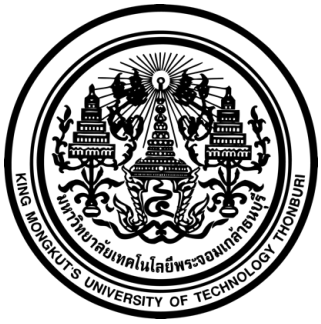

TREATMENT OF INK FACTORY WASTEWATER BY CHEMICAL PRECIPITATION COUPLED WITH *Cyperus alternifolius*

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Abstract

Wastewater from ink production is complex, as it contains pigments, acrylic resin, monoethanolamine (MEA), NH_4OH. These chemicals have the effect of increasing chemical oxygen demand (COD), ammonium nitrogen (NH_4^+−N), suspended solids (SS), and especially dark color. Therefore, this study investigated the potential of wastewater treatment by chemical precipitation coupled with *Cyperus alternifolius*. From preliminary screening of plants, we found that *C. alternifolius* (Umbrella papyrus) had the highest efficiency to remove ink factory wastewater higher than *Echinodorus cordifolius* (Creeping Burrhead), *Thalia geniculata* (Alligator Flag), *Acorus calamus* (Sweet Flag), and *Dracaena sanderiana* (Lucky Bamboo). The results showed that original wastewater treated by *C. alternifolius* took a long time (about 45 days) to reduce COD from 32336 mg/L to 237 mg/L and total dissolved solids (TDS) from 12581 mg/L to 717 mg/L (under soil conditions). After the treatment, the plant was still in good health but the wastewater color was appeared. Firstly, chemical precipitation by H_2SO_4 was used to treat the original wastewater. The COD was reduced to 987 mg/L from an initial 12601 mg/L while SS was reduced to 30 from an initial 1176 mg/L. After that the phytoremediation with *C. alternifolius* was used as a secondary treatment. At day 7, COD and total kjedalh nitrogen (TKN) concentrations were reduced to 171 mg/L and 45 mg/L from an initial 987 mg/L and 156 mg/L, respectively. Notably, the color of the wastewater was disappeared.

The results of our study showed that plant absorption, soil adsorption, and microbial activities had an effect in treating wastewater and especially plant-microbe interaction. COD removal efficiency in the system was attributed as follows: 45% to *C. alternifolius* and microorganisms in plants, 26% to soil absorption, 19% to soil microorganisms, and 10% to wastewater microorganisms. In this study, using the denaturing gradient gel electrophoresis (DGGE) method, it was confirmed that the microbial community was made up of aerobic and facultative−anaerobic groups. Phylogenetic diversity analyses of eubacterial genes were carried out on wastewater, through which the 16S rRNA gene. It revealed that the major microorganism under plants grown in soil−containing wastewater conditions was Azospirillum. It was nitrogen fixing bacteria and it utilized nitrate as a respiratory electron acceptor, which was then reduced to molecular nitrogen. These results revealed that plant−microbe interactions are an efficient process for the treatment of wastewater containing high COD, TKN, and color. In addition, the data of microbial diversity can be useful for understanding plant-microbe interactions to improve system performance.

The potential of using sulfuric acid and magnesium and phosphate coupled with *C. alternifolius* for removal of ammonium nitrogen was also investigated. The results showed that when original wastewater was treated with H_2SO_4, the color was reduced from a dark color to an orange color while COD and SS decreased from 28827 mg/L to 1354 mg/L, and 2917 mg/L to 32 mg/L, respectively. NH_4^+−N also decreased from 458 mg/L to 295 mg/L. Precipitation with various types of MAPs such as (i) $MgCl_2.6H_2O$ + $Na_2HPO_4.12H_2O$, (ii) MgO + 85% H_3PO_4 and (iii) $MgSO_4.7H_2O$ + $Ca(H_2PO_4)_2$ and then phytoremediation by *C. alternifolius* were investigated. The results revealed that treatment with MgO + 85% H_3PO_4 and adjusting pH with $Ca(OH)_2$ was the best condition for *C. alternifolius* to grow effectively. This condition could change the orange color to colorless and decrease COD and NH_4^+−N to 329 mg/L and 13 mg/L, respectively, within 3 days. However, this method had a problem in the cost treatment. Therefore chemical precipitation by H_2SO_4 coupled with *C. alternifolius* was appropriated in treating of this wastewater.

In addition, research was also done on the degradation of ethanolamines such as monoethanolamine (MEA), diethanolamine (DEA), and triethanolamine (TEA) by *C. alternifolius*. We found that plants could degrade TEA into DEA, then into MEA, and then further into acetic acid. The accumulation of ethanolamines was found mainly in plant stems, which had the highest biomass. It demonstrated that the molecular size affected the removal rate through plant. A smaller molecular weight—MEA (MW=61.08 g/mol) was the fastest taken up, followed by DEA (MW=105.14 g/mol) and TEA (MW=149.19 g/mol), the highest molecular weight. The toxicity to the plant when exposed to ethanolamines elucidated that MEA had the highest toxicity, followed by DEA and TEA. Moreover, the application of *C. alternifolius* in ink factory wastewater revealed that the plant could completely uptake MEA at day 5 from an initial MEA concentration of 540 mg/L. The result indicated that *C. alternifolius* has a potential to remove ethanolamines and can be applied to ethanolamines-contaminated wastewater.

Keywords: Chemical precipitation / *Cyperus alternifolius* / Ethanolamine / Microorganism / Pigments / Phytoremediation

หัวข้อวิทยานิพนธ์	การบำบัดน้ำเสียจากการผลิตหมึกโดยใช้วิธีการตกตะกอนทางเคมีร่วมกับต้นกกราชินี
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ผู้เขียน	นางสาวรุจิรา ดลเพ็ญ
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หลักสูตร	ปรัชญาดุษฎีบัณฑิต
สาขาวิชา	เทคโนโลยีชีวภาพ
ภาควิชา	เทคโนโลยีชีวภาพ
คณะ	ทรัพยากรชีวภาพและเทคโนโลยี
ปีการศึกษา	2557

บทคัดย่อ

น้ำเสียจากโรงงานผลิตหมึกมีความซับซ้อน เนื่องจากประกอบด้วยเม็ดสี อะไครลิคเรซิน โมโนเอทธานอลเอมีน แอมโมเนียมไฮดรอกไซด์และอื่นๆ ซึ่งสารเคมีเหล่านี้ส่งผลต่อการเพิ่มความเข้มข้นของค่าซีโอดี แอมโมเนียมไนโตรเจน สารแขวนลอย โดยเฉพาะอย่างยิ่งส่งผลทำให้น้ำเสียมีสีเข้ม ดังนั้นงานวิจัยนี้จึงทำการศึกษาความเป็นไปได้ในการบำบัดน้ำเสียโดยการตกตะกอนเคมีร่วมกับการประยุกต์ใช้พืช ซึ่งพืชที่ใช้ในการศึกษาคือ กกราชินี (*Cyperus alternifolius*) เนื่องจากการศึกษาเบื้องต้นพบว่าต้นกกราชินีมีความสามารถในการบำบัดน้ำเสียสูงที่สุด ซึ่งสูงกว่าต้นอเมซอน (*Echinodorus cordifolius*) ต้นคล้าน้ำ (*Thalia geniculata*) ต้นว่านน้ำ (*Acorus calamus*) และต้นกวนอิม (*Dracaena sanderiana*) ผลการทดลองแสดงให้เห็นว่าการบำบัดน้ำเสียตั้งต้นโดยใช้ต้นกกราชินีในระบบปลูกพืชแบบมีดินโดยตรงนั้นใช้ระยะเวลาในการบำบัดค่อนข้างนานประมาณ 45 วัน ในการลดค่าซีโอดีจาก 32336 มิลลิกรัมต่อลิตร เหลือเท่ากับ 237 มิลลิกรัมต่อลิตร และลดปริมาณของแข็งที่ละลายเจือปนอยู่ในน้ำจาก 12581 มิลลิกรัมต่อลิตร เหลือเท่ากับ 717 มิลลิกรัมต่อลิตร หลังจากการบำบัดน้ำเสียดังกล่าวพบว่าพืชยังคงแข็งแรงดีแต่สีของน้ำเสียยังคงหลงเหลืออยู่ในระบบ ดังนั้นจึงทำการศึกษาโดยใช้การตกตะกอนเคมีร่วมกับการประยุกต์ใช้พืชในการบำบัดน้ำเสียดังกล่าว การตกตะกอนทางเคมีด้วยกรดซัลฟูริกในขั้นตอนแรกสามารถลดค่าซีโอดีจาก 12601 มิลลิกรัมต่อลิตร เหลือเท่ากับ 987 มิลลิกรัมต่อลิตร ขณะที่สารแขวนลอยลดลงจาก 1176 มิลลิกรัมต่อลิตร เหลือเท่ากับ 30 มิลลิกรัมต่อลิตร หลังจากนั้นนำต้นกกราชินีมาประยุกต์ใช้ในการบำบัดน้ำเสียดังกล่าว พบว่าในวันที่ 7 ของการทดลอง สามารถลดค่าซีโอดีและค่าทีเคเอ็นจาก 987 มิลลิกรัมต่อลิตร และ 156 มิลลิกรัมต่อลิตร ตามลำดับ เหลือเท่ากับ 171 มิลลิกรัมต่อลิตร และ 45 มิลลิกรัมต่อลิตร ตามลำดับ และเป็นที่น่าสังเกตว่าไม่พบสีของน้ำเสียหลงเหลืออยู่ในระบบ

ผลการศึกษาที่ได้แสดงให้เห็นว่า การดูดซับโดยพืช ดินและกิจกรรมของจุลิทรีย์มีผลต่อการบำบัดน้ำเสีย ซึ่งประสิทธิภาพในการลดค่าซีโอดีในระบบเกี่ยวข้องกับพืชและจุลินทรีย์จากพืช ร้อยละ 45 การดูดซับโดยดิน ร้อยละ 26 จุลินทรีย์ในดิน ร้อยละ 19 และจุลินทรีย์ในน้ำเสีย ร้อยละ 10 นอกจากนี้ยังได้นำการวิเคราะห์ด้วยวิธี denaturing gradient gel electrophoresis (DGGE) มายืนยันกลุ่มประชากรจุลินทรีย์ที่เกี่ยวข้องในระบบ ซึ่งพบกลุ่มจุลินทรีย์ที่สามารถเจริญเติบโตได้ในสภาพที่มีอากาศและกลุ่มจุลินทรีย์ที่สามารถเจริญได้ทั้งในสภาวะที่มีและไม่มีอากาศปริมาณมากที่สุด การวิเคราะห์การกระจายของจุลินทรีย์ในน้ำเสียโดยอาศัยความสัมพันธ์ทางสายวิวัฒนาการผ่านทางยีน 16S rRNA พบว่ากลุ่มจุลินทรีย์ที่โดดเด่นภายใต้สภาวะการปลูกพืชแบบมีดินคือ กลุ่ม Azospirillum ซึ่งเป็นแบคทีเรียตรึงไนโตรเจนและสามารถใช้ไนเตรตเป็นตัวรับอิเล็กตรอนในการหายใจ ผลการทดลองเหล่านี้เปิดเผยว่าความสัมพันธ์ของพืชกับจุลินทรีย์เป็นกระบวนการที่มีประสิทธิภาพในการบำบัดน้ำเสียที่มีสีปนเปื้อนและมีค่าซีโอดีและทีเคเอ็นสูง ซึ่งข้อมูลของความหลากหลายของจุลินทรีย์สามารถนำไปใช้ประโยชน์เพื่อเข้าใจความสัมพันธ์ของพืชกับจุลินทรีย์ในการปรับปรุงประสิทธิภาพของระบบ

นอกจากนี้ยังทำการศึกษาการกำจัดแอมโมเนียมไนโตรเจนในน้ำเสียโดยการใช้กรดซัลฟูริกและแมกนีเซียมและฟอสเฟต ร่วมกับการใช้ต้นกกราชินี ผลการทดลองแสดงให้เห็นว่าเมื่อบำบัดน้ำเสียตั้งต้นด้วยกรดซัลฟูริกสามารถลดสีของน้ำเสียจากสีเข้มไปเป็นสีส้ม ขณะที่ค่าซีโอดีลดลงจาก 28827 มิลลิกรัมต่อลิตร เหลือเท่ากับ 1354 มิลลิกรัมต่อลิตร สารแขวนลอยลดลงจาก 2917 มิลลิกรัมต่อลิตร เหลือเท่ากับ 32 มิลลิกรัมต่อลิตร และแอมโมเนียมไนโตรเจนก็ลดลงจาก 458 มิลลิกรัมต่อลิตร เหลือเท่ากับ 295 มิลลิกรัมต่อลิตร จากนั้นเปรียบเทียบการตกตะกอนทางเคมีด้วยสาร 3 ชนิด ได้แก่ แมกนีเซียมคลอไรด์เฮกซะไฮเดรตกับไดโซเดียมฟอสเฟต แมกนีเซียมออกไซด์กับกรดฟอสฟอริก และแมกนีเซียมซัลเฟตเฮปตะไฮเดรตกับโมโนแคลเซียมฟอสเฟต ร่วมกับการบำบัดน้ำเสียโดยใช้ต้นกกราชินี ผลการทดลองเปิดเผยว่าการตกตะกอนด้วยแมกนีเซียมออกไซด์กับกรดฟอสฟอริก และปรับค่าพีเอชด้วยแคลเซียมไฮดรอกไซด์เป็นสภาวะที่ดีที่สุดในการเจริญเติบโตของพืช และสภาวะนี้สามารถลดความเข้มข้นของค่าซีโอดีและแอมโมเนียมไนโตรเจนเท่ากับ 329 มิลลิกรัมต่อลิตร และ 13 มิลลิกรัมต่อลิตร ตามลำดับ และสามารถกำจัดสีได้หมดภายใน 3 วัน อย่างไรก็ตามวิธีการนี้มีค่าใช้จ่ายในการบำบัดน้ำเสียที่สูง ดังนั้นการตกตะกอนทางเคมีด้วยกรดซัลฟูริกร่วมกับต้นกกราชินีจึงเป็นวิธีที่เหมาะสมที่สุด

นอกจากนี้งานวิจัยได้ศึกษาการย่อยสลายของสารกลุ่มเอทธานอลเอมีน เช่น โมโนเอทธานอลเอมีน ไดเอทธานอลเอมีน และไตรเอทธานอลเอมีนโดยต้นกกราชินี พบว่าพืชสามารถย่อยสลายสารไตรเอทธานอลเอมีนเป็นไดเอทธานอลเอมีนแล้วเปลี่ยนเป็นโมโนเอทธานอลเอมีน จากนั้นถูกย่อยสลาย

ต่อเป็นกรดอะซิติก การสะสมของสารเอทธานอลเอมีนพบในส่วนของลำต้นพืชเป็นส่วนใหญ่ซึ่งมีปริมาณชีวมวลสูงสุด นอกจากนี้ยังพบว่าขนาดของโมเลกุลมีผลต่ออัตราการเคลื่อนที่ของสารไปยังส่วนต่าง ๆ ของพืช โดยพบว่าสารโมโนเอทธานอลเอมีนซึ่งมีน้ำหนักโมเลกุลต่ำที่สุดจะถูกดูดขึ้นไปในพืชเร็วที่สุด (น้ำหนักโมเลกุล = 61.08 กรัมต่อโมล) ตามด้วยสารไดเอทธานอลเอมีน (น้ำหนักโมเลกุล = 105.14 กรัมต่อโมล) และสารไตรเอทธานอลเอมีน (น้ำหนักโมเลกุล = 149.19 กรัมต่อโมล) ซึ่งมีน้ำหนักโมเลกุลใหญ่ที่สุด เมื่อเทียบความเป็นพิษของสารเอทธานอลเอมีนต่อพืช พบว่าพิษของสารโมโนเอทธานอลเอมีนรุนแรงที่สุด รองลงมาคือสารไดเอทธานอลเอมีน และสารไตรเอทธานอลเอมีน นอกจากนี้การประยุกต์ใช้ต้นกกราชินีในน้ำเสียจากอุตสาหกรรมการผลิตหมึก พบว่าพืชสามารถดูดซับสารโมโนเอทธานอลเอมีนได้หมดภายใน 5 วัน จากความเข้มข้นสารโมโนเอทธานอลเอมีนเริ่มต้น 540 มิลลิกรัมต่อลิตร ผลการทดลองชี้ให้เห็นว่ามีความเป็นไปได้ที่จะนำต้นกกราชินีไปประยุกต์ใช้ในน้ำเสียที่มีการปนเปื้อนสารโมโนเอทธานอลเอมีน

คำสำคัญ: การตกตะกอนทางเคมี / การบำบัดสารมลพิษโดยใช้พืช / จุลินทรีย์ / ต้นกกราชินี / เม็ดสี / เอทธานอลเอมีน

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ABBREVIATIONS

AC	=	Activated carbon
Annamox	=	Anaerobic ammonium oxidation
ANOVA	=	Analysis of variane
AOB	=	Ammonia oxidixing bacteria
AOP	=	Advanced oxidation processes
BGFA	=	Bagasse fly ash
BOD	=	Biochemical oxygen demand
^{o}C	=	Degree celsius
CANON	=	Completely autotrophic nitrogen removal over nitrite
CDEO	=	Conductive diamond electrooxidation
COD	=	Chemical oxygen demand
DEA	=	Diethanolamine
DGGE	=	Denaturing gradient gel electrophoresis
DO	=	Dissolved oxygen concentration
g	=	Gram
g/mol	=	Gram per mole
MAP	=	Magnesium ammonium phosphate
MEA	=	Monoethanolamine
mg/L	=	Milligram per liter
mL	=	Milliliter
mM	=	Millimole
NOB	=	Nitrite oxidizing bacteria
ORP	=	Oxidation reduction potential
PAC	=	Polyaluminium chloride
PCR	=	Polymerase chain reaction
SBR	=	Sequencing batch reactor
SDFA	=	Sawdust fly ash
SEM/EDX	=	Scanning electron microscope connected with energy dispersive
SHARON	=	Single reactor system for high ammonia removal over nitrite
SND	=	Simultaneous nitrification and denitrification
SS	=	Suspended solids
TEA	=	Triethanolamine
TDS	=	Total dissolved solids
TKN	=	Total kjedalh nitrogen
TN	=	Total nitrogen
TOC	=	Total organic carbon
TOC/MLVSS	=	Total organic carbon to mixed liquor volatile suspended solids
XRF	=	X-ray fluorescence spectrometry
XRS	=	X-ray spectroscopy

CHAPTER 1
INTRODUCTION

1.1 Rational/Problem statement

The demand of ink increase with the development of the economy, especially the consumption of flexographic printing ink. Flexographic printing is used for the production of labels, foods, toys, wine and other packaging materials (Ma and Xia, 2009). The process of ink production associated with the use of colorants (pigments), vehicles (acrylic resin), solvents (monoethanolamine and ammonium hydroxide), and additives (waxes, and defoamer). Theses composition caused in high concentration of organic matter, ammonium nitrogen and suspended solids (SS) in wastewater. Moreover, flexographic printing ink has been change from using of organic solvent-based to water-based inks, resulting in potentially increased wastewater discharges from factory (Larsen et al., 1996).

The quality and quantity of ink factory wastewaters varies with the different processes of ink production such as the kinds of inks, pigments, and additives used. In flexographic printing industry, ink pigments are the primary source of adhesive problems because it contained high pigments and organic matter that affect to SS, total dissolved solids (TDS) and chemical oxygen demand (COD) contents. From these reasons, the effluent wastewater obtained after cleaning/washing of the equipment cannot be directly discharged to the environment without treatment not only deleterious effects on human health and the environment, but also colored wastewater may also be an aesthetic concern.

Biological processes have been developed based on biological nitrification and denitrification such as short-cut nitrification and denitrification, anaerobic ammonium oxidation (Annamox), completely autotrophic nitrogen removal over nitrite (CANON) process, oxygen-limited autotrophic nitrification–denitrification process. However, it was difficult for controlling the system such as levels of dissolved oxygen concentration (DO), pH, substrates, temperature, sludge age, etc. Nevertheless, the sequencing batch reactor (SBR) process has been widely used in wastewater treatment. Some researchers have reported that for a nitrogen removal system, the on-line monitoring parameters, such as, oxidation-reduction potential (ORP), DO, and pH, indicated the end of the biodegradation processes (Charpentier et al., 1998; Lee et al., 2001). However, the most severe weakness of the SBR process is the complexity of operation. Moreover, acrylic resin used in water-based ink formulations and pigments are often very difficult to break down by microorganism (Ma and Xia, 2009).

There are many methods of chemical process were used in wastewater treatment and remove pigments. Some examples include coagulation with $AlCl_3.6H_2O$ and $FeCl_3.6H_2O$ (Meteš et al., 2000; Meteš et al., 2004), precipitation with alum ($Al_2(SO_4)_3 \cdot 18H_2O$) coagulation using polyaluminium chloride (PAC) and iron (Diamadopoulos et al., 2009), coagulation/flocculation process using biopolymers

(chitosan and tannin) (Roussy et al., 2005), Fenton's reaction combined with polyaluminium chloride (PAC) (Meteš et al., 2000; Ma and Xia, 2009), ultrasonication in combination with Fenton's reaction (Chua and Loh, 2008; Chen, 2010), electrochemical oxidation (Leshem et al., 2006). These methods could reduce color and turbidity of wastewater but color, COD and nitrogen compound concentrations still appeared at higher values. Although activated carbon has been till now the most used adsorbent (Zhang et al., 2010; Meteš et al., 2004), owing to its high price, using natural waste materials were considered but it has a limited amount of time to use. Therefore, finding an effective and lower cost treatment method to remove color, COD, and NH_4^+−N from wastewater is interesting.

Even though, there are various methods of biological (Lay-Son and Drakids, 2008) and physico-chemical processes can be applied to treat high concentrations of ammonium nitrogen, such as air stripping (Marttinen et al., 2002), ion exchange (Jorgensen and Weatherley, 2003), membrane separation (Di Palma et al., 2002), and chemical precipitation using MAP (magnesium ammonium phosphate) (Li et al., 1999; Zhang et al., 2009). However, the pigments and acrylic resin in this wastewater were still affected to many methods. Therefore, wastewater was important for decreasing acrylic resin and pigments before using other methods.

Therefore, the ultimate goals of complete treatment of wastewater are simplicity, efficiency, and low cost. In this study, pretreatment with chemical substances was performed at first step for reducing the pigments, COD, and SS values in wastewater. After that, phytoremediation was applied for cleanup of pretreatment wastewater by *Cyperus alternifolius* (Umbrella papyrus). From screening of plants, *Cyperus alternifolius*, *Echinodorus cordifolius* (Creeping Burrhead), *Thalia geniculata* (Alligator Flag), *Acorus calamus* (Sweet Flag), and *Dracaena sanderiana* (Lucky Bamboo) were used for performed tests and it was found that *C. alternifolius* had the highest efficiency for color and COD removal.

Using a chemical process and following up with phytoremediation for a reduction of color, COD, SS, and TN are highlighted as a new processes for removal of ink factory wastewater. While pH, DO, and ORP, as parameters for observed information about the optimization of biological process and improved biological nitrogen removal efficiency. In addition, the diversity of microbial structures was observed by the denaturing gradient gel electrophoresis (DGGE) method for observing the relation of microorganisms in the system. The identification of groups of microbes could explain numerically dominant organisms, which may be of critical importance to system performance.

1.2 Literature reviews

1.2.1 Treatment methods of ink factory wastewater

Treatment of pigments is the major problem in the printing ink industry that is difficult

to remove. The organic pigment widely used in flexographic printing industry. Organic pigments produce dust pollution in the process of production (grinding), organic pigment wastewater contains high COD, high chroma, and characteristics sewage discharged by the pigment in the production process often contains carcinogenic material.

Conventional mechanical wastewater treatment is a combination of physical and biological processes designed to remove organic matter and solids from solution. There are many methods of wastewater treatment. The earliest mechanical method was plain sedimentation in septic tanks but it has limited effectiveness. Since less than half of the wastewater organic contents is typically settleable (Hammer and Hammer, 2008). The initial attempt at secondary treatment involved chemical coagulation at the primary clarifier to improve settleability of the wastes. Some examples include coagulation, precipitation, and adsorption method.

Meteš et al., 2000, proposed the method for treating ink factory wastewater that they used different kinds of components. The wastewater was a mixture of the flexographic inks, based on a polyacrylic binder (ammonium salt of terpolymer acrylic acid, metylmetacrylate, and ethylacrylate), in which approximately 7% of the product was propanol. The best destabilization was achieved by adding of $AlCl_3.H_2O$, $FeCl_3.6H_2O$, and $Al_2(SO_4).18H_2O$. The results showed significant reductions of approximately 80% of COD. While wastewater that contained printing inks based on a methylmetacrylate co-polymer with no solvent added. Similar results were obtained for adding of $AlCl_3.H_2O$, $FeCl_3.6H_2O$, and $Al_2(SO_4).18H_2O$, the results showed 92% of COD removal. Moreover, for the both found that colorless and clear water was obtained. Meanwhile Nandy et al. (2003) studied coagulant agents such as ferrous sulphate, ferric chloride, aluminium sulphate and polyaluminium chloride (PAC) for the treatment of printing ink wastewater. They found that a combination of PAC with a cationic polyelectrolyte could reduce color, SS, BOD and COD of 95.9–96.5%, 96.5–97.0%, 61.3–65.8% and 54.8– 61.8%, respectively, for the treatment of printing ink wastewater at an optimum concentration of 1500 mg/L.

Adsorption is the process which is most frequently employed for the treatment of colored effluents, using for example activated carbon, resins, agricultural wastes, micro-organisms and biopolymers. Roussy et al. (2005) investigated the use of both chitosan and tannins (in combination) for adsorption ink-containing effluent generated in the processing of packaging. The optimum conditions were pH controlled to pH 5, chitosan concentration close to 20 mg/L and tannin concentration in the range 70 to 100 mg/L. The COD concentration was decreased by 80 to 85% from an initial COD around 8000 to 9000 mg O_2/L and it decreased to 1800 to 2000 mg O_2/L (Roussy et al., 2005). The results from Meteš et al. (2004) studied adsorption of residual organic pollutants from flocculated printing ink wastewater onto synthetic zeolites (Meteš et al., 2004). They reported that coagulation with $AlCl_3$ and $FeCl_3$ had received 88% reduction of total organic carbon (TOC) removal while the combination of flocculation and adsorption with zeolite which resulted in the overall TOC efficiency of 95% for printing ink wastewater of about 1000 mg/L initial TOC. Santos and co workers (2007) use a metal hydroxide sludge absorbed color from textile dyeing effluents because it as a low-cost adsorbent. They revealed that the removing a reactive textile dye (Remazol Brilliant Blue) in solution had the maximum adsorption capacities obtained varied between 275

mg/g (at 25^{o}C and pH 4) (Silvia C.R. Santos). Noonpui et al. (2010) use bagasse fly ash (BGFA), sawdust fly ash (SDFA) and activated carbon (AC) as adsorbent for color removal from wastewater from printing ink industry after water-based ink coagulation. Color removal by SDFA (90%) was higher than commercial AC (80%) at the same dosage of 0.3% (w/v) while the efficiency of BGFA was 85% at 0.5% (w/v).

The color removal from a synthetic textile effluent containing a reactive dye (C.I. Reactive Orange 4) was studied by Vilaseca et al. (2010). They use an electrochemical cell with titanium covered by platinum oxide (Ti/PtO_x) electrodes to remove color. Meanwhile, Vlyssides and Israilides studied electrochemical degradation of organic matter from textile dye and finishing wastewater using Ti/Pt as anode and Stainless Steel 304 as cathode. At the end of 40 min of electrolysis, there was 92% COD, 92.2% BOD and 94% color (ADMI color units) reduction with energy consumption of 44 kWh/kg of COD removal. In the effluents of ink-manufacturing processes, Cañizares and co workers studied the conductive diamond electrooxidation (CDEO) remove several dyes (methylene blue and rhodamine B), solvents (monoethylene glycol, diethylene glycol and glycerol) and surfactants (sodium dodecylbenzenesulfonate). For all the compounds tested, boron doped diamond (BDD) was used as anode and stainless steel (AISI 304) as cathode. The results showed the almost complete removal of COD while the mineralization rate (TOC removal) is 40-80% (Cañizares et al., 2007).

Ma and Xia (2009) used Fenton process combined with coagulation PAC and ferrous sulfate ($FeSO_4$)) for treatment water-based printing ink wastewater. The overall color, COD and SS removal reached 100%, 93.4% and 87.2% at pH 9, 700 mg/L PAC and 300 mg/L of $FeSO_4$ and 30 min settling time. He et al. (2009) studied the combination of ultrasonic and Fenton oxidation (UV-Fenton) for treats the organic pollutants in ink wastewater. The results showed that the removal rate of COD and color of the combined technology reach 81.38% and 100% while single Fenton oxidation are 16.0% and 5.5%, respectively (De-wen et al., 2009). Meanwhile, Chua and Loh (2008) used the same technique investigated for treating cyan ink effluent from circuit board production received COD removal about 98%. However, the oxidation processes may cause pollution hazards since in some cases the products generated during the oxidation are more hazardous for the environment than original contaminants.

1.2.2 Treatment of ethanolamines

Lai and Shieh (1996) showed that monoethanolamine (MEA) was highly degraded via nitrate respiration using NO_3^- as an electron acceptor when ratio of initial total organic carbon to mixed liquor volatile suspended solids (TOC/MLVSS) was below 0.35. Under tested condition, the medium solution that contained cobalt (0.45 mg/ I), copper (0.5 mg/1), molybdenum (0.5 mg/l), and yeast extract (1.0 mg/l). They found that ammonia was formed as an end product via nitrate respiration.

Breakdown of ethanolamine requires a coenzyme B_{12}-dependent ethanolamine ammonia-lyase, this enzyme splits ethanolamine to acetaldehyde and ammonia as described in *Clostridium* sp. , *Escherichia coli* (Blackwell and Turner, 1978), *Klebsiella aerogenes* (Scarlett and Turner, 1976). In one strain of *Pseudomonas* sp., ethanolamine was degraded by an ammonia-lyase whereas its homologue 1-aminopropan-2-ol was degraded by the kinase and phospho-lyase route (Faulkner and Turner, 1974). Ohtaguchi et al. (1995) have demonstrated the MEA degradation via aerobic degradation of amine wastes from CO_2 capture using pure cultures of *Escherichia coli*

K 12. After that Ohtaguchi and Yokoyama (1997) also demonstrate of chat *Clostridium formicoaceticum* could degrade MEA to formic and acetic acids. The authors described biotransformation of MEA to ammonium ion for biomass formation, acetaldehyde and acetic acid by the following reactions (Ohtaguchi et al., 1995):

$$\underset{\text{monoethanolamine}}{H_3N{+}CH_2CH_2OH} \xrightarrow{\text{ethanolamine ammonia – lyase}} \underset{\text{ammonium}}{NH_4^+} + \underset{\text{acetaldehyde}}{CH_3CHO} \quad (1.1)$$

$$CH_3CHO + CoA + NAD^+ \xrightarrow{\text{aldehydedehydrogenase}} NH_4^+ + CH_3CHO \quad (1.2)$$

coenzyme

$$\underset{\text{acetyl CoA}}{CH_3CO\text{-}CoA} + Pi \xrightarrow{\text{phosphateacetyltransferase}} CH_3CO_2PO_3^{2-} + CoA + 2H^+ \quad (1.3)$$

$$CH_3CO_2PO_3^{2-} + 2H^+ + ADP \xrightarrow{\text{acetate kinase}} CH_3COOH + ATP \quad (1.4)$$

acetic acid

In contrast the coenzyme B_{12}-independent deamination of MEA, in which ethanolamine O-phosphate appears as intermediate, was known to occur in *Erwina* species (Jones and Turner, 1973; Jones et al., 1973) and *Pseudomonas* strains (Jones and Turner, 1973). Acetaldehyde formation of these organisms from MEA is shown by the following equations:

$$H_3N^+CH_2CH_2OH + ATP \xrightarrow{\text{ATP – amino alcohol phosphotransferase}} H_3N^+CH_2CH_2OPO_3^{2-} + ADP \quad (1.5)$$

$$H_3N^+CH_2CH_2OPO_3^{2-} + OH^- \xrightarrow{\text{amino alcohol O – phophate phospholyase}} CH_3CHO + NH_4^+ + PO_4^{3-} \quad (1.6)$$

Moreover, they presented several different metabolic alternatives for MEA biodegradation with reference to the previous works (Blaekwell et al., 1976; Atkinson et al., 1980; Krieg and Holt, 1984; Sneath et al., 1986). They found that acetic acid is the major end product in most of the aerobic metabolic pathway. Other intermediate degradation products such as ethanol, acetaldehyde, amino acid, and H_2 are also candidates for further anaerobic degradation under methanogenic conditions (Möiler et al., 1986).

Moreover, Frings and coworkers (1994) are reported that adenosylcobalamin (coenzyme B_{12}) is recognized to be a major cofactor initiating this kind of eliminase reaction. Triethanolamine (TEA) is converted into acetaldehyde and diethanolamine, and then diethanolamine is cleaved to ethanolamine and acetaldehyde. In cell-free extracts of strain LuTria 3, ethanolamine ammonia-lyase activity forming acetaldehyde and ammonia was present acetate and ammonia by a strictly anaerobic, gram-positive *Acetobacterium* strain LuTria3.

Ndegwa et al., 2004 have done on biodegradation of MEA in soil (about 1500 mg/kg) under aerobic and anaerobic conditions. The suggested degradation pathways involves hydrolysis to ammonium and acetaldehyde. Under aerbic conditions, ammonium can be oxidized to nitrite and then nitrate while acetaldehyde was hydrolysed to ethanol and acetic acid and turned to CO_2 as showed in Fig. 1.1. In aerobic conditions, acetic acid and ethanol are readily consumed by bacteria. They revealed that MEA could completely remove under aerobic within 10 days while under anareobic within 12 days. Cold temperatures (5°C) reduced significantly the biodegradation rates compared to rates at room temperature.

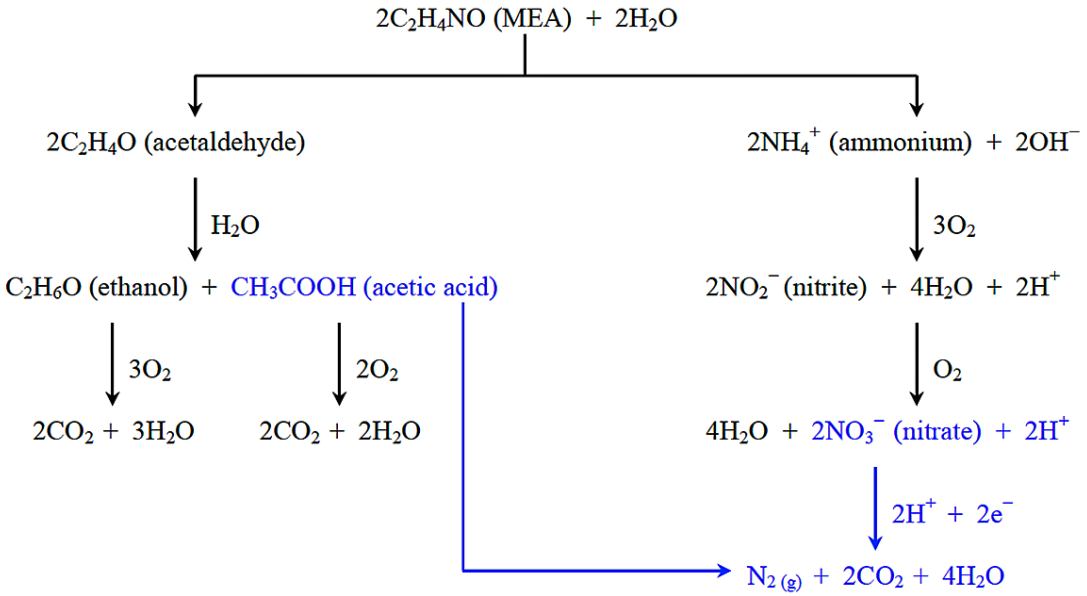


Figure 1.1 Biodegradation of monoethanolamine (Ndegwa et al., 2004).

Williams and Callely (1982) revealed that a yellow Gram-negative rod-shaped organism that could grow with MEA, diethanolamine (DEA) or TEA as the sole carbon and energy source. They showed the pathway for the degradation of these compounds that TEA is converted via triethanolamine N-oxide, into DEA and glycolaldehyde while DEA is converted to MEA and glycolaldehyde. Ethanolamine is converted into acetyl units via ethanolamine O-phosphate and acetaldehyde. By this reaction, cleavage of the alkyl-nitrogen linkage is prepared because the hemiaminal thus formed is unstable and decomposes easily to acetaldehyde and DEA. In the same way, DEA is cleaved to MEA and acetaldehyde. In cell-free extracts of strain LuTria 3, ethanolamine ammonia-lyase activity forming acetaldehyde and ammonia was present. In contrast, the ethanolamine ammonia lyase of other bacteria, e.g.,*Clostridium* sp., *Escherichia coli*, *Klebsiella aerogenes*, or *Salmonella typhimurium*, are coenzyme B_{12}-dependent (Babior, 1982). These observations indicate that a free terminal hydroxyl group is required to allow cleavage of the alkyl-nitrogen linkage through a hydroxyl shift reaction.

Speranza et al., 2006 further noticed a strictly anaerobic bacterium (*Acetobacterium* sp., from sewage sludge) and summarized four different mechanisms to explain anaerobic biodegradation of MEA, DEA, and TEA. All four mechanisms suggest that acetyldehyde and ammonia are products. They further reported that a homoacetogenic bacterium isolated from sewage sludge (*Acetobacterium* sp., strain LuTria3) was able to ferment TEA to acetate and ammonia by a strictly anaerobic. They suggested that the recognized bacterium could use a

redical mechanism to process all three types of ethanolamines.

West and Gonsior (1996) determined the fate and lifetime of [^{14}C] triethanolamine in an aerobic surface soil, freshwater river systems, and in activated sludge waste treatment. The study has demonstrated TEA biodegradation in a variety of environmental depended on microbial diversity and an initial TEA concentration. Moreover, adding TEA as organic carbon in each of experiment could recovery CO_2 between 46-64% and an insoluble organic carbon species, most likely biomass.

1.2.3 Nitrogen removal

Ammonium nitrogen removal from wastewater has been of considerable concern for several decades. The wastewater usually contains quite high NH_4^+−N concentration, which is well known to inhibit nitrification in biological treatment processes. A common pre-treatment for reducing high strength of ammonium (NH_4^+−N) is by an air-stripping process. However, there are some operational problems such as carbonate scaling in the process of stripping. Some technical alternatives for NH_4^+−N removal were investigated that was precipitating NH_4^+−N as magnesium ammonium phosphate (MAP). In the experiment of MAP, the combinations of chemicals were used with the different stoichiometric ratios to generate the MAP precipitate effectively such as $MgCl_2.6H_2O$ + $Na_2HPO_4.12H_2O$, MgO + 85%H_3PO_4, and $Ca(H_2PO_4)2.H_2O$ + MgSO4.7H_2O (Li et al., 1999; Li and Zhao, 2001; Tengrui et al., 2007), $MgCl_2.6H_2O$ + $Na_2HPO_4.12H_2O$ (Zhang et al., 2009).

The biological nitrogen removal (BNR) process is the most common method for removing low quantities of ammonium from wastewater, but this is not the usual treatment for high-strength ammonium wastewater. Because of high concentration of ammonium or nitrite inhibit the nitrification (Anthonisen et al., 1976). Normally, physical-chemical system such as stripping is more frequently used. However, Carrera et al. (2003) showed that it is possible to remove the ammonium of an industrial wastewater with up to 5000 mg/L using two sludge systems with nitrification and denitrification for 450 days.

The microbial community varied with the different concentrations of organic matter (sodium acetate); the dominant populations shifted from autotrophic and oligotrophic bacteria (NOB, and some strains of Bacteroidetes, Alphaproteobacteria, Actinobacteria, and green nonsulfur bacteria) to heterotrophic and denitrifying bacteria (strains of Gammaproteobacteria, especially *Pseudomonas stutzeri* and *P. nitroreducens*) when sodium acetate changed from 0.5 or 1 g/L to 5 or 10 g/L (Jie and Daping, 2008). Usually, Nitrospira species are able to use low concentrations of nitrite more efficiently than Nitrobacter species. They were found to be the dominant nitrite oxidizers in nitrifying fluidized bed reactors (Schramm et al., 1998), activated sludge (Juretschko et al., 1998) and in biofilm airlift reactors (Nogueira and Melo, 2006). In contrast to this, Coskuner and Curtis (2002) found that Nitrobacter species were more dominant in high nitrite concentrations in an activated sludge plant, and sequencing batch reactors (Kim and Kim, 2006). Haseborg et al., 2010 found Nitrobactor in fixed-bed biofilm reactors during high nitrite concentrations (5–10 mg/L).

NO_3^-−N removal from paper mill wastewater was successful by treatments with denitrifying bacteria. The initial NO_3^-−N concentration was decreased to 1.90 mg/L (89.34%) from an initial concentration 18.94 mg/L for 1.5 days. *Stenotrophomonas* sp.

ZZ15 and *Oceanimonas* sp. YC13 showed efficient NO_3^-−N removing abilities under a semi-anaerobic condition without obvious accumulation of NO_2^-−N, N_2O−N and NH_4^+−N (Yu et al., 2009). *Azospirillum* was confirmed that it played role in N_2-fixation (C_2H_2−reduction) and denitrification activities with cereal. The results showed that *Azospirillum* performed N_2−fixation in dependence of an association with wheat. It showed wheat−*Azospirillum* association reduce nitrate to gaseous nitrogen products, depending on the concentrations of nitrate and oxygen (Neuer et al., 1985). Moreover, nitrification/denitrification processes have been used to remove ammonia and nitrate from municipal and industrial processes (Koren et al., 2000; Yang et al., 2011). Aerobic/anoxic membrane biofilm reactor combination accomplished simultaneous COD oxidation and nitrogen removal (Hasar et al., 2008). Recently, several new processes for nitrogen removal have been developed. The new processes are based on partial nitrification of ammonium to nitrite combined with anaerobic ammonium oxidation. These processes include the single reactor system for high ammonia removal over nitrite (SHARON) process, which involves part conversion of ammonium to nitrite; the anaerobic ammonium oxidation (ANAMMOX) process, which involves anaerobic ammonium oxidation; and the completely autographic nitrogen removal over nitrite (CANON) process, which involves nitrogen removal within one reactor under oxygen limited conditions. These new processes target the removal of nitrogen from wastewaters containing significant quantities of ammonium (Khin and Annachatre, 2004).

Since nitrogen−rich discharges into receiving water system are responsible for a variety of environment problem. Activated sludge and biofilm process have been the main focus for biological nitrogen removal. However, constructed wetland has also proven potential for nitrogen removal. The major nitrogen treatment mechanisms of constructed wetland include microbial interactions with nitrogen, sedimentation, chemical adsorption, and plant uptake (Spieles and Mitsch, 2000). The central pathway for nitrogen removal is nitrification followed by denitrification. In constructed wetland, nitrogen removal range from 25 to 85%. The denitrification process may remove 60−70% of total removal nitrogen and 20-30% of that is derived from plant uptake (Reddy et al., 1997; Spieles and Mitsch, 2000).

1.3 Research objectives

1.3.1 To study the efficiency of *C. alternifolius* for treating ink factory wastewater.
1.3.2 To study the mechanism of monoethanolamine, diethanolamine, and triethanolamine removal by *C. alternifolius*.
1.3.3 To study the microbial community in ink factory wastewater by molecular technique.

1.4 Scope of research works

1.4.1 To pretreatment of ink factory wastewater by chemical substances for remove color, COD, SS, and TDS.
1.4.2 To study the efficiency of *C. alternifolius* for treating ink factory wastewater
1.4.3 To study chemical precipitation for ammonium nitrogen removal by various type of magnesium ammonium phosphate (MAP)
1.4.4 To study the microbial community in ink factory wastewater by PCR-DGGE and phylogenetic tree.
1.4.5 To study the degradation of monoethanolamine, diethanolamine, and triethanolamine by *C. alternifolius*.

CHAPTER 2
THEORIES

2.1 Ink production

The factory produces ink for flexographic printing that use water-based inks to produce packaging materials. Water-based ink is nonflammable, produces less objectionable vapors in the workplace, and does not contaminate packaged products (Gecol et al., 2001). The major components were pigments, acrylic resins, solvents, and additives. These resulted in an increased concentration of organic wastewaters, ammonia value and suspended solids. Obviously, the wastewater generated from this process was highly colored and had strong odor. The quality and quantity of wastewaters varies with different processes of ink production such as kinds of inks, pigments and additives.

2.1.1 Raw Materials for Printing ink formulations

2.1.1.1 Colorants

Colorants are compounds that reflect and absorb certain wavelengths of light. Wavelengths that are reflected by a colorant are seen by the eye and perceived as colors. The two types of colorants used in printing are dyes and pigments. Dyes are soluble in the vehicle, and the final product can be transparent. The most common dyes are basic, amino-based compounds. The transparent properties of dyes can be beneficial when transparency is desired, and the colors of dyes are often quite strong. However, dyes can be susceptible to attack by chemicals and water, and they can also be toxic.

Pigments are small, insoluble particles, color the ink and make it opaque. It occurs as a colloidal suspension in ink and retains a crystal or particulate structure throughout the coloring or printing process. They form a wide range of organic compounds, and as a result, have a variety of properties. Particle size and chemical stability are two variable properties that can yield differing ink characteristics. Color printing inks primarily consist of linseed oil, soybean oil, or a heavy petroleum distillate as the solvent (called the vehicle) combined with organic pigments made up of salts of nitrogen-containing compounds (dyes), such as yellow lake, peacock blue, phthalocyanine green, and diarylide orange. Inorganic pigments (used to a lesser extent) in printing inks include chrome green (Cr_2O_3), Prussian blue ($Fe_4[Fe(CN)_6]_3$), cadmium yellow (CdS), and molybdate orange. White pigments, such as titanium dioxide, are used either by themselves or to adjust characteristics of color inks. Black ink is made using carbon black. Most red writing inks are a dilute solution of the red dye eosin. Blue color can be obtained with substituted triphenylmethane dyes.

2.1.1.2 Resins

Resin is the comprehensive expression for a broad selection, semi-synthetic or synthetic materials which are employed as binders for printing inks. Resins cause ink to adhere to the substrate, disperse the pigment, provide gloss to the finished coating, and resistance to heat, chemicals and water. Resins are solid compounds that are soluble in the solvent and often have complex molecular structures. Common category of resins includes ketones, epoxides, polyvinylbutyral, alkyds, maleics, fumarics, polyamides, cellulose derivatives, formaldehydes, hydrocarbons, shellac, rubber resins, phenolics, isocyanate free polyurethanes, and acrylic resins.

2.1.1.3 Solvents

Solvents are important in delivering the ink to the substrate. The solvent allows the ink to flow through the printing mechanism, and then evaporates so that the ink forms a solid coating on the substrate. Typically, inks are manufactured and transported in a concentrated form, and the printer must add solvent to the ink to attain the desired viscosity. A solvent must display several important characteristics. It must adequately disperse or dissolve the solid components of the ink, but must not react with the ink or with any part of the press. It must dry quickly and thoroughly, and have low odor. Finally, it is desirable for the solvent to have minimal flammability and toxicity concerns. Common solvents in solvent-based inks are ethanol, propanol, and propyl acetate. In water-based inks, the solvent is water, which is amended with monoethanolamine that controls the viscosity/solubility of acrylic resins, and NH_4OH for adjusting pH.

2.1.1.4 Additives

Additives are used to improve the performance of the finished products such as wax, deforamer, and biocide. Wax, which promotes rub resistance; e.g., Polyethylene Wax (PE Wax), Carnuba-an exudate from the leaves of *Copernicia prunifera* consisting of esters of hydroxylated unsaturated fatty acids with at least twelve carbon atoms in the acid chain. Defoamer, which reduces the surface tension in water-based inks so that stable bubbles cannot exist; e.g., hydrocarbon emulsions. Biocides use to inhibit the fungal and bacterial growth, e.g., Biopol FI/31.

2.2 Manufacturing of ink production

The pigments, additives, and vehicle (resin) are each produced separately. The pigments are ground or milled to particles sizes between 5μm to 10nm depending on what color strength, coating thickness, and dispersion properties are wanted. The pigment is then mixed in a high-speed mixer with glue. The additives are then added to the mixer to achieve the desired properties, which completes the ink making it ready for use in the industrial of flexographic printing.

2.3 Characteristics of ink production wastewater

2.3.1 High concentration of organic matter and nitrogen compounds

Wastewater are kind of high concentration organic wastewater (Cai and Shanghai, 2006; Li et al., 2007). This effluent wastewater is characterized by alkaline pH that effect from ammonium hydroxide (NH_4OH) used for adjusting pH system. Moreover, NH_4OH is effect to high levels of nitrogen compounds in wastewater. The COD is generally above 20000 mg/L and sometimes more than 100000 mg/L, SS about 1000-5000 mg/L, and TDS about 2000-13000 mg/L.

2.3.2 High colorants (dyes and pigments)

The color of wastewater has dark blue, purple, and black with high pigments that depends on the different processes of ink production. Pigments have low biodegradation, high concentrations of organic matter, metals and suspended solids (Ma and Xia, 2009)

2.3.3 Complex of wastewater composition

The major compositions were pigments (30%), glue (60%; acrylic resins (70%), monoethanolamine (MEA; 8%), and NH_4OH (2%)) and additives (10%). Moreover, acrylic resin is main component of COD in wastewater and it also contains of additives such as stabilizers, defoaming agent, blockers, surfactants and preservative.

2.3.4 Poor biodegradability

Most of the components of wastewater are synthetic organic polymer with strong stability, which bring on high concentration of COD value of wastewaters. Moreover, acrylic resin and heavy metal ions has effect to inhibiting biological reaction, making for difficulty to be microbial decomposed and transformed.

2.4 Treatment methods of ink production wastewater

2.4.1 Coagulation and flocculation

Coagulation and flocculation processes are used to separate the suspended solids portion from the water. They occur in successive steps intended to overcome the forces stabilizing the suspended particles, allowing particle collision and growth of floc. It is usually cost effective to apply them to remove colloidal and small particles that settle slowly.

The commonly used metal coagulants based on aluminum and iron. The aluminum coagulants include aluminum sulfate, aluminum chloride and sodium aluminate. The iron coagulants include ferric sulfate, ferrous sulfate, ferric chloride and ferric chloride sulfate. Other chemicals used as coagulants include hydrated lime and magnesium carbonate. The efficiency of rapid mixing, the pH, and the coagulant dosage determine which hydrolysis species is effective for treatment. There has been considerable development of pre-hydrolyzed inorganic coagulants, based on both aluminum and iron because they are able to function efficiently over wide ranges of pH and raw water temperatures. They are less sensitive to low water temperatures; lower dosages are required to achieve water treatment goals; less chemical residuals are produced; and lower chloride or sulfate residuals are produced, resulting in lower final water TDS. They also produce lower metal residuals. These include aluminum chlorohydrate, polyaluminum chloride, polyaluminum sulfate chloride, polyaluminum silicate chloride and forms of polyaluminum chloride with organic polymers. Iron forms include polyferric sulfate and ferric salts with polymers.

2.4.2 Adsorption

The change in concentration of a given substance at the interface as compared with the neighbouring phases is referred to as adsorption. Depending on the type of phases in contact, this process in the following systems: liquid-gas, liquid-liquid, solid-liquid and solid-gas. The material in the adsorbed state is defined as the ‘adsorbate’. The penetration by the adsorbate molecules into the bulk solid phase is determined as ‘absorption’ (Dąbrowski, 2001). It is operative in most natural physical, biological, and chemical system. For example, activated carbon and synthetic resin are used widely in industrial application and for purification of water and wastewater (Weber, 1985). However, adsorption with zeolites has great importance due to its simplicity and comparable low cost of application.

2.4.3 Electrolysis method

Electrolysis method to treat wastewater mainly includes oxidation, reduction, agglomeration, air floating in the electrolytic process to make pollutants transfer, degradation, mineralization, and reduce BOD, COD, NH_4^+−N. In the electrochemical process, either direct or indirect oxidation process destroys the pollutants. In direct anodic oxidation process, the pollutants are first adsorbed on the anode surface and then destroyed by the anodic electron transfer reaction. In indirect oxidation process, strong oxidants such as hypochlorite/chlorine, ozone, and hydrogen peroxide are electrochemically generated. The pollutants are then destroyed in the bulk solution by oxidation reaction of the generated oxidant (Rajkumar and Palanivelu, 2004). In addition, the conductive diamond electrochemical oxidation (CDEO) that uses treat aqueous wastes by forming of hydroxyl radicals as an advanced oxidation processes (AOP). This radical is a very powerful oxidant, which leads to a very effective oxidation process. This mechanism, the global electrochemical oxidation process with conductive-diamond anodes is known to be complemented by direct electrooxidation on the surface and also mediated oxidation by other oxidants electrogenerated on the surface from the electrolyte salts (Cañizares et al., 2005).

2.4.4 Chemical oxidation

Chemical oxidation modifies the structure of pollutants in wastewater to similar, but less harmful, compounds through the addition of an oxidizing agent. During chemical oxidation, one or more electrons transfer from the oxidant to the targeted pollutant, causing its destruction.

Chemical oxidation can also be performed with other oxidants such as NaClO, $KMnO_4$, O_3, and $C_2H_2O_4.H_2O$. They can reduce most organic to a specified concentration, but sometime they cannot complete and the cost is high. Meanwhile, advanced oxidations such as Fenton oxidation, ultrasonic wave radiation oxidation, and photochemical catalytic oxidation are new and effective chemical oxidation process in treating organic wastewater. The reaction mechanism is generally considered as the free radical oxidation composite oxidant, illumination, electricity or catalyst to induce and produce various forms of strong oxidation active substances. Especially oxyhydrogen free radicals can make most of the organic pollutants completely mineralization or partial decomposition.

2.4.5 Biodegradation

Biodegradation is the process that broken down organic substance into smaller compounds by the enzymes produced by living microbial organisms. The microbial organisms transform the substance through metabolic or enzymatic processes. Biodegradable matter is generally organic material such as plant and animal matter and other substances originating from living organisms, or artificial materials that are similar enough to plant and animal matter to be put to use by microorganisms. The major biological treatment processes for wastewater include activated sludge processes, aerated lagoons or stabilization ponds, trickling filters or fixed-film reactors, and anaerobic processes. The major groups of biological processes include aerobic, anaerobic and a combination of both. The systems are divided into suspended or attached growth processes for the removal of BOD, nitrification, denitrification, stabilization and phosphorus removal. Some microorganisms have catabolic diversity to degrade, transform or accumulate a huge range of compounds including hydrocarbons

(e.g. oil), polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), pharmaceutical substances, radionuclides and metals.

Aerobic biodegradation is the breakdown of organic contaminants by microorganisms when oxygen is present. More specifically, it refers to occurring or living only in the presence of oxygen; therefore, the chemistry of the system, environment, or organism is characterized by oxidative conditions. Many organic contaminants are rapidly degraded under aerobic conditions by aerobic bacteria called aerobes. Aerobic bacteria (aerobe) have an oxygen-based metabolism. Aerobes, in a process known as cellular respiration, use oxygen to oxidize substrates in order to obtain carbon dioxide, water, energy and biomass. This process includes activated sludge, trickling filtration, oxidation ponds, aerated lagoons, and aerobic digestion.

The anaerobic treatment processes is a processes in which microorganisms break down biodegradable material in the absence of oxygen (and thus molecular/free oxygen) by those microorganisms (also called anaerobes), which do not require air (molecular/free oxygen) to assimilate organic impurities. Anaerobic organisms work together to degrade the organic sludges and wastes in three steps, consisting of hydrolysis of high-molecular-mass compounds, acidogenesis and methanogenesis. The final products of organic assimilation in anaerobic treatment are methane, carbon dioxide gas and biomass. Anaerobic includes anaerobic digestion, septic tanks, landfill, and lagoons.

2.5 Ethanolamines

Monoethanolamine (MEA, 2−aminoethanol, CAS 141−43−5), diethanolamine (DEA, 2, 2′−iminodiethanol, CAS 111−42−2) and triethanolamine (TEA, 2, 2′, 2′′−nitrilotriethanol, CAS 102−71−6) belong to the ethanolamine family and have a broad range of applications from industry to daily domestic use (e.g. cosmetics and personal care products) (Zurita et al., 2005; Libralato et al., 2008). Alkanolamines are commonly used by the natural gas industry to remove hydrogen sulfide, carbon dioxide, and other acid gases from the natural gas in which they occur (sour gas if hydrogen sulfide is present) (Wong and bioletti, 2002; Eide-Haugmo et al., 2009). The physical and chemical properties of them are summarized in Table 2.1. Alkanolamines are bifunctional molecules with amine and alcohol functional groups occurring in the same compound. As a result, they undergo a wide variety of useful reactions common to amines and alcohols as shows in Table 2.2 (Dow, 2003).

Table 2.1 Physical and chemical properties of ethanolamines (Dean, 1992).

Property	Monoethanolamine	Diethanolamine	Triethanolamine
CAS number	141−43−5	111−42−2	102−71−6
Chemical name	Ethanol, 2−amino	Ethanol, 2, 2−iminobis	Ethanol, 2, 2, 2−nitrilotris
Structure	C_2H_7NO	$C_4H_{11}NO_2$	$C_6H_{15}NO_3$
Molecular weight	61.08	105.13	149.18
Physical state (25°C)	Liquid above 10.5°C	Liquid above 28°C	Liquid above 20.5°C
Melting point (°C)	10.5°C	28.0	20.5
Boiling point (°C)	171	268.8	335.4
Density (g/cm^3)	1.0180 at 20°C	1.0966 at 20°C	1.1242 at 20°C
Refractive index	1.4542 at 20°C	1.4776 at 20°C	1.4853 at 20°C
Solubility	Water, alcohol, ethanol, benzene, chloroform, glycerol, ligroin	Water, alcohol, ethanol, benzene	Water, alcohol, ethanol, benzene, chloroform, ligroin
pK_a	9.5 at 25°C	8.88 at 25°C	7.76 at 25°C

Table 2.2 The application of monoethanolamine, diethanolamine, and triethanolamine in various industries and consumer products (Dow, 2003).

Typical application	Monoethanolamine	Diethanolamine	Triethanolamine
Adhesives	−	−	−
Agricultural chemical	−	+	−
Cement grinding aids	−	−	+
Concrete additives	−	−	+
Detergents, Specialty cleaners personal care products	+	+	+
Gas treating	+	+	+
Metalworking	+	−	+
Oil well chemicals	+	−	+
Packaging and printing inks	+	−	−
Photographic chemical	−	+	+
Rubber	−	−	−
Textile finishing	+	+	+
Urethane foams	−	+	+

Remark: −, present; +, absent

As a result of their use in these applications (Table 2.2), ethanolamines may be released into a variety of industrial and municipal wastewaters, which may then be introduced to surface soil and aquatic environments, leading to relevant potential for human exposure and environmental degradation (Davis and Carpenter, 1997).

2.5.1 Toxicity of ethanolamines

Ethanolamines are completely miscible with water and the toxicity is exposed at high concentrations (Gong et al., 2000). Health hazards associated with exposures to ethanolamines include irritation and necrosis of the skin and central nervous system as showed in Table 2.3.

Table 2.3 Symptoms of ethanolamines exposure from the International Chemical Safety Cards (ICSC).

Route of Exposure	Symptoms		
	MEA	**DEA**	**TEA**
Inhalation	Cough Headache Shortness of breath Sore throat	−	Cough Sore throat
Skin	Redness Skin burns Pain	−	Redness
Eyes	Redness Pain Severe deep burns	Redness Pain Severe deep burns	Redness
Ingestion	Abdominal pain Burning sensation Shock or collapse	Abdominal pain Burning sensation	−

Moreover, the remaining portion of the dose was retained in tissues, with the greatest concentrations residing in the liver and kidneys. Available data indicated that most ethanolamines accumulated in the liver, followed by the heart and brain (Knaak et al., 1997).

In addition, the review of literature toxicity data as EC50 or LC50 for MEA, DEA and TEA as showed in Table 2.4. This means that MEA is always more toxic than DEA, as well as TEA (EC50 (MEA) < EC50 (DEA) < EC50 (TEA)). From the PAN Pesticide database (Kegley et al., 2008), MEA shows a moderate acute toxicity, DEA presents no available of evidence toxicity summary assessment and TEA is considered as not acutely toxic.

Table 2.4 Review of existing literature toxicity data as EC50 or LC 50 for MEA, DEA and TEA.

Test species	Effect	Toxicity endpoint	Study time	MEA (mg/L)	DEA (mg/L)	TEA (mg/L)	Reference
Bacterium							
Vibrio fischeri	Bioluminescence inhibition	EC 50	5 min	26.37	122	547	(Libralato et al., 2008)
Vibrio fischeri		EC 50	15 min	23.37	111	503	(Libralato et al., 2008)
Vibrio fischeri		EC 50	30 min	21.50	95.51	425	(Libralato et al., 2008)
Algae							
Chlorella vulgaris	Growth inhibition	EC 50	72 h	–	776	–	(Zurita et al., 2005)
Skeletonema costatum		EC 50	72 h	–	523	–	(Davis and Carpenter, 1997)
Skeletonema costatum		EC 50	72 h	100–200	200–400	–	(Eide-Haugmo et al., 2009)
Phaeodactylum tricornutum		EC 50	72 h	24.70	86.96	204	(Libralatoa et al., 2010)
Crustaceans							
Artemia salina	Immobilisation	EC 50	24 h	–	–	5600	(Davis and Carpenter, 1997)
Artemia salina		EC 50	96 h	–	2800	–	(Davis and Carpenter, 1997)
Artemia franciscana		EC 50	24 h	43	378	577	(Libralatoa et al., 2010)
Asellus intermedius	Mortality	LC50	96 h	100	–	100	(Portmann and Wilson, 1971)
Crangon crangon	Mortality	LC50	48 h	43	378	577	(Libralatoa et al., 2010)
Mollusc							
Crassostrea gigas	Embryo larval development	EC 50	48 h	27.57	82.68	236	(Libralatoa et al., 2010)
Mytilus galloprovincialis		EC 50	48 h	18.17	71.72	112	(Libralatoa et al., 2010)
Amphibians							
Xenopus laevis	Mortality	LC 50	48 h	220	1174	–	(Kegley et al., 2008)
Annelida							
Lumbriculus variegatus	Mortality	LC50	96 h	–	100	–	(Kegley et al., 2008)
Fish							
Carassius auratus	Mortality	LC50	24	190	800	5000	(Bridie et al., 1979)
Phytoplankton							
Chlorococcales	Physiology	EC 50	24 h	70	1000	–	(Harrison et al., 1987)
Skeletonema costatum	Population	EC 50	5 d	–	522.8	–	(Cowgill et al., 1989)
Zooplankton							
Daphnia magna	Behavior	EC 50	24 h	–	289	–	(Bringmann and Kühn, 1977)
	Intoxication	LC50	24 h	140	180	–	(Bringmann and Kühn, 1977)

2.6 Nitrogen compounds

Nitrogen exists in marine and freshwater aquatic systems at oxidation states from −3 to +5. There are four stable forms of inorganic nitrogen: ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), and $N_{2(g)}$. The first three forms are highly soluble, although ammonium can also lose a proton as pH increase above neutral to become ammonia (NH_3), which exist primarily as an insoluble gas. N_2 is the most abundant form of nitrogen on earth, although it can be biologically fixed to ammonia, the rate of fixation is slow.

Nitrogen is the most prominent macronutrient in aquatic systems as same as phosphorus, which can act as limiting nutrient or result in phytoplankton production. Excessive decreased phytoplankton production can result in eutrophication, a condition cause decreased dissolved oxygen concentrations and a severe reduction in aquatic life diversity.

2.6.1 Nitrogen removal

Table 2.5 shows various options for removing or converting nitrogen from one species to another.

1) Conversion of nitrogen to mostly nitrogen gas but also a small amount of nitrous oxide, $N_2O(g)$, and nitric oxide, NO(g), which escapes into the atmosphere. This is achieved in biological treatment systems (nitrification followed by denitrification or simultaneous nitrification/denitrification) or chemically (using breakpoint chlorination).
2) Biological uptake of nitrogen for the growth of biomass followed by efficient solids removal.
3) Removal of ammonia from the water though $NH_3(g)$ stripping at a high pH.
4) Ion exchange to chemically exchange nitrogen ions as NH_4^+ or as NO_3^- using a cation or an ion-exchange resin, respectively.
5) Membrane separation process such as nanofiltration or reverse-osmosis membranes to remove particulate and dissolved nitrogen species. Efficiency varies with the type of membrane and nitrogen species. Ion separation membrane process such as reverse osmosis will remove all particulate and many soluble nitrogen species from water. Particulate nitrogen such as organic particles, including bacteria, is removed with all solids separation processes.

Table 2.5 Nitrogen removal and conversion process.

Initial species	Process	Ultimate species
Organic-N	Ammonification−biological conversion of organic nitrogen to ammonia	Ammonia, NH_4^+−N
Ammonia, NH_4^+−N	Biological nitrification	Nitrate, NO_3^-−N
	Biological nitrition	Nitrite, NO_2^-−N
	Stripping at high pH	Ammonia gas, $NH_3(g)$
	Biological uptake during bacteria growth	Organic nitrogen (biomass)
	Breakpoint chlorination	Nitrogen gas, $N_2(g)$
	Ion exchange will exchange ammonia for another cation	Chemically bound
Nitrite, NO_2^-−N	Denitritation	Nitrogen gas, $N_2(g)$
Ammonia, NH_4^+−N and Nitrite, NO_2^-−N	Anammox	Nitrogen gas, $N_2(g)$
Nitrate, NO_3^-−N	Biological denitrification	Nitrogen gas, $N_2(g)$
	Ion exchange will exchange nitrate for another anion	Chemically bound
Refractory dissolved organic nitrogen (rDON)	Reverse osmosis	Phase separation of rDON
	Chemical coagulation/filtration	Adsorb/filter
	Oxidizer rDON	Convert to biodegradable nitrogen

2.6.2 Biological nitrogen removal

2.6.2.1 Ammonia and nitrite oxidation

Bacterial ammonia oxidation typically is an autotrophic (inorganic electron donor, or energy source), aerobic process that occurs though a two−step metabolism known as nitrification. In the first step, ammonia oxidixing bacteria (AOB) oxidize NH_4^+ to NO_2^- in the process that consumes oxygen both for respiration (as a terminal electron acceptor) and to support enzymatic ammonia monooxygenase reactions. The AOB have been found in fresh water or low salinity systems include α-Proteobacteria, like *Nitrosomonas* and *Nitrosospira* (include *Nitrosolobus* and *Nitrosovibrio*) and also to the γ−proteobacteria (*Nitrosococcus oceanus*). The second step involves the continued oxidation of NO_2^- to NO_3^-via nitrite oxidizing bacteria (NOB) with oxygen serving as electron acceptor that include *Nitrobacter* (α-Proteobacteria), Nitrococcus (γ− Proteobacteria), Nitrospina (δ- Proteobacteria), and *Nitrospira* (Nitrospina phylum) (Jie and Daping, 2008). Recent new advance process configurations that allow NH_4^+oxidation but prevent NO_2^- oxidation require use of the term "nitritation".

2.6.2.2 Denitrification

Denitrification involves the removal of nitrogen in the form of nitrate by conversion to nitrogen gas under anoxic conditions. In denitrifying systems, DO is a critical parameter. Its presence suppresses the enzyme system needed for denitrification. The optimal pH lies between 7 and 8. Denitrification is not specific to any one phylogenetic group; the trait is found in about 50 genera, mostly in the Proteobacteria (Zumft, 1999).

For example, member of the genera *Alcaligenes*, *Pseudomonas*, *Methylobacterium*, *Bacillus*, *Paracoccus*, and *Hyphomicrobium* were isolated as part of the denitrifying microcial flora from wastewater treatment process (Wagner et al., 2002).

Denitrification is the reduction of NO_3^- or nitrite (NO_2^-) to N_2O or dinitrogen (N_2) in the absence of O_2, in the pathway: $NO_3^- \xrightarrow{Nar} NO_2^- \xrightarrow{Nir} NO \xrightarrow{Nor} N_2O \xrightarrow{Nos} N_2$; with abbreviations for the individual N-oxide reductases shown above arrows, e.g. Nos for N_2O reductase. Although many individual denitrifying bacteria reduce NO_3^- to N_2, denitrification, in principle, consists of three modules of reactions [i.e. (1) $NO_3^- \rightarrow NO_2^-$, (2) $NO_2^- \rightarrow NO \rightarrow N_2O$, and (3) $N_2O \rightarrow N_2$ that can occur independently (Zumft, 1999).

2.6.2.3 Ammonification

Heterotrophic bacteria are involved in the regeneration of ammonium from soluble or particulate organic nitrogen in a process called ammonification. This occurs when amino groups are released from organic nitrogen compounds, either because of intracellular or extracellular enzymatic activity. Ammonification is important in wastewater treatment because it makes organic nitrogen bioavailable for nitrification. Biological process can be described in term of the nitrogen species consumed or produced in the process. The key nitrogen species and transformation are shown in Table 2.6.

Table 2.6 Biological nitrogen removal and conversion process.

Initial species	Process	Ultimate species
Organic-N	Ammonification−biological conversion of organic nitrogen to ammonia decay products from biological treatment	Ammonia, NH_4^+−N
	A fraction of the DON is not biodegradable in the process and appears in the effluent as rDON	Dissolved organic nitrogen, both bDON and rDON
Ammonia, NH_4^+−N	Biological ammonia oxidation, first step in nitrification using AOB	Nitrate, NO_2^-−N
	Biological nitrification−in reality the sum of ammonia and nitrite oxidation	Nitrate, NO_3^-−N
	Biological uptake during bacterial growth	Organic nitrogen (biomass)
	Anammox−direct oxidation of ammonia to nitrogen gas using nitrate	Nitrogen gas, N_2(g)
Nitrate, NO_2^-−N	Nitrite oxidation using NOBs	Nitrate, NO_3^-−N
	Denitrification of nitrite	Nitrogen gas, N_2(g)
Nitrate, NO_3^-−N	Biological denitrification	Nitrogen gas, N_2(g)
	Biological uptake during bacterial growth	Organic nitrogen (biomass)

AOB=ammonia oxidizing bacteria; bDON=biodegradable dissolved organic nitrogen; rDON=refractory dissolved organic nitrogen; and NOB=nitrite oxidizing organism.

2.7 Phytoremediation

The term "Phytoremediation" is derived from the Greek word "phyto" (which means plant) and the Latin word "remedium" (which means restoring balance). Phytoremediation is involving into a cost-effective means of managing wastes. It is the use of plant species for in situ risk reduction and/or removal of contaminants from contaminated soil, water, sediments, and air. The mechanisms by which plants promote the removal of pollutants are varied, including uptake and concentration, transformation of pollutants, stabilization, and rhizosphere degradation, in which plants promote the growth of bacteria underground in the root zone that in turn break down pollutants. Specially selected or engineered plants are used in the process. The wastes that potentially can be manage using phytoremediation can be both inorganic and organic chemicals including petroleum hydrocarbons, chlorinated solvents, pesticides, explosives, heavy metals, radionuclides, salts, nutrients, xenobiotic organic chemical, sewage, landfill leachates and air pollutions. Phytoremediation is an energy efficient, aseptically pleasing method of remediating sites with low to moderate levels of contamination and it can be used in conjunction with other more traditional remedial methods as a finishing step to the remedial process. Plant metabolism using sunlight energy and atmospheric carbon dioxide to produce organic matter is fundamentally different from heterotrophic microbial respiration requiring energy, carbon, and nutrients from soil or water. As a result, green plant transformation, conjugation, and sequestration are vital new tools in waste management that are categorized along with methods of vegetative of pollutions to manage contaminated.

Advantages of phytoremediation compared to classical remediation.

- It is more economically viable using the same tools and supplies as agriculture.
- It is less disruptive to the environment.
- Disposal sites are not needed.
- It is more likely to be accepted by the public, as it is more aesthetically pleasing then traditional methods.
- It avoids excavation and transport of polluted media thus reducing the risk of spreading the contamination.
- It has the potential to treat sites polluted with more than one type of pollutant.

Disadvantages of phytoremediation compared to classical remediation.

- It is dependent on the growing conditions required by the plant (e.g. climate, geology, altitude, temperature).
- Large-scale operations require access to agricultural equipment and knowledge.
- Success is dependent on the tolerance of the plant to the pollutant.
- Contaminants collected in senescing tissues may be released back into the environmental.
- Contaminants may be collected in woody tissues used as fuel.
- Time taken to remediate sites far exceeds that of other technologies.

2.7.1 Mechanisms of phytoremediation

Phytoremediation requires a detailed knowledge about plant physiology, acceptable doses, and about transport and metabolism inside plants and in the root zone. Some of the factors affecting chemical uptake and distribution within living plants include: (1) physical and chemical properties of the compound (e.g. water solubility, vapor pressure, molecular weight, and octanol−water partition coefficient, K_{ow}); (2) environmental

characteristics (e.g. temperature, pH, organic matter, and soil moisture content); (3) plant characteristics (e.g. type of root system, and type of enzymes).

Plants affect the water balance of a site; they change redox potential and pH, and stimulate microbial activity of the soil. These indirect influences may accelerate degradation in the root zone or reduce leaching of compounds to groundwater. Compounds taken up into plants may be metabolized, accumulated, or volatilized into air. Based on these processes, several phytoremediation methods have been developed as below (Trapp and Karlson, 2001).

2.7.1.1 Phytoextraction/phytoaccumulation

Phytoextraction is the removal of a contaminant from the soil, ground water or surface water by live plants and translocate them to their above soil tissues. Phytoaccumulation occurs when the contaminant taken up by the plant is not degraded rapidly or completely, resulting in an accumulation in the plant. As different plant have different abilities to uptake and withstand high levels of pollutants many different plants may be used. This occurs when metal contaminants in the soil are taken up by roots and translocate to the aboveground tissues. The plants can then be removed from the site, or if removal of the entire plant is not practical, then the aboveground tissues can be removed for continual remediation of the soil. Removal of these tissues can occur multiple times during the growing season, thus increasing the rate of contamination removal. The harvested tissues can then be incinerated and the ash can is stored in a hazardous waste landfill. The volume of ash stored would be significantly less than excavating the soil and storing it in the same hazardous waste landfill (Succuro et al., 2009). Hyperaccumulator plant species (species which absorb higher amounts of pollutants than most other species) are used on may sites due to their tolerance of relatively extreme levels of pollution. Certain plants hyperaccumulate metals (e.g. nickel, zinc, copper, chromium), and radionuclides. Heavy metal hyperaccumulation is defined as accumulation of more than 0.1% by dry weight in plant tissue (0.01% for cadmium. Hyperaccumulation of more common elements such as iron and manganese is defined as more than 1% of the element by dry weight in plant tissue (0.01% for Cadmium). In the process of hyperaccumulating contaminants (Dushenkov et al., 1995), some plants can remediate the contaminated soils to acceptable levels. Other plants may die or experience severe stress under conditions of hyperaccumulation. Less tolerant plants can still be used in areas of contamination then harvested and disposed after these plants have hyperaccumulated the contaminant to their maximum extent. Such crops can be replanted, if necessary, to complete this remediation. If the remediation goal is to harvest after these plants hyperaccumulate a contaminant, then it is desirable for the selected plants to be able to translocate the contaminant from the root into above ground tissue, such as shoots and leaves (Nellessen and Fletcher, 1993). If the contaminant remains in the roots, harvesting for disposal or recovery may be more difficult.

2.7.1.2 Rhizofiltration

Rhizofiltration is the adsorption or precipitation of contaminants onto plant roots or the absorption into the roots of contaminants that are in solution surrounding the root zone due to biotic or abiotic processes, e.g. heavy metals or lipophilic compounds can be extracted from water by this technique. The uptake, concentration, and translocation of contaminants by the plants may occur and will depend on the contaminant and the type of plant. Exudates from the plant roots may cause precipitation of some organics.

Rhizofiltration first results in decontamination, a process by which the contaminants are immobilized or accumulated on or within the plant. Contaminants are then removed via plant harvesting (Succuro et al., 2009).

2.7.1.3 Phytopumping and water balance control

Phytopumping is another mechanism that can be used to remove or minimize migration of contaminants. In this case, plants are used as organic 'pumps' to pull-in large volumes of the contaminated water as part of the transpiration process. The result is reduced migration of the contaminant in ground water, in addition to potential uptake. Plants that are capable of removing large amounts of water from the soil are best for this purpose. For example, the willow tree (S*alix* spp.) may use up to 200 liters of water per day (Gatliff, 1994). Plants having these characteristics may provide an inexpensive alternative to mechanical pump and treat systems for contaminated ground water in shallow aquifers.

2.7.1.4 Phytostabilization

Phytostabilisation is the use of certain plants to immobilize soil and water contaminants. Contaminant are absorbed and accumulated by roots, adsorbed onto the roots, or precipitated in the rhizosphere. This process takes advantage of plant roots ability to alter soil environment conditions, such as pH and soil moisture content. Phytostabilization is the use of a plant's root system to stabilize the metal-contaminated soil thus preventing the spread of the contaminant. Many root exudates cause metals to precipitate, thus reducing bioavailability. One advantage of this strategy over phytoaccumulation is the disposal of the metal-laden plant material is not required. By choosing and maintaining an appropriate cover of plant species, coupled with appropriate soil amendments, it may be possible to stabilize certain contaminants (particularly metals) in the soil, and reduce the interaction of these contaminants with associated biota. This reduces or even prevents the mobility of the contaminants preventing migration into the groundwater or air, and also reduces the bioavailibility of the contaminant thus preventing spread through the food chain. This technique can also be used to reestablish a plant community on sites that have been denuded due to the high levels of metal contamination. Once a community of tolerant species has been established the potential for wind erosion (and thus spread of the pollutant) is reduced and leaching of the soil contaminants is also reduced.

2.7.1.5 Phytodegradation (Phytotransformation)

Phytodegradation is the process of using plants to convert toxic contaminants into less toxic forms. Phytodegradation is the degradation or breakdown of organic contaminants by internal and external metabolic processes driven by plant enzymes or enzyme co-factors. Plant metabolic processes hydrolyse organic compounds into smaller units that can be absorbed by the plant. Some contaminants can be absorbed by the plant and are then broken down by plant enzymes. These smaller pollutant molecules may then be used as metabolites by the plant as it grows, thus becoming incorporated into the plant tissues.

2.7.1.6 Phytovolatilization

Phytovolatilization is the process where plants uptake contaminants that are water-soluble and release them into the atmosphere as they transpire the water. The contaminant may become modified along the way, as the water travels along the plant's vascular system from the roots to the leaves, whereby the contaminants evaporate or *volatilize* into the air surrounding the plant. For example, plants, possibly in association with microorganisms, can convert selenium to dimethyl selenide. Dimethyl selenide is a less toxic, volatile form of selenium. Phytovolatilization may be a useful, inexpensive means of removing selenium from sites contaminated with high concentration selenium wastes.

2.7.1.7 Rhizodegradation

Rhizodegradation is also called enhanced rhizosphere biodegradation, phytostimulation, and plant-assisted bioremediation. This is the degradation of contaminants in the root zone, either due to microbial activity or by roots, or by both. This degradation is appropriate for in situ degradation of a variety of organic contaminants. During rhizosphere degradation plants naturally promote increased microbial growth in their root zone. In the root zone, several processes accelerate degradation of some compounds. Phyto and rhizodegradation are frequently used for the remediation of organic contaminations, among them petroleum, PAH, BTEX, TNT, chlorinated solvents and pesticides (EPA 2000). Certain soil dwelling microbes digest organic pollutants such as fuels and solvents, producing harmless products through a process known as *Bioremediation*. Plants can moderate the geochemical environment in the rhizosphere, providing ideal conditions for bacteria and fungi to grow and degrade organic contaminants. Plant root exudates provide nutrients such as nitrate and phosphate that reduce or eliminate the need for costly fertilizer additives and sugars, alcohols, and organic acids act as carbohydrate sources for the soil microflora and enhance microbial growth and activity. Some of these compounds may also act as chemotactic signals for certain microbes. The plant roots also loosen the soil and transport water to the rhizosphere thus additional enhancing microbial activity. Moreover, plant roots penetrate the soil, providing zones of aeration and stimulate aerobic biodegradation.

2.7.1.8 Hydraulic control of Pollutants

Hydraulic control is the term given to the use of plants to control the migration of subsurface water through the rapid uptake of large volumes of water by the plants. Very often, water is pumped to prevent leaching or movement of pollutants. Trees or other plants, saving costs, may do hydraulic control partly or completely. In principle, this method can be applied for all contaminants, as long as the plants have no contact with the toxicants. The harvest products can be used without limitation. The main purpose of this technique is to combine it with mechanical pumping to reduce energy consumption and costs. The plants are effectively acting as natural hydraulic pumps which when a dense root network has been established near the water table can transpire up to 300 gallons of water per day. This fact has been utilized to decrease the migration of contaminants from surface water into the groundwater and drinking water supplies.

2.8 Plant material: *Cyperus alternifolius*

2.8.1 Scientific classification

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Liliopsida
Order:	Poales
Family:	Cyperaceae
Genus:	*Cyperus*
Species:	*Cyperus alternifolius*
Binomial name	*Cyperus alternifolius*
Synonym:	*Cyperus alternifolius*, *Cyperus alternifolius subsp. Flabelliformis*, *Cyperus flabelliformis*
Common Name:	Umbrella papyrus; Umbrella palm

The leaves of the plant are narrow and flattened and 10−25 cm of length. All the leaves are arranged atop triangular stems. Flower grows at the center of the leaves and has small greenish flowers that form an attractive umbrella-like head. It appears at the end of the stems, on top of the leaf-shaped bracts. A bract of inflorescence conspicuous and it has a cluster of 10−25, mostly longer than the inflorescence. Inflorescence is a central of spikelet cluster including 15−25 stalked clusters, each cluster having 8−15 spikelets. While spikelets are approximately 0.9−1.0 cm long and has 15−30 flowers. Stems has clump-forming, solid, triangular and 50-300 cm of height and root is rhizome white. Plant growth well in Sandy, Well-Drained, Wet, Alkaline, Clay, and Tolerates Acid with soil pH requirements was 5.6 to 6.0 (acidic), 6.1 to 6.5 (mildly acidic). Plant was bloom all year and very high moisture needs on partial shade of sun. Propagation can perform by seed. However, it can growth by take cuttings from the top of the plant and then place them upside down in water and when new plants emerge and then repot in soil. Moreover, dividing of rhizomes, corms or bulbs with place the old growth in the center of the pot for new plant is one method for propagation.

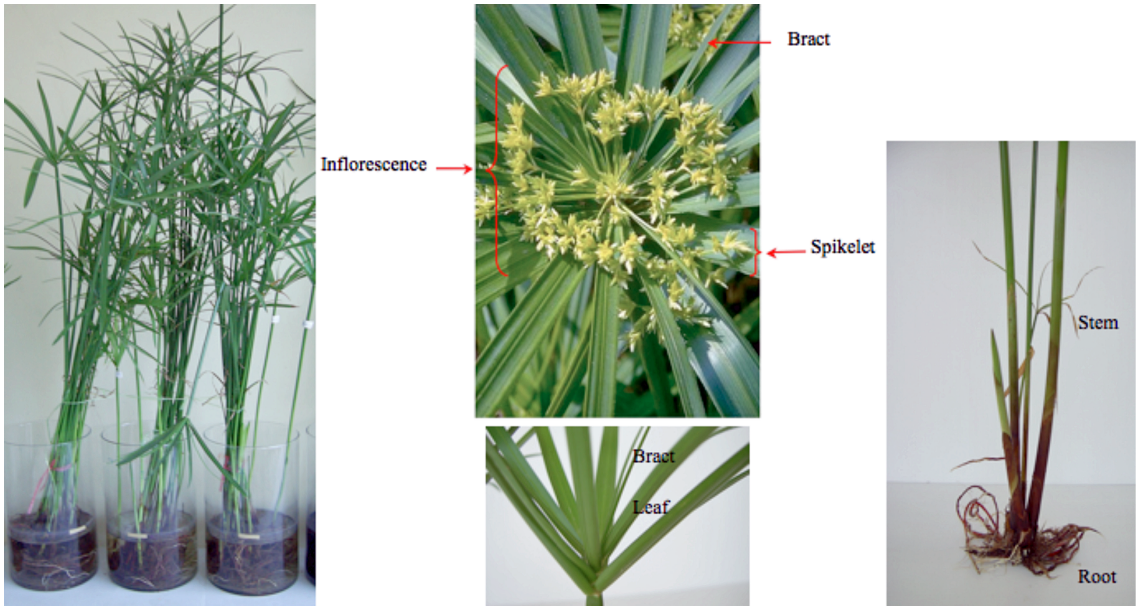


Figure 2.1 Characteristic of *Cyperus alternifolius*.

CHAPTER 3
METHODOLOGY

3.1 Equipments and apparatus

3.1.1 pH/ORP meter: Delta 340, METTLER TOLEDO, Switzerland
3.1.2 DO Meter, OM-14, Horiba, Japan
3.1.3 Vortex Mixer, SA8, STUART, UK
3.1.4 Vortex Mixer, FINEVOTEX, FINEPCR, Korea
3.1.5 Centrifuge: Labofuge 200, Heraeus, England
3.1.6 Centrifuge: Nanofuge, HF-120, Tomy Seiko, Japan
3.1.7 Centrifuge: Microfuge® 16 centrifuge, Backman coulter, Germany
3.1.8 Orbital Incubator Shaker, Gyromax 737 R, Amerex Instruments, USA
3.1.9 Mini centrifuge: 6K-22V, ExtraGene, Taiwan
3.1.10 Weighing, CP 323P, Sartorius, UK
3.1.11 Sterile membrane filter: Whatman cellulose nitrate membrane filters. Whatman, Camlab, UK
3.1.12 Syringe filter, VertiPure™ Nylon Syringe Filter, 13 mm, 0.45 μm, Vertical Chromatograp hy, Thailand
3.1.13 Sterile scalpel
3.1.14 Gel cutting tips, Gel Excision Tip 6.5x1mm, Cleaver Scientific, UK,
3.1.15 COD reactor, C 9800, Hanna, UK
3.1.16 Scanning Electron Microscope connected to an Energy Dispersive X-ray spectroscope (SEM/EDX), JSM-6400, Japan
3.1.17 Hot Air Oven, DIN 12880-KI, MEMMERT,Germany
3.1.18 10 μL Pipet Tips, ExtraGene, USA
3.1.19 200 μL Pipet Tips, ExtraGene, USA
3.1.20 1000 μL Pipet Tips, ExtraGene, USA
3.1.21 PCR tube, 0.2 mL
3.1.22 Filter Pipette Tips, Transparent 0.1-10 μl, GENTAUR, USA
3.1.23 Filter Pipette Tips, Transparent 1-200 μl, GENTAUR, USA
3.1.24 Mx3000P Optical Strip Caps, Agilent Technology, USA
3.1.25 Mx3000P Strip Tube, Agilent Technology, USA
3.1.26 Filter paper No.1: Whatman, UK
3.1.27 Eppendorf® Mastercycler Gradient (Eppendorf, Germany)
3.1.28 DGGE–2401 system apparatus, CBS Scientific Company, USA
3.1.29 Peristaltic Pump, 323, Watson-Marlow Bredel Pump, England
3.1.30 UV transilluminator and captured using Biovision CN 1000/26M, Vilber Lourmat, France
3.1.31 Lamina: Holten LaminAir, Safe 2000, Denmark
3.1.32 Spectrophotometer: NanoDrop 1000 Spectrophotometer, Thermo Scientific, USA
3.1.33 Realtime-PCR instrument: Stratagene Mx3005P, Agilent technologies, USA
3.1.34 Gas chromatograph: GC-17A, Shimadza
3.1.35 DNase/Rnase free distilled water (Invitrogen, U.S.A.)
3.1.36 Gel/PCR DNA fragments extraction kits (Geneaid, Taiwan)
3.1.37 ICP, JY/ICP-AES
3.1.38 Laboratory bottle 100 mL
3.1.39 Loop

3.1.40 Spreader
3.1.41 Petri dish
3.1.42 Test tube
3.1.43 Burette
3.1.44 Beaker
3.1.45 Volumetric flask
3.1.46 Pipette
3.1.47 Cylinder, beaker
3.1.48 Sealed glass bottle
3.1.49 Borosilicate Glass 250 mL, distillation flask

3.2 Chemicals and reagents

3.2.1 Monoethanolamine ($H_2NC_2H_4OH$, MEA, 99% purity), Carlo Erba, Italy
3.2.2 Diethanolamine ($HN(CH_2CH_2OH)_2$, DEA, 99% purity), Sigma-Aldrich, USA
3.2.3 triethanolamine ($N(CH_2CH_2OH)_3$, TEA, 98% purity), Sigma-Aldrich, USA
3.2.4 Ferrous sulphate
3.2.5 Calcium chloride
3.2.6 Magmesium sulphate 7 H2O
3.2.7 Di Potassium hydrogen phosphate
3.2.8 Magnesium oxide, QREC
3.2.9 Agar powder, Biomark
3.2.10 Calcium dihydrogen phosphate mono, Aldrich
3.2.11 Reagent for Test Nitrate, HR, Nitraver 5 Reagent, Hach, U.S.A.
3.2.12 KAPA SYBR® FAST Master Mix (2X) Universal 1 x 1mL, kapabiosystems, USA
3.2.13 PGEM-T Easy Vector System I, Promega, USA
3.2.14 High-Speed Plasmid Mini Kit (10-50 Kb), Geneaid, USA
3.2.15 Favorgen™ Gel/PCR Purification Mini, Favorgen, USA
3.2.16 Taq DNA Polymerase, Invitrogen, USA
3.2.17 Dnase/Rnase free distilled water, Invitrogen, USA

3.3 Original wastewater

The original wastewater used in this study was taken from a factory located in Thailand. The factory produces pigments and ink for flexographic printing to produce packaging materials. The major compositions were a mixture of water-soluble dyes and/or pigments as coloring agents (30%), acrylic resin (42%), MEA (4.8%), NH_4OH (1.2%), water (12%), and additives (10%). Wastewater had turbidity, a dark color, a bad smell, and contained high COD and ammonia concentrations.

3.4 Soil material

Soil was used for support the root that prepared from wet clay soil. It was air-dried and ground and then the size of soil was sieved through 0.47 mm and mixed to homogeneous texture as showed in Fig. 3.1.

Figure 3.1 An appearance of soil.

The soil is used as a nutrient reservoir. It varies widely with respect to composition, structure, and nutrient supply. Especially important from the nutrient perspective are inorganic and organic soil particles called colloids. Soil colloids retain nutrient for release into the soil solution where they are available for uptake by root. Therefore, the study of chemical properties of soil and X-ray fluorescence spectrometry (XRF) could explain composition of soil as showed in Table 3.1 and Table 3.2.

Table 3.1 Chemical properties of soil.

Soil chemical properties									
pH	Sand (%)	Silt (%)	Clay (%)	Organic carbon (%)	P (mg/kg)	K (mg/kg)	Ca (mg/kg)	Mg (mg/kg)	CEC (cmol/kg)
4.3	16	16	68	1.78	6	169	3501	395	30.20

Table 3.2 The percentage of elements in clay soil by X-ray fluorescence spectrometry (XRF).

Compound	% Element
SiO_2	52.0
Al_2O_3	19.9
Fe_2O_3	5.48
K_2O	2.23
TiO_2	0.918
CaO	0.857
MgO	0.767
SO_3	0.361
Na_2O	0.192
P_2O_5	0.0602
BaO	0.0519
MnO	0.0502
ZrO_2	0.0265
V_2O_5	0.0225

Compound	% Element
WO_3	0.0223
Rb_2O	0.0213
ZnO	0.0135
Cl	0.0119
CuO	0.0118
SrO	0.0085
NiO	0.0074
Y_2O_3	0.0037
Ga_2O_3	0.0035

3.5 Plant material

C. alternifolius, about 3–4 months-old, was obtained from a garden market. Plants were washed carefully with tap water to remove soil and any contaminated substances from their roots. After that, they were pre-cultured in tap water for 5 days and the healthy plants were selected. Plants of similar sizes and similar fresh weights were used in the experiment.

3.6 Research methodology

3.6.1 Chemical precipitation of wastewater by sulfuric acid and ferric sulfate

This experiment compared the efficiency of COD removal between sulfuric acid (H_2SO_4) and ferric sulfate ($Fe_2(SO_4)_3$). They were added in raw wastewater to the dispersion and mixed slowly for 1 minute. The resulting flocs were allowed to settle. Afterwards, the supernatant was collected to adjust the pH to neutral using calcium hydroxide ($Ca(OH)_2$, in order for the plant to survive at this neutral pH.

3.6.2 Chemical precipitation of wastewater by magnesium ammonium phosphate (MAP)

Experiments for MAP precipitation were performed with various chemicals such as $MgCl_2.6H_2O$ + $Na_2HPO_4.12H_2O$ (substance A), MgO + 85% H_3PO_4 (substance B), and $MgSO_4.7H_2O$+ $Ca(H_2PO_4)_2$ (substance C). Firstly, chemical substances were added to the wastewater (1000 mL) at Mg^{2+}: NH_4^+: PO_4^{3-} molar ratios of 1:1:1. Secondly, the reaction solution was agitated by magnetic stirrers for 15 min and adjusted pH for the minimum MAP solubility with various alkaline chemicals (NaOH, $Ca(OH)_2$, and KOH) at pH 8.5-9.0. Thirdly, the formed MAP was allowed to settle in the reaction solution for 15 min. Lastly, the supernatant was collected.

3.6.3 Phytoremediation method

This study was performed under various conditions; wastewater conditions (W), wastewater+soil conditions (WS), wastewater+plant conditions (WP), and wastewater+soil+plant conditions (WSP). The weight of plants and soil were increased higher than plant screening for increasing the efficiency of wastewater removal. The healthy plants and soil about 300 g of weights each and 2 L of wastewater were used in the experiment. The experiment was performed at 30±2°C, 68±5% humidity and a 12 h white light/12 h dark.

3.6.4 Removal of synthetic MEA, DEA, and TEA wastewater by *C. alternifolius*

C. alternifolius was cultured in 1400 mg/L of synthetic MEA, DEA, and TEA wastewater at a volume of 2 L for each pot. The high concentration of MEA is as high as the level found in wastewater effluent from the factory produces pigments and ink (Effluent samples are collected from one site). Therefore, this research used the concentration of synthetic ethanolamines wastewater was 1400 mg/L. Plants used were about 300 g fresh weights. Plants were grown in a greenhouse at 30±2°C with 70±5% humidity in a 12 h white light/12 h dark cycle. Plants were grown in synthetic ethanolamine wastewater under hydroponic conditions for a period of 12 days. Synthetic ethanolamine wastewater was used as controls. The volume of water evaporation and water transpiration by plants during the experiment was compensated by adding with tap water. MEA, DEA, and TEA were sterilized by filtered through

0.42 μm of sterile filter paper in sterilized bottles. The equipments were autoclaved at a temperature of 121 °C under 15 pounds of pressure per square inch (Psi) for 20 min. The sample solution was analyzed for ethanolamines and organic acids by gas chromatograph (GC)(Shimadzu, GC 17A, Kyoto, Japan) and pH change was measured using a pH meter (Metler Toledo Delta 304, USA).

3.7 Sampling and analytical methods

Before sampling the solution, the volume of water evaporate was compensated by adding tap water. All samples were rapidly filtered using Whatman GF/A filter paper to remove solids. Concentrations of TKN, COD and BOD were measured according to the standard methods (APHA, 1995.). NH_4^+–N and NO_2–N were measured according to the standard methods (APHA, 1998).

TKN was measured by the digestion-titration method using an analyzer (Kjeldatherm, Gerhardt, Germany). NH_4^+–N was measured by the titration method using a Rapid Distillation System (Vapodesk 20, Gerhardt, Germany). COD was measured by the reactor digestion method using COD digestion vials (Odyssey DR/2500, Hach, USA), NO_2–N was measured by diazotization method (Odyssey DR/2500, Hach, USA), and NO_3–N was measured by the cadmium reduction method using NitraVer 5 reagent (Odyssey DR/2500, Hach, USA), and all procedures were followed according to the Hach DR/2500 spectrophotometer manual (Hach Company, 2001). Dissolved oxygen (DO) concentrations under various conditions were measured inside pots using an oxygen meter (OM-14, Horiba, Japan). ORP was measured with reference to Ag/AgCl in this study (pH/ORP meter: Delta 340, METTLER TOLEDO, Switzerland).

3.7.1 pH (SM 4500-H^+: Electrometric method)

Buffers solution of pH 4, 7, and 9.2 are used to calibrate the pH meter before measure sample. Remove electrodes from storage solution, rinse thoroughly with distilled water, blot dry with soft tissue. Remove electrodes from the first buffer, rinse thoroughly with distilled water, blot dry and immerse in second buffer. After that determine pH of the sample by dipping the electrode in three or four successive portions of the sample and read pH.

3.7.2 Oxidation-reduction potential (ORP) (SM: 2580)

This probe is a combination/double junction electrode consisting of a silver/silver chloride junction and a platinum wire junction. Saturated potassium chloride is used as a fill solution for the silver junction. The readout is obtained through the pH meter set in the millivolt mode. Standardize the electrode system against redox solutions that provide stable known Eh values over a range of temperature. If the reading is within ± 10 mV from the theoretical redox standard value at that temperature, record it and temperature. Then rinse the electrode with distilled water and proceed with the sample measurement.

3.7.3 Chemical oxygen demand (COD) (SM: 5220 D: closed reflux, colorimetric method)

COD assay solutions were prepared according to the closed reflux. Briefly, the digestion solution was prepared by mixing 2.6 g $K_2Cr_2O_7$, 8.33 g $HgSO_4$, and 42 ml H_2SO_4 (concentrated), and then diluting to a total volume of 250 ml with distilled water. The catalyst solution was prepared by adding 5.06 g Ag_2SO_4 to 500 ml of H_2SO_4

(concentrated); this solution was mixed for 48 h to ensure that the Ag_2SO_4 dissolved completely.

Calibration curve is prepared at least five standards from potassium hydrogen phthalane solution with COD equivalents to cover concentration range. The sample solutions in each pot were taken about 5 ml for COD analysis. Before testing COD, the sample solutions were centrifuge at a speed of 4,500 rpm for 10 min. After that, sample solutions were diluted (if necessary) and then taking 2.5 ml in crew-cap test tube (10 ml). Distilled water used for blank COD. Then adding 3.5 ml of the catalyst solution and 1.5 ml of the digestion solution. Preferably analyze samples in duplicate. COD samples were then incubated at 150^{o}C for 2 h in a dry incubator. After allowing the COD tubes to cool at room temperature, COD levels were determined by measuring the absorbance of the digested assay solution at λ=600 nm.

3.7.4 Nitrogen (organic) (SM: 4500-N_{org} B)

Storage of Samples; Preserve samples by acidifying to pH <2 and storing at 4°C for preservation for up to 28 days.

Digestion; Place a measured volume of sample in a 300 mL kjeldahl flask. 10 mL of sample solution is contained in distillation flask and then added carefully 10 mL sulfuric acid and 1 tablet of Kjelbtabs (K_2SO_4, $CuSO_4$) under a hood. Then continue to digest at 385^{o}C for an additional 45-60 minutes. As digestion continues, colored or turbid samples will become transparent and pale green.

Distillation; Connect distillation flask to a steamed-out distillation apparatus and swirl flask to insure complete mixing. The pH of the solution should exceed 11.0. After that distillation flask are amino nitrogen and free ammonia are converted to ammonium ion. A solution sodium hydroxide and sodium thiosulfate is added to the digestate, resulting in the conversion of all ammonium to ammonia. The ammonia is then distilled into a solution of boric acid that also contains methyl red and methylene blue indicators.

Titration; At least 200 mL of distillate is collected, and then titrated with standardized 0.02 M sulfuric acid.

Adding known concentrations of ammonium chloride solution to samples before digestion is begun carries out spikes. Duplicate samples and distilled water blanks are carried through the digestion and distillation processes.

Calculation

$$\text{mg} - \text{N} / \text{L} = \frac{(\text{A} - \text{B}) \text{ x N x } 14.007 \text{ x } 1000}{\text{Volume of sample (mL)}}$$

Where:
A = volume of H_2SO_4 titrate for sample, mL
B = volume of H_2SO_4 titrate for blank, mL
N = Normality of H_2SO_4

3.7.5 Nitrogen (ammonia) (SM: 4500-NH_3)

Storage of Samples; Preserve samples by acidifying to pH <2 and storing at 4°C for preservation for up to 28 days. 10 mL of sample solution is contained in distillation flask and then added 25 mL borate buffer solution. Connect flask to a distillation

apparatus. A solution sodium hydroxide and sodium thiosulfate is added to the digestate, resulting in the conversion of all ammonium to ammonia. The ammonia is then distilled into indicating boric acid solution that also contains methyl red and methylene blue indicators.

Titration; Sample solution of distillate is collected, and then titrated with standardized 0.02 M sulfuric acid. Adding known concentrations of ammonium chloride solution to samples before ditillation is begun carries out spikes. Duplicate samples and DI blanks are carried through the distillation processes.

Calculation

$$\text{mg} - \text{N} / \text{L} = \frac{(\text{A} - \text{B}) \text{ x N x } 14.007 \text{ x } 1000}{\text{Volume of sample } (\text{mL})}$$

Where:
A = volume of H_2SO_4 titrate for sample, mL
B = volume of H_2SO_4 titrate for blank, mL
N = Normality of H_2SO_4

3.7.6 Nitrogen (Nitrite) (SM 4500-NO_2^- B: sulphanilamide spectrophotometric)

Sample solutions were filtered though a 0.45 μm pore size (Whatman GF/A filter paper) before analysis for removal of suspended solids. To 50 mL sample or to a portion diluted to 50 mL in a volume flask and then add 2 mL color reagent and mix. Blank is 50 mL distilled water. Wait between 10 min and 2 hr after adding color reagent to samples and standard, measure absorbance at 543 nm using a 1 cm path length cuvette. Prepare standard curve by diluting 1, 2, 3, 4 and 5 mL of standard nitrite solution to 100 m L to give 5, 10, 15, 20 and 25 μg/L concentration, respectively. Calculations compute sample concentration directly from the curve, taking in consideration dilution of the sample if applicable.

Color reagent

To 800 mL water add 100 mL 85% phosphoric acid and 10 g sulfanilamide. After dissolving sulfanilamide completely, add 1 g N-(1-naphthyl)-ethylenediamine dihydrochloride. Mix to dissolved, then dilute to 1 L with water. Solution is stable for about a month when stored in a dark bottle in refrigerator.

Calculation

$$NO_2^- \ (\mu\text{gN} / \text{L}) = \frac{\mu\text{gN } (\text{involume 50 mL}) \text{ x } 1000}{\text{Volume of sample } (\text{mL})}$$

3.7.7 Nitrogen (Nitrate)

Nitrate (NO_3^-) was measured by the cadmium reduction method using NitraVer 5 reagent (Odyssey DR/2500, Hach, USA), and all procedures were followed according to the Hach DR/2500 spectrophotometer manual (Hach Company, 2001)

3.7.8 Total dissolved solids (SM: 2540)

Pre-dry evaporating dishes (porcelain) by placing them at 105±3ºC in drying oven for at least 1 h. Store and cool the dishes in a desiccator to balance temperature. Using gloves

or tweezers to handle the dishes, record this weight.

Assemble filtering apparatus and filter and suction. Thoroughly mix sample solution and then measurement amount to the 50 mL. Liquor samples should be passed through a GF/C filter paper and then wash filter with distilled water, and continue suction for about 1-3 min after filtration is complete. Transfer total filtrate (with washing) to the evaporating dishes and then evaporate to dryness in an oven at 105 ± 3°C for at least 1 h. Remove evaporating dishes from the oven, allow it to cool at room temperature in a desiccator. Weigh the dish containing the oven-dried sample and record this weight.

Calculation

$$\text{mg total solid / L} = \frac{(A - B) \times 10^6}{\text{Sample volume (mL)}}$$

Where:
A = weight of dried residue + dish (mg)
B = weight of dish (mg)

3.7.9 Suspended solids (SM: 2540)

Pre-drying GF/C filter paper at 105±3ºC on glass plate for at least 1 h. Store and cool filter paper in a desiccator to balance temperature, record this weight. Assemble filtering apparatus and filter paper and suction. Wash filter with distilled water, allowing complete drainage between washing. Thoroughly mix the sample solution and then measurement amount to the 50 mL, and continue suction. Carefully remove filter paper from filter apparatus and transfer to a glass plate as a support. Dry for at least 1 h at 105 ± 3°C in an oven. Store and cool in a desiccator to balance temperature, and weight.

Calculation

$$\text{mg suspended solids / L} = \frac{(A - B) \times 10^6}{\text{Sample volume (mL)}}$$

Where:
A = weight of filter paper + dried residue (mg)
B = weight of filter paper (mg)

3.7.10 Gas chromatography (GC)

Ethanolamines and organic acids were measured with a Shimadzu GC-17A gas chromatograph equipped with a split/splitless injector, a flame ionization detector (FID), and a Shimadzu AOC-20i GC auto-injector (Shimadzu Scientific Instruments, Columbia, MD, USA). The gas chromatograph used the capillary column Rtx®−200 with a 30m length x 0.32mm I.D. x 1 μm film thickness (Restek, Bad Homburg, Germany). The column oven temperature was 150°C. The detector temperature was 250°C. Helium was used as the carrier gas with a flow rate of approximately 1.93 mL min^{-1}. The injector volume was 1.0 μL with a split ratio of 20:1 and a temperature of 200°C. The sample solution was filtered through VertiPure™ nylon syringe filters 0.2 μm (Vertical chromatography Co., Ltd., Thailand) prior to analysis. The accumulation of ethanolamines in plant tissues were analyzed from 10 g wet weight of plant tissues that were cut into small pieces with a scissors and then ground with liquid nitrogen

using a hand blender. The sample was placed into a centrifuge tube with 20 mL of distilled water, agitated for 12 hours in shaker (Gallenkamp Environmental, Model 10X 400, UK). The samples were centrifuged at 2185 g for 15 min and the solution was then filtered through VertiPure™ nylon syringe filters 0.2 μm (Vertical chromatography Co., Ltd., Thailand) and stored at −20°C prior to determination.

3.7.11 Fe, Mn and Al concentrations from a rust-colored iron oxide scum on the surface of soil-contained wastewater condition

A rust-colored iron oxide scum on the surface of wastewater+soil conditions was separated and oven-dried at 70°C for 2 days. After that 1.0 g of dried sample was ground, weighed, and digested with 0.50 mL of HNO_3 and distilled water 0.5 mL. After that the solution was filtered through Whatman filter paper No. 542 and then digestion solution was adjusted the volume with deionized water before analysed cadmium concentration by ICP-OES.

3.7.12 Relation of plant, soil and microorganisms in treatment of wastewater treatment

The efficiency of plant absorption, soil adsorption, and microbial activity in systems was investigated. This experiment was observed after 7 days under various conditions: wastewater, sterilized wastewater, soil in sterilized wastewater, sterilized soil in sterilized wastewater, and plants grown in sterilized soil and sterilized wastewater conditions. Sterilized soil and wastewater samples were prepared by being autoclaved at a temperature of 121 °C under 15 pounds of pressure per square inch (Psi) for 20 min. All experiments were performed in replicates of 3. After that, the COD concentration was measured and then the percentage removal of COD by plant, soil, and microorganisms were shown in equations as follows:

% COD removal by microorganisms in wastewater = ([A-B]/A) x 100 (3.1)
% COD removal by microorganisms in soil = ([C-D]/C) x 100 (3.2)
% COD removal by soil adsorption = ([B-D]/B) x 100 (3.3)
% COD removal by plant absorption and microorganisms in plant
= ([D-E]/D) x 100 (3.4)

Whereas:

A = COD of non-sterile wastewater
B = COD of sterile wastewater
C = COD of non-sterile soil in sterilized wastewater
D = COD of sterile soil in sterilized wastewater
E = COD of plants grown in sterile soil and sterilized wastewater

3.7.13 Scanning electron microscope connected with energy dispersive X-ray spectroscopy (SEM/EDX)

Plant tissue samples were performed under various conditions such as plant grown in tap water (control), the synthetic MEA solution+plant, the synthetic MEA solution+soil+plant, wastewater+plant, and wastewater+soil+plant. The leaves and roots of plant under various conditions was harvested after 7 days, and dried at 70°C for 2 days. A small piece of leaves and roots were put on aluminium straps, and coated with carbon before it was analysed by a Scanning Electron Microscope connected with Energy Dispersive X-ray spectroscopy (SEM/EDX) (JSM-6400, Japan). The percentages of elements on the plant surfaces were calculated from at least three areas of scanning.

3.8 Molecular microbiology methods

3.8.1 Sample preparation

Wastewater from wastewater at day 0 (W_0) and wastewater (W_7), wastewater+soil conditions (WS_7), wastewater+plant conditions (WP_7) and wastewater+soil+plant conditions (WSP_7) were collected at day 7. Collected samples were centrifuged at 4000 rpm for 20 min and then the pellets were collected while supernatant was filled with 0.45 μm sterile membrane filter. The pellets and samples-contained in sterile membrane filter were stored at –20^{o}C for preparation cell lysis at the next step.

3.8.2 Genomic DNA extraction

Genomic DNA from sample solutions and plant tissue were obtained using the chloroform method (Moore 1996).

3.8.2.1 Solution

Tris-HCl 1M (pH 8.0) buffer

Dissolved 121.1 g of Tris in 800 mL of distilled water. Adjust pH to 8.0 with concentrated HCl (≈42 mL), and set the final volume to 1 L. Autoclave at 121°C for 15 minutes.

Ethylenediaminetetraacetic Acid (EDTA) 0.5M (pH 8.0)

Dissolved 186.1 g of sodium-EDTA $2H_2O$ in 800 mL distilled water. Adjust pH to 8.0 with NaOH (≈20 g), and autoclave.

NaCl 5M

Dissolved 292.2 g of sodium chloride in 800 mL of distilled water. Adjust the final volume to 1 L and autoclave.

S buffer (pH 8.0)

Composition: For a final volume of 500 mL, mix into a sterile bottle: 50 mL of Tris-HCl 1M, 50 mL of EDTA 0.5M, 150 mL of NaCl 5M, 50 mL of CTAB 10%, and 200 mL of sterile distilled water.

10% CTAB (Cetyl Trimethyl Ammonium Bromide) (100 mL)

10% CTAB (10 g)
0.7M NaCl (14 mL, 5M)

3.8.2.2 Cell lysis

Cell was lysed with S buffer proceeding to the DNA extraction through chloroform extraction.

-Transfer the sample to a 50 mL sterile tube and resuspend the pellet in 10 mL of S buffer.

-Add proteinase K (10 μg/μL) and vortex.

-Add lysozyme (10 μg/μL) and vortex.

-Incubate in a water-bath at 37^{o}C for 2 hours and invert the tube every 15 to 20 minutes. Cool to room temperature.

-Centrifuge at 5,000 rpm for 15 minutes. Transfer the aqueous phase into a clean 50 mL tube.

3.8.2.3 Chloroform extraction

-Add 1V Chloroform:isoamyl alcohol 24:1 (v/v) and gently mix by inversion.

-Centrifuge at 14,000 rpm for 5 minutes. Transfer the upper phase into a clean 50 mL tube.

-Add 0.6V of isopropanol and mix by inversion. In this step sometimes DNA can be seen as a white filamentous precipitate.

-Store the tube at room temperature for 1 hour or at 4°C overnight.

-Centrifuge the sample at 14,000 rpm for 20 minutes.

-Discard the supernatant and wash the pellet with 100% ethanol. Mix gently by inversion.

-Centrifuge the sample at 14,000 rpm for 20 minutes and discard the supernatant and then allow air-drying.

-Resuspend the purified DNA with 20 μL sterile distilled water.

3.8.3 DNA cleaning methods

The Gel/PCR DNA Fragments Extraction Kit (Geneaid) was designed to recover or concentrate DNA fragments (100 bp→10 Kb) from agarose gel, PCR, or other enzymatic reactions. This method provides a quick and efficient purification of DNA.

3.8.3.1 DNA cleaning with PCR DNA Fragments Extraction

-Transfer 100 μL of the sample into a 1.5 microcentrifuge tube.

-Add 5 volumes of DF buffer to 1 volume of the sample and mix by vortex.

-Place a DF column in a 2 mL Collection Tube.

-Transfer the sample mixture from step 1 to the DF Column and Centrifuge at 13,000 rpm for 30 seconds.

-Discard the flow-through and place the DF column back in the 2 mL collection tube.

-To wash the DNA, add 600 μL of wash buffer (ethanol added) into the center of the column and let stand for 1 minute. Centrifuge at 13,000 rpm for 30 seconds.

-Discard the flow-through and place the DF column back in the 2 mL collection tube.

-Centrifuge the column again for 3 minutes at 13,000 rpm to dry the column matrix.

-Insert the DF column to a new 1.5 mL microcentrifuge tube.

-To elute the DNA, add 20-50 μL of elution buffer or TE onto the center of the column matrix. Let stand for 2 minutes and centrifuge for 2 minutes at 13,000 rpm to elute the purified DNA.

-DNA products were checked by loading 3 μL of the reaction product on 0.8% agarose gel and then stored at -20°C.

3.8.3.2 DNA cleaning with Gel Extraction

-Run the samples in a 1% agarose gel at 100V for 25 minutes. A molecular marker (1 Kb or 100 bp ladder-Fermentas) should also be run in order to have a size reference to recognize the DNA band to recover.

-Cut the agarose gel slice containing the desired DNA fragments with a sterile scalpel and drop it into a new 1.5 mL microcentrifuge tube previously weighted.

-Add 1.67V of DF buffer (Geneaid) to 1V of gel (consider 300 mg of gel as 500 μL) and mix by votex.

-Incubate at 55-60°C for 10-15 minutes or until the agarose has been completely dissolved. During incubation, invert the tube every 2-3 minutes. Cool the dissolved

sample mixture to room temperature.

-Place the DF column in a 2 mL collection tube. Transfer the sample mixture from the previous step to the DF column. Centrifuge at 14,000 rpm for 30 seconds. Now the DNA is retained on the menbrane of the column. Discard the effluent.

-To wash the DNA, add 400 μL of W1 buffer into the DF column. Centrifuge at 14,000 rpm for 30 seconds and then discard the effluent.

-Add 600 μL of wash buffer (ethanol added) into the DF column and let stand for 1 minute. Centrifuge at 14,000 rpm for 30 seconds and then discard the effluent.

-Centrifuge the column again at 14,000 rpm for 3 minutes to dry the column matrix.

-Transfer the dried DF column to a new 1.5 mL microcentrifuge tube.

-Add 20-50 μL of elution buffer or TE onto the center of the column matrix. Let stand for 2 minutes or until the elution buffer or TE is absorbed by the matrix. Centrifuge for 2 minutes at 14,000 rpm to elute the purified DNA. Store at -20°C.

3.8.4 DNA quantification methods

3.8.4.1 Spectrophotometric determination

Purified and concentration of extracted genomic DNA was determined by spectrophotometer. An absorbance spectrum from 200 to 400 nm was performed and absorbance was measured at 230, 260, and 280 nm (A230, A260, and A280, respectively) by using NanoDrop spectrophotometer. The coefficient A260/A280 provided an analytical measure of the genomic DNA purity (Sambrok 1989). Thus, a DNA sample is considered to be pure when A260/A280 value ranges from 1.8 to 1.9 (Gallagher 1989). Beside, a coefficient A260/A230 was more than 2.2 denote a protein-free DNA sample.

3.8.5 DNA Polymerase chain reaction (PCR)

3.8.5.1 PCR conditions

PCR mixes used in the reactions were prepared on cleaned table by 70% ethanol. All the material was sterilized and used to perform polymerase chain reactions only.

3.8.5.1.1 16S rDNA targeted primers

-8F and 1492R, this universal set of the standard primers was used to amplify a fragment of 16S rDNA gene of all the Eubacteria. Eubac27F or 8F and 1492R (Lane 1991) are complementary to the position 8-27 and 1492-1513 of 16S rDNA according E. coli position.

-338F and P518R, the primer pair 338f, P518r (Muyzer et al., 1993) was used for amplification of the V3 region part of 16S rDNA genes.

Table 3.3 Primers used in this study.

Primer		Sequence	
Eub8F	5’	AGA GTT TGA TCC TGG CTC AG	3’
Eub1492R	5’	ACG GTT ACC TTG TTA CGA CTT	3’
318F		ACT CCT ACG GGA GGC AGC AG	
318F-GC	5’	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG	3’
P518R	5’	ATT ACC GCG GCT GCT GG	3’

3.8.5.2 PCR programs for 16S rDNA targeted primer

Polymerase chain reaction was carried out in total volumes of 25 µL, 50 ng template DNA was used in each reaction, and non-template control was carried out every time. PCR products were checked by loading 3 µL of the reaction product on 1.0% agarose gel and then PCR products were stored at -20^{o}C.

The PCR reaction mixture:

Buffer (10X)	2.5	µL
$MgCl_2$ (25 mM)	1.5	µL
dNTPs (containing 5 µM dATP, dCTP, dGTP, dTTP)	0.25	µL
Primer forward (10 µM)	0.25	µL
Primer reverse (10 µM)	0.25	µL
Taq polymerase	0.2	µL
DNA template	1.0	µL
Sterile distilled water	to 25	µL

-Program for partial amplification of 16S rDNA genes with primers 8F and 1492R

Condition	Temperature	Time
1. Denaturing	94°C	5 min
2. 24 cycles:	95°C	50 sec (denaturing)
	55°C	30 sec (annealing)
	72°C	2 min (extension)
3. Extension	72°C	7 min
4. Conservation	4°C	Pause

-Program for partial amplification of 16S rDNA genes with primers 338F and P518R

Condition	Temperature	Time
1. Denaturing	95°C	5 min
2. 29 cycles:	95°C	50 sec (denaturing)
	60°C	30 sec (annealing)
	72°C	50 sec (extension)
3. Extension	72°C	7 min
4. Conservation	4°C	Pause

3.8.6 Electrophoresis methods

3.8.6.1 Solutions and reactives

TAE buffer 50x

Dissolve 242 g Tris in 800 mL of distilled water and 100 mL of EDTA 0.5M. Adjust pH by adding glacial acetic acid (≈57 mL); fill up to a final volume to 1 L, and autoclave.

Ethidium bromide (10 mg/mL)

Dissolved 0.1 g ethidium bromide in 10 mL of distilled water and mix with a magnetic stirrer overnight. Store at 4°C protected from the light. This compound is both mutagenic toxic.

Loading buffer

The loading buffer was used for increasing the sample density in order to ease the loading of the samples in electrophoresis gels. Besides, the colorants of the loading buffer also migrate in the electrophoresis, which helps to locate the samples in the gel along the run. The loading proportion was 4 µL sample: 1µL loading buffer. Loading buffer composition in distilled water was: 0.25% Bromophenol blue, 0.25% Cyanol Xylene FF, and 30% glycerol.

Molecular weight ladder

100bp DNA ladder (Fermentas), the ladder consists of eleven fragments that range in size from 100-1,000 bp in 100 bp increments, plus an additional fragment at 1,500bp. The 500 bp fragment is present at increased intensity to allow easy identification.

1Kb DNA ladder (Fermentas), the ladder consist of thirteen blunt-ended fragments with sizes ranging from 250 bp to 10,000 bp. The 1,000 bp and 3,000 bp fragments are present at increased at intensity for easy identification.

3.8.6.2 Agarose gels

Horizontal electrophoresis in agarose gels was performed for both the analysis of PCR or restriction products, and for purification DNA preparations. The agarose gels were prepared by dissolving agarose power in TAE 0.5x buffer. Electrophoresis device used was MiNi-SUB® CELL GT (BioRad). The electric source was a Power-PAC300 (BioRad). Agarose concentration varied depending on the experimental needs. Raging from 0.8% to 1.0% agarose in TAE 0.5x buffer.

Gel casting

Weigh the amount of agarose needed according to the desired agarose percentage and mix with the indicated volume of TAE 0.5x buffer. Melt the agarose in a microwave oven; make sure the agarose is completely melted. After cooling the solution to about 60°C add ethidium bromide (10 mg/mL) (sample 25 mL:ethidium bromide 10 µL) and mix. Pour agarose into the casting tray containing a comb to form the wells. Let it cool until the agarose is completely solid and the wells are formed. Carefully remove the comb by lifting it gently at one end.

Sample loading gel running

Cover the gel with TAE 0.5x buffer. Add ¼ (vol/vol) loading buffer to the samples and load them into the wells using a micropipette. Load the molecular weight ladder too. Apply a voltage 2-10V cm2, which corresponds to approximately 100V in the MINI-

SUB®CELL GT gel box. Let the sample run until the first blue colorant of the loading buffer (bromophenol blue) reach ¾ of the length of the gel (≈25 minutes).

-DNA bands image, DNA bands can be seen on a transiluminator providing UV light of 312 nm (TFX-20M). Image can be digitalized with a PC image capturing system (Scion Image, TDI)

3.8.6.3 Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is an electrophoresis method to distinguish among DNA sequences having the same length but differing in base composition. This method allows the electrophoresis separation and screening of heterogeneous PCR product mixtures and it has been used to assess the diversity and composition of environmental communities. DGGE performance requires one of the two primers to contain a GC clamp at 5' end. Template DNA from 8F-1492R 1400 bp products was PCR amplified using primer set: 338F and P518R to produce PCR products of approximately 200 bp.

Sample preparation

Samples used in DGGE must be obtained by PCR with a primer containing a GC clamp at the 5'end.

The PCR reaction mixture:

Buffer (10X)	2.5	µL
$MgCl_2$ (25 mM)	1.5	µL
dNTPs (containing 5 µM dATP, dCTP, dGTP, dTTP)	0.25	µL
Primer forward (10 µM)	0.25	µL
Primer reverse (10 µM)	0.25	µL
Taq polymerase	0.2	µL
DNA template	2.0	µL
Sterile distilled water	to 25	µL

PCR program used for DGGE with primer 338f and P518r:

Condition	Temperature	Time
1. Denaturing	95°C	5 min
2. 29 cycles:	95°C	50 sec (denaturing)
	60°C	30 sec (annealing)
	72°C	50 sec (extension)
3. Extension	72°C	7 min
4. Conservation	4°C	Pause

Gel casting

Wash the glasses with water, detergent and ethanol. Wash the combs and spacers with water, detergent and distilled water. Assemble the two glasses with the spacers and place the gel sandwich with the clamps in the casting stand. Prepare the acrylamide solution.

Two solutions must be prepared to cast a 7% acrylamide.

	A solution (0%)	B solution (80%)
Acrylamide/Bisacrylamide 40%	2.63 mL	2.63 mL
TAE 50X (50%)	0.3 mL	0.3 mL
Urea	5 g	5 g
Formamide		4.8 mL
Distilled water	24.14 mL	2.27 mL

After that 30-60% denaturing gradient gel was prepared by calculation from A and B solution. Ammonium persulfate (10%) and TEMED cause a quick polymerization of acrylamide, they must be added in the last minutes. And then the two concentration (30% and 60% denaturing gradient) are introduced into the gel sandwich. Place the comb, and allow the gel to polymerize for about 5 hours at room temperature.

Sample loading and gel running

Put the polymerized gel sandwich inside the electrophoresis tank with the TAE buffer 0.5X preheated to 60^{o}C, and check the upper chamber filled up with TAE buffer.

Prepared the samples with ¼ (V/V) loading buffer. Wash every well with the help of a syringe and load the samples with a micropipette. Run the gel at 200V for about 5 hour.

Gel staining

Submerge the gel in 20 mL of TAE buffer 1X with 0.20 µL Syber Green (SYBR Gold nucleic acid stain) for 30 minutes. DNA bands can be seen on a transiluminator providing UV light and images can be digitalized with a PC images capturing system by Biovision CN 1000/26M (Vilber Lourmat, France).

3.8.7 Analysis of DNA sequences

3.8.7.1 Comparison of sequences against the databases

To identify DGGE bands, individual DGGE bands were cut from gels using a sterile scalpel in new eppendorf and dissolved in 20 µL of DNase/RNase free distilled water (Invitrogen, U.S.A.). The eluted DNA was used as DNA template following PCR amplification with primers 338F (devoid of a GC clamp) and P518R under the same PCR condition. PCR products were purified using Gel/PCR DNA fragments extraction kits (Geneaid, Taiwan) before sequencing by 1st BASE (Malaysia).

After that, the sequences and chromatograms were initially checked with ChromasPro program, then sequences were compared to the available databases of the NCBI (National Center for Biotechnology Information) to determine their approximate phylogenetic affiliations. Sequence similarities were checked using the BLAST (Basic Local Aliment Search Tool) software, which is available at the web page: http:// us.expasy.org/tools/blast/and http://www.ncbi.nlm.nih.gov/BLAST/.

3.8.7.2 phylogenetic analyses

The nucleotide and deduced amino acid sequences of samples were compared with entries in the GenBank database. To determine the phylogenetic affiliation, a similarity search was performed using the Megablast program (Geneious 6.1.2). The nucleotide sequences were aligned with the ClustalW (Thompson, Gibson et al. 1997) and the phylogenetic trees were constructed by the neighbor-joining method with outgroup with

Archaeal bacteria (*Methanocaldococcus jannadchii*). The Tamura-Nei genetic distance model was selected (Tamura and Nei, 1993) and all trees were resampled using the bootstrap method and 1000 replicates (Felsenstein, 1985).

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Screening of plants

Phytoremediation is a convention method that has efficiency for removal of organic carbon and nitrogen compounds. It has evolved to become a potential technology for cleanup of contaminated site (Macek et al., 2000). However, the successful phytoremediation of removal of contaminants requires biological availability for absorption or uptake and metabolism by plants or associated microbial systems (Cunnungham et al., 1995). Uptake also depends on the plant species and on the physicochemical properties of the chemical (Bokern and Harms, 1993; Harms, 1996). Therefore, in order to use this environmental remediation technique most efficiently, knowledge of the metabolic potential of suitable plant species for transforming and degrading chemicals is essential. Therefore, screening and selection of plants for an optimal capability of remediation of this wastewater is also important.

This experiment was screen plants that could be adapted to living on aquatic environments because of living on wastewater require numerous special adaptations and could grow in permanently saturated soil. Moreover, these plants were inexpensive and general distribution. The purpose of this experiment is to compare the effectively of 7 plants such as *Echinodorus cordifolius* (Creeping Burrhead), *Cyperus alternifolius* (Umbrella papyrus), *Thalia geniculata* (Alligator Flag), *Acorus calamus* (Sweet Flag), *Dracaena sanderiana* (Lucky Bamboo); (silver), *Dracaena sanderiana* (gold), and *Dracaena sanderiana* (greenz) to reduce COD value of ink factory wastewater (Fig.4.1).

Echinodorus cordifolius

Cyperus alternifolius

Thalia geniculata

Acorus calamus

Dracaena sanderiana (Silver)

Dracaena sanderiana (Gold)

Dracaena sanderiana (Green)

Figure 4.1 Screening of various plants for treatment of ink factory wastewater.

In this experiment, plants were grown under soilless condition for 7 days. The weight of plants was 35−40 g and a volume 500 mL of wastewater. Plants were grown in wastewater at an initial COD concentration about 1114 mg/L. At day 7, the results showed that remaining COD concentraation of original wastewater condition (control) was decreased to 855 mg/l while treatment of wastewater with *E. cordifolius*, *C. alternifolius, A. calamus*, *T. geniculata, D. sanderiana* (Gold), *sanderiana* (Silver), and *D. sanderiana* (green), remaining COD were 388 mg/L, 380 mg/L, 481 mg/L, 495 mg/L, 785 mg/L, 811 mg/L, and 855 mg/L, respectively (Fig. 4.2). The results showed that the highest efficiency of COD reduction in wastewater was *E. cordifolius*, followed by *C. alternifolius*, *A. calamus*, *T. geniculata*, *D. sanderiana* (Gold), *D. sanderiana* (Silver), and *D. sanderiana* (green), respectively. However, the toxicity of wastewater to *E. cordifolius* showed that it could be not survived as same as *A. calamus* and *T. geniculata* although they could decrease COD in the wastewater (Table 4.1). Meanwhile, *D. sanderiana* (Gold), *D. sanderiana* (Silver), and *D. sanderiana* (green) still survived but they cannot reduce COD of the wastewater. However, *C. alternifolius* could reduce higher COD concentration. Therefore, *C. alternifolius* was selected to study in this research.

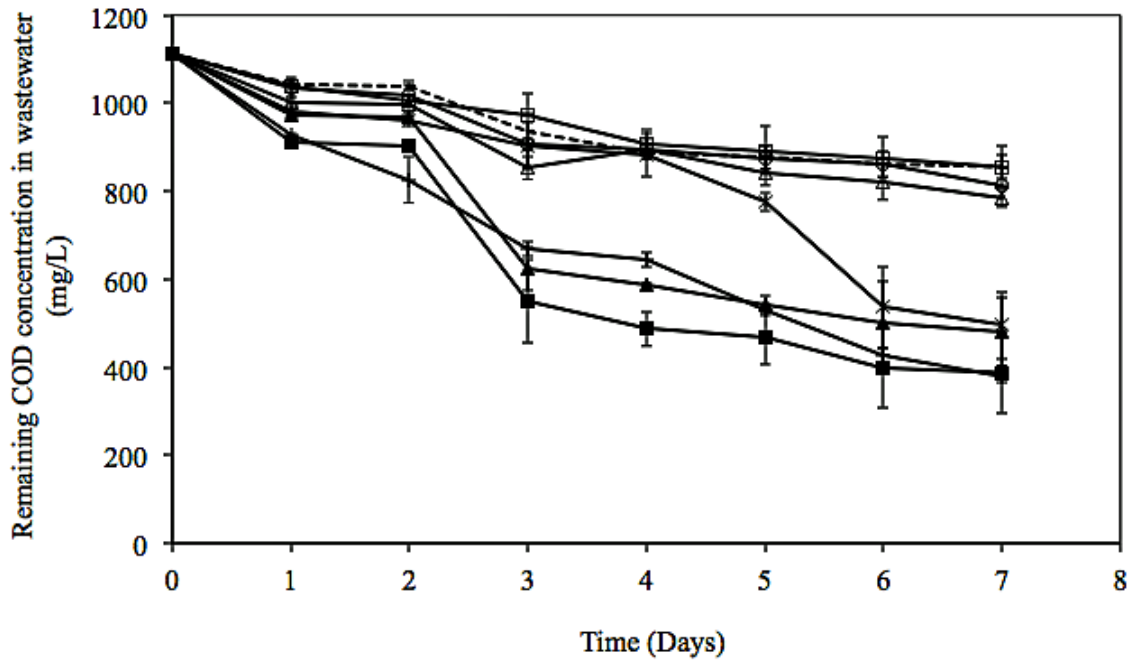


Figure 4.2 Remaining COD concentration after treatment of pretreated ink factory wastewater by various plants for 7 days (- - - wastewater, —△— *D. sanderiana* (Gold), —○— *D. sanderiana* (Silver), —□— *D. sanderiana* (Green), —✳— *T. geniculata*, —▲— *A. calamus*, —■— *E. cordifolius*, —+— *C. alternifolius*).

Table 4.1 Screening of various plants and toxicity after treatment of ink factory wastewater for 7 days.

Plants	Time (Days)			
	0	2	4	7
E. cordifolius				
C. alternifolius				
A. calamus				
T. geniculata				

Table 4.1 Screening of various plants and toxicity after treatment of ink factory wastewater for 7 days (cont.).

Plants	Time (Days)			
	0	2	4	7
D. sanderiana (Gold)				
D. sanderiana (Silver)				
D. sanderiana (green)				

4.2 Chateractics of *Cyperus alternifolius*

4.2.1 The leaf

Transverse section (TS) of leaf of *C. alterniforius*, it shows the size of epidermal cells was different on both adaxial and abaxial surfaces, and those on adaxial surface were much larger than the abaxial cells. A large vein from the leaf, vascular cambium is lacking between the adaxially situated xylem (b) and abaxial phloem (a). The vein is partially separated from the mesophyll (d) by a sheath of fiber (e).

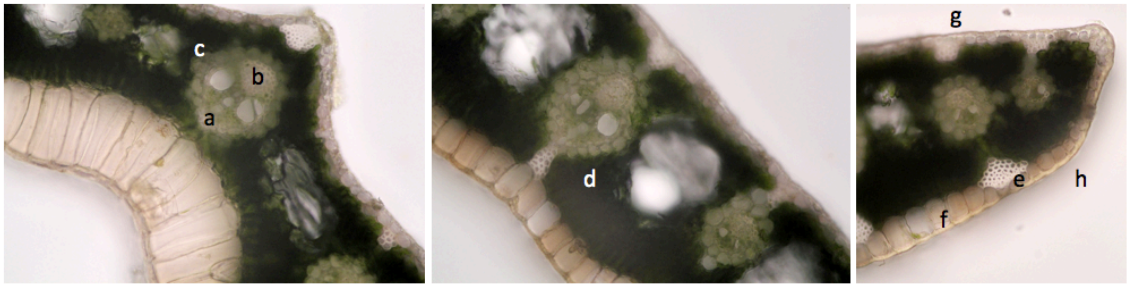


Figure 4.3 Microscopy of transverse section of *C. alternifolius* leaf (x100). a = adaxial xylem, b = abaxial phloem, c= bundle sheath, d = mesophyll cells, e = fibers, f = upper epidermis, g = lower epidermis, h = cuticle.

Looking through a microscope at the leaf of *C. alternifolius*, it shows stomata lie in neatly parallel rows and it has parallel veins running its length. The maximum stomatal area of the adaxial surface (Fig. 4.4a) was lower than abaxial surface (Fig. 4.4b).

(a) (b)

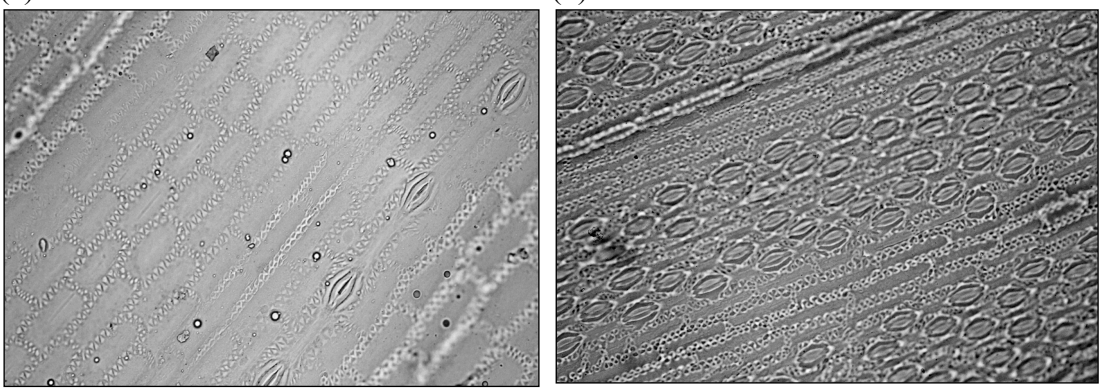


Figure 4.4 The image under microscope of stoma of (a) the upper leaf (adaxial) and (b) lower leaf (abaxial) tissues of *C. alternifolius* (x400).

4.2.2 The stem

Transverse section of the stem of the monocotyleden *C. alternifolius*, it has a smooth waxy cuticle that protects it and gives it a smooth feeling. The epidermal cells are lignified which gives them strength and resistance to decay. Bundles of sclerenchyma fibers are located at interval just beneath the epidermis. These are lignified and provide a lot of strength to the stem. Its vascular bundle is distributed throughout its cross-sectional area. This shows that there are two kinds of vascular bundles. Small circular bundles are located near the outside of the stem while large bundles occur towards the interior. They contain well-defined areas of phloem and xylem and are involved in long-distance transport. Small vascular bundles with associated photosynthetic tissue are

located just beneath the fibers (h). This is where photosynthesis occurs in *C. alterniforius*. Most of the cells in these vascular bundles are lignified and thus contribute to the support of the stem. The internal ground tissue is unlignified. It also has prominent air cavities. These probably aid in gas diffusion and they are commonly found in semi aquatic plants. They are also distributed throughout the stem. These give the stems a spongy quality, which may have provided some level of extra comfort in mats made from them.

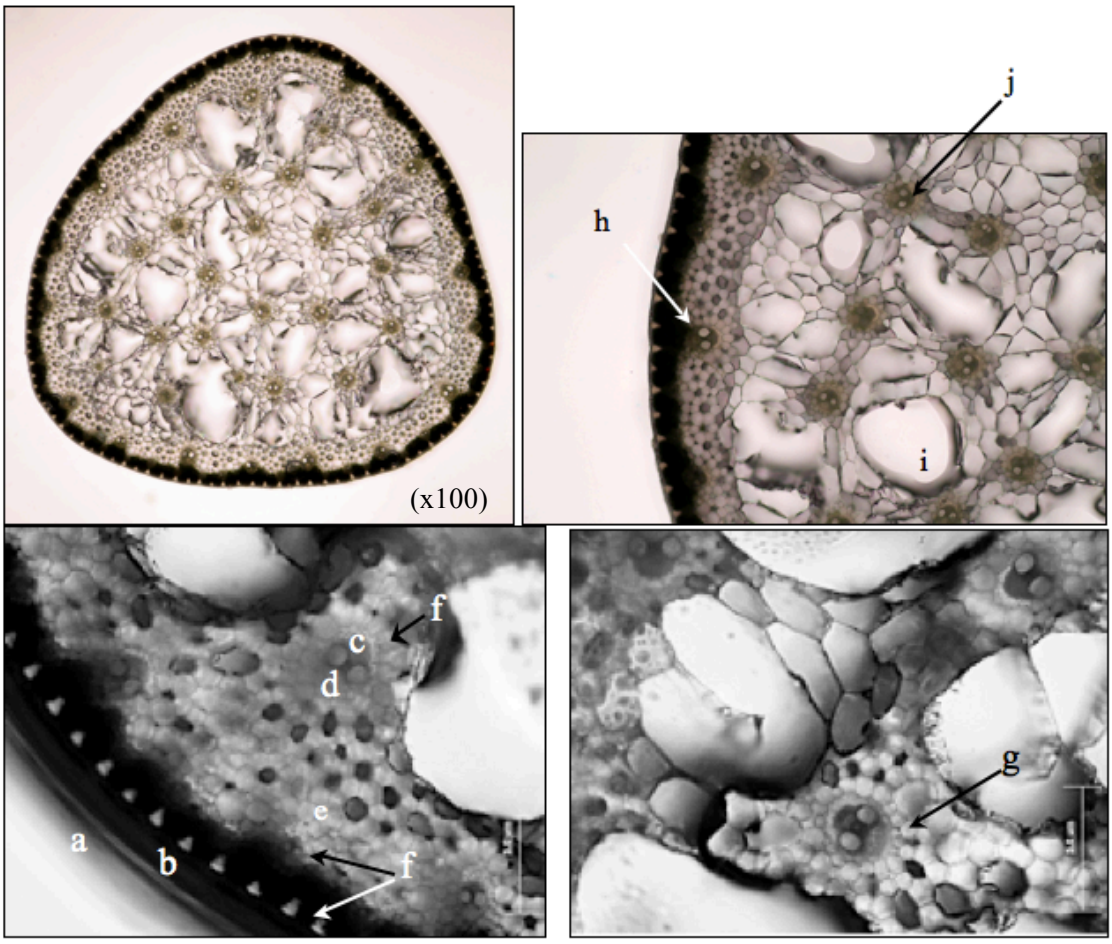


Figure 4.5 Microscopy of transverse section of *C. alternifolius* stem (x400); a = cuticle, b = epidermis, c = xylem, d = Phloem, e = pith, f = fibers, g = photosynthetic tissue, h = small vascular bundle, i = air cavity, j = large vascular bundle.

4.2.3 The root

Microscope of the root tip of *C. alternifolius* shows root cap (Fig. 4.6 a) and root hair (Fig. 4.6 b). Transverse section of root shows several-layered, exodermis (a), enclosed the wide parenchymatous cortex (b). The layered endodermis (c) is also present and the vascular tissue enclosed within it shows a polyarch arrangement (metaxylem, e). Xylem (f) is surrounded by radially aligned of metaxylem vessel, while phloem stands (d) occur between metaxylem (Fig. 4.6 c and Fig. 4.6 d).

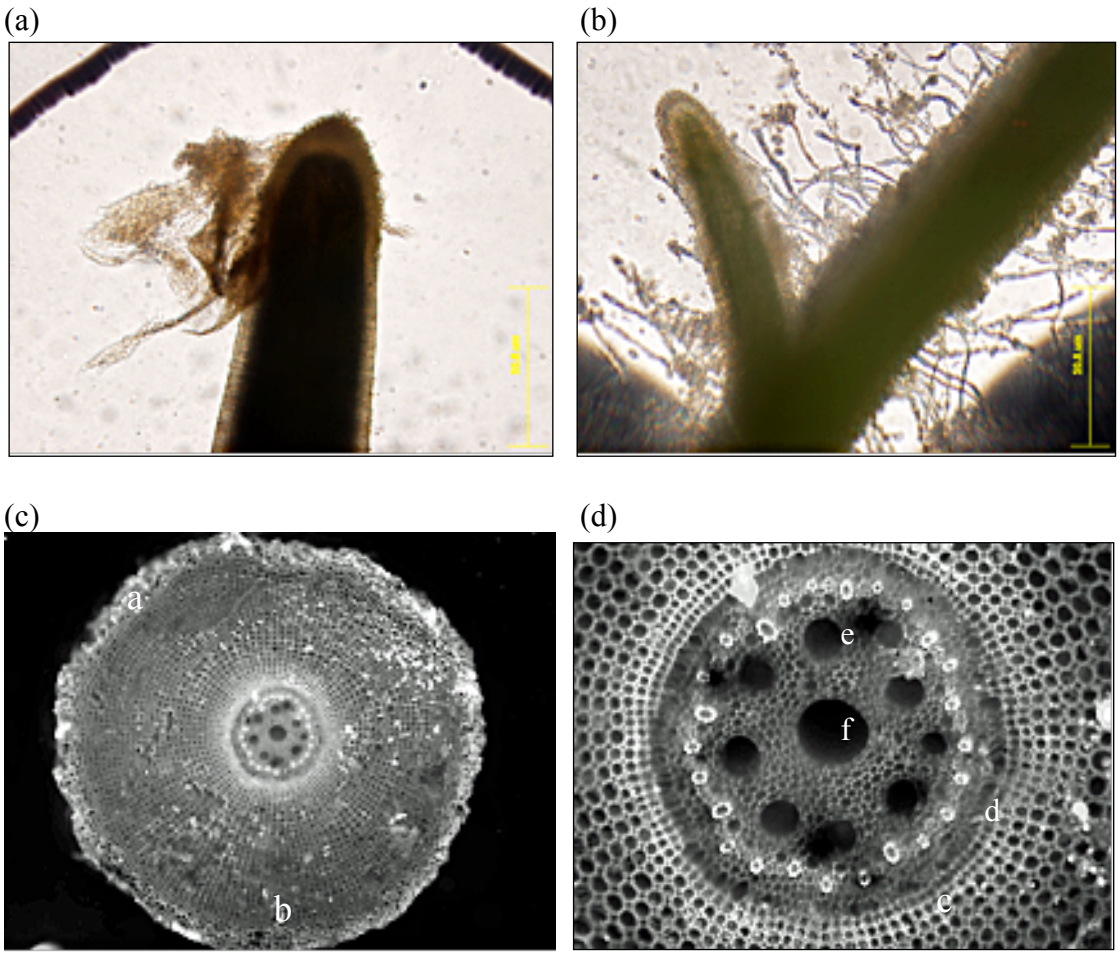


Figure 4.6 Micrograph (a and b, x100) and transverse section (c: x100 and d: x400) of root tissue of *C. alternifolius*; a = exodermal cells, b = parenchymatous cortex, c = endodermal cells, d = phloem, e = metaxylem vessels, f = xylem.

4.3 Treatment of original wastewater by phytoremediation

4.3.1 Study of COD removal

Phytoremediation of original wastewater with *C. alternifolius* was investigated. The experiment used the well growth plants and soil were 300 g of weights and 2 L of wastewater. The ink factory wastewater appeared dark colored and had an initial COD concentration of 32336 mg/L. After treatment for 45 days, the results showed the remaining COD concentration under wastewater conditions was decreased approximately 15419 mg/L that reach to 52% of COD removal. It could be possible due to microorganisms that affected to reduce organic matters. Under wastewater+plant and wastewater+soil conditions, the remaining COD was decreased to 12950 mg/L and 885 mg/L that equal to 60% and 97%, respectively. While wastewater+soil+plant conditions rapidly reduced COD to 237 mg/L, which receive to 99% of COD removal (Fig. 4.7). Plants were still healthy and new plants were adapted for survival, though a small amount of wilted leaves did appeared (Fig 4.8).

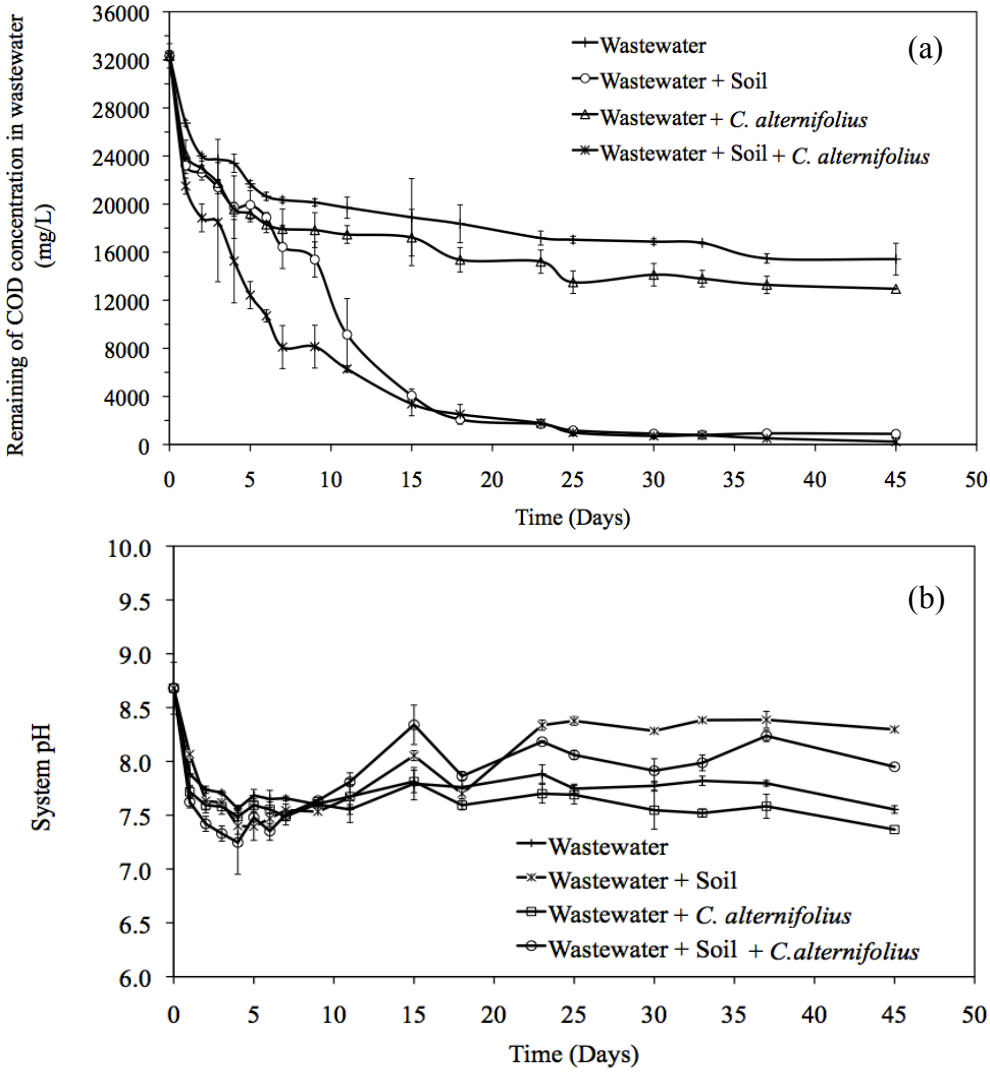


Figure 4.7 Remaining COD concentration (a) and system pH (b) of original wastewater after treatment for 45 days various conditions.

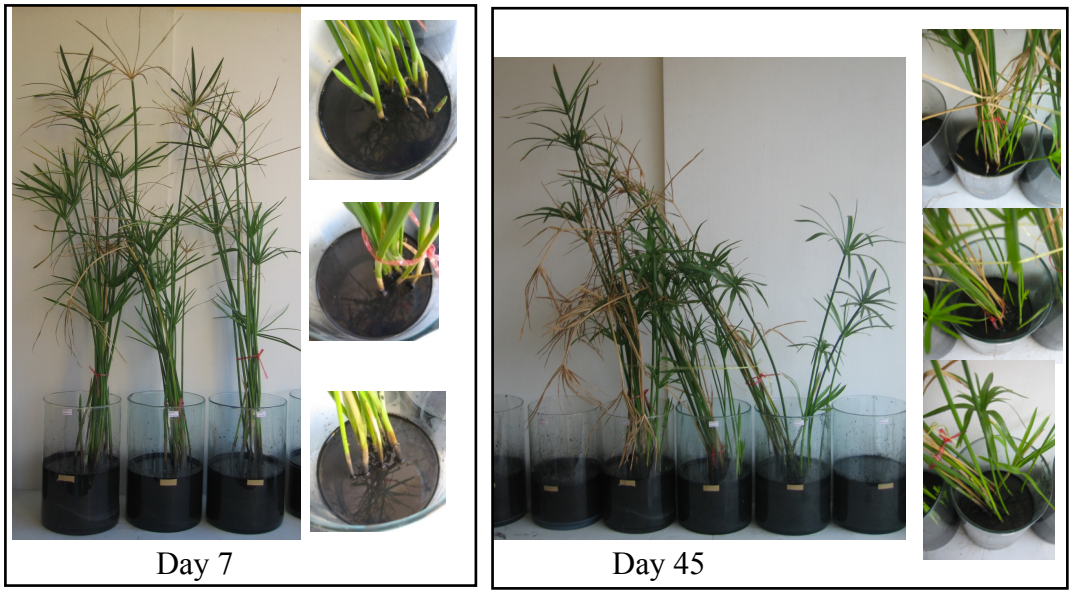


Figure 4.8 Appearance of plant health after grown in wastewater+soil+plant conditions at day 7 and day 45.

4.3.2 Monoethanolamine removal from original wastewater

Monoethanolamine (MEA) is one composition in the ink production, a solvent for dissolved acrylic resin was also affected to increase COD concentration. After treating original wastewater for 14 day, the results indicated that MEA could be degraded from microorganism that it was rapidly reduced from 540 mg/L to 0 mg/L. While wastewater+ soil conditions could be completely degraded on day 12 that it was possible due to adsorption of soil and microbial activity. Moreover, wastewater+plant conditions and wastewater+soil+plant conditions could be completely degraded on day 7 and day 5, respectively (Fig. 4.9). They were possible due to absorption of plant, adsorption of soil and microbial activity from soil, plants, and wastewater.

Moreover, the result from GC analysis was appeared peaks of ethanol and acetic acid (Fig. 4.9). Initial ethanol content of wastewater condition was 85 mg/L that it was possible due to microbial activity in wastewater. While wastewater+soil conditions, microorganism from wastewater and soil could be degraded MEA to ethanol but it could be adsorbed with soil and readily consumed by bacteria in aerobic condition. Plant grown in wastewater conditions was the highest ethanol but it rapidly decreased to 0 mg/L on day 5. Meanwhile, the initial acetic acid content of wastewater condition was 723 mg/L and it was increased about 1459 mg/L on day 2 and then rapidly decreased to 0 mg/L on day 5. While wastewater+soil conditions was occurred acetic acid on day 1 about 283 mg/L and it was the highest value about 1562 mg/L on day 2 and then it was completely consumed by microbial activity on day 3. Moreover, wastewater+plant conditions and wastewater+soil+plant conditions were rapidly increased about 3620 mg/L and 3507 mg/L, respectively on day 3 and then disappeared on day 8.

These results shows acetic acid is the major end product in most of metabolic pathways of MEA aerobic degradation. MEA has two functional groups, an alcoholic group and an amino group and it easily oxidized with acid and biodegradation (Mrklas et al., 2004) (Ndegwa et al., 2004) by a process of hydrolysis to ammonium and acetataldehyde (Eq.

4.1) and then acetataldehyde was hydrolysed to ethanol and acetic acid (Eq. 4.2) while ammonium can be oxidized to nitrite and then nitrate (Eq. 4.3).

$$\underset{\text{(MEA)}}{2C_2H_7NO} + 2H_2O \longrightarrow \underset{\text{(acetaldehyde)}}{2C_2H_4O} + \underset{\text{(ammonium)}}{2NH_4^+} + 2OH^- \quad (4.1)$$

$$2C_2H_4O + H_2O \xrightarrow{O_2} C_2H_6O + CH_3COOH \quad (4.2)$$
(acetaldehyde) (ethanol) (acetic acid)

$$2NH_4^+ + 2OH^- + 3O_2 \xrightarrow{3O_2} 2NO_2^- + 4H_2O + 2H^+ \xrightarrow{O_2} 4H_2O + 2NO_3^- + 2H^+ \quad (4.3)$$

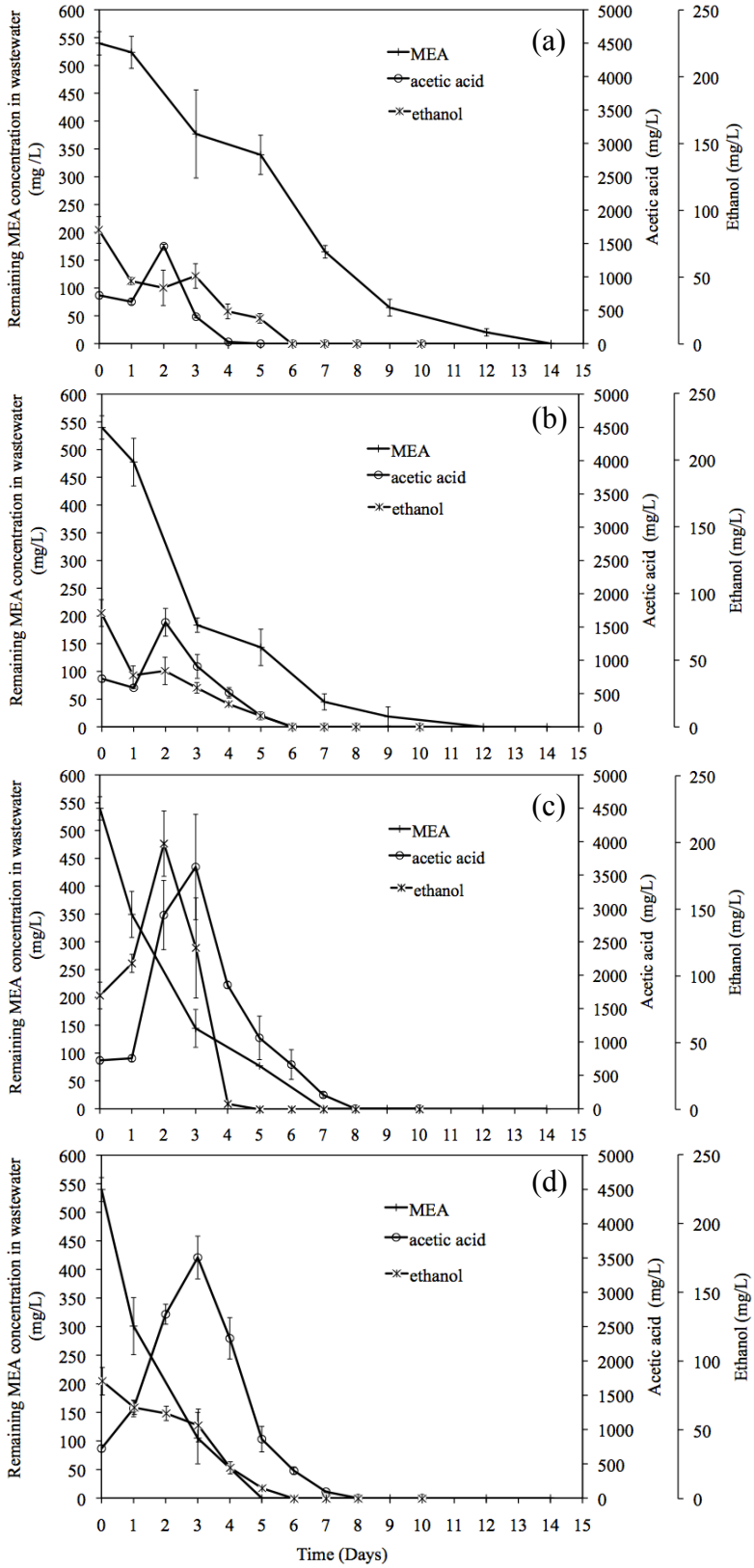


Figure 4.9 Treatment of monoethanolamine (MEA), acetic acid, and ethanol under various conditions; wastewater conditions (a), wastewater+soil conditions (b), wastewater+plant conditions (c), and wastewater+soil+plant conditions (d) after treatment for 14 days.

4.3.3 Color and total dissolved solids (TDS) removal from original wastewater

Total dissolved solids is an important problem for treatment process of wastewater. This wastewater is highly color by the pigments and acrylic resin that impact to an increasing of TDS content. An initial TDS concentration was 12581 mg/L, after treatment under various conditions, the appearance showed in Table 4.2. The results indicated that wastewater+soil+plant conditions could decrease TDS to 3175 mg/L at day 7 that less than the effluent standard of Thailand (less than 5000 mg/L) and almost clear color at day 45. However, under wastewater+soil conditions showed the high efficiency of TDS removal. Soil could adsorb pigments and other pollutants; it could reduce color of wastewater that it was appeared pink color at day 45 (Table 4.2). From chemical properties of soil showed it was an acid soil (pH 4.3) and a highly clay fraction (Table 3.1). Moreover, CEC values (30.20 cmol/kg) showed strong adsorption. Therefore, it was possible that soil had a great capacity to attract and hold cation because of its chemical structure. At day 15, soil could decrease TDS value less than the effluent standard that approximately 1874 mg/L (Fig. 4.10). Meanwhile, under wastewater and wastewater+plant conditions were still appeared black dark color. Plants could not decrease TDS lower than industrial effluent standard because a limit of high concentration of TDS and amount of plant. The efficiency of wastewater removal and the effect to plants were continuously investigated for a longer time.

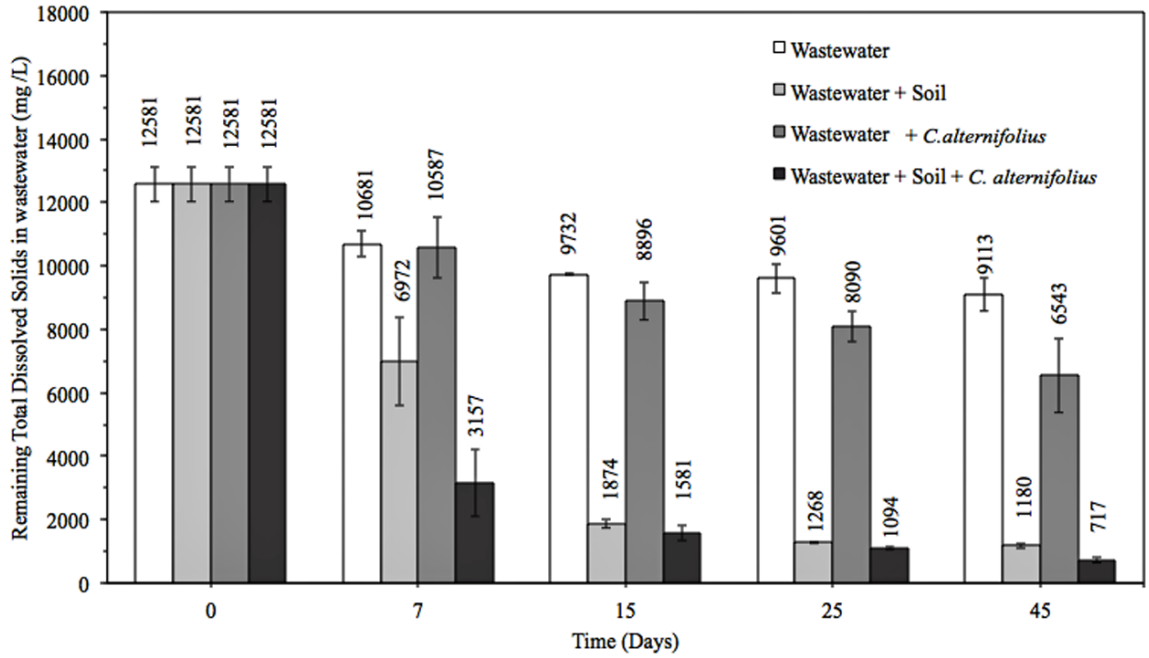


Figure 4.10 Treatment of total dissolved solids (TDS) under various conditions; wastewater conditions (a), wastewater+soil conditions (b), wastewater+plant conditions (c), and wastewater+soil+plant conditions (d) after treatment for 45 days.

Table 4.2 Appearance of color of ink factory wastewater after treatment under various conditions for 45 days.

Time (Days)	Treatment conditions			
	Wastewater	Wastewater + plant	Wastewater + soil	Wastewater + soil + plant
7				
15				
33				
45				

The results suggested that a problem of treatment is the amount of time it took (45 days) and plants might not be tolerant to the toxicity from wastewater in the next cycle. Although, soil conditions had efficiency for wastewater removal but soil has a limit in adsorption of wastewater. Therefore, pretreatment original wastewater with chemical precipitation was an alternative option for reducing high color, COD, SS, TDS, and reduce toxicity before using the plant.

4.4 Pretreatment of ink factory wastewater by chemical substances

Original wastewater contained high COD, SS, TDS and NH_4^+–N concentrations. In addition, it had a dark color from the pigments that was a major problem of wastewater. Although there are many methods for the removal of COD and NH_4^+–N in a short time, it is difficult to treat pigments and acrylic resin from ink production wastewater by phytoremediation. Therefore, the treatment of wastewater for decreasing pigments and acrylic resin before using other methods was investigated.

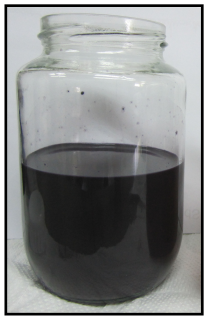


Figure 4.11 Appearance of ink factory wastewater.

For the first step, treatment of original wastewater by sulfuric acid and ferric sulfate as precipitants for pigments removal was compared. The result revealed that the supernatant of both precipitants after precipitation was very clear which indicates that these chemicals could precipitate pigments and other suspended solids (Fig. 4.12). The characteristics of wastewater before and after treatment, including adjusted pH were summarized in Table 4.4. A comparison of the quality of treated wastewater indicated that there was no significant difference between these two agents. However, using $Fe_2(SO_4)_3$ appeared as an iron color in treated effluent. For these results, H_2SO_4 was chosen for the treatment of wastewater.

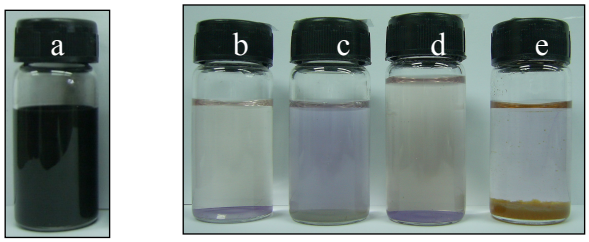


Figure 4.12 Color of original wastewater (a), pretreatment of wastewater by H_2SO_4 (b), pretreatment of wastewater by $Fe_2(SO_4)_3$ (c), pretreatment of wastewater by H_2SO_4 and adjusted pH with $Ca(OH)_2$ (d), pretreatment of wastewater by $Fe_2(SO_4)_3$ and adjusted pH with $Ca(OH)_2$ (e).

Table 4.3 Characteristics of wastewater before and after pretreatment by sulfuric acid and ferric sulfate and then adjusting pH with $Ca(OH)_2$.

Sample	Remaining COD concentration (mg/L)	%COD removal	System pH
Wastewater	32336	-	8.56
Wastewater+ H_2SO_4+ $Ca(OH)_2$	1427	96	7.12
Wastewater+$Fe_2(SO_4)_3$+ $Ca(OH)_2$	1223	96	7.10

4.5 Treatment of wastewater by chemical precipitation (sulfuric acid) coupled with *C. alternifolius*

4.5.1 Treatment of COD, total nitrogen (TN), and total kjedalh nitrogen (TKN) from wastewater

Before treatment of wastewater with *Cyperus alternifolius*, the pretreatment with chemical precipitation (sulfuric acid) was necessary because of the large amount of pigments and COD was affected to plant survival. However, after precipitation by sulfuric acid, $Ca(OH)_2$ was used for adjusting system pH for suitable growth of plants.

Table 4.4 characteristics of original wastewater and after precipitation with sulfuric acid and adjusting pH with $Ca(OH)_2$.

Parameters	Value		Industrial Effluent Standard values[a]
	Original wastewater	Precipitation by H_2SO_4 and adjusting pH with $Ca(OH)_2$	
Color and Odor	Dark color and strong odor	Transparent oranged red	Not objectionable
COD (mg/L)	12601 ± 151	987 ± 86	120-400
pH	8.45 ± 0.01	7.05 ± 0.01	5.5-9.0
SS (mg/L)	1176 ± 13	30 ± 6	50-150
TDS (mg/L)	6781 ± 34	6926 ± 45	3000-5000
TKN (mg-N/L)	165 ± 26	156 ± 4	100-200
BOD (mg/L)	-	258±11	20-60
Ammonium (mg-N/L)	-	146 ± 2	-
Organic nitrogen (mg-N/L)	-	10 ± 2	-
Nitrite (mg-N/L)	-	0.014 ± 0	-
Nitrate (mg-N/L)	-	28 ± 0.07	-
TN (mg-N/L)	-	184 ± 1	-
MEA (mg/L)	173 ± 21	39 ± 0.39	-

[a]Industrial Effluent Standard from Notification the Ministry the Ministry of Science, Technology and Environment, 1996,Thailand

After that, phytoremediation of wastewater under various conditions were investigated for 100 days. The results of COD concentrations were rapidly decreased at day 13. At day 13, remaining COD was about 217, 171, 162 and 117 mg/L from initial COD of 987 mg/L under wastewater, wastewater+soil, wastewater+plant, and wastewater+soil+plant conditions, respectively (Fig. 4.13). COD removals under various conditions were slowly reduced after 13 days. At day 100, remaining COD was 89, 86, 74 and 67 mg/L under wastewater, wastewater+soil, wastewater+plant, and wastewater+soil+plant conditions, respectively. While at day 7, TN was reduced to 96, 74, 90 and 52 mg/L from initial TN of 184 mg/L under wastewater, wastewater+soil, wastewater+plant, and wastewater+soil+plant conditions, respectively. Moreover, remaining TKN concentrations of various conditions showed lower than the effluent

standard that were 21 mg/L, 21 mg/L, 38 mg/L, and 19 mg/L under wastewater, wastewater+soil, wastewater+plant, and wastewater+soil+plant conditions, respectively. The result confirmed that under wastewater+soil+plant conditions was the highest efficiency and good performance in organic carbon and nitrogen compound removal.

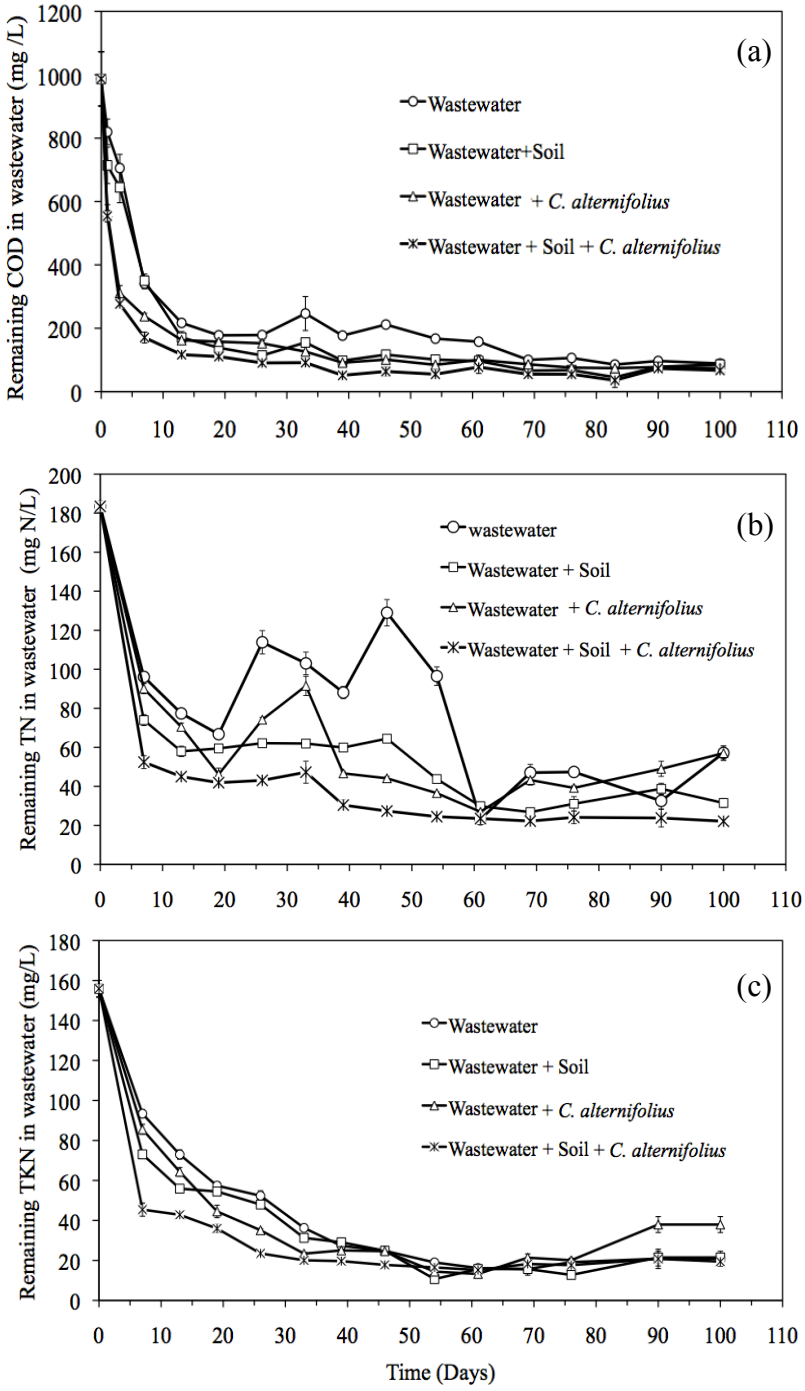


Figure 4.13 Remaining COD (a), TN (b), and TKN (c) in wastewater under various conditions for 100 days.

4.5.2 Treatment of BOD from wastewater

BOD of wastewater after pretreatment with chemical substances was 258 mg/L. After 7 days, the remaining BOD under wastewater and wastewater+plant conditions were 93 mg/L and 7 mg/L equal to 64% and 97% of BOD removal, respectively. The results confirmed that microorganisms, soil and plant involved in reducing BOD, especially plants had higher efficiency than microorganisms (Fig. 4.14).

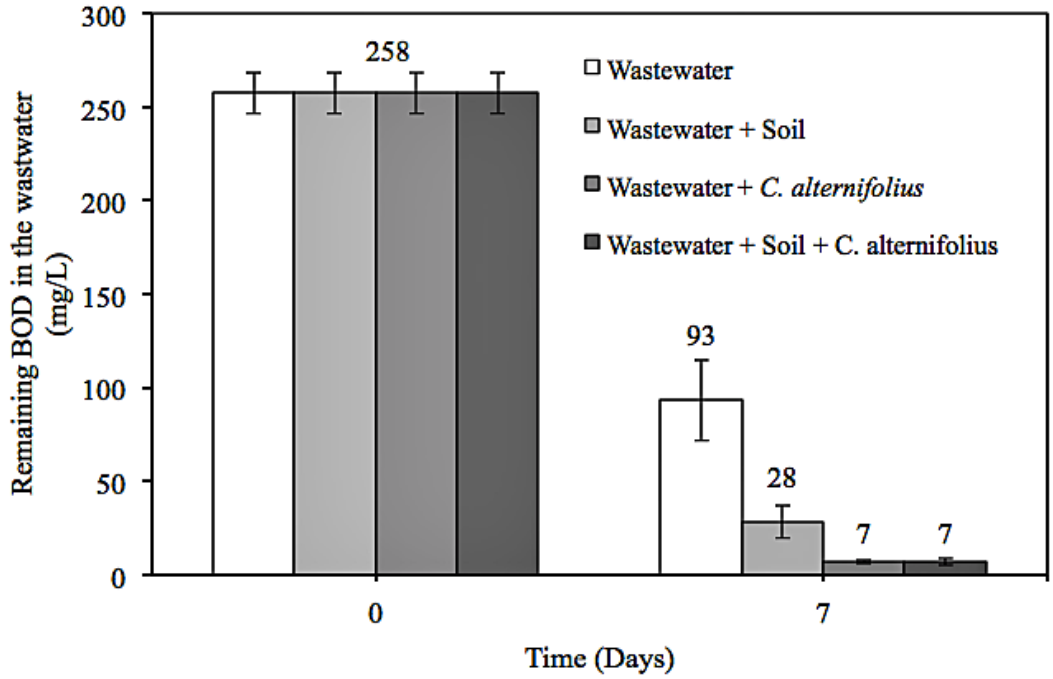


Figure 4.14 Remaining BOD from wastewater under various conditions after treatment for 7 days.

4.5.3 Treatment of inorganic nitrogen compounds and the relationship of DO, pH and ORP

The presence of inorganic nitrogen compounds is one of the major problems associated with wastewater treatment. The concentrations of NH_4^+–N and NO_3^-–N in the sample wastewater after pretreatment were approximately 146 mg/L and 28 mg/L, respectively. Therefore, NH_4^+–N, NO_2^-–N, and NO_3^-–N were analyzed to observe efficiency of removal. In this study, pH, DO, and ORP were investigated to determine the biological nutrient removal of denitrification-nitrification processes. The ORP profile is very effective for anoxic phase control and it also reflects the concentration of DO (Kishida et al., 2003). The pH demonstrates the characteristics of microbial reactions, increasing pH indicates ammonification and denitrification, while decreasing pH indicates nitrification (Chang and Hao, 1996; Kishida et al., 2003). The results can be explained in three stages as follows (Fig. 4.15).

At stage I, as substrate was consumed, reduction reaction took place for both aerobic and anoxic conditions. NH_4^+–N and NO_3^-–N were rapidly reduced while the ORP and DO curves dropped down sharply at the beginning. The ORP values rapidly declined, lower than –50 mV, followed by a sudden increase. A large amount of DO was demanded for microbial activity, which uses organic matters for building cell tissue, then the anoxic phase occurred. After, DO values maintained lower values, less than 1 mg/L, then NO_3^-–N was reduced to a lower value. Nitrate is the most critical form of

nitrogen and it has an active role in the eutrophication process and methaemoglobinaemia. However, the NO_3^-–N under various conditions was completed and reduced to the lowest value under wastewater conditions approximately 2.69 mg/L of NO_3^-–N at day 7 and under plant condition was 0.08 mg/L of NO_3^-–N at day 13. The hydrolysis of organic matters generated alkalinity, causing the raise of pH. At this stage, it was possible that anoxic condition was due to the biological characteristics of facultative heterotrophic bacteria. The range of time at this stage was about 7 days under wastewater conditions and it took about 13 days under wastewater+plant conditions.

At the stage II, The changes on the slope of ORP curves increased continuously. While DO values were higher than 1 mg/L, it was confirmed that the system under various conditions was an aerobic process. Meanwhile, NO_2^-–N and NO_3^-–N were detected at a higher value while NH_4^+–N decreased continuously. The depletion of substrate decreased bacterial respiration activity and resulted in increasing DO, and could be directly related to COD and NH_4^+–N concentrations. This stage showed the break point of ammonium that ammonium was decreased to the lowest value. The oxidation of NH_4^+–N to nitrite produces acidity or consumes alkalinity during the nitrification that affect to pH declined to a lower level. At this stage, the end of the process under wastewater conditions was at day 46, while it took 26 days under wastewater+plant conditions.

At stage III, the end of the nitrification process showed nitrifying bacteria completely oxidized nitrite under various treatment conditions (Fig 4.16). However, amount of organic matter still occurred in the system, and also NH_4^+–N, therefore the changing of NH_4^+–N to NO_3^-–N was still detected. The pH curves were rapidly increased, primarily due to consumption of acid products during process (Chen et al., 2002).

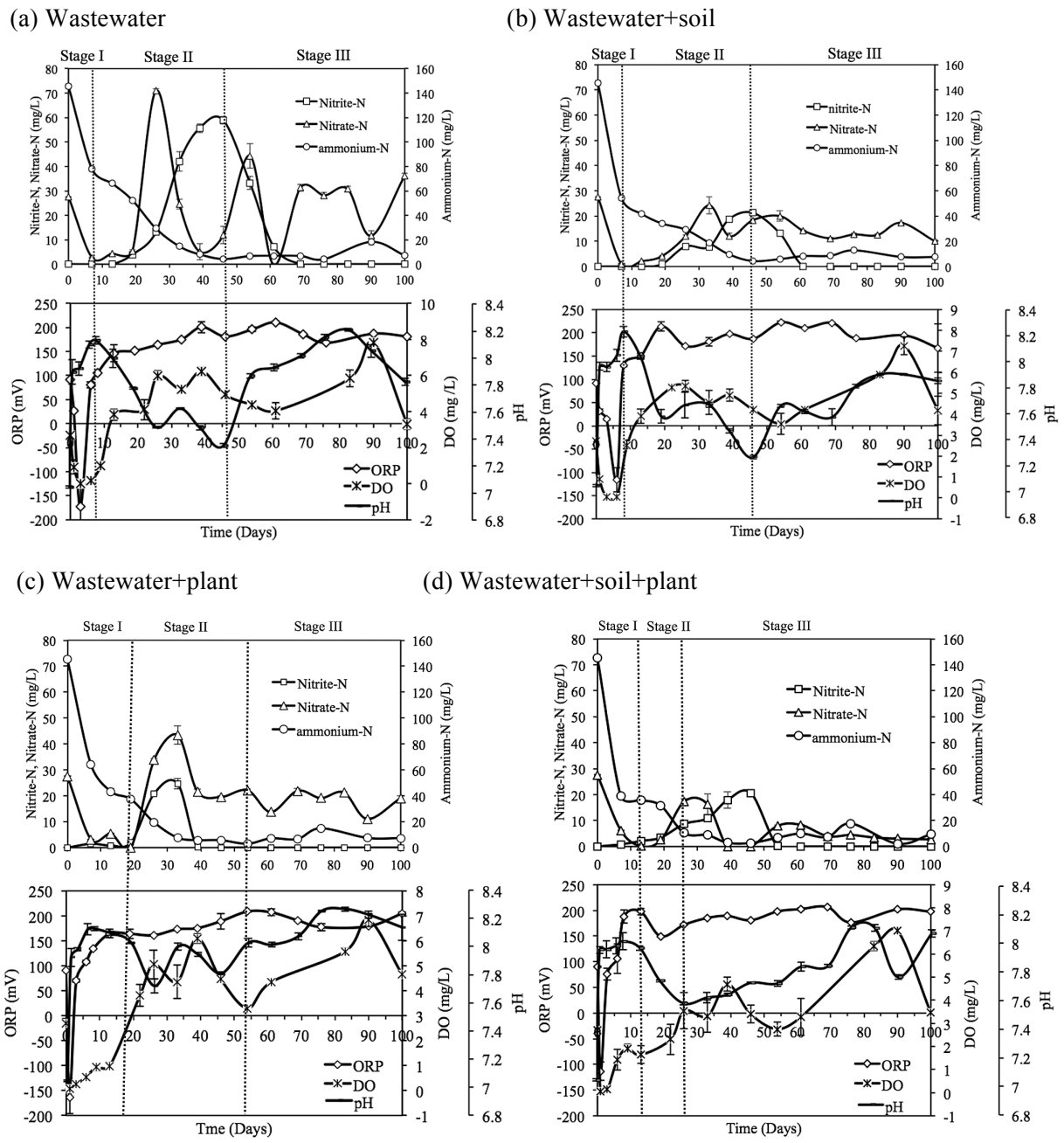


Figure 4.15 Correlation of concentration of ammonium, nitrite, nitrate and the profiles of pH, ORP and DO under wastewater conditions (a), wastewater+soil conditions (b), wastewater+plant conditions (c) and wastewater+soil+plant conditions (d) after treatment for 100 days.

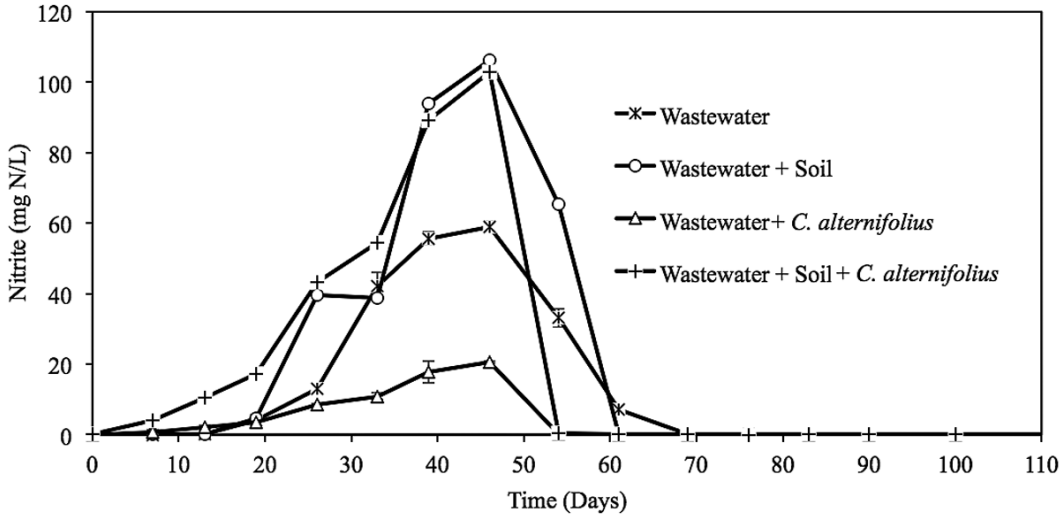


Figure 4.16 Nitrite concentration under various conditions after treatment for 100 days.

4.5.4 Treatment of monoethanolamine (MEA) and acetic acid from pretreated wastewater

MEA was contaminated in original wastewater about 173 mg/L. After pretreatment original wastewater by sulfuric acid, MEA was reduced to about 39 mg/L. This confirmed that MEA could be degraded by acid.

The wastewater after pretreatment still contained MEA. This wastewater was then treated by plant under various conditions (wastewater conditions, wastewater+soil conditions, wastewater+plant conditions, and wastewater+soil+plant conditions). The result found that under wastewater conditions, MEA could be decreased by microbial activity. While under wastewater+soil conditions, MEA can be decreased by both soil adsorption and microbial activities in soil and wastewater (Fig. 4.17). In addition, under both of wastewater+plant conditions and wastewater+soil+plant conditions confirmed that plant uptake MEA in roots. The result revealed that under plants conditions could decrease MEA and acetic acid faster than others conditions.

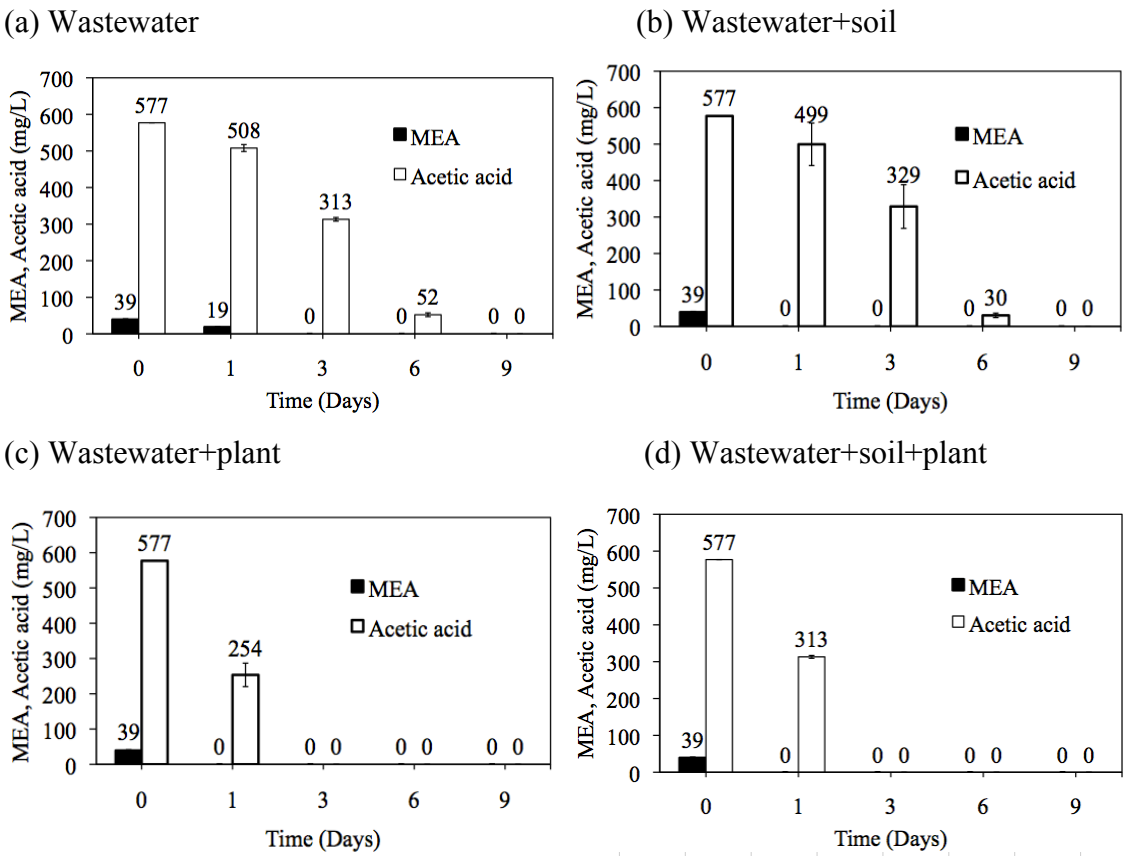


Figure 4.17 Treatment of monoethanolamine and acetic acid under various conditions: wastewater conditions (a), wastewater+soil conditions (b), wastewater+soil conditions (c), and wastewater+soil+plant conditions (d) after treatment for 9 days.

Table 4.5 Summary of characteristics of original wastewater and pretreated wastewater before and after treatment by H_2SO_4 + $Ca(OH)_2$ and coupled with phytoremediation.

Parameter	Original wastewater	Pretreated wastewater by H_2SO_4 and adjusting pH with $Ca(OH)_2$	Pretreated of wastewater by *C. alternifolius* under various conditions (At Day 7)				Industrial effluent standards[a]
			Wastewater	Wastewater + soil	Wastewater + plant	Wastewater + soil + plant	
Color and Odor	Strong color and odor	Transparent orange red color	Transparent orange red color	Clear color	Clear color	Clear color	Not objectionable
COD (mg/L)	12601 ± 151	987 ± 86	510±21	350±21	238±9.00	171±17	≤120
pH	8.45 ± 0.01	7.05 ± 0.01	8.10±0.03	8.12±0.04	8.10± 0.05	7.89±0.06	5.5-9.0
TKN (mg/L)	165 ± 26	156 ± 4	93.40±2 .05	73.07±2.75	85.56±2.52	45.42±3.29	≤100
NH_4^+−N (mg/L)	-	145.57±2.03	77.83±1.9	54.22±2.0	64.01±1.56	38.7±0.91	-
NO_2^-−N (mg/L)	-	0.0135±0.001	0.0137±0.001	0.0073±0.00	1.42±0.204	0.80±0.003	-
NO_3^-−N (mg/L)	-	27.54±0.07	2.69±0.02	0.86±0.01	3.08±0.06	6.13±0.10	-
MEA (mg/L)	173 ± 21	39 ± 0.39	0	0	0	0	-
SS (mg/L)	1176 ± 13	30 ± 6	-	-	-	-	≤50
TDS (mg/L)	6781 ± 34	6426 ± 187	5386±105	3839±68	4725±74	1147±143	≤3000
BOD (mg/L)	-	258 ± 11	93±22	28±8	7±1	7±2	≤20

[a]Industial Effluent Standard from Notification of the Ministry of Science, Technology and Environment, 1996,Thailand.

4.5.5 Scanning Electron Microscope connected with Energy Dispersive X-ray spectroscopy (SEM/EDX) of plant tissues

A Scanning Electron Microscope (SEM) connected to an Energy Dispersive X-ray spectroscopy (EDX) was used to study the images of the plants' surface and the percentages of elements. The SEM images of the leaves and roots of *C. alternifolius* after treatment under wastewater conditions (wastewater+plant conditions) and wastewater+soil+plant conditions for 7 days are shown in Tables 4.6 and 4.7. Under wastewater+soil conditions, SEM images show no toxic symptoms of plant leaves and roots that it possible due to soil could support the root and as a nutrient reservoir and adsorption material. Under wastewater+plant conditions, SEM image showed plant leaves still healthy (Table 4.6) while plant roots were damaged in the cortex tissue (Table 4.7). It possible due to the toxic symptoms of wastewater was exposed in the roots tissue and then leaves tissue. Because of plant grown in wastewater found that a part of plant leaves had green wilting and then yellow.

Although the SEM images show unclear but the percentages of elements in the leaves and roots of plant were analysed by EDX could explain relation between elements and symptom in plant tissues. When the concentration of nutrient or an essential element is low enough to limit yield, the deficiency symptoms are visible. Extreme deficiencies can result in plant death. With moderate or slight deficiencies, symptoms may not be visible, but yields will still be reduced. The percentages of elements in the leaves and roots of plant were analysed by EDX as listed in Table 4.8 and 4.9.

Table 4.6 Scanning electron micrograph (x200) and EDX spectra of the leaves of *C. alternifolius* after cultivation in tap water conditions (Control-Plant), wastewater+plant conditions and wastewater+soil+plant conditions for 7 days.

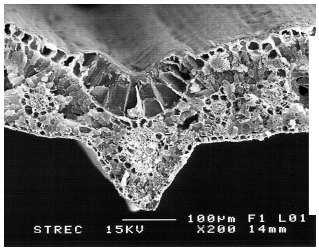
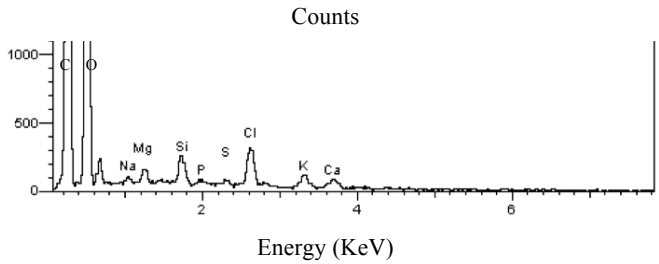
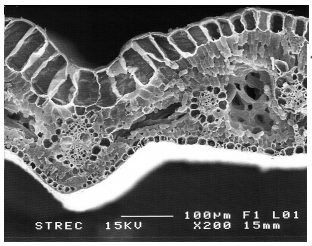
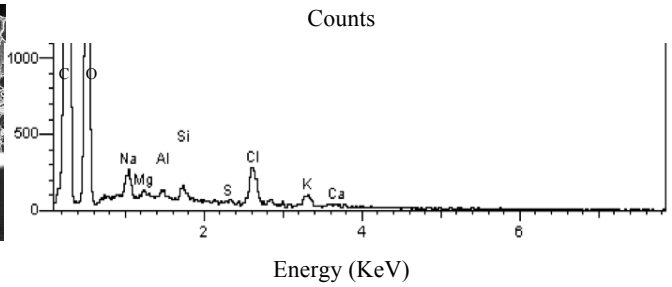
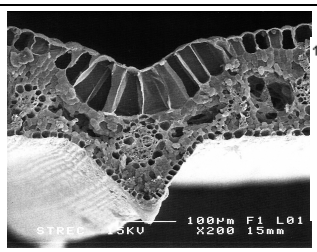
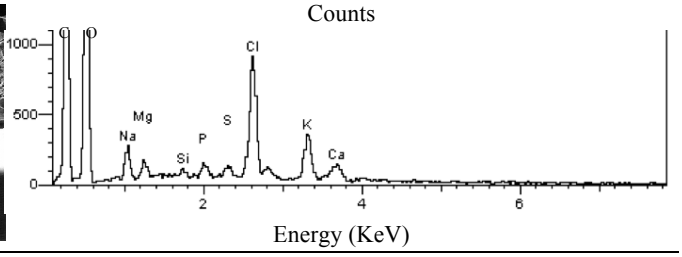
Conditions	SEM micrograph	EDX spectra
Control-Plant		
Wastewater +Plant		
Wastewater +Soil+Plant		

Table 4.7 Scanning electron micrograph (x200) and EDX spectra of the roots of *C. alternifolius* after cultivation in tap water conditions (Control-Plant), wastewater+plant conditions, and wastewater+soil+plant conditions for 7 days.

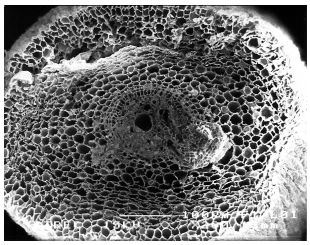
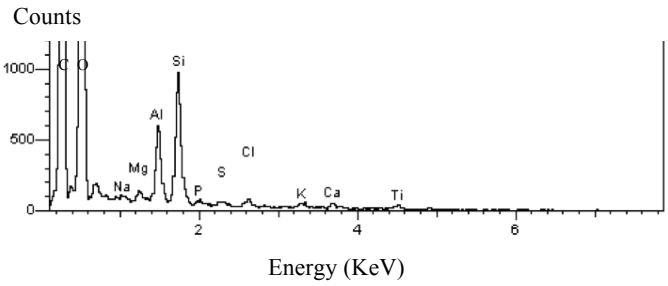
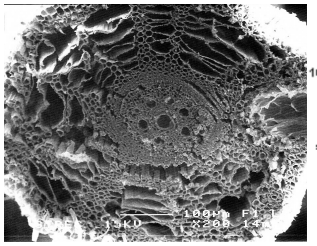
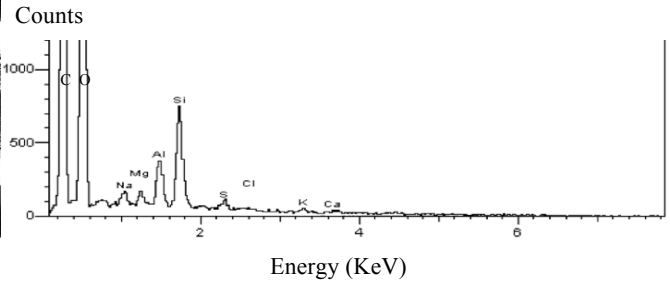
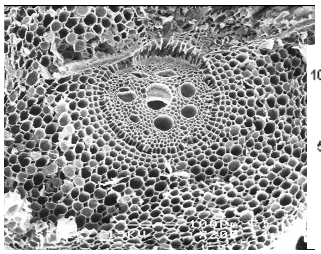
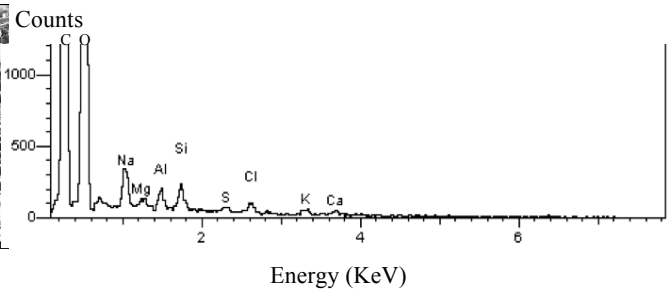
Conditions	SEM micrograph	EDX spectra
Control-Plant		
Wastewater +Plant		
Wastewater +Soil+Plant		

Table 4.8 SEM/EDX of the leaves of *C. alternifolius* after cultivation in tap water conditions, wastewater+plant conditions, and wastewater+soil+plant conditions for 7 days.

Element (%)	Conditions		
	Control−Plant	Wastewater+plant	Wastewater+soil+plant
C	49.22	61.50	44.23
O	48.14	36.87	47.84
Na	0.23	0.54	1.33
Mg	0.31	0.08	0.48
Al	-	0.1	-
Si	0.51	0.15	0.14
P	0.08	-	0.32
S	0.08	0.03*	0.26
Cl	0.81	0.53	3.29
K	0.35	0.19	1.51
Ca	0.26	0.02*	0.6

*=<2 sigma

From Table 4.8 shows carbon element in leaves of wastewater+plant conditions was higher than the control (tap water conditions) and wastewater+soil+plant conditions while oxygen elements were lower than the control and wastewater+soil+plant conditions. It was possible due to plant was stressed under wastewater conditions because plants use oxygen for organic compounds production higher than control conditions. The results of the deficiency of elements on plant could explain as below (Raven et al., 1986; Elliott and Elliott, 2001; Hopkins 2004).

The symptom of magnesium deficiency is chlorosis due to a breakdown of chlorophyll. It is readily withdrawn from the older leaves and transported to the younger leaves that are more actively growing and synthesizing chlorophyll. Magnesium, the function is part of the chlorophyll molecule and activator of many enzymes.

Silicon has a large number of functions in plants such as the strengthening of epidermal cells in leaves and stems. It is important constituent of DNA and RNA, decreasing toxicity, water balance, and improves plant growth and yield. Silicon can increase root mass, photosynthesis rate and increase pest and disease resistance. Low silicon content increases the transpiration rate (water loss through leaves) creating poor water use efficiency. Silicon deficiency decreases the synthesis of proteins and chlorophyll that found in leaves of pretreated wastewater conditions.

Phosphorus is available in the soil solution. The function is formation of high−energy phosphate compound (ATP and ADP), nucleic acids, phosphorylation of sugars (which

play such an important role in photosynthesis and intermediary metabolism), and several essential enzymes and phospholipids. Therefore, phosphate deficiency is an intense green coloration of the leaves, leading to the rapid senescence and death of older leaves.

Sulfur is concerned with some amino acids, proteins, and coenzyme (an important component in respiration and fatty acids metabolism). Sulfur deficiency is not a common problem because there are numerous microorganisms capable of oxidizing sulfides or decomposing organic sulfur compounds. Therefore, under soil-contained pretreated wastewater conditions had not effected from sulfur deficiency because of microbial activity in systems. Meanwhile, plant leaves under plant grown in pretreated wastewater condition could not detect sulfur that results in a generalized chlorosis of the leaf, including the tissue surrounding the vascular bundles. This is due to reduced protein synthesis rather than a direct impairment of chlorophyll synthesis.

Chlorine has a role in photosynthetic reaction that produces oxygen and charge balance across cellular membrane. Chlorine ion appears to be acquired for cell division in both leaves and shoots. Plants deprived of chloride tend to exhibit reducing growth, wilting of the leaf tips, and a general chorosis.

Moreover, potassium and calcium in plant leaf under pretreated wastewater were low values that shows lose function of enzyme, amino acids, and protein synthesis that starch and protein synthesis are also affected by potassium deficiency. Moreover, It is an activator for a number of enzymes, most notably those involved in photosynthesis and respiration. Potassium serves an important function in regulating the osmotic potential of cells, such as the opening and closure of stomatal guard cells and the sleep movement. Deficiency symptoms, the necrotic lesion begin at the older tips of the leaves and gradually progress along the margins to the younger cells near the leaf base, stem are shortened and weakened.

Calcium is function to calcium of cell walls, enzyme cofactor, and cell permeability, a regulator of membrane and enzyme activities. Because of its role in dividing cell, calcium deficiency symptoms characteristically appear in the meristhematic regions where cell division is occurring and new cell walls are being laid down. It affected to young leaves are deformed and necrotic.

Table 4.9 SEM/EDX of the roots of *C. alternifolius* after cultivation in tap water conditions, wastewater+soil conditions, and wastewater+soil+plant conditions for 7 days.

Element (%)	Conditions		
	Control-Plant	Wastewater+plant	Wastewater+soil+plant
C	43.48	45.60	44.92
O	53.05	51.85	53.37
Na	0.15	0.36	0.96
Mg	0.16	0.19	0.14
Al	1.05	0.62	-
Si	1.62	1.15	0.31
P	0.05	-	-
S	0.07	0.1	-
Cl	0.13	0.03*	0.14
K	0.07	0.05	0.09
Ca	0.07	0.05	0.06
Ti	0.11	-	-

*=<2 sigma

In Table 4.9, it found chlorine deficiency under wastewater+plant conditions lower than control conditions. It might be effect to diffusible cations, thus maintaining electrical neutrality across membrane and osmotically active solutes in the vacuole.

4.5.6 Microbial communities in wastewater

In the system, the complex processes caused by the plant absorption, soil adsorption, and microorganisms affected wastewater treatment. Therefore, the efficiency of plants, soil, and microorganisms involved in COD reduction of wastewater was investigated. The result showed that plants and microorganisms in plants achieved the highest efficiency for COD reduction in wastewater treatment (Fig. 4.18). The COD removal efficiency in the system was attributed as follows: 45% from *C. alternifolius* and microorganisms in plants, 26% from soil adsorption, 19% from microorganisms in soil, and 10% from microorganisms of wastewater. Microorganisms were one of the major factors in reducing the COD value that included microbes in wastewater, soil, and microbial association with the root plant. Therefore, it is extremely important to understand the microbial community in the system. Targeted approaches were aimed at the identification of groups of microbes that performed well-defined functions in a process or, in the case where organisms were unknown, the identification of numerically dominant organisms, which may be of critical importance to the treatment performance of ink factory wastewater.

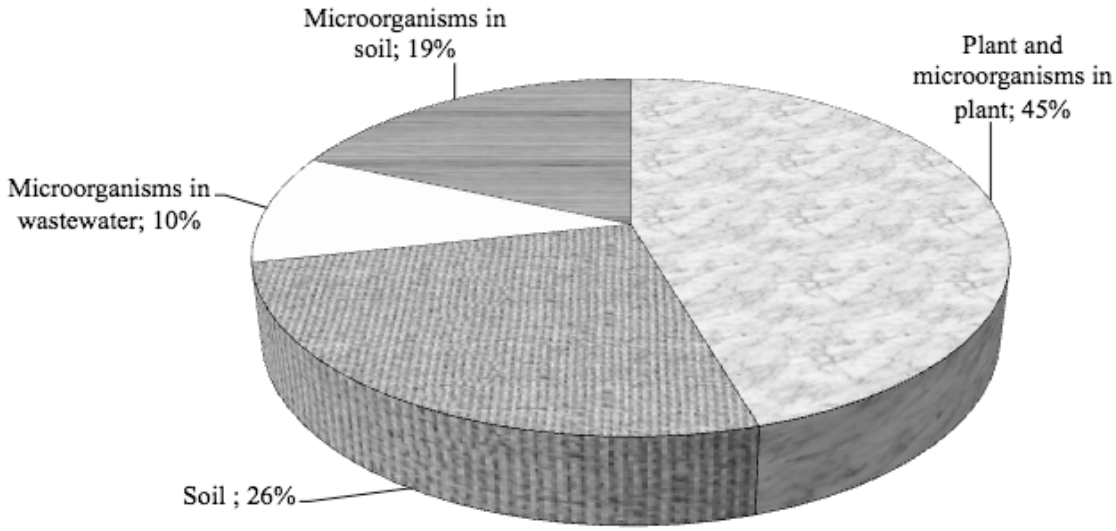


Figure 4.18 The percentage of COD reduction in wastewater involved plant absorption and microorganisms in plant, soil adsorption and microorganisms in soil and wastewaer after treatment for 7 days.

This research use molecular biology techniques to link an environmental sample from ink production wastewater. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and phylogenetic relationships are reliable and suitable techniques to obtain such community data. In this study, DNA was extracted from 5 conditions: wastewater at day 0 (W_0), wastewater at day 7 (W_7), wastewater+soil at day 7 (WS_7), wastewater+plant at day 7 (WP_7), and wastewater+soil+plant at day 7 (WSP_7). They were used to amplify the 16S rRNA gene with primers 8F and 1492R, 338F–GC and 518R for DGGE analysis.

The sample sequences recovered for each of these groups were novel. The denaturing pattern of 16S rDNA fragments from samples is displayed in Fig. 4.19. Meanwhile, sequencing of predominant bacteria that were present in the DGGE bands under various conditions is summarized in Table 4.10. DGGE can separate sequences of the same length with only a base difference. The 49 predominant bacteria associated with the 49 bands in DGGE gel were selected under various conditions. The results showed that some bands were at the same position in the DGGE gel but had different microbial strains (e.g., bands No. 3 and 7 were the closest matched to *Pseudomonas* sp. 3BC6 and *Methylobacterium populi* strain L3-774, respectively). This was possibly due to bands at the same position in the gel having the same melting behavior that was mainly based on different GC-content affecting the gene sequence (Muyzer et al., 1993). Moreover, the same sequence may have different bands in the DGGE gel or have different locations (e.g., bands No. 37, 46, 47, and 49 were closest matched to *Azospirillum amazonense*). This may result from the different conformation of one sequence, which provides an overestimation of microbial diversity (Fasoli et al., 2003). Therefore, DGGE should be combined with other molecular methods such as gene sequencing and phylogenetic analysis in microbial diversity analysis and community comparisons (Li et al., 2006)

The DGGE pattern shows 49 microorganisms isolated from various conditions. After that, sequencing of the bands of DNA excised from gels allowed the identification of different members of the microbial community. The relationships of microorganisms

that were isolated from various conditions were heterogeneous and categorized as 17 of α−Proteobacteria, 4 of β−Proteobacteria, 11 of γ−Proteobacteria, 1 of δ−Proteobacteria, 1 of ε−Proteobacteria, 3 of *Bacteroidetes*, and 12 of *Firmicutes*.

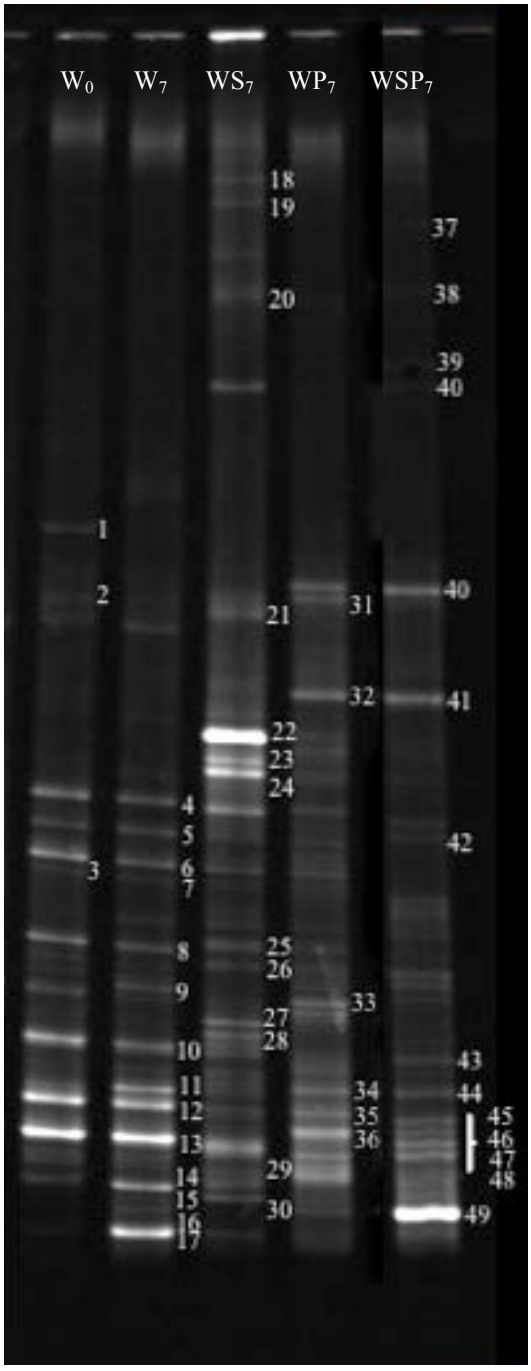


Figure 4.19 PCR-DGGE pattern of wastewater at day 0 (W_0), wastewater at day 7 (W_7), wastewater+soil conditions at day 7 (WS_7), wastewater+plant conditions at day 7 (WP_7), and wastewater+soil+plant conditions at day 7 (WSP_7).

Table 4.10 The analysis of the sequenced excised bands in the DGGE by NCBI Blast.

Band No.	GenBank accession No.	Strain	Eubacterial classification	% Similarity	Samples				
					W_0	W_7	WS_7	WP_7	WSP_7
Out group	AB603516	*Methanocaldococcus jannaschii*	Archaea	–	–	–	–	–	–
1	HQ663061	Gamma proteobacterium SCGC AAA024-E17	γ-proteobacteria	89	+	–	–	–	–
2	JN050957	*Enterobacter* sp. N0-20R2A	γ-proteobacteria	92	+	–	–	–	–
3	AF229885	*Pseudomonas* sp. 3CB6	γ-proteobacteria	92	+	–	–	–	–
4	HQ288939	*Pseudomonas aeruginosa* strain A12	γ-proteobacteria	98	+	+	+	+	–
5	EU620069	*Pseudomonas putida* strain GNA5	γ-proteobacteria	94	+	+	+	+	–
6	JN941348	*Diaphorobacter nitroreducens* strain AW3	β-proteobacteria	100	+	+	+	+	–
7	JQ659342	*Methylobacterium populi* strain L3-774	α-proteobacteria	82	–	+	+	–	–
8	HQ003420	*Acidovorax delafieldii* strain NBGD35	β-proteobacteria	100	+	+	–	–	–
9	HM103365	*Enterobacter* sp. AJAR-A2	γ-proteobacteria	94	–	+	–	–	–
10	EF061452	*Stenotrophomonas maltophilia* strain DTQ-CRS31	γ-proteobacteria	94	+	+	–	–	–
11	EU057829	*Flexibacteraceae bacterium* CH30#7	Bacteroidetes	100	+	+	–	–	–
12	AY752939	*Enterobacter sakazakii* strain z759	γ-proteobacteria	100	+	+	–	–	–
13	HM103366	*Enterobacter* sp. AJAR-A3	γ-proteobacteria	100	+	+	–	–	–
14	FN293046	*Azospirillum* sp. Z012	α-proteobacteria	100	+	+	–	–	–

Table 4.10 The analysis of the sequenced excised bands in the DGGE by NCBI Blast (cont.).

Band No.	GenBank accession No.	Strain	Eubacterial classification	% Similarity	Samples				
					W_0	W_7	WS_7	WP_7	WSP_7
15	NR_042090	*Dechloromonas denitrificans* strain ED1	β-proteobacteria	100	−	+	−	−	–
16	JF508370	*Azospirillum irakense* strain L-6	α-proteobacteria	100	−	+	−	−	–
17	EF394925	*Candidatus Azospirillum massiliensis* strain URAM1	α-proteobacteria	100	−	+	−	−	–
18	AB547221	*Lysinibacillus fusiformis*	Firmicutes	96	−	−	−	−	–
19	GU143900	*Lysinibacillus sphaericus* strain T12-16	Firmicutes	100	−	−	+	−	–
20	HQ396802	*Lysinibacillus* sp. CH-N5	Firmicutes	96	−	−	+	−	–
21	JN700165	*Lysinibacillus sphaericus* strain P16	Firmicutes	94	−	−	+	−	–
22	JN215512	*Lysinibacillus sphaericus* strain VB7	Firmicutes	100	−	−	+	−	–
23	JQ312065	*Lysinibacillus sphaericus* strain AIMST Ehe33	Firmicutes	100	−	−	+	−	–
24	GU214826	*Bacillus cereus* strain Q-hrb05	Firmicutes	99	−	−	+	−	–
25	JF683591	*Acinetobacter calcoaceticus* strain MCMB868	γ-proteobacteria	99	−	−	+	−	–
26	CP000698	*Geobacter uraniireducens* Rf4	δ-proteobacteria	100	−	−	+	−	–
27	NR_041641	*Bacillus azotoformans* strain NBRC 15712	Firmicutes	99	−	−	+	−	–
28	NR_025125	*Desulfitobacterium metallireducens* strain 853-15	Firmicutes	93	−	−	+	−	–
29	FN870348	*Bacillus pocheonensis*	Firmicutes	100	–	–	+	–	–

Table 4.10 The analysis of the sequenced excised bands in the DGGE by NCBI Blast (cont.).

Band No.	GenBank accession No.	Strain	Eubacterial classification	% Similarity	Samples				
					W_0	W_7	WS_7	WP_7	WSP_7
30	EF143993	*Clostridium* sp. 'HY-129-11 T'	Firmicutes	98	−	−	+	−	−
31	JN409060	Uncultured alpha proteobacterium clone HG-J02177	α-proteobacteria	88	−	−	−	+	−
32	GQ420909	Uncultured *Flexibacter* sp. clone RUGL1-414	Bacteroidetes	98	−	−	−	+	−
33	DQ072907	Uncultured *Rhizobium* sp.	α-proteobacteria	93	−	−	−	+	−
34	AF431208	Uncultured alpha proteobacterium clone S52.37PG	α-proteobacteria	100	−	−	−	+	−
35	HM755803	Uncultured type II methanotroph	α-proteobacteria	100	−	−	−	+	−
36	FJ817479	*Bacillus* sp. DST4	Firmicutes	99	−	−	−	+	−
37	AB568111	*Azospirillum amazonense*	α-proteobacteria	100	−	−	−	−	+
38	JN941348	*Diaphorobacter nitroreducens* strain AW3	β-proteobacteria	100	−	−	−	−	+
39	AF307869	*Pseudomonas putida* 5IIANH	γ-proteobacteria	99	−	−	−	−	+
40	NR_041642	*Bacteroides graminisolvens* strain XDT-1	Bacteroidetes	95	−	−	−	−	+
41	CP001816	*Sulfurospirillum deleyianum* DSM 6946	Epsilonproteobacteria	97	−	−	−	−	+
42	FJ916802	Uncultured alpha proteobacterium clone LT1bE8	α-proteobacteria	95	−	−	−	−	+
43	DQ387436	*Azospirillum* sp. 21R	α-proteobacteria	98	−	−	−	−	+

Table 4.10 The analysis of the sequenced excised bands in the DGGE by NCBI Blast (cont.).

Band No.	GenBank accession No.	Strain	Eubacterial classification	% Similarity	Samples				
					W_0	W_7	WS_7	WP_7	WSP_7
44	EF422177	*Azospirillum* sp. 2456	α-proteobacteria	100	−	−	−	−	+
45	GU048666	*Azospirillum* sp. YC6995	α-proteobacteria	100	−	−	−	−	+
46	AB568112	*Azospirillum amazonense*	α-proteobacteria	100	−	−	−	−	+
47	AB568111	*Azospirillum amazonense*	α-proteobacteria	100	−	−	−	−	+
48	EF422192	*Azospirillum* sp. 12812	α-proteobacteria	96	−	−	−	−	+
49	AB568112	*Azospirillum amazonense*	α-proteobacteria	98	−	−	−	−	+

Note: a = +, visible band; -, no visible band.
W_0 = wastewater at day 0
W_7 = wastewater at day 7
WS_7 = wastewater+soil conditions at day 7
WP_7 = wastewater+plant conditions at day 7
WSP_7 = wastewater+soil+plant conditions at day 7

Both of the bacterial groups recovered molecularly, *Proteobacter* and *Bacterioderes*, represent organisms that are metabolically diverse in pretreated wastewater at day 0 and day 7 (Table 4.10). Isolates from the genus *Proteobacter* belong to the α-, *β*-, and γ-Proteobacteria. Under wastewater+soil conditions, two major groups are shown to belong to genera *Firmicutes* and *Proteobacter*. Meanwhile, the microorganisms that were isolated from wastewater+plant and wastewater+soil+plant conditions showed a dominant microorganism belonging to genera *Proteobacteria* such as *α*-, *β*-, γ, and ε-Proteobacteia.

Figure 4.19 indicated that the dominant bands of the DGGE pattern were bands No. 12 and 13 that closely related to *Enterobacter* sp., which are facultative anaerobic. Moreover, bands No. 2 and 9 are also identified as being affiliated with the *Enterobacter* bacteria that is found only in wastewater conditions. This species was reported as a polysaccharide-producing bacterium (Shimada et al., 1997) and a proportion of the nitrate-reducing bacteria of the denitrifying process (Guynot et al., 1998). The result was confirmed with the appearance of mucilage in the solution under wastewater conditions at day 7, which was possibly polysaccharide (Fig. 4.20). Besides *Enterobacter*, the predominant bacteria in wastewater at day 0 and day 7 were bands No. 3, 4, and 5 that were identified as being affiliated with the *Pseudomonas* species. However, *Pseudomonas* sp. was also found in wastewater+soil and wastewater+plant conditions at day 7. Many researchers have reported that *Pseudomonas* sp. could reduce nitrate, especially *Pseudomonas aeruginosa*, which has been extensively studied genetically with regard to denitrification (Curtis and John, 1983; Silvestrini et al., 1989; Gilbert et al., 2008). In addition*, Pseudomonas putida* demonstrated a very diverse metabolism, including the ability to degrade organic solvents (Pinkart and White, 1997). However, these microorganisms had the efficiency of only 10% of COD removal and actually took a long time to degrade organic contaminants in wastewater if only microbial activity was used.

Figure 4.20 The mucilage was found under wastewater conditions after treatment for 7 days.

Figures 4.21 and 4.22 shows the phylogenetic trees of microorganisms in wastewater at day 0 and day 7. The strains, categorized into two major groups, belong to genera *Proteobacter* and *Bacterioderes*. Isolates from the genus *Proteobacter* belong to the α-, *β*-, and γ-Proteobacteria. The α−Proteobacteria as *Azospirillum* sp., *Azospirillum irakense*, and *Candidatus Azospirillum massiliensis* were nearly identical (100% similarity), but *Methylobacterium populi* was the most identical (82% similarity to GenBank accession no. JQ659342). Isolates from the *β*−Proteobacteria,

Diaphorobacter nitroreducens, *Acidovorax delafieldii*, and *Dechloromonas denitrificans* had rRNA gene sequences that were nearly identical (100% sequence similarity). Class γ−Proteobacteria such as *Enterobacter* sp., *Pseudomonas* sp., *Pseudomonas aeruginosa*, *Pseudomonas putida*, and *Stenotrophomonas maltophilia* had sequence isolates that were the most identical (92-100% similarity).

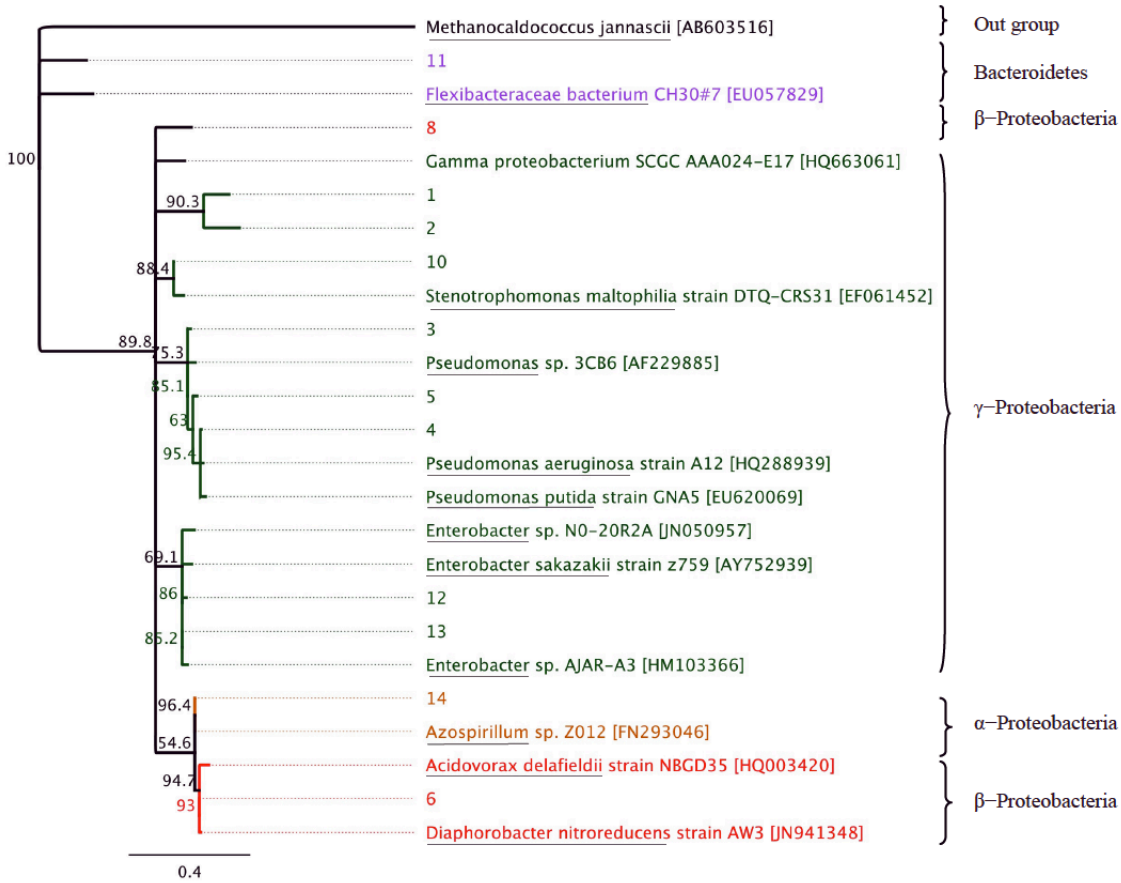


Figure 4.21 Phylogenetic analyses of archaeal (*Methanocaldococcus jannadchii*; out group) and bacterial 16S rRNA gene sequences of *Eubacteria* from wastewater at day 0. The phylogenetic tree was constructed by the neighbor-joining method with 1,000 bootstrap replicates, using the Geneious® Pro 5.6.4. software. Scale bar correspond to nucleotide sequence differences position. Accession numbers are given in parentheses.

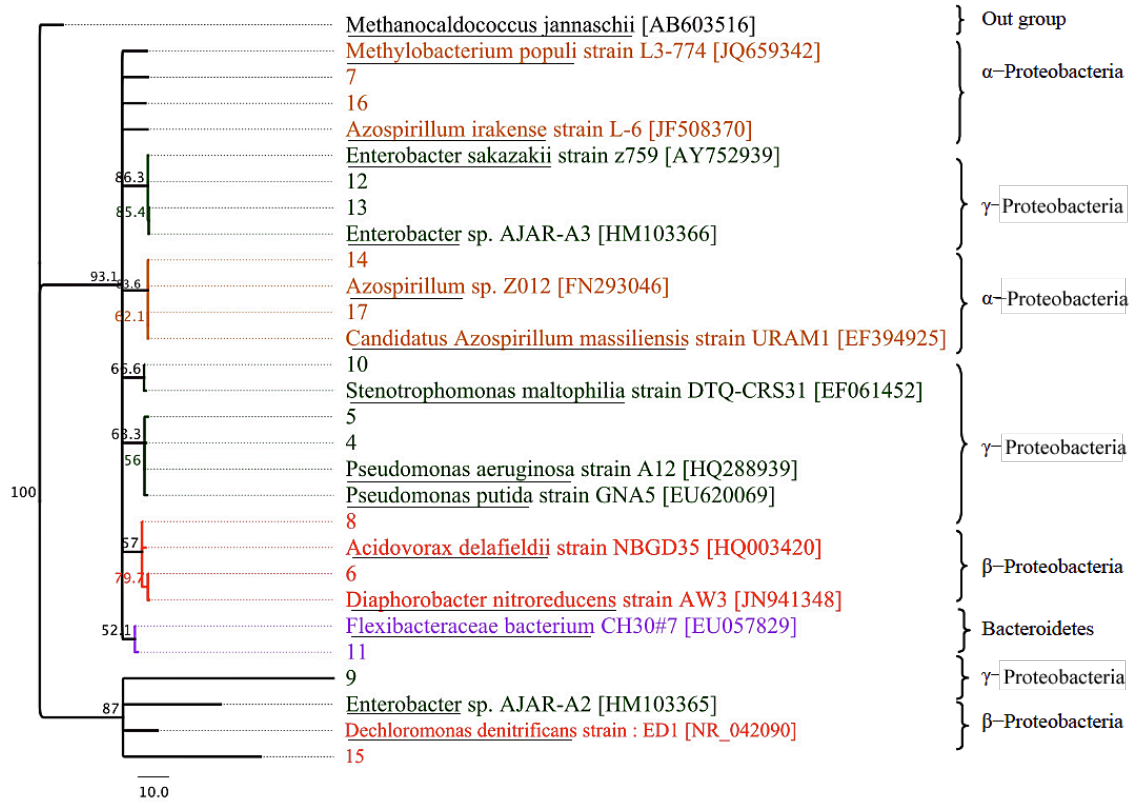


Figure 4.22 Phylogenetic analyses of archaeal (*Methanocaldococcus jannadchii*; out group) and bacterial 16S rRNA gene sequences of *Eubacteria* from wastewater at day 7. The phylogenetic tree was constructed by the neighbor-joining method with 1,000 bootstrap replicates, using the Geneious® Pro 5.6.4. software. Scale bar correspond to nucleotide sequence differences position. Accession numbers are given in parentheses.

On the phylogenetic tree under wastewater+soil conditions (Fig. 4.23), two major groups are shown to belong to genera *Firmicutes* and *Proteobacter*. Isolates from the genus *Firmicutes* were very close phylogenetically to *Clostridium* sp., *Desulfitobacterium metallireducens*, *Bacillus azotoformans*, *Bacillus pocheonensis, Lysinibacillus sphaericus*, *Bacillus cereus*, *Lysinibacillus fusiformis*, and *Lysinibacillus* sp. (93-100% similarity). Meanwhile, isolates from the *Proteobacter* such as *Methylobacterium populi* (α-proteobacteria), *Diaphorobacter nitroreducens* (β-proteobacteria), *Geobacter uraniireducens* (δ-Proteobacteria), *Psedomonas putida*, *Pseudomonas aeruginosa,* and *Acinetobacter calcoaceticus* (γ-proteobacteria) were the most identical (99-100% sequence similarity).

The 19% of COD removal received from microorganisms in soil is possibly due to clay soils as the nutrient source which creates large amounts of small pores that offer protection for bacteria (Six et al. 2004). Under wastewater+soil conditions, it was found that bands No. 25 and 26 were *Proteobacteria* while the other bands were *Firmicutes*; a major bacterium of the *Firmicutes* group was *Lysinibacillus* sp. (bands No. 18, 19, 20, 21, 22, and 23). Li and co-worker reported that *Firmicutes*-related Fe (III)-reducing bacteria might also be an important group of Fe (III) reducers besides the well-known *Geobacter* species (Li et al., 2011). Microorganisms could oxidize organic compounds with Fe (III) serving as the electron acceptor, as well as degrade organic contaminants (Canfield et al., 1993; Lovley and Anderson, 2000). These microorganisms were related

to the presence of a rust-colored iron oxide scum, which was possibly due to clay soils being contaminated with iron as showed in Fig. 4.24. The result of digestion of the solid showed in Table 4.11. Meanwhile, *Desulfitobacterium metallireducens* (Band No. 28), an obligate anaerobe that can reduce Fe(III), may have a competitive advantage in anaerobic subsurface environments in which Fe(III) is obtained abundantly (Finneran et al., 2002), but often lacks significant quantities of the other electron acceptors such as nitrate, thiosulfate and sulfite. Most denitrifying bacteria are aerobic heterotrophic groups that can oxidize a carbon source to an N oxide under anaerobic conditions while autotrophic denitrifiers can utilize inorganic sulfur compounds, hydrogen, ammonia, or nitrite (Zumft, 1997). Recently, it was found that the oxidation of Fe (II) was also coupled to a complete denitrification process as shown in Eq. 4.4 (Straub et al., 1996). It is suggested that Fe (III)-reducing microorganisms may be beneficial to reduce nitrate under wastewater+soil conditions.

$$10FeCO_3 + 2NO_3^- + 24H_2O \rightarrow 10Fe(OH)_3 + N_2 + 10HCO_3 + 8H^+ \qquad (4.4)$$

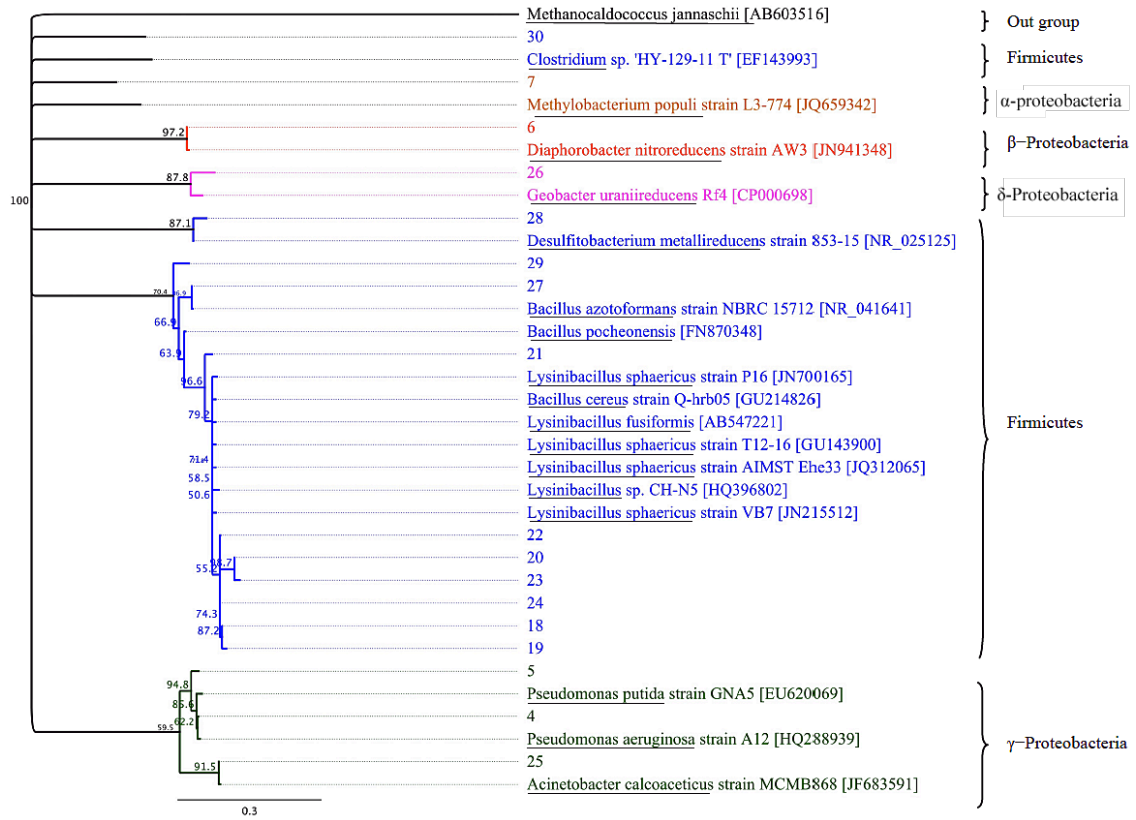


Figure 4.23 Phylogenetic analyses of archaeal (*Methanocaldococcus jannadchii*; out group) and bacterial 16S rRNA gene sequences of *Eubacteria* from wastewater+soil conditions at day 7 (WS_7). The phylogenetic tree was constructed by the neighbor-joining method with 1,000 bootstrap replicates, using the Geneious® Pro 5.6.4. software. Scale bar correspond to nucleotide sequence differences position. Accession numbers are given in parentheses.

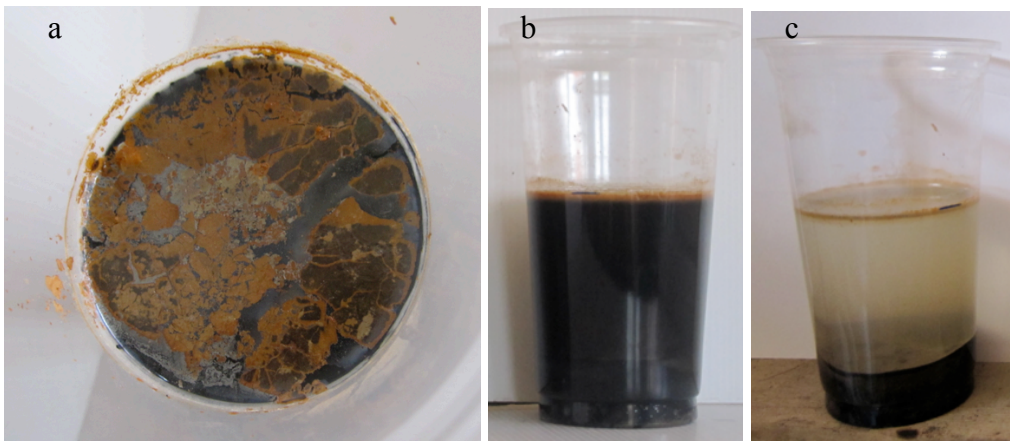


Figure 4.24 A rust-colored iron oxide scum on the surface of wastewater+soil conditions (a), appearance of black colored solution from sulfate-reducing bacteria after treatment for 7-10 days (b) and 10-20 days (c).

Table 4.11 Fe, Mn, and Al concentrations from a rust-colored iron oxide scum on the surface of wastewater+soil conditions after treatment for 7 days.

Elements	Concentration (g/kg dry weight)
Fe	9.57±0.25
Mn	3.15±0.09
Al	3.32±0.19

The phylogenetic analyses of microorganisms that were isolated from wastewater+plant conditions are shown in Fig. 4.25. The results showed a dominant microorganism belonging to genera *Proteobacteria* such as *α*-, *β*-, and γ-Proteobacteia. Classification from *α*−Proteobacteria was closely related to uncultured type II methanotroph, uncultured alpha proteobacterium, and uncultured *Rhizobium* sp. that were the most identical (88-100% sequence similarity). Betaproteobacteria belong to *Diaphorobacter nitroreducens* and were nearly identical (100% similarity). γ−Proteobacteia *Pseudomonas aeruginosa* and *Pseudomonas putida* were the most identical (94-98% sequence similarity). Moreover, this condition appeared in *Bacteroidetes* and *Firmicutes* groups belonging to uncultured *Flexibacter* sp. and *Bacillus* sp., respectively (98-99% sequence similarity).

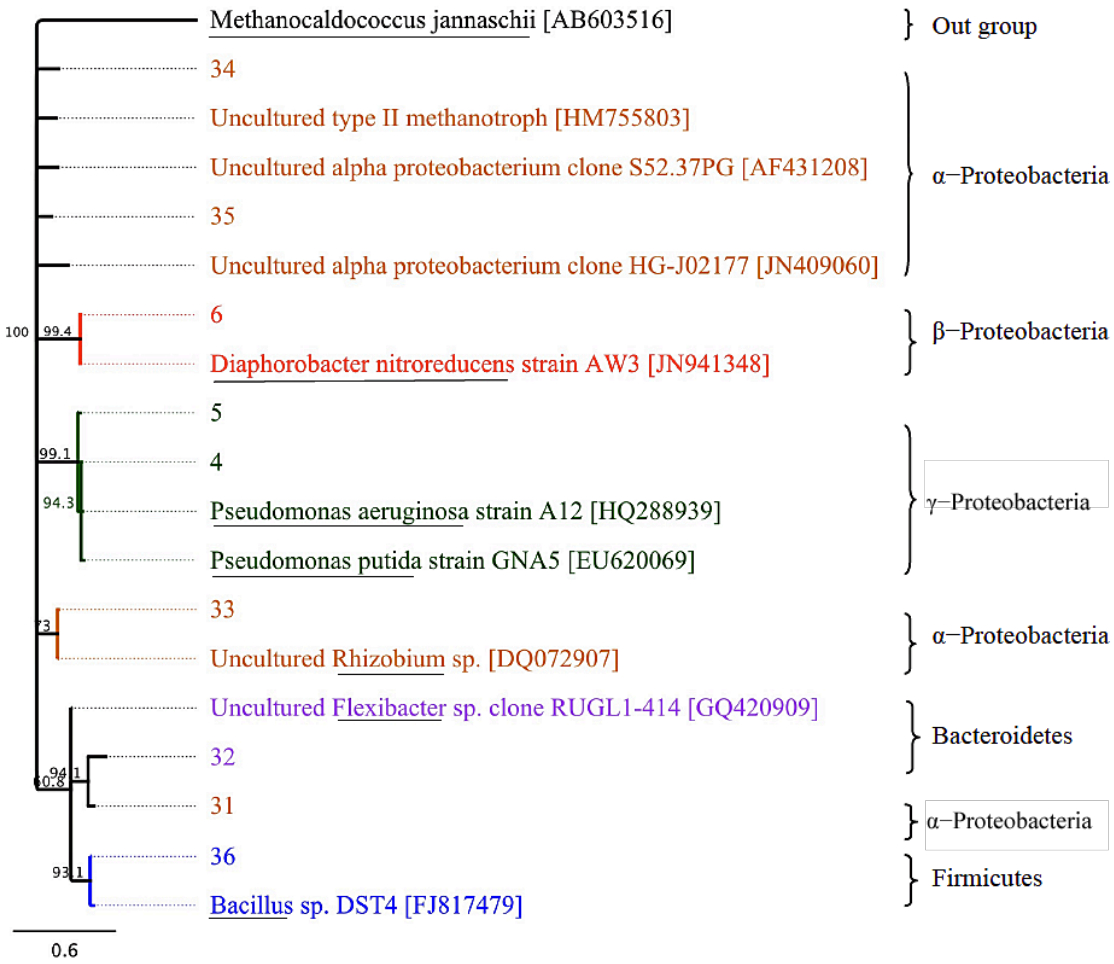


Figure 4.25 Phylogenetic analyses of archaeal (*Methanocaldococcus jannadchii*; out group) and bacterial 16S rRNA gene sequences of *Eubacteria* from wastewater+plant conditions at day 7 (WP_7). The phylogenetic tree was constructed by the neighbor-joining method with 1,000 bootstrap replicates, using the Geneious® Pro 5.6.4. software. Scale bar correspond to nucleotide sequence differences position. Accession numbers are given in parentheses.

The phylogenetic analyses of microorganisms that were isolated from wastewater+soil+plant conditions are shown in Fig. 4.26. The results showed that a dominant microorganism was genera *Proteobacteria* as *α*-, *β*-, γ, and ε-Proteobacteia. *Alphaproteobacteria* was a major group in this condition where *Azospirillum sp.* was the most identical (96-100% sequence similarity). Betaproteobacteria *Diaphorobacter nitroreducens* were nearly identical (100% similarity) while γ−Proteobacteria and ε-Proteobacteria *Pseudomonas putida* had 99% similarity and *Sulfurospirillum deleyianum* had 97% similarity, respectively.

Nevertheless, under wastewater+soil+plant conditions, it was found that the highest COD removal of wastewater came from plant-microbe interactions. The bacterial identity abundance found was the highest for *Azospirillum* species (Bands No. 37, 46, 47, and 49). Rare sequences identified as *Diaphorobacter nitroreducens, Pseudomonas putida, Bacteroides graminisolvens,* and *Sulfurospirillum deleyianum* were also found. The most likely genetic relationship between 16S rDNA sequences PCR-amplified is shown in Fig. 4.19. Bacteria of the genus *Azospirillum* are highlighted as plant growth

promoting rhizobacteria (PGPR) (Okon and Vanderleyden, 1997) and N_2-fixers bacteria which can perform all the reactions of the nitrogen cycle, especially denitrification (Danneberg et al., 1986). It can utilize nitrate as a respiratory electron acceptor and reduce it to molecular nitrogen via nitrite and nitrous oxide (Rösch et al., 2002; Rich et al., 2004). However, *Azospirillum* is not only able to fix atmospheric N, but can also mineralize nutrients from the soil to sequester Fe and to survive harsh environmental conditions or abiotic stresses (Bashan et al., 2004). In addition, Azospirilla exhibits positive chemotaxis towards organic acids, sugars, amino acids, and aromatic compounds as well as root exudates (Heinrich and Hess, 1985). Based on the results reported here, for taxonomic purposes we strongly recommend that *Azospirillum* detected in association with plant roots that represents the best-characterized genus should be highlighted as a natural habitat, plant root interaction, nitrogen fixation and biosynthesis of plant growth hormones (Khan and Hiraishi, 2002).

In addition, it was interesting to find *Diaphorobacter nitroreducens* under various conditions and also wastewater+soil+plant conditions. It had the ability to perform simultaneous nitrification and denitrification (SND) under both aerobic and anaerobic conditions (Khardenavis et al. 2007). The process of SND can be considered economical provided nitrification stops at the nitrite formation level, which instead of nitrate serves as a substrate for denitrification and is reduced to N_2 (Horn et al. 2005). Moreover, the other microorganism closely related to *Acidovorax delafieldii*, *Stenotrophomonas maltophilia*, *Dechloromonas denitrificans*, and *Sulfurospirillum deleyianum* such as bands No. 8, 10, 15, and 41 are also involved in nitrate reduction and nitrogen removal (Eisenmann et al. 1995 ; Horn et al. 2005 ; Heylen et al. 2007; Khardenavis et al. 2007).

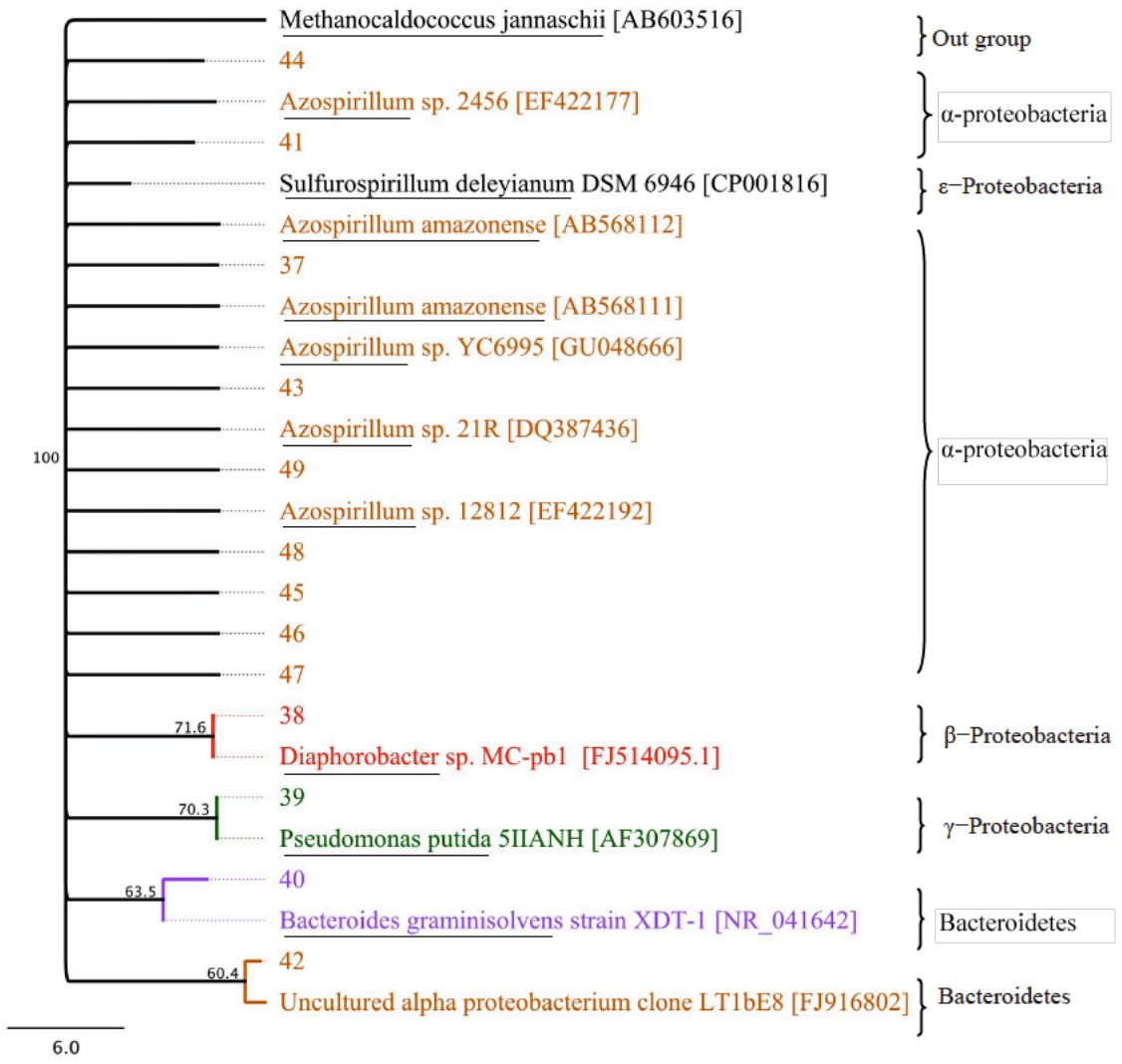


Figure 4.26 Phylogenetic analyses of archaeal (*Methanocaldococcus jannadchii*; out group) and bacterial 16S rRNA gene sequences of *Eubacteria* from wastewater+soil+plant conditions at day 7 (WSP_7). The phylogenetic tree was constructed by the neighbor-joining method with 1,000 bootstrap replicates, using the Geneious® Pro 5.6.4. software. Scale bar correspond to nucleotide sequence differences position. Accession numbers are given in parentheses.

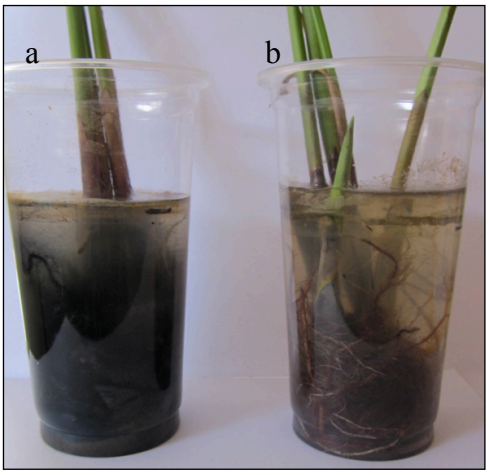


Figure 4. 27 Appearance of wastewater + plant conditions (a) and wastewater + soil + plant conditions (b) after treatment for 7-10 days.

In addition, the microbial communities of W_0, W_7, WS_7 and WSP_7 could be concluded as Table 4.12.

Table 4.12 Summary of microbial communities and functions under various conditions.

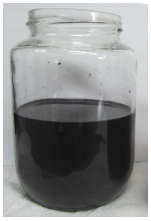
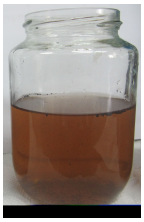
Microbial communities	Strain	Conditions				
		W_0	W_7	WS_7	WP_7	WSP_7
Polysaccharide-producing bacteria	*Enterobacter* sp.	+	+	−	−	−
Fe (III)-reducing bacteria	Firmicutes : *Lysinibacillus* sp.	−	−	+	−	−
	Geobacter sp.	−	−	+	−	−
	Desulfitobacterium metallireducens	−	−	+	−	−
Nitrate reducing bacteria	*Enterobacter* sp.	+	+	−	−	−
	Peudomonas sp.	+	+	+	+	+
	Bacillus sp.	−	−	+	+	−
	Stenotrophomonas sp	+	+	−	−	−
	Diaphorobacter sp.	+	+	+	+	+
	Azospirillum sp.	+	+	−	+	+
	Sulfurospirillum deleyianum	−	−	−	−	+
	Dechloromonas denitrificans	−	+	−	−	−
Degrade organic solvents	*Pseudomonas* sp.	+	+	+	+	+
Nitrogen-fixing bacteria	*Methylobacterium* sp.		+	+	−	−
	Azospirillum sp.	+	+	+	+	+
Sulfate-reducing bacteria	*Desulfitobacterium* sp.	−	−	+	−	−

4.6 Ammonium nitrogen removal

4.6.1 Treatment of wastewater by magnesium ammonium phosphate (MAP)

Ammonium nitrogen is one major problem in ink production wastewater. Although, there are many methods for ammonium nitrogen removal but it is difficult to treating ammonium nitrogen in ink factory wastewater because the effect of pigments and acrylic resin. Therefore, pretreatment of wastewater for decreasing acrylic resin and pigments before using other methods was investigated. From our study found that sulfuric acid had the efficiency for removal of acrylic resin, pigments, COD, SS, and also ammonium nitrogen as showed in Table 4.13. The result showed that initial NH_4^+−N concentration of wastewater and pretreated wastewater with sulfuric acid was 458 mg/L and 295 mg/L, respectively. While remaining COD and SS were declined to 1354 mg/L and 32 mg/L, respectively. However, the treatment of high NH_4^+−N using plants directly might take a long time to solve this problem. The method of chemical precipitation has been studied widely for the treatment of high strength ammonium nitrogen wastewater under an equal molar ratio of Mg_2^+:NH_4^+:PO_4^{3-}. Therefore, chemical precipitation of ammonium nitrogen removal by adding magnesium and phosphate to form magnesium ammonium phosphate hexahydrate (MAP) was investigated. The precipitation with various types of MAP such as substance A [$MgCl_2.6H_2O$ + $Na_2HPO_4.12H_2O$], substance B [MgO + 85% H_3PO_4], and substance C [$MgSO_4.7H_2O$ + $Ca(H_2PO_4)_2$].

Table 4.13 The characteristics of wastewater before and after treatment with sulfuric acid.

Parameter	Wastewater	Pretreated wastewater (Wastewater + H_2SO_4)	Effluent standard value[a]
Appearance			Not objectionable
COD (mg/L)	28827±385	1354±49	120-400
pH	7.45±0.01	1.87±0	5.5-9
TKN (mg/L)	514±32	369±27	100-200
NH_4^+−N (mg/L)	458±15	295±23	-
TDS	11183±157	11345±162	3000-5000
SS	2917±34	32±6	50-150

Source: [a]Industrial Effluent Standard from Notification the Ministry the Ministry of Science, Technology and Environment, 1996,Thailand.

Firstly, the various types of MAP directly compared with the treatment of sulfuric acid and MAP was studied. After the direct treatment of MAP, the results showed that the original wastewater still appeared dark color (Table 4.14). It was confirmed that MAP could not precipitate pigments in wastewater. However, it showed that treatment with substance A directly could decrease NH_4^+−N more than substance B and C to approximately 160 mg/L from an initial NH_4^+−N of 458 mg/L. In treatments by substances B and C, NH_4^+−N values were still high at about 309 mg/L and 380 mg/L, respectively (Table 4.14).

In addition, the treatment of original wastewater by sulfuric acid and then MAP received a higher efficiency than direct treatment with MAP alone. The result indicated that substance A had the highest efficiency of NH_4^+−N removal as the remaining NH_4^+−N concentration was approximately 61 mg/L from an initial 458 mg/L (Table 4.14). Substances B and C could remove NH_4^+−N to 98 mg/L and 201 mg/L, respectively (Table 4.14). The results suggested that the treatment of wastewater by sulfuric acid and then using the MAP method to reduce NH_4^+−N was the best way to treat this wastewater.

However, these chemical reactions to form MAP have been expressed in Eqs. 4.5-4.7. Equation 4.5 shows that the product of substance A was two molecules of NaCl, which might have affected to the survival of the plant while substance C had the worst efficiency of NH_4^+−N removal (201 mg/L). Therefore, substance B was selected for the next study (phytoremediation) because it showed that the product was water that was not toxic to the plant and it had the second highest efficiency for NH_4^+−N removal at about 98 mg/L (See Eqs. 4.6).

$$MgCl_2.6H_2O + Na_2HPO_4.12H_2O + NH_4^+ \rightarrow MgNH_4PO_4.6H_2O \downarrow + 2NaCl + 12H_2 + H^+ \quad (4.5)$$

$$MgO + H_3PO_4 + NH_4^+ + 6H_2O \rightarrow MgNH_4PO_4.6H_2O \downarrow + H_2O + H^+ \quad (4.6)$$

$$MgSO_4.7H_2O + Ca(H_2PO_4)_2.H_2O + NH_4^+ \rightarrow MgNH_4PO_4.6H_2O \downarrow + CaSO_4 \downarrow + PO_4^{3-} + 2H_2O \quad (4.7)$$

Moreover, the pH range affected the minimum MAP solubility (pH 8.5−9.0). Therefore, before using phytoremediation method, the three most common alkalis, NaOH, KOH, and $Ca(OH)_2$ (Table 4.15), were observed for selecting the most suitable chemical for the highest efficiency. The result showed that adjusting pH with NaOH and KOH increased the COD values to 1564 mg/L and 1488 mg/L, respectively, from an initial 1354 mg/L. It was possible that these alkalis enhanced its solubilization with hydrolyzed organic matter in wastewater samples resulting in an increase in the concentration of soluble chemical oxygen demand (SCOD). In general, the concentration of added reagent sodium hydroxide increased solubilization of the samples of COD while using $Ca(OH)_2$ did not affect solubilization (Li et al. 2008; Doğan and Sanin, 2009; Zheng et al. 2009). The results found that $Ca(OH)_2$ had the highest efficiency for COD and NH_4^+−N removal which were 1320 mg/L and 29 mg/L from an initial COD of 1354 mg/L and NH_4^+−N of 295 mg/L, respectively.

4.6.2 Phytoremediation study

Although Table 4.15 shows that the three alkaline chemicals could decrease NH_4^+−N values, the amount of organic matter in effluent wastewater still affected the COD value to be higher than the effluent standard and also had the color of wastewater. It was necessary to treat the effluent before discharging into the environment. Therefore, the three alkaline chemicals were also observed for their effects on plants and their removal efficiency in phytoremediation studies.

NH_4^+−N concentrations of wastewater after adjusting pH with NaOH, KOH, and $Ca(OH)_2$ were approximately 35 mg/L, 32 mg/L, and 29 mg/L, respectively (Table

4.15). At day 3, wastewater conditions (control) had the remaining concentrations of NH_4^+−N equal to 18 mg/L, 20 mg/L, and 32 mg/L while under wastewater+plant conditions were 16 mg/L, 17 mg/L, and 13 mg/L by adjusting pH with NaOH, KOH, and $Ca(OH)_2$, respectively (Table 4.17). The data shows that adjusting the system pH with $Ca(OH)_2$ had the highest efficiency for NH_4^+−N removal when compared to other reagents. This might be because $Ca(OH)_2$ and NH_4^+−N was used as a nutrient source that did not affect the plant. In addition, under wastewater+plant conditions could also reduce nitrate nitrogen higher than under wastewater conditions (control) (Table 4.16). At day 3, the remaining COD concentration of wastewater conditions (control) were approximately 1252 mg/L, 1092 mg/L, and 950 mg/L by adjusting pH with NaOH, KOH, and $Ca(OH)_2$, respectively. Meanwhile, under plant grown in soil contained wastewater conditions with the adjustment of pH with $Ca(OH)_2$ could decrease COD concentrations lower than the effluent standard. It was approximately 329 mg/L that reached to 78% of COD removal. The remaining COD concentrations of adjusting pH with NaOH and KOH were 554 mg/L and 495 mg/L that reached to 65% and 63% of COD removal, respectively. These results confirmed that under wastewater+soil+plant conditions that adjusted pH with $Ca(OH)_2$ was the best condition with the efficiency for COD removal while other conditions were higher than the effluent standard. It was possible that $Ca(OH)_2$ did not enhance COD solubilization and was also a good nutrient for plants to grow.

In addition, the toxic alkaline chemicals used in adjusting pH in plants were observed. The results found that NaOH and KOH had an effect on plants, as the plant leaves yellowed and wilted, followed by the plant stems beginning to wither and dry. While plants were exposed to $Ca(OH)_2$, plant leaves and plant stems were still healthy and new roots and shoots appeared. Therefore, $Ca(OH)_2$ did not affect the plant and was suitable for plant growth. Moreover, adjusting the system pH with $Ca(OH)_2$ had the highest efficiency for COD removal (Table 4.16). It was possible that, due to the exposure of Ca^+, the plant could tolerate and adapt, and use $Ca(OH)_2$ as a nutrient. Calcium ions (Ca^{2+}) act as an osmoticum within vacuoles, a stabilizing element of membranes, a strengthening agent in cell walls, and a secondary messenger for a multitude of signals (White and Broadley, 2003; Dodd et al., 2010; Marschner, 1995). With a high concentration of Na^+ and K^+ as ionic stress, plants could not tolerate and adapt to the stress. An increase in Na^+ content during treatment is thought to result from the competition between the transport and accumulation of Na^+ that leads to osmotic shock in roots, then to molecular damage, inhibited plant growth and then death. Because they inhibit many enzymes, it is important to prevent Na^+ accumulating to a high level in the cytoplasm or in organelles other than the vacuole (Zhu, 2001). Meanwhile, the excess potassium is not toxic to plants but it limits the efficient absorption of other minerals and nutrients leading to lots of other deficiencies such as magnesium, manganese, zinc and iron, and can cause problems with calcium as well. This in turn results in poorly developed plants with a yellowish color and stunted growth due to a molecular imbalance (Hopkins and Hüner, 2004).

Table 4.14 Characteristics of wastewater and pretreated wastewater after precipitation with three combination chemicals.

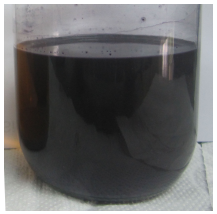
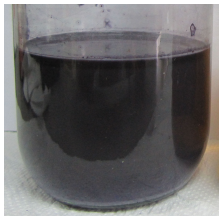
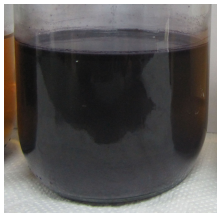
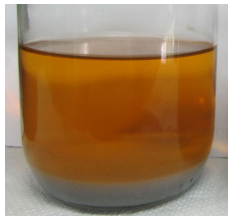
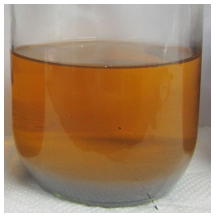
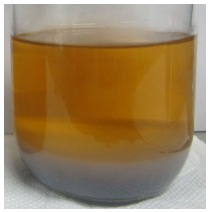
Parameter	Wastewater + magnesium and phosphate to form MAP			Wastewater + H_2SO_4 + magnesium and phosphate to form MAP		
	Substance A	Substance B	Substance C	Substance A	Substance B	Substance C
Appearance						
NH_4^+−N (mg/L)	160	309	380	61	98	201

Table 4.15 Efficiency of effluent after using various alkaline chemicals for adjusting the pH in wastewater after treating with sulfuric acid and MgO + 85% H_3PO_4.

Parameter	Wastewater + H_2SO_4	Wastewater + sulfuric acid + MgO + 85% H_3PO_4			
		Adjusting pH with NaOH	Adjusting pH with KOH	Adjusting pH with $Ca(OH)_2$	Effluent standard value[a]
COD (mg/L)	1354±49	1564±55	1320±12	1488±15	120-400
pH	1.87±0	8.81±0.2	8.50±0	8.74±0.01	5.5-9
TKN (mg/L)	369±27	57±6	44±8	49±7	100-200
NH_4^+−N (mg/L)	295±23	35±4	32±7	29.41±9	-
NO_2^-−N (mg/L)	-	0.013±0.004	0.05±0.002	0.029±0.001	-
NO_3^-−N (mg/L)	-	12.34 ±2	10.18±2.55	11.50±1.1	-
SS	32±6	59±7	40±7	66±13	50-150
TDS	11345±162	13940±119	14750±212	19932±57	3000-5000

Source: [a]Industrial Effluent Standard from Notification the Ministry the Ministry of Science, Technology and Environment, 1996, Thailand.

Table 4.16 Phytoremediation of wastewater by sulfuric acid and $MgO + H_3PO_4$ coupled with *C. alternifolius* for 3 days.

Parameter	Wastewater (control)			Wastewater + Soil + Plant		
	NaOH	KOH	$Ca(OH)_2$	NaOH	KOH	$Ca(OH)_2$
COD (mg/L)	1252±49	1092±5	950±26	554±24	495±8	329±37
pH	8.48±0.036	8.37±0.02	8.44±0.02	7.83±0.021	7.09±0.01	7.04±0.01
TKN (mg/L)	45.08±3.06	36±7	39±13	20.10±1.85	20±7	19±7
NH_4^+−N (mg/L)	17.54±1.12	19.61±1.51	32±1	16.06±0.87	17.24±1.86	13±2
NO_2^-−N (mg/L)	0.03±0.005	0.02±0.001	0.05±0.003	0.36±0.126	0.67±0.027	0.204±0.001
NO_3^-−N (mg/L)	11.60±1.64	6.98±1.99	8.13±0.40	4.72±0.38	1.069±0.33	2.26±0.25

4.7 Treatment of synthetic monoethanolamine, diethanolamine, and triethanolamine wastewater by *C. alternifolius*

4.7.1 Efficiency of COD removal

Remaining COD concentrations of sterilized MEA, DEA, and TEA were still constant while non-sterilized samples slowly decreased. This could be due to microbial activity. Moreover, *C.alternifolius* could reduce COD in synthetic ethanolamines wastewater. The results showed that the COD of synthetic MEA wastewater decreased below the standard effluent of Thailand (less than 120 mg/L) by day 12 (Fig. 4.28a), a level equal to 97% COD removal. Meanwhile, DEA and TEA were still higher than the standard effluent at about 424 mg/L and 586 mg/L that was equal to 70% and 61% COD removal, respectively (Fig. 4.28b-c). However, the efficiency of COD removal increased with an increase of the amount of plants and the period of time.

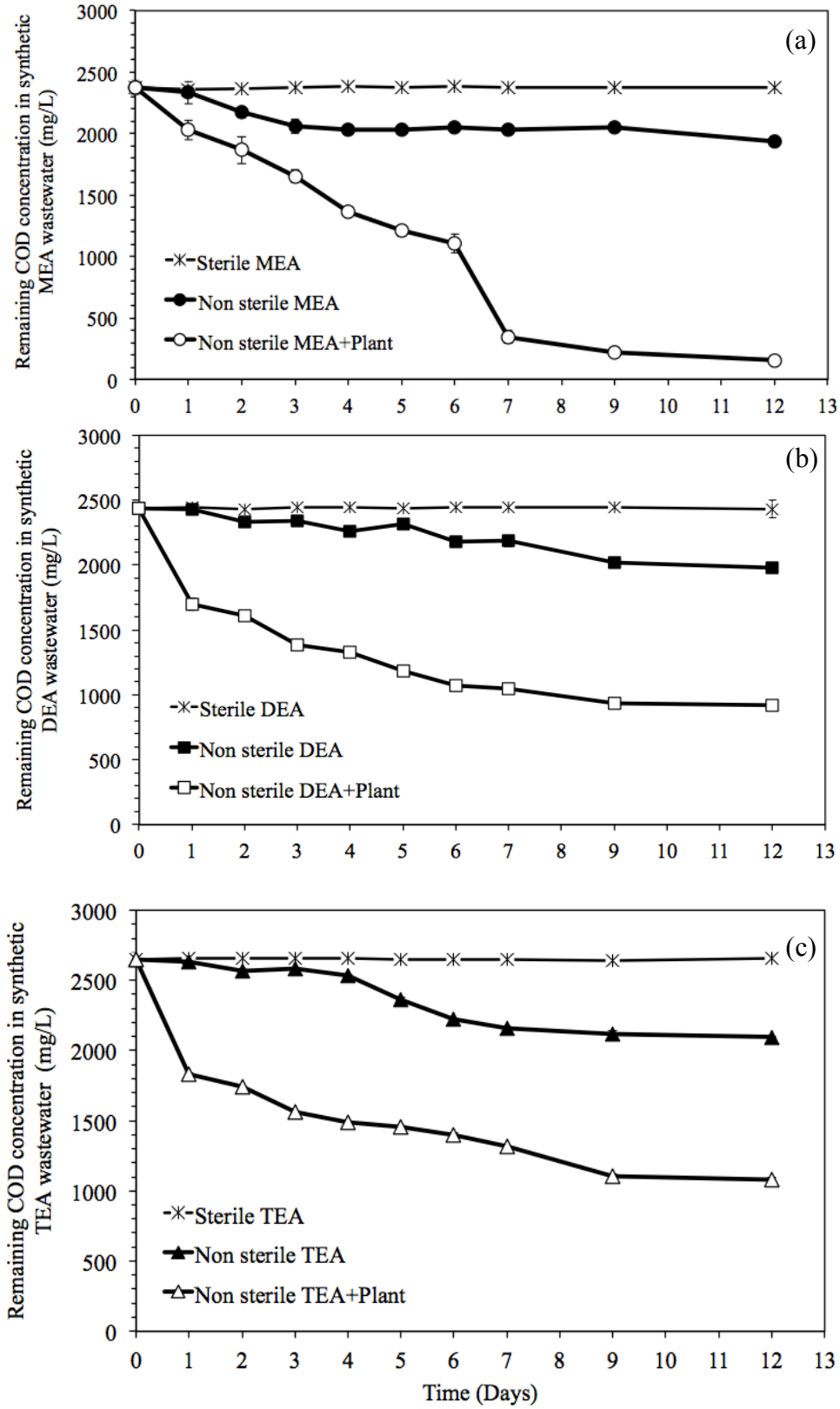


Figure 4.28 Remaining COD concentrations in synthetic MEA (a), DEA (b), and TEA (c) wastewater after treatment by *C. alternifolius*.

4.7.2 Monoethanolamine, diethanolamine, and triethanolamine removal by *C. alternifolius*

The initial concentration of MEA, DEA, and TEA was about 1400 mg/L in a volume of 2 L, which was equivalent to 47, 27, and 19 mmol, respectively. At the end of the 12-day exposure period, under plant-free conditions of non-sterile MEA, DEA, and TEA were removed—about 9%, 18%, and 12%, respectively, while sterile samples were still unchanged (Fig. 4.29). This confirmed that ethanolamines were degraded by microorganisms. Many researchers reported that microorganisms could degrade ethanolamines. They were capable of utilizing ethanolamines as sole carbon and energy sources. For example, triethanolamine was converted via triethanolamine N-oxide into diethanolamine and glycolaldehyde while diethanolamine was converted to ethanolamine and glycolaldehyde by a yellow gram-negative rod-shaped organism (Williams and Callely 1982). As a strictly anaerobic, gram-positive *Acetobacterium* strain LuTria3, triethanolamine was converted to acetate and ammonia (Speranza *et al*. 2006). Monoethanolamine was hydrolyzed into ammonium and acetataldehyde and acetataldehyde was then hydrolyzed to ethanol and acetic acid while ammonium was oxidized to nitrite and then nitrate (Mrklas *et al*. 2004, Ndegwa *et al*. 2004).

An initial system pH of synthetic ethanolamines wastewater was alkaline (Fig. 4.30). After 12 days of treatment, the system pH of sterile wastewater of MEA, DEA, and TEA were still constant value at pH 10.20, 9.77 and 9.41 from an initial pH at 10.20, 9.77, and 9.37, respectively. While the system pH of non-sterile wastewater of MEA, DEA, and TEA were gradually decreased to pH 9.15, 8.53, and 8.31, respectively that possible due to microorganism's activity. However, the results illustrated that microbial could negligible growth under non-sterile wastewater of ethanolamines. Bakalova et al (2008) reveals the limited ability of microbial growth at pH 9.4, the concentrations of 50 mM of MEA, DEA, and TEA were inhibited the microbial growth while at the lowest concentration of 20 mM, inhibition did not reach 50%. Therefore, the synthetic ethanolamine wastewater concentration of 1400 mg/L at pH values greater than 9.4, they might be effect to the ability of microbial growth.

After plant treatment, *C. alternifolius* showed potential to remove ethanolamines. Plant conditions could reduce MEA, DEA and TEA to 126 mg/L, 550 mg/L, and 622 mg/L—equal to 91% MEA, 61% DEA, and 56% TEA removal, respectively (Fig. 4.29). These were possible due to plant absorption and microorganisms in plant and microorganisms in wastewater. After 12 days of treatment, the system pH gradually decreased under plants grown in synthetic wastewater conditions. It decreased to neutral values. This was possibility due to the cation-anion balance by the roots when plants receiving more cation (as MEA contains an amine group) than anion were taken up. Plants will counterbalance the corresponding excess of positive charges by releasing equivalent amounts of H^+ by the roots in the rhizosphere, thereby decreasing the rhizosphere pH (Hinsinger *et al*. 2003).

The solubility value of ethanolamines showed TEA had high water solubility (6.273×10^5 mg/L) followed by DEA (3.180×10^5 mg/L) and MEA (2.465×10^5 mg/L), respectively (Lyman et al. 1982). However, Remaining MEA, DEA, and TEA concentration in the solution was confirmed that MEA was removed faster than DEA and TEA, respectively. This was possibly due to the molecular size of MEA (61.08 g/mol) was smaller than DEA (105.14 g/mol) and TEA (149.19 g/mol). Although the initial mole of MEA was higher than DEA and TEA, the amount of MEA uptake in

plants was the highest (Fig. 4.31).

Diffusion is involved in many plant process that concluding the movement of substance or nutrients toward root surfaces. To express diffusion quantitatively, a diffusive flux was considered with Fick's first law for leading to the net molecular movement as showed in equation 4.8.

$$\text{Fick's first law of diffusion}: J = -D\frac{dc}{dx} \tag{4.8}$$

Where J (mol/m^2 s) is the diffusion flux, D (m^2/s) is the diffusion coefficient, c (mol/m^3) is the concentration in dimensions, and x (m) is the position. The negative sign in this relationship indicates that particle flow occurs in a down gradient direction, i.e. from regions of higher to regions of lower concentration.

In applications, in this experiment was performed in the same an initial concentration of MEA, DEA, and TEA that were 1400 mg/L and the same conditions of temperature and pressure. The gradient itself is the rate of change of the concentration with distance (dc/dx) as a constant. Therefore, diffusivities or diffusion coefficient (*D*) of MEA, DEA, and TEA in aqueous solution was referred to as molecular diffusion. Hikita et al. (1980) measured values of the diffusivities of the three ethanolamines in aqueous solution. They reported that the diffusivities of MEA, DEA, and TEA were 11.6, 8.2, and 7.7×10^{10} m^2/s, at temperature of 25°C, respectively. These data was indicated that MEA could diffuse or move toward root surfaces fastest than DEA and TEA. It was confirmed that the molecular size of ethanolamines has an effect on its absorption. Plants could take up smaller molecules faster than larger molecules.

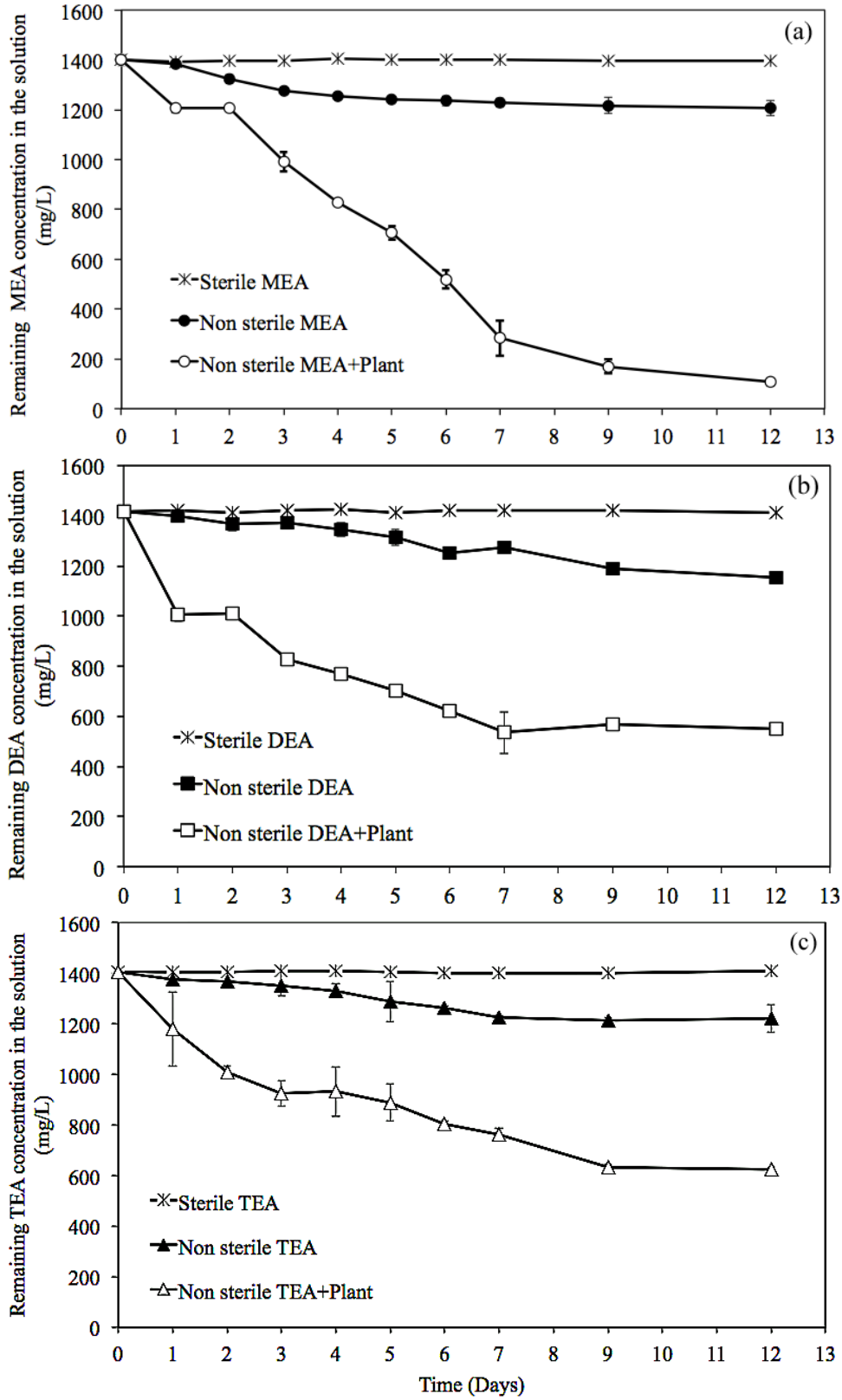


Figure 4.29 Remaining MEA (a), DEA (b), and TEA (c) concentrations in synthetic ethanolamine wastewater after treatment by *C. alternifolius* under hydroponic conditions.

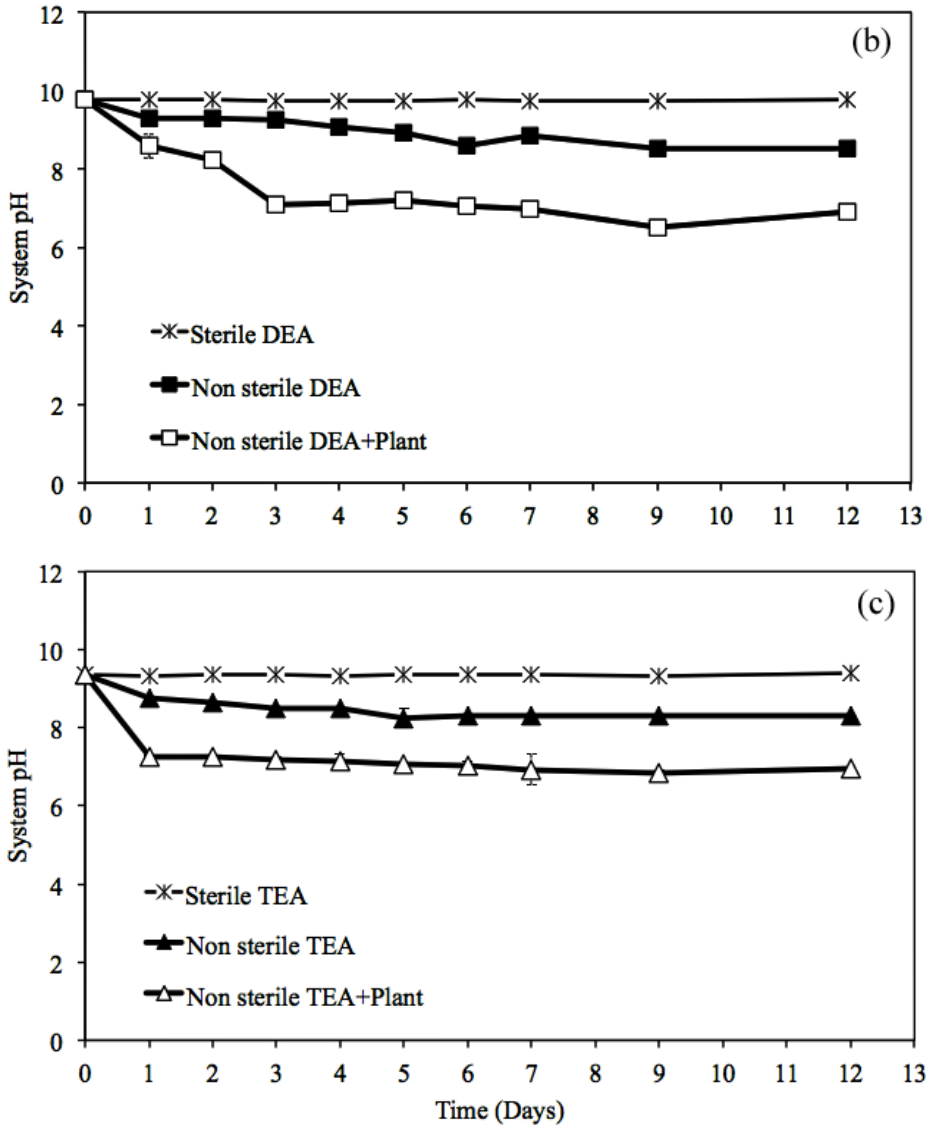


Figure 4.30 System pH of synthetic MEA (a), DEA (b), and TEA wastewater (c) in synthetic ethanolamine wastewater after treatment by *C. alternifolius* under hydroponic conditions.

When calculated in mole concentrations, the concentration of MEA (1400 mg/L) used in this experiment was higher than DEA and TEA. The results showed that the toxicity of MEA was higher than DEA followed by TEA that influence on plant growth and survival. The toxic symptoms of MEA were exhibited by the plant leaves yellowing and wilting, followed by plant stems beginning to wither and dry (Fig.4.32a). While plants were exposed to DEA, plant leaves wilted, but plant stems were still healthy and new roots and shoots appeared (Fig. 4.32b). However, no toxic symptoms of plants under TEA conditions were recorded (Fig.4.32c).

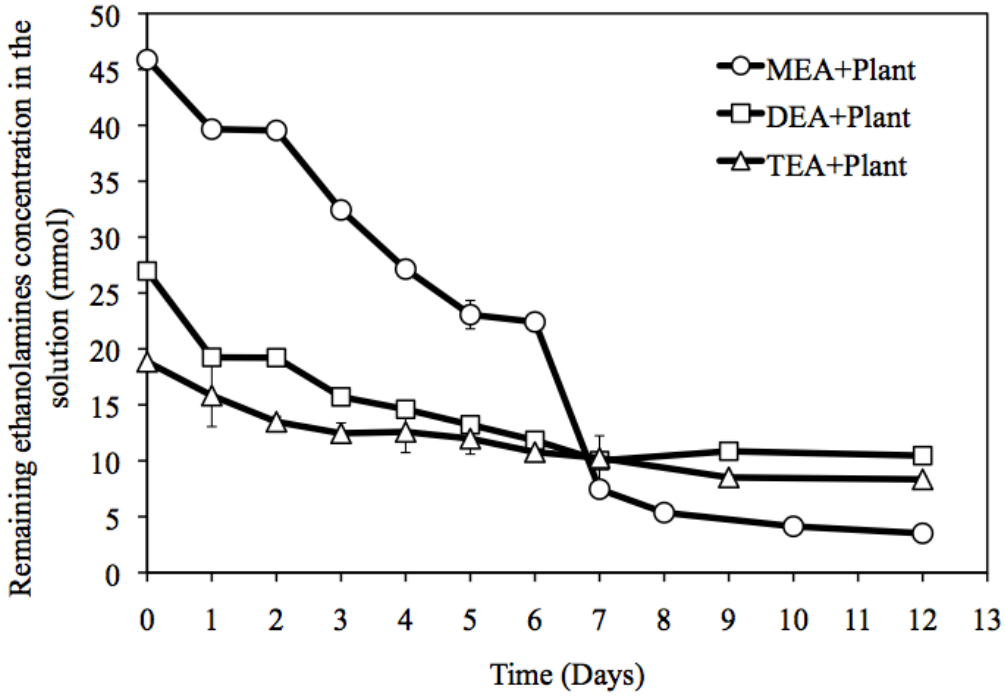


Figure 4.31 Remaining MEA, DEA, and TEA concentrations in synthetic ethanolamines wastewater after treatment by *C. alterniferlious* under soilless condition.

Figure 4.32 Appearance of the toxic symptoms of plant tissues in synthetic MEA (a), DEA (b), and TEA (c) wastewater after treatment for 12 days under hydroponic conditions.

4.7.3 Monoethanolamine degradation in plant tissues

MEA was used as a model in this experiment. We found that it was accumulated mainly in stems. It was also found that stems had the largest biomass (ratio of fresh weight of leaf:stem:root = 1:7:1). This confirmed that MEA was easily moved through roots, then accumulated in plant stems and plant leaves (Fig.4.33a). In addition, MEA was degraded to acetic acid in plant tissues (Fig. 4.33b) while under plant grown in tap water (control) acetic acid was not found.

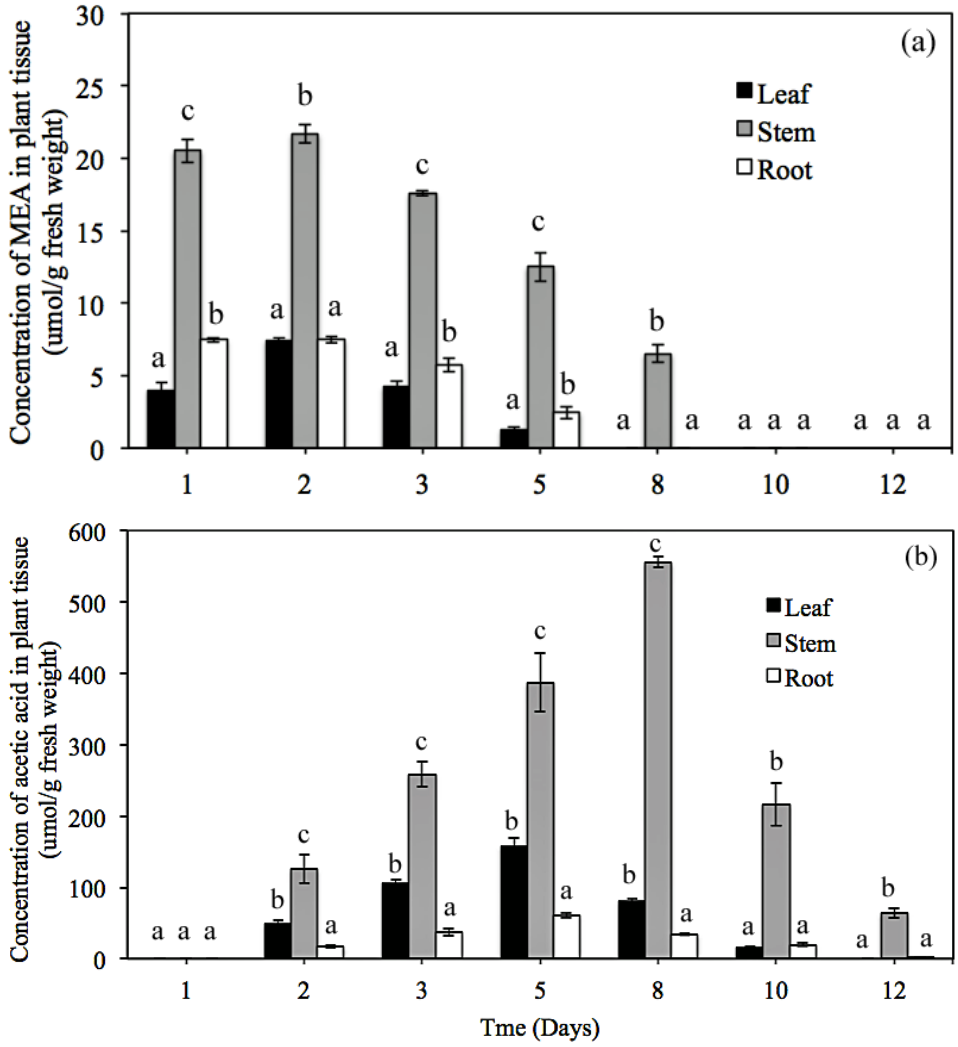


Figure 4.33 Concentration of monoethanolamine (a) and acetic acid (b) in leaves, stems, and roots after treatment of synthetic monoethanolamine wastewater by *C. alternifolius* for 12 days. Different letters above bar indicate statistically significant differences between time points (one-way ANOVA, $p < 0.05$).

4.7.4 Monoethanolamine, diethanolamine, and triethanolamine degradation on *C. alternifolius*

Due to the toxicity of ethanolamines in plants, the experiment was set at 3mM MEA, 4 mM DEA, and 40 mM TEA, and the degradation of MEA, DEA, and TEA in plant tissues was studied. The result indicated that plants can uptake MEA, DEA, and TEA in their tissues (Fig. 4.34). The results confirmed that plants could degrade TEA to DEA then MEA. Moreover, acetic acid, the intermediate degradation product, appeared at all conditions (Fig. 4.34). Base on the results, the degradation pathway of TEA by *C. alternifolius* might be demonstrated in Fig. 4.35.

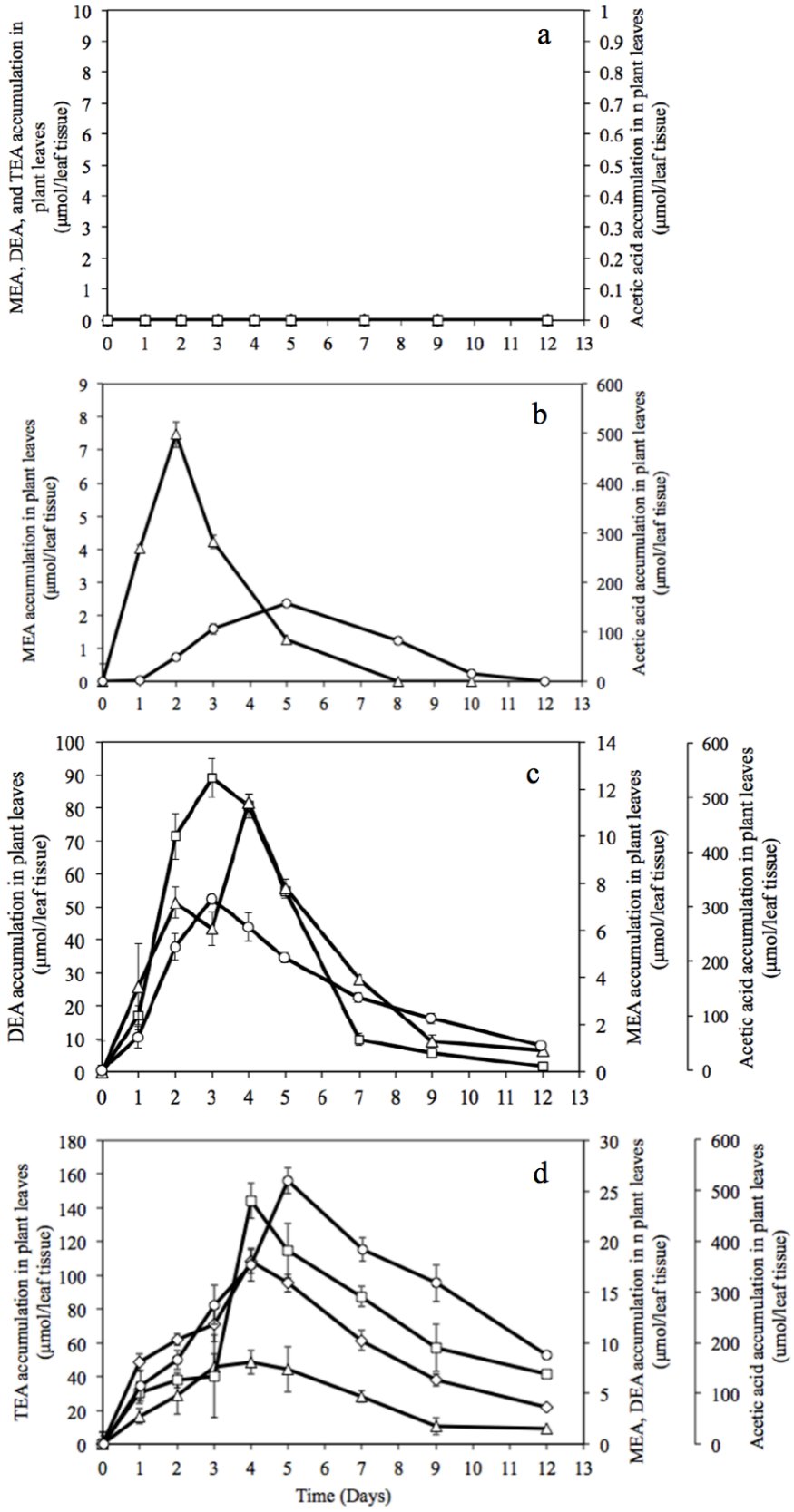


Figure 4.34 MEA (△), DEA (□), TEA (◇), and acetic acid (○) accumulation in plant leaves under plants grown in tap water (controls, a), synthetic MEA (b), DEA (c), and TEA wastewater (d) after treatment for 12 days.

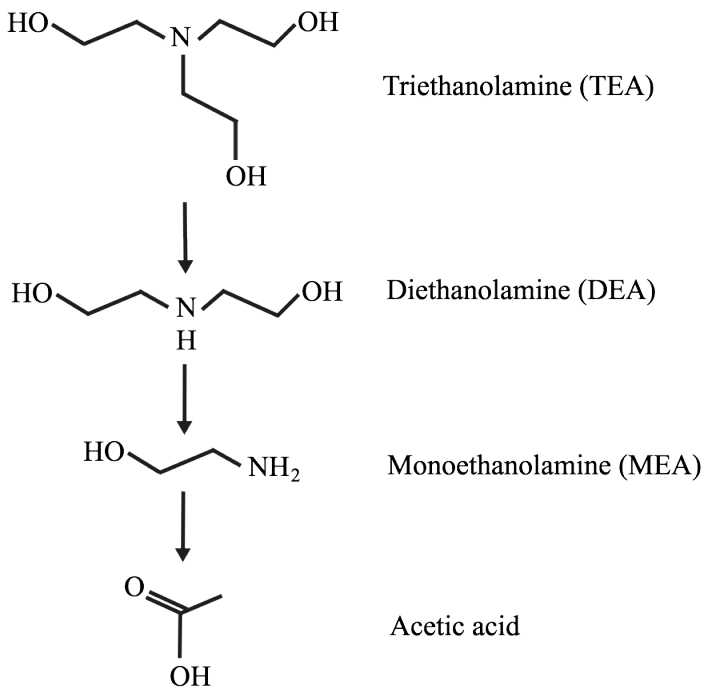


Figure 4.35 Proposed pathway for triethanolamine, diethanolamine, and ethanolamine degradation by *Cyperus alternifolius*.

4.7.5 Scanning Electron Microscope connected with Energy Dispersive X-ray spectroscopy (SEM/EDX)

From Table 4.18 shows carbon element in leaf under the synthetic MEA solution+plant conditions was higher values than control (tap water conditions) and the synthetic MEA solution+soil+plant conditions. While oxygen elements was lower than control and under the synthetic MEA solution+soil+plant conditions. The results suggested that under stress conditions, plants use oxygen for organic compounds production higher than control condition.

Moreover, sodium, magnesium, silicon, phosphorus, sulfur, potassium, and calcium under the synthetic MEA solution+plant were lower value than control and the synthetic MEA solution+soil+plant conditions. The deficiencies of these elements were affected to plant growth, yield, and yellow color and wilting of the leaf. More details are listed below (Raven et al., 1986; Elliott and Elliott, 2001; Hopkins 2004).

Sodium deficiency in the leaf of MEA condition was affected to photosynthetic reaction that effect to wilting leaves. Sodium has a role in osmotic and ionic balance, probably not essential for many plants. Required by some desert and salt marsh species. May be required by all plants that utilize C_4 photosynthesis.

Magnesium deficiency is loss function in part of the chlorophyll molecule and activator of many enzymes that affect to withdraw from the older leaves and transported to the younger leaves that are more actively growing and synthesizing chlorophyll. Silicon deficiency decreases the synthesis of proteins, chlorophyll and decrease pest and disease

resistance. Phosphorus is available in the soil solution. Phosphate deficiency is an intense green coloration of the leaves, leading to the rapid senescence and death of older leaves. Sulfur deficiency was effect to plant leaves in synthetic MEA solution could not detected sulfur that results in a generalized chlorosis of the leaf, including the tissue surrounding the vascular bundles. This is due to reduced protein synthesis rather than a direct impairment of chlorophyll synthesis. Chlorine deficiency was reduced the growth, wilting of the leaf tips, and a general chorosis. Because of chlorine has a role in photosynthetic reaction. Deficiency symptoms of potassium, the necrotic lesion begin at the older tips of the leaves and gradually progress along the margins to the younger cells near the leaf base, stem are shortened and weakened. In addition, Calcium deficiency effect to young leaves is deformed and necrotic.

Table 4.17 Scanning electron micrograph and EDX spectra of the leaves of *C. alternifolius* after cultivation in tap water (Control-Plant), the synthetic MEA solution+plant (MEA+plant), and the synthetic MEA solution+soil+plant (MEA+soil+plant) for 7 days.

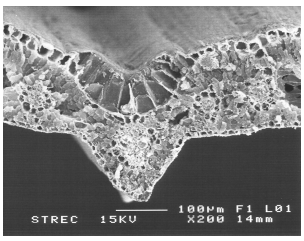
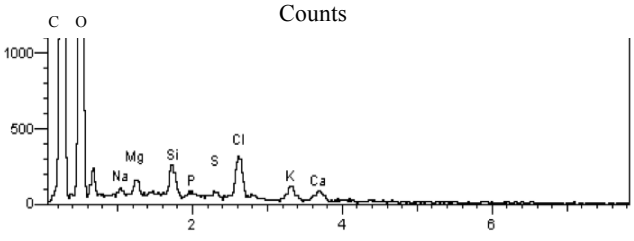
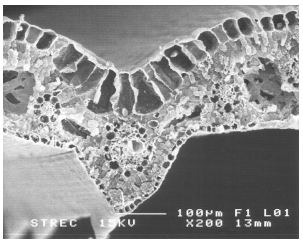
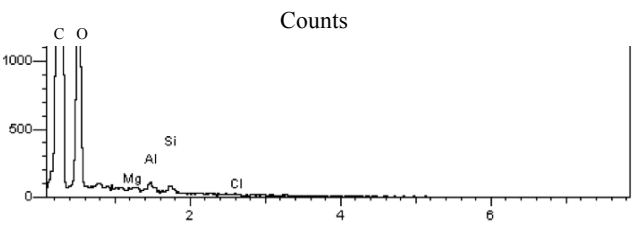
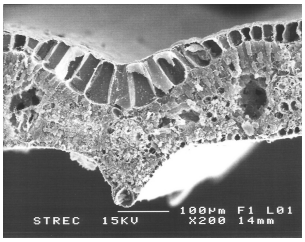
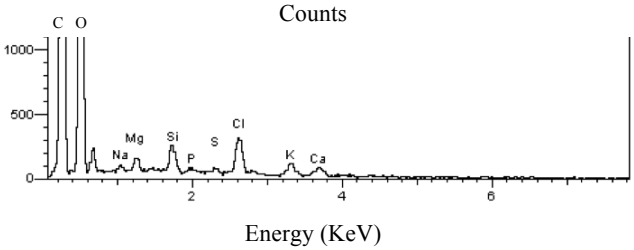
Conditions	SEM micrograph	EDX spectra
Control-Plant		
MEA+plant		
MEA+soil+plant		

Table 4.18 SEM/EDX of the leaves of *C. alternifolius* after cultivation in tab water conditions, the synthetic MEA solution+plant (MEA+plant), and the synthetic MEA solution+soil+plant (MEA+soil+plant) for 7 days.

Element (%)	Conditions		
	Control-Plant	MEA+plant	MEA+soil+plant
C	49.22	70.49	48.52
O	48.14	29.27	45.10
Na	0.23	-	0.20
Mg	0.31	0.04*	0.23
Al	-	0.11	-
Si	0.51	-	0.25
P	0.08	-	0.22
S	0.08	-	0.08
Cl	0.81	0.02*	2.70
K	0.35	-	1.65
Ca	0.26	-	1.13

*=<2 sigma

The roots under the synthetic MEA solution+plant and the synthetic MEA solution+soil+plant conditions found that sodium was lost. It had effected to osmotic and ionic balance of plants. Meanwhile, aluminium has no known physiological function in plants, but will accumulate in roots and leaves tissue under the synthetic MEA solution+plant conditions. Chlorine, under the synthetic MEA solution+plant conditions was lower value than control. It might be effect to diffusible cations, thus maintaining electrical neutrality across membrane and osmotically active solutes in the vacuole. Moreover, a calcium deficiency symptom was also appeared under the synthetic MEA solution+plant conditions, it was affected to cell walls and cell permeability especially parenchymatous cortex (Table 4.20).

Table 4.19 Scanning electron micrograph and EDX spectra of the roots of *C. alternifolius* after cultivation in tap water (Control-Plant), the synthetic MEA solution+plant (MEA+plant), and the synthetic MEA solution+soil+plant (MEA+soil+plant) for 7 days.

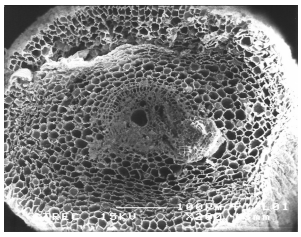
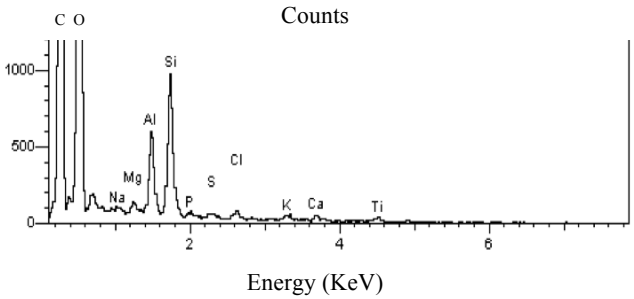
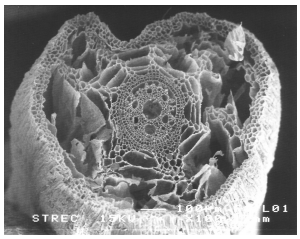
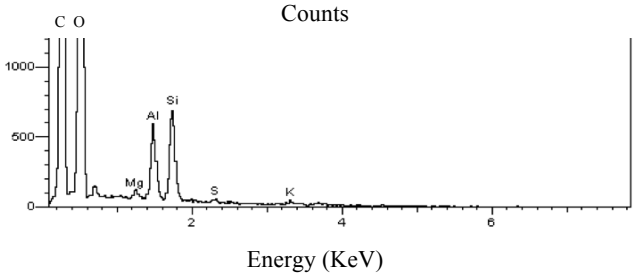
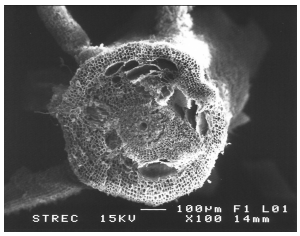
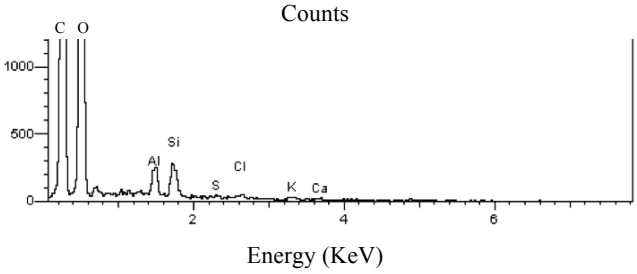
Conditions	SEM micrograph	EDX spectra
Control-Plant		
MEA+plant		
MEA+soil+ plant		

Table 4.20 SEM/EDX of the roots of *C. alternifolius* after cultivation in tab water conditions, the synthetic MEA solution+plant (MEA+plant), and the synthetic MEA solution+soil+plant (MEA+soil+plant) for 7 days.

Element (%)	Conditions		
	Control-Plant	MEA+plant	MEA+soil+plant
C	43.48	40.09	43.95
O	53.05	57.09	55.15
Na	0.15	-	-
Mg	0.16	0.14	-
Al	1.05	1.18	-
Si	1.62	1.41	0.67
P	0.05	-	-
S	0.07	0.05	0.05
Cl	0.13	-	0.07
K	0.07	0.04	0.08
Ca	0.07	-	0.03
Ti	0.11	-	-

*=<2 sigma

CHAPTER 5
CONCLUSIONS

5.1 Conclusions

In the flexography industry, pigments are the main problem of wastewater treatment because it causes dark color and increases the concentration of COD, SS, and TDS. Moreover, pigments are difficult to degrade by microorganisms. However, other contaminated compositions in wastewater such as acrylic resin, NH_4OH, MEA, and additives were affected to biodegradation as well as increasing of organic compounds and ammonium nitrogen. At present, there are many methods for treatment of ink factory wastewater but can not remove the color completely. However, activated carbon and agricultural waste products have been developed to be efficient at absorbing the color, but these treatment has a limit of economic cost and disposal of sludge waste.

The result showed that *Cyperus alternifolius* could not remove color, COD, and SS lower than the standard effluent within 7 days if using this plant directly to treat ink factory wastewater. It still had dark color and high value of COD and SS. The plant took a longer time to remove COD lower than the standard effluent (under wastewater+soil+plant conditions, COD reduced to 237 mg/L from an initial 32336 mg/L at day 45). However, the color still appeared in the system. The plants were able to adapt to survive, although its leaves wilted. If the same lot of plants were used a second time of treatment with a new lot of wastewater, the plants may not be able to withstand the toxicity of the wastewater. Pigments, acrylic resin, and a high COD value had an effect on plant absorption and survival. Therefore, the chemical precipitation of wastewater to remove pigments before coupled with plants was applied.

Based on detailed experimentation on the precipitation process, H_2SO_4 has proven to be an appropriate pretreatment option for ink factory wastewater as it provides efficient color removal. Moreover, removal of suspended solids is also very high with stable performance achieved for removal of organic substances in terms of COD. After that, phytoremediation with *C. alternifolius* can uptake organic and inorganic compounds continuously less than the effluent standards of Thailand within day 7.

However, NH_4^+−N removal was also investigated. The precipitation method with three combinations of chemicals such as $MgCl_2.6H_2O$ + $Na_2HPO_4.12H_2O$, MgO + 85% H_3PO_4, and $MgSO_4.7H_2O$+ $Ca(H_2PO_4)_2$ were studied for the removal of NH_4^+−N in a short time. The results revealed that precipitation with MgO + 85% H_3PO_4 was most efficient for NH_4^+−N removal and NaCl did not occur in the products to affect the plants. Moreover, adjusting pH with $Ca(OH)_2$ was the best alkaline because $Ca(OH)_2$ can be used as a nutrient for plant growth.

From the results mentioned above, it can be concluded that the treatment of ink factory wastewater by chemical precipitation (H_2SO_4) can reduce pigments, SS, and COD values. After that *C. alternifolius* (under soil condition) was most efficient for organic and nitrogen compounds removal, especially the color in a short time (about 7 days). Therefore, precipitation original wastewater by H_2SO_4 coupled with *C. alternifolius* was an appropriate method for ink factory wastewater. Although, the precipitation method with H_2SO_4 + MgO + 85% H_3PO_4 coupled with *C. alternifolius* (under soil condition) was most efficient for NH_4^+−N and TKN removal to 13 mg/L and 19 mg/L, respectively, at day 3 but the quantity of chemical substances that are used for

precipitation process might be too costly. However, MAP precipitation is an appropriate method for removing high ammonium concentration contaminated in wastewater.

In phytoremediation, the results of our study confirmed that the system involved plant-microbe interactions. The COD removal efficiency in the system was attributed as follows: 45% from *C. alternifolius* and microorganisms, 26% from soil absorption, 19% from microorganisms in soil, and 10% from microorganisms in wastewater. The PCR-DGGE pattern obtained indicates the structure of the community present under various conditions. The results clearly indicated differences in the microbial community composition amongst the systems studied. We found *Enterobacter* groups which are polysaccharide-producing bacterium, *Pseudomonas* species which are nitrate reducing bacteria and degrade organic solvents, *Stenotrophomonas maltophila* and *Diaphorobacter nitroreducens* which are denitrifying bacteria and nitrate reducing bacteria, and *Azospirillum* species which appeared under wastewater after precipitation by H_2SO_4 at day 0 and day 7. Meanwhile, Firmicutes groups such as *Lysinibacillus* and *Geobacter* groups (Fe (III)-reducing microorganism) were found under wastewater+soil conditions. Moreover, in this condition was also found *Diaphorobacter nitroreducens*, *Pseudomonas* species, and *Desulfitobacterium metallireducens*. However, the major microorganisms in the system under wastewater+soil+plant, we found that the *Azospirillum* species (nitrate reducing bacteria and nitrogen-fixing bacteria) were the dominant bacteria. Therefore, the results indicated that it was suitable to apply this plant to ink factory wastewater treatment.

In addition, *C. alternifolius* could remove and degrade MEA, DEA, and TEA. The molecular weight had an effect on absorption through plant tissues. The removal rate of MEA was faster than that of DEA and TEA, suggesting that the molecular size of the ethanolamines affected its rate of removal. Plants were able to degrade TEA to DEA, then to MEA, then further to acetic acid. The accumulation of ethanolamines was mainly in the plant stems. However, MEA had the highest toxicity for plants when compared to DEA and TEA.

5.2 Suggestions

The treatment of ink factory wastewater was treated by precipitation of original wastewater by H_2SO_4 followed by *C. alternifolius*. Therefore, the treatment process of wastewater can be separated into 3 steps; (1) chemical precipitation by H_2SO_4, (2) the adjusting system pH with $Ca(OH)_2$ before phytoremediation, and (3) constructed wetland as showed in Fig. 5.1.

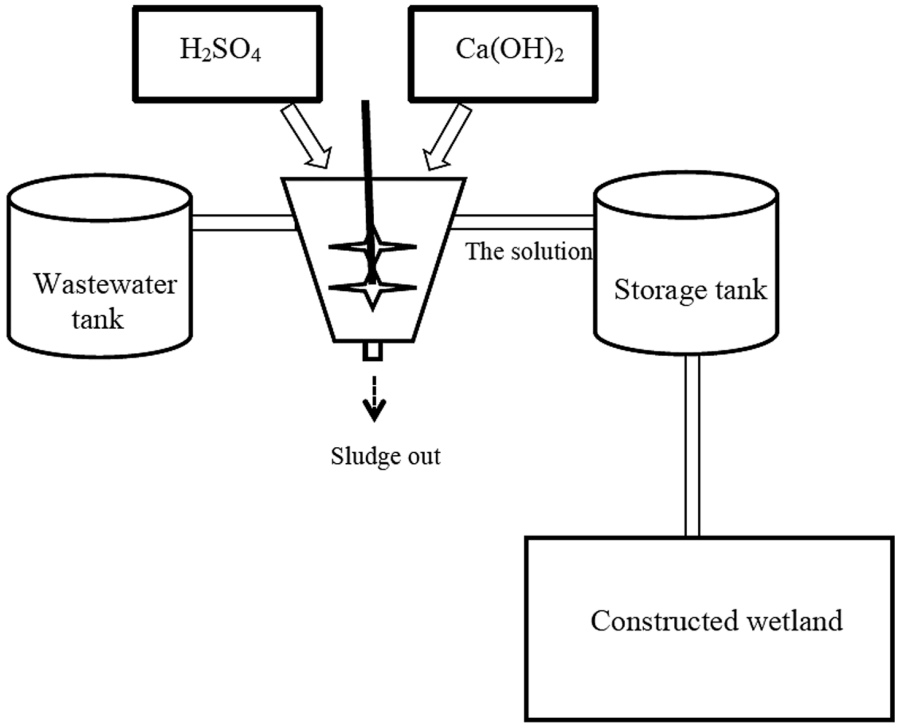


Figure 5.1 The flowchart of treatment of ink factory wastewater.

The precipitation of wastewater by H_2SO_4 coupled with *C. alternifolius* could remove COD, SS, TDS, and NH_4^+−N, as well as the color on day 7. However, the experiment was performed in a batch process with only one cycle of the wastewater through the system, through which that plant still adapts to survive. For the highest efficiency over a longer period of time, the tolerance of the plant when exposed to wastewater more than one cycle should be confirmed.

In addition, the amount of the plant and the weight of soil can increase in constructed wetland for increasing the efficiency of wastewater treatment and time period. Moreover, the result of a major microorganism in the system such as *Azospirillum* species can increase the efficiency of organic matter and nitrogen compounds removal.

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APPENDICES

Appendix A Characteristics of wastewater before and after chemical precipitation by sulfuric acid and ferric acid and then adjusting pH with calcium hydroxide.

Table A-1 Characteristics of wastewater before and after chemical precipitation by H_2SO_4 and $Fe_2(SO_4)_3$ and then adjusting pH with $Ca(OH)_2$.

Sample	Abs	Remaining COD concentration (mg/L)	%COD removal	System pH
Wastewater	12.375	32336	–	8.56
Wastewater+ H_2SO_4	0.696	1819	94	1.80
Wastewater+ H_2SO_4+ $Ca(OH)_2$	0.546	1427	96	7.12
Wastewater+$Fe_2(SO_4)_3$	0.606	1583	95	2.93
Wastewater+$Fe_2(SO_4)_3$+ $Ca(OH)_2$	0.468	1223	96	7.10

Note: All data are mean of triplicate.

Appendix B Screening of plants

Table B-1 Remaining COD concentration and system pH of treatment of ink factory wastewater by H_2SO_4 precipitation coupled with various plants for 7 days.

Time (Days)	Parameters	Treatment Conditions							
		Wastewater + H_2SO_4	Wastewater+ H_2SO_4+ *D. sanderiana* (Silver)	Wastewater+ H_2SO_4+ *D. sanderiana* (Gold)	Wastewater+ H_2SO_4+ *D. sanderiana* (Green)	Wastewater+ H_2SO_4+ *C. alternifolius*	Wastewater+ H_2SO_4+ *E. cordifolius*	Wastewater+ H_2SO_4+ *A. calamus*	Wastewater+ H_2SO_4+ *T. geniculata*
0	COD (mg/L)	1114	1114	1114	1114	1114	1114	1114	1114
	SD	6.50	6.50	6.50	6.50	6.50	6.50	6.50	6.50
	pH	6.66	6.66	6.66	6.66	6.66	6.66	6.66	6.66
	SD	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
1	COD (mg/L)	1042	1033	1000	1040	926	911	974	981
	SD	18.31	20.92	15.69	10.46	5.23	5.23	15.69	52.30
	pH	7.00	6.93	6.96	6.69	6.66	6.99	7.20	6.93
	SD	0.10	0.06	0.08	0.11	0.11	0.02	0.08	0.08
2	COD (mg/L)	1040	1018	998	1005	826	904	968	961
	SD	10.46	26.15	18.31	13.08	52.30	5.23	13.08	65.38
	pH	7.03	6.90	6.80	6.20	6.95	6.85	6.71	6.56
	SD	0.04	0.10	0.16	0.46	0.08	0.11	0.10	0.20
3	COD (mg/L)	935	906	853	974	669	549	624	905
	SD	86.30	13.95	25.89	3.74	17.43	94.15	50.56	12.20
	pH	7.04	7.24	7.27	7.04	6.87	7.00	6.88	6.73
	SD	0.06	0.05	0.22	0.09	0.16	0.33	0.13	0.30

Note: All data are mean of triplicate.

Table B-1 Remaining COD concentration and system pH of treatment of ink factory wastewater by H_2SO_4 precipitation coupled with various plants for 7 days (cont.).

Time (Days)	Parameters	Treatment Conditions							
		Wastewater + H_2SO_4	Wastewater+ H_2SO_4+ *D. sanderiana* (Silver)	Wastewater+ H_2SO_4+ *D. sanderiana* (Gold)	Wastewater+ H_2SO_4+ *D. sanderiana* (Green)	Wastewater+ H_2SO_4+ *C. alternifolius*	Wastewater+ H_2SO_4+ *E. cordifolius*	Wastewater+ H_2SO_4+ *A. calamus*	Wastewater+ H_2SO_4+ *T. geniculata*
4	COD (mg/L)	887	896	895	907	645	487	587	884
	SD	54.92	10.46	22.66	25.89	17.43	38.36	5.23	31.38
	pH	7.47	7.32	6.98	7.06	7.00	7.05	5.17	7.20
	SD	0.06	0.09	0.04	0.48	0.01	0.04	0.14	0.23
5	COD (mg/L)	878	876	843	892	530	470	544	775
	SD	26.15	27.90	29.64	54.05	12.20	62.76	20.92	80.20
	pH	7.13	7.51	7.33	7.17	7.04	6.35	7.07	5.11
	SD	0.01	0.09	0.06	0.02	0.05	0.09	0.09	0.18
6	COD (mg/L)	861	860	821	876	426	398	503	536
	SD	28.77	4.74	40.10	45.33	19.18	90.66	92.40	8.00
	pH	7.08	7.47	7.20	7.15	6.23	6.89	6.81	5.95
	SD	0.03	0.18	0.01	0.03	0.04	0.04	0.06	0.04
7	COD (mg/L)	855	811	785	855	380	388	481	495
	SD	26.15	1.74	20.92	47.07	15.69	94.15	76.35	12.20
	pH	7.10	7.32	7.24	7.10	7.08	6.28	7.07	5.22
	SD	0.01	0.06	0.04	0.08	0.03	0.04	0.06	0.08

Note: All data are mean of triplicate.

Appendix C Treatment of original wastewater by *C. alternifolius*.

Appendix C-1 Study of COD removal

Table C-1.1 Remaining COD concentration from original wastewater after treatment by *C. alternifolius* under various conditions (wastewater, wastewater+soil, wastewater+plant and wastewater+soil+plant) for 45 days.

Treatment conditions	Remaining COD concentration (mg/L)																	
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 9	Day 11	Day 15	Day 18	Day 23	Day 25	Day 30	Day 33	Day 37	Day 45
W	32336	26731	24000	23713	23391	21677	20644	20344	20134	19702	18901	18361	17173	17038	16876	16768	15482	15419
SD	421	245	184	1673	760	270	339	233	296	879	3217	1573	584	284	234	81	392	1318
WS	32336	23212	22610	21400	19745	19927	18891	16427	15391	9142	4071	2082	1698	1176	899	807	927	885
SD	421	309	677	611	545	2600	1195	406	1784	1462	3001	550	246	27	93	124	86	68
WP	32336	24144	22968	21753	19570	19211	18306	17927	17837	17471	17220	15370	15213	13501	14122	13798	13283	12950
SD	421	413	1178	611	62	563	686	680	1666	1452	742	2341	1018	966	939	939	692	726
WSP	32336	21498	18852	18500	15249	12427	10698	8106	8142	6284	3376	2511	1769	990	711	760	514	237
SD	421	1013	670	1160	4959	3467	1131	506	1789	1781	260	984	823	333	266	221	78	63

Note: W=wastewater, WS = wastewater+soil, WP = wastewater+plant, WSP = wastewater+soil+plant. All data are mean of triplicate.

Table C-1.2 System pH from original wastewater after treatment by *C. alternifolius* under various conditions (wastewater, wastewater+soil, wastewater+plant and wastewater+soil+plant) for 45 days.

Treatment conditions	Remaining COD concentration (mg/L)																	
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 9	Day 11	Day 15	Day 18	Day 23	Day 25	Day 30	Day 33	Day 37	Day 45
W	8.68	7.88	7.74	7.71	7.56	7.68	7.65	7.66	7.60	7.55	7.79	7.76	7.88	7.75	7.77	7.82	7.80	7.55
SD	0.02	0.11	0.03	0.02	0.01	0.06	0.08	0.02	0.02	0.05	0.15	0.07	0.09	0.04	0.04	0.04	0.03	0.04
WS	8.68	8.07	7.63	7.62	7.40	7.39	7.47	7.55	7.53	7.66	8.05	7.70	8.34	8.38	8.28	8.38	8.39	8.30
SD	0.02	0.24	0.01	0.11	0.03	0.08	0.13	0.05	0.03	0.03	0.23	0.05	0.05	0.05	0.04	0.03	0.02	0.08
WP	8.68	7.72	7.59	7.58	7.49	7.59	7.55	7.49	7.61	7.67	7.81	7.59	7.70	7.69	7.55	7.52	7.58	7.37
SD	0.02	0.04	0.06	0.04	0.07	0.11	0.06	0.07	0.04	0.02	0.10	0.10	0.04	0.09	0.09	0.18	0.04	0.11
WSP	8.68	7.62	7.42	7.33	7.25	7.48	7.35	7.51	7.64	7.81	8.34	7.86	8.18	8.06	7.91	7.99	8.24	7.95
SD	0.02	0.01	0.06	0.07	0.07	0.30	0.12	0.09	0.10	0.03	0.03	0.18	0.04	0.01	0.04	0.11	0.07	0.05

Note: W=wastewater, WS = wastewater+soil, WP = wastewater+plant, WSP = wastewater+soil+plant. All data are mean of triplicate.

Appendix C-2 Study of monoethanolamine, ethanol, and acetic acid removal from ink factory wastewater.

Table C-2.1 Monoethanolamine (a), acetic acid (b), and ethanol (c) concentrations in wastewater after treatment by *C. alternifolius* under various conditions for 15 days.

(a)

Treatment conditions	Remaining MEA concentration in wastewater (mg/L)							
	Day 0	Day 1	Day 3	Day 5	Day 7	Day 9	Day 12	Day 15
W	539.80	523.48	376.88	339.34	165.12	64.70	20.29	0.00
SD	21.00	28.82	79.18	35.32	11.33	15.05	6.47	0.00
WS	539.80	477.41	183.46	143.28	44.99	18.66	0.00	0.00
SD	21.00	42.90	12.99	32.80	14.46	17.35	0.00	0.00
WP	539.80	349.26	144.37	77.00	0.00	0.00	0.00	0.00
SD	21.00	41.37	33.97	0.00	0.00	0.00	0.00	0.00
WSP	539.80	300.89	105.00	0.00	0.00	0.00	0.00	0.00
SD	21.00	49.88	45.00	0.00	0.00	0.00	0.00	0.00

(b)

Treatment conditions	Ethanol concentration in wastewater (mg/L)							
	Day 0	Day 1	Day 3	Day 5	Day 7	Day 9	Day 12	Day 15
W	85.37	47.10	42.04	50.90	24.40	19.21	0.00	0.00
SD	10.00	2.82	13.25	9.19	5.50	3.53	0.00	0.00
WS	85.37	38.72	42.02	29.32	17.13	8.33	0.00	0.00
SD	10.00	7.04	10.26	3.94	0.94	2.97	0.00	0.00
WP	85.37	109.42	199.14	121.04	4.11	0.00	0.00	0.00
SD	10.00	6.79	24.48	37.49	0.48	0.00	0.00	0.00
WSP	85.37	66.14	61.93	53.19	22.45	7.55	0.00	0.00
SD	10.00	4.92	5.24	11.94	4.35	1.89	0.00	0.00

(c)

Treatment conditions	Acetic concentration in wastewater (mg/L)							
	Day 0	Day 1	Day 3	Day 5	Day 7	Day 9	Day 12	Day 15
W	723.40	627.72	1458.53	403.12	26.19	0.00	0.00	0.00
SD	51.00	43.85	30.81	17.53	4.08	0.00	0.00	0.00
WS	723.40	586.93	1570.97	908.22	512.11	167.55	0.00	0.00
SD	51.00	12.40	208.14	179.34	77.71	32.84	0.00	0.00
WP	723.40	754.79	2900.19	3620.4	1853.54	1060.47	661.05	206.10
SD	51.00	48.72	518.09	788.40	52.53	325.98	222.32	43.97
WSP	723.40	1307.4	2680.93	3507.4	2329.95	860.40	399.90	92.55
SD	51.00	119.55	145.82	311.99	301.20	186.08	49.50	10.40

Note: W=wastewater, WS = wastewater+soil, WP = wastewater+plant, WSP = wastewater+soil+plant. All data are mean of triplicate.

Appendix C-3 Study of total dissolved solids (TDS).

Table C-3.1 Remaining total dissolved solids (TDS) after treatment by *C. alternifolius* under various conditions for 45 days.

Treatment conditions	Remaining TDS in wastewater (mg/L)				
	Day 0	Day 7	Day 15	Day 25	Day 45
W	12581	10681	9732	9601	9113
SD	553	412	28	460	533
WS	12581	6972	1874	1268	1180
SD	553	1394	136	16	80
WP	12581	10587	8896	8090	6543
SD	553	973	598	499	1166
WSP	12581	3157	1581	1094	717
SD	553	1054	245	40	78

Note: W=wastewater, WS = wastewater+soil, WP = wastewater+plant, WSP = wastewater+soil+plant. All data are mean of triplicate.

Appendix D Treatment of ink factory wastewater by H_2SO_4 precipitation coupled with *C. alternifolius.*

Appendix D-1 Remaining COD, total Kjedalh nitrogen (TKN), total nitrogen (TN) from ink factory wastewater by H_2SO_4 precipitation coupled with *C. alternifolius*.

Table D-1.1 Remaining COD from ink factory wastewater after treatment by H_2SO_4 precipitation coupled with *C. alternifolius* under various conditions for 100 days.

Treatment conditions	Remaining COD concentration in wastewater (mg/L)								
	Day 0	Day 1	Day 3	Day 7	Day 13	Day 19	Day 26	Day 33	Day 39
W	987	820	706	410	217	178	179	246	177
SD	86	41	43	21	9	7	3	54	9
WS	987	714	645	350	171	138	114	155	98
SD	86	58	48	21	19	7	9	4	4
WP	987	565	311	238	162	158	152	126	91
SD	86	25	24	9	9	5	8	4	8
WSP	987	554	277	171	117	111	91	91	51
SD	86	18	8	17	5	5	4	3	7

Table D-1.1 Remaining COD from ink factory wastewater after treatment by H_2SO_4 precipitation coupled with *C. alternifolius* under various conditions for 100 day (cont.).

Treatment conditions	Remaining COD concentration in wastewater (mg/L)							
	Day 46	Day 54	Day 61	Day 69	Day 76	Day 83	Day 90	Day 100
W	212	167	158	100	106	85	97	89
SD	9	3	9	12	3	8	5	3
WS	117	101	97	66	68	45	78	86
SD	4	8	18	9	3	8	3	8
WP	101	84	100	86	76	74	78	74
SD	4	15	7	12	3	17	4	6
WSP	64	55	78	55	55	36	73	67
SD	13	9	20	9	7	23	3	7

Note: W=wastewater, WS = wastewater+soil, WP = wastewater+plant, WSP = wastewater+soil+plant. All data are mean of triplicate.

Table D-1.2 Remaining TKN from ink factory wastewater after treatment by H_2SO_4 precipitation coupled with *C. alternifolius* under various conditions for 100 days.

Treatment conditions	Remaining TKN in wastewater (mg/L)						
	Day 0	Day 7	Day 13	Day 19	Day 26	Day 33	Day 39
W	183.45	96.10	77.37	66.71	113.83	103.01	88.07
SD	1.32	1.85	2.20	1.07	5.97	5.78	3.05
WS	183.45	73.94	57.94	59.45	62.12	61.95	59.89
SD	1.32	2.81	2.66	0.78	0.97	1.64	1.32
WP	183.45	90.06	70.46	46.17	74.18	91.49	46.64
SD	1.32	2.58	1.96	3.19	1.17	4.92	1.02
WSP	183.45	52.42	44.99	41.87	43.07	47.25	30.41
SD	1.32	3.28	1.03	2.12	0.84	5.64	2.64

Table D-1.2 Remaining TKN from ink factory wastewater after treatment by H_2SO_4 precipitation coupled with *C. alternifolius* under various conditions for 100 days (cont.).

Treatment conditions	Remaining TKN in wastewater (mg/L)						
	Day 46	Day 54	Day 61	Day 69	Day 76	Day 90	Day 100
W	128.98	96.50	23.14	47.02	47.35	32.55	57.13
SD	6.76	4.74	2.12	4.25	1.10	4.29	3.57
WS	64.44	43.75	29.85	26.79	31.07	38.74	31.47
SD	0.76	1.97	0.64	0.44	3.68	2.85	2.30
WP	44.13	36.51	26.97	43.24	39.20	48.98	57.01
SD	0.95	0.70	1.33	2.63	1.14	3.87	3.75
WSP	27.35	24.47	23.50	22.20	24.11	23.79	22.11
SD	0.75	1.24	0.87	0.97	3.21	4.55	1.86

Note: W=wastewater, WS = wastewater+soil, WP = wastewater+plant, WSP = wastewater+soil+plant. All data are mean of triplicate.

Table D-1.3 Remaining TN from ink factory wastewater after treatment by H_2SO_4 precipitation coupled with *C. alternifolius* under various conditions for 100 days.

Treatment conditions	Remaining TN in wastewater (mg/L)						
	Day 0	Day 7	Day 13	Day 19	Day 26	Day 33	Day 39
W	183.45	96.10	77.37	66.71	113.83	103.01	88.07
SD	1.32	1.85	2.20	1.07	5.97	5.78	3.05
WS	183.45	73.94	57.94	59.45	62.12	61.95	59.89
SD	1.32	2.81	2.66	0.78	0.97	1.64	1.32
WP	183.45	90.06	70.46	46.17	74.18	91.49	46.64
SD	1.32	2.58	1.96	3.19	1.17	4.92	1.02
WSP	183.45	52.42	44.99	41.87	43.07	47.25	30.41
SD	1.32	3.28	1.03	2.12	0.84	5.64	2.64

Table D-1.3 Remaining TN from ink factory wastewater after treatment by H_2SO_4 precipitation coupled with *C. alternifolius* under various conditions for 100 days (cont.).

Treatment conditions	Remaining TN in wastewater (mg/L)						
	Day 46	Day 54	Day 61	Day 69	Day 76	Day 90	Day 100
W	128.98	96.50	23.14	47.02	47.35	32.55	57.13
SD	6.76	4.74	2.12	4.25	1.10	4.29	3.57
WS	64.44	43.75	29.85	26.79	31.07	38.74	31.47
SD	0.76	1.97	0.64	0.44	3.68	2.85	2.30
WP	44.13	36.51	26.97	43.24	39.20	48.98	57.01
SD	0.95	0.70	1.33	2.63	1.14	3.87	3.75
WSP	183.45	52.42	44.99	41.87	43.07	47.25	30.41
SD	0.75	1.24	0.87	0.97	3.21	4.55	1.86

Note: W=wastewater, WS = wastewater+soil, WP = wastewater+plant, WSP = wastewater+soil+plant. All data are mean of triplicate.

Appendix D-2 Treatment of BOD from ink factory wastewater by H_2SO_4 precipitation coupled with *C. alternifolius.*

Table D-2.1 Remaining BOD from ink factory wastewater after treatment by H_2SO_4 precipitation coupled with *C. alternifolius* under various conditions for 7 days.

Sample	% Dilution	Sodium thiosulfate titrant (mL)		BOD_5 (mg/L)			
		I	II	I	II	Average	SD
Original wastewater	0.2	7.05	7.00	237.50	262.50	257.50	10.84
	0.5	6.25	6.20	255.00	265.00		
	1	4.70	4.80	267.50	257.50		
Wastewater+ H_2SO_4	0.5	6.60	6.85	85.00	135.00	93.33	21.56
	1	6.55	6.70	87.50	72.50		
	2	6.65	6.80	93.75	86.25		
WS	0.5	7.65	7.72	35.00	21.00	28.08	7.90
	1	7.40	7.35	32.50	37.50		
	2	7.30	7.20	18.75	23.75		
WP	0.5	7.69	7.69	6.50	6.70	6.87	0.49
	1	7.60	7.61	7.50	6.90		
	2	7.40	7.65	6.25	7.37		
WSP	1	7.64	7.67	8.30	5.40	7	1.29
	2	6.60	6.59	6.20	6.70		
	5	7.14	7.25	8.80	6.60		

Note: W=wastewater, WS = wastewater+soil, WP = wastewater+plant, WSP = wastewater+soil+plant. All data are mean of triplicate.

Appendix D-3 Treatment of inorganic nitrogen compounds from ink factory wastewater by H_2SO_4 precipitation coupled with *C. alternifolius*.

Table D-3.1 Remaining nitrite nitrogen from ink factory wastewater after treatment by H_2SO_4 precipitation coupled with *C. alternifolius* under various conditions for 100 days.

Treatment conditions	Remaining nitrite nitrogen in wastewater (mg/L)						
	Day 0	Day 7	Day 13	Day 19	Day 26	Day 33	Day 39
W	0.014	0.014	0.029	3.976	13.153	42.106	55.657
SD	0.001	0.001	0.004	0.050	0.523	4.052	1.830
WS	0.014	0.007	0.012	0.908	7.910	7.766	18.791
SD	0.001	0.000	0.003	0.006	0.185	0.663	0.457
WP	0.014	1.421	0.787	1.923	20.826	24.649	0.038
SD	0.001	0.204	0.076	0.050	0.421	2.046	0.002
WSP	0.014	0.799	2.087	3.426	8.644	10.884	17.855
SD	0.001	0.003	0.576	0.045	0.291	1.024	3.148

Table D-3.1 Remaining nitrite nitrogen from ink factory wastewater after treatment by H_2SO_4 precipitation coupled with *C. alternifolius* under various conditions for 100 days (cont.).

Treatment conditions	Remaining nitrite nitrogen in wastewater (mg/L)						
	Day 46	Day 54	Day 61	Day 69	Day 76	Day 90	Day 100
W	58.967	33.187	7.220	0.036	0.038	0.059	0.031
SD	1.560	2.654	0.407	0.002	0.002	0.001	0.003
WS	21.250	13.109	0.020	0.007	0.003	0.005	0.004
SD	0.191	0.215	0.001	0.001	0.000	0.001	0.001
WP	0.024	0.014	0.006	0.009	0.006	0.009	0.009
SD	0.001	0.002	0.001	0.001	0.001	0.002	0.002
WSP	20.562	0.081	0.010	0.007	0.002	0.006	0.005
SD	0.254	0.002	0.001	0.001	0.000	0.001	0.001

Note: W=wastewater, WS = wastewater+soil, WP = wastewater+plant, WSP = wastewater+soil+plant. All data are mean of triplicate.

Table D-3.2 Remaining nitrate nitrogen from ink factory wastewater after treatment by H_2SO_4 precipitation coupled with *C. alternifolius* under various conditions for 100 days.

Treatment conditions	Remaining nitrate nitrogen in wastewater (mg/L)						
	Day 0	Day 7	Day 13	Day 19	Day 26	Day 33	Day 39
W	27.540	2.686	4.371	5.324	70.947	24.644	5.101
SD	0.071	0.200	0.100	0.529	0.854	2.046	3.278
WS	27.540	0.857	2.021	4.066	11.923	24.234	12.017
SD	0.071	0.058	0.058	0.162	0.535	3.464	1.164
WP	27.540	3.079	5.347	0.000	33.890	43.434	21.595
SD	0.071	0.100	0.153	0.152	0.404	3.591	0.924
WSP	27.540	6.132	0.080	2.477	17.282	16.282	0.000
SD	0.071	0.100	0.058	0.100	1.010	4.072	0.000

Table D-3.2 Remaining nitrate nitrogen from ink factory wastewater after treatment by H_2SO_4 precipitation coupled with *C. alternifolius* under various conditions for 100 days (cont.).

Treatment conditions	Remaining nitrate nitrogen in wastewater (mg/L)						
	Day 46	Day 54	Day 61	Day 69	Day 76	Day 90	Day 100
W	11.866	44.313	-0.254	31.298	28.196	30.774	11.636
SD	3.539	5.000	0.651	1.405	1.124	1.233	2.082
WS	18.334	19.974	14.140	11.093	12.630	12.428	17.263
SD	0.629	2.082	0.216	0.458	0.551	0.404	0.252
WP	19.476	22.086	13.727	21.858	19.094	21.225	11.024
SD	0.500	0.458	0.208	0.808	0.361	0.208	0.351
WSP	0.000	7.886	8.290	3.959	4.398	3.428	2.995
SD	0.000	0.252	0.200	0.252	0.361	0.208	0.300

Note: W=wastewater, WS = wastewater+soil, WP = wastewater+plant, WSP = wastewater+soil+plant. All data are mean of triplicate.

Table D-3.3 Remaining ammonium nitrogen from ink factory wastewater after treatment by H_2SO_4 precipitation coupled with *C. alternifolius* under various conditions for 100 days.

Treatment conditions	Remaining ammonium nitrogen in wastewater (mg/L)						
	Day 0	Day 7	Day 13	Day 19	Day 26	Day 33	Day 39
W	145.57	77.83	66.09	52.32	29.25	14.57	8.07
SD	2.03	1.90	0.72	0.73	0.23	0.26	0.06
WS	145.57	54.22	41.71	34.05	29.19	18.69	9.43
SD	2.03	2.00	0.62	0.35	0.24	0.32	0.12
WP	145.57	64.01	43.24	37.20	19.33	7.63	5.71
SD	0.17	0.74	0.34	0.59	1.51	2.54	1.67
WSP	145.57	38.70	35.79	31.33	10.30	8.66	2.80
SD	2.03	0.91	0.80	1.21	0.31	0.16	0.19

Table D-3.3 Remaining ammonium nitrogen from ink factory wastewater after treatment by H_2SO_4 precipitation coupled with *C. alternifolius* under various conditions for 100 days (cont.).

Treatment conditions	Remaining ammonium nitrogen in wastewater (mg/L)						
	Day 46	Day 54	Day 61	Day 69	Day 76	Day 90	Day 100
W	4.30	6.67	6.96	6.77	4.22	18.34	7.26
SD	0.17	0.74	0.34	0.59	1.51	2.54	1.67
WS	4.24	5.78	8.14	8.43	12.75	7.55	7.45
SD	0.22	0.45	0.95	0.45	0.85	1.19	1.22
WP	5.63	2.94	7.06	6.57	14.81	7.45	7.26
SD	0.23	0.29	0.29	0.61	0.74	1.62	1.33
WSP	2.65	6.67	9.90	7.26	17.65	2.06	9.31
SD	0.11	0.74	0.34	0.45	1.47	1.06	1.67

Note: W=wastewater, WS = wastewater+soil, WP = wastewater+plant, WSP = wastewater+soil+plant. All data are mean of triplicate.

Table D-3.4 The profiles of pH from ink factory wastewater after that by H_2SO_4 precipitation coupled with *C. alternifolius* under various conditions for 100 days.

Treatment conditions	System pH								
	Day 0	Day 1	Day 3	Day 7	Day 9	Day 13	Day 19	Day 26	Day 33
W	7.05	7.90	7.92	8.10	8.13	8.00	7.78	7.49	7.63
SD	0.01	0.09	0.05	0.03	0.03	0.07	0.01	0.01	0.01
WS	7.05	7.97	7.96	8.05	8.23	8.04	7.58	7.67	7.67
SD	0.01	0.03	0.04	0.05	0.04	0.02	0.05	0.10	0.02
WP	7.05	7.92	7.98	8.12	8.13	8.09	8.03	7.72	8.00
SD	0.01	0.07	0.01	0.04	0.02	0.02	0.01	0.05	0.03
WSP	7.05	7.95	7.96	7.98	8.01	7.97	7.74	7.58	7.62
SD	0.01	0.03	0.06	0.06	0.06	0.02	0.01	0.01	0.04

Table D-3.4 The profiles of pH from ink factory wastewater after treatment by H_2SO_4 precipitation coupled with *C. alternifolius* under various conditions for 100 days (cont.).

Treatment conditions	System pH								
	Day 39	Day 46	Day 54	Day 61	Day 69	Day 76	Day 83	Day 90	Day 100
W	7.48	7.35	7.87	7.93	8.02	8.15	8.21	8.05	7.82
SD	0.02	0.02	0.02	0.03	0.02	0.02	0.01	0.05	0.03
WS	7.47	7.27	7.66	7.62	7.57	7.81	7.90	7.90	7.85
SD	0.02	0.02	0.01	0.02	0.06	0.02	0.01	0.01	0.02
WP	7.94	7.81	8.03	8.01	8.07	8.25	8.27	8.23	8.14
SD	0.02	0.01	0.03	0.02	0.03	0.01	0.02	0.03	0.09
WSP	7.64	7.72	7.72	7.84	7.84	8.11	8.11	7.76	8.07
SD	0.01	0.01	0.02	0.03	0.01	0.01	0.02	0.02	0.03

Note: W=wastewater, WS = wastewater+soil, WP = wastewater+plant, WSP = wastewater+soil+plant. All data are mean of triplicate.

Table D-3.5 The profiles of dissolved oxygen (DO) from ink factory wastewater after treatment by H_2SO_4 precipitation coupled with *C. alternifolius* under various conditions for 100 days.

Treatment conditions	DO (mg/L)								
	Day 0	Day 1	Day 3	Day 7	Day 9	Day 13	Day 19	Day 26	Day 33
W	2.71	0.93	0.00	0.16	1.01	3.82	4.11	5.99	5.24
SD	0.16	0.38	0.00	0.03	0.03	0.30	0.57	0.26	0.14
WS	2.71	0.90	0.05	0.03	2.49	3.91	5.28	5.36	4.57
SD	0.16	0.07	0.02	0.03	0.08	0.36	0.13	0.25	0.58
WP	2.71	0.06	0.24	0.55	0.93	0.99	3.82	5.05	4.36
SD	0.16	0.03	0.09	0.03	0.14	0.14	0.44	0.57	0.67
WSP	2.71	0.05	0.14	1.42	1.91	1.64	2.31	3.55	3.32
SD	0.16	0.01	0.03	0.44	0.17	0.38	0.67	0.78	0.70

Table D-3.5 The profiles of dissolved oxygen (DO) from ink factory wastewater after treatment by H_2SO_4 precipitation coupled with *C. alternifolius* under various conditions for 100 days (cont.).

Treatment conditions	DO (mg/L)						
	Day 39	Day 46	Day 54	Day 61	Day 83	Day 90	Day 100
W	6.24	4.93	4.38	4.05	5.85	7.83	3.31
SD	0.25	0.19	0.21	0.45	0.47	0.19	0.29
WS	4.91	4.20	3.54	4.22	5.89	7.27	4.19
SD	0.30	0.11	0.50	0.08	0.14	0.42	0.69
WP	6.10	4.46	3.30	4.34	5.57	6.83	4.66
SD	0.19	0.06	0.07	0.13	0.14	0.26	0.03
WSP	4.69	3.40	2.75	3.27	6.34	7.02	3.47
SD	0.30	0.39	0.30	0.80	0.19	0.12	0.11

Note: W=wastewater, WS = wastewater+soil, WP = wastewater+plant, WSP = wastewater+soil+plant. All data are mean of triplicate.

Table D-3.6 The profiles of oxidation-reduction potential (ORP) from ink factory wastewater after treatment by H_2SO_4 precipitation coupled with *C. alternifolius* under various conditions for 100 days.

Treatment conditions	ORP (mg/L)								
	Day 0	Day 1	Day 3	Day 7	Day 9	Day 13	Day 19	Day 26	Day 33
W	91	27	-173	80	105	146	151	164	175
SD	2	2	48	6	2	17	1	2	1
WS	91	31	14	-116	130	150	214	172	180
SD	2	5	2	26	3	6	10	2	9
WP	91	-164	70	108	134	165	164	161	173
SD	2	32	4	3	1	8	10	1	0
WSP	91	-113	76	106	188	198	150	172	185
SD	2	18	12	29	13	6	2	1	1

Table D-3.6 The profiles of oxidation-reduction potential (ORP) from ink factory wastewater after treatment by H_2SO_4 precipitation coupled with *C. alternifolius* under various conditions for 100 days (cont.).

Treatment conditions	ORP (mg/L)							
	Day 39	Day 46	Day 54	Day 61	Day 69	Day 76	Day 90	Day 100
W	201	181	196	211	186	168	187	181
SD	12	1	4	2	1	1	2	1
WS	197	187	222	211	221	188	194	167
SD	2	2	3	1	2	2	1	52
WP	175	189	209	207	191	177	179	205
SD	1	15	2	7	1	8	1	3
WSP	189	181	198	203	207	177	202	198
SD	1	1	2	3	2	1	2	1

Note: W=wastewater, WS = wastewater+soil, WP = wastewater+plant, WSP = wastewater+soil+plant. All data are mean of triplicate.

Appendix D-4 Concentration of monoethanolamine (MEA) and acetic acid from ink production wastewater by H_2SO_4 precipitation coupled with *C. alternifolius*.

Table D-4.1 Concentration of MEA under various conditions after treatment for 9 days.

Treatment conditions	MEA concentration in wastewater (mg/L)				
	Day 0	Day 1	Day 3	Day 6	Day 9
W	39.06	18.55	0	0	0
SD	0.39	0.22	0	0	0
WS	39.06	0	0	0	0
SD	0.39	0	0	0	0
WP	39.06	0	0	0	0
SD	0.39	0	0	0	0
WSP	39.06	0	0	0	0
SD	0.39	0	0	0	0

Table D-4.2 Concentration of acetic acid under various conditions after treatment for 9 days.

Treatment conditions	Acetic acid concentration in wastewater (mg/L)				
	Day 0	Day 1	Day 3	Day 6	Day 9
W	577	508	313	52	0
SD	2	9	5	5	0
WS	577	499	329	30	0
SD	2	58	60	6	0
WP	577	254	0	0	0
SD	2	33	0	0	0
WSP	577	313	0	0	0
SD	2	4	0	0	0

Note: W=wastewater, WS = wastewater+soil, WP = wastewater+plant, WSP = wastewater+soil+plant. All data are mean of triplicate.

Appendix E Microbial communities in ink factory wastewater after treatment by H_2SO_4 precipitation coupled with *C. alternifolius.*

>AB603516 *Methanocaldococcus jannaschii* (Out group)
TCCTGCCGGAGGCCACTGCTATCGGGGTCCGACTAAGCCATGCGAGTCAAG
GGGCTCCCTTCGGGGAGCACCGGCGCACGGCTCAGTAACACGTGGCTAACC
TACCCTCGGGTGGGGGATAACCTCGGGAAACTGAGGCTAATCCCCCATAGG
GGAGGAGGTCTGGAATGATCCCTCCCCGAAAGGCGTAAGCYGCCCGAGGAT
GGGGCTGCGGCGGATTAGGTAGTTGGTGGGGTAACGGCCCACCAAGCCTAC
GATCCGTACGGGCCCTGAGAGGGGGAGCCCGGAGATGGACACTGAACACG
GTCCAGGCCCTACGGGGCGCAGCAGGCGCGAAACCTCCGCAATGCGCGAAA
GCGCGACGGGGGGACCCCGAGTGCCCACGCCCTGCGTGGGCTTTTCCGGAG
TGTAAACAGCTCCGGGAATAAGGGCTGGGCAAGTCCGGTGCCAGCAGCCGC
GGTAATACCGGCGGCCCAAGTGGTGGCCACTGTTATTGGGCCTAAAGCGTC
CGTAGCCGGCCCGGTAAGTCTCTGCTTAAATCCTGCGGCTCAACCGCAGGCT
GGCAGAGATACTGCCGGGCTTGGGACCGGGAGAGGCCGGGGGTACCCCAG
GGGTAGCGGTGAAATGCGTTGATCCCTGGGGGACCACCTGTGGCGAAGGCG
CCCGGCTGGAACGGGTCCGACGGTGAGGGACGAAGGCCAGGGGAGCAAAC
CGGATTAGATACCCGGGTAGTCCTGGCTGTAAACTCTGCGGACTAGGTGTC
GCGTCGGCTTCGGGCCGACGCGGTGCCGAAGGGAAGCCGTTAAGTCCGCCG
CCTGGGGAGTACGGTCGCAAGACTGAAACTTAAAGGAATTGGCGGGGGAG
CACTACAACGGGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGGCATCTT
ACCAGGGGCGACGGCAGGATGAAGGCCAGGTTGACGACCTTGCCAGACGC
GCCGAGAGGTGGTGCATGGCCGTCGTCAGCTCGTACCGTGAGGCGTCCTGT
TAAGTCAGGTAACGAGCGAGACCCGTGCCCCATGTTGCTACCTCCTCCTCCG
GGAGGAGGGCACTCATGGGGGACCGCCGGCGCTAAGCCGGAGGAAGGTGC
GGGCAACGACAGGTCCGCATGCCCCGAATCCCCTGGGCTACACGCGGGCTA
CAATGGCCGGGACAATGGGACGCGACCCCGAAAGGGGGAGCGAATCCCCT
AAACCCGGTCGTAGTCCGGATCGAGGGCTGTAACTCGCCCTCGTGAAGCCG
GAATCCGTAGTAATCGCGCCTCACCATGGCGCGGTGAATGCGTCCCTGCTCC
TTGCACACACCGCCCGTCACGCCACCCGAGTTGAGCCCAAGTGAGGCCCTG
TCCGCAAGGGCAGGGTCGAACTTGGGTTCAGCGAGGGGGGCGAAGTCGTAA
CAAGGTAGCCGTAGGGGAACCTGCGGCTG

>HQ663061 Gamma proteobacterium SCGC AAA024-E17 (Band No.1)
GGCGCAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTT
GTAAAGCTCTTTTGTTGGGGAGGAAGGGGTGTNGGTTAATACCNTGNGGTT
TTGACGTTACCCACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCG
GTAAT

>JN050957 *Enterobacter* sp. N0-20R2A (Band No. 2)
CAAGCCTGATCCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAA
AGTACTTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAACCTGCTGTTTTGAC
GTTACCCGCAGAATAAGCACCGGCTAACTCCGTGCC

>AF229885 *Pseudomonas* sp. 3CB6 (Band No. 3)
AATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCG
GATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCATTTGGYTAATACCCAGG
AGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGC
CGCGGTAATA

>HQ288939 *Pseudomonas aeruginosa* strain A12 (Band No. 4)
GGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGAT
TGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGT
TTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGC
GGTA

>EU620069 *Pseudomonas putida* strain GNA5 (Band No. 5)
ATTGGACAATGGGCGAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGG
TCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATA
CCTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCA
GCAGCCGCGGTAAT

>JN941348 *Diaphorobacter nitroreducens* strain AW3 (Band No. 6)
AATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGCAGGATGAAGGCCTTCG
GGTTGTAAACTGCTTTTGTACGGAACGAAAAGCCTCTTTCTAATAAAGAGG
GGTCATGACGGTACCGTAAGAATAAGCACCGGCTAACTACGTGCCAGCAGC
CGCGGTAATA

>JQ659342 *Methylobacterium populi* strain L3-774 (Band No. 7)
TGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGC
CTTAGGGTTGTAAAGCTCTTTTGTCCGGGACGATAATGACGGTACCGGAAG
AATAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACG

>HQ003420 *Acidovorax delafieldii* strain NBGD35 (Band No. 8)
TGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGATGAAGGC
CTTCGGGTTGTAAACTGCTTTTGTAACGGAAACGAAAAAGACTCTGGGTTA
ATACCTGGGGTCCCATGACGGTACCGTAAAGAAATAAGCACCGGGCTAACT
ACGTGCCAGCAGCCGCGG

>HM103365 *Enterobacter* sp. AJAR-A2 (Band No. 9)
GGGGATGCATGGGGCGCAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAG
GCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGTGGTTAAT
AACCGCAGCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGC
CAGCAGCCGCGGTAATA

>EF061452 *Stenotrophomonas maltophilia* strain DTQ-CRS31 (Band No. 10)
CGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGT
AAAGCCCTTTTGTTGGGAAAGAAATCCATCTGGTTAATACCCGGGTGGGAT
GACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGT
AAT

>EU057829 *Flexibacteraceae bacterium* CH30#7 (Band No. 11)
AAGCCTGACCCAGCCACGCCGCGTGCAGGAAGAAGGCCCTCTGGGTTGTAA
ACTGCTTTTGACTGGGAAGAAAAGACTCCTTGCGAGGAGAGTTGACGGTAC
CAGTGGAATAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAAT

>AY752939 *Enterobacter sakazakii* strain z759 (Band No. 12)
CAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTC
GGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAACCAC

AGCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAG
CCGCGGTAAT

>HM103366 *Enterobacter* sp. AJAR-A3 (Band No. 13)
AATGGGCGCAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGG
GTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAACCGCAG
CAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCC
GCGGTAATA

>FN293046 *Azospirillum* sp. Z012 (Band No. 14)
AATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTCG
GGTTGTAAAGCTCTTTCGCACACGACGATGATGACGGTAGTGTGAGAAGAA
GCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATA

>NR_042090 *Dechloromonas denitrificans* strain : ED1 (Band No. 15)
ATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGG
GTTGTAAAGCTCTTTCGGCCGGGAAGAAATCGCATGGGTTAATACCCTGTGC
GGATGACGGTACCGGCATAAGAAGCACCGGCTAACTACGTGCCAGCAGCCG
CGGTAATA

>JF508370 *Azospirillum irakense* strain L-6 (Band No. 16)
CCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTCGGGTTGTAAAGCT
CTTTCGCACGTGACGATGATGACGGTAACGTGAGAAGAAGCCCCGGCTAAC
TTCGTGCCAGCAGCCGCGGTAA

>EF394925 *Candidatus Azospirillum massiliensis* strain URAM1 (Band No. 17)
GGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTCGGGTT
GTAAAGCTCTTTCGCACACGACGATGATGACGGTAGTGTGAGAAGAAGCCC
CGGCTAACTTCGTGCCAGCAGCCGCGGTAAT

>AB547221 *Lysinibacillus fusiformis* (Band No. 18)
CCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACT
CTGTTGTAAGGGAAGAACAAGTACAGTAGTAACTGGCTGTACCTTGACGGT
ACCTTATTAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGATAAT

>GU143900 *Lysinibacillus sphaericus* strain T12-16 (Band No. 19)
AATGGGCGAAGCCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGG
ATCGTAAAACTCTGTTGTAAGGGAAGAACAAGTACAGTAGTAACTGGCTGT
ACCTTGACGGTACCTTATTAGAAAGCCACGGCTAACTACGTGCCAGCAGCC
GCGGTAAT

>HQ396802 *Lysinibacillus* sp. CH-N5 (Band No. 20)
CAATGGGCGAAAGCCTGATGGAGCATCGCCGCGTGAGTGAAGAAGGTTTTC
GGATCGTAAAACTCTGTTGTAAGGGAAGAACAAGTACAGTAGTAACTGGCT
GTACCTTGACGGTACCTTATTAGAAAGCCACGGCTAACTACGTGCCAGCAG
CCGCGGTAATA

>JN700165 *Lysinibacillus sphaericus* strain P16 (Band No. 21)
CCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGATTTCGGTTCGTAAAACT
CTGTTGTAAGGGAAGAACAAGTACAGTAGTAACTGGCTGTACCTTGACGGT
ACCTTATTAAAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAA

>JN215512 *Lysinibacillus sphaericus* strain VB7 (Band No.22)
TCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG
TTTTCGGATCGTAAAACTCTGTTGTAAGGGAAGAACAAGTACAGTAGTAAC
TGGCTGTACCTTGACGGTACCTTATTAGAAAGCCACGGCTAACTACGTGCCA
GCAGCCGCGGTAATA

>JQ312065 *Lysinibacillus sphaericus* strain AIMST Ehe33 (Band No. 23)
CAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTC
GGATCGTAAAACTCTGTTGTAAGGGAAGAACAAGTACAGTAGTAACTGGCT
GTACCTTGACGGTACCTTATTAGAAAGCCACGGCTAACTACGTGCCAGCAG
CCGCGGTAATA

>GU214826 *Bacillus cereus* strain Q-hrb05 (Band No. 24)
CCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACT
CTGTTGTAAGGGAAGAACAAGTACAGTAGTAACTGGCTGTACCTTGACGGT
ACCTTATTAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATAA

>JF683591 *Acinetobacter calcoaceticus* strain MCMB868 (Band No. 25)
CGCAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTATGGTTGT
AAAGCACTTTAAGCGAGGAGGAGGCTACTTTAGTTAATACCTAGAGATAGT
GGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGG
TAATA

>CP000698 *Geobacter uraniireducens* Rf4 (Band No. 26)
TGACGCAGCAACGCCGCGTGAGTGATGAAGGCCTTCGGGTCGTAAAGCTCT
GTCAGAGGGGAAGAAGTGTATGGGTGCTAATACCATCTTTACTTGACGGTA
CCCTCAAAGGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAA

>NR_041641 *Bacillus azotoformans* strain NBRC 15712 (Band No. 27)
GCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGCGATGAAGGCCTT
CGGGTCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCAGTTAACTGCTG
GTACCTTGACGGTACCTAACGAGAAAGCCACGGCTAACTACGTGCCAGCAG
CCGCGGTAATA

>NR_025125 *Desulfitobacterium metallireducens* strain 853-15 (Band No. 28)
AGTCTGACGGAGCAACGCCGCGTGTATGATGAAGGCCTTCGGGTTGTAAAG
TACTGTTTTCAGGGACGAACGGTAGATATGCAAATAGTGTATTTACATGACG
GTACCTGAGGAGGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATA

>FN870348 *Bacillus pocheonensis* (Band No. 29)
CGCCGCGTGAGTGATGAAGGCCTTCGGGTCGTAAAACTCTGTTGTCAGGGA
AGAACAAGTATCGGAGTAACTGCCGGTACCTTGACGGTACCTGACCAGAAA
GCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATA

>EF143993 *Clostridium* sp. 'HY-129-11 T' (Band No. 30)
TGGGGGAAACCCTGATGCAGCAACGCCGCGTGAGTGATGAAGGCCTTCGGG
TTGTAAAGCTCTGTCTTTGGGGACGATAATGACGGTACCCAAGGAGGAAGC
CACGGCTAACTACGTGCCAGCAGCCGCGGTAATA

>JN409060 Uncultured alpha proteobacterium clone HG-J02177 (Band No. 31)
CTGATCCAGCCATGCCACGTGAATGATGAAGGCCTTCGGGTTGTAAAATTCT
TTTAGTGGGGAAGATAATGACGGTACCCACAGAAAAAGCTCCGGCTAACTT
CGTGCCAGCAGCCGCGGTAATA

>GQ420909 Uncultured *Flexibacter* sp. clone RUGL1-414 (Band No. 39)
ATGGGCGAAAGCCTGACGCAGCAACGCCGCGTGAGGGATGAAGGCCTTCG
GGTTGTAAACCTCTTTTCTCAGGGAAGATAATGACGGTACCTGAGGAATAA
GCACCGGCTAACTCCGTGCCAGCAGCCGCGGT

>DQ072907 Uncultured *Rhizobium* sp. (Band No. 33)
GGAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTT
AGGGTTGTAAAGCTCTTTCACCGGAGAAGATAATGACGGTACTCGGAGAAG
AAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATA

>AF431208 Uncultured alpha proteobacterium clone S52.37PG (Band No. 34)
AATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTAG
GGTTGTAAAGCTCTTTTGGCGGGGAAGATAATGACGGTACCCGCAGAATAA
GCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATA

>HM755803 Uncultured type II methanotroph (Band No. 35)
TGGGCGCAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGT
TGTAAAGCTCTTTCGGCGGGGACGATAATGACGGTACCCGCAGAAGAAGCC
CCGGCTAACTTCGTGCCAGCAGCCGCGGTAATAA

>FJ817479 *Bacillus* sp. DST4 (Band No. 36)
GGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTG
TAAGGGAAGAACAAGTACAGTATTAACTGGCTGTACCTTGACGGTACCTTA
TTAAAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAAT

>AB568111 *Azospirillum amazonense* gene (Band No. 37)
ATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGG
GTTGTAAAGCTCTTTCGCACGTGACGATGATGACGGTAACGTGAGAAGAAG
CCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATA

>JN941348 *Diaphorobacter nitroreducens* strain AW3 (Band No. 38)
GGGCGAAAGCCTGATCCAGCCATGCCGCGTGCAGGATGAAGGCCTTCGGGT
TGTAAACTGCTTTTGTACGGAACGAAAAGCCTCTTTCTAATAAAGAGGGGTC
ATGACGGTACCGTAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCG
GTAATA

>AF307869 *Pseudomonas putida* 5IIANH (Band No. 39)
GGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTC
TTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGTCAGTAAGTTAATACC

TTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGC
AGCCGCGGTAATA

>NR_041642 *Bacteroides graminisolvens* strain XDT-1 (Band No. 40)
AGTCTGAACCAGCCAAGTAGCGTGAAGGATGACTGCCCTATGGGTTGTAAA
CTTCTTTTATAAGGGAATAAAGTGAGGGACGTGTCCCTTTTTGTATGTACCT
TATGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT

>CP001816 *Sulfurospirillum deleyianum* DSM 6946 (Band No. 41)
AATGGAGGAAACTCTGATGCAGCAACGCCGCGTGGAGGATGACGCATTTCG
GTGTGTAAACTCCTTTTATAAGGGAAGATAATGACGGTACCTTATGAATAA
GCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATA

>FJ916802 Uncultured alpha proteobacterium clone LT1bE8 (Band No. 42)
GGCGAAAGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTATGGTT
GTAAAGCTCTTTTACCAGGGATGATAAT

>DQ387436 *Azospirillum* sp. 21R (Band No. 43)
CCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCT
CTTTCGCACGTGACGATGATGACGGTAACGTGAGAAGAAGCCCCGGCTAAC
TTCGTGCCAGCAGCCGCGGTAATA

>EF422177 *Azospirillum* sp. 2456 (Band No. 44)
CAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTCGC
ACGTGACGATGATGACGGTAACGTGAGAAGAAGCCCCGGCTAACTTCGTGC
CAGCAGCCGCGGTAATAC

>GU048666 *Azospirillum* sp. YC6995 (Band No. 45)
GGGCGCAAGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGT
TGTAAAGCTCTTTCGCACGTGACGATGATGACGGTAACGTGAGAAGAAGCC
CCGGCTAACTTCGTGCCAGCAGCCGCGGTAATAC

>AB568112 *Azospirillum amazonense* (Band No. 46)
AATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAG
GGTTGTAAAGCTCTTTCGCACGTGACGATGATGACGGTAACGTGAGAAGAA
GCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATAC

>AB568111 *Azospirillum amazonense* gene (47)
GGGCGCAAGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGT
TGTAAAGCTCTTTCGCACGTGACGATGATGACGGTAACGTGAGAAGAAGCC
CCGGCTAACTTCGTGCCAGCAGCCGCGGTAATA

>EF422192 *Azospirillum* sp. 12812 (Band No. 48)
CTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTC
TTTCGCACGTGACGATGATGACGGTAACGTGAGAAGAAGCCCCGGCTAACT
TCGTGCCAGCAGCCGCGGTA

>AB568112 *Azospirillum amazonense* (Band No. 49)
AATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAG
GGTTGTAAAGCTCTTTCGCACGTGACGATGATGACGGTAACGTGAGAAGAA
GCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATA

Appendix F Treatment of synthetic monoethanolamine, diethanolamine, and triethanolamine wastewater by *C. alternifolius* and its degradation.

Appendix F-1 Efficiency of COD removal

Table F-1.1 Remaining COD concentrations in synthetic MEA (a), DEA (b), and TEA (c) wastewater after treatment by *C. alternifolius* for 12 days.

(a)

Treatment conditions	Remaining COD concentration in synthetic MEA wastewater (mg/L)									
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 9	Day 12
MEA-steriled	1831	1818	1824	1827	1834	1828	1834	1827	1827	1827
SD	31	27	14	10	8	5	14	10	13	7
MEA-non steriled	1831	1797	1677	1586	1569	1569	1580	1569	1583	1489
SD	31	68	17	43	26	26	10	26	9	17
MEA non steriled - Plant	1831	1563	1438	1272	1050	930	853	266	170	121
SD	31	77	103	144	70	36	62	103	55	18

(b)

Treatment conditions	Remaining COD concentration in synthetic DEA wastewater (mg/L)									
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 9	Day 12
DEA-steriled	2546	2554	2542	2553	2556	2550	2554	2559	2553	2543
SD	20	12	11	9	9	15	9	7	11	73
DEA-non steriled	2546	2544	2438	2444	2364	2421	2279	2286	2110	2066
SD	20	15	12	9	32	5	9	45	11	7
DEA non steriled - Plant	2546	1773	1687	1450	1388	1237	1118	1092	981	962
SD	20	52	15	10	41	13	5	25	12	13

(c)

Treatment conditions	Remaining COD concentration in synthetic TEA wastewater (mg/L)									
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 9	Day 12
TEA-steriled	2890	2895	2895	2899	2896	2891	2887	2889	2877	2896
SD	40	16	20	7	25	9	13	13	17	15
TEA-non steriled	2890	2867	2802	2818	2762	2580	2421	2353	2310	2286
SD	40	13	13	6	8	12	7	15	28	9
TEA non steriled - Plant	2890	1994	1895	1701	1622	1584	1527	1433	1206	1175
SD	40	15	13	13	8	10	15	5	16	16

Note: All data are mean of triplicate.

Appendix F-2 Monoethanolamine, diethanolamine, and triethanolamine removal by *C. alternifolius.*

Table F-2.1 Remaining MEA (a), DEA (b), and TEA (c) concentrations in synthetic ethanolamines wastewater after treatment by *C. alternifolius* for 12 days.

(a)

Treatment conditions	Remaining MEA concentration in synthetic MEA wastewater (mg/L)									
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 9	Day 12
MEA-steriled	1401	1391	1395	1398	1403	1399	1399	1399	1397	1398
SD	21	21	11	8	6	4	4	4	10	20
MEA-non steriled	1401	1383	1323	1276	1255	1241	1235	1229	1217	1206
SD	21	3	15	14	9	6	19	16	32	30
MEA non steriled - Plant	1401	1206	1207	990	830	707	520	283	170	106
SD	21	15	6	39	6	28	36	70	27	13

(b)

Treatment conditions	Remaining DEA concentration in synthetic DEA wastewater (mg/L)									
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 9	Day 12
DEA-steriled	1417	1420	1413	1422	1424	1413	1422	1420	1423	1412
SD	11	6	3	5	4	10	8	3	10	4
DEA-non steriled	1417	1399	1367	1374	1343	1313	1253	1276	1190	1154
SD	11	6	28	13	27	31	9	10	22	9
DEA non steriled - Plant	1417	1005	1010	826	769	700	621	535	569	550
SD	11	25	9	4	11	10	4	84	9	9

(c)

Treatment conditions	Remaining TEA concentration in synthetic TEA wastewater (mg/L)									
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 9	Day 12
TEA-steriled	1406	1406	1405	1410	1408	1406	1402	1401	1400	1407
SD	9	9	4	3	6	5	4	4	3	4
TEA-non steriled	1406	1377	1365	1348	1331	1287	1264	1225	1213	1220
SD	9	11	25	38	27	80	8	8	12	54
TEA non steriled - Plant	1406	1179	1008	923	932	889	803	764	634	622
SD	9	146	25	50	98	74	12	22	12	12

Note: All data are mean of triplicate.

Table F-2.2 System pH of synthetic MEA (a), DEA (b), and TEA wastewater (c) in synthetic ethanolamines wastewater after treatment by *C. alternifolius* for 12 days.

(a)

Treatment conditions	System pH									
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 9	Day 12
MEA-steriled	10.20	10.20	10.16	10.13	10.17	10.18	10.17	10.15	10.20	10.20
SD	0.02	0.09	0.02	0.04	0.03	0.03	0.02	0.05	0.02	0.01
MEA-non steriled	10.20	10.11	10.20	9.82	9.56	9.41	9.37	9.21	9.19	9.15
SD	0.02	0.02	0.25	0.14	0.08	0.03	0.05	0.02	0.07	0.05
MEA non steriled - Plant	10.20	9.71	9.31	9.01	7.49	7.30	7.40	7.35	7.50	7.66
SD	0.02	0.02	0.01	0.04	0.10	0.06	0.06	0.02	0.25	0.02

(b)

Treatment conditions	System pH									
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 9	Day 12
DEA-steriled	9.77	9.79	9.78	9.75	9.76	9.76	9.76	9.74	9.76	9.77
SD	0.03	0.02	0.03	0.02	0.02	0.01	0.01	0.04	0.01	0.02
DEA-non steriled	9.77	9.30	9.30	9.26	9.08	8.92	8.62	8.87	8.52	8.53
SD	0.03	0.02	0.02	0.02	0.03	0.03	0.01	0.02	0.01	0.01
DEA non steriled - Plant	9.77	8.59	8.23	7.11	7.14	7.19	7.07	6.97	6.52	6.92
SD	0.03	0.30	0.17	0.09	0.13	0.18	0.08	0.05	0.17	0.08

(c)

Treatment conditions	System pH									
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 9	Day 12
TEA-steriled	9.37	9.32	9.34	9.34	9.32	9.37	9.35	9.36	9.33	9.41
SD	0.04	0.03	0.03	0.04	0.02	0.03	0.05	0.01	0.03	0.01
TEA-non steriled	9.37	8.76	8.63	8.51	8.51	8.25	8.32	8.32	8.32	8.31
SD	0.04	0.01	0.04	0.02	0.02	0.25	0.02	0.02	0.02	0.02
TEA non steriled - Plant	9.37	7.27	7.24	7.18	7.14	7.08	7.02	6.93	6.83	6.94
SD	0.04	0.04	0.03	0.06	0.21	0.01	0.13	0.39	0.08	0.07

Note: All data are mean of triplicate.

Appendix F-3 Monoethanolamine degradation in plant tissues.

Table F-3.1 Concentration of monoethanolamine (a) and acetic acid (b) in leaves, stems, and roots after treatment for 12 days.

(a)

MEA in plant tissue	Concentration of MEA (umol /tissue)						
	Day 1	Day 2	Day 3	Day 5	Day 8	Day 10	Day 12
Leaf	4.02	7.47	4.21	1.25	0.00	0.00	0.00
SD	0.25	0.46	0.26	0.08	0.00	0.00	0.00
Stem	20.52	21.69	17.59	12.51	6.51	0.00	0.00
SD	1.25	1.32	1.07	0.76	0.40	0.00	0.00
Root	7.47	7.48	5.73	2.44	0.00	0.00	0.00
SD	0.46	0.46	0.35	0.15	0.00	0.00	0.00

(b)

MEA in plant tissue	Concentration of MEA (umol /tissue)						
	Day 1	Day 2	Day 3	Day 5	Day 8	Day 10	Day 12
Leaf	0.00	48.96	105.38	158.05	81.33	16.33	0.00
SD	0.00	5.54	5.15	10.62	3.45	0.87	0.00
Stem	0.00	125.78	258.68	387.48	555.70	216.12	63.86
SD	0.00	19.67	17.31	41.68	7.74	29.45	7.19
Root	0.00	16.77	37.18	61.12	34.57	19.81	2.19
SD	0.00	1.46	5.32	3.46	1.46	2.05	0.32

Note: All data are mean of triplicate.

Appendix F-4 Monoethanolamine, diethanolamine, and triethanolamine degradationon on *C. alternifolius.*

Table F-4.1 MEA and acetic acid accumulation in plant leaves under synthetic MEA wastewater after treatment for 12 days.

Sample	The accumulation in plant leaves (umol/tissue)						
	Day 1	Day 2	Day 3	Day 5	Day 8	Day 10	Day 12
MEA	4.02	7.47	4.21	1.25	0.00	0.00	0.00
SD	0.54	0.10	0.39	0.20	0.00	0.00	0.00
Acetic acid	1.46	48.96	105.38	158.05	81.33	16.33	0.00
SD	0.00	5.45	5.07	10.44	3.39	0.85	0.00

Note: All data are mean of triplicate.

Table F-4.2 MEA, DEA, and acetic acid accumulation in plant leaves under synthetic DEA wastewater after treatment for 12 days.

Sample	The accumulation in plant leaves (umol/tissue)							
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7	Day 9	Day 12
DEA	17.10	71.44	89.10	80.49	54.67	9.76	5.75	1.70
SD	1.83	2.92	6.99	5.88	3.55	1.16	1.92	0.13
MEA	3.61	7.17	6.07	11.41	7.78	3.91	1.29	0.90
SD	1.32	1.83	0.66	0.70	0.39	0.40	0.22	0.26
Acetic acid	60.35	226.04	314.52	262.13	206.38	133.58	95.21	45.39
SD	8.34	19.85	23.91	7.48	25.63	9.09	9.34	9.92

Note: All data are mean of triplicate.

Table F-4.3 MEA, DEA, TEA, and acetic acid accumulation in plant leaves under synthetic TEA wastewater after treatment for 12 days.

Sample	The accumulation in plant leaves (umol/tissue)							
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7	Day 9	Day 12
TEA	48.40	61.91	71.25	108.18	95.56	61.41	37.95	22.09
SD	7.14	5.22	3.37	10.56	7.11	5.31	5.87	3.77
DEA	5.06	6.32	6.74	24.05	19.08	14.56	9.50	6.92
SD	1.13	1.06	0.76	4.11	1.75	2.72	1.00	2.31
MEA	2.75	4.76	7.63	8.10	7.40	4.72	1.76	1.51
SD	0.53	0.73	1.80	1.32	1.21	2.25	0.58	0.82
Acetic acid	115.09	166.09	273.86	353.87	519.12	382.95	316.74	176.72
SD	16.26	29.91	19.02	38.67	32.67	25.93	22.66	35.80

Note: All data are mean of triplicate.

Appendix G The ANOVA summary

Appendix G-1 The ANOVA summary for the mean values of concentration of monoethanolamine in leaves, stems, and roots.

Table G-1.1 The ANOVA summary for the mean values of concentration of monoethanolamine in leaves, stems, and roots after treatment of synthetic monoethanolamine wastewater by *C. alternifolius* on day 1.

Source of variance	Sum of Squares	df	Mean Square	F	P-value
MEA	454.642	2	227.321	674.023	0.000*
Within Groups	2.024	6	0.337		
Total	456.666	8			

Note: * = the mean values of MEA concentration were significantly different at the 95% confidence level (P≤0.05), Duncan's Multiple Range Test for the mean values are show in Figure 4.34

Table G-1.2 The ANOVA summary for the mean values of concentration of monoethanolamine in leaves, stems, and roots after treatment of synthetic monoethanolamine wastewater by *C. alternifolius* on day 2.

Source of variance	Sum of Squares	df	Mean Square	F	P-value
MEA	404.093	2	202.046	1412.277	0.000*
Within Groups	0.858	6	0.143		
Total	404.951	8			

Note: * = the mean values of MEA concentration were significantly different at the 95% confidence level (P≤0.05), Duncan's Multiple Range Test for the mean values are show in Figure 4.34

Table G-1.3 The ANOVA summary for the mean values of concentration of monoethanolamine in leaves, stems, and roots after treatment of synthetic monoethanolamine wastewater by *C. alternifolius* on day 3.

Source of variance	Sum of Squares	df	Mean Square	F	P-value
MEA	321.834	2	160.917	1224.590	0.000*
Within Groups	0.788	6	0.131		
Total	322.623	8			

Note: * = the mean values of MEA concentration were significantly different at the 95% confidence level (P≤0.05), Duncan's Multiple Range Test for the mean values are show in Figure 4.34

Table G-1.4 The ANOVA summary for the mean values of concentration of monoethanolamine in leaves, stems, and roots after treatment of synthetic monoethanolamine wastewater by *C. alternifolius* on day 5.

Source of variance	Sum of Squares	df	Mean Square	F	P-value
MEA	229.745	2	114.873	282.093	0.000*
Within Groups	2.443	6	0.407		
Total	232.189	8			

Note: * = the mean values of MEA concentration were significantly different at the 95% confidence level (P≤0.05), Duncan's Multiple Range Test for the mean values are show in Figure 4.34

Table G-1.5 The ANOVA summary for the mean values of concentration of monoethanolamine in leaves, stems, and roots after treatment of synthetic monoethanolamine wastewater by *C. alternifolius* on day 8.

Source of variance	Sum of Squares	df	Mean Square	F	P-value
MEA	84.804	2	42.402	332.745	0.000*
Within Groups	0.765	6	0.127		
Total	85.569	8			

Note: * = the mean values of MEA concentration were significantly different at the 95% confidence level (P≤0.05), Duncan's Multiple Range Test for the mean values are show in Figure 4.34

Appendix G-2 The ANOVA summary for the mean values of concentration of acetic acid in leaves, stems, and roots.

Table G-2.1 The ANOVA summary for the mean values of concentration of acetic acid in leaves, stems, and roots after treatment of synthetic monoethanolamine wastewater by *C. alternifolius* on day 2.

Source of variance	Sum of Squares	df	Mean Square	F	P-value
Acetic acid	18821.003	2	9410.502	67.241	0.000*
Within Groups	839.706	6	139.951		
Total	19660.709	8			

Note: * = the mean values of MEA concentration were significantly different at the 95% confidence level (P≤0.05), Duncan's Multiple Range Test for the mean values are show in Figure 4.33

Table G-2.2 The ANOVA summary for the mean values of concentration of acetic acid in leaves, stems, and roots after treatment of synthetic monoethanolamine wastewater by *C. alternifolius* on day 3.

Source of variance	Sum of Squares	df	Mean Square	F	P-value
Acetic acid	77212.471	2	38606.236	326.585	0.000*
Within Groups	709.272	6	118.212		
Total	77921.743	8			

Note: * = the mean values of MEA concentration were significantly different at the 95% confidence level (P≤0.05), Duncan's Multiple Range Test for the mean values are show in Figure 4.33

Table G-2.3 The ANOVA summary for the mean values of concentration of acetic acid in leaves, stems, and roots after treatment of synthetic monoethanolamine wastewater by *C. alternifolius* on day 5.

Source of variance	Sum of Squares	df	Mean Square	F	P-value
Acetic acid	168548.387	2	84274.193	135.803	0.000*
Within Groups	3723.373	6	620.562		
Total	172271.760	8			

Note: * = the mean values of MEA concentration were significantly different at the 95% confidence level (P≤0.05), Duncan's Multiple Range Test for the mean values are show in Figure 4.33

Table G-2.4 The ANOVA summary for the mean values of concentration of acetic acid in leaves, stems, and roots after treatment of synthetic monoethanolamine wastewater by *C. alternifolius* on day 8.

Source of variance	Sum of Squares	df	Mean Square	F	P-value
Acetic acid	498792.145	2	249396.072	10120.150	0.000*
Within Groups	147.861	6	24.644		
Total	498940.006	8			

Note: * = the mean values of MEA concentration were significantly different at the 95% confidence level (P≤0.05), Duncan's Multiple Range Test for the mean values are show in Figure 4.33

Table G-2.5 The ANOVA summary for the mean values of concentration of acetic acid in leaves, stems, and roots after treatment of synthetic monoethanolamine wastewater by *C. alternifolius* on day 10.

Source of variance	Sum of Squares	df	Mean Square	F	P-value
Acetic acid	78466.546	2	39233.273	134.957	0.000*
Within Groups	1744.253	6	290.709		
Total	80210.799	8			

Note: * = the mean values of MEA concentration were significantly different at the 95% confidence level (P≤0.05), Duncan's Multiple Range Test for the mean values are show in Figure 4.33

Table G-2.6 The ANOVA summary for the mean values of concentration of acetic acid in leaves, stems, and roots after treatment of synthetic monoethanolamine wastewater by *C. alternifolius* on day 12.

Source of variance	Sum of Squares	df	Mean Square	F	P-value
Acetic acid	7887.804	2	3943.902	228.196	0.000*
Within Groups	103.698	6	17.283		
Total	7991.502	8			

Note: * = the mean values of MEA concentration were significantly different at the 95% confidence level (P≤0.05), Duncan's Multiple Range Test for the mean values are show in Figure 4.33

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