 70	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70	COD 140	COD 140	COD 140
0	424.4550	425.0474	423.7990	424.5739	425.8857	422.5801	422.4679	423.7270	423.0858	419.1384	423.0500	428.0435
1	407.5701	408.8181	401.8272	406.6547	-	-	395.6690	-	-	392.8393	-	-
4	408.8185	407.5701	401.8276	353.7234	425.0880	423.2150	357.2189	420.0586	420.2413	364.8756	410.5662	417.8068
5	367.3724	372.1162	361.8795	342.1918	-	-	330.6501	-	-	339.0392	-	-
6	325.6566	339.8382	311.4751	283.1119	420.1815	420.2298	307.4802	406.0311	413.8807	310.6761	400.0799	410.3166
7	303.6851	326.6553	288.7047	240.2343	-	-	260.7410	-	-	275.5218	-	-
8	276.9100	292.8143	269.5695	207.7583	417.6605	419.3072	230.4787	403.2372	410.5007	251.6845	387.5961	405.0734
9	224.4681	235.1040	236.9020	171.7867	-	-	201.1485	-	-	220.7230	-	-
10	200.3495	220.4234	186.5672	134.5351	416.7564	418.4822	177.0795	404.7629	407.9058	197.9527	390.5922	402.8263
11	173.9139	-	179.3069	106.4682	-	-	148.6797	-	-	174.8460	-	-
12	171.0165	202.6183	182.2851	80.1084	418.3514	419.5869	122.3119	401.6926	406.1928	156.4246	390.9296	403.3547
15	145.3070	164.4953	159.6671	56.4778	-	-	97.1118	-	-	118.8426	-	-
18	114.9042	140.2337	135.2801	40.0381	417.4824	415.9035	69.2749	398.5855	404.9422	93.3068	384.0244	398.3533

Symbols and Abbrivations: WW = Wastewater.

Figure A-4.3 Average concentration of nitrite in EE2 degradation (3.5 mg/l) by AOB in NAS with municipal wastewater under 30 mM ammonia concentrations.

Day	Media	WW	WW	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 140	COD 70	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70	COD 140	COD 140	COD 140
0	0.0000	1.0753	0.8948	0.0000	0.0000	0.0000	0.6913	0.9289	0.6878	1.5271	1.2038	0.8267
1	2.4040	3.4619	1.3811	2.6550	-	-	2.1533	-	-	2.4400	-	-
4	7.1663	4.5333	2.5369	4.6877	1.2860	1.0355	6.7719	1.8353	1.1958	6.1673	1.6186	1.0199
5	7.4009	9.4843	8.1009	9.5194	-	-	13.4856	-	-	13.7850	-	-
6	14.3923	14.5535	11.9269	12.7035	0.7586	1.4108	15.0188	1.5599	0.9781	14.8374	3.1566	1.3941
7	14.0145	17.6371	13.2752	14.5442	-	-	17.3697	-	-	17.5495	-	-
8	15.1813	15.6899	12.6508	15.7111	1.7066	2.0175	14.9750	2.9822	1.6318	18.6594	3.5263	1.0526
9	10.4543	8.6326	12.1000	12.7837	-	-	14.2902	-	-	15.5152	-	-
10	12.6266	11.1167	14.5632	13.8040	1.0449	0.4537	13.4271	2.0242	0.5539	18.1722	2.2150	1.3952
11	11.9125	-	9.7762	10.7748	-	-	12.2602	-	-	13.5498	-	-
12	10.0364	6.5474	9.0471	3.9110	1.9643	1.0187	11.3968	0.4940	0.2771	13.9201	0.8974	0.5614
15	7.0472	8.3899	5.6201	4.9612	-	-	8.5679	-	-	9.3758	-	-
18	9.5095	6.7127	7.8269	4.1217	0.0167	1.0109	3.5727	0.0433	0.4928	7.1675	2.2042	1.0404

Symbols and Abbrivations: WW = Wastewater.

Figure A-4.4 Average concentration of nitrate in EE2 degradation (3.5 mg/l) by AOB in NAS with municipal wastewater under 30 mM ammonia concentrations.

Day	Media	WW	WW	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 140	COD 70	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70	COD 140	COD 140	COD 140
0	0.0000	1.8425	1.0200	0.0000	0.0000	0.0000	0.9669	1.2752	0.9695	2.2037	1.2280	0.9708
1	8.7653	14.3121	8.7196	10.0516	-	-	21.9811	-	-	26.1441	-	-
4	38.8478	56.9299	50.0441	52.0112	1.4411	0.8117	57.4189	1.6171	0.9289	60.7231	6.3699	2.5663
5	56.4572	74.8339	71.5807	70.7250	-	-	78.5461	-	-	90.8669	-	-
6	74.1811	97.8730	82.4532	92.7655	3.4835	3.7346	111.7674	19.2442	5.5925	138.0874	15.0451	7.6169
7	91.2183	114.4329	105.0391	128.4182	-	-	142.7025	-	-	184.7623	-	-
8	118.8092	140.9164	134.1438	155.1518	2.0940	2.6664	180.4113	16.0562	7.4997	207.1842	25.5878	17.4671
9	136.5345	183.0295	143.4612	195.2420	-	-	214.7054	-	-	250.2868	-	-
10	154.1771	192.4595	182.1957	217.3087	3.1388	0.6607	245.3122	17.2067	16.6050	287.5524	27.9307	17.2992
11	169.9762	-	206.3727	247.2564	-	-	274.2297	-	-	316.4358	-	-
12	196.8915	240.2288	224.0880	274.6839	2.8157	2.0342	299.2396	24.5242	10.5798	355.7930	34.3900	29.9775
15	257.5867	272.3842	249.5160	305.5110	-	-	333.3373	-	-	386.7045	-	-
18	294.9409	295.0116	277.0585	341.1568	6.3085	2.2099	369.7937	39.0368	18.0575	397.0899	43.8249	23.8735

Symbols and Abbrivations: WW = Wastewater.

Figure A-4.5 Average concentration of total nitrogen in EE2 degradation (3.5 mg/l) by AOB in NAS with municipal wastewater under 30 mM ammonia concentrations.

Day	Media	WW	WW	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 140	COD 70	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70	COD 140	COD 140	COD 140
0	423.7990	427.9652	426.3698	424.5739	425.8857	422.5801	423.5733	425.9311	424.7430	421.6256	425.4818	429.8409
1	412.9965	426.5920	417.6707	419.3612	-	-	419.8033	-	-	421.4234	-	-
4	447.8417	469.0333	461.3994	410.4224	427.8150	425.0622	421.4097	423.5109	422.3659	431.7661	418.5547	421.3930
5	425.7376	456.4344	447.0539	422.4361	-	-	422.6818	-	-	443.6911	-	-
6	396.7424	452.2646	420.0367	388.5809	424.4236	425.3752	434.2664	426.8351	420.4512	466.6636	418.2816	419.3276
7	393.9374	449.7317	421.9994	383.1968	-	-	420.8132	-	-	477.8336	-	-
8	403.5600	449.4206	423.7045	378.6212	421.4611	423.9910	425.8650	422.2755	419.6321	477.5281	416.7101	423.5931
9	383.8907	426.7660	380.0293	379.8123	-	-	430.1440	-	-	486.5250	-	-
10	353.3708	423.9996	397.1083	365.6478	420.9400	419.5966	435.8188	423.9937	425.0646	503.6772	420.7378	421.5207
11	337.3266	-	395.7797	364.4994	-	-	435.1696	-	-	504.8316	-	-
12	389.2129	417.7926	415.4202	358.7032	423.1313	422.6398	432.9484	426.7107	417.0496	526.1377	426.2169	433.8936
15	424.3009	445.2694	414.8031	366.9501	-	-	439.0170	-	-	514.9229	-	-
18	439.7305	441.9580	420.1655	385.3166	423.8076	419.1243	442.6413	437.6656	423.4925	497.5641	430.0535	423.2672

Symbols and Abbrivations: WW = Wastewater.

Figure A-4.6 Average pH of EE2 degradation (3.5 mg/l) by AOB in NAS with municipal wastewater under 30 mM ammonia concentration.

Day	Media	WW	WW	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 140	COD 70	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70	COD 140	COD 140	COD 140
0	7.8500	8.2700	8.2250	8.3867	8.3250	8.4150	8.3833	8.3600	8.4150	8.3567	8.3850	8.3950
1	7.6950	8.0900	8.0300	8.3300	-	-	8.3300	-	-	8.3600	-	-
4	7.5350	7.8250	7.8750	8.1133	8.2550	8.3250	8.1433	8.3450	8.3500	8.0500	8.2650	8.3650
5	7.3400	7.7550	7.6600	7.9833	-	-	7.8100	-	-	7.8167	-	-
6	7.1400	7.5667	7.5433	7.5833	8.2800	8.3200	7.5967	8.2350	8.3450	7.5733	8.0700	8.3650
7	7.1350	7.4400	7.5250	7.5367	-	-	7.5600	-	-	7.5333	-	-
8	7.0050	7.4333	7.4167	7.4933	8.1750	8.2850	7.4400	8.0450	8.2500	7.4133	8.0350	8.2100
9	6.9533	7.3050	7.3600	7.3600	-	-	7.3067	-	-	7.2667	-	-
10	6.8550	7.2150	7.2550	7.2567	8.0850	8.2450	7.2800	7.9700	8.2550	7.2100	7.9950	8.2850
11	6.8450	7.1000	7.1633	7.1967	-	-	7.1267	-	-	7.0833	-	-
12	6.9150	7.1300	7.0800	7.0967	8.1050	8.2150	7.0433	7.9700	8.1950	6.9500	7.9500	8.2000
15	6.8650	7.1000	6.9400	7.0400	-	-	6.9400	-	-	6.8533	-	-
18	6.5800	7.0200	6.9100	6.9400	8.0500	8.1700	6.8800	7.9300	8.2500	6.8250	7.9500	8.2500

Symbols and Abbrivations: WW = Wastewater.

Figure A-5.1 Average concentration of EE2 in EE2 degradation (10 mg/l) by AOB in NAS with municipal wastewater under 2 mM ammonia concentrations.

Day	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70	COD 140	COD 140	COD 140
0	11.1760	11.5754	11.6122	11.2124	11.4201	11.1462	11.2218	11.6056	11.0075
1	10.2747	-	-	10.4671	-	-	11.2247	-	-
3	8.9531	11.0183	10.7662	9.1377	10.8441	10.9805	11.1562	11.2937	10.8906
4	8.8035	-	-	9.0540	-	-	9.8060	-	-
5	7.8704	11.1454	10.9372	8.8327	11.0634	11.3761	9.7692	11.2108	10.9408
6	6.8979	-	-	7.8721	-	-	8.8818	-	-
7	4.8012	10.1817	11.0435	7.1696	10.3662	11.3791	8.2239	9.9899	11.6793
8	4.3126	-	-	5.8665	-	-	7.4775	-	-
9	3.4635	10.6310	11.0608	4.7935	10.4360	10.2577	6.2696	10.1181	10.4167
10	3.1903	-	-	3.8280	-	-	5.3796	-	-
12	3.1847	10.1034	10.9633	3.4120	10.1636	10.8600	4.0991	10.2000	10.9594
13	2.5647	-	-	3.0345	-	-	3.6271	-	-
14	2.2011	10.1078	10.6823	2.9966	10.1260	11.3246	3.3324	10.3769	10.7774
18	2.9074	-	-	3.1512	-	-	3.2884	-	-

Figure A-5.2 Average concentration of ammonia in EE2 degradation (10 mg/l) by AOB in NAS with municipal wastewater under 2 mM ammonia concentrations.

Day	Media	WW	WW	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 140	COD 70	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70	COD 140	COD 140	COD 140
0	29.3459	28.7399	29.0259	28.9330	29.3208	28.8855	29.0435	29.1608	28.9931	29.1410	28.8935	28.7455
1	22.1898	20.5782	22.5903	20.9777	-	-	20.8942	-	-	20.4507	-	-
3	8.8696	6.0414	8.4355	9.5844	27.4936	28.0186	8.0831	27.6122	27.9979	7.1284	27.0328	28.0893
4	4.8103	3.0710	3.9642	7.0826	-	-	5.3711	-	-	4.6678	-	-
5	1.6151	2.3862	2.8887	3.3421	27.0094	27.5175	3.0168	26.8142	27.5454	1.2752	25.9942	27.2756
6	0.3573	0.3840	0.4588	2.4363	-	-	2.4664	-	-	0.6472	-	-
7	0.3798	0.6102	0.3332	1.9329	27.9399	28.5001	1.0940	26.8436	28.0634	0.1560	25.9787	28.0749
8	0.0000	0.0000	0.0000	0.9547	-	-	0.8095	-	-	0.0749	-	-
9	0.0000	0.0000	0.0000	0.7726	25.9607	28.0139	0.3167	25.8112	27.8619	0.0000	26.3194	27.8139
10	0.0000	0.0000	0.0000	0.0777	-	-	0.0491	-	-	0.0000	-	-
12	0.0000	0.0000	0.0000	0.0000	25.8110	28.2082	0.0000	25.9370	27.7674	0.0000	25.5845	27.9068
13	0.0000	0.0000	0.0000	0.0000	-	-	0.0000	-	-	0.0000	-	-
14	0.0000	0.0000	0.0000	0.0000	26.0161	28.0798	0.0000	25.5608	28.1128	0.0000	25.6157	27.6983
18	0.0000	0.0000	0.0000	0.0000	-	-	0.0000	-	-	0.0000	-	-

Symbols and Abbrivations: WW = Wastewater.

Figure A-5.3 Average concentration of nitrite in EE2 degradation (10 mg/l) by AOB in NAS with municipal wastewater under 2 mM ammonia concentrations.

Day	Media	WW	WW	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 140	COD 70	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70	COD 140	COD 140	COD 140
0	0.0000	0.0318	0.0400	0.0000	0.0000	0.0000	0.0873	0.6441	0.0685	1.0439	0.8369	1.4434
1	1.8889	1.8189	0.6571	1.1918	-	-	1.3698	-	-	2.1616	-	-
3	2.2861	2.3179	1.5528	1.0219	0.3154	0.0027	2.3044	0.5391	0.2984	2.0139	1.4615	0.5300
4	1.2485	4.1447	2.6011	1.6470	-	-	2.3613	-	-	3.2803	-	-
5	2.6209	1.5782	2.2186	1.9212	0.4553	0.0039	3.7116	1.4921	0.1082	3.6821	0.5521	1.0355
6	1.1154	1.8811	1.4868	1.7475	-	-	3.2365	-	-	2.9729	-	-
7	0.4849	1.4801	0.8855	2.4652	0.1896	0.1975	2.7813	0.5363	0.3438	2.5316	1.0379	0.7108
8	0.0604	0.5634	0.5373	3.1926	-	-	2.9419	-	-	2.3606	-	-
9	0.0325	0.4200	0.0895	2.4205	0.2385	0.0349	2.0544	1.3968	0.5298	1.5435	0.6339	0.7996
10	0.0485	0.0362	0.2160	1.6909	-	-	1.9462	-	-	1.5595	-	-
12	0.0000	0.0000	0.0000	1.3563	0.1866	0.0237	0.7289	1.2834	1.0657	0.6672	0.8276	0.5351
13	0.0000	0.0000	0.0000	0.6956	-	-	0.9592	-	-	0.0524	-	-
14	0.0000	0.0000	0.0000	0.1757	0.5159	0.4506	0.0000	0.3864	0.8330	0.1577	0.8620	0.6276
18	0.0000	0.0000	0.0000	0.0000	-	-	0.0000	-	-	0.0000	-	-

Symbols and Abbrivations: WW = Wastewater.

Figure A-5.4 Average concentration of nitrate in EE2 degradation (10 mg/l) by AOB in NAS with municipal wastewater under 2 mM ammonia concentrations.

Day	Media	WW	WW	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 140	COD 70	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70	COD 140	COD 140	COD 140
0	0.0000	0.1478	0.0284	0.0000	0.0000	0.0000	0.0237	0.1112	0.0492	0.1408	0.1997	0.1519
1	4.1564	5.8578	5.0999	6.2932	-	-	6.3407	-	-	6.7650	-	-
3	16.7843	20.5704	18.5845	16.7889	0.6011	0.0312	18.7133	0.5410	0.4450	20.0450	1.5352	0.1198
4	22.9713	21.8096	21.1221	19.2685	-	-	21.2031	-	-	21.7223	-	-
5	26.1791	25.0205	23.0791	22.7243	0.7395	0.6147	23.0412	0.8002	1.7831	25.6755	2.4561	0.5214
6	28.8011	26.5330	26.0646	24.2510	-	-	24.2203	-	-	29.2060	-	-
7	28.9902	27.2327	26.5648	24.1031	0.6351	0.0905	26.0258	1.0351	0.0457	26.9911	1.0949	0.0098
8	29.0681	29.0875	28.2315	25.0239	-	-	26.8582	-	-	28.2395	-	-
9	30.2411	30.0767	29.0800	25.6884	2.2116	0.9896	27.2333	1.0451	0.0237	28.3987	3.4902	1.5301
10	29.8880	30.5822	30.4212	26.4522	-	-	28.5464	-	-	29.7390	-	-
12	30.1329	30.5498	30.2770	27.3188	1.9290	0.0664	28.9895	1.5298	0.0488	30.6332	3.4494	1.0090
13	30.6557	30.8998	30.1517	28.1864	-	-	29.8107	-	-	30.7100	-	-
14	30.0731	30.6126	30.5335	29.8265	1.5459	0.4175	30.5246	2.9768	0.6152	31.2734	3.0166	0.1196
18	30.5790	31.3553	30.6062	30.1999	-	-	30.8196	-	-	31.4848	-	-

Symbols and Abbrivations: WW = Wastewater.

Figure A-5.5 Average concentration of total nitrogen in EE2 degradation (10 mg/l) by AOB in NAS with municipal wastewater under 2 mM ammonia concentrations.

Day	Media	WW	WW	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 140	COD 70	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70	COD 140	COD 140	COD 140
0	29.3459	28.9195	29.0942	28.9330	29.3208	28.8855	29.1175	29.9160	29.1108	29.9307	29.9300	30.3408
1	28.2351	28.2549	28.3472	28.4627	-	-	28.6047	-	-	29.3773	-	-
3	28.3920	28.9296	28.5727	27.3952	28.4101	28.0525	29.1009	28.6922	28.7413	29.1873	30.0294	28.7390
4	29.0301	29.0252	27.6874	27.9981	-	-	28.9356	-	-	29.6704	-	-
5	30.4150	28.9848	28.1863	27.9877	28.2041	28.1360	29.7695	29.1065	29.4367	30.6328	29.0024	28.8324
6	30.2738	28.7981	28.0101	28.4348	-	-	29.9233	-	-	32.8785	-	-
7	29.8548	29.3229	27.7835	28.5012	28.7646	28.7880	29.9011	28.4150	28.4528	30.1200	28.1114	28.7955
8	29.1284	29.6509	28.7687	29.1712	-	-	30.6096	-	-	30.8459	-	-
9	30.2736	30.4967	29.1695	28.8815	28.4108	29.0384	29.6045	28.2530	28.4153	30.7593	30.4434	30.1435
10	29.9364	30.6184	30.6371	28.2208	-	-	30.5416	-	-	31.2824	-	-
12	30.1329	30.5498	30.2770	28.6751	27.9265	28.2983	29.7184	28.7501	28.8819	32.1927	29.8615	29.4508
13	30.6557	30.8998	30.1517	28.8819	-	-	30.7699	-	-	31.3772	-	-
14	30.0731	30.6126	30.5335	30.0022	27.8728	29.0762	30.5246	29.3001	29.2156	31.3258	29.4631	28.6540
18	30.5790	31.3553	30.6062	30.1999	-	-	30.8196	-	-	31.6424	-	-

Symbols and Abbrivations: WW = Wastewater.

Figure A-5.6 Average pH of EE2 degradation (10 mg/l) by AOB in NAS with municipal wastewater under 2 mM ammonia concentration.

Day	Media	WW	WW	EE2	Inhibition	Control	EE2	Inhibition	Control	EE2	Inhibition	Control
	COD 0	COD 140	COD 70	COD 0	COD 0	COD 0	COD 70	COD 70	COD 70	COD 140	COD 140	COD 140
0	7.5067	8.5067	8.3033	7.8267	7.7400	7.6950	7.8433	8.4700	8.4900	8.0533	8.6500	8.4050
1	7.0767	7.0100	7.6333	7.5133	-	-	7.5300	-	-	7.5767	-	-
3	7.7500	7.0400	7.1833	8.0467	8.1400	8.1100	7.2867	8.0200	8.0750	7.2700	7.5950	7.6850
4	6.9467	6.7700	7.0900	7.2633	-	-	7.3133	-	-	6.9833	-	-
5	7.4667	7.1933	7.2133	7.2633	7.6050	7.7800	7.3800	7.6100	7.9350	7.2567	7.7000	8.5250
6	7.0367	7.2067	7.1800	7.2467	-	-	7.2633	-	-	6.9833	-	-
7	6.9133	6.7700	7.0100	7.1933	7.4900	7.5850	7.2267	7.7000	7.9250	7.1333	7.7000	8.4550
8	6.9167	6.8333	7.0267	7.1467	-	-	7.2800	-	-	7.0367	-	-
9	6.8800	6.8767	7.0633	7.0833	7.4950	7.6950	7.2167	7.6350	7.9200	6.9600	7.6200	8.4300
10	7.0733	6.8533	6.9867	7.1467	-	-	7.2267	-	-	7.1167	-	-
12	6.9433	6.8933	6.8567	7.1600	7.5150	7.7100	7.1733	7.6500	7.9400	7.0500	7.5000	8.4450
13	6.7267	6.7267	6.8867	7.1100	-	-	7.1600	-	-	7.1067	-	-
14	6.8100	6.8967	6.7833	6.9967	7.4200	7.6150	7.1867	7.7850	7.9900	6.9967	7.5100	8.5900
18	6.6667	6.6667	6.7033	6.9567	-	-	6.9467	-	-	6.9167	-	-

Symbols and Abbrivations: WW = Wastewater.

BIOGRAPHY

Miss Likitmongkonsakun was born on October 28, 1984 in Bangkok province, Thailand. She obtained her Diploma in Analytical Chemistry Training and B.Sc. Degree in General Science from the Faculty of Science, Chulalongkorn University in 2004 and 2006, respectively. She pursued her Master Degree at The International Postgraduate Programs in Environmental Management (Hazardous waste management), Inter-Department of Environment Management, Faculty of Graduate School, Chulalongkorn University, Bangkok, Thailand since May 2007. She finished her Master of Science Degree in Environmental Management in May 2009.