การย่อยสลายทางชีวภาพของสาร4-คลอโรอะนีลีน และ 3,4-ไดคลอโรอะนีลีน ที่ปนเปื้อนในดิน ภายใต้การปนเปื้อนร่วมของสารคอปเปอร์

นางสาวนฤมล ชุ่มใจ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

สาขาวิชาการจัดการสิ่งแวดล้อม (สหสาขาวิชา)

บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2554

BIOREMEDIATION OF 4-CHLOROANILINE AND 3,4-DICHLOROANILINE CONTAMINATED SOIL UNDER COPPER FUNGICIDE CO-CONTAMINATION

Miss Naruemon Chumjai

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Environmental Management

(Interdisciplinary Program)

Graduate School

Chulalongkorn University

Academic Year 2011

Thesis	BIOREMEDIATION OF 4-CHLOROANILINE AND 3,4-DICHLOROANILINE CONTAMINATED SOIL UNDER COPPER FUNGICIDE CO-CONTAMINATION
By	Miss Naruemon Chumjai
Field of Study	Environmental Management
Thesis Advisor	Associate Professor Alisa Vangnai, Ph.D.

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

…….. Dean of the Graduate School

(Associate Professor Pornpote Piumsomboon, Ph.D.)

THESIS COMMITTEE

…….. Chairman

(Assistant Professor Ekawan Luepromchai, Ph.D.)

…….. Thesis Advisor

(Associate Professor Alisa Vangnai, Ph.D.)

…….. Examiner

(Assistant Professor Onruthai Pinyakong, Ph.D.)

…….. Examiner

(Assistant Professor Sumana Siripattanakul, Ph.D.)

…….. External Examiner

(Associate Professor Duangrat Inthorn, Ph.D.)

นฤมล ชุ่มใจ : การย่อยสลายทางชีวภาพของสาร 4-คลอโรอะนีลีน และ 3,4-ไดคลอโรอะนีลีน ที่ปนเปื้อนในดินภายใต้การปนเปื้อนร่วมของสารฆ่าเชื้อราคอปเปอร์. (BIOREMEDIATION OF 4-CHLOROANILINE AND 3,4-DICHLOROANILINE CONTAMINATED SOIL UNDER COPPER FUNGICIDE CO-CONTAMINATION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ดร. อลิสา วังใน, 150 หน้า

4-คลอโรอะนีลีน และ3,4-ไดคลอโรอะนีลีน ถูกใช้อย่างกว้างขวางในกระบวนการผลิตของอุตสาหกรรมหลายอย่าง และ 4-คลอโรอะนีลีน และ3,4-ไดคลอโรอะนีลีน ยังเป็นสารตัวกลางของการย่อยสลายยาปราบศัตรูพืชโดยจุลชีพ เนื่องจากความเป็นพิษและความคงทนในสิ่งแวดล้อม วิธีการย่อยสลายสารทางชีวภาพจึงจำเป็นในการบำบัด 4-คลอโรอะนีลีน และ3,4-ไดคลอโรอะนีลีนที่ตกค้างในสิ่งแวดล้อม ผลการทดลองในอาหารเลี้ยงเชื้อพบว่า การเติมสารอาหารที่มาจากแหล่งธรรมชาติยับยั้งการย่อยสลายสาร 4-คลอโรอะนีลีนของเชื้อ *Acinetobacter baylyi* strain GFJ2 แต่เพิ่มการย่อยสลายของสาร3,4-ไดคลอโรอะนีลีน นอกจากนั้นการเติมสารฆ่าเชื้อราคอปเปอร์ที่ความเข้มข้น 5, 10, 50 และ 100 ส่วนในล้านส่วน พบว่าการย่อยสลายของสาร4-คลอโรอะนีลีนโดยเชื้อ *A. baylyi* strain GFJ2 ถูกยับยั้งโดยยาฆ่าเชื้อราคอปเปอร์ ที่ทุกความเข้มข้น แต่ยาฆ่าเชื้อราคอปเปอร์บางตัว ได้แก่ คอปเปอร์ซัลเฟต, โคปินา-85, โคปินาไฮดร็อกไซด์ และบอร์โดเอ็ม ที่ความเข้มข้น 5 และ 10 ส่วนในล้านส่วน เพิ่มการย่อยสลายสาร3,4-ไดคลอโรอะนีลีน วิธีการบำบัดทางชีวภาพประกอบด้วย การสลายตามธรรมชาติ, การใช้สารเร่งการสลายทางชีวภาพ และการเติมเชื้อจุลินทรีย์ ถูกนำมาใช้เพื่อการศึกษาการย่อยสลาย 4-คลอโรอะนีลีน และ 3,4-ไดคลอโรอะนีลีนที่มีความเข้มข้น 500 ส่วนในล้านส่วน ซึ่งปนเปื้อนในดิน 2 ชนิด ในดิน S3 พบว่าวิธีการเติมเชื้อจุลินทรีย์ของการรวมกันของจุลินทรีย์เดี่ยว *A. baylyi* strain GFJ2 และจุลินทรีย์ผสมจากปุ๋ย การย่อยสลายของสารคลอโรอะนีลีนเกิดมากที่สุดภายใน 4 สัปดาห์ สำหรับดิน S5 ไม่มีความแตกต่างในการย่อยสารคลอโรอะนีลีนในแต่และวิธี การย่อยสลายภายใต้การปนเปื้อนร่วมของคอปเปอร์ซัลเฟตที่ความเข้มข้น 150 ส่วนในล้านส่วน พบว่าไม่มีความแตกต่างจากไม่ปนเปื้อนร่วมกับคอปเปอร์ การศึกษาการเปลี่ยนแปลงกลุ่มเชื้อจุลินทรีย์ในดินS3 พบว่ามีการเปลี่ยนแปลงและการปรับสภาพของเชื้อจุลินทรีย์แตกต่างกันในแต่ละวิธีการบำบัด และพบว่า *A. baylyi* strain GFJ2 ไม่สามารถมีชีวิตอยู่ภายในดินภายหลังจากการเติมลงไป

สาขาวิชาการจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต .....

ปีการศึกษา2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

5287539020: MAJOR ENVIRONMENTAL MANAGEMENT
KEYWORDS: 4-CHLOROANILINE / 3,4-DICHLOROANILINE / BIOREMEDIATION / COPPER CO-CONTAMINATION / SOIL

NARUEMON CHUMJAI: BIOREMEDIATION OF 4-CHLOROANILINE AND 3,4-DICHLOROANILINE CONTAMINATED SOIL UNDER COPPER CO- CONTAMINATION. ADVISOR: ASSOC. PROF. ALISA VANGNAI, Ph.D., 150 pp.

4-chloroaniline and 3,4-dichloroaniline have been widely used in many production processes of industries such as dye, rubbers, pharmaceutical products, pesticides and herbicides. Besides, 4CA and 3,4-DCA are the main intermediates of herbicide degradation by microorganisms. Due to their toxicity and recalcitrant in environment, bioremediation technique is necessary to reduce and clean up them. In liquid medium, addition of nutrients from natural sources inhibited 4CA degradation of *Acinetobacter baylyi* strain GFJ2 but enhanced 3,4-DCA biodegradation. Moreover, the addition copper fungicides (5, 10, 50 and 100 ppm) inhibited biodegradation of 4CA by *A. baylyi* strain GFJ2. Some copper fungicides (copper sulfate, copina-85, copinahydroxide and bordeaux-M) at low concentrations (5 and 10 ppm) enhanced 3,4-DCA biodegradation. Bioremediation including natural attenuation, biostimulation and bioaugmentation with 1) *A. baylyi* strain GFJ2; 2) *A. baylyi* strain GFJ2 and supplementary nutrients and 3) combination of *A. baylyi* strain GFJ2 and natural bacterial consortium from fertilizer were carried out to degrade 500 ppm CAs in two soils. In S3 soil, the highest CAs degradation was observed after 4 week treatment with the bioaugmentation having a combination of *A. baylyi* strain GFJ2 and natural bacterial consortium. For S5 soil, there was no difference of CAs degradation in each treatment. Biodegradation of CAs with and without copper sulfate (150 ppm) co-contamination was not different. The bacterial community during each treatment was monitored using the denaturing gradient gel electrophoresis (DGGE). The result showed that bacteria community in each treatment was changed and some bacteria were adapted and recovered. From DGGE result showed that *A. baylyi* strain GFJ2 could not survive in soil.

Field of Study: Environmental Management Student's Signature……

Academic Year: 2011 Advisor's Signature……

ACKNOWLEDGEMENTS

I would like to sincere gratitude to my advisor, Associate Professor Alisa Vangnai, Ph.D. for her valuable advice, guidance, useful comment, great encouragement and helpfulness throughout this thesis. Without her kindness and understanding, this work could not be accomplished.

My gratitude is also extended to Assistant Professor Ekawan Luepromchai, Assistant Professor Sumana Siripattanakul, Associate Professor Duangrat Inthorn, and Assistant Professor Onruthai Pinyakong for serving as thesis committee, for valuable comments and also for useful suggestion.

There are so many friends to mention from so little space to fill. I would like to give a deep thank to all staff members of room 604 and friends in the Department of Biochemistry, Program in environmental management and Biotechnology for their friendship, kind assistance and support.

Finally, the greatest gratitude is expressed to my lovely family for their infinite love, willpower, support, encouragement and everything giving to my life.

This work was financially supported by 90^{th} anniversary of Chulalongkorn University Fund, Graduate School, and Program in Environmental Management, Faculty of Science, Chulalongkorn University.

CONTENTS

Page

Page

Page

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS

4CA	=	4-Chloroaniline
3,4-DCA	=	3,4-dichloroaniline
CAs	=	Chloroanilines
EPA	=	Environmental Protection Agency
ml	=	Milliliter
ul	=	Microliter
mg	=	milligram
ppm	=	Part per million
OD	=	Optical density
rpm	=	Revolution per minute
MM	=	Mineral medium
LB	=	Luria bertani
bp	=	base pairs
HPLC	=	High performance liquid chromatography
min	=	Minutes
hr	=	Hour
DGGE	=	Denaturing gradient gel electrophoresis
TTC	=	2,3,5-triphenyltetrazolium chloride
TPF	=	Triphenylformazan
NS	=	Nutrients from soil
NF	=	Nutrients from fertilizer
N	=	Natural attenuation
S	=	Biostimulation with fertilizer nutrients

A	=	Bioaugmentation with the natural mixed culture
J2	=	Bioaugmentation with *Acinetobacter baylyi* strain GFJ2
J2S	=	Bioaugmentation with *Acinetobacter baylyi* strain GFJ2 and supplemented with fertilizer nutrients
J2A	=	Bioaugmentation with *Acinetobacter baylyi* strain GFJ2 and the natural mixed culture
NCu	=	Natural attenuation under copper sulfate co-contamination
SCu	=	Biostimulation with fertilizer nutrients under copper sulfate co-contamination
ACu	=	Bioaugmentation with the natural mixed culture under copper sulfate co-contamination
J2Cu	=	Bioaugmentation with *Acinetobacter baylyi* strain GFJ2 under copper sulfate co-contamination
J2SCu	=	Bioaugmentation with *Acinetobacter baylyi* strain GFJ2 and supplemented with fertilizer nutrients under copper sulfate co-contamination
J2ACu	=	Bioaugmentation with *Acinetobacter baylyi* strain GFJ2 and the natural mixed culture under copper sulfate co-contamination

CHAPTER I

INTRODUCTION

1.1 Statement of problem

Due to human activities and developments of various industries, large quantities of hazardous chemical have been released into the environment every year (Ibarrolaza et al., 2009). Chloroanilines (CAs) are chlorinated aromatic amine, which are widely used in various production of dyes, rubbers, pharmaceutical products, photographic chemicals, vanishes, herbicides and pesticides (Zhang et al., 2010). Extensive use of these industrial products containing CAs resulted in many environmental contaminations (Bartha, 1968). In addition, CAs are intermediates generated by microbial degradation of herbicides (Hongsawat and Vangnai, 2011).

Several herbicides have been used for weed control in agricultural practices such as phenylurea, phenylcarbamate and acylanilide. In Thailand, the phenylurea herbicides such as diuron are the one of top ten ranks of herbicides has been used in agriculture (Vangnai and Petchkroh, 2007). Diuron has been detected in environment, besides itself, the main intermediate 4-chloroaniline (4CA)6 and 3,4-dichloroaniline (3,4-DCA) have been accumulated in environment too (Giacomazzi and Cochet, 2004). These intermediate more harmful to human and animals than diuron itself (Sorensen et al., 2008). Therefore, the more phenylurea, phenylcarbamate and acylanilide have been used, the more CAs has been contaminated in the environment.

In agricultural practices, not only herbicides are used, copper-based fungicides such as $Cu(OH)_2$, $CuSO_4$, copper-oxychloride and bordeaux mixture are also applied

to control fungal diseases (Komárek et al., 2010). These lead to the co-contamination of copper-based fungicides and CAs in the environment.

Chloroanilines are toxicity to human health and nontarget organisms. CAs irritates and burns eyes and skin, also causes methmoglobinemia disease, potential endocrine-disrupting properties and carcinogen in human (Breugelmans et al., 2010). Due to the toxicity and persistency in the environment of these CAs, they are classified as the hazardous substance by the environmental protection agency (EPA) (Tongarun et al., 2008). Therefore, it is necessary to clean up contaminated CAs in the environment by appropriate techniques.

Several methods have been used to clean up contaminated site. Physical and chemical treatments have been used are high-temperature incineration and various types of chemical decomposition (base-catalyzed, dechlorination and UV oxidation). However, these treatments are high-cost, require energy and heavy equipment and lack of public acceptance (Mrozik and Piotrowska-Seget, 2010).

Bioremediation is an alternatively technique that useful treatments to clean up CAs in the environment. Bioremediation treatment uses bacteria, fungi or plants to degrade or detoxify hazardous substances to less toxic or non toxic form. However, bioremediation processes is a complex system and can be influenced by many factors such as the presence of suitable microbial population with the ability to degrade pollutants, soil type, temperature, pH, moisture, the presence of oxygen and nutrients (Vidali, 2001).

There is an abundant literature on degrading mono-chloroaniline as sole carbon and energy sources. At the same time, there are limited study the biodegradation of 3,4-DCA and co-contamination of mono- and di-chloroaniline.

Some of researchers to report on the microbial degradation of 3,4-DCA were Travkin and Golovleva (2003), who described the *Pseudimonas fluorescences* strain 26-K that was able to degrade 3,4-DCA as the sole source of carbon, nitrogen and energy. You and Bartha (1982), who described the *Pseudomonas putida* that was able to degrade 3,4-DCA in the presence of unchlorinated analogue substrate. Hongsawat and Vangnai (2011), who described *Acinetobacter baylyi* strain GFJ2, which was isolated from soil, exhibited effective degradation of 4CA and 3,4-DCA.

Therefore, this study used *A. baylyi* strain GFJ2 for bioremediation of 4CA, 3,4-DCA and co-occurrence of 4CA-34DCA under copper-based fungicides co-contamination in soil. Bioremediation treatments including natural attenuation, biostimulation and bioaugmentation were investigated. Moreover, the concentration of CAs, the number of CAs degrading bacteria, the total microbial activity and microbial community during bioremediation were determined.

1.2 Objectives

The main objective of this study was to develop a bioremediation technique to clean up 4CA and 3,4-DCA contaminated soil. The sub-objectives were as followed:

1.2.1. To determine factors affecting 4CA and 3,4-DCA biodegradation including concentrations, supplementary nutrients, co-occurring substrates and co-contamination of copper, in liquid.

1.2.2. To determine factors affecting 4CA and 3,4-DCA biodegradation including supplementary nutrients, co-occurring substrate and co-contamination of copper in soil.

1.3 Hypothesis

1.3.1. Co-occurring substrate, 3,4-DCA may influence 4CA biodegradation and *vice versa*.

1.3.2. 4CA, 3,4-DCA and 4CA-3,4-DCA were biodegraded more effectively in bioaugmentation and biostimulation than natural attenuation.

1.3.3. Co-contamination of copper can influence CAs degradation.

1.4 Scope of study

1.4.1. Factors affecting degradation of 4CA and 3,4-DCA in liquid medium

1.4.1.1. Concentrations of 4CA, 3,4-DCA and co-occurring 4CA-3,4-DCA

1.4.1.2. Effect of supplementary nutrients

1.4.1.3. The co-contamination of copper fungicide

1.4.1.3.1. Effect of copper fungicide types

1.4.1.3.2. Effect of copper fungicide concentrations

1.4.2. The study of 4CA, 34DCA and 4CA-3,4-DCA degradation in soil

1.4.2.1. Contamination of CAs

1.4.2.2. Co-contamination of CAs and copper

For 1.4.2.1 and 1.4.2.2, three bioremediation treatments were applied as followed:

i. Natural attenuation: 4CA, 3,4-DCA and 4CA-3,4-DCA degradation were conducted using natural soil microorganisms.

ii. Biostimulation: 4CA, 3,4-DCA and 4CA-3,4-DCA degradation were conducted by using indigenous microorganisms with supplementary nutrients.

iii. Bioaugmentation: 4CA, 3,4-DCA and 4CA-3,4-DCA degradation were conducted by inoculating pure bacterial culture (*Acenetobacter baylyi* strain GFJ2) and bacterial consortium from fertilizer.

1.4.2.3. The study of bacterial populations

1.4.2.3.1. Determination of the amount of CAs degrading bacteria by 10-fold dilution method.

1.4.2.3.2. Determination of total microbial activity by dehydrogenase activity

1.4.2.3.3. Determination of microbial community using the denaturing gradient gel electrophoresis (DGGE)

CHAPTER II

THEORETICAL BACKGROUND AND LITERATURE REVIEWS

2.1 Chloroanilines production and usage

Chloroanilines are widely used in production processes of several industries such as polyurethanes, rubbers, dyes, pharmaceutics, photographic chemicals, vanishes and pesticides (Zhang et al., 2010). As a result, they have been distributed and accumulated in the environment by industrial effluents. In addition, microbial degradation of several of herbicides such as phenylcarbamate, phenylurea and acylanilide can generate and accumulate CAs in the environment as well (Vangnai and Petchkroh, 2007). The amount of 4CA and 3,4-DCA production is summarized in Table 2.1.

The application of pesticides, insecticides and herbicides such as monolinuron, diflubenzuron, buturon, propanil, chlorofenpropmethyl, benzoylpropmethyl, chloroaniformmethane, chlorobromuron, neburon, oxadiazon and especially phenylureas can release 4CA to environment as a degradation product (Boehncke et al., 2003). The intermediate product of biodegradation of phynylurea, phynylcarbamate and acylanilide herbicides is 3,4-DCA (Bureau, 2006). However, under anaerobic conditions 3,4-DCA can release 4CA into soils (Boehncke et al., 2003).

Therefore, CAs has been contaminated both from industrial productions that contain residual CAs and agricultural practices as a degradation product.

Table 2.1 The amount of 4-chloroaniline and 3,4-dichloroaniline production (Boehncke et al., 2003; Bureau, 2006)

Year	Source	Amount
1988	The global annual production of 4CA	3500 tones
1990	4CA production in The former Federal of Germany	1350 tones
	The processing of 4CA at the German manufacturers	1000 tones
	The production of 4CA released into air at German manufacturers	<20g/tone
	The production of 4CA released into surface water at German manufacturers	13 g/tone
	The annual wastes of 4CA at German manufacturers (in special company incinerators)	400 g/tone
1991	The annual US production of 4CA	45 – 450 tones
	3,4-DCA production in Western Europe	12,000 tones
1995	4CA production in Western European and Japanese	3000 – 3300 tones
	Total releases of 4CA in the USA	500 kg/tone
1996	4CA production in India and China	800 – 1300 tones
1997	4CA production in France	≥ 1000 tones
1998	Total releases of 4CA in the USA	2814 kg/tone
1996 - 1998	The total production of 3,4-DCA in Western Europe	13,500 – 15,500 tones
1999	Total releases of 4CA in the USA	212 kg/tone

In Thailand, agricultural practices widely used phenylurea herbicides such as diuron. They were the sixth rank of the imported hazardous chemical substances of department of agriculture in 2003 which is presented in the Table 2.2 (Tongarun et al., 2008). Moreover, EUROPA (2006) reported that clothes made in Thailand contained 4-chloroaniline at level of 39 mg/kg which this chemical prohibited under Directive 76/769/EEC.

Table 2.2 The imported hazardous chemicals by value in 2003 (Department of Agriculture, 2003)

Rank	Herbicide name	Quality (kg)	Value (Bath)
1	Glyphosate isopropylammonium	24,812,105	1,824,107,984
2	Paraquat dichloride	8,366,582	1,385,300,727
3	Ameyhrn	2,374,950	488,492,508
4	2,4-D	5,114,724	392,071,423
5	Atrazine	2,364,450	309,974,446
6	Diuron	984,245	178,858,920
7	Bromacil	304,309	150,816,860
8	Fenoxaprop-P-ethyl	210,658	150,816,860
9	Butachlor	1,309,267	126,341,644
10	Propanil	827,333	118,439,010

2.2 4-chloroaniline and 3,4-dicloroaniline properties

Chloroanilines have three isomers such as monochloroaniline, dichloroaniline and trichloroaniline.

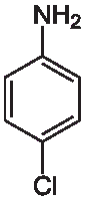
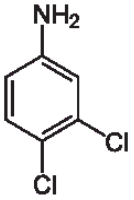
Monochloroaniline isomers have 1 branch of chloride at the 2, 3 and 4 (ortho, *meta* and *para*) position called 2CA, 3CA and 4CA, respectively. 4CA has the cruelest genotoxic effect of animals among of three monochloroanilines (Boehncke et al., 2003)

Dichloroaniline isomers have 2 branches of chloride such as 2,3-DCA, 2,4-DCA, 2,5-DCA, 2,6-DCA and 3,4-DCA.

Trichloroaniline isomers have 3 branches of chloride such as 2,3,4-TCA, 2,4,5-TCA, 2,4,6-TCA and 3,4,5-TCA.

This study focused on 4CA and 3,4-DCA because 4CA and 3,4-DCA are the main intermediate of diuron degradation in environment. Therefore, it is necessary to clean up 4CA and 3,4-DCA in the environment by appropriate technique. Physical and chemical properties of 4CA and 3,4-DCA is shown in the Table 2.3 (Boehncke et al., 2003; Bureau, 2006).

Table 2.3 Physical and chemical properties of 4-chloroaniline and 3,4-dichloroaniline (Boehncke et al., 2003; Bureau, 2006).

Property	**4CA**	**3,4-DCA**
Name	1-amino-4-chlorobenzene, PCA, *p*-chloroaniline, 1-chloro-4-aminobenzene, 4-chloro-1-aminobenzene, 4-chlorobenzeneamine, 4-chloroaminobenzene and 4-chlorophenylamine	3,4-dichlorophenylamine, 3,4-dichloroaniline and 3,4-dichlorobenzeneamine
Chemical formula	C_6H_6ClN	$C_6H_5Cl_2N$
Chemical structure		
Molecular weight (g/mol)	127.57	162.02
Physical state	Crystalline solid	Solid at 20 ˚C
Color	Colorless to slightly amber color	Brown
Melting point	69-73 ˚C	70-72.5 ˚C
Boiling point	232 ˚C	272 ˚C
Water solubility	2.6 $g.l^{-1}$ at 20 ˚C	580 $mg.l^{-1}$ at 20 ˚C
Vapor pressure	0.5 Pa at 10 ˚C 1.4 - 2.1 Pa at 20 ˚C	0.184 Pa at 20 ˚C
Henry's law constant	0.1 $Pa.m^3.mol^{-1}$ at 20 ˚C	0.05 $Pa.m^3.mol^{-1}$ at 20 ˚C
Oral rat LD_{50}	310 mg/kg	648 mg/kg
Kow	1.83	2.69

2.3 Environmental fate

Chloroanilines have been released into the hydrosphere from industrial productions and the extensive use of products involving CAs. Besides, the releases from the industrial production, the source of chloroanilines have been reported from agricultural area. Chloroanilines can be contaminated in air, surface water, groundwater, sediment, soil and aquatic organisms.

In 1990, the German manufacturer reported 4CA released into air at concentration <20 $g.tone^{-1}$ (Boehncke et al., 2003). 4CA can be degrade by photolysis, reacting with photochemically produced hydroxyl radicals or adsorbed on particulate matter in air (Tongarun et al., 2008).

In 1980 to 1990, 4CA was released in surface water from the German and Dutch parts of river Rhine with the concentrations between 0.1-1 $\mu g.l^{-1}$. In the same period, Japan reported 4CA in surface water was between 0.024-0.39 $\mu g.l^{-1}$ and also found 4CA at concentrations between 0.007-0.013 $\mu g.l^{-1}$ in German drinking-water samples. In 1995-1996, from three sites in an industrialized area near Milan found 4CA in groundwater at concentrations 0.01-0.06 $\mu g.l^{-1}$. The half-life of 4CA in water was 151 days at a water depth of 1 meter and a temperature of 20 ˚C and 3 years at 55 ˚C, pH 3, 7 and 11 which initial concentration 129 $mg.l^{-1}$ by OECD Guideline (Boehncke et al., 2003).

In 1976, 4CA was detected in Japanese sediment at concentrations 1-270 $\mu g.kg^{-1}$. Only a few percentage of 4CA can be evaporated from the soil in range of 0.11-3.65%. In German agricultural soil detected 4CA with maximum concentration of 968 $\mu g.kg^{-1}$ (Boehncke et al., 2003).

In Finland 1984, after used herbicide (diflubenzuron) for treated a forest area found the concentration of 4CA in wild mushrooms, blueberries and cranberries at concentration 10-20 $\mu g.kg^{-1}$. For 19 days after used diflubenzuron found 4CA at concentration between 0.9-1.3 $\mu g.kg^{-1}$ in tissue samples of the bluegill (Boehncke et al., 2003).

3,4-DCA was released to atmospheric which half-life was 9 hours which cannot volatilize because of the low Henry's law constant and no hydrolysis. 3,4-DCA was released to surface waters and can be degraded with photolysis which half-life was 18 days and no biodegradation occurs in WWTPs and surface waters. 3,4-DCA was released into soils. The reaction of 3,4-DCA with humic substances in soils and sediments lead to 3,4-DCA accumulated which half-life was 1,000 days because of the very low biodegradation (Bureau, 2006).

The major part of CAs was released in agriculture soils. When CAs are released into soil, it can combine rapidly with soil components and accumulates in soil (Tongarun et al., 2008). The sorption rate increases with increasing of organic matter and decreasing of pH in the soil. The sorption of CAs in soil depends on soil types and soil characteristics (Boehncke et al., 2003). The amount of CAs in environment is presented in Table 2.4.

Table 2.4 The amount of 4-chloroaniline and 3,4-dichloroaniline in environment

Phase	Country	Substance	Concentration	Reference
Air	Germany	4CA	< 20 $g.ton^{-1}$	Boehncke et al., 2003
Surface water	Germany	4CA	240 $g.ton^{-1}$	Boehncke et al., 2003
	Germany	4CA	0.1 – 1 $\mu g.L^{-1}$	Boehncke et al., 2003
	Japan	4CA	0.024 – 0.39 $\mu g.L^{-1}$	Fattore et al., 1998
	Netherlands	3,4-DCA	0.68 $\mu g.L^{-1}$	Buearu, 2006
	Spain	3,4-DCA	470 $\mu g.L^{-1}$	Carvlho et al., 2010
Groundwater	Denmark	4CA	< 10 $\mu g.L^{-1}$ (depth 5.5 m)	Holm et al., 1995
	Denmark	4CA	50 $\mu g.L^{-1}$ (depth 8.5 m)	Holm et al., 1995
	Italy	4CA	0.01 – 0.06 $ug.L^{-1}$	Boehncke et al., 2003
	-	4CA	2 $\mu g.L^{-1}$	ALS, 2010
Sediment	Japan	4CA	1 – 270 $\mu g.kg^{-1}$	Boehncke et al., 2003
	Netherlands	3,4-DCA	0.15 $mg.kg^{-1}$	Buearu, 2006
	USA	4CA	3,300 $\mu g.kg^{-1}$	EPA, 2010

Phase	Country	Substance	Concentration	Reference
Soil	Germany	4CA	< 5 μg.kg^{-1}	Boehncke et al., 2003
	Germany	4CA	5 – 10 μg.kg^{-1}	Boehncke et al., 2003
	Germany	4CA	10 – 30 μg.kg^{-1}	Boehncke et al., 2003
	Germany	4CA	30 – 50 μg.kg^{-1}	Boehncke et al., 2003
	Germany	4CA	> 200 μg.kg^{-1}	Boehncke et al., 2003
	Germany	4CA	968 μg.kg^{-1}	Boehncke et al., 2003
	-	4CA	10 mg.kg^{-1}	ALS, 2010
	-	4CA	30 μg.kg^{-1}	Boehncke et al., 2003
	Japan	4CA	1 – 270 μg.kg^{-1}	Boehncke et al., 2003
Aquatic organism				
Bluegill	Finland	4CA	0.9 – 1.3 μg.kg^{-1}	Boehncke et al., 2003
Brachydanio rerio	-	3,4-DCA	2 μg.L^{-1}	Buearu, 2006
Daphnia magna	Germany	4CA	0.01 mg.L^{-1}	Boehncke et al., 2003
Daphnia magna	-	3,4-DCA	0.16 - 0.23 mg.L^{-1}	Buearu, 2006
Worm	-	3,4-DCA	0.0026 – 6.3 mg.kg^{-1}	Buearu, 2006

2.4 Toxicity of chloroanilines

Chloroanilines are harmful to human which bind covalently to haemoglobin and to protein which the main target tissues being liver, kidney and urinary bladder. In addition, CAs are harmful to mammals and animals which absorbed at gastrointestinal and respiratory systems (Giacomazzi and Cochet, 2004). CAs can interfere with the ability of blood to carry oxygen causing headache, dizziness and a blue color to skin and lips (methaemoglobinaemia disease) and hyperplasia of bone marrow in female rats (Boehncke et al., 2003). Beside, they were found to be carcinogenic in male rats and may be a potential carcinogen in human (Gonza'lez-Pradas et al., 2005).

The toxicity towards microorganisms which the growth of bacteria was inhibited by 4CA has been reported at 3 mM (Radianingtyas et al., 2003) and 4 mM (Zeyer and Kearney, 1982).

For 4CA, the oral LD_{50} in animals were reported, LD_{50} in rats is 300-420 $mg.kg^{-1}$ body weight, in mice is 228-500 $mg.kg^{-1}$ body weight and in guinea-pigs is 350 mg.kg-^{1} body weight. For intraperitoneal and dermal LC_{50} in rat is 2340 $mg.m^{-3}$ (Boehncke et al., 2003).

The toxicity of 3,4-DCA on various organisms has been reported such as protozoan (NOEC = 5100 $\mu g.l^{-1}$), unicellular algae (NOEC = 700 to 4400 $\mu g.l^{-1}$), rotifer (NOEC = 700 $\mu g.l^{-1}$), insect (NOEC = 3.1 to 760 $\mu g.l^{-1}$), crustacean (NOEC = 1 to 60 $\mu g.l^{-1}$), pisces (NOEC = 2.1 to 360 $\mu g.l^{-1}$) and mammalian (LOEC = 324 $mg.kg^{-1}$) (Giacomazzi and Cochet, 2004).

2.5 Legislation

Chloroanilines are the intermediate by microbial degradation of diuron which diuron is on the list of the European Commission's list of priority substances for European freshwater resources and on the U.S. Environmental Protection Agency's Second Drinking Water Contaminant Candidate List (Sorensen et al., 2008). Because of their toxicity and recalcitrant property, they are classified as the Pollutant List of the U.S. Environment Protection Agency and subject to legislative control by the 76/464/EEC Directive (Zhang et al., 2010).

The products or the marketing that containing 4CA-based azo dyes were banned by the European Union (EU) (Boehncke et al., 2003). 4CA was one of the 129 priority pollutants on black list of EEC (de Wolf et al., 1994).

In addition, 3,4-DCA is on the list of European Economic Community 1982 which this substance concern to be contaminate and accumulate in the environment (Livigston and Willacy, 1991).

2.6 Treatment

The hazardous chemicals were increased and accumulated in the environment due to the carelessness and negligence in using of hazardous chemicals by human. So, it is necessary to have the treatment to clean up hazardous chemicals. The treatment can categorize into physical, chemical and biological treatment.

2.6.1 Physical and chemical treatments

There are several physical and chemical treatments which have been used to remove CAs.

The radiation- induced degradation of 4CA by γ-rays in aqueous solution under conditions saturated with air, pure oxygen, N_2O, argon and argon in the presence of *t*-Butanol. The result showed that in the presence of N_2O (oxidation by OH-radicals) have the highest degradation yield of final products such as ammonia, chloride, 4-aminophenol, 4-chlorophenol, phenol, aniline, mixture of aldehydes and carboxylic acids (Sánchez et al., 2002).

Boron-doped diamond (BDD) anode with high efficiency could be used to degrade diuron or 3,4-DCA and intermediate products were short-chained organic acids. The degradation process was followed by HPLC and ionic chromatography analyses. Values of efficiencies greater than 20% were operating with low reactant concentration, 0.17 mM for diuron and 2 mM for 3,4-DCA (Polcaro et al., 2004).

Batch experiments of two soil clays kaolinite KGa-1 and Na-montmorillonite SWy-1 showed their capacity to adsorb CAs such as 3CA, 3,4-DCA and 2,4,6-TCA at concentration range from 0.05-0.485 $mg.g^{-1}$ clay. The result showed that montmorillonite has a higher sorption capacity than kaolinite. The greather sorption

was the lipophilic TCA and the sorption increase when concentration increasing. X-ray analysis suggests that CAs is sorption on the mineral surface of kaolinite (Angioi et al., 2005).

However, disadvantage of the physical and chemical treatments is their high-cost and lack of public acceptance. In addition, these treatments do not solve the contamination problem. The contaminants are incompletely treated, transfer from one phase to another phase which might become more toxic (Vidali, 2001).

2.6.2 Biological treatment

Biological treatment is an effective way to transformation or mineralization of contaminants to less toxic or nontoxic by microorganisms and plants.

Phytoremediation is treatment that used plants to remove harmful chemicals in the environment which their roots take pollutant in soil, streams and groundwater. Time to clean up using phytoremediation depends on type and number of plants, type and amount of pollutant, size and depth of contaminated area and type of soil. The advantages of this treatment are requires less equipment and labor and avoid contact with hazardous chemicals (EPA, 2001).

Bioremediation is a good alternative treatment that used microorganisms to clean up harmful chemicals using natural biological activity. The limited of bioremediation is bacteria growth and activity on contaminated area (Mrozik and Piotrowska-Seget, 2010). The microorganisms which used for treatment may be from indigenous to contaminated site or may be isolated from another site and brought to the contaminated site. Bioremediation is more economical than other techniques because it is a natural and safe process (Vidali, 2001). In addition, it has public

acceptance and complete degradation process which metabolized to carbon dioxide and water. This treatment can occur via aerobic and anaerobic conditions which aerobic condition is faster than anaerobic condition (Evans and Furlong, 2003).

2.7 Bioremediation strategies

Bioremediation can be dividing to three main strategies including natural attenuation, biostimulation and bioaugmentation.

2.7.1 Natural attenation

Natural attenuation is natural processes that act without human enhancement. The natural processes included biodegradation, dispersion, dilution, sorption, volatilization, biological transformation and stabilization of contaminants (EPA, 1999). Natural attenuation is an easy method and low cost. In contrast, these method takes a long time to completely degrade the contaminants because of low population of indigenous degrading microorganisms (Tongarun et al., 2008).

2.7.2 Biostimulation

Biostimulation is treatment that added nutrients and electron acceptors such as carbon, nitrogen, phosphorus and oxygen to a contaminated site to enhance the growth and stimulate the indigenous microorganisms for degradation (Mrozik and Piotrowska-Seget, 2010). This treatment enhances natural attenuation.

2.7.3 Bioaugmentation

Bioaugmentation is the application of single strains or consortia of microorganisms which capable to degrade toxic compound at contaminated site. Because of indigenous microorganisms not always has ability to degrade hazardous substance therefore it is necessary to add exogenous microorganisms or specialized microorganisms into environment to accelerate bioremediation (Watanabe, 2001). One of the most difficult of bioaugmentation is the survival of microorganism decreased when inoculated to soil. The effectiveness of this treatment depends on biotic and abiotic factors. Abiotic factors such as temperature, moisture, pH and organic matter. In addition, aeration, nutrient content and soil type have an effect on bioaugmentation (Mrozik and Piotrowska-Seget, 2010).

2.8 Chloroanilines degradation

2.8.1 Biodegradation by pure culture

For CAs degradation, various bacterial were applied including, *Moraxella* sp. strain G, *Pseudomonas* sp. strain JL2, *Brevundimonas siminuta* INMI KS-7, *Pseudomonas acidovarans* CA28, *Comamonas testosterone* I2, *Aquaspirillum* sp. strain 2C, *Acinetobacter baumannii* CA, *Klebsiella* sp. CA17, *Pseudomonas putida* CA16 (Zhang et al., 2010).

Zeyer and Kearney, (1982) reported that *Pseudomonas* sp. strain G was able to degrade 0.05 mM 3,4-DCA to CO_2 in presence of 1.5 mM 4CA under pure culture conditions.

You and Bartha, (1982) reported that *Pseudomonas putida* was unable to degrade monochloroaniline or dichloroaniline but was able to degrade 3,4-DCA in the

presence of propionanilide and aniline for the source of carbon, nitrogen and energy. Pathway of 3,4-DCA biodegradation occurred through 4,5-dichlorocatechlo, 3,4-dichloromuconate, 3-chlorobutenolide, 3-chloromaleylacetate and 3,chloro-4-ketadipate which the end product was inorganic. The comparison of mono- and dichloroaniline in resting cell technique showed dechlorination rates in order of 4CA < 3CA < 3,4-DCA < 2CA.

Vangnai and Petchkron, (2007) isolated three gram-negative bacteria from agricultural soil which have a potential to utilize 4CA such as *Acinetobacter baumannii* CA2, *Pseudomonas putida* CA16 and *Klebsiella* Sp. CA17. The result showed that the isolates grew well at 0.2 mM of 4CA without supplemented with aniline or another cosubstrate. These bacteria can survive at concentration up to 1.2 mM and 4CA was degraded via modified ortho-cleavage pathway.

Zhang et al., (2010) studied that a new strain *Delfla tsuruhatensis* H1 has ability to degrade individual CAs compounds or a mixture (2CA, 3CA and 4CA) as the sole carbon, nitrogen and energy source at concentration 150, 150 and 100 $mg.l^{-1}$, respectively. Individually, 3CA and 4CA were almost completely degraded within 23 and 25 hours, respectively, but 2CA was not degraded. As a mixture, 3CA degradation was faster, followed by 4CA and then 2CA. This indicated that strain H1 was unable degrade 2CA alone, but able degrade 2CA when mixed with other CAs.

Zeyer et al., (1985) reported *Moraxella* sp. strain G was able to degrade 4-fluoroaniline, 2CA, 3CA, 4CA and 4-bromoaniline as sole carbon and nitrogen source but not 3,4-DCA. The pathway of 4CA degradation was investigated by analysis of catabolic intermediates and enzyme activities which found that 4CA degradation occurred via modified ortho-cleavage pathway (Figure 2.1).

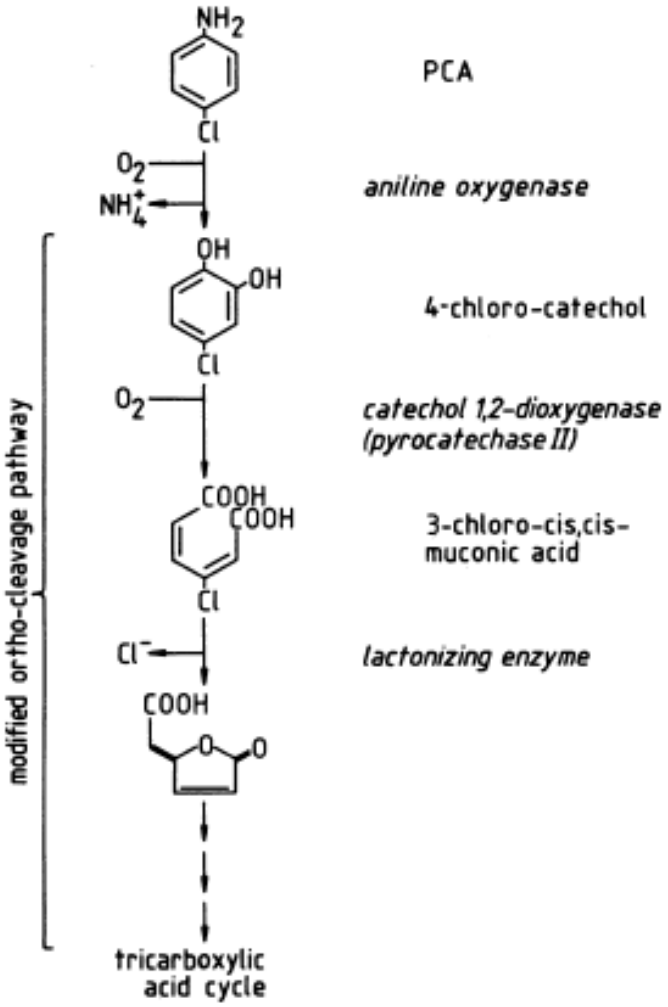


Figure 2.1 4-chloroaniline degradation pathway of *Moraxella* sp. strain G occurred *via* modified ortho-cleavage pathway (Zeyer et al., 1985).

Hongsawat and Vangnai, (2011) reported that *Acinetobacter baylyi* strain GFJ2 was able to degrade 3,4-DCA, 4CA, other monohalogeneted anilines (chloro-, bromo-, and fluoro-aniline) and DCAs. Moreover, the pathways of 4CA and 3,4-DCA degradation were detected by analysis of intermediates and enzyme activities. 4CA was found to be the first intermediate of 3,4-DCA degradation and after that the degradation of 4CA by strain GFJ2 proceeds with two distinct pathways (Figure 2.2).

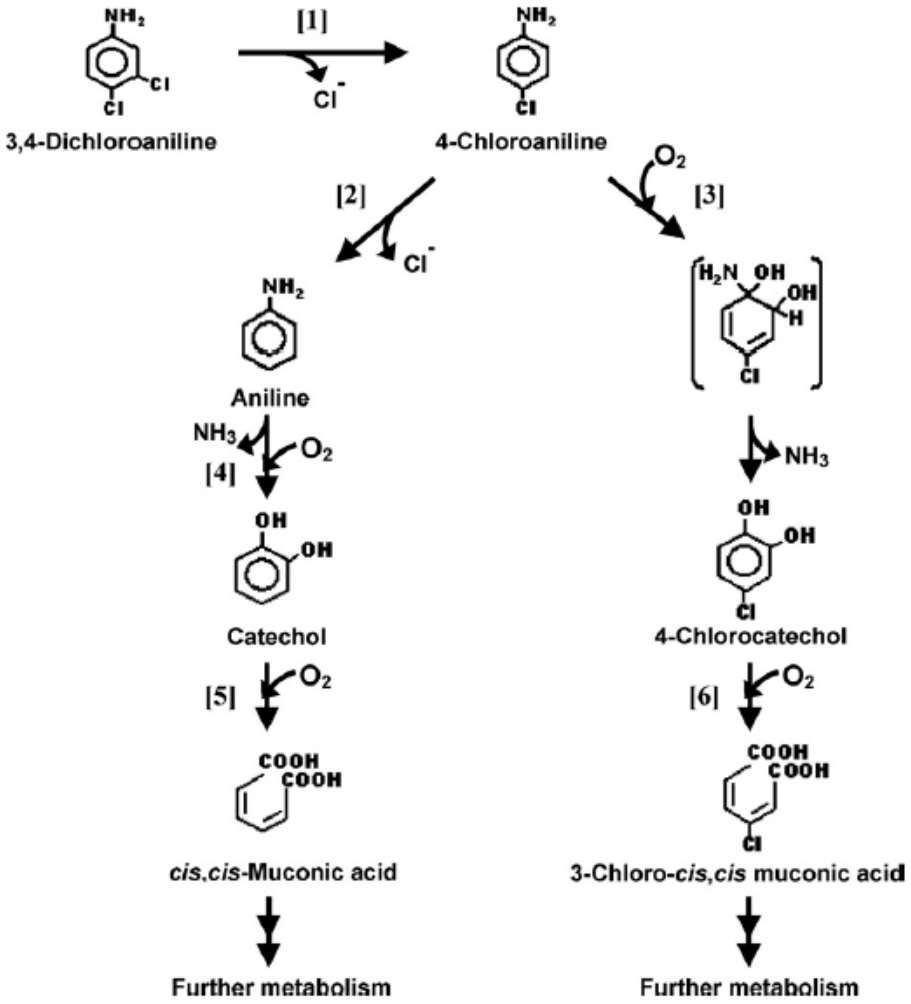


Figure 2.2 The pathways of 3,4-DCA and 4CA degradation by *Acinetobacter baylyi* strain GFJ2 (Hongsawat and Vangnai, 2011).

2.8.2 Treatments of CAs in environment

2.8.2.1 Water

Miille and Crosby, (1983) used 3,4-DCA as a model to investigate photochemical reactions in seawater. The result suggests that photolysis could be a major reaction in seawater to breakdown organic pollutants and other chemicals. The products of 3,4-DCA photolysis in seawater is shown in Figure 2.3. The products contained 70 – 80% of 2,-chloro-5-aminophenol (11), 5% of 3-chloroaniline (12) and the smallest amount of 3,3',4,4'-tetrachloroazobenzene (13).

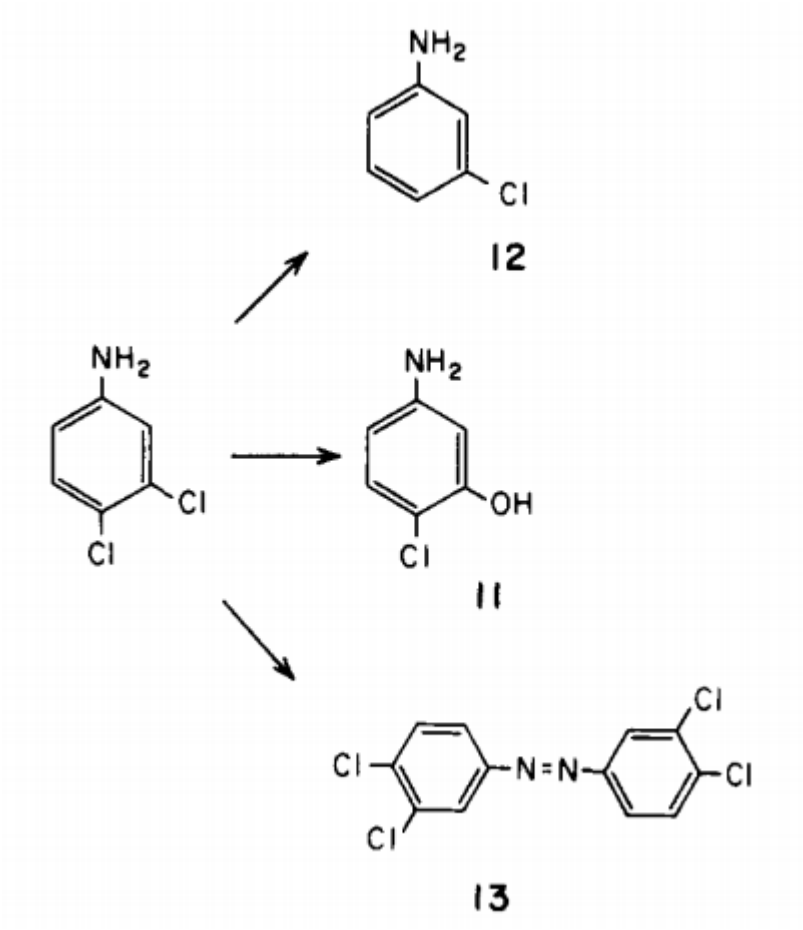


Figure 2.3 Photodegradation of 3,4-DCA in seawater (Miille and Crosby, 1983).

Lyones et al., (1985) reported that the 250 μg.ml^{-1} substituted anilines were recovered only 10% after 2 week which showed high persistence in pond water samples. The degradation of 4CA and 3,4-DCA in pond water were 4% and 3%, respectively, in 14 days. The degradation of 4CA and 3,4-DCA in pond water with sewage sludge were 7% and 6%, respectively, in 14 days. Which pond water contained 90 – 117 μg.ml^{-1} microbial biomasses and sewage sludge contained 8 ug.ml^{-1} biomasses.

Livingdtons and Willacy, (1991) studied the degradation of 3,4-DCA in aqueous solution by mixed cultures free and immobilized cells in a packed-bed reactor. The result showed that 3,4-DCA at concentration 250 mg.l^{-1} was degraded over 98% which degradation rate was 90 mg.l^{-1}.h^{-1}. For industrial wastewater that contained 3,4-DCA, aniline, 4CA, 2,3-DCA and 3,4-dichloronitrobenzene, the biomass can be degraded these compounds excepted 2,3-DCA.

2.8.2.2 Soil

Burge, (1972) studied that in five soils showed that 30 – 60 % of added propanil had been hydrolyzed to 3,4-DCA within 2 – 4 hr. and some 3,4-DCA was still accumulated in all soils after 83 days of incubation.

Brunsbanch et al., (1993) reported the indigenous soil population were degrading 3CA and 3,4-DCA more rapidly than 4CA and 2CA within 6 week. The addition of supplements buffer, mineral salts and acetate influence the degradation of indigenous soil populations. The selected CA-degrading bacteria such as *Pseudomonas acidovorans* strain BN3.1 could enhance the degradation of CAs within 10 days.

Tongarun et al., (2008) studied three bioremediation approaches including, natural attenuation (NA), biostimulation (BS) and bioaugmentation (BA) toward the degradation of 4CA contaminated in two types of agricultural soil. The result showed that 4CA degradation in soil depends on soil properties, characteristics of the indigenous microorganisms and the stability of population density of the bioaugmentation culture.

Sorensen et al., (2008) reported the degradation of 2 $mg.kg^{-1}$ diuron in soil after inoculation of *Arthrobacter globiformis* strain D47 and *Variovarax* sp. strain SRS16 individually or in a co-culture. The result showed that 79% of the added diuron was remained in soil without any inoculums after incubated for 26 days. Inoculation with strain D47 and strain SRS16 individually, 17% and 23% of diuron were still present in soil, respectively. The result of co-culture showed only 1% of initial amount of diuron was still present. The researcher suggest that soil and water contaminated with diuron and linuron could be clean up by combining of strain D47 and strain SRS16 which their shared metabolite 3,4-DCA.

Ding et al., (2009) used wetland soil at Yancheng institute of technology, Yancheng, China to study on bioremediation actions of 4CA. The conditions were sterilized soil and avoid light, non-sterilized soil and avoid light and sterilized soil and natural light. The result showed that sterilized soil and avoid light condition was the slowest degradation. These reports suggest that the important factors for 4CA degradation in wetland soil were microorganism and light.

2.9 Parameters involving soil bioremediation

Microorganisms in soil have limits for environmental conditions, as well as optimal conditions are useful for microorganisms to degrade the pollutants. The factors that affect the success of bioremediation as followed (Vidali, 2001);

2.9.1 Environmental factors

2.9.1.1 pH

Bioremediation can occur in the wide range of pH; however, best range is from 6.5 – 7.5. Soil pH may affect the solubility of phosphorus and important nutrient which pH 6.5 is optimal for biological system, pH that is higher or lower may decrease the solubility.

2.9.1.2 Temperature

Temperature affects the rate of bioremediation by controlling the rates of enzyme catalyzed reactions. Temperature also affects on log growth rate of microorganisms, appropriate temperatures for microbial growth are 0 – 40 ˚C.

2.9.1.3 Moisture

Moisture or water will disperse and dilute essential nutrients for the microorganisms. The moisture in pore spaces of soil may affect the exchange of oxygen. The optimal soil moisture should be from 50 – 80% of the water holding capacity of the soil.

2.9.1.4 Electron acceptors

Oxygen is the terminal electron acceptor in aerobic processes. The contaminant degradation rate is limited by the availability of oxygen. Oxygen is supplied by diffusion from the air or dissolved in and transported by water. When oxygen is consumed, other organic and inorganic compounds are used as terminal electron acceptors.

2.9.1.5 Energy source

Energy source affects the activity of microorganisms which contaminant will be used as an energy source for microorganisms. Pollutants will be carbon and nitrogen sources for microbial degradation.

2.9.1.6 Nutrient

A good microbial growth can be support by nitrogen, phosphorus, sulfur and other nutrients. The rate of contaminant degradation can influence by nitrogen available to microorganisms in the form of organic nitrogen, ammonia (NH_4^+), nitrate (NO_3^-) and nitrite (NO_2^-).

2.9.2 Microorganisms

For bioremediation to be successful depend on having the right microbes which have the potentially to degrade the pollutants in the right place with the right environmental factors for degradation to occur.

2.9.2.1 Total microbial activity study

Dehydrogenases were intracellular enzymes, as respiratory chain enzymes in microbial respiratory metabolism. Dehydrogenase activity can be used as an indicator and measure for the total oxidative activities of soil microorganisms (Alef, 1995).

Dehydrogenase activity in soil measured by either the reduction of TTC (2,3,5-triphenyltetrazolium chloride) to TPF (triphenylformazan) or INT (2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyl-tetrazolium chloride) to iodonitrotetrazolium formazan (INF). The extraction solvent was important for estimates of soil dehydrogenase activity. Methanol was extraction solvent for TPF. In case of INT, the extraction solvent were methanol, tetrachlorethylene:acetone mixture (1:1.5) or tetradrofuran (Ping, 1997).

In some case, the INF concentration of autoclaved soil (control condition) was higher than INF concentration of the test condition. TTC and TPF were light sensitive, therefore there were recommended measurement and incubate in the dark. The reduction of TTC to TPF is shown in Figure 2.4 (Alef, 1995).

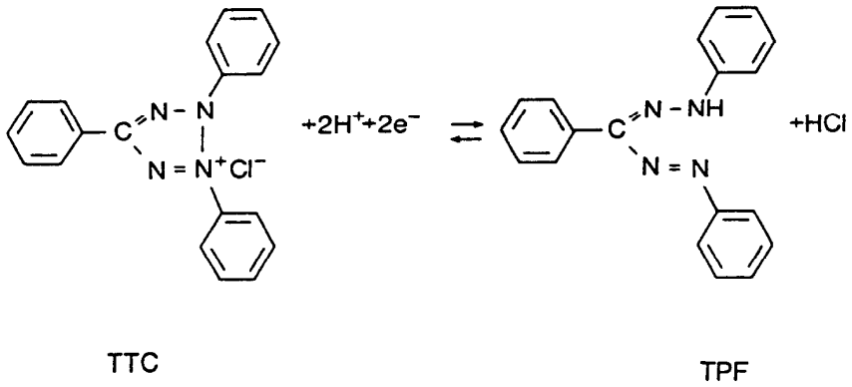


Figure 2.4 The reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to triphenylformazan (TPF) (Alef, 1995).

Ibarrolaza et al., (2009) studied the combined effect of phenanthrene and Cr(VI) on soil microbial activity and the efficiency of bioremediation processes. The results showed that Cr(VI) inhibited the phenanthrene mineralization. In addition, the soil dehydrogenase activity was determined by colorimetric measurement of the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to triphenyltetrazolium formazan which have creaming red-colored formazan (TPF). In the presence of Cr(VI)(25, 50 and 100 $mg.kg^{-1}$) were retarded the dehydrogenase activity and Cr(VI) 100 $mg.kg^{-1}$ was inhibited soil dehydrogenase activity.

Doi and Ranamukhaarachchi, (2009) determined the dehydrogenase activity by colorimetric measurement of the reduction of 2,3,5-triphenyltetrazolium chloride to triphenyltetrazolium formazan with a wet and dry seasons. The result showed that soil dehydrogenase activity was not significantly different between the wet and dry seasons. In contrast, soil dehydrogenase activity was significantly different between bare ground and the forests.

Chander and Brookes, (1991) reported soil dehydrogenase activity increased in the soil amended with sludge but decreased when soil containing Cu-rich sludge. Cu-contaminated soil decreased dehydrogenase activity because of the abiological reaction between TPF and Cu.

2.9.2.2 Microbial community analysis

Many herbicides are applied to the agricultural practices in which soil microorganisms may be affected by herbicide applications either directly or indirectly. Therefore, it is important to understand about microbial community in soil after the application of herbicides. To date, modern molecular techniques provide a

good opportunity to understanding the diversity of microbial community and to monitor population dynamics (Malik et al., 2008).

Extraction pure nucleic acids from soil has been difficult because of the complex of soil matrix, strong binding between microorganisms and soil particles or organic matter and coextraction substances such as humic acids which interfered DNA measurement, inhibited enzyme polymerase in PCR and reduced transformation efficiency (Zhou et al., 1996). One of the genetic fingerprinting techniques which attracted the attention of many environmental microbiologists and used in many laboratories is denaturing gradient gel electrophoresis (DGGE) (Muyzer and Smalla, 1998).

The DGGE technique is an electrophoretic method based on differential denaturation (melting) profile of double-strand DNA fragments. DNA fragments of the same size but different sequence will be separate in DGGE. The DNA fragments to be loaded on DGGE are PCR products which not longer than 500 bp. The most commonly target for PCR amplification is the ribosomal DNA (rDNA). This is because it is a highly conserved sequence between all organisms and different bacterial species have differences 16S rDNA (Ercolini, 2004).

Fantroussi et al., (1999) studied the effect of three phenylurea herbicides; diuron, linuron and chlorotoluron by using soil sample 10-year history of treatment. DGGE was used for analysis of 16s rRNA genes (16s rDNA). The results showed that microbial community was significantly different between treated and non-treated with phenylurea herbicides which bacterial diversity decreased in treated soil. The

sequence of DGGE fragments showed that the most affected bacterial species in the diuron and linuron treated soil was an uncultivated bacterial group.

Li et al., (2006) used PCR-DGGE based on 16S rDNA to assess the changes in microbial community in seven field sites contaminated by heavy metal (Cu, Zn and Cd). The results showed that increasing distance from the Cu and Zn smelter increased the number of DGGE bands in soils, so heavy metal contamination decreased diversity of bacterial community in the soil.

Lipthay et al., (2004) studied three DNA extraction protocol (sonication, grinding-freezing-thawing and bead beating) applied to three different soils. The result showed that bead beating protocol gave the highest number of DNA band which this protocol was obtained the highest soil bacterial diversity. In contrast, bead beating gave more sheared DNA and many fractions of low molecular weight DNA.

Lai et al., (2006) studied bacterial community composition in deep-sea sediments of the South China Sea which DNA was extracted by five different methods. DGGE was used to analyze the 16S rRNA gene diversity of samples. The DNA extraction method contained glass bead-beating (GB), enzymeatic lysis (EL), chemical lysis (CL), SN kit and improved DNA extraction method (IM) which used bead-beating and polyethylene glycol (PEG) to precipitate and improve the purity of DNA. The result showed the lowest DNA yield in SN kit and using the IM method was obtained more diverse genomic DNA. The DNA fragment from IM method was in range of 30 – 100 kb with many sheared DNA. The sheared DNA could influence the sensitivity of PCR detection and increase the chimeric PCR products. The humic acid substances could influence the PCR reactions. Therefore, to reduce the humic

acid effect, dilution the crude DNA (1:100) before used as templates for PCR amplification is necessary.

2.9.3 Copper fungicides co-contamination

Beside that herbicide was applied to agricultural field, other soil amendments may be applied and affected the fate of herbicides such as copper-base fungicides ($Cu(OH)_2$, $CuSO_4$, copper oxychloride, copper hydroxide and Bordeaux-M). Copper-base fungicides have been widely used in Europe since the end of the 19^{th} century to control fungal diseases, bactericidal sprays, fertilizer and stimulants of animal growth (Komárek et al., 2010). Copper has ability to interact with soil mineral and soil organic matter to form precipitates with sulfide, carbonate, hydroxide and other anions (Dumestre et al., 1999). The presence of organic and inorganic pollutants, soil type and cation-exchange capacity of soil are the factors affecting metal retention in soil (Palma and Medici, 2002).

On health effects, copper is absorbed through the gastrointestinal tract and excreted within faeces or transported to the liver. There have been evidences that vineyard workers have acute and chronic respiratory problems, lung carcinoma caused inhalation of copper fungicides (Araya et al., 2003).The European directive 98/83/CE sets copper concentrations for drinking water below 2.0 $mg.l\text{-}^{1}$. At concentration 0.8 mg $Cu.l^{-1}$ or maximum 4.2 mg $Cu.l^{-1}$ gave chronic exposure of children. Concentration more than 10 mg $Cu.l^{-1}$ gave acute exposure of adult and led to rapid gastric diseases (Komárek et al., 2010).

Increased copper concentrations affect adversely on soil biota and plants due to their non-biodegradable nature and long-term biological half-lives (Komárek et al.,

2009). Copper have adverse effects on earthworm growth at concentrations of 100 $mg.kg^{-1}$ or higher than 300 and 320 mg $Cu.kg-^{1}$ soil and reproduction at concentrations of 53.3-150 mg $Cu.kg^{-1}$ soil (Helling et al., 2000). Copper concentration in soil around the world as published is shown in Table 2.5.

Table 2.5 Copper concentration in soil as published (Komárek et al., 2010)

Country	Depth (cm)	mg Cu kg^{-1} (ppm)	Method used
Australia	0-1	9-249	15.5 M HNO_3
Australia	0-10	6-223	HNO_3+HCl
Brazil	0-5	37-3216	HNO_3+$HClO_4$+HF
Bulgaria	0-10	72	HNO_3+HCl
Canada	0-15	10-77	HNO_3
Croatia	0-10	30-700	HNO_3+HCl
Croatia	0-20	105-553	$HClO_4$+HNO_3+HCl+HF
France	0-10	15-430	$HClO_4$+HF
France	0-2	323	HF
France	0-20	57-332	$LiBO_2$ at 550 ˚C+ HNO_3
Greece	0-30	<157	HNO_3+HCl
Italy	0-15	50-300	HNO_3+$HClO_4$
New Zealand	0-10	4-259	HNO_3+HCl
Slovenia	0-20	87-120	HNO_3+HCl
Spain	0-20	25-272	HNO_3+HCl+HF
Spain	0-20	38-63	HNO_3+HCl
Thailand	0-10	115-238	X-ray fluorescence

2.9.3.1 Copper sulfate

Copper sulfate is the chemical compounds had bright blue color and widely used in organic agriculture as fungicide, pesticide, herbicide, algicide and source of copper in animal nutrition. The U.S. Environmental Protection Agency (EPA) is classified copper sulfate as a General Use Pesticide (GUP). It is allowed for use in crop disease in the US and internationally, but it has banned in the EU since May 2002 (NOSB, 2001).

Copper sulfate is used in rice production system which it is applied into flooded fields. This situation common occurred in the Thailand, Malaysia, Sri Lanka, India, Australia, south Europe and America. In addition, mosquito fish, pond snails and other organisms in rice paddies were killed by copper sulfate for beneficial rice productions. 15 lb/acre copper sulfate was applied to rice field which it was accumulation in rice seed for 3.5 to 5.7 mg/kg. China reported, people daily intake copper to 1.4 mg/person from rice (NOSB, 2001).

Copper sulfate could be accumulated in soil and groundwater. For soil, copper sulfate adsorption to clay, organic materials and mineral surfaces depends on property of soil (the acidity or alkalinity). In water, copper sulfate bind with sediment and water particulates (NOSB, 2001).

High dose of copper causes toxicity in human and animals. Copper sulfate burns skin and eyes; as well as it shows acute toxicity in animals at the spleen, liver and kidneys (NOSB, 2001). Copper sulfate is found to be more toxic than copper hydroxide and copper oxychloride on snail by 13- and 22-fold, respectively (El-Gendy et al., 2009).

2.9.3.2 Copina-85

Copina-8 is a copper based fungicide commonly knows in the name of copper oxychloride with green or blue-green power color which contained 85% copper as wetting powder. Copper oxychloride is widely used for the control of fungus diseases which preventing black spot on vegetable and fruits. To reduce the toxicity of copper in soils, soil microorganisms transformed copper oxychloride into insoluble copper oxalate (Komárek et al., 2010).

2.9.3.3 Copina hydroxide

Copina hydroxide is a copper based fungicide commonly knows in the name of copper hydroxide with blue color contained 77% copper as wetting powder. Copper hydroxide is a protective fungicide of both fungi and bacteria and also applied in the agricultural to prevent copper insufficiency.

2.9.3.4 Poltiglia

Poltiglia or commonly name of bordeaux mixture is the commercial name of copper based fungicide, a chemically undefined mixture of copper sulfate and hydrated lime. Poltiglia contained 74% copper sulfate as wetting powder. The mixture of copper sulfate and lime have been used more than two centuries and it is still widely used (Pietrzak and McPhail, 2004).

2.9.3.5 Bordeaux-M

Bordeaux-M is a commercial name of copper based fungicide which contained 48% copper sulfate as wetting powder, manganese ethylenbis dithiocarbamate and surfactants.

CHAPTER III

MATERIALS AND METHODS

3.1 Laboratory equipment and chemicals

3.1.1 Laboratory equipment

Laboratory equipments	Company	Country
Aspirator pumps	Cole-Parmer Instruction Co.	Korea
Autoclave NLS-3020	Sanyo Electric Co., Ltd	Japan
Autoclave HV-110	Hirayama	Japan
Bead ruptor 24	Omni	USA
Centrifuge (Prism R)	Labnet	USA
Cooling & heating block	Bioer Technology	USA
C18 column (Hyperclone 5u BDS C18 130A, a 250 x 4.6 mm)	Phenomenex	USA
Denaturing Gradient Gel Electrophoresis (DGGE)	Scie-Plas	England
Digestion Unit, K-424	Buchi	Switzerland
Digital dry bath	Labnet	USA
Distillation Unit, B-324	Buchi	Switzerland

Laboratory equipments	**Company**	**Country**
Finemixer (SH 2000, Finemould)	Precision Ind. Co.	USA
Gel Doc™ XR + Image Lab™ Software	Bio-Rad	USA
GeneAmp® PCR system 2700	AB Applied Biosystems	USA
High Performance Liquid Chromatography (HPLC) LC-20	Shimazu	Japan
Hotplate stirrer	Lab Tech	Korea
Incubator shaker, innova 4000	New Brunswick scientific	USA
Incubator shaker, innova 4340	New Brunswick scientific	USA
Micropipette 20,100, 200, 1000 ul	Gilson	France
Microscopy, SK-500	Seekscope	China
MiniRun Gel Electrophoresis	Bioer Technology	USA
pH meter	Mettler Toledo	USA
Protector Laboratory Hood	Science Technology	USA
Quick spin (Micro one)	TOMY	Japan
Refrigerated Centrifuge, Avanti ™ J-301	Beckman Coulter	USA
Refrigerated Centrifuge, 5804R	Eppendorf	USA
Scrubber Unit, B-414	Buchi	Switzerland

Laboratory equipments	Company	Country
Spectrophotometer DU 650	Beckman	USA
Ultrasonic	Banderlin	Germany
UV transilluminater, BioDoc-It™ System	UVP	USA
Vacuum pumps	GAST	USA
Vortex (Touch mixer model 232)	Fisher Scientific	USA

3.1.2 Laboratory chemicals

(1) Chemicals

Chemicals	Company	Country
Acrylamide	Bio basic Inc	USA
Acryl/Bis™ 37.5:1	Amresco	USA
Agar	Scharlau Chemic Microbiology	Spain
Agarose	BMA	USA
Ammonium persulfate	Bio-Rad	USA
Bis-acrylamide	Bio basic Inc	USA
Bordeaux-M (48% copper sulfate)	Erawan	Thailand
Bovine serum albumin (BSA)	Sigma	USA
$CaCl_2.2H_2O$	Merck	Germany

Chemicals	**Company**	**Country**
$C_{12}H_{25}O_4SNa$ (SDS)	Sigma	USA
Copina Hydroxide (77% copper hydroxide)	Erawan	Thailand
Copina 85 (85% copper oxychloride)	Erawan	Thailand
Cetyltrimethylammonium (cetrimonium) bromide (CTAB)	Bio basic Inc	USA
$CoCl_2.6H_2O$	Merck	Germany
Crystal violet	BDH	England
$CuSO_4.5H_20$	Scharlau Microbiology	Spain
EDTA	BDH	England
$Fe(NH_4)_2(SO_4).6H_2O$	BDH	England
$FeSO_4.7H_2O$	BDH	England
Folin-Ciocalteu's reagent	Carlo Erba Reagenti	Italy
Formamide	Amresco	USA
Glycerol	Univar	Australia
H_3BO_3	Merck	Germany
Iodine crystal	BDH	England
$K_2Cr_2O_7$	Riedel	Germany

Chemicals	**Company**	**Country**
KH_2PO_4	Carlo Erba Reagenti	Italy
K_2HPO_4	Riedel	Germany
Methyl red	Fluka	Germany
$MgSO_4.7H_2O$	Carlo Erba Reagenti	Italy
$MnSO_4.H_2O$	Merck	Germany
MoO_3	Merck	Germany
NaCl	BDH	England
Na_2CO_3	BDH	England
NaF	BDH	England
$NaNO_3$	Carlo Erba Reagenti	Italy
NaOH	Merck	Germany
Na_2HPO_4	Fluka	Germany
Na_2SO_4	Fluka	Germany
Peptone	Merck	Germany
Phenol/Chloroform/Isoamyl Alcohol (25:24:1	Research Organics Inc	USA
Poltiglia (74% copper sulfate)	Erawan	Thailand
Poly(ethylene glycol)	Alorich	Germany
Safanin O	Fluka	Germany

Chemicals	**Company**	**Country**
Succinic acid	Merck	Germany
Triphenylformazan (TPF)	TCI	Japan
Taq DNA polymerase	Fermentas	USA
Tetramethylethylenediamine (TEMED)	Bio Basic Inc	USA
Tris	USB	USA
Tryptone	Himedia	India
Urea	Merck	Germany
Yeast extracts	Scharlau Chemic Microbiology	Spain
$ZnSO_4.7H_2O$	Merck	Germany
2,3,5-triphenyltetrazolium chloride (TTC)	TCI	Japan
3,4-dichloroaniline (99.5% purity)	Chem Service, Inc.	USA
4-chloroaniline (99.5% purity)	Chem Service, Inc.	USA

(2) Analytical grade organic solvents

Organic solvents	**Company**	**Country**
Absolute ethanol (99.9% purity)	Merck	Germany
Acetic acid, glacial	J.T. Baker	USA
Organic solvents	**Company**	**Country**
Methanol	Merck	Germany

(3) HPLC grade organic solvents

Chemicals	**Company**	**Country**
Acetonitrile (99.9% purity)	Lab-scan	Thailand
Methanol (99.9 % purity)	Lab-scan	Thailand

3.2 Culture medium

3.2.1 The mineral medium (MM)

The mineral medium was used for screening, isolation, cultivation and degradation. The mineral medium was comprised of media and trace element.

(1) Media

Na_2HPO_4	1.4196	g
KH_2PO_4	1.3609	g
$MgSO_4.7H_2O$	0.0985	g
$CaCl_2.H_2O$	0.0059	g

The component was dissolved in 1 liter of distilled water and adjusted pH to 7 by 1 N NaOH. The mineral medium was autoclaved at 121 ˚C for 15 minutes.

(2) Trace element

H_3BO_4	0.116	g
$FeSO_4.7H_2O$	0.278	g
$ZnSO_4.7H_2O$	0.115	g
$MnSO_4.H_2O$	0.169	g
$CuSO_4.H_2O$	0.038	g
$CoCl_2.6H_2O$	0.024	g
MoO_3	0.010	g

Trace element solution was separately prepared from media as a stock solution. They were dissolved in 100 ml of distilled water and it was autoclaved at 121 ˚C for 15 minutes. Before using, 0.1% (v/v) sterile trace element was supplemented in mineral medium.

3.2.2 Luria bertani medium (LB)

Tryptone	10	g
Yeast extract	5	g
NaCl	10	g

LB medium was dissolved in 1 liter of distilled water, adjusted pH to 7 and autoclaved at 121 ˚C for 15 minutes.

3.3 Preparation of 4CA and 3,4-DCA solution

4CA and 34DCA stock solution were prepared in absolute ethanol at a concentration of 10,000 ppm. CAs at each concentration was obtained by doing a serial dilution from CAs stock solution.

3.4 Preparation of copper solution

The stock solution of copper was prepared in distilled water at concentration of 1,000 ppm. Copper at each concentration was obtained by doing a serial dilution from the copper stock solution.

3.5 Preparation of supplementary nutrient

250 grams of fertilizer and preserved soil were dissolved in 1 liter of distilled water. After 30 minutes, the solution was centrifugation for 20 minutes at 8,000 rpm and supernatant was sterilized by autoclaving at 121 ˚C for 15 minutes.

3.6 Biodegradation in liquid medium

3.6.1 Effect of CAs concentrations by *Acinetobacter baylyi* strain GFJ2

3.6.1.1 Chloroanilines utilization

(1) Preparation of bacterial inoculums

The cell inoculums were prepared by transfer one loop of *A. baylyi* strain GFJ2 from LB agar plate into 20-mL glass bottle containing 5-mL LB medium, pH 7.0 and cultured on the rotary shaker at 150 rpm under room temperature for 12 hours.

(2) Study of CAs degradation

The cell inoculums (1% v/v) were transferred to 250-mL Erlenmeyer flask that contained 100-mL of MMSAY (the mineral medium containing 4-mM succinate, 1-mM ammonium sulfate and 0.1% (w/v) yeast extract), pH 7.0 and 0.2, 0.4, 0.8 and 4.0-mM 4CA, 34DCA and 4CA-34DCA on the rotary shaker at 150 rpm under room temperature. 1-mL of culture medium was collected at different incubation times in order to determine cell growth by measuring cell optical density at 560 nm, the CAs residual by HPLC and cell protein by a modified Lowry method.

3.6.1.2 Chloroanilines utilization *via* resting cell technique

(1) Preparation of bacteria inoculums

The cell inoculums were prepared by transfer one loop of *A. baylyi* strain GFJ2 from LB agar plate into 20-mL glass bottle containing 5-mL MMSAY (the mineral medium containing 4-mM succinate, 1-mM ammonium sulfate and 0.1% (w/v) yeast extract), pH 7.0 and cultured on the rotary shaker at 150 rpm under room temperature for 12 hours. Then, the cell inoculums (1% v/v) were transferred to 250-mL Erlenmeyer flask that contained 100-mL of MMSAY, pH 7.0. The CAs at concentration 0.2-mM was alternatively added as an inducer. After 16-18 hours of incubation on the rotary shaker at 150 rpm under room temperature, the cells were harvested by centrifugation for 20 minutes at 8,000 rpm 4 ˚C, washed twice with 0.85% (w/v) NaCl and resuspended in MMSAY, pH 7.0.

(2) Study of CAs degradation

5-mL bacterial suspension was added into 20-mL glass bottle, after that CAs at various concentrations (0.2, 0.4, 0.8 and 4.0-mM) was added in order to test biodegradability of the *A. baylyi* GFJ2. The sample (500-uL) was collected at interval incubation times to determine the CAs residual by HPLC and cell protein by a modified Lowry method.

3.6.2 Effect of supplementary nutrient

According to the previous CAs biodegradation method (3.6.1), each additional nutrient source which preparation by method 3.5 (Fertilizer nutrients (FSN), manure nutrients and soil nutrients (PSN)) was supplemented to make the final C/N ratio as indicated (at approximately 5% (v/v)).

3.6.3 Effect of CAs and copper fungicide co-contamination

3.6.3.1 Effect of copper fungicide types and concentrations

According to the previous CAs biodegradation method (3.6.1), each additional copper fungicide (copper sulfate, copina-85, copina hydroxide, poltiglia and Bordeaux-M) was prepared by doing a serial dilution from the copper stock solutions and supplemented at 5, 10, 50 and 100-ppm.

3.7 Biodegradation in soil

3.7.1 Recovery of CAs from soil

Five grams of sieved soil were placed in 20-mL glass bottle and spiked with CAs (500-ppm). Then CAs was extracted by adding 10-mL of methanol (50%, 80% and 100%). The sample bottles were rotated by the rotator overnight at room temperature. The soil suspension sample was allowed to settle and spin before the extracted liquid solution was collected. Then, the amount of CAs recovered from each extraction was analyzed by high performance liquid chromatography (HPLC).

3.7.2 Bioremediation of CAs

Three biodegradation process including natural attenuation, biostimulation and bioaugmentation were studied to evaluate the efficiency of 4CA and 34DCA degradation.

3.7.2.1 Natural attenuation

Five grams of 0.2 mm sieved soil were placed in 20-mL glass bottle and spiked with 500-ppm CAs. Then the soil samples were incubated at room temperature and moisture content was maintained by monitoring the using the weight of the microcosm every week. The samples were collected at different incubation times to determine the CAs residual by HPLC and soil microbiological analysis. The spiked CAs was degraded by the ability of the indigenous microorganisms in soil.

3.7.2.2 Biostimulation

Five grams of 0.2 mm sieved soil were placed in 20-mL glass bottle and spiked with 500-ppm CAs. Then, 5% (v/w) of fertilizer nutrients was added in the glass bottle. The soil sample were incubated at room temperature and moisture content was maintained at 50% as its initial value by monitoring the using the weight of the microcosm every week. The samples were collected at different incubation times to determine the CAs residual by HPLC and soil microbiological analysis.

3.7.2.3 Bioaugmentation

Four conditions of bacterial inoculums were carried out for the experiment:

(1) Pure bacterial culture; *A. Baylyi* GFJ2 (10^{10} CFU/g soil)

(2) Bacterial consortium from fertilizer (25% w/w)

(3) Combination of *A. Baylyi* GFJ2 (10^{10} CFU/g soil) and bacterial consortium (25% w/w)

(4) *A. Baylyi* GFJ2 (10^{10} CFU/g soil) supplemented with 5% (v/w) fertilizer nutrient

Preparation of bacterial culture

The cell inoculums were prepared by transfer one loop of *A. baylyi* strain GFJ2 from LB agar plate into 20-mL glass bottle containing 5-mL MMSAY, pH 7.0 and cultured on the rotary shaker at 150 rpm under room temperature for 12 hours. Then, the cell inoculums (1% v/v) were transferred to 250-mL Erlenmeyer flask that

contained 100-mL of MMSAY, pH 7.0. The CAs at concentration 0.2-mM was alternatively added as an inducer. After 16-18 hours of incubation on the rotary shaker at 150 rpm under room temperature, the cells were harvested by centrifugation for 20 minutes at 8,000 rpm 4 ˚C, washed twice with 0.85% (w/v) NaCl and resuspended in MMSAY, pH 7.0. (Approximately of 10^{10} CFU/ g soil)

Bioaugmentation treatment

Five grams of sieved soil were placed in 20-mL glass bottle and spiked with 500-ppm CAs. 10^{10} CFU/ g soil of *A. Baylyi* GFJ2 and 25% (w/w) of bacterial consortium from fertilizer were added into the bottles and well mixed with the soil. The soil sample were incubated at room temperature and moisture content was maintained by monitoring the using the weight of the microcosm every week. The samples were collected at different incubation times to determine the CAs residual by HPLC and soil microbiological analysis.

3.7.3 Effect of CAs and copper fungicide co-contamination

According to the previous 4CA and 34DCA biodegradation method (3.7.2), then, the effect of 150 ppm copper sulfate co-contamination of 4CA and 3,4-DCA degradation was examines.

3.7.4 Controls of the experiment

Five grams of 0.2 mm sieved soil were sterilized by autoclaving 3 times at 121 ˚C for 15 minutes and were placed in 20-mL glass bottle. The moisture content was maintained at 50% as its initial value by adding autoclaved distilled water indicated to initial weight. CAs was spiked into the bottles to the give final concentration of 500-ppm. The soil sample was incubated at room temperature and moisture content was maintained and adjusted by monitoring the using the weight of the microcosm every week. The samples were collected at interval incubation times to determine the CAs residual by HPLC and soil microbiological analysis.

3.8 Analytical procedures

3.8.1 Cell growth determination

The cell growth was determined by spectrophotometer at wavelength 560 nm and using the culture medium as a blank.

3.8.2 Cell protein determination

(1) Protein extraction from cell

The sample (500 μL) was centrifuged at 10,000 rpm, 5 minutes. Then the supernatant was removed and washed twice with 0.85% (w/v) NaCl. Protein was resuspended in 0.85% (w/v) NaCl. Protein was extracted from cells by heating at 100 ˚C for 10 minutes. The supernatant was used to determine protein concentration.

(2) Protein determination

Protein concentration was determined by a modified Lowry method (Lowry et al. 1951). Bovine serum albumin (BSA) was used as a standard protein.

- **Reagent preparation**

Reagent A: 2% sodium carbonate in 0.1 M sodium hydroxide containing 0.5% sodium dodecyl sulfate (SDS)

Reagent B: 0.5% copper sulfate in 1% potassium sodium tartrate

Reagent C: phenol solution (Folin-Clocahen's reagent)

Adjust volume of sample to 0.4-mL using autoclaved deionized water. Add 2-mL of fresh mixed solution A and B (A: B, 50:1, v/v) and rapidly mixed. The mixture was incubated at 30 ˚C for 10 minutes. After that added 0.2-mL of solution C, mixed immediately, and incubated at 30 ˚C for 30 minutes. Finally, the quantity of protein was measured by spectrophotometer at wavelength 750 nm and using deionized water as bank.

3.8.3 Chloroanilines residual determination

Chloroanilines residual were determined by using High Performance Liquid Chromatography (HPLC).

(1) Chloroaniline calibration curve

Calibration curve was used to analyze chromatogram of HPLC result in order to calculate the concentration of substance. CAs standard was prepared by dilution to various concentrations (0.025, 0.05, 0.1, 0.2 and 0.4-mM) of CAs. The prepared

standard solutions were then filtered through 0.45 μm nylon filter and analyzed in HPLC (Appendix A).

(2) Sample preparation from liquid medium

Cell samples were harvested to remove cells by centrifugation at 10,000 rpm for 5 minutes. The cell-free supernatant was collected, mixed with acetronitrile (HPLC mobile phase) at ratio 1:10 (v/v), and filtered through 0.45 μm-nylon syringe filter.

(3) Sample preparation from soil

CAs was extracted from soil by adding 10-mL of 80% methanol into the sample bottle. The sample bottles were rotated by rotator overnight at room temperature. After that, the samples were allowed to settle, spin and collected the liquid solution. The liquid solution was mixed with acetronitrile (HPLC mobile phase) at ratio 1:10 (v/v), and filtered through 0.45 μm-nylon syringe filter.

(4) HPLC analytical condition

The prepared samples were quantitatively analyzed by a reverse phase HPLC with a UV detector at a wavelength of 240 nm. The separation was performed on C18 HPLC column (Inersil ODS-3, 250×4.6 mm) using a mixture of acetronitrile:water (70:30, v/v) as a mobile phase with a flow rate 1 ml/min. The injection volume was 15 μL. The retention times of CAs under the condition test were 3.5 and 4.1 minutes, respectively.

3.8.4 Dehydrogenase activity determination

Total microbial activity in soil was determined with 2,3,5-triphenyltetrazolium chloride (TTC). Soil sample (1 g) was mixed with 2-mL of substrate solution (1% TTC solution; TTC was dissolved in tris buffer solution, pH 7.6) in 20-mL glass bottle with rubber stopper and incubated at 30 ˚C in the dark for 24 hours. 5-mL of methanol was added to sample bottle and future incubated at 30 ˚C in the dark for 2 hours. The soil suspension was centrifuged at 4,500 rpm for 10 minutes and measured by spectrophotometer at wavelength 485 nm.

(1) TPF calibration curve

Calibration solution of TPF standard was prepared by dissolved TPF in methanol as following concentrations: 0, 3.33, 6.67, 16.7 and 33.3 ug.mL^{-1}, then measured by spectrophotometer at 485 nm (Appendix D).

3.8.5 Amount of CAs-degrading bacteria determination

Colony Forming Unit (CFU) was used for determining the amount of CAs-degrading bacteria. Ten-fold serial dilution of bacteria was prepared by diluting soil sample with sterile water. The serial dilution of cell suspension was spreaded on MMSAY agar plates which 100-ppm CAs (with or without 150-ppm copper sulfate) as sole carbon source. The plates were incubated at 30 ˚C. Number of bacteria colony on the agar plate was accounted for the CAs-degrading bacteria.

3.8.6 Microbial community determination

Bacterial identification based on 16S rDNA was studied bacterial communities using denaturing gradient gel electrophoresis (DGGE).

(1) Soil DNA extraction

Soil sample 0.5 g was mixed with 0.4 g 1.4 mm ceramic beads in 2 mL plastic tube with screw cap and added 500 μL phosphate buffer, 500 μL SDS lysis mixture and 300 μl chloroform-isoamyl alcohol (CIA, 24:1). Sample was mixed in bead ruptor at speed 2 M/s, 10 sec for 5 cycles. Sample was centrifuged at 8,000 rpm for 10 minutes and transferred 800 μL supernatant to a new tube. Added 600 μL phenol/chloroform/isoamyl alcohol (25:24:1) and mixed by inverting 8 times. Sample was centrifuged and transferred 600 μL upper phase to a new tube. Added 600 μL CIA and mixed by inverting 8 times, after that, transferred 400 μL aqueous phase and mixed with 450 μL precipitation solution, then incubated at 37 ˚C for 1 hour. DNA was precipitated by centrifugation at 12,000 rpm for 30 minutes, room temperature. Pellet was washed twice with ice-cold 70% (v/v) ethanol, then, removed all the liquid and air dried for 20 minutes. Added 50-μL TE buffer and gently flicking to resuspend.

(2) Polymerase chain reaction (PCR) of 16S rDNA

Polymerase chain reaction (PCR) using 341F+GC clamp (5' CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG 3') and 520 R (5' ACCGCGGCTGCTGGC 3') primers (Dar et al. 2005) were carried out to amplify 16S rDNA. The expected PCR product size was 200 bp long. The PCR reaction contained 5 μl 10x Taq buffer with $(NH_4)_2SO_4$, 0.2-ul 5 $U.ul^{-1}$

Taq polymerase, 4 µl 25 mM $MgCl_2$, 5 µl 2 mM dNTP mix, 2 µl 10 µM of both primer, 2 µl 0.4 mg/ml BSA and 4 µl dilute DNA template and deionized water to a total of 50 µl (Edenborn and Sexstone 2007).

The condition was

1. Pre-denaturation at 95 ˚C for 5 min
2. Denaturation step at 94 ˚C for 0.45 min
3. Annealing step at 60 ˚C for 0.45 min
4. Extension step at 72 ˚C for 2 min

Go to step 2-4 for 30 cycles

The final extension at 72 ˚C for 10 min

5 µL of PCR product was run in 2% agarose gel with 1x TAE at 100 V. Then, the gel was stained in ethidium bromide and destained with water. The PCR product band was detected under UV transilluminater.

(3) Ethanol precipitation of PCR product

Measure the volume of the PCR product after that added equal volume of phenol/chloroform, mixed by vortex and spin at 12,000 rpm, 4 ˚C for 10 min. Take the supernatant (upper layer), added 1/10 volume of 3 M sodium acetate, pH 5.2 and added 2.5 volumes of cold 99.9% ethanol, mixed well after that keep at -20 ˚C for 2 hr. or overnight. Spin at 12,000 rpm, 4 ˚C for 15 min, carefully decant supernatant, rinsed with 1 mL 70% ethanol (cold), mixed and spin again. Airs dry for 15 min after that resuspend pellet in appropriate volume of water.

(4) Denaturing gradient gel electrophoresis (DGGE)

PCR product was loaded onto 8% polyacrylamide gel with a denaturing gradient ranging from 50 – 70%. The gel was run at 60°C for 7 hr at 130 V with 1xTAE buffer. DGGE gel was stained in 50 $\mu g.mL^{-1}$ ethidium bromide for 1 hr. DNA band profiles can be detected under the UV transilluminater.

3.8.7 Available copper content in soil

Five grams of 0.2 mm sieved soil was placed in 250 ml Erlenmeyer flask and spiked with 150 ppm copper sulfate after that mixed well. Added 50 ml distilled water to sample flask, mixed soil sample and allowed, spin it to settle. Supernatant was collected and analyzed available copper using Atomic Absoption Spectrophotometer (AAS), Varian Model AA280FS (air-C_2H_2 flame atomic absorption spectrophotometry) by Scientific and Technological Research Equipment Centre Chulalongkorn University.

CHAPTER IV

RESULTS

4.1 Biodegradation of 4CA, 3,4-DCA and co-occurring 4CA-34DCA in liquid medium

4.1.1 Effect of CAs concentrations by *Acinetobacter baylyi* strain GFJ2

Bacterial used in this study was *Acinetobacter baylyi* strain GFJ2, a gram negative coccus-shaped bacterium which was isolated from soil by our laboratory (Hongsawat and Vangnai 2011). *A. baylyi* strain GFJ2 was initially tested for CAs biodegradability by using various concentrations. It was grown on the mineral medium containing 4 mM succinate, 1 mM ammonium sulfate and 0.1% (w/v) yeast extract (MMSAY) supplemented with 0.2, 0.4, 0.8 and 4.0 mM CAs at room temperature.

- **Chloroanilines Utilization**

The preliminary result showed that *A. baylyi* strain GFJ2 was able to degrade CAs. The result of CAs utilization ability and maximum growth is shown in Table 4.1. *A. baylyi* strain GFJ2 was exactly showed the highest degradation (expressed as % degradation) and maximum growth expressed as what unit on 0.2 mM 4CA (90±2%; 1.2708±0.0337 unit) and 0.2 mM 3,4-DCA (71±11%; 0.3990±0.0178 unit), after incubation at room temperature for 48 hours. Increasing CAs concentrations decreased maximum growth of *A. baylyi* strain GFJ2. The result of maximum growth of single substrate suggested that *A. baylyi* strain GFJ2 was

preferred to use 4CA as the carbon and nitrogen source more than 3,4-DCA. Interestingly, mixed substrates condition, total degradation of 4CA and 3,4-DCA was decreased compared with single substrate degradation at the same concentration.

- **Chloroanilines utilization *via* resting cell technique**

Resting cell technique was applied to increase the quantity of bacteria capable of degrading CAs. The result of CAs biodegradability and specific degradation *via* resting cell technique is shown in Table 4.1. The highest total degradation (expressed as % degradation) of 4CA was 84±4% at concentration 0.2 mM. While, the total degradation of 3,4-DCA was not significantly different at concentrations 0.2, 0.4 and 0.8 mM (68±2%, 71±0% and 70±2%, respectively), after incubation at room temperature for 12 hours. For 4CA-34DCA co-existence, total biodegradation of 4CA and 34DCA was decreased compared with single substrate degradation.

The result of both utilization and resting cell technique showed the same tendency of degradation in which CAs biodegradation was adversely affected with the increasing of CAs concentration. In co-occurring condition, 34DCA biodegradation was much decreased due to inhibitory effect from co-existence of 4CA. This result agreed with the previous report that showed 4CA was inhibitor of 3,4-DCA (Hongsawat and Vangnai 2011). CAs at concentration 0.2 mM was selected for further study because cell achieved high percentage of total biodegradation.

Comparing total degradation of 0.2 mM 4CA and 0.2 mM 3,4-DCA by utilization (90±2% and 71±11%, respectively) and resting cell technique (84±4% and 68±2%, respectively) was not significantly different. In contrast, specific degradation

rate (nmole.mg $prot^{-1}.hr^{-1}$) of 0.2 mM 4CA and 0.2 mM 3,4-DCA by resting cell technique (88.7±5.3 and 166.6±9.0, respectively) was higher than utilization (24.4±5.2 and 96.8±68.1, respectively). This result indicated that resting cell technique acceptable as the determination technique. So, resting cell technique was selected for further study.

Table 4.1 Chloroanilines biodegradability and specific degradation rate of *A. baylyi* strain GFJ2 by utilization and resting cell technique

		Utilization with in 48 hr.			Resting cell technique with in 12 hr.	
substrate	**Conc. (mM)**	**Maximum growth (Unit)**	**% Total degradation**	**Specific degradation rate (nmole.mg prot^{-1}.hr^{-1})**	**% Total degradation**	**Specific degradation rate (nmole.mg prot^{-1}.hr^{-1})**
4CA	0.2	1.2708-1.3045	88-90	19.2-24.4	84±4	88.7±5.3
	0.4	0.9663-1.2106	47-59	46.4-64.0	68±3	154.3±16.4
	0.8	0.9210-0.9510	34-36	39.5-64.7	63±5	213.1±26.1
	4.0	0.6726-0.6849	17-20	3.5-5.8	2±3	39.6±14.3
3,4-DCA	0.2	0.3990±0.0178	60-71	68.1-96.8	68±2	166.6±9.0
	0.4	0.3662±0.0706	46-49	43.0-81.4	71±0	378.4±5.7
	0.8	0.0296±0.0022	2-5	52.6-59.7	70±2	788.4±20.5
	4.0	0.0195±0.0011	0	0	14±3	155.4±35.7
4CA:34DCA	0.2:0.2	0.5481±0.2123	10-17[a]	19.0-22.4[a]	61±5[a]	96.8±7.0[a]
			7-8[b]	6.2-12.0[b]	24±6[b]	16.4±1.8[b]
	0.4:0.4	0.3841±0.0413	33-43[a]	15.6-16.4[a]	45±4[a]	158.3±6.2[a]
			5-6[b]	10.2-12.7[b]	22±2[b]	25.6±3.5[b]
	0.8:0.8	0.0323±0.0110	3-8[a]	8.2-9.9[a]	40±0[a]	260.8±16.4[a]
			2-7[b]	3.1-4.1[b]	15±3[b]	28.6±6.9[b]
	4.0:4.0	0.0284±0.0033	6-7[a]	10.3-13.5[a]	8±2[a]	106.2±7.9[a]
			0[b]	0[b]	14±4[b]	135.8±89.5[b]

[a] and [b] were amount of 4CA and 3,4-DCA, respectively, at mix substrates condition

4.1.2 Effect of supplementary nutrients on CAs biodegradability *via* resting cell technique

Chloroanilines can serve as a sole carbon and nitrogen source for *A. baylyi* strain GFJ2. Previous study of Zeyer and Kearney (1982) reported *Pseudomonas* Sp. strain G was able to degrade 3,4-DCA when supplemented with 1% succinate and 0.05% ammonium nitrate as additional substrates. Petchkroh (2006) reported increasing 4CA degradation when added 4 mM citrate or 4 mM succinate for carbon source, 4mM NH_4Cl or $NaNO_3$ for nitrogen source and 1 mM aniline for carbon and nitrogen source. In this study, nutrients from soil and fertilizer were used as supplemented nutrients in order to improve bacteria growth and degradation efficiency. In general, the farmers add fertilizer to increase mineral nutrients in the soil for plant to grow healthy and to improve agricultural produce. Therefore, the effect of these nutrients on CAs degradation and on CAs-degrading bacteria was examined.

A. baylyi strain GFJ2 was cultured in MMSAY containing 0.2 mM CAs and supplemented with nutrients from soil (NS) or fertilizer (NF) at room temperature. The result of total degradation and specific degradation rate supplemented with nutrients is shown in Table 4.2. The nutrients from natural sources, both soil and fertilizer reduced the 4CA biodegradation by 24-53% but enhanced biodegradation of 3,4-DCA up to 17-22% from non-supplemented with natural nutrients. The nutrients from NF2 had 4CA biodegradation and specific degradation rate (nmole.mg $prot^{-1}.hr^{-1}$) (60±6 and 52.9±1.6, respectively) more than nutrients from other sources. This may be because NF2 with higher N content (Appendix H) can provide more N which is required for the bacterium. The total degradation and

specific degradation rate of 4CA when supplemented with natural nutrients lower than non-supplemented with natural nutrients. This may be because nutrients from soil and fertilizer had many un-identified nutrients; some nutrients may be inhibiting 4CA degradation pathway. In contrast, total degradation of 3,4-DCA when supplemented with natural nutrients was higher than non-supplemented. This indicated that 3,4-DCA had different degradation pathway from 4CA. So, NF2 was selected as the supplementary nutrients for further study.

Table 4.2 Chloroanilines biodegradability and specific degradation rate of *A. baylyi* strain GFJ2 supplemented with additional nutrients *via* resting cell technique

Substrate	**Nutrient (5%, v/v)**	**% Total degradation**	**Specific degradation rate (nmole.mg prot^{-1}.hr^{-1})**
0.2 mM 4CA	-	84±4	88.7±5.3
	NS1	43±2	38.0±1.0
	NS2	41±3	40.3±5.6
	NS3	31±0	36.7±7.9
	NS4	38±2	31.8±0.9
	NS5	37±6	37.9±5.9
	NF1	39±7	30.3±5.5
	NF2	60±6	52.9±1.6
0.2 mM 3,4-D4CA	-	68±2	166.6±9.0
	NS1	90±2	89.8±1.2
	NS2	87±3	88.8±24.8
	NS3	85±2	95.6±19.4
	NS4	87±1	83.4±9.4
	NS5	87±0	104.8±32.7
	NF1	89±1	132.1±2.2
	NF2	88±1	107.3±45.2

NS1-NS5 = nutrients from various soil (C:N ratio of NS1= 12.57, NS2= 17.13, NS3= 15.76, NS4= 15.27 and NS5= 6.90)

NF1 and NF2 = nutrients from various fertilizer (C:N ratio of NF1= 11.32 and NF2= 7.44)

4.1.3 Effect of copper fungicide types and concentrations co-contamination on CAs biodegradation *via* resting cell technique

Chloroanilines were the important intermediates generated by microbial degradation of herbicides. These CAs derivatives may be contaminated in agricultural practices. One thing to consider was that beside these CAs, other chemicals such as copper fungicides were also applied to these areas.

Copper is an essential micronutrient and required by all living microorganisms but, at high concentration, it is a toxic heavy metal in the environment and toxic for microorganisms. Copper fungicides work as fungicides and bactericides because when they contactd water, they release copper ion which is nonspecific denaturation of cellular proteins. Biodegradation of *A. baylyi* strain GFJ2 of CAs under co-contamination with different copper fungicides at various concentrations (5, 10, 50 and 100 ppm) were investigated.

Under copper fungicides co-contamination, *A. baylyi* strain GFJ2 remained active for 4CA and 3,4-DCA biodegradation. 4CA degradation under copper fungicides co-contamination was relatively retarded compared to the treatment without copper fungicides. This result agree with previous research showed that metal can inhibit the contaminant degradation or mineralization of toxic compounds when co-contamination (Ibarrolaza et al. 2009). Nevertheless, some copper fungicides at low concentration enhanced 34DCA degradation compared with the treatment without copper fungicide, as shown in Table 4.3.

The concentration of copper fungicides affected 4CA and 3,4-DCA biodegradation in which total degradation and specific degradation rate were

decreased when increasing copper concentration. Interestingly, copper fungicides inhibited 4CA degradation but at low concentration enhanced 3,4-DCA degradation.

The type of copper fungicides which had different copper form (copper sulfate, copper oxychloride and copper hydroxide) affected 4CA and 3,4-DCA biodegradation. Addition of copper sulfate, poltiglia and bordeaux-M having copper sulfate as active ingredient at low concentration showed that total degradation of 4CA and 3,4-DCA under co-contamination with copper sulfate (69±1% and 86±3%, respectivelt) was higher than poltiglia (60±22% and 67±9%, respectively) and bordeaux-M (60±1% and 86±2%, respectively). This may be because poltiglia and bordeaux-M contained other chemicals which may be inhibiting degradation of CAs. In the presence of copper sulfate, copper oxychloride and copper hydroxide at low concentrations, 4CA biodegradation of Copper sulfate (60±2%) was higher than Copper oxychloride (58±0%) and Bordeaux-M (54±0%).

Total degradation of 4CA under co-contaminated with copper fungicides was decreased comparing with non-present of copper fungicides. In contrast, in the present of copper fungicides at low concentration enhanced 3,4-DCA biodegradability up to 13-24%, as shown in Table 4.3.

Copper sulfate showed the high total degradation more than other copper form, so copper sulfate was selected as the copper fungicide for further study.

Table 4.3 Chloroanilines biodegradability and specific degradation rate of of *A. baylyi* strain GFJ2 under co-contamination with copper fungicides

Copper sulfate (active ingredient; >99% copper sulfate as powder)						
Substrate (0.2 mM)	**Copper conc. (ppm)**	**% Total degradation**	**Specific degradation rate (nmole.mg prot^{-1}.hr^{-1})**	**Substrate (0.2 mM)**	**% Total degradation**	**Specific degradation rate (nmole.mg prot^{-1}.hr^{-1})**
4CA	-	84±4	88.7±5.3	3,4-DCA	68±2	166.6±9.0
	5	69±1	99.9±14.6		86±3	80.3±4.4
	10	62±9	73.0±18.1		85±1	74.1±6.8
	50	67±4	60.8±2.6		47±2	53.2±2.1
	100	0	0		13±10	18.3±2.6
Copina-85 (active ingredient; 85% copper oxychloride as wetting powder)						
4CA	5	58±0	108.7±10.0	3,4-DCA	78±1	75.5±20.3
	10	58±2	93.7±11.4		81±12	90.1±13.4
	50	30±2	117.4±19.7		92±1	81.4±4.9
	100	43±5	55.0±0.1		46±18	37.3±3.7

Copina hydroxide (active ingredient; 77% copper hydroxide as wetting powder)						
Substrate (0.2 mM)	**Copper conc. (ppm)**	**% Total degradation**	**Specific degradation rate (nmole.mg prot^{-1}.hr^{-1})**	**Substrate (0.2 mM)**	**% Total degradation**	**Specific degradation rate (nmole.mg prot^{-1}.hr^{-1})**
4CA	5	54±0	98.4±7.5	3,4-DCA	88±6	99.7±3.9
	10	49±6	93.0±4.3		42±6	56.9±4.9
	50	36±3	53.0±23.8		39±5	33.4±20.1
	100	18±4	7.2±0.2		27±2	15.9±18.9
Poltiglia (active ingredient; 74% copper sulfate as wetting powder and amixture with or without stabilizing agent of calcium hydroxide)						
4CA	5	60±22	118.7±22.7	3,4-DCA	67±9	58.5±22.4
	10	53±6	106.2±5.3		67±11	68.0±4.7
	50	53±6	88.8±3.4		34±2	53.5±9.7
	100	43±12	88.8±12.7		33±6	39.7±0.5
Bordeaux-M (active ingredient; 48% copper sulfate as wetting powder, manganese ethylenebis dithiocarbarmate and surfactants)						
4CA	5	60±1	97.2±6.4	3,4-DCA	86±2	54.3±27.5
	10	51±8	106.9±28.6		32±6	55.4±16.2
	50	43±5	92.5±10.7		0	0
	100	31±24	89.6±0.2		0	0

4.2 Biodegradation of 4CA, 3,4-DCA and co-occurring 4CA-34DCA in soil

4.2.1 Soil properties

Two types of soil samples; S3 and S5, as shown in Figure 4.1 were used for agricultural practices. The soil samples were sieved using ASTM E11, soil samples sieved through 2 mm mesh (sieve No. 10, 2000 microns and 0.0787 inches) and 0.85 mm mesh (sieve No.20, 850 microns and 0.0331 inches). They were analyzed for their physical and chemical properties at Department of Agriculture, Kasetsart University. The soil properties are provided in Table 4.4.

S3 soil is classified as sandy clay with black color. pH of S3 was 6.82. Percentage of organic matter, organic carbon, nitrogen and water holding capacity were 5.34, 37.4, 2.45 and 23.94, respectively. The concentration of phosphorus, potassium and copper were 59, 640 and 1.62 mg/kg, respectively. C:N ratio was 15.27. The indigenous microorganism involving 4CA and 3,4-DCA in S3 soil were $4.20\times10^{5} \pm 1.70\times10^{5}$ and $5.95\times10^{4} \pm 3.32\times10^{4}$ CFU/ g soil, respectively.

S5 is loam soil with dark brown color. pH of S5 was 6.92. Percentage of organic matter, organic carbon, nitrogen and water holding capacity were 3.94, 37.21, 5.39 and 27.91, respectively. The concentration of phosphorus, potassium and copper were 171, 1880 and 0.98 mg/kg, respectively. C:N ratio was 6.90. The indigenous microorganism involving 4CA and 3,4-DCA in S5 soil were $2.05\times10^{6} \pm 3.54\times10^{5}$ and $2.43\times10^{5} \pm 1.87\times10^{5}$, respectively.

The properties of soil samples showed that both of soil samples had different physical and chemical properties. Therefore, two types of soil were used for study to determine soil properties affected CAs degradation during bioremediation.

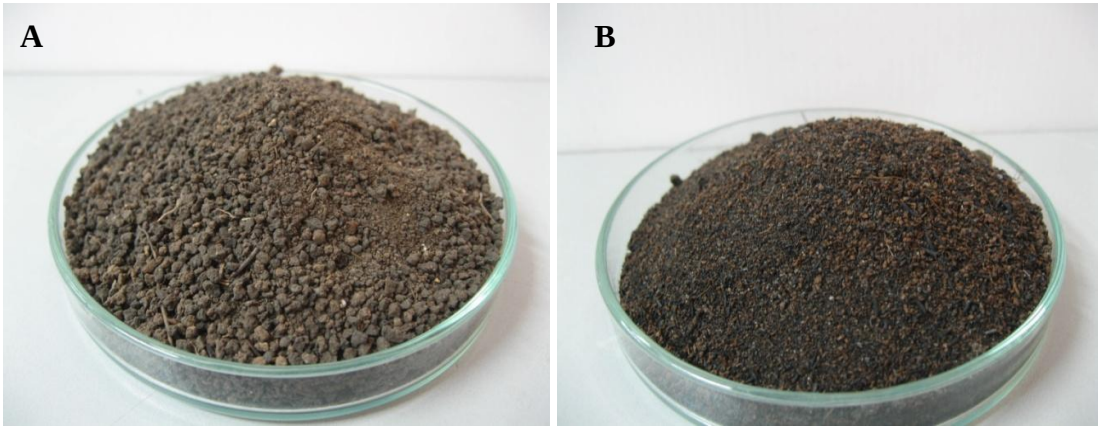


Figure 4.1 S3 soil sample (A) and S5 soil sample (B)

Table 4.4 Properties of soil samples

Properties	S3 soil	S5 soil
Soil texture	Sandy clay	Sandy loam
Color	Brown	Dark brown
pH	6.82	6.92
Organic matter (%)	5.34	3.94
Phosphorus (mg/kg)	59	171
Potassium (mg/kg)	640	1880
Cu (mg/kg)	1.62	0.98
Organic carbon (%)	32.92	37.21
Nitrogen (%)	2.10	5.39
C:N ratio	15.76	6.90
Water holding capacity (%)	23.94	27.91

4.2.2 Recovery of CAs from soil

Chloroanilines contaminated in soil was extracted by organic solvent such as methanol at concentration 50%, 80% and 100 % (v/v). The extracted CAs was analyzed by high performance liquid chromatography (HPLC). The recovery percentage of CAs is shown in Table 4.5 which methanol at concentration 80% (v/v) showed the highest percentage of 4CA and 3,4-DCA recovery in both of soils. For S3 soil, percentage of 4CA and 3,4-DCA recovery were 88±1 and 100±1, respectively. For S5 soil, percentage of 4CA and 3,4-DCA recovery were 72±8 and 91±5, respectively. Therefore, 80% (v/v) methanol was used for extract CAs from soil in the following experiments.

Table 4.5 The recovery percentage of CAs from soils

	S3 soil		**S5 soil**	
Extraction solvent	**% 4CA recovery**	**% 3,4-DCA recovery**	**% 4CA recovery**	**% 3,4-DCA recovery**
100% methanol	53±5	64±8	70±16	75±7
80% methanol	88±1	100±1	72±8	91±5
50% methanol	77±10	90±1	62±25	72±23

4.2.3 Bioremediation of CAs in S3 soil

Chloroanilines biodegradation was conducted at the laboratory scale in which 5 g S3 soil was spiked with 500 ppm CAs before three bioremediation treatments including natural attenuation, biostimulation and bioaugmentation. Natural attenuation was the process that CAs was reduced by sorption, chemical reaction and degradation by indigenous microorganisms in soil. Nutrients from fertilizer were added into soil contaminated with CAs for biostimulation. According to previous result (Table 4.2), nutrients from fertilizer (NF2) was able to stimulate the total degradation of 3,4-DCA. For bioaugmentation, *A. baylyi* GFJ2 (approximately 10^{10} CFU/g soil) and bacterial consortium from fertilizer were used in bioaugmentation treatment.

In S3 soil, the result of CAs biodegradability is shown in Table 4.6. The fast degradation was occurred during 1 week after that the degradation was slowly degraded until 4 week. The highest degradation (expressed as % degradation) of 4CA (97±0%) was observed in bioaugmentation in combination with *A. baylyi* GFJ2 and the natural mixed culture after 4-week. Additional nutrients promoted 94±2% of 4CA degradation. Only 85±3% of 4CA could be reduced via natural attenuation, while bioaugmentation and biostimulation processes could be reduced 4CA in range 92-97%.

For 3,4-DCA biodegradation, the highest percentage degradation was shown in bioaugmentation (81±1%) in combination with *A. baylyi* GFJ2 and the natural mixed culture after 4-week. 3,4-DCA could be reduced in range 75-81% by

bioaugmentation and biostimulation, only 72±5% could be reduced by natural attenuation treatment.

The mixed substrate of 4CA and 3,4-DCA which contaminated in S3 soil showed that the highest percentage degradation was showed in bioaugmentation (96±0% and 74±1% of 4CA and 3,4-DCA, respectively) in combination with *A. baylyi* GFJ2 and the natural mixed culture after 4-week. 85-96% of 4CA and 58-74% of 3,4-DCA could be reduced by bioaugmentation and biostimulation. Only 80±1% of 4CA and 51±3% of 3,4-DCA could be reduced via natural attenuation treatment. Total degradation after 4 week of 4CA and 3,4-DCA co-occurring was decreased when comparing with single substrate.

4.2.3.1 Total microbial activity in S3 soil

Total microbial activity in soil has been detected by dehydrogenase activity. Among bioremediation treatments of S3 soil, the highest value of dehydrogenase activity was found in bioaugmentation with combination of *A. baylyi* GFJ2 and bacterial consortium (Table 4.6). The addition of *A. baylyi* GFJ2 at 10^{10} CFU/g soil and 25% (w/w) of bacterial consortium from fertilizer increased the total dehydrogenase activity in the end of incubation time (4-week) to 2.96, 1.32 and 1.11 folds (4CA, 3,4-DCA and mixed substrates, respectively). The fold of dehydrogenase activity was compared with the attenuation treatment value. The highest value of dehydrogenase activity in bioaugmentation treatment in combination with *A. baylyi* GFJ2 and the natural mixed culture relatively corresponded to the efficiency of CAs biodegradation.

4.2.3.2 Number of CAs-degrading bacterial in S3 soil

Plate count technique used to determine the populations of CAs-degrading bacteria by supplementation of 100 ppm CAs on agar plate. In the end of incubation time (4-week), the number of 4CA-degrading bacteria increased approximately 10 times through natural attenuation (N) and bioaugmentation in combination with *A. baylyi* GFJ2 and the natural mixed culture (J2A) (from $1.15\times10^{5}\pm4.95\times10^{4}$ to $3.00\times10^{6}\pm1.41\times10^{6}$ and $8.00\times10^{8}\pm2.83\times10^{8}$ to $1.45\times10^{9}\pm3.54\times10^{8}$, respectively). The number of 4CA-degrading bacteria increased approximately 1,000 times through biostimulation with fertilizer nutrients (S) and bioaugmentation with fertilizer mixed culture (A) (from $9.00\times10^{4}\pm4.24\times10^{4}$ to $1.05\times10^{7}\pm3.54\times10^{6}$ and $5.00\times10^{6}\pm4.24\times10^{6}$ to $1.30\times10^{9}\pm5.66\times10^{8}$, respectively). In contrast, when bioaugmented in bacterial pure culture of *A. Baylyi* GFJ2 (J2) and *A. Baylyi* GFJ2 supplemented with fertilizer nutrient (J2S), the number of 4CA-degrading bacteria decreased approximately 10 times (from $4.50\times10^{8}\pm3.45\times10^{8}$ to $1.00\times10^{7}\pm2.83\times10^{6}$ and $6.50\times10^{8}\pm2.12\times10^{8}$ to $1.10\times10^{7}\pm1.41\times10^{6}$, respectively).

For 3,4-DCA contaminated soil showed that the number of 3,4-DCA-degrading bacteria increased approximately 10 times through bioaugmentation with fertilizer mixed cuture (A) (from $3.50\times10^{6}\pm2.12\times10^{6}$ to $1.3\times10^{7}\pm5.66\times10^{6}$), *A. Baylyi* GFJ2 supplemented with fertilizer nutrient (J2S) (from $6.50\times10^{7}\pm2.12\times10^{7}$ to $1.10\times10^{8}\pm1.41\times10^{7}$) and the combination with *A. baylyi* GFJ2 and the natural mixed culture (J2A) (from $5.00\times10^{7}\pm1.41\times10^{7}$ to $1.30\times10^{8}\pm5.66\times10^{7}$) . The number of 3,4-DCA-degrading bacteria increased approximately 100 times through natural attenuation (N) and biostimulation with fertilizer nutrients (S) (from

$7.00\times10^{4}\pm1.41\times10^{4}$ to $3.00\times10^{6}\pm1.41\times10^{6}$ and $6.50\times10^{4}\pm7.07\times10^{3}$ to $2.50\times10^{6}\pm7.07\times10^{5}$, respectively). There was no significant difference on bioaugmentation with bacterial pure culture of *A. Baylyi* GFJ2 (J2) (from $2.50\times10^{7}\pm7.07\times10^{6}$ to $6.60\times10^{7}\pm1.98\times10^{7}$).

The result of the mixed CA substrates (4CA:34DCA) contaminated soil showed that the number of CAs-degrading bacteria increased approximately 10 times through natural attenuation (N) (from $6.00\times10^{4}\pm1.41\times10^{4}$ to $1.45\times10^{5}\pm3.54\times10^{4}$). The number of CAs-degrading bacteria increased approximately 100 times through bioaugmentation with fertilizer mixed culture (A) (from $7.00\times10^{4}\pm1.41\times10^{4}$ to $7.00\times10^{6}\pm1.41\times10^{6}$). Mean while, the number of CAs-degrading bacteria decreased approximately 10 times through bioaugmentation with bacterial pure culture of *A. Baylyi* GFJ2 (J2) (from $6.50\times10^{6}\pm7.07\times10^{5}$ to $2.00\times10^{5}\pm4.24\times10^{4}$). The number of CAs-degrading bacteria decreased approximately 100 times through *A. Baylyi* GFJ2 supplemented with fertilizer nutrient (J2S) (from $4.50\times10^{6}\pm2.12\times10^{6}$ to $7.00\times10^{4}\pm1.41\times10^{4}$). There were no significant differences on biostimulation with fertilizer nutrients (S) (from $7.00\times10^{4}\pm1.41\times10^{4}$ to $3.00\times10^{4}\pm1.41\times10^{4}$) and the combination with *A. baylyi* GFJ2 and the natural mixed culture (J2A) (from $1.05\times10^{7}\pm7.07\times10^{5}$ to $1.40\times10^{7}\pm2.83\times10^{6}$).

Table 4.6 Chloroanilines biodegradability and dehydrogenase activity of *A. baylyi* GFJ2 in S3 soil

Substrate	Condition	% Total degradation (1wk)	% Total degradation (4wk)	Degradation rate at 0-1 wk (uM.hr^{-1})	Dehydrogenase activity (fold)
4CA	Natural attenuation **(N)**	69±0	85±3	5.4±0.8	1.00±0.00
	Biostimulation with fertilizer nutrients **(S)**	84±7	94±2	9.8±1.8	1.57±0.06
	Bioaugmentation				
	- Bacterial consortium from fertilizer **(A)**	84±4	94±1	7.0±0.3	2.91±0.02
	- *A. Baylyi* GFJ2 **(J2)**	83±6	94±1	8.9±0.6	1.81±0.08
	- *A. Baylyi* GFJ2 supplemented with fertilizer nutrient **(J2S)**	79±1	92±1	6.8±1.0	1.30±0.01
	- Combination of *A. Baylyi* GFJ2 and bacterial consortium **(J2A)**	90±3	97±0	8.7±0.0	2.96±0.03
3,4-DCA	Natural attenuation **(N)**	47±10	72±5	7.2±1.3	1.00±0.00
	Biostimulation with fertilizer nutrients **(S)**	64±1	77±0	9.0±0.0	1.19±0.05
	Bioaugmentation				
	- Bacterial consortium from fertilizer **(A)**	51±7	71±0	6.9±0.4	1.03±0.00
	- *A. Baylyi* GFJ2 **(J2)**	53±2	75±1	11.1±0.4	1.17±0.04
	- *A. Baylyi* GFJ2 supplemented with fertilizer nutrient **(J2S)**	55±3	72±3	11.0±1.1	1.13±0.08
	- Combination of *A. Baylyi* GFJ2 and bacterial consortium **(J2A)**	63±1	81±1	8.3±0.4	1.32±0.02

Substrate	Condition	% Total degradation (1wk)		% Total degradation (4wk)		Degradation rate at 0-1 wk (uM.hr^{-1})		Dehydrogenase activity (fold)
		4CA	**3,4-DCA**	**4CA**	**3,4-DCA**	**4CA**	**3,4-DCA**	
4CA:34DCA	Natural attenuation **(N)**	51±4	24±6	80±1	51±3	6.5±0.4	6.3±0.4	1.00±0.00
	Biostimulation with fertilizer nutrients **(S)**	59±14	34±22	85±5	58±13	9.6±0.3	7.8±1.5	0.83±0.01
	Bioaugmentation							
	- Bacterial consortium from fertilizer **(A)**	70±3	38±6	91±1	66±3	7.1±0.7	6.0±0.2	1.08±0.01
	- *A. Baylyi* GFJ2 **(J2)**	62±1	31±3	90±0	65±1	6.8±0.1	5.9±0.6	0.81±0.02
	- *A. Baylyi* GFJ2 supplemented with fertilizer nutrient **(J2S)**	56±4	26±6	90±1	60±2	5.9±0.3	5.2±0.3	0.85±0.01
	- Combination of *A. Baylyi* GFJ2 and bacterial consortium **(J2A)**	90±1	56±2	96±0	74±1	5.6±0.2	4.1±0.4	1.11±0.02

The fold of dehydrogenase activity was compared with the values determined in natural attenuation treatment (132±16, 169±5 and 184±0 ug.g^{-1} dry soil for 4CA, 3,4-DCA and the mix substrate treatment, respectively), after incubation for 4 week.

4.2.4 Bioremediation of CAs in S3 soil under copper sulfate co-contamination

In S3 soil under co-contamination with copper sulfate, the result of CAs biodegradation is shown in Table 4.7. The fast degradation was occurred during 1 week after that the degradation was slowly occurred until 4 week. The highest degradation (expressed as % degradation) of 4CA (96±0%) was observed in bioaugmentation with *A. baylyi* GFJ2, after 4 week. Additional nutrients promoted 4CA degradation to 90±0%. Natural attenuation treatment could be reduced 4CA to 93±0%.

For 3,4-DCA biodegradation, the highest percentage degradation was shown in bioaugmentation of *A. baylyi* GFJ2 (83±0), after 4 week, while it could be degraded to 78±2% by natural attenuation treatment. On the other hand, 61±10% could be degraded by biostimulation supplemented with fertilizer nutrients.

The mixed substrate of 4CA and 3,4-DCA contaminated in S3 soil treatment in the presence of copper sulfate showed the highest percentage degradation with bioaugmentation (95±0% and 77±1% of 4CA and 3,4-DCA, respectively) in combination with *A. baylyi* GFJ2 and the natural mixed culture after 4 week. 80-95% of 4CA and 40-77% of 3,4-DCA could be reduced by bioaugmentation and biostimulation, while, 82±1% of 4CA and 53±3% of 3,4-DCA could be reduced *via* natural attenuation treatment. Total degradation after 4 week of 4CA and 3,4-DCA co-occurring was decreased when comparing with single substrate.

4.2.4.1 Total microbial activity in S3 soil under copper sulfate co-contamination

The total microbial activity of S3 soil under copper sulfate co-contamination is shown in Table 4.7. In the end of incubation time (4 weeks), the highest value of dehydrogenase activity was found in bioaugmentation in combination with *A. baylyi* GFJ2 and the natural mixed culture (J2A). The fold of dehydrogenase activity was compared with the attenuation treatment value. The addition of *A. Baylyi* GFJ2 and bacterial consortium from fertilizer increased the total microbial activity 1.09±0.01, 1.15±0.03 and 1.14±0.05 fold under contaminated with 4CA, 3,4-DCA and the mixed substrates, respectively.

4.2.4.2 Number of CAs-degrading bacterial in S3 soil under copper sulfate co-contamination

After incubation time for 4 week, the result of 4CA-degrading bacteria under copper sulfate co-contamination showed that, 4CA-degrading bacteria on biostimulation with fertilizer nutrients (SCu) and natural attenuation (NCu) treatment increased approximately 100 and 1,000 times, respectively (from $4.50\times10^{4}\pm7.07\times10^{3}$ to $4.50\times10^{6}\pm3.54\times10^{6}$ and $6.50\times10^{4}\pm4.95\times10^{4}$ to $1.05\times10^{7}\pm2.12\times10^{6}$, respectively), while, the number of 4CA-degrading bacteria decreased approximately 100 and 1,000 times through bioaugmentation treatment with *A. Baylyi* GFJ2 (J2Cu) and *A. Baylyi* GFJ2 supplemented with fertilizer nutrient (J2SCu), respectively (from $3.50\times10^{8}\pm2.12\times10^{8}$ to $2.50\times10^{6}\pm7.07\times10^{5}$ and $2.50\times10^{8}\pm7.07\times10^{7}$ to $1.30\times10^{5}\pm2.83\times10^{4}$, respectively). There were no significant difference on bioaugmentation treatment of bacterial consortium from

fertilizer (ACu) and the combination with *A. baylyi* GFJ2 and the natural mixed culture (J2ACu) (from $9.00\times10^{6}\pm4.24\times10^{6}$ to $7.00\times10^{6}\pm1.41\times10^{6}$ and $4.00\times10^{8}\pm2.83\times10^{8}$ to $4.85\times10^{8}\pm7.07\times10^{7}$, respectively).

The 3,4-DCA contaminated soil under copper sulfate co-contamination treatment showed that the number of 3,4-DCA-degrading bacteria increased approximately 10 and 100 times through biostimulation with fertilizer nutrients (SCu) and bioaugmentation treatment with fertilizer mixed culture (ACu), respectively (from $5.00\times10^{5}\pm1.41\times10^{5}$ to $5.00\times10^{6}\pm2.83\times10^{6}$ and $7.00\times10^{6}\pm1.41\times10^{6}$ to $3.50\times10^{8}\pm7.07\times10^{7}$, respectively). In contrast, the 3,4-DCA-degrading bacteria decreased approximately 100 times through *A. Baylyi* GFJ2 supplemented with fertilizer nutrient (J2SCu) (from $3.00\times10^{8}\pm1.41\times10^{8}$ to $2.55\times10^{6}\pm6.36\times10^{5}$). No significant difference on natural attenuation treatment (NCu) (from $5.00\times10^{5}\pm1.41\times10^{5}$ to $7.50\times10^{5}\pm2.12\times10^{5}$), bioaugmentation of *A. Baylyi* GFJ2 (J2Cu) (from $3.50\times10^{8}\pm2.12\times10^{8}$ to $6.00\times10^{8}\pm1.41\times10^{8}$) and the combination of *A. baylyi* GFJ2 and bacterial consortium (J2ACu) (from $3.50\times10^{8}\pm7.07\times10^{7}$ to $6.50\times10^{8}\pm2.12\times10^{8}$) was detected.

The treatment of mixed CA substrates (4CA:34DCA) contaminated soil under copper sulfate co-contamination showed that the number of CAs-degrading bacteria increased approximately 10 times on natural attenuation treatment (NCu) (from $4.00\times10^{4}\pm1.41\times10^{4}$ to $1.15\times10^{5}\pm7.07\times10^{3}$) and increased 100 times on bioaugmentation treatment of fertilizer mixed culture (ACu) (from $5.00\times10^{4}\pm1.41\times10^{4}$ to $6.00\times10^{6}\pm1.41\times10^{6}$). The CAs-degrading bacteria decreased approximately 10 times on bioaugmentation of *A. Baylyi* GFJ2 (J2Cu) (from $6.50\times10^{6}\pm7.07\times10^{5}$ to $2.00\times10^{5}\pm4.24\times10^{4}$) and the combination of *A. baylyi*

GFJ2 and the natural mixed culture (J2ACu) (from $1.05\times10^{7}\pm7.07\times10^{5}$ to $7.50\times10^{6}\pm2.12\times10^{6}$). The CAs-degrading bacteria decreased approximately 100 times on *A. Baylyi* GFJ2 supplemented with fertilizer nutrient (J2SCu) (from $4.50\times10^{6}\pm2.12\times10^{6}$ to $7.00\times10^{4}\pm1.41\times10^{4}$). No significant difference on biostimulation with fertilizer nutrients (SCu) treatment (from $8.00\times10^{4}\pm5.66\times10^{4}$ to $4.50\times10^{4}\pm7.07\times10^{3}$) was detected.

Table 4.7 Chloroanilines biodegradability and dehydrogenase activity of *A. baylyi* GFJ2 in S3 soil under copper sulfate co-contamination

Substrate	Condition	% Total degradation (1wk)	% Total degradation (4wk)	Degradation rate at 0-1 wk (uM.hr^{-1})	Dehydrogenase activity (fold)
4CA	Natural attenuation **(NCu)**	57±1	93±0	6.2±0.0	1.00±0.00
	Biostimulation with fertilizer nutrients **(SCu)**	62±1	90±0	6.6±0.2	0.84±0.00
	Bioaugmentation				
	- Bacterial consortium from fertilizer **(ACu)**	70±1	94±0	6.4±0.2	0.99±0.01
	- *A. Baylyi* GFJ2 **(J2Cu)**	68±1	96±0	5.8±0.1	0.85±0.06
	- *A. Baylyi* GFJ2 supplemented with fertilizer nutrient **(J2SCu)**	51±2	78±1	5.9±0.1	0.63±0.01
	- Combination of *A. Baylyi* GFJ2 and bacterial consortium **(J2ACu)**	76±1	91±1	6.5±0.4	1.09±0.01
3,4-DCA	Natural attenuation **(NCu)**	47±5	78±2	8.2±0.6	1.00±0.00
	Biostimulation with fertilizer nutrients **(SCu)**	52±13	61±10	9.4±2.4	1.05±0.02
	Bioaugmentation				
	- Bacterial consortium from fertilizer **(ACu)**	31±12	72±5	3.8±0.6	1.14±0.01
	- *A. Baylyi* GFJ2 **(J2Cu)**	58±1	83±0	5.1±0.1	1.15±0.01
	- *A. Baylyi* GFJ2 supplemented with fertilizer nutrient **(J2SCu)**	26±0	66±0	4.3±0.0	1.10±0.02
	- Combination of *A. Baylyi* GFJ2 and bacterial consortium **(J2ACu)**	64±1	78±0	4.6±0.1	1.15±0.03

Substrate	Condition	% Total degradation (1wk)		% Total degradation (4wk)		Degradation rate at 0-1 week (uM.hr^{-1})		Dehydrogenase activity (fold)
		4CA	**3,4-DCA**	**4CA**	**3,4-DCA**	**4CA**	**3,4-DCA**	
4CA:34DCA	Natural attenuation **(NCu)**	54±5	26±7	82±1	53±3	7.1±0.5	6.1±0.3	1.00±0.00
	Biostimulation with fertilizer nutrients **(SCu)**	60±4	28±7	80±1	54±3	6.5±0.4	5.6±0.3	0.87±0.6
	Bioaugmentation							
	- Bacterial consortium from fertilizer **(ACu)**	71±1	32±2	91±0	61±1	5.8±0.1	4.7±0.5	1.14±0.02
	- *A. Baylyi* GFJ2 **(J2Cu)**	59±3	24±5	89±1	62±2	6.2±0.4	5.3±0.3	0.97±0.02
	- *A. Baylyi* GFJ2 supplemented with fertilizer nutrient **(J2SCu)**	63±3	33±6	80±1	40±4	6.1±0.3	5.3±0.3	0.73±0.03
	- Combination of *A. Baylyi* GFJ2 and bacterial consortium **(J2ACu)**	80±1	40±4	95±0	77±1	5.4±0.3	4.6±0.3	1.14±0.05

The fold of dehydrogenase activity was compared with the values determined in natural attenuation treatment (231±3, 165±12 and 174±3 ug.g^{-1} dry soil for 4CA, 3,4-DCA and the mix substrate treatment, respectively), after incubation for 4 week.

4.2.5 Bioremediation of CAs in S5 soil

Chloroanilines biodegradation was conducted through three bioremediation treatments including natural attenuation, biostimulation and bioaugmentation using S5 soil.

In S5 soil, the result of CAs biodegradability is shown in Table 4.8. The fast degradation was occurred during 1 week after that the degradation was slowly occurred until 4 week. The degradation of natural attenuation, biostimulation and augmentation were not significantly different which in the range 95-98%, after incubation for 4 weeks. The highest degradation (expressed as % degradation) of 4CA (98±0%) was observed in natural attenuation, after 4 week. Additional nutrients promoted 96±1% of 4CA degradation, which 95-97% could be reduced via bioaugmentation.

For 3,4-DCA biodegradation, the degradation of three bioremediation were not significantly different which in the range 89-91%, after incubation for 4 weeks. The highest percentage degradation was showed biostimulation with fertilizer nutrients (S), after 4 week.

The mixed substrate of 4CA and 3,4-DCA which contaminated in S5 soil showed that the degradation of three bioremediation were not significantly different which in the range 93-99% of 4CA and 91-97% of 3,4-DCA, after incubation for 4 weeks. However, the degradation of 3,4-DCA in biostimulation treatment (S) and bioaugmentation treatment of bacterial consortium from fertilizer (A) was different from others in which the degradation was 77±3% and 72±3%, respectively.

4.2.5.1 Total microbial activity in S5 soil

After incubation time for 4 weeks, the total microbial activity of S5 soil is shown in Table 4.8. The highest value of dehydrogenase activity was found in bioaugmentation with combination of *A. baylyi* GFJ2 and bacterial consortium (J2A). The fold of dehydrogenase activity was compared with the attenuation treatment value. The addition of *A. Baylyi* GFJ2 and bacterial consortium from fertilizer increased the total microbial activity 1.12±0.00, 1.21±0.01 and 1.25±0.00 fold under contaminated with 4CA, 3,4-DCA and mix substrates, respectively.

4.2.5.2 Number of CAs-degrading bacterial in S5 soil

After incubation for 4 week, the number of 4CA-degrading bacteria increased approximately 10 times through bioaugmentation with bacterial consortium from fertilizer (A) and bioaugmentation with combination of *A. baylyi* GFJ2 and bacterial consortium (J2A) (from $4.10\times10^{7}\pm2.9597\times10^{7}$ to $7.500\times10^{8}\pm2.12\times10^{68}$ and $5.500\times10^{8}\pm2.12\times10^{8}$ to $1.65\times10^{9}\pm7.5407\times10^{7}$, respectively). The number of 4CA-degrading bacteria increased approximately 100 times through natural attenuation (N) biostimulation with fertilizer nutrients (S) (from $1.35\times10^{5}\pm2.12\times10^{4}$ to $4.40\times10^{7}\pm5.37\times10^{6}$ and $1.45\times10^{5}\pm3.2454\times10^{4}$ to $5.05\times10^{7}\pm4.17\times10^{7}$, respectively). In contrast, bioaugmentation with bacterial pure culture of *A. Baylyi* GFJ2 (J2), the number of 4CA-degrading bacteria decreased approximately 10 times (from $3.10\times10^{8}\pm1.56\times10^{8}$ to $6.50\times10^{7}\pm2.12\times10^{7}$). No significant difference on *A. Baylyi* GFJ2 supplemented with fertilizer nutrient (J2S) (from $6.50\times10^{8}\pm2.12\times10^{8}$ to $1.10\times10^{8}\pm1.41\times10^{7}$) was detected.

The 3,4-DCA contaminated soil treatment showed that the number of 3,4-DCA-degrading bacteria increased approximately 10 times through natural attenuation (N) (from $3.00\times10^{5}\pm1.41\times10^{5}$to $3.00\times10^{6}\pm1.41\times10^{6}$), biostimulation with fertilizer nutrients (S) (from $3.50\times10^{5}\pm7.07\times10^{4}$ to $8.50\times10^{6}\pm7.07\times10^{5}$) and bioaugmentation treatment. Bioaugmentation treatment including bacterial consortium from fertilizer (A) (from $5.50\times10^{6}\pm7.07\times10^{5}$ to $5.35\times10^{7}\pm5.16\times10^{7}$) and *A. Baylyi* GFJ2 supplemented with fertilizer nutrient (J2S) (from $3.50\times10^{8}\pm2.12\times10^{8}$ to $1.10\times10^{9}\pm1.41\times10^{8}$). While, no significant difference on bacterial pure culture of *A. Baylyi* GFJ2 (J2) (from $3.00\times10^{8}\pm1.41\times10^{8}$ to $8.00\times10^{8}\pm0.00$) and the combination of *A. baylyi* GFJ2 and bacterial consortium (J2A) (from $5.00\times10^{8}\pm1.41\times10^{8}$ to $9.00\times10^{8}\pm0.00$) was detected.

The result of the mixed CA substrates (4CA:34DCA) contaminated soil showed that the number of CAs-degrading bacteria increased approximately 10 times through natural attenuation (N) (from $6.00\times10^{4}\pm1.41\times10^{4}$ to $8.95\times10^{5}\pm1.06\times10^{5}$), bioaugmentation with bacterial consortium from fertilizer (A) (from $5.00\times10^{6}\pm0.00$ to $7.00\times10^{7}\pm1.41\times10^{7}$), bioaugmentation with bacterial pure culture of *A. Baylyi* GFJ2 (J2) (from $4.50\times10^{6}\pm7.07\times10^{5}$ to $2.00\times10^{7}\pm4.24\times10^{6}$) and *A. Baylyi* GFJ2 supplemented with fertilizer nutrient (J2S) (from $2.50\times10^{6}\pm7.07\times10^{5}$ to $7.00\times10^{7}\pm1.41\times10^{7}$). The number of CAs-degrading bacteria increased approximately 100 times through biostimulation with fertilizer nutrients (S) (from $7.00\times10^{4}\pm1.41\times10^{4}$ to $3.00\times10^{6}\pm1.41\times10^{6}$). No significant difference on the combination of *A. baylyi* GFJ2 and bacterial consortium (J2A) (from $1.35\times10^{7}\pm7.07\times10^{5}$ to $1.40\times10^{7}\pm2.83\times10^{6}$) was observed.

Table 4.8 Chloroanilines biodegradability and dehydrogenase activity of *A. baylyi* GFJ2 in S5 soil

Substrate	Condition	% Total degradation (1wk)	% Total degradation (4wk)	Degradation rate at 0-1 wk (uM.hr^{-1})	Dehydrogenase activity (fold)
4CA	Natural attenuation **(N)**	89±1	98±0	3.2±0.7	1.00±0.00
	Biostimulation with fertilizer nutrients **(S)**	93±1	96±1	3.2±1.1	0.98±0.01
	Bioaugmentation				
	- Bacterial consortium from fertilizer **(A)**	95±1	95±2	2.6±0.9	1.12±0.07
	- *A. Baylyi* GFJ2 **(J2)**	93±1	96±1	4.2±1.2	1.02±0.00
	- *A. Baylyi* GFJ2 supplemented with fertilizer nutrient **(J2S)**	94±0	97±1	3.3±1.0	1.01±0.04
	- Combination of *A. Baylyi* GFJ2 and bacterial consortium **(J2A)**	94±0	97±1	3.1±1.0	1.09±0.00
3,4-DCA	Natural attenuation **(N)**	76±2	90±1	3.4±0.3	1.00±0.00
	Biostimulation with fertilizer nutrients **(S)**	83±1	93±1	3.9±0.3	1.02±0.06
	Bioaugmentation				
	- Bacterial consortium from fertilizer **(A)**	84±1	89±0	3.4±0.2	1.11±0.01
	- *A. Baylyi* GFJ2 **(J2)**	80±0	91±0	5.7±0.2	1.13±0.03
	- *A. Baylyi* GFJ2 supplemented with fertilizer nutrient **(J2S)**	88±1	91±1	4.4±0.3	1.07±0.01
	- Combination of *A. Baylyi* GFJ2 and bacterial consortium **(J2A)**	80±1	89±1	4.2±0.3	1.21±0.01

Substrate	Condition	% Total degradation (1wk)		% Total degradation (4wk)		Degradation rate at 0-1 wk (uM.hr^{-1})		Dehydrogenase activity (fold)
		4CA	**3,4-DCA**	**4CA**	**3,4-DCA**	**4CA**	**3,4-DCA**	
4CA:34DCA	Natural attenuation **(N)**	69±2	40±5	97±0	91±1	5.1±0.3	4.9±0.4	1.00±0.00
	Biostimulation with fertilizer nutrients **(S)**	73±3	36±8	94±1	77±3	4.3±0.4	3.6±0.0	0.87±0.13
	Bioaugmentation							
	- Bacterial consortium from fertilizer **(A)**	80±2	39±7	93±1	72±3	5.4±0.6	4.9±0.0	0.97±0.02
	- *A. Baylyi* GFJ2 **(J2)**	82±1	58±4	96±0	91±1	5.1±0.3	4.5±0.1	1.08±0.00
	- *A. Baylyi* GFJ2 supplemented with fertilizer nutrient **(J2S)**	82±2	56±6	97±0	93±1	4.3±0.4	3.6±0.0	1.03±0.01
	- Combination of *A. Baylyi* GFJ2 and bacterial consortium **(J2A)**	87±2	61±6	99±0	97±0	5.4±0.6	4.9±0.0	1.25±0.00

The fold of dehydrogenase activity was compared with the values determined in natural attenuation treatment (223±4, 197±7 and195±12 ug.g^{-1} dry soil for 4CA, 3,4-DCA and the mix substrate treatment, respectively), after incubation for 4 week.

4.2.6 Bioremediation of CAs in S5 soil under copper sulfate co-contamination

In S5 soil under co-contamination with copper sulfate, the result of CAs biodegradability is shown in Table 4.9. The fast degradation was occurred during 1 week after that the degradation was slowly occurred until 4 week. The degradation of natural attenuation, biostimulation and augmentation were not significantly different which in the range 94-97%, after incubation for 4 weeks.

For 3,4-DCA biodegradation, the degradation of three bioremediation were not significantly different in which in the range 90-93%, after incubation for 4 weeks.

The mixed substrate of 4CA and 3,4-DCA which contaminated in S5 soil showed that the degradation of three bioremediation were not significantly different in which in the range 93-98% of 4CA and 87-93% of 3,4-DCA, after incubation for 4 weeks.

4.2.6.1 Total microbial activity in S5 soil under copper sulfate co-contamination

The total microbial activity of S5 soil under copper sulfate co-contamination is shown in Table 4.9. In the end of incubation time (4 weeks), the highest value of dehydrogenase activity under contaminated with 4CA and copper sulfate was found in biostimulation of nutrients from fertilizer (S) (1.07±0.02 fold). In 3,4-DCA co-contaminated with copper sulfate, the highest total microbial activity in bioaugmentation of *A. Baylyi* GFJ2 (J2) (1.19±0.03 fold) was observed. While, mix substrates co-contaminated with copper sulfate, the highest total microbial activity in

the combination with *A. baylyi* GFJ2 and the natural mixed culture (J2A) (1.06±0.01 fold) was observed. The fold of dehydrogenase activity was compared with the attenuation treatment value.

4.2.6.2 Number of CAs-degrading bacterial in S5 soil under copper sulfate co-contamination

In the end of incubation time (4 weeks), the result of 4CA-degrading bacteria under copper sulfate co-contamination showed that, 4CA-degrading bacteria on biostimulation with fertilizer nutrients (SCu) and natural attenuation (NCu) treatment increased approximately 100 and 1,000 times, respectively (from $4.50\times10^{4}\pm7.07\times10^{3}$ to $4.50\times10^{6}\pm3.54\times10^{6}$ and $6.50\times10^{4}\pm4.95\times10^{4}$ to $1.05\times10^{7}\pm2.12\times10^{6}$, respectively). While, the number of 4CA-degrading bacteria decreased approximately 10 times through the combination of *A. baylyi* GFJ2 and bacterial consortium (J2ACu) (from $4.00\times10^{8}\pm2.83\times10^{8}$ to $4.50\times10^{7}\pm7.07\times10^{6}$). The number of 4CA-degrading bacteria decreased approximately 100 times through bioaugmentation treatment of *A. Baylyi* GFJ2 (J2Cu) and *A. Baylyi* GFJ2 supplemented with fertilizer nutrient (J2SCu) (from $3.50\times10^{8}\pm2.12\times10^{8}$ to $2.50\times10^{6}\pm7.07\times10^{5}$ and $2.50\times10^{8}\pm7.07\times10^{7}$ to $2.05\times10^{6}\pm2.12\times10^{5}$, respectively). No significance different on bioaugmentation treatment of bacterial consortium from fertilizer (ACu) (from $9.00\times10^{6}\pm4.24\times10^{6}$ to $7.00\times10^{6}\pm1.41\times10^{6}$) was detected.

The 3,4-DCA contaminated soil under copper sulfate co-contamination showed that the number of 3,4-DCA-degrading bacteria increased approximately 10 times through natural attenuation treatment (NCu) and biostimulation with fertilizer

nutrients (SCu) (from $5.00\times10^{5}\pm1.41\times10^{5}$ to $7.50\times10^{6}\pm2.12\times10^{6}$ and $5.00\times10^{6}\pm2.83\times10^{6}$ to $5.00\times10^{7}\pm2.83\times10^{7}$, respectively). The number of 3,4-DCA-degrading bacteria increased approximately 100 times through bioaugmentation treatment of bacterial consortium from fertilizer (ACu), respectively (from $7.50\times10^{6}\pm7.07\times10^{5}$ to $3.50\times10^{8}\pm7.07\times10^{7}$). In contrast, the 3,4-DCA-degrading bacteria decreased approximately 10 times through the combination of *A. baylyi* GFJ2 and bacterial consortium (J2ACu) (from $3.50\times10^{8}\pm7.07\times10^{7}$ to $3.50\times10^{7}\pm2.12\times10^{7}$). No significance different on bioaugmentation of *A. Baylyi* GFJ2 (J2Cu) (from $7.00\times10^{8}\pm1.41\times10^{8}$ to $6.00\times10^{8}\pm1.41\times10^{8}$) and *A. Baylyi* GFJ2 supplemented with fertilizer nutrient (J2SCu) (from $3.50\times10^{8}\pm7.07\times10^{7}$ to $7.50\times10^{8}\pm7.07\times10^{7}$) was detected.

For mixed CA substrates (4CA:34DCA) contaminated soil under copper sulfate co-contamination showed that, the number of CAs-degrading bacteria increased approximately 10 times on natural attenuation treatment (NCu) (from $3.50\times10^{5}\pm7.07\times10^{4}$ to $4.65\times10^{6}\pm6.36\times10^{5}$) and the combination of *A. baylyi* GFJ2 and bacterial consortium (J2ACu) (from $8.50\times10^{6}\pm7.07\times10^{5}$ to $1.15\times10^{7}\pm7.07\times10^{5}$). The number of CAs-degrading bacteria increased approximately 100 times on biostimulation with fertilizer nutrients (SCu) treatment (from $8.00\times10^{4}\pm5.66\times10^{4}$ to $4.50\times10^{6}\pm7.07\times10^{5}$) and bioaugmentation treatment of bacterial consortium from fertilizer (ACu) (from $7.50\times10^{4}\pm7.07\times10^{3}$ to $2.00\times10^{6}\pm1.41\times10^{6}$). In contrast, the CAs-degrading bacteria decreased approximately 10 times on bioaugmentation of *A. Baylyi* GFJ2 (J2Cu) (from $8.50\times10^{6}\pm7.07\times10^{5}$ to $6.50\times10^{5}\pm1.70\times10^{5}$). No significance different on *A. Baylyi*

GFJ2 supplemented with fertilizer nutrient (J2SCu) (from $5.00\times10^{6}\pm1.41\times10^{6}$ to $7.00\times10^{6}\pm1.41\times10^{6}$) was observed.

Table 4.9 Chloroanilines biodegradability and dehydrogenase activity of *A. baylyi* GFJ2 in S5 soil under copper sulfate co-contamination

Substrate	Condition	% Total degradation (1wk)	% Total degradation (4wk)	Degradation rate at 0-1 wk ($uM.hr^{-1}$)	Dehydrogenase activity (fold)
4CA	Natural attenuation **(N)**	86±4	94±2	2.3±0.9	1.00±0.00
	Biostimulation with fertilizer nutrients **(S)**	94±0	97±0	3.7±0.3	1.07±0.02
	Bioaugmentation				
	- Bacterial consortium from fertilizer **(A)**	97±0	97±0	3.8±0.6	1.04±0.05
	- *A. Baylyi* GFJ2 **(J2)**	90±1	94±2	2.9±0.9	0.85±0.04
	- *A. Baylyi* GFJ2 supplemented with fertilizer nutrient **(J2S)**	89±2	95±1	2.7±0.9	0.86±0.01
	- Combination of *A. Baylyi* GFJ2 and bacterial consortium **(J2A)**	91±0	96±1	2.5±0.8	0.86±0.02
3,4-DCA	Natural attenuation **(N)**	57±0	91±0	4.7±0.0	1.00±0.00
	Biostimulation with fertilizer nutrients **(S)**	86±0	93±0	5.0±0.1	1.00±0.01
	Bioaugmentation				
	- Bacterial consortium from fertilizer **(A)**	66±0	90±1	4.4±0.0	1.17±0.03
	- *A. Baylyi* GFJ2 **(J2)**	82±0	93±0	3.8±0.0	1.19±0.03
	- *A. Baylyi* GFJ2 supplemented with fertilizer nutrient **(J2S)**	88±0	91±1	4.0±0.0	1.10±0.04
	- Combination of *A. Baylyi* GFJ2 and bacterial consortium **(J2A)**	59±0	91±0	2.9±0.0	1.16±0.03

Substrate	Condition	% Total degradation (1wk)		% Total degradation (4wk)		Degradation rate at 0-1 wk (uM.hr^{-1})		Dehydrogenase activity (fold)
		4CA	**3,4-DCA**	**4CA**	**3,4-DCA**	**4CA**	**3,4-DCA**	
4CA:34DCA	Natural attenuation **(N)**	70±0	20±0	93±0	85±1	3.3±0.1	3.6±0.2	1.00±0.00
	Biostimulation with fertilizer nutrients **(S)**	68±2	42±5	96±0	92±1	3.5±0.2	3.6±0.2	0.83±0.03
	Bioaugmentation							
	- Bacterial consortium from fertilizer **(A)**	75±0	34±1	97±0	87±0	2.4±0.3	3.5±0.4	0.99±0.01
	- *A. Baylyi* GFJ2 **(J2)**	77±0	42±0	96±0	89±0	3.6±0.0	3.5±0.4	1.02±0.04
	- *A. Baylyi* GFJ2 supplemented with fertilizer nutrient **(J2S)**	84±1	47±5	98±0	93±1	3.6±0.2	3.3±0.1	0.90±0.02
	- Combination of *A. Baylyi* GFJ2 and bacterial consortium **(J2A)**	90±1	69±3	98±0	93±1	5.1±0.3	4.5±0.2	1.06±0.01

The fold of dehydrogenase activity was compared with the values determined in natural attenuation treatment (205±2, 190±34 and 187±1 ug.g^{-1} dry soil for 4CA, 3,4-DCA and the mix substrate treatment, respectively), after incubation for 4 week.

4.2.7 Microbial community analysis in soil

Denaturing gradient gel electrophoresis (DGGE) is a method to evaluate of bioremediation treatment on microbial diversity in complex environments (Petersen et al. 2007). The method initially starts from extraction DNA from soils. The DNA extracted from S3 soil (white color) and S5 soil (blown color) had different color, as shown in Figure 4.1. After that, 200 bp fragment of 16S rDNA was amplified using 341F+GC and 520R primers (Dar et al. 2005) in PCR reaction as described in method 3.8.6 but only S3 soil DNA yielded PCR product (Figure 4.2). So, only the PCR products from S3 soil with different nucleotide sequences were run in DGGE to investigate the bacterial community change during 4 weeks of each biological treatment.

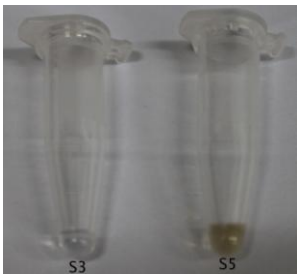


Figure 4.2 DNA extractions from S3 and S5 soil

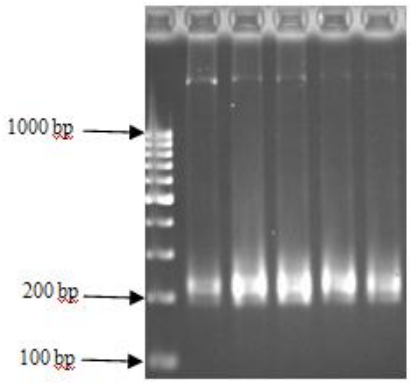


Figure 4.3 PCR amplification of S3 soil

DGGE profile of bioremediation treatments during 4 weeks of incubation time is shown in Figure 4.3. The bioremediation treatments of S3 soil including natural attenuation (N), biostimulation with fertilizer nutrient (S) and bioaugmentation; fertilizer mixed culture (A), *A. Baylyi* strain GFJ2 (J2), *A. Baylyi* strain GFJ2 supplemented with fertilizer nutrient (J2S) and combination with *A. Baylyi* strain GFJ2 and the natural mixed culture (J2A). The control was *A. Baylyi* strain GFJ2 in which DNA was extracted from liquid culture ($J2_C$).

For 4CA contaminated soil, the result of DGGE profiles in natural attenuation treatment (N) illustrated the decreased number of bacteria populations, which represented by decreasing of DNA bands from 0 to 1^{st} week and 2^{nd} to 4^{th} week. In contrast, DNA bands were increasing from 1^{st} to 2^{nd} week which representing the increased number of bacteria population. The intensity of each band was different between each time point (Figure 4.3a). In biostimulation treatment with fertilizer nutrients (S), DNA bands and intensities of bands at 0 week were less than natural attenuation treatment (N) at the same time. DNA bands from 0 to 4^{th} week were decreased. Interestingly, at 2^{nd} and 3^{rd} week, some new DNA band were occurred and the DNA intensities were increased (Figure 4.3b). In bioaugmentation with fertilizer mixed culture treatment (A), DNA band intensities were less than natural attenuation treatment (N) but some new DNA bands were occurred with high intensities at 0 week. DNA bands and intensities from 0 to 3^{rd} week were decreased. From 3^{rd} to 4^{th} week, some new DNA bands were occurred and the intensities of bands were increased (Figure 4.3c). In bioaugmentation with *A. baylyi* GFJ2 treatment (J2), DNA bands and intensities of bands at 0 week was less than natural attenuation treatment (N) at the same time. DNA bands from 0 to 4^{th} week were decreased. At 0 week,

DGGE profile showed the same DNA bands of *A. baylyi* GFJ2 was extracted from liquid culture ($J2_C$) but at 2^{nd} to 4^{th}, these DNA bands were disappeared (Figure 4.3d). In bioaugmentation with *A. baylyi* GFJ2 supplemented with fertilizer nutrients treatment (J2S), DNA bands and intensities of bands at 0 week were less than natural attenuation treatment (N) at the same time. DNA bands and intensities from 1^{st} to 4^{th} were increased. At 1^{st} week, DGGE profile showed the same DNA bands of *A. baylyi* GFJ2 was extracted from liquid culture ($J2_C$) but at 2^{nd} to 4^{th}, these DNA bands were disappeared (Figure 4.3e). Bioaugmentation with *A. baylyi* GFJ2 and the natural mixed culture treatment (J2A), DNA band from 0 to 4^{th} week were decreased. The intensity of each band was different between each time point. At 1^{st} week, DGGE profile showed the same DNA bands of *A. baylyi* GFJ2 was extracted from liquid culture ($J2_C$) but at 2^{nd} to 4^{th}, these DNA bands were disappeared (Figure 4.3f).

For 3,4-DCA contaminated soil, In natural attenuation treatment (N) the bacteria population were increased, which represented by increasing DNA bands from 0 to 1^{st} week. From 1^{st} to 4^{th} week, DNA bands not much different (Figure 4.3g). In biostimulation treatment (S), DNA bands were increased but low intensities at 0 to 1^{st} week. At 1^{st} to 3^{rd} week, DNA bands were decreased which intensities of some DNA bands was higher. DNA bands were increased from 3^{rd} to 4^{th} week (Figure 4.3h). In bioaugmentation with fertilizer mixed culture treatment (A), DNA bands were decreased from 0 to 4^{th} week. The intensities of each band were different between each time point (Figure 4.3i). In bioaugmentation with *A. baylyi* GFJ2 treatment (J2), DNA bands and intensities were decreased from 0 to 4^{th} week. At 1^{st} week, DGGE profile showed the same DNA bands of *A. baylyi* GFJ2 was extracted from liquid culture ($J2_C$) but at 2^{nd} to 4^{th}, these DNA bands were disappeared (Figure 4.3j). In

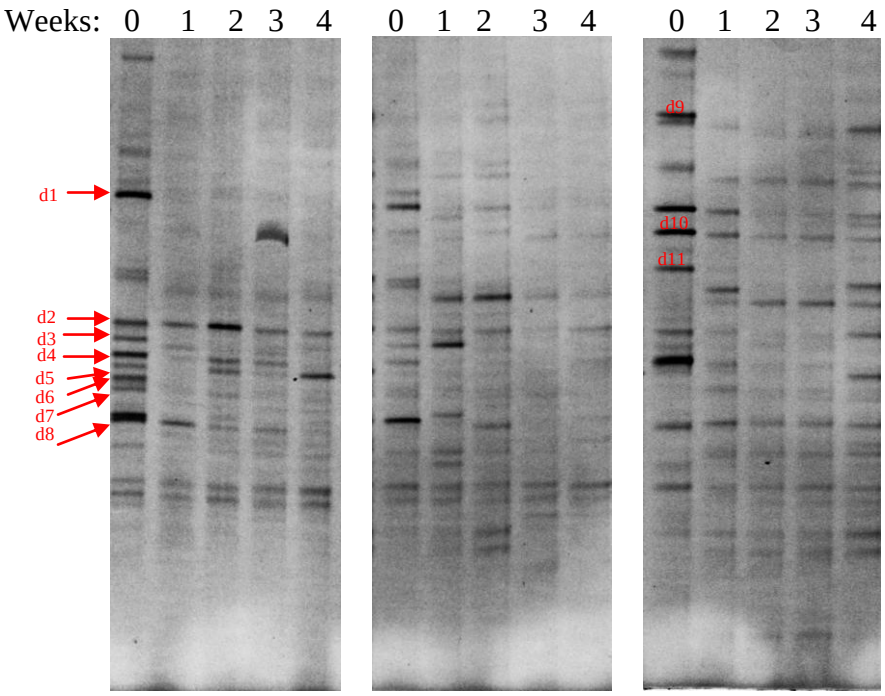
bioaugmentation with *A. baylyi* GFJ2 supplemented with fertilizer nutrients treatment (J2S), at each week DGGE profile showed some new DNA band occurred which the intensities of each band was different between each time point. At 1st week, DGGE profile showed the same DNA bands of *A. baylyi* GFJ2 was extracted from liquid culture ($J2_C$) but at 2nd to 4th, these DNA bands were disappeared (Figure 4.3k). In bioaugmentation with *A. baylyi* GFJ2 and the natural mixed culture treatment (J2A), at 1st and 2nd week some new bands were occurred with low intensities. At 1st week, DGGE profile showed high intensities DNA bands of *A. baylyi* GFJ2 was extracted from liquid culture ($J2_C$) but at 2nd to 4th the DNA intensities was decreased (Figure 4.3l).

For the mixed substrate 4CA and 3,4-DCA contaminated soil, in bioaugmentation with *A. baylyi* GFJ2 treatment (J2) the intensities of bands were increased from 0 to 1st week. The intensities of some bands at 2nd week were increased. The DNA intensities were not different from 3rd to 4th week. At 1st week, DGGE profile showed high DNA intensities of *A. baylyi* GFJ2 was extracted from liquid culture ($J2_C$) but at 2nd to 4th the DNA intensities was decreased (Figure 4.3m). In bioaugmentation with *A. baylyi* GFJ2 supplemented with fertilizer nutrients treatment (J2S), at 1st week showed the increased number of bacteria populations, which represented by increasing of DNA bands from 0 to 1st week. The DNA bands were decreased from 1st to 4th week. At 1st week, DGGE profile showed the same DNA bands of *A. baylyi* GFJ2 was extracted from liquid culture ($J2_C$) but at 2nd to 4th, these DNA bands were disappeared (Figure 4.3n). In bioaugmentation with *A. baylyi* GFJ2 and the natural mixed culture treatment (J2A), the DNA bands were increased from 0 to 1st week. From 1st to 4th week, the DNA bands were not much different but

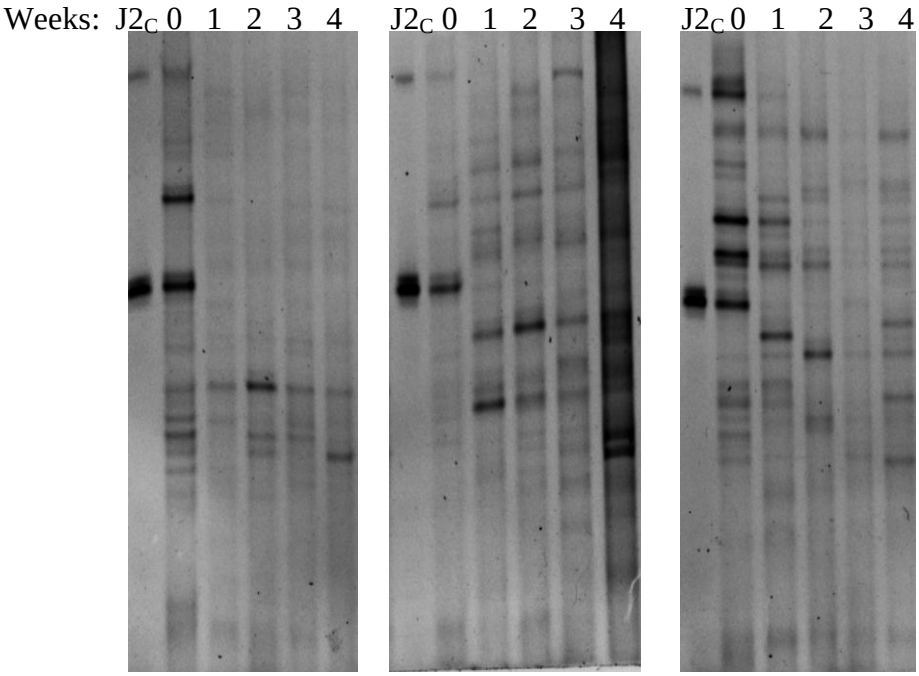
intensity at each point was different. At 1st week, DGGE profile showed the same DNA bands of *A. baylyi* GFJ2 was extracted from liquid culture ($J2_C$) but at 2nd to 4th, these DNA bands were disappeared (Figure 4.3o).

Comparison of the dominant bands in each treatment of 4CA contaminated in S3 soil showed that at 0 week the eight dominant bands (d1-d8) in natural attenuation treatment (N) were observed, as shown in Figure 4.4. Addition fertilizer nutrients (S), the result showed that at 0 week the intensities of eight dominant bands decreased comparing with natural attenuation treatment. Bioaugmentation with fertilizer mixed culture (A) showed that the intensities of dominant bands such as d2, d3, d5, d6, d7 and d8 decreased but the intensity of d4 increased. Some new bands was occurred such as d9, d10 and d11 comparing with natural attenuation treatment.

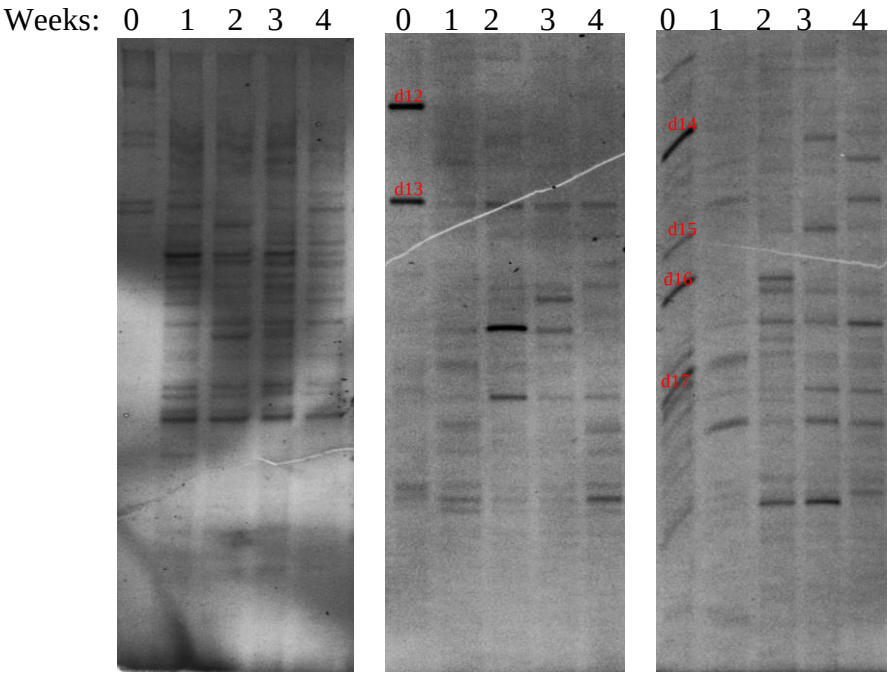
For 3,4-DCA contaminated in S3 soil at 0 week, addition fertilizer nutrients (S) the result showed that at one new band was occurred (d12) and the intensity of d13 increased comparing with natural attenuation treatment. Bioaugmentation with fertilizer mixed culture (A) showed that some new bands were occurred such as d14, d15, d16 and d17 comparing with natural attenuation treatment.



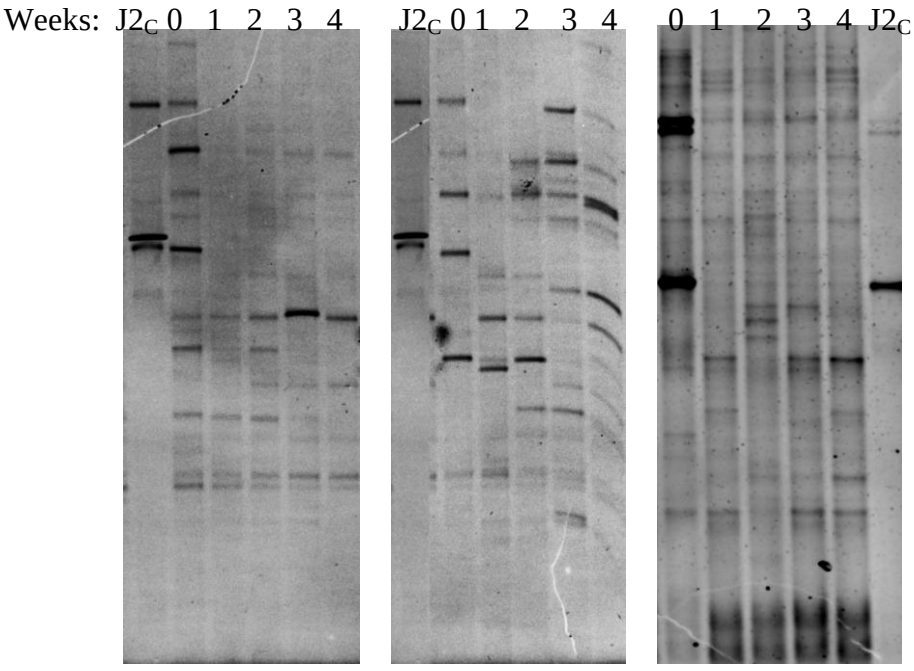
Treatment: (a) 4CA-N (b) 4CA-S (c) 4CA-A



Treatment: (d) 4CA-J2 (e) 4CA-J2S (f) 4CA-J2A



Treatment: (g) 34DCA-N (h) 34DCA-S (i) 34DCA-A



Treatment: (j) 34DCA-J2 (k) 34DCA-J2S (l) 34DCA-J2A

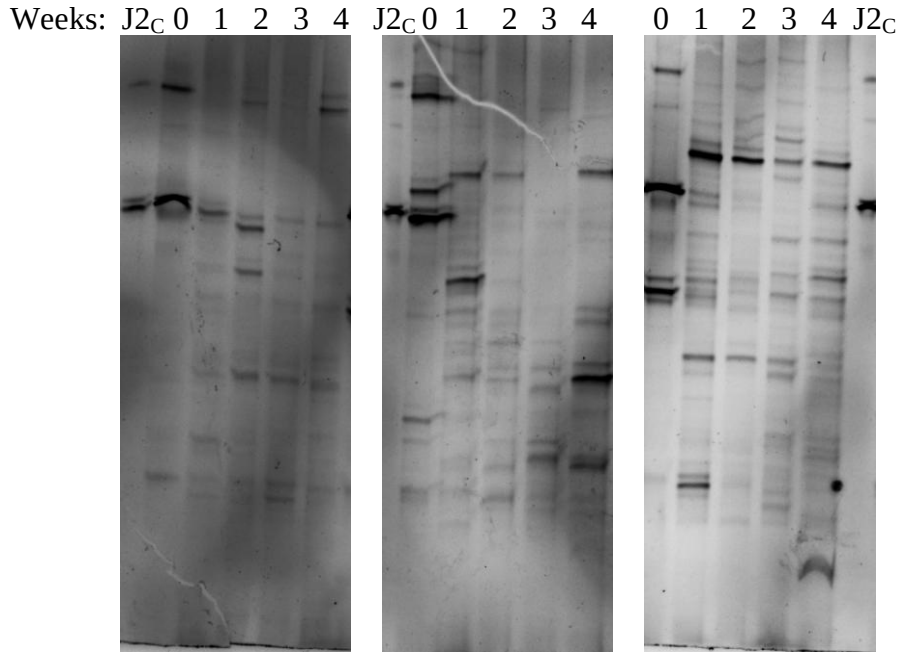


Figure 4.4 DGGE profiles of the bioremediation treatments of S3 soil during 4 weeks of incubation time. (a), (b), (c), (d), (e) and (f) were DGGE profiles of the bioremediation treatments of 4CA contaminated soil. (g), (h), (i), (j), (k) and (l) were DGGE profiles of the bioremediation treatments of 3,4-DCA contaminated soil. (m), (n) and (o) were DGGE profiles of the bioremediation treatments of mix substrate 4CA and 3,4-DCA. $J2_C$ was *A. baylyi* strain GFJ2 which DNA extraction from liquid medium. Numbers represent the week of incubation.

4.2.8 Copper content in soil

Copper is one of the heavy metal most often accumulated in agricultural soil. Copper can exist in soil depends on acidity or alkalinity of the soil with different forms such as primary and/or secondary minerals, adsorbed on surfaces of organic matter, exchanged with other cations in clay and other minerals (Tsang et al. 2007). The method to determine copper content in soil is acid digestion with HNO_3-HCl-HF in a microwave oven and then the digestion sample was analyzed by flame Atomic Absorption Spectrometry (AAS). Copper sulfate is water soluble which it is one of mobile metals in soil. In this study available copper content in soil was investigated.

Soil sample was spiked with 150 ppm copper sulfate then washing soil sample with distilled water. Copper in supernatant was analyzed using AAS. The result showed that 150 ppm copper sulfate almost adsorbed in soil, as shown in Table 4.10.

Table 4.10 Available copper content in soils using Atomic Absorption Spectrometry

Sample	Incubation time (week)	Cu concentration (ppm)
S3	0	0.089
S3	2	0.131
S3	4	0.040
S5	0	0.209
S5	2	0.121
S5	4	0.159

CHAPTER V

DISCUSSIONS

5.1 Effect of CAs concentrations by *Acinetobacter baylyi* strain GFJ2 in liquid medium

Bacteria with ability to degrade aniline or chloroaniline were previously reported such as *Moraxella* sp. strain G which was the first strain that could degrade 4CA as a sole carbon and nitrogen source (Zeyer and Kearney 1982). Surovtseva et al. (1985) reported that *Pseudomonas diminuta* was able to degrade 3CA, 4CA and 3,4-DCA at concentration up to 0.3 mM. Other bacteria such as *Delftia acidovorans* CA28 (Loidl et al. 1990), *Delftia acidovorans* BN3.1 (Brunsbach and Reineke 1993) and *Comamonas testosterone* I2 (Boon et al. 2000) were reported for their degradation ability as well.

In this study *Acinetobacter baylyi* strain GFJ2, a gram negative bacterium was used 4CA and 3,4-DCA degradation. This experiment determined if *A. baylyi* strain GFJ2 was able to tolerate and to degrade 4CA and 3,4-DCA at higher concentrations in the range of 0.2, 0.4, 0.8 and 4.0 mM. The increasing of 4CA and 3,4-DCA concentrations decreased total degradation. The result exhibited that 4CA and 3,4-DCA at 4.0 mM inhibited total degradation of *A. baylyi* strain GFJ2 (Table 4.1)6. Total degradation of 4CA was higher than 3,4-DCA at the same concentration by utilization technique. This result suggested that molecule of 3,4-DCA was more complex than 4CA. Microbial degradation and toxicity of chloroanilines depend on

the position of chlorine atoms and number on the aromatic ring (Hongsawat and Vangnai 2011).

The result of specific degradation rate of this study agreed with the previou reports in which increasing concentrations increased the specific degradation rate of *A. baylyi* strain GFJ2. The specific degradation rate of 4CA was slowly increased when increasing concentration of 4CA up to 0.8 mM. On the other hand, the specific degradation rate of 3,4-DCA was fast increased (faster than 4CA) as 3,4-DCA concentration was increased up to 0.8 mM, but significantly dropped at concentration higher than 0.8 mM, suggesting a substrate inhibition (Hongsawat and Vangnai 2011).

In the combination treatment of 4CA and 3,4-DCA, the result showed that the degradation of 4CA and 3,4-DCA was decrease compared with treatment of a single substrate. This result agreed with Hongsawat and Vangnai (2011) which reported that 4CA was strong mixed-competitive inhibitor for 3,4-DCA. 3,4-DCA biodegradation by *A. baylyi* strain GFJ2 was much decreased or non-degrade when co-occurring with 4CA. This result was different from Zeyer and Kearney (1982) which reported *Pseudomonas* sp. strain G was able to degrade 3,4-DCA in the presence of 4CA as additional substrate. Other report about substrate inhibitor was in Zhang et al. (2010) where *Delftia tsuruhatensis* H1 could degrade several substituted aniline compounds such as 2CA, 3CA, 4CA, 3,4-DCA, 2,3-DCA and 2,4-DCA. Interestingly, aniline was inhibitor for CA degradation of strain H1 which was different from the previous reports regarding the aniline as an inducer for cometabolic biodegradation. Radianingtyas et al. (2003) reported that aniline enhanced bacteria growth and 4CA biodegradation. You and Bartha (1982) reported that 3,4-DCA biodegradation by *Pseudomonas putida* was increased in the presence of aniline.

This result suggested that *A. baylyi* strain GFJ2 have efficiency biodegradation of 4CA and 3,4DCA at a broad-range concentration. The efficiency biodegradation of *A. baylyi* strain GFJ2 of 4CA and 3,4-DCA was decreased when co-occurring 4CA and 3,4-DCA which 4CA was inhibitor of 3,4-DCA biodegradation.

5.2 Effect of supplementary nutrients on CAs biodegradation in liquid medium

This study was to examine the effect of supplementary nutrients from natural sources (nutrients from soil, NS and nutrients from fertilizer, NF) as another carbon and nitrogen source on the biodegradation of 4CA and 3,4-DCA. Addition of nutrients as a growth stimulation of microorganisms can enhance biodegradation, however, the biodegradation of organic contaminants may be inhibited (Lee et al. 2003).

The supplementary nutrients came from various sources, which had different C:N ratio (Appendix H). The result showed that the total degradation and specific degradation rate of 4CA supplemented with nutrients was decreased compared with non-supplemented condition. This result was different from previous studied which Petchkron (2006) reported total degradation of 4CA was increased when added carbon and nitrogen source at 4 mM. Zeyer et al. (1985) reported that 4CA degradation of *Moraxella* sp. strain G was enhanced in the presence of 10 mM ammonium or nitrate as additional nitrogen source. The addition of nutrients does not always enhanced the microbial activity because other factors may inhibit microbial activity or slow degradation by microbial nutrient limitation or the degradation of test compound was reduced by other substrates (Lee et al. 2003). In contrast, the total degradation of 3,4-DCA supplemented with nutrients was increased compared with

non-supplemented condition. Pritchard and Costa (1991) studied bioremediation of oil spill and application fertilizer on a test beach. The natural bioremediation rate on the test beach was occurred quite high because it was stimulated by small concentration of ammonia and phosphate in seawater. However, addition fertilizers was enhanced two to three fold of bioremediation (Pritchard and Costa 1991). Zhang et al. (1998) reported mineral nutrients (N, P and other mineral) enhanced biodegradation of wastewater. Other nutrient that increased biodegradation such as P. Steffensen and Alexander (1995) reported that *Pseudomonas putida* was slowly mineralization benzylamine but when P was added in solution, the mineralization was enhanced.

Additional nutrients enhanced 3,4-DCA biodegradation but inhibited 4CA biodegradation. This result suggested that *A. baylyi* strain GFJ2 had different pathways to degrade 4CA and 3,4-DCA. Hongsawat and Vangnai (2011) reported that 3,4-DCA biodegradation pathway of *A. baylyi* strain GFJ2 was a dechlorination reaction after that 4CA was formed as the first intermediate which had two distinct 4CA degradation pathways.

Total degradation of 4CA supplemented with NF2 having the highest N (6.02%) showed the highest total degradation than other sources. In contrast, biodegradation of 4CA supplemented with NS3 which had the lowest N (2.10%) showed the lowest total degradation than other sources. This result suggested that the 4CA degradation of *A. baylyi* strain GFJ2 was influenced by N which agreed with the previous reports, which showed that the addition of ammonium sulfate as a nitrogen source increased the 4CA degradation of *A. baylyi* strain GFJ2 (Hongsawat and Vangnai 2011).

5.3 Effect of copper fungicide on CAs biodegradation in liquid medium

Copper has been reported for its contamination in environment, including soils, silts, water sources and wastewater from the many uses of copper in industrial applications. Copper tends to accumulate in soils and cannot be destroyed. Copper as an important trace elements in biochemical reactions but at high concentrations these metal are toxic (Colin et al. 2012). Copper is one of the heavy metals most often encountered in site contamination which have been reported in the United States and Europe (Tsang et al. 2007). This experiment was to determine if the *A. baylyi* strain GFJ2 was able to degrade CAs in the presence of copper-based fungicide at various concentrations in the range of 5, 10, 50 and 100 ppm.

The result showed that 4CA degradation of *A. baylyi* strain GFJ2 in the presence of copper fungicides was decreased compared with non-presence condition. Copper fungicide inhibited the 4CA mineralization. This result was similar of Ibarrolaza et al. (2009) who found that chromium(VI) is heavy metal inhibited phenathrene mineralization and toxic to microbial community. Sprocati et al. (2011) reported the co-contamination of both heavy metal and organic compounds is a difficulty because the presence of toxic metals inhibits the activity of the degrader bacteria. Said and Lewis (1991) reported that heavy metal present in soil is also toxic to the microbial community and in co-contaminated site, the biodegradation of organic pollutants could inhibit.

The 3,4-DCA degradation of *A. baylyi* strain GFJ2 in the presence of copper fungicides was increased compared with non-presence condition. Copper fungicides at low concentrations (5ppm and 10 ppm) enhanced 3,4-DCA biodegradation but at

higher concentrations copper fungicides inhibited the degradation. This result corresponded to Mejare and Bulow (2001), which reported that many heavy metals are essential as trace elements but they become toxic at higher concentrations. Copper at high concentrations inhibits cell metabolism (Cervantes and Gutierrez Corona 1994). Alisi et al. (2009) reported that biodegradation of organic pollutants was inhibited by high metal concentrations, affecting a double stress on microbial populations. Komarek et al. (2009) reported that high Cu concentrations can have adverse effects on soil biota and plants.

The result suggested that *A. baylyi* strain GFJ2 under co-contamination with copper fungicides as certain concentration remained active for 4CA and 3,4-DCA biodegradation. Total degradation of 4CA was decreased but total degradation of 3,4-DCA was increased in the presence of copper fungicides at low concentration. This indicated that copper fungicides had different effect on 4CA and 3,4-DCA biodegradation pathway.

Under co-contamination of various copper fungicides, copper sulfate showed the least adverse effect total 4CA and 3,4-DCA degradation than other copper-based fungicides. This suggested those other copper-based fungicides, containing copper as active ingredient, and other organic and inorganic chemicals which may affect the degradation pathway or interrupted the degradation pathway.

5.4 Effect of biological treatment in soil

4CA and 3,4-DCA are hazardous chemicals, which are metabolites of microbial degradation of herbicides and pesticides. They have been contaminated in environment, which mainly accumulating in agricultural soils. So, appropriate technique to clean up these chemicals is necessary. In this study, bioremediation technique such as natural attenuation, biostimulation and bioaugmentation were investigated to determine the suitable option for 4CA and 3,4-DCA biodegradation in the contaminated soils.

In S3 soil, the comparison of three bioremediation technique including natural attenuation, biostimulation and bioaugmentation showed that total degradation of 4CA, 3,4-DCA and mix substrates (4CA:34DCA) of natural attenuation was different from biostimulation and bioaugmentation, after 4-week treatment. Total degradation of natural attenuation was the lowest than biostimulation and bioaugmentation. This result showed that indigenous microorganisms in S3 soil had ability to degrade 4CA, 3,4-DCA and co-contamination of 4CA and 3,4-DCA but total degradation was enhanced when nutrients and CAs-degrading bacteria or natural exogenous microorganisms were supplemented into contaminated soil.

The biostimulation results agreed with the previous studies in that when organic matter and nutrients helped the treatment of the herbicide isoproturon in soil. It was shown that addition of nitrogen and phosphorus had the greatest effect on isoproturon degradation (Perrin-Ganier et al. 2001). Chaineau C.H. et al. (2005) studied effect of nutrients on the biodegradation of crude oil in soil. The result showed that addition of low level nutrients was efficient for the biodegradation of

saturated hydrocarbon when compared with natural attenuation but high level nutrients inhibited the assimilation of hydrocarbons. Indigenous microorganisms in S3 soil was enhanced 4CA biodegradation when nutrients were supplemented. This addition nutrients may stimulate the growth of indigenous microorganisms (Zhang et al. 2005) or increase the biomass of soil microorganisms (Lee et al. 2003).

Bioaugmentaion treatment with the combination of *A. baylyi* GFJ2 and natural bacterial consortium from fertilizer (J2A) showed the highest total degradation in 4CA, 3,4-DCA and mix substrate contaminated soils because the number of bacteria able to degrade CAs into soil (such as *A. baylyi* strain GFJ2 and bacterial consortium from fertilizer) was increased. Beside these, nutrients from fertilizer were added into soil. These were the combination of biostimulation and bioaugmentation treatment. These results agreed to the previous report in which bioaugmentation was the best treatment compared with biostimulation and natural attenuation for the treatment of petroleum hydrocarbon from Long Beach soil samples (Bento et al. 2005). Olaniran et al. (2006) reported that indigenous microorganisms in soils and water samples were able degraded dichloroethanes (DCEs). The biodegradation was increased with the combination of biostimulation and bioaugmentation within two weeks (14% for *cis*-DCE and 18% for *trans*-DCE).

In some cases, bioaugmentation treatment was not completely succeeded. For example, Yu et al. (2005) studied biodegradation of mixed PAHs. The result showed that bacterial consortium was able to degrade mixed PAHs in liquid medium. In contrast, augmentation bacterial consortium in mangrove sediments, these bacterial were not effective in enhancing biodegradation of mixed PAHs. The biodegradation ability of the bacterial consortium was suppressed by other indigenous

microorganisms in the sediment. Several factors influenced bioaugmentation such as physicochemical pollutant characteristics (temperature, humidity and ionic strength) may decrease the microbial activity; limit the mass transfer (clay and organic matter content) of pollutants to microorganisms; microbial ecology (energy flux, indigenous activity, predators, competitions, co-substrates and enzyme stability) and methodology (strains selection, concentration and methods of inoculation) (Sprocati et al. 2011).

Comparison of total degradation of 4CA contaminated in S3 soil by *A. baylyi* GFJ2 (J2) and *A. baylyi* GFJ2 supplemented with fertilizer nutrients (J2S) showed that total degradation of J2 was higher than total degradation of J2S. This result agreed to the previous result in liquid medium experiment in which total degradation of 4CA of *A. baylyi* GFJ2 was decreased when added nutrients from fertilizer (NF2) which some nutrients may be inhibited 4CA degradation pathway of *A. baylyi* GFJ2. Chaineau C.H. et al (2005) reported that addition of high fertilization inhibited hydrocarbon degradation.

DGGE result from bioremediation of S3 soil showed that bacterial community was changed after incubation. During 0 week and 1st week, bacterial community was decreased, these result corresponded with biodegradation of 4CA and 3,4-DCA fast decrease in first week, after that the degradation was slowly occurred. This may be because microorganisms used up nutrients from soils for their growth. Then, when nutrients were un-available, they could use another C, N from pollutant present in the soil. Some microorganisms cannot tolerate and utilize pollutant, so they cannot survive. The amount of 4CA and 3,4-DCA continuously decreased because number of survived CAs-degrading bacteria was enough to degrade CAs. However, the added

bacteria pure culture (*A. baylyi* GFJ2) was not found in DGGE profile after 1-week incubation. This result may be because *A. baylyi* GFJ2 had some biotic stress (predator etc.) and abiotic stress (pH, soil characteristic and environment) (Alexander 1994). Previous studies in liquid medium showed that *A. baylyi* GFJ2 could use 4CA and 3,4-DCA as sole carbon and nitrogen sources. When applied *A. baylyi* GFJ2 in soil, biodegradation of 4CA and 3,4-DCA were carried out at much higher concentration than that in liquid medium, probably because 4CA and 3,4-DCA can combine rapidly with soil component. This may be another reason that *A. baylyi* GFJ2 cannot survive in soil. Sprocati et al. (2011) reported the major problem with soil bioaugmentation was the survival of inoculants (microbiostasis), owing to biotic and biotic stresses.

The DGGE profile showed that some bands of DNA were decreased or disappeared, but when increased treatment times, those bands of DNA were appeared again or some new bands were occurred. These results may be because microorganisms could recover themselves or adapt to new environment. Wang et al. (2004) study effect of fungicide iprodione on bacterial community in soil. The result showed that soil bacterial community were changed and recovered to the initial status when incubation at 15 ˚C. The impact of iprodione on soil bacterial community was recoverable and impermanent.

For 4CA and 3,4-DCA biodegradation in S5 soil, total degradation of CAs were not different in natural attenuation, biostimulation and bioaugmentation. This result may be because that S5 soil had many indigenous microorganisms, which could degrade CAs and/or nutrient in S5 soil was available for the microorganisms. Klier et al. (1999) reported that indigenous microorganism (natural attenuation treatment) in

soil and groundwater was able to degrade *cis*-1,2-, *trans*-1,2- and 1,2-DCE without supplementation of nutrients.

We could not see DGGE profile from S5 soil because we could not amplify PCR product. The color of DNA extraction from S3 soil and S5 soil was different of which S5 soil DNA had blown color but S3 soil DNA had white color. One of the reasons that we could not amplify PCR product from S5 soil may due to the inhibition by pigments, humic acids or other unidentified organic matter in S5 soil. Braid et al. (2003) reported soils contain humic substances which were a major component of soil organic matter. These compounds were very difficult to remove from DNA extraction from soil for PCR and they inhibited *Taq* DNA polymerase and restriction endonucleases.

5.5 Effect of soil type

S3 soil was sandy clay soil with pH 6.82. Biodegradation of 500 ppm CAs was enhanced when supplemented with nutrients and augmented with *A. baylyi* GFJ2 and/or natural bacteria consortium. Another soil was S5, sandy loam soil with pH 6.92. There was no different efficiency in natural attenuation, biostimulation and bioaugmentation treatments. Table 4.4 showed that S3 and S5 soil had different organic matter, nutrients (P, K and N) and C:N ratio. These results indicate that environment condition was one of the factors that may affect biodegradation.

Biodegradation rate of CAs in S3 soil by natural attenuation was the lowest. This may be because the environmental conditions were not suitable for the indigenous microorganisms. The environment condition such as nutrients and natural

exogenous microorganisms should be adjusted in bioremediation to enhance growth and amount of bacteria able degrade CAs. Alexander (1994) reported factors that affect degradation which include pollutant concentration and environment condition. Hamby (1996) also reported that biodegradation in soil could be influenced by environmental conditions such as pH, temperature, oxygen, nutrients and soil moisture. Several literatures showed that soil type is a factor which influences the biodegradation (Hamby 1996; Klier et al. 1999; Scow et al. 1995).

Comparison of nutrients from S3 and S5 soil showed that nutrient from S3 soil was lower than that of S5 soil. This agreed to the fact that total degradation by natural attenuation of S3 soil was lower than that of S5 soil. After supplemented nutrient to S3 soil, total degradation was enhanced. This may be because nutrients limit was occurred in S3 soil. Nutrients limiting was not occurred in S5 soil because total degradation of natural attenuation was not different from biostimulation treatment.

5.6 Effect of copper fungicide for bioremediation

Copper sulfate at 150 ppm was added into CAs contaminated soil to investigate the biodegradation under co-contamination of CAs and copper (heavy metal) condition. The result showed that total degradation of the 4 week treatments were not much different between with and without copper condition. Copper did not affect CAs biodegradation in soil. This may be because copper almost absorbed into soil no available copper on surface soil which could affect biodegradation of microorganisms. Other reason was that microorganism could adapt and survive in copper environment that co-contamination with copper. Umrania (2006) reported that 72 acidothemophilic autotrophic microbes were isolated from industrial soil samples

of Rajkot city. Soil samples contained Ag, Au, Cr, Cu, Ni, Pb, Zn and others heavy metals and the indigenous microbes were able to adapt for metal tolerance and biosorption potentiality. At higher levels of metals, bacteria and fungi which were isolated from contaminated environment were frequently tolerant than microorganisms isolated from uncontaminated areas (Arnebrant et al. 1987; Bååth 1991). To make toxic metals harmless to microorganisms in contaminated soils and waters, these microorganisms have developed resistance mechanisms such as exclusion, compartmentalization and synthesis or making complexes binging proteins (Mejáre and Bülow 2001). Komarek et al. (2010) reported that fertilizer such as phosphate could reduce the mobility of risk metals through sorption mechanisms on newly formed phased, for example, Pb-phosphates.

The results of microbial to activity or tolerance copper in soil and in liquid medium were different in which in liquid medium copper was inhibited CAs at high concentration (50-100 ppm). This may be because that in liquid medium the toxicity of copper was higher than in soil, where absorption occurred to reduce the toxicity.

CHAPTER VI

CONCLUSIONS

In liquid medium, *A. baylyi* strain GFJ2 have ability to degrade CAs at various concentrations but biodegrade ability was if 4CA and 3,4-DCA are co-contaminated. This may be because 4CA is an inhibitor of 3,4-DCA biodegradation. Supplementation of nutrients from natural sources affected CAs biodegradation by reducing 4CA but enhancing 3,4-DCA biodegradation. *A. baylyi* strain GFJ2 has different pathways to degrade 4CA and 3,4-DCA and therefore nutrients may influence 4CA and 3,4-DCA biodegradation pathways differently. Under copper fungicide co-contamination with CAs, copper fungicide inhibited 4CA biodegradation of *A. baylyi* strain GFJ2, but at low concentration of some copper fungicide (i.e. copper sulfate, copina-85, copina hydroxide and bordeaux-M) enhanced 3,4-DCA biodegradation of *A. baylyi* strain GFJ2. This result supports that *A. baylyi* strain GFJ2 have different pathway to degrade 4CA and 3,4-DCA.

For soil experiment, the comparison of three biological treatments of S3 soil after 4-weeks treatment showed that the total degradation of natural attenuation was the lowest but it was improved by addition of nutrients (biostimulation) and addition of microorganisms (bioaugmentation), which increased total degradation by 9-12%. This result suggested that indigenous microorganisms in S3 soil have ability to degrade CAs but nutrients in S3 soil were limited. So, nutrients addition could stimulate indigenous microorganisms to enhance more CAs degradation. Addition of natural exogenous microorganisms improved the degradation as well as bacteria able

to degrade CAs or other intermediate chemicals were increased.

For biological treatment of S5 soil after 4-week treatment showed that total degradations by natural attenuation, biostimulation and bioaugmentation were not different. This may be because nutrients in S5 soil are available for indigenous microorganisms and amount of CAs-degrading bacteria was sufficient.

Two different soil types which contained different C:N ratio, nutrients and organic matters showed different bioremediation rate. S5 was sandy loam soil which contained higher N, P, K than S3 soil (sandy clay soil). Bioremediation in S5 soil occurred at higher rate and efficiency than that in S3 soil.

In soil S3's DGGE profile showed that bands of *A. baylyi* strain GFJ2 were disappeared at 1^{st} to 4^{th} week. This result suggested that *A. baylyi* strain GFJ2 could not survive in S3 soil due to biotic and abiotic stresses. So, biodegradation of CAs contaminated soil was occurred via indigenous microorganisms and/or natural exogenous microorganism (natural bacterial consortium) from fertilizer.

Biodegradation efficiency of CAs was not different between the condition with and without copper sulfate at 150 ppm. This result may be because available copper was not toxic to microorganisms or copper rapidly combined into soil. Another reason was microorganism could adapt and resistance to copper environment.

CHAPTER VII

SUGGESTIONS AND FUTURE WORKS

The results illustrate that nutrients and copper fungicides inhibited 4CA degradation but nutrients and some copper fungicides such as copper sulfate, copina-85, copina hydroxide and bordeaux-M at low concentrations (5 and 10 ppm) enhanced 3,4-DCA degradation of *A. baylyi* strain GFJ2. To clarify this finding, the enzymes involving in 4CA and 3,4-DCA degradation should be studied and the inhibition effect from these nutrients should be performed.

In this study, nutrients were added to stimulate bacterial growth and enhance biodegradation of CAs. Other conditions such as pH, oxygen, moisture content and temperature should be further studied to improve degradation.

The inhibitors such as other heavy metals or/and toxic substances in soil should be considered. In addition, predators or competitors in soil affect the inoculated bacteria survival. Therefore, the repeating addition of the inoculated bacteria should be considered in order to increased the survival of the inoculated bacteria and to continue the pollutant degradation (Alexander 1994).

A. baylyi strain GF2 was inoculated into soil as free cells. The result showed that the bacteria could not adapte and survives in soil. Other method for bacteria inoculation is immobilization or entrapment of bacteria or bacterial consortium. Hall et al. (1998) studied transport and survival of *Pseudomonas aeruginosa* UG2Lr in soil by alginate-encapsulated and unencapsulated. The result showed that alginate-

encapsulation enhanced survival and distribution of inoculate bacteria in the soil environment.

This research study demonstrated the importance of supplementation of bioavailable nutrients in order to improve ability of indigenous microbes or bioaugmented microbe for the degradation of herbicide (organic pollutant) either as a single soil contaminant or as mixed contaminants with metal fungicide. The knowledge can be applied to treat contaminated agricultural soil by treating soil after crop cultivation with organic fertilizer with proper composition of bioavailable nutrients. Then, the cycle of plant cultivation and harvesting can start all over again after the soil treatment.

REFERENCES

Alef K. 1995. Method in applied soil microbiology and biochemistry. New York: Academic Press.

Alexander M. 1994. Biodegradation and Bioremediation. New York: Academic Press.

Alisi C, Musella R, Tasso F, Ubaldi C, Manzo S, Cremisini C, and Sprocati AR. 2009. Bioremediation of diesel oil in a co-contaminated soil by bioaugmentation with a microbial formula tailored with native strains selected for heavy metals resistance. Science of The Total Environment 407(8):3024-3032.

ALS. 2010. A solution for your organuc compound environmental monitoring, both volatile (VOC) and semi-volatile (SVOC) [online]. Available from : http://www.alsglobal.com/upload/environment/downloads/hong_kong/envirmail%20and%20foodforum/Thailand/Enviromail/Enviromail-14-ALSTH-VOC-and-SVOC.pdf [2012, January 2]

Angioi S, Polati S, Roz M, Rinaudo C, Gianotti V, and Gennaro MC. 2005. Sorption studies of chloroanilines on kaolinite and montmorillonite. Environmental Pollution 134(1):35-43.

Araya M, Pena C, Pizarro F, and Olivares M. 2003. Gastric response to acute copper exposure. Science of The Total Environment 303:253-257.

Arnebrant K, Bååth E, and Nordgren A. 1987. Copper Tolerance of Microfungi Isolated from Polluted and Unpolluted Forest Soil. Mycologia 79(6):890-895.

Bååth E. 1991. Tolerance of copper by entomogenous fungi and the use of copper-amended media for isolation of entomogenous fungi from soil. Mycological

Research 95(9):1140-1142.

Bartha R. 1968. Biochemical transformation of aniline herbicides in soil. Journal of Agricultural & Food Chemistry 16:602-604.

Bento FM, Camargo FAO, Okeke BC, and Frankenberger WT. 2005. Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. Bioresource Technology 96(9):1049-1055.

Boehncke A, Kielhorn J, Könnecker G, Pohlenz-Michel C, and Mangelsdorf I. 2003. 4-chloroaniline[online]. Available from : www.inchem.org/documenr/cicads/cicads/cicads48.htm [2011, May, 21]

Boon N, Goris J, Vos PD, Verstraete W, and Top EM. 2000. Bioaugmentation of Activated Sludge by an Indigenous 3-Chloroaniline-Degrading Comamonas testosteroni Strain, I2gfp. Applied and environmental microbiology 66(7):2906–2913.

Braid MD, Daniels LM, and Kitts CL. 2003. Removal of PCR inhibitors from soil DNA by chemical flocculation. Journal of Microbiological Methods 52(3):389-393.

Breugelmans P, Leroy B, Bers K, Dejonghe W, Wattiez R, Mot DR, and Springael D. 2010. Proteomic study of linuron and 3,4-dichloroaniline degradation by *Variovorax* sp. WDL1: evidence for the involvement of an aniline dioxygenase-related multicomponent protein. Research in Microbiology 161:208-218.

Brunsbach FR, and Reineke W. 1993. Degradation of chloroanilines in soil slurry by specialized organisms. Applied Microbiology and Biotechnology 40(2):402-407

Bureau EC. 2006. 3,4-dichloroaniline (3,4-DCA) [Online]. Available from: http://esis.jrc.ec.europa.eu/doc/existingchemicals/risk_assessment/REPORT/34dichloroaniline_DCAreport048.pdf [2001, January 21]

Burge WD. 1972. Microbial populations hydrolyzing propanil and accumulation of 3,4-dichloroaniline and 3,3',4,4'-tetrachloroazobenzene in soils. Soil Biology and Biochemistry 4:379-386.

Carvalho G, Marques R, Lopes AR, Faria C, Noronha JP, Oehmen A, Nunes OC, and Reis MAM. 2010. Biological treatment of propanil and 3,4-dichloroaniline: Kinetic and microbiological characterisation. Water Research 44(17):4980-4991.

Cervantes C, and Gutierrez Corona F. 1994. Copper resistance mechanism in bacteria and fungi. FEMS Microbiol Reviews 14:121-137.

Chaîneau CH, Rougeux G, Yéprémian C, and Oudot J. 2005. Effects of nutrient concentration on the biodegradation of crude oil and associated microbial populations in the soil. Soil Biology and Biochemistry 37(8):1490-1497.

Chander K, and Brookes PC. 1991. Is the dehydrogenase assay invalid as a method to estimate microbial activity in copper-contaminated soils? Soil Biology and Biochemistry 23(10):909-915.

Colin VL, Villegas LB, and Abate CM. 2012. Indigenous microorganisms as potential bioremediators for environments contaminated with heavy metals. International Biodeterioration & Biodegradation 69(0):28-37.

Dar AS, Kuenen GJ, and Muyzer G. 2005. Nested PCR-denaturing gradient gel electrophoresis approach to determine the diversity of sulfate-reducing bacteria in complex microbial communities. Applied Microbiology and Biotechnology 71:2325-2330.

De Wolf W, Mast B, Yedema ESE, Seinen W, and Hermens JLM. 1994. Kinetics of 4-chloroaniline in guppy, Poecilia reticulata. Aquatic Toxicology 28(1-2):65-78.

Ding C, Zhang Z, Yang C, and Yao C. 2009. Studies on the bioremediation Actions of *p*-chloroaniline on Wetland Soil. International conference on environmental science and information application technology.

Doi R, and Ranamukhaarachchi LS. 2009. Soil dehydrogenase in a land degradation-rehabilitation gradient:observations from a savanna site with a wet/dry seasonal cycle. Rev Biol Trop 57:223-234.

Dumestre A, Sauve S, McBride M, Baveye P, and Berthelin J. 1999. Copper speciation and microbial activity in long-term contaminated soils. Archives of Environmental Contamination and Toxicology 36:124-131.

Edenborn SL, and Sexstone AJ. 2007. DGGE fingerprinting of culturable soil bacterial communities complements culture-independent analyses. Soil Biology and Biochemistry 39(7):1570-1579.

El-Gendy KS, Radwan MA, and Gad AF. 2009. In vivo evaluation of oxidative stress biomarkers in the land snail, Theba pisana exposed to copper-based pesticides. Chemosphere 77(3):339-344.

EPA. 1999. Monitored natural attenuation of petroleum hydrocarbons [Online]. Available from : http://www.epa.gov/nrmrl/pubs/600f98021/600f98021.pdf [2012, January 1]

EPA. 2001. A citizen's guide to phytoremediation. p 1-2 [Online]. Available from : http://www.clu-in.org/download/citizens/citphyto.pdf [2012, January 1]

EPA US. 2010. SAUGET AREA 2 [Online]. Available from : http://www.epa.gov/R

5Super/npl/illinois/ILD000605790.htm [2012,January,2011]

Ercolini D. 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. Journal of Microbiological Methods 56:297-314.

EUROPA. 2006. The rapid alert system for non-food products (RAPEX) [online]. Available from: http://ec.europa.eu/consumer/dyna/rapex/rapex_archives.cfm

Evans GM, and Furlong JC. 2003. Environmental Biotechnology: Theory and Application. England: John Wiley and Sons.

Fantroussi S, Verschuere L, Verstraete W, and Top EM. 1999. Effect of Phenylurea Herbicides on Soil Microbial Communities Estimated by Analysis of 16S rRNA Gene Fingerprints and Community-Level Physiological Profiles. Applied Microbiology and Biotechnology 65(3):982–988.

Fattore E, Müller L, Davoli E, Castelli D, and Benfenati E. 1998. Industrial pollutants in ground waters from Northern Milan. Chemosphere 36(9):2007-2017.

Giacomazzi S, and Cochet N. 2004. Environmental impact of diuron transformation: a review. Chemosphere 56(11):1021-1032.

Gonza'lez-Pradas E, Ferna'ndez-Pe'rez M, Flores-Ce'spedes F, Villafranca-Sa'nchez M, Uren a-Amate M, Soci'as-Viciana M, and Garrido-Herrera F. 2005. Effect of dissolved organic carbon on sorption of 3,4-dichloroaniline and 4-bromoaniline in a calcareous soil. Chemosphere 59:721-728.

Hall BM, McLoughlin AJ, Leung KT, Trevors JT, and Lee H. 1998. Transport and survival of alginate-encapsulated and free lux-lac marked Pseudomonas aeruginosa UG2Lr cells in soil. FEMS Microbiology Ecology 26(1):51-61.

Hamby DM. 1996. Site remediation techniques supporting environmental restoration activities--a review. The Science of the Total Environment 191(3):203-224.

Helling B, Reinecke SA, and Reinecke AJ. 2000. Effects of the fungicide copper oxychloride on the growth and reproduction of *Eisenia fetida* (oligochaeta). Ecotoxicology and Environmental Safety 46:108-116.

Holm J, Rugge K, Bjerg P, and Christensen T. 1995. Occurrence and distribution of phamaceutical organic compounds in the groundwater downgradient of a landfill (Grinsted, Denmark). Environmental Science and Technology 29(5):1414-1420.

Hongsawat P, and Vangnai AS. 2011. Biodegradation pathways of chloroanilines by Acinetobacter baylyi strain GFJ2. Journal of Hazardous Materials 186(2-3):1300-1307.

Ibarrolaza A, Coppotelli MB, Del Panno MT, Donati ER, and Morelli IS. 2009. Dynamics of microbial community during bioremediation of phenanthrene and chromium(VI)-contaminated soil microcosms. Biodegradation 20:95-107.

Klier NJ, West RJ, and Donberg PA. 1999. Aerobic biodegradation of dichloroethylenes in surface and subsurface soils. Chemosphere 38(5):1175-1188.

Komárek M, Cadková E, Chrastný V, Bordas F, and Bollinger J-C. 2010. Contamination of vineyard soils with fungicides: A review of environmental and toxicological aspects. Environment International 36(1):138-151.

Komárek M, Vanek A, Chrastny V, Szakova J, Kubova K, Drahota P, and Balik J. 2009. Retention of copper from different fungicides in contrasting soil types. Journal of Hazardous Materials 166:1395-1402.

Lai X, Zeng X, Fang S, Huang Y, Cao L, and Zhou S. 2006. Denaturing gradient gel electrophoresis (DGGE) analysis of bacteria community composition in deep-

sea sediments of the South China Sea. World J Microbiol Biotechnol 22:1337-1345.

Lee K, Park J-W, and Ahn I-S. 2003. Effect of additional carbon source on naphthalene biodegradation by Pseudomonas putida G7. Journal of Hazardous Materials 105(1–3):157-167.

Li Z, Xu J, Tang C, Wu J, Muhammad A, and Wang H. 2006. Application of 16S rDNA-PCR amplification and DGGE fingerprinting for detection of shift in microbial community diversity in Cu-, Zn-, and Cd-contaminated paddy soils. Chemosphere 62(8):1374-1380.

Lipthay JRd, Enzinger C, Johnsen K, Aamand J, and Sorensen SJ. 2004. Impact of DNA extraction method on bacterial community composition measured by denaturing gradient gel electrophoresis. Soil Biology and Biochemistry 36:1607-1614.

Livigston AG, and Willacy A. 1991. Degradation of 3,4-dichloroaniline in synthetic and industrially produced wastewaters by mixed cultures freely suspended and immobilized in a packed-bed reactor. Applied Microbiology and Biotechnology 35(4):551-557.

Loidl M, Hinteregger C, Ditzelmüller G, Ferschl A, and Streichsbier F. 1990. Degradation of aniline and monochlorinated anilines by soil-born <i>Pseudomonas acidovorans</i> strains. Archives of Microbiology 155(1):56-61.

Lyons CD, Katz SE, and Bartha R. 1985. Persistence and mutagenic potential of herbicide-derived aniline residues in pond water. Bulletin of Environmental Contamination and Toxicology 35(1):696-703.

Malik S, Beer M, Megharaj M, and Naidu R. 2008. The use of molecular techniques to characterize the microbial communities in contaminated soil and water. Environment International 34:265-276.

Mejáre M, and Bülow L. 2001. Metal-binding proteins and peptides in bioremediation and phytoremediation of heavy metals. Trends in Biotechnology 19(2):67-73.

Miille MJ, and Crosby DG. 1983. Pentachlorophenol and 3,4-dichloroaniline as models for photochemical reactions in seawater. Marine Chemistry 14(2):111-120.

Mrozik A, and Piotrowska-Seget Z. 2010. Bioaugmentation as a strategy for cleaning up of soils contaminated with aromatic compounds. Microbiological Research 165(5):363-375.

Muyzer G, and Smalla K. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie van Leeuwenhoek 73:127-141.

NOSB T. 2001. Copper Sulfate, as algicide and invertebrate pest control. p 1-17 [Online]. Available from : http://www.ams.usda.gov/AMSv1.0/getfile?dDocName=STELPRDC5067070

Olaniran AO, Pillay D, and Pillay B. 2006. Biostimulation and bioaugmentation enhances aerobic biodegradation of dichloroethenes. Chemosphere 63(4):600-608.

Palma DL, and Medici F. 2002. Recovery of copper from contaminated soil by flushing. Waste Management 22:883-886.

Perrin-Ganier C, Schiavon F, Morel JL, and Schiavon M. 2001. Effect of sludge-amendment or nutrient addition on the biodegradation of the herbicide isopro-

turon in soil. Chemosphere 44(4):887-892.

Petchkroh W. 2006. Isolation and characterization of 4-chloroaniline-degrading bacteria. Thailand: Chulalongkorn University.

Pietrzak U, and McPhail DC. 2004. Copper accumulation, distribution and fractionation in vineyard soil of Victoria, Australia. Geoderma 122:151-166.

Ping G. 1997. Dehydrogenase activity in soil: A comparison between the TTC and INT assay under their optimum conditions. Soil Biology and Biochemistry 29(2):211-214.

Polcaro AM, Mascia DC, Palmas S, and Vacca A. 2004. Electrochemical degradation of diuron and dichloroaniline at BDD electrode. Electrochimica Acta 49(4):649-656.

Pritchard PH, and Costa CF. 1991. EPA's Alaska oil spill bioremediation project. Part 5. Environmental Science & Technology 25(3):372-379.

Radianingtyas H, Robinson GK, and Bull AT. 2003. Characterization of a soil-derived bacterial consortium degrading 4-chloroaniline. Microbiology 149:3279–3287.

Said W, and Lewis DL. 1991. Quantitative assessment of the effects of metals on microbial degradation of organic chemicals. Applied and environmental microbiology 57:1498-1503.

Sánchez M, Wolfger H, and Getoff N. 2002. Radiation-induced degradation of 4-chloroaniline in aqueous solution. Radiation Physics and Chemistry 65(6):611-620.

Scow KM, Fan S, Johnson C, and Ma GM. 1995. Biodegradation of Sorbed Chemicals in Soil. Environmental Health Perspectives 103(ArticleType:

research-article / Issue Title: Supplement 5: Biodegradation / Full publication date: Jun., 1995 / Copyright © 1995 The National Institute of Environmental Health Sciences (NIEHS)):93-95.

Sorensen RS, Albers NC, and Aamand J. 2008. Rapid mineralization of the phenylurea herbicide diuron by *Variovorax* sp. strain SRS16 in pure culture and within a two-menber cinsortium. Applied and environmental microbiology 74(8):2332-2340.

Sprocati AR, Alisi C, Tasso F, Marconi P, Sciullo A, Pinto V, Chiavarini S, Ubaldi C, and Cremisini C. 2011. Effectiveness of a microbial formula, as a bioaugmentation agent, tailored for bioremediation of diesel oil and heavy metal co-contaminated soil. Process Biochemistry(0).

Steffensen SW, and Alexander M. 1995. Role of competition for inorganic nutrients in the biodegradation of mixtures of substrates. Applied and environmental microbiology 61(8):2859-2862.

Surovtseva EG, Ivoilov VS, Karasevich YN, and Vacileva GK. 1985. Chlorinated anilines, a sources of carbon, nitrogen and energy for *Pseudomonas diminuta*. Microbiologiya 54:948-952.

Tongarun R, Luepromchai E, and Vangnai AS. 2008. Natural Attenuation, Biostimulation, and Bioaugmentation in 4-Chloroaniline-Contaminated Soil. Current Microbiology 56:182-188.

Travkin VVM, Solyanikova IIP, Rietjens IIMCM, Vervoort JJ, van Berkel WWJH, and Golovleva LLA. 2003. Degradation of 3,4-Dichloro- and 3,4-Difluoroaniline by Pseudomonas fluorescens 26-K. Journal of Environmental Science and Health, Part B 38(2):121-132.

Tsang CWD, Zhang W, and Lo MCI. 2007. Copper extraction effectiveness and soil dissolution issues of EDTA-flushing of artificially contaminated soils. Chemosphere 68:234-243.

Umrania VV. 2006. Bioremediation of toxic heavy metals using acidothermophilic autotrophes. Bioresource Technology 97(10):1237-1242.

Vangnai AS, and Petchkroh W. 2007. Biodegradation of 4-chloroaniline by bacteria enriched from soil. FEMS Microbiology Lett 268:209-216.

Vidali M. 2001. Bioremediation. An overview. Pure and Applied Chemistry 73:1163-1172.

Wang Y-S, Wen C-Y, Chiu T-C, and Yen J-H. 2004. Effect of fungicide iprodione on soil bacterial community. Ecotoxicology and Environmental Safety 59:127-132.

Watanabe K. 2001. Microorganisms relevant to bioremediation. Current Opinion in Biotechnology 12(3):237-241.

You IS, and Bartha R. 1982. Metabolism of 3,4-dichloroaniline by Pseudomonas putida. Journal of Agricultural and Food Chemistry 30(2):274-277.

Yu KSH, Wong AHY, Yau KWY, Wong YS, and Tam NFY. 2005. Natural attenuation, biostimulation and bioaugmentation on biodegradation of polycyclic aromatic hydrocarbons (PAHs) in mangrove sediments. Marine Pollution Bulletin 51(8–12):1071-1077.

Zeyer J, and Kearney PC. 1982a. Microbial degradation of para-chloroaniline as sole carbon and nitrogen source. Pesticide Biochemistry and Physiology 17(3):215-223.

Zeyer J, and Kearney PC. 1982b. Microbial metabolism of propanyl- and 3,4-dichloro

aniline. Pesticide Biochemistry and Physiology 17:224-277.

Zeyer J, Wasserfallen A, and Timmis KN. 1985. Microbial Mineralization of Ring-Substituted Anilines through an *Ortho*-Cleavage Pathway. Applied and environmental microbiology 50(2):447-453.

Zhang C, Valsaraj KT, Constant WD, and Roy D. 1998. Nutrient and surfactant enhancement for the biodegradation of chlorinated hydrocarbons in the wastewater from a Louisiana Superfund site. Journal of Hazardous Materials 62(1):41-58.

Zhang L-l, He D, Chen J-m, and Liu Y. 2010. Biodegradation of 2-chloroaniline, 3-chloroaniline, and 4-chloroaniline by a novel strain Delftia tsuruhatensis H1. Journal of Hazardous Materials 179(1-3):875-882.

Zhou J, Bruns AM, and Tiedje MJ. 1996. DNA Recovery from Soils of Diverse Composition. Applied and environmental microbiology 62(2):316-322.

APPENDICES

APPENDIX A

4CA and 3,4-DCA standard curve

Standard curve was used to analyze chromatogram of HPLC result in order to calculate the concentration of substance. The CAs standard was freshly prepared in ethanol as a stock solution at 1 M. Then, CAs at concentration of 0.025 mM, 0.05 mM, 0.1 mM, 0.2 mM and 0.4 mM was obtained by doing a serial dilution from CAs stock solution. The CAs residual was determined by using the same method as described in topic 3.8.3. The standard curve of 4CA and 3,4-DCA were showed in Figure C1and C2, respectively.

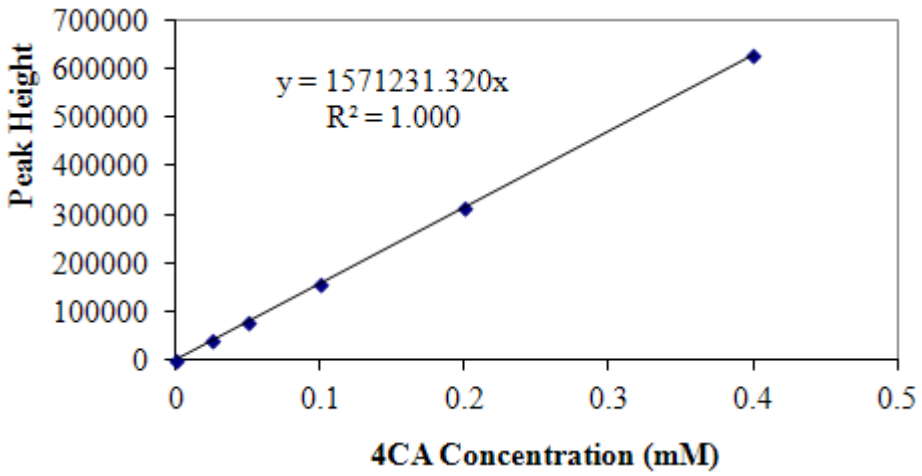


Figure A1 4CA standard curve for calculation 4CA concentration, n=3.

$$\text{The amount of 4CA} = \frac{\text{Peak height}}{1571231.320}$$

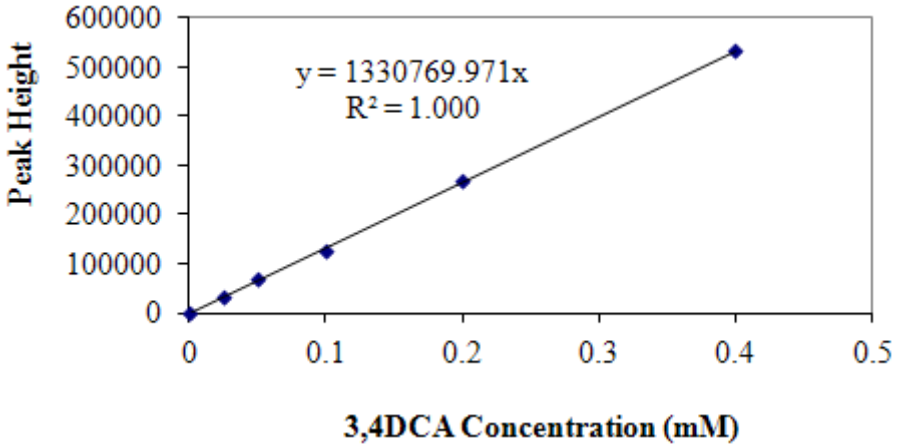


Figure A2 3,4-DCA standard curve for calculation of 3,4-DCA concentration, n=3.

$$\text{The amount of 3,4-DCA} = \frac{\text{Peak height}}{1330769.971}$$

APPENDIX B

Retention times of 4CA and 3,4-DCA

CAs was analyzed using reverse phase HPLC equipped with a UV detector (at 240 nm). The separation was performed at 28.5 °C on a C18 column using an acetonitrile: water mixture (70:30, v/v) as a mobile phase at a flow rate of 1 ml/min. The injection volume was 15 µl. The retention time of 4CA and 3,4-DCA under the condition tested were 3.6 and 4.1 minute, respectively as shown in Figure B1 and B2.

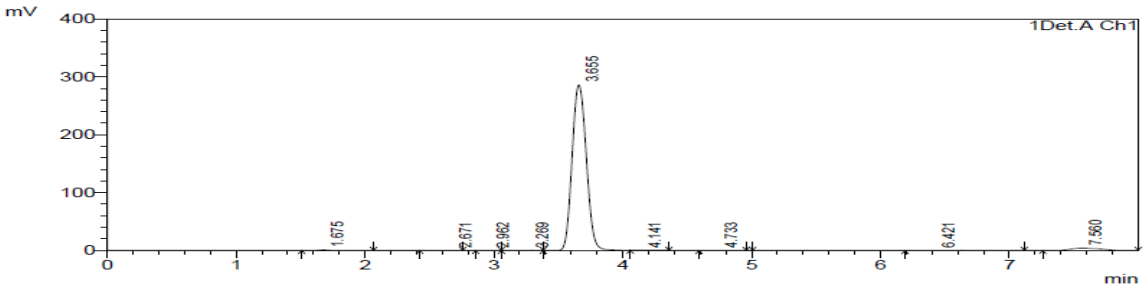


Figure B1 HPLC chromatogram of 4CA at concentration 0.2 mM. The retention time of 4CA was 3.655.

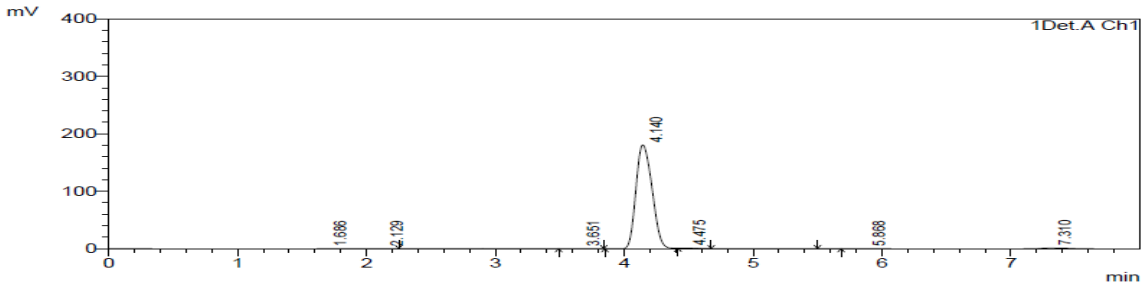


Figure B2 HPLC chromatogram of 3,4-DCA at concentration 0.2 mM. The retention time of 3,4-DCA was 4.140.

APPENDIX C

Protein calibration curve

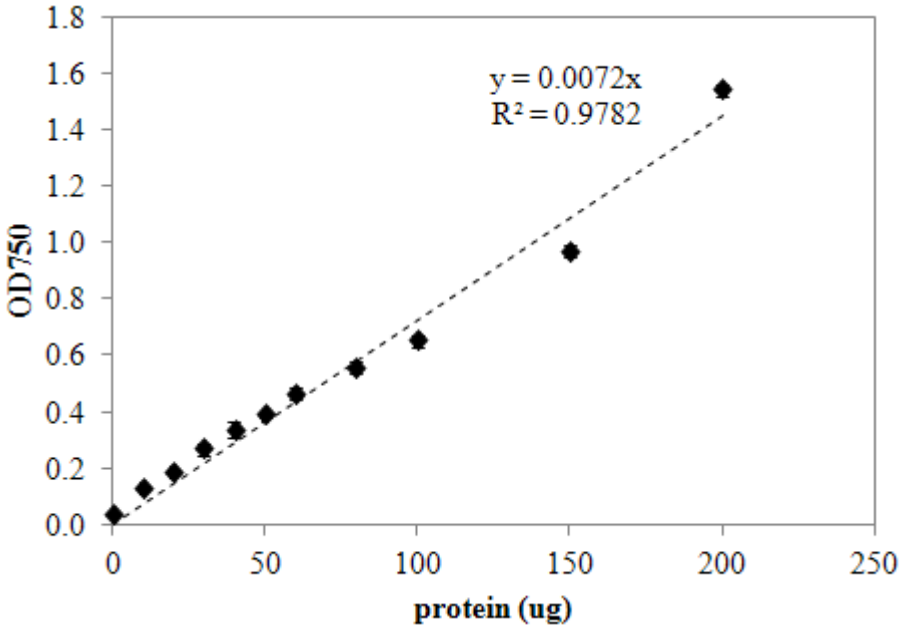


Figure C Standard curve of modified Lowry method used to determine cell protein, n=3.

APPENDIX D

TPF calibration curve

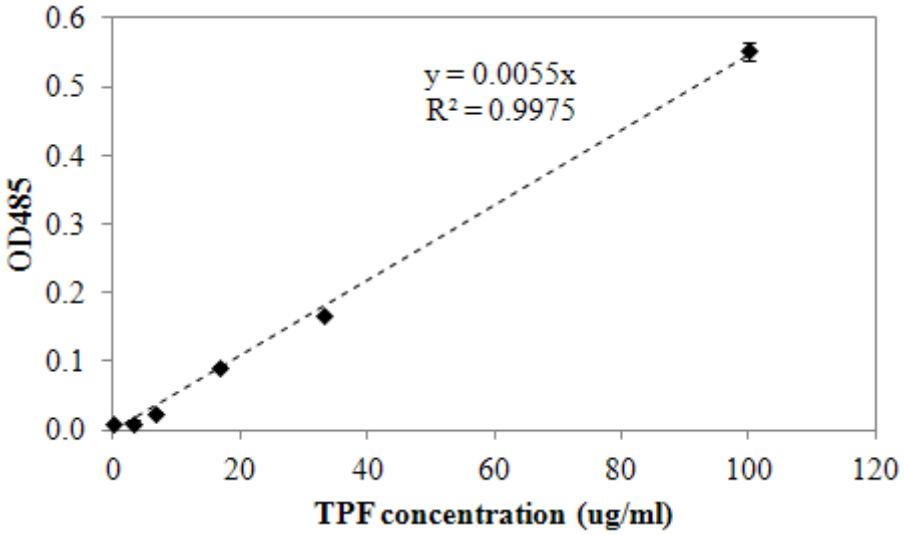


Figure D TPF calibration curve for the calculation of microbial activity (dehydrogenase activity), n=3.

$$TTC + 2H^{+} + 2e^{-} \leftrightarrow TPF + HCl$$

$$\text{Dehydrogenase activity TPF (µg)/dwt (g)} = \frac{\text{TPF (µg/ml)} \times 7}{\text{Dwt} \times 1}$$

When 7 = the volume of solution added to the soil sample in the assay (ml)

1 = the moist soil used (g)

Dwt = the dry weight of 1 g moist soil: S3 = 0.823 and S5 = 0.744

APPENDIX E

Soil pH analysis

Equipment

MP 220 pH meter (Mettler Toledo, Model SevenEasy^{TM}pH)

Reagent

Buffer solution pH 4, 7 and 10

Methodology

1. Weigh 10 g of 2 mm sieved soil into 50 ml beaker.
2. Add 25 ml distilled water to sample beaker.
3. Mix soil sample and allow it to settle for 30 minutes.
4. Calibrate pH meter with buffer solution pH 4, 7 and 10 before taking a measure of the sample pH.

APPENDIX F

Total carbon analysis

Reagents

1. $K_2Cr_2O_7$
2. Conc.H_2SO_4
3. Conc.H_3PO_4
4. NaF
5. Redox indicator (Dissolve 0.5 g diphenylamine with 20 ml distilled water and 100 ml conc.H_2SO_4)
6. $Fe(NH_4)_2(SO_4).6H_2O$

Methodology

1. Weigh 0.1 g of 0.5 mm sieved soil into 250 ml flask (The blank flask without soil sample).
2. Pipette 13 ml of 1 N $K_2Cr_2O_7$ into flask and gently mixing.
3. Add 20 ml conc.H_2SO_4 and mix 1 minute.
4. Allow sample to cool down at room temperature, 30 minutes.
5. Add 100 ml distilled water, 10 ml conc.H_3PO_4 and 0.2 g NAF.
6. Add 2-3 droplets of redox indicator, mix well. Color of sample solution will be change from orange to purple as blue or purple red.
7. Titrate sample solution with 0.5 N $Fe(NH_4)_2(SO_4).6H_2O$

8. At the end point the color of sample solution will be brilliant green
9. For the real end point, add 0.5 ml 1N $K_2Cr_2O_7$ and titrate until the color change to brilliant green again.

$$\% \text{ total carbon} = \frac{13.5 \times (B\text{-}S) \times 12 \times 100}{B \times G \times 4000 \times 0.77}$$

When 13.5 = volume of $K_2Cr_2O_7$ (ml)

B = volume of $Fe(NH_4)_2(SO_4).6H_2O$ titrated with blank (ml)

S = volume of $Fe(NH_4)_2(SO_4).6H_2O$ titrated with sample (ml)

G = soil weight (g)

APPENDIX G

Total nitrogen analysis

Equipments

1. Digestion Unit
2. Scrubber Unit
3. Distillation Unit

Reagents

1. Na_2SO_4
2. $CuSO_4$
3. Conc.H_2SO_4
4. NaOH
5. Methyl red

Methodology

1. Weigh 2 g of 0.5 mm sieved soil into distilled glass tube. Then add 6 g Na_2SO_4 and 0.4 g $CuSO_4$. The blank tube contains only Na_2SO_4 and $CuSO_4$ (without soil sample).
2. Add 20 ml conc.H_2SO_4. Scrubber unit must be on when the digestion unit programme is running.

3. Run the digestion programme until the sample solution color turn to clear blue-green. Stop the programme, cool down the tube in fume hood (scrubber unit must be on).
4. Prepare the distillation unit by insert a bare distilled glass tube into the distillation unit. Calibrate pH probe, run pre-heating and priming the distillation unit follow by manuscript.
5. Pipette 25 ml of 0.1 N H_2SO_4 into 250 ml flask and add 5-7 droplets of methyl red.
6. Set the machine to fill 50 ml distilled water and 90 ml 35% NaOH for titration.
7. Take flask for back titration with 0.1 N NaOH until sample solution color turn to yellow at end point.

$$\% \text{ total nitrogen} = \frac{(B\text{-}S) \times 0.1\ NH_2SO_4 \times 1.4}{\text{Soil weight (g)}}$$

When B = volume of 0.1 N H2SO4 titrated with blank (ml)

S = volume of 0.1 N H2SO4 titrated with sample (ml)

APPENDIX H

Soil samples properties

Their properties were determined using Appendices E, F and G (pH, total carbon and total nitrogen, respectively)

Soil properties

Soil sample	pH	Organic carbon (%)	Nitrogen (%)	C:N ratio
S1	6.77	29.03	2.31	12.57
S2	6.94	38.38	2.24	17.13
S3	6.82	32.92	2.10	15.76
S4	7.34	37.40	2.45	15.27
S5	6.92	37.21	5.39	6.90
F1	8.60	60.78	5.46	11.32
F2	6.19	44.81	6.02	7.44

APPENDIX I

Preparation for denaturing gel gradient electrophoresis (DGGE)

Reagents

1. Acry/Bis 37.5:1, 40% (w/v) solution

For 500 ml volume used 187.5 g of acrylamide and 5 g of bis-acrylamide, after that add distilled H_2O to 500 ml in a plastic 1 liter beaker or more accurately cylinder.

2. 50x TAE buffer

Trisma-base	:	121 g
Glacial acetic acid	:	28.6 ml
0.5 ml EDTA, pH 8.0	:	50 ml

Fill with distilled H_20 to 500 ml in a beaker or cylinder. Use the same batch of DGGE/TAE buffer to prepare running buffer and gels.

3. 7M urea

42.042 g urea in 100 ml of H_2O

4. 10% ammonium persulfate (APS)

0.1 g ammonium persulfate in 1 ml of H_2O

Pouring the gel

Reagent	**Final Conc.**	**0%**	**10%**	**20%**	**30%**	**40%**	**50%**	**60%**	**70%**
H_2O	N/A	10.8 ml	10.5 ml	9.5 ml	8.5 ml	7.5 ml	6.5 ml	5.5 ml	4.5 ml
40% acry:bis (37.5:1)	8%	3.9 ml	3.0 ml	3.0 ml	3.0 ml	3.0 ml	3.0 ml	3.0 ml	3.0 ml
50x TAE buffer	1x	0.3 ml	0.3 ml	0.3 ml	0.3 ml	0.3 ml	0.3 ml	0.3 ml	0.3 ml
7 M urea	Variable	0	0.7 ml	1.3 ml	1.9 ml	2.5 ml	3.1 ml	3.7 ml	4.3 ml
Formamide	Variable	0	0.6 ml	1.2 ml	1.8 ml	2.4 ml	3.0 ml	3.6 ml	4.2 ml
Glycerol	2%	0	0.3 ml	0.3 ml	0.3 ml	0.3 ml	0.3 ml	0.3 ml	0.3 ml
10% APS		100 µl	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl
TEMED		10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl

Note: APS and TEMED used to polymerization of the acrylamide gel which have approximately 15 minutes to pour the gel.

APPENDIX J

Calculation

Table J The raw data

Time (hr)	Height	Concentration (mM)
0	281230	0.18
3	215480	0.14
6	102640	0.07
9	43960	0.03
12	36490	0.02

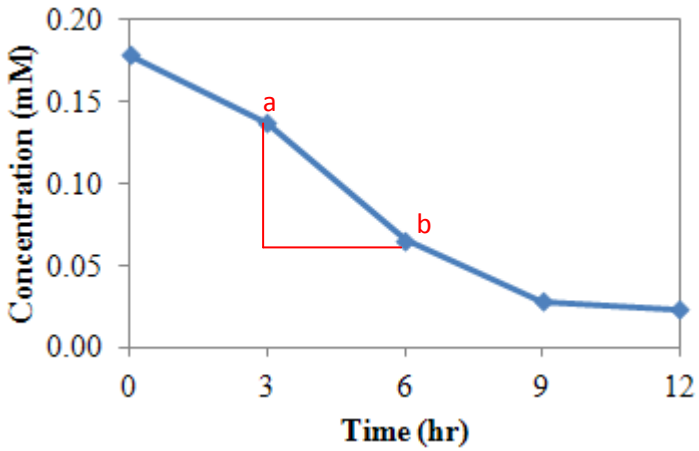


Figure J The concentration remaining of 0.2 mM 4-chloroaniline of *A. baylyi* strain GFJ2 in MMSAY medium.

Protein = 282 $mg.l^{-1}$ (using modified Lowry method)

Degradation rate ($mM.hr^{-1}$) = $-\frac{\text{slope (Ya-Yb)}}{\text{(Xb-Xa)}}$ } Choose the highest slope

Specific degradation rate ($mM.hr^{-1}.mg$ cell $protein^{-1}$) = $\frac{\text{Degradation rate}}{\text{Cell protein}}$

BIOGRAPHY

Miss Naruemon Chumjai was born on February 28th, 1987 in Chiang Rai province, Thailand. She received Bachelor's Degree in Biochemistry, Faculty of science, Chulalongkorn University in 2008. She pursued her Master degree study in the inter-Department of Environmental Management, Chulalongkorn University, Bangkok, Thailand in May 2009. She finished Master Degree of Science in Environmental Management in 2011.