ความหลากหลายของแบคทีเรียในดินที่มีการปนเปื้อนแคดเมียมจากอำเภอแม่สอด จังหวัดตาก

นางสาวอุรัจฉวี อุณหเลขกะ

MICROBIAL DIVERSITY IN CADMIUM-CONTAMINATED SOILS

FROM MAE SOT DISTRICT, TAK PROVINCE

Miss Uratchwee Unhalekhaka

A Dissertation Submitted in Partial Fulfillment of the Requirements

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อุรัจฉวี อุณหเลขกะ : ความหลากหลายของแบคทีเรียในดินที่มีการปนเปื้อนแคดเมียม จากอำเภอแม่สอด จังหวัดตาก. (MICROBIAL DIVERSITY IN CADMIUM-CONTAMINATED SOILS FROM MAE SOT DISTRICT, TAK PROVINCE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร. ชาญวิทย์ โฆษิตานนท์, 101 หน้า.

งานวิจัยนี้ได้ศึกษาความหลากหลายของแบคทีเรียในดินที่มีการปนเปื้อนแคดเมียม บริเวณอำเภอแม่สอด จังหวัดตาก ซึ่งเป็นพื้นที่ที่มีการทำเหมืองแร่สังกะสี โดยทำการเก็บตัวอย่าง บริเวณห้วยแม่ตาว, ห้วยแม่ตาวแง่ซ้าย, ห้วยแม่กุ และห้วยหนองเขียวในเดือนพฤษภาคม พ.ศ. 2551 จากการศึกษาพบว่า บริเวณต้นน้ำห้วยแม่ตาวมีค่าแคดเมียมและสังกะสีทั้งหมด 8.45 และ 193.05 mg/kg ตามลำดับ และโลหะทั้งสองมีค่าสูงขึ้นจนถึงบริเวณท้ายน้ำหรือหมู่บ้านแม่ตาว ใหม่ โดยมีความเข้มข้น 16.85-22.50 และ 954.00-1,998.00 mg/kg ตามลำดับ ห้วยแม่ตาวแง่ ซ้ายซึ่งอยู่แถบที่ราบสูงตะวันออกเฉียงเหนือของเหมืองพบปริมาณแคดเมียม 3.05 mg/kg และ สังกะสี 97.45 mg/kg บริเวณห้วยแม่กุซึ่งตั้งอยู่อีกด้านของภูเขา พบการปนเปื้อนแคดเมียมและ สังกะสี 7.55-34.95 และ 316.05-789.00 mg/kg ตามลำดับ และห้วยหนองเขียวซึ่งอยู่บริเวณที่ ราบสูงตะวันตกเฉียงใต้พบค่าแคดเมียมและสังกะสี 1.10 และ 27.05 mg/kg ตามลำดับ ปริมาณ แคดเมียมมีความสัมพันธ์กันในเชิงลบ(ตรงข้ามกัน)กับการแลกเปลี่ยนประจุบวก (r = -0.749) ที่ ระดับนัยสำคัญทางสถิติ 0.05 แต่ไม่พบความสัมพันธ์กับลักษณะสมบัติอื่นๆ ตัวอย่างดินที่มีการ ปนเปื้อนของแคดเมียมมากที่สุด (34.95 mg/kg) มีค่าดัชนีความหลากหลายของแบคทีเรียสูงสุด และปริมาณแคดเมียมเป็นเพียงตัวแปรเดียวที่ส่งผลกระทบต่อความหลากหลายของแบคทีเรียใน ดินตัวอย่างโดยมีค่าสัมประสิทธิ์สหสัมพันธ์ในเชิงบวก(ตามกัน) (r = 0.768) โดยมีนัยสำคัญทาง สถิติที่ 0.05 จากลำดับเบสของแบคทีเรียเด่นในดินตัวอย่างพบว่ากลุ่มแบคทีเรียที่มีจำนวนมาก ที่สุดคือ beta-proteobacteria และแบคทีเรียหลักคือ Actinobacteria ชนิด *Rhodococcus yunnanensis* เมื่อพิจารณารูปแบบของประชากรแบคทีเรียด้วย DGGE พบว่ามีแบคทีเรีย 2 ชนิด คือ uncultured *Aquicella* sp. และ *Xenophilus* sp. ที่อาจนำมาใช้เป็นทางเลือกหนึ่งในการ ตรวจสอบปริมาณแคดเมียมในดินได้

สาขาวิชา___วิทยาศาสตร์สิ่งแวดล้อม___ ลายมือชื่อนิสิต___อุรัจฉวี อุณหเลขกะ___
ปีการศึกษา___2552___ ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก___

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KEYWORDS : SOIL MICROBIAL DIVERSITY / CADMIUM / ZINC / 16S rRNA / DGGE

URATCHWEE UNHALEKHAKA : MICROBIAL DIVERSITY IN CADMIUM-CONTAMINATED SOILS FROM MAE SOT DISTRICT, TAK PROVINCE. THESIS ADVISOR: ASST. PROF. CHARNWIT KOSITANONT, Ph.D., 101 pp.

This research studied the diversity of active soil bacteria in cadmium (Cd) contaminated soil near zinc (Zn) mine at Mae Sot district, Tak province. The soil samples were collected from 4 creeks; Huai Mae Tao, Huai Mae Tao Ngae Sai, Huai Mae Ku and Huai Nong Khiao on May 2008. The total Cd and Zn quantities of the upstream samples of Huai Mae Tao were 8.45 and 193.05 mg/kg soil, respectively. Concentrations were increased to 16.85-22.50 and 954.00-1,998.00 mg/kg soil in the downstream (Ban Mae Tao Mai) samples. The Cd and Zn levels 3.05 and 97.45 mg/kg soil were found in the sample from Huai Mae Tao Ngae Sai, the north-eastern highland of the Zn mine. The metals concentration at Huai Mae Ku, the creek on the other side of the mountain, were 7.55-34.95 and 316.05-789.00 mg/kg soil whereas only 1.10 mg/kg soil of Cd and 27.05 mg/kg soil of Zn were found in a creek in the south-western highland. Cd was found negatively (inversely) correlated to only CEC in all soil (r = -0.749) at the 0.05 confidential level. The maximum Cd amount (34.95 mg/kg soil) was resulted in the highest soil bacterial diversity index (H). From Pearson correlation, the quantity of Cd was positively correlated to the bacterial diversity with correlation coefficient (r) of 0.768. Due to the DNA sequencing results, major group of bacteria was beta-proteobacteria and dominant strain was, Actinobacteria, *Rhodococcus yunnanensis*. Considering from bacterial community pattern by using DGGE, there were 2 bacterial strains, uncultured *Aquicella* sp. and *Xenophilus* sp., which might be used as an alternative detector for Cd in soil.

Field of Study : Environmental Science   Student's Signature *Uratchwee Unhalekhaka*

Academic Year : 2009   Advisor's Signature *C. Kositanont*

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LIST OF ABBREVIATIONS

μg	=	microgram
μl	=	microliter
μm	=	micrometer
μM	=	micromolar
bp	=	base pair
°C	=	degree celsius
cm	=	centimeter
cmol	=	centimole
g	=	gram
kb	=	kilo base pair
kg	=	kilogram
km^2	=	square kilometer
L	=	liter
M	=	molar
m^3	=	cubic meter
mg	=	milligram
ml	=	milliliter
mM	=	millimolar
mm	=	millimeter
mol	=	mole
ng	=	nanogram
ppm	=	part per million
U	=	unit
V	=	volt

CHAPTER I

INTRODUCTION

1.1 General Statement

Soil is one of the most important natural resources for life as it is the origin of the four requisites in both direct and indirect ways. In addition, it also serves as the place that holds wastes and pollutants. In Thailand, crises of the soil contamination with chemicals such as pesticides, heavy metals, etc. are remain unsolved.

At present heavy metals, cadmium (Cd) in particular, are one of the major contaminants in soils,. Though it is a highly toxic element, its effect to biological role in environment is still unclear. Cd can be absorbed into the body through intake and respiration then impact on various systems of man for example nervous, respiratory, digestive and reproductive systems. Since Cd is widely used in industries, it source of contamination is varied.

The earliest case of Cd pollution occurred in 1950 at Jinzu river basin, Toyama prefecture, Japan. Wastewater from Kamioka mine, operated by Mitsui Mining and Smelting Co., Ltd., was flowed into the river which was used for consumption, irrigation, and fishery by the surrounding people. From this phenomenon, most villagers were ill with fragile bone, kidney dysfunction and over one hundred lives departed. After investigation, the Cd concentration in this area was found as high as 4.85 mg/kg soil or 14 times of background level (0.34 mg/kg soil) (International Center for Environmental Technology Transfer [ICETT], 1998).

Cd is always found together with Zinc (Zn) in the deposited; therefore zinc mining is usually having Cd as a byproduct. The Zinc mines in Thailand are situated at Tak province due to the biggest Zn deposited. The production capacity in the latest year was 50,000 metric tons (Padaeng Industry Public Company Limited, 2008). In mining several activities for example drilling, ores transportation, smelting, tailings disposal and wastewater discharge may spread out heavy metal to the surroundings especially in case of poor handling and inadequate waste management as displayed in the research of Soil Analysis Division, Land Development Department, Thailand that the average Cd level in the sediment came from the collapsed stacking of cinders at the Zn mining was as high as 228.50 mg/kg (Nitayaporn Tunmanee and Jurai Thongmarg, 1994).

The occupation of people who live at Mae Sot is farming and the major cultivated produce are rice, soy beans and garlic. They irrigated water from natural water sources which parts of them pass through the Zn mine, therefore the area situated below is in risk of the heavy metals contamination and might lead to the accumulation within the vegetables that cropped in the contaminated fields then transfer to human food chain bringing harm to consumers, eventually.

Nowadays, methods for heavy metal detection in soil is chemical approaches with an atomic adsorption spectrophotometer (AAS) or inductively coupled plasma-optical emission spectrometry (ICP-OES). The organisms which not only have an important role in soil but also have a large quantity, bacteria, have not been adequately investigated to understand the relationship between the Cd level and soil bacteria.

Thus, this research aims to examine the active bacterial diversity in Cd-contaminated soils by using molecular techniques i.e. denaturing gradient gel electrophoresis (DGGE) of complementary DNA (cDNA) that synthesized from 16S ribosomal RNA (16S rRNA). The data acquired from this study could be used as basic knowledge as alternative options to indicate Cd amounts in soil besides chemical methods.

1.2 Objectives

1.2.1 To characterize soil properties of Cd polluted soils around Mae Sot district, Tak province

1.2.2 To investigate the correlation between Cd concentration and soil properties.

1.2.3 To investigate the effects of Cd concentration on the bacterial communities.

1.3 Hypothesis

Soil Cd concentrations could cause the differences of bacterial diversity.

1.4 Expected Benefits

1.4.1 The research results could reveal some soil properties which affect heavy metal contamination.

1.4.2 The soil properties which correlated to Cd concentration could be used as a hint for Cd accumulation in soils.

1.4.3 Bacterial composition in Cd-contaminated soils could be used as preliminary information indicating Cd contamination in soil.

CHAPTER II

LITERATURE REVIEW

2.1 Heavy Metals

Heavy metals are metallic elements with high density such as arsenic (As), lead (Pb), zinc (Zn), mercury (Hg) and cadmium (Cd). Small quantities of some heavy metals are required for the living organisms such as iron (Fe), copper (Cu) and manganese (Mn) but it might toxic when accumulated in the bodies.

At present, the heavy metals contamination in environment is a serious problem and needs to be solved. Sources of heavy metal could be natural or human activities e.g. fertilizers, industries, wastes, mining and smelting. Any of the sources may lead to the distribution from sources to soils and water resources then entering food chain and finally endanger human.

2.1.1 Cadmium (Cd)

Cd is a metal that can be found in earth's crust and almost everywhere in the environment (Table 2.1). It was firstly discovered in 1817 by Friedrich Strohmeyer, a German scientist. This bluish-white metal has atomic number 48 and 112.411 g/mol in atomic weight. It is usually associated with Zn, Pb and Cu ores and mostly found in sphalerite (ZnS) in form of greenockite (CdS). For this reason, Cd is mostly recovered as a by-product of Zn mining. Cd amount in ores is related to content of Zn which Zn:Cd ratio ranging from 100:1 to 1,000:1 (Babich and Stotzky, 1978). Cd are widely applied in industries for instance nickel-cadmium (Ni-Cd) batteries, electroplating, polyvinylchloride (PVC) stabilizers and pigments.

Many human activities cause emission and accumulation of anthropogenic Cd in environment (Table 2.2). The fates of Cd accessed into surrounding environments are varied as shown in Table 2.3.

Table 2.1 Cd concentrations in environment (International Cadmium Association [ICdA], 2009).

Sources	Amounts
Air	
- rural areas	0.1-5 ng/m^3
- urban areas	2-15 ng/m^3
- industrial areas	15-150 ng/m^3
Soil	
- earth's crust	0.1-0.5 mg/kg
- metamorphic rocks	0.02-0.2 mg/kg
Water	
- oceans	< 5 ng/L
- fresh waters	10 ng/L
- sediments	0.2-0.9 mg/kg

Table 2.2 Sources of Cd in the environment (Babich and Stotzky, 1978).

Air pollution	Soil pollution
Mining and smelting process	Dust fall
Galvanizing	Mine wastewater
Pigment manufacture	Sewage sludge fertilizers
Battery production	Super phosphate fertilizers
Electroplating	Pesticides
Alloy manufacturing	Water pollution
Fertilizer production	Mine wastewater
Fossil fuel combustion	Sewage treatment effluents
Pesticide application	Industrial effluents
Cigarette smoke	Leaching of landfills

Table 2.3 Fate of Cd within air, soil and water (Agency for Toxic Substances and

Disease Registry [ATSDR] and Environmental Protection Agency [EPA], 2008).

Sources	Fate
Air	exist as particles or vapors and fall into soils and water
Soil	bind to organic matter and absorbed by plant
Water	exist as hydrated ion or ionic complexes with other inorganic or organic substances. Soluble forms dissolve in water and insoluble forms deposit to sediments

Cd is absorbed into human body through ingestion, inhalation and dermal exposure. For short-term exposure, effects on human health are vomiting, nausea, diarrhea and muscle cramps etc. while long-term symptoms, Cd accumulated within human kidney causes kidney dysfunction and osteomalacia (bone disease).

2.1.2 Zinc (Zn)

Zn is a metallic element that generally found in air, water and soil as shown in Table 2.4

Table 2.4 Zn amounts in natural (Emsley, 2001).

Sources	Amounts
Atmosphere	0.1-4 $\mu g/m^3$
Soil	5-770 mg/kg
Sea water	30 $\mu g/L$
Earth's crust	75 mg/kg

This bluish-pale grey metal's atomic number and atomic weight are 30 and 65.409 g/mol. Zn is found together with Cu and Pb ores. Extraction of Zn is normally done in the form of Zn blende (ZnS). Zn is used in many industries such as galvanizing, brass making, dry cell battery and die-casting alloy products.

Zn production produces large quantities of sulfur dioxide (SO_2) and Cd vapor. The concentration of itself that leaks into the environment is also higher and should be aware. Only small amount of Zn (2 ppm) within rivers impact to the oxygen level of fish blood while the absorbent ability of plants are interfered when Zn level in soil is higher than 500 ppm (Emsley, 2001).

Even though Zn is an essential element for human to use in biological reactions within bodies but higher doses also cause many health problems for instance stomach cramps, nausea, headache and pancreas damage (Parada Maneewong, 2005).

2.2 Impact of Soil Characteristics on Cadmium

Because of the complexity, there are several properties in both physical and chemical that affect on the distribution of Cd in soil.

2.2.1 pH

Chemical reactions in soils relate to H^+ and OH^- which measured by soil pH. Solubility of numerous substances i.e. heavy metals depend on pH and might lead to mobility of Cd throughout environment. The higher pH the more Cd is adsorbed in soil or decreased Cd bioavailability form (Anderson and Nilsson, 1974; Zhao and Saigusa, 2007).

Naidu *et al.* (1997) stated that adsorption of Cd in soils decreases when pH ranging from 2-3, this corresponds with research by Römkens and Salomons (1998) who found increase in solubility of Cd and Zn when soil pH below 5.5.

Apart from pH, Cd adsorption in soil also depends on Cd content, organic matter, moisture and clay particles (Wu, Morel and Guckert, 1994; Karak *et al.*, 2005).

Table 2.5 Classification system of soils based on pH (soil:water = 1:1) (Department of
Land Development [LDD], 2004).

Rating	Range
Ultra acid	< 3.5
Extremely acid	3.5-4.5
Very strongly acid	4.6-5.0
Strongly acid	5.1-5.5
Moderately acid	5.6-6.0
Slightly acid	6.1-6.5
Neutral	6.6-7.3
Slightly alkaline	7.4-7.8
Moderately alkaline	7.9-8.4
Strongly alkaline	8.5-9.0
Very strongly alkaline	> 9.0

2.2.2 Texture

Relative proportions of particle size separates which are sand (2.0-0.050 mm), silt (0.050-0.002 mm) and clay (< 0.002 mm) used for dividing soil textural classes. Fine textured soils are described when dominant proportion is clay while coarse texture is soils that have sand as dominant. United States Department of Agriculture (USDA) groups soil textures into 12 classes as shown in Figure 2.1.

Clay is soil particle that carry a negative charge then it enables to adsorb water, nutrient ions such as magnesium (Mg^{2+}), potassium (K^+) and calcium (Ca^{2+}) and positive charge contaminants (Coyne and Thompson, 2006) which indicated by the decrease in the solubility of Cd occurred in the addition of clay content (Eriksson, 1990).
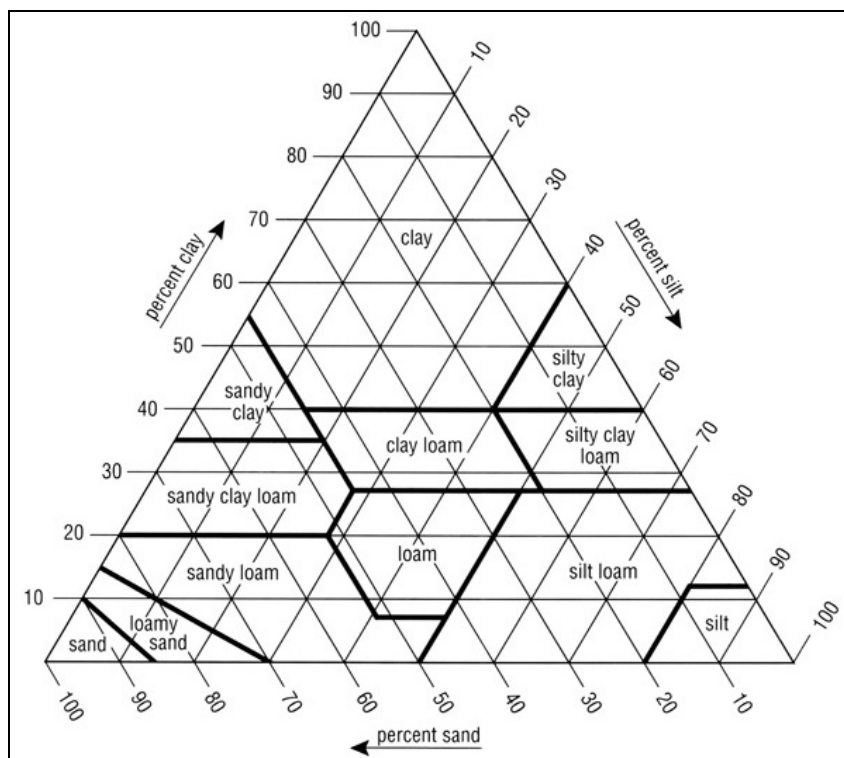
Figure 2.1 Basic textural classes by USDA (2009).

2.2.3 Organic matter (OM)

It is organic portion composed of microbial cells, plant and animal residues in decomposition stage. The product at the end of decomposition process is called humus which can be divided into 2 groups, humic; the compounds that synthesized residues by microorganisms and non-humic substances; the products that come from metabolism of living things i.e. amino acids, carbohydrates, lipids, etc. Because humus is the component that has negative charge, therefore it is able to adsorb cation or positive charge element (Tan, 2005) and prefers bivalent ions e.g. Mn^{2+}, Ca^{2+}, Zn^{2+} than trivalent ions e.g. Cr^{3+}, Al^{3+} (Enerex Botanicals, 2009).
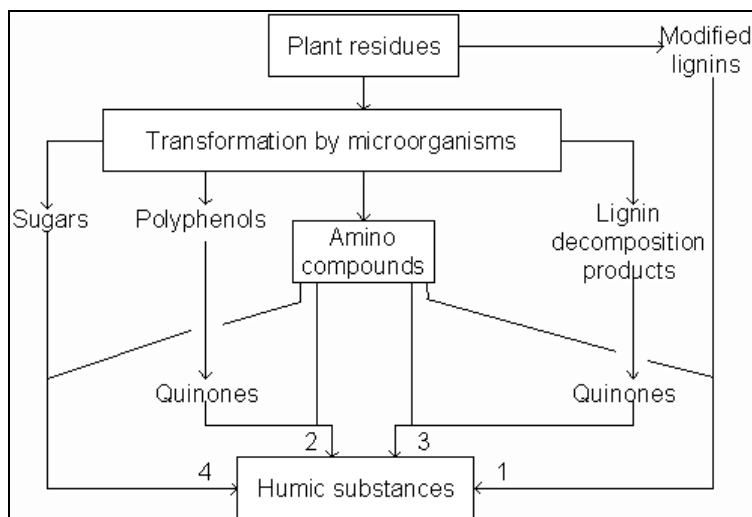
Figure 2.2 Mechanisms for humic formation in soil (Enerex Botanicals, 2009).

There are some researches indicated that the capability of soil to adsorb Cd depends on both quantity and composition of OM (Singh, 2005) and Mahara *et al.* (2007) also found that low molecular weight of OM (< $1x10^3$ Daltons) bound more Cd than the higher fractions.

Table 2.6 Categories of organic matter (OM) in soils (LDD, 2004).

Rating	%
Very low (VL)	< 0.5
Low (L)	0.5-1.0
Moderately low (ML)	1.0-1.5
Moderate (M)	1.5-2.5
Moderately high (MH)	2.5-3.5
High (H)	3.5-4.5
Very high (VH)	> 4.5

2.2.4 Cation exchange capacity (CEC)

Soil has capability to adsorb and exchange cations by clay minerals and OM which their surfaces are negative charge. This ability occurs between soil and its solution, the strength prevents cations from water leaching but this is a temporary stage because they can be replaced by other cations within soils.

CEC is usually used for measuring soil quality and fertility and expressed as milliequivalents (meq)/100 g or cmol/kg which demonstrates amount of cations that can be adhered.

Normally, CEC is varied in different type of soils and depends on quantity of OM and clay minerals for example it would be < 1 meq/100 g for sandy soils but as high as 200 meq/100 g in humus (Babich and Stotzky, 1978; Cooper, 2009).

Due to this chelating property, Cd accumulation in soils is likely to occur by increasing CEC and this cause the less Cd distribution into environments.

Table 2.7 Rating of cation exchange capacity (CEC) (LDD, 2004).

Rating	CEC (cmol/kg)
Very low	< 3.0
Low	3.0-5.0
Slightly low	5.0-10.0
Moderate	10.0-15.0
Slightly high	15.0-20.0
High	20.0-30.0
Very high	> 30.0

2.3 Effects of Cadmium on Soil Bacteria

Due to the fact that soil is a complex habitat which contains soil particles, OM, water and plant roots then soil microorganisms ex. bacteria, fungi, virus, algae and actinomycetes are highly diverse and numerous.
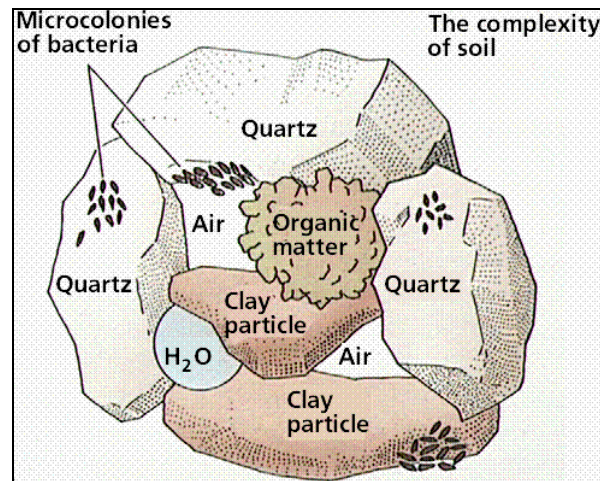
Figure 2.3 Structure of soil (Purves, 1998).

Numbers of microbes in soil differ in sizes, amounts and biomass as shown in Table 2.8. Bacteria are the most plentiful microbes with populations in excess of 10^8 individuals/g soil and more than 10^4 different species.

Table 2.8 Sizes and numbers of major microbial soil (Zuberer and Wollum, 2005).

Microbial group	Example	Size (µm)	Numbers (no./g soil)
Viruses	Tobacco mosaic	0.02 x 0.3	10^{10}-10^{11}
Bacteria	*Pseudomonas*	0.5 x 1.5	10^8-10^9
Actinomycetes	*Streptomyces*	0.5-2.0*	10^7-10^8
Fungi	*Mucor*	8.0*	10^5-10^6
Algae	*Chlorella*	5 x 13	10^3-10^6
Protozoa	*Euglena*	15 x 50	10^3-10^5

∗diameter of hyphae

Soil bacteria live commonly in the film of water around soil particles and play important roles in recycling plant nutrients by decompose organic compounds, maintenance soil structure with substances that produce to bind particles into small aggregates and also detoxify pollutants and wastes that contaminated within soils.

Mechanisms that bacteria develop themselves to react with heavy metals include (1) methylation and demethylation (2) extracellular binding, complexation and alter cell permeability (3) extracellular precipitation (4) intracellular accumulation and (5) oxidation and reduction reactions (Brierley, 1990). Nevertheless, at high level of heavy metals, they are lethal to abundant soil bacteria and impact further to the ecosystem since their functions are failure.

Focus on Cd, there are several researches mentioned about the response of enzyme activity to Cd for example Dar (1996) found decreasing of dehydrogenase, alkaline phosphatase and arginine-ammonification activities at 50 mg/kg soil which corresponds with Lorenz *et al.* (2006) who observed reducing of enzyme activities for protease, urease, alkaline phosphatase and arylsulphatase while xylanase activity was higher but no significant effect on invertase activity after Cd contamination at 34.2 mg/kg soil as well as 134.4 mg/kg soil.

Sensitivity of the bacteria to Cd quantities is variability such as the eubacteria are more sensitive of Cd than the actinomycetes and the gram-negative eubacteria are more resistant to Cd than gram-positive eubacteria (Babich and Stotzky, 1978) for this reason, shift of bacterial communities are a good indicator to assess Cd contaminations in soils.

2.4 Determination of Bacterial Diversity in Soil

Due to limited understanding of molecular analysis in the past, effects of Cd to soil bacterial communities are measured through changes in these parameters; carbon mineralization (Gupta *et al.*, 1984; Hattori, 1989), nitrogen transformation (Bewley and Stotzky, 1983; Dar and Mishra, 1994), enzyme activities (Lorenz *et al.*, 2006), biomass (Fritze *et al.*, 2000; Landi *et al.*, 2000) and bacterial amounts (Olson and Thornton, 1982; Ellis *et al.*, 2003; Qing *et al.*, 2007).

Because techniques that used to determine these factors are cultural-based methods which many limitations occur since laboratory conditions, then these means might not provide the information about uncultivable bacteria or major segment of soil microbial populations (Torsvik *et al*., 1998).

In order to remove the disadvantages and biases in culturable methods, molecular techniques which based on nucleic acids extracted directly from soil are applied (Figure 2.4). Knowledge that obtains from these methods will improve the understanding of how heavy metals impact on sensitive soil bacterial populations.
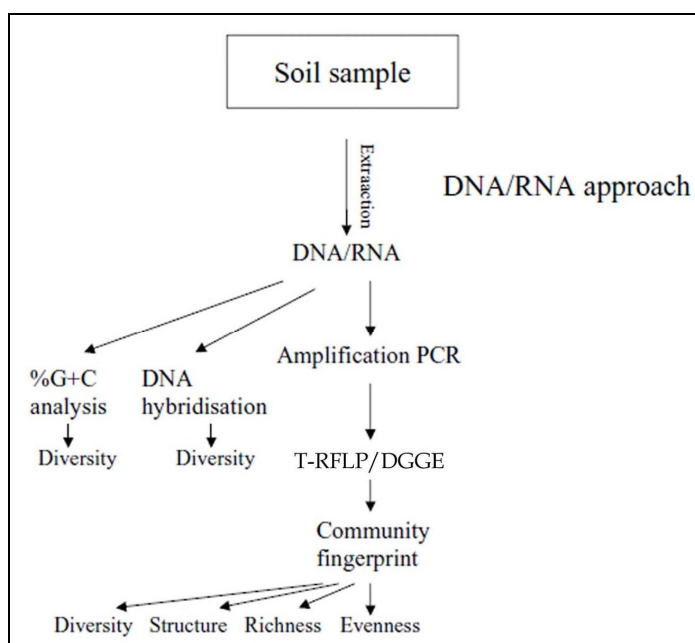
Figure 2.4 Approaches for bacterial community fingerprinting in soil polluted with Cd
(modified from Walker, 2008).

There are a lot of molecular approaches have been developed e.g. nucleic acid reassociation and hybridization (Torsvik *et al*., 1996), DNA micro arrays (Greene and Voordouw, 2003), guanine plus cytosine (G+C) content (Nusslein and Tiedje, 1999), single strand conformation polymorphism; SSCP (Peters *et al*., 2000), denaturing and temperature gradient gel electrophoresis; DGGE and TGGE (Kozdroj and van Elsas, 2001; Vilchez *et al*., 2007), amplified ribosomal DNA restriction analysis; ARDRA or restriction fragment length polymorphism; RFLP (Liu *et al*., 1997), terminal restriction fragment length polymorphism; T-RFLP (Lazzaro *et al*., 2008) and

(automated) ribosomal intergenic spacer analysis; ARISA or RISA (Lear *et al*., 2009). Among these techniques, DGGE is one of genetic fingerprinting methods which allow higher throughput and profile comparison between each sample.

Comparing DGGE with other fingerprinting approaches which are SSCP, T-RFLP and RISA as shown in Table 2.9, it is found that SSCP and DGGE are highly sensitive methods due to the ability of 1-bp different detection but when compare with RISA and T-RFLP, they require more sensitive conditions for reproducible profiles. Furthermore, techniques in T-RFLP and RISA can be easily automatically perform because their simple procedure but SSCP and DGGE are more effective and responsive when the finding of slightly different in sequence is required (Ikeda *et al*., 2006).

Table 2.9 Comparison molecular-based methods for microbial diversity analysis

(Kirk *et al*., 2004; Ikeda *et al*., 2006).

Method	Facility cost	Processing time	Technical requirements	Advantages	Disadvantages
RISA	Low	Short	Low	Highly reproducible	Require DNA large quantities
DGGE	Medium	Medium	Medium	Reliable, reproducible, rapid	Co-migration might occur
SSCP	Medium	Medium	Medium	No GC clamp & gradient	Some ssDNA can form > 1 stable conformation
T-RFLP	High	Medium	Medium	Automated, highly reproducible	Choice of restriction enzyme will influence

2.4.1 Theoretical basic of DGGE

The earliest work of DGGE was demonstrated by Fischer and Lerman (1979), thereafter Muyzer *et al*. (1993) were a pioneered group that adapt this method for using with the environmental samples (Margesin and Schinner, 2005) and widely used in present-day.

Differ from normal electrophoresis which separate nucleic acid fragments by size, DGGE separates fragments by their sequences (Nakatsu, 2007) because of chemical denaturants, a mixture of urea and formamide, are used.

Same length of nucleic acids are amplified with specific primers by PCR and these product mixture which derived from each individual bacteria in sample are separated in a polyacrylamide gel containing a linear gradient of denaturants at constant running temperature (usually 60°C).

When melting domain of nucleic acids reaches its melting temperature, double-stranded PCR fragment denatures into single-stranded because hydrogen (H) bonds within complementary nucleotides are broken and this retard its migration on gel. Difference in sequence of each fragment makes mobility of DNA stops at various positions on gel, therefore the more guanine (G) and cytosine (C) in strands, the further migration of molecule (Marschner, 2007).

Complete strand departure is avoided by adding a GC-rich sequence (generally 40 bp) at the 5'-end of a PCR forward primer to act as a high temperature melting domain and this leads to the sharp band production (Muyzer and Smalla, 1998). Outcome profiles represent diversity of sample which each band and its intensity are amounts of populations and their abundance, respectively.

2.4.2 General DGGE procedure

Mainly protocol to acquire DGGE profiles composes of 5 steps which are nucleic acid extraction, PCR amplification of target gene, separation of PCR products by DGGE, visualization of fingerprinting and data analysis (Figure 2.5).
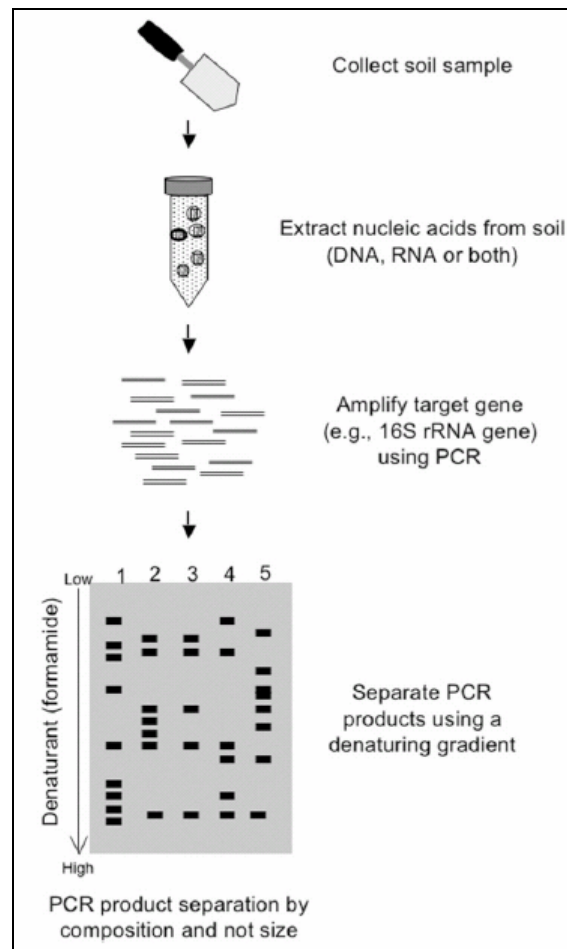
Figure 2.5 Flow diagram of the DGGE for soil bacteria analysis (Nakatsu, 2007).

For microbial community investigation, DNA or RNA can be isolated from various habitats which ranging from natural samples i.e. soil, water, sediment to synthetic systems i.e. bioreactor, laboratory microcosm etc. There are a lot of extraction protocols with different techniques are found in the literature (Moran *et al*., 1993; Borneman and Triplett, 1997; Duarte *et al*., 1998; Hurt *et al*., 2001; Orsini and Romano-Spica, 2001; Costa *et al*., 2004; Griffiths *et al*., 2004; de Lipthay *et al*., 2004; Solaiman and Marschner, 2007; Xiaoqi *et al*., 2007).

Basics of this step are efficiency of nucleic acid recovery and their purity especially for soil samples that some chemicals contamination ex. humic substances might interfere other molecular techniques (Tebbe and Vahjen, 1993; Jackson *et al*., 1997).

The next step after nucleic acid isolation is PCR or RT-PCR which a target sequence is amplified at an exponential rate. Within PCR program, the annealing temperature is suitably chosen based on primers of interest to avoid nonspecific products. Modified methods can be used to improve the specificity for instance hot start procedure which is performed by heating PCR reaction mix lacking a key component such as target DNA or polymerase enzyme and starting the reaction by adding the missing part at denaturing temperature and touch-down PCR is another technique that using the annealing temperature 5-10°C above the optimum in the early few cycles and decreased 1-2°C every cycle until it reaches the specified temperature (van Elsas and Boersma, 2004; Nakatsu, 2007).

For DGGE, any gene can be used as a target such as functional genes for coding proteins which perform metabolic reactions but it is more generally to study in ribosomal RNA (rRNA) gene which provides phylogenetic information of community analysis. It is ideal for determining taxonomy, phylogeny and species divergence because it has conserved regions between all bacteria which have not changed over time due to their critical function and other regions that variable between different groups (Devereux and Wilkinson, 2004).

In bacteria, ribosome composes of 2 subunits defined by their sedimentation rate (Svedberg unit; S) as large and small, large subunit contains 5S and 23S rRNA while small subunit or 16S rRNA is the most commonly marker for demonstrating phylogenetic relationships between bacterial groups.
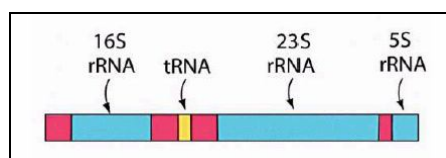
Figure 2.6 rRNA unit of bacteria (Naresuan University, Faculty of Agriculture Natural Resources and Environment, 2008).

Furthermore, there are some researches (Duarte *et al.*, 1998; Savaporn Supaphol *et al.*, 2006; Hoshino and Matsumoto, 2007; Izumi *et al.*, 2007; Anderson, Parkin, and Campbell, 2008) focused on rRNA-based methods instead of rDNA (DNA coding for rRNA) because rRNA analysis reveals the information about active bacteria since amount of rRNA synthesis roughly correlates with growth of functioning bacteria (Wagner, 1994).

Before starting 16S rRNA amplification process, nucleic acids derived from bacterial cells lysis in environmental samples are treated with RNase-free DNase to remove DNA residues, afterwards rRNA is used as a template for complementary DNA (cDNA) synthesis combines with reverse transcriptase enzyme, this method is known as reverse transcriptase-polymerase chain reaction (RT-PCR). Then, cDNA is amplified further by using primers which designed for binding to conserved regions and expand variable regions by means of PCR step and the outcome products are investigated further with DGGE.

The same length of PCR product mixture from multiple bacterial species which differ in each sequence are loaded into DGGE. DNA fragments migrate through the gel and form a band when reach the certain concentration of denaturants creating a banding pattern that varies according to microbial composition of total community in the habitat.

After electrophoresis, the polyacrylamide gel is visualized by detecting with ethidium bromide (EtBr), SYBR green or silver stain (Baker and Harayama, 2004). Then, the gel is transilluminated with light sources that appropriate to the staining methods and photographed using gel imaging system.
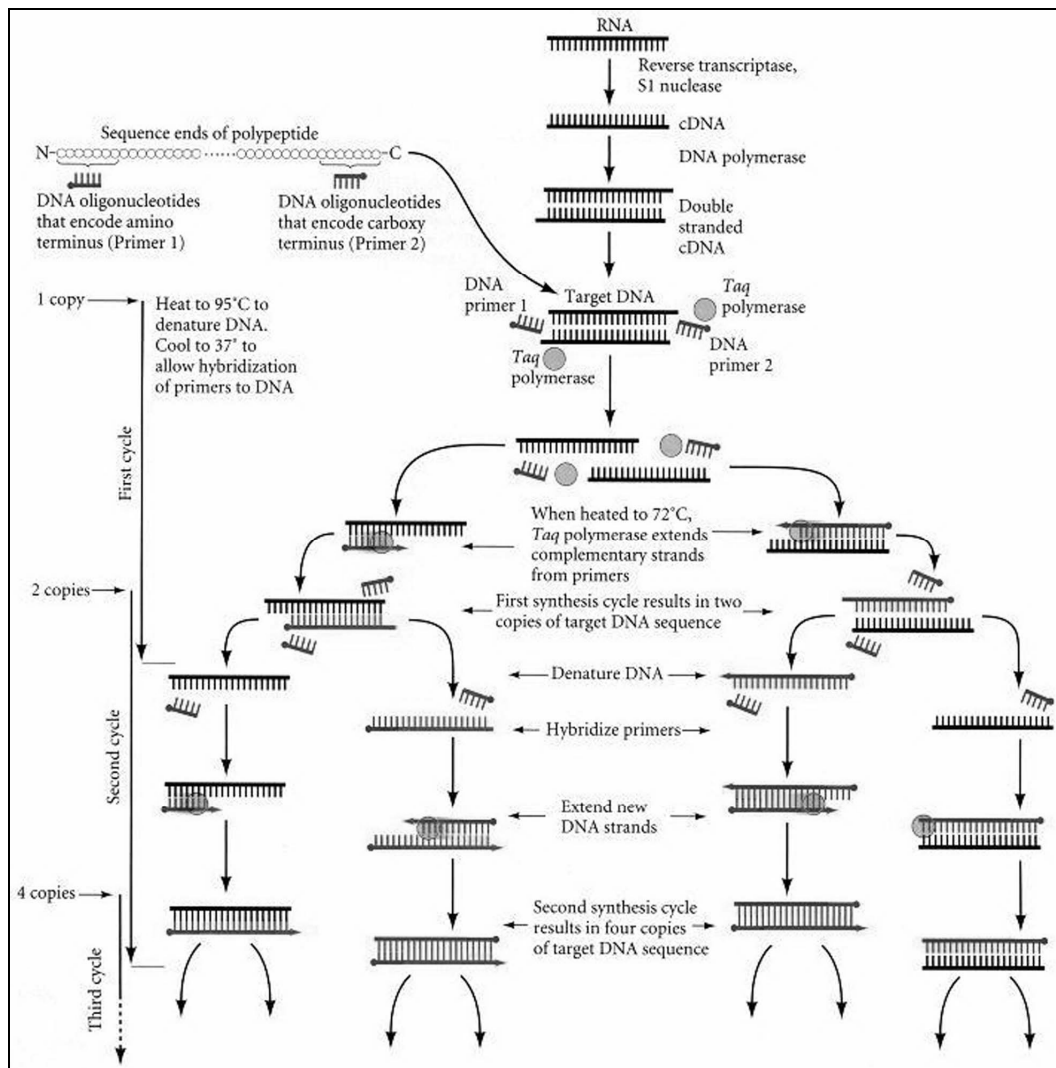
Figure 2.7 Schematic of amplification of rRNA (National Center for Biotechnology
Information [NCBI], 2000).

To interpret the banding patterns, number and band intensities can be
analyzed by visually or using computer programs for instance Quantity One, GelCompar
II and Bio-Profil (Green, 2005). These data might be illustrated as dendograms for
representing the relationship between each sample by cluster analysis e.g. unweighted
pair group method using arithmetic averages (UPGAMA) (Nakatsu, 2007).

It should be noticed that one specie might produce many bands in
DGGE due to it contains several gene copy numbers whereas an observed band may
compose of different bacterial species because they varied only a few base within the
sequence (Farrelly, Rainey and Stackebrandt, 1995).

Each DGGE band can be excised and sequenced to identify microbial type by comparing the results with available databases such as Ribosomal Database Project (RDP; http://rdp.cme.msu.edu/) and National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/).

2.4.3 Applications of DGGE

It is widely used to investigate microbial populations in various matrices (Muyzer *et al.*, 2004) for example screen clone libraries (Izumi *et al.*, 2007), compare nucleic acids extraction methods (Lipthay *et al.*, 2004; Xiaoqi *et al.*, 2007), community structure analysis (Muyzer, de Waal, and Uitterlinden, 1993; Borneman *et al.*, 1996; Duarte *et al.*, 1998; Duineveld *et al.*, 2001; Dilly *et al.*, 2004; Collins *et al.*, 2006; Edenborn and Sexstone, 2007; Wang *et al.*, 2008) and determining changes of bacterial populations (Handschur *et al.*, 2005; Hoshino and Matsumoto, 2007; Wenhui *et al.*, 2007) etc.

There are a lot of researches that used DGGE as a technique to examine the effect of pollutants on soil microbial diversity i.e. heavy metals exposure (Moffett *et al.*, 2003; Wang *et al.*, 2007; Anderson, Parkin, and Campbell, 2008), hydrocarbon (Röling *et al.*, 2006; Savaporn Supaphol *et al.*, 2006) and herbicide contaminated soil (Roongnapa Tongarun, Ekawan Luepromchai, and Alisa S. Vangnai, 2008) etc.

Concentrate on Cd, several studies are demonstrated that Cd contamination influences over soil microbial communities for instance Macnaughton *et al.* (1999) found changes in DGGE profile of eubacterial community in toxic metal salts addition soil (caesium; Cs, strontium; Sr, Cd and cobalt; Co) when compared with controls and 2 types of bacteria were detected as *Acinetobacter* and *Burkholderia*. This result corresponds to the work of Lorenz *et al.* (2006) which showed that reduction of bacterial composition in Cd contaminated soils was occurred.

The impacts were clearly exhibited in soils around heavy metals smelter, as shown by Li *et al*. (2006) and Qing *et al*. (2007). They suggested that number of bacteria in soil samples increased with increasing distance from mines and concluded that heavy metal contamination included Cd, decreased bacterial diversity in soils.

2.5 Monitoring Area

The study site situated in Mae Sot district, Tak province, northern region of Thailand, and connected to Myanmar through Moei river at western part.

2.5.1 Geology of Mae Sot

Mineral resources around Mae Sot district are shown in Figure 2.8. Padaeng deposit or supergene nonsulfide Zn deposit was found in this area (Reynolds, 2003). Source of this ore originated from both primary and secondary deposit which primary Zn is sphalerite (ZnS) and secondary, the dominant type, are hemimorphite ($Zn_4(Si_2O_7)(OH)_2 \cdot 2H_2O$) and smithsonite ($ZnCO_3$) (Department of Primary Industries and Mines [DPIM], 2006).

For this reason, the area has been developed into Zn mines, operated by Padaeng Industry Public Co. Ltd. and Tak Mining Co. Ltd. which closed down since 2003.

2.5.2 Hydrology of Mae Sot (DPIM, 2006)

Water source of main creeks or Huai, in Thai, comes from Thanon Thongchai mountain range and drainage form of these creeks is dendritic pattern that branching similar to roots of a tree and flows from east to west into Moei river. There are 3 major creeks that related to this investigating site which are

2.5.2.1 Huai Mae Tao has 4 branches which are Huai Mae Tao Ngae Khao, Huai Mae Tao Ngae Sai, Huai Pa Pu and Huai Pong that running through Ban Mae Tao into the Moei river with 33 kilometers in length.

2.5.2.2 Huai Mae Ku flows through Ban Mae Ku Nuea and separate into 3 brooks including Huai Muang, Huai Nam Mueang, and Huai Mae Ku.

2.5.2.3 Huai Nong Khiao runs from eastern hill and join with Huai Luang at Ban Pha Lat into Moei river.

Along with these creeks, the dykes were constructed intervals for the irrigation of agriculture within the area and usually floods in the rainy season.
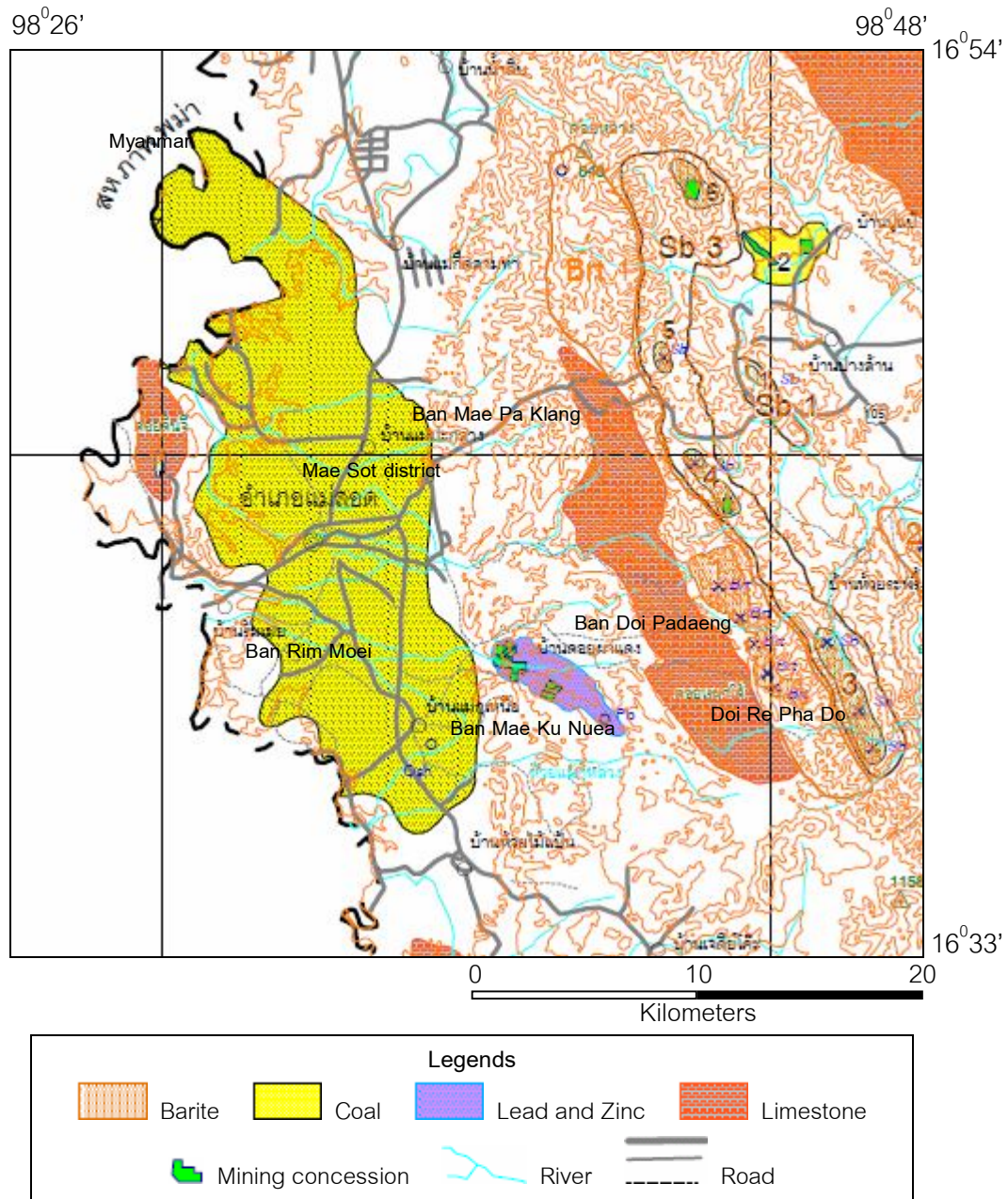
Figure 2.8 Map of mineral resources at Mae Sot district, scale 1:250,000 sheet NE 47-14 (Moulmein) (Department of Mineral Resources [DMR], 2001).

2.5.3 Land utilization within Mae Sot

Major areas within the district are paddy fields that irrigation sources from Huai Mae Tao. In dry season, crop rotation for soy beans, garlic and corn also found after the well-known Jasmine rice is gathered.

2.5.4 Contamination problem at Mae Sot

In 2000, the primary investigation of Cd quantities in rice field soils that irrigate from Huai Mae Tao at Ban Pha Te by the International Water Management Institute (IWMI) showed that total Cd levels of 154 samples ranged from 3.4-284 mg/kg soil which was 94 times higher than European Union (EU) Maximum Permissible (MP) for soil (3.0 mg/kg soil) or 1,800 times when compared with Thai Investigation Level for Cd (0.15 mg/kg soil) (Zarcinas *et al*., 2004).

A year later, the sampling sites were expanded along Huai Mae Tao for 334 fields and the investigation found total Cd concentrations ranged from 0.5-218 mg/kg soil. In case of total Zn amounts, they found 197-8,036 mg/kg soil or 26 times of EU MP for Zn concentration in soil (300 mg/kg soil) and greater than Thai Investigation Level for Zn around 114 times (70 mg/kg soil) (Zarcinas *et al*., 2004). For extensive study, total Zn ranged from 100 to 7,918 mg/kg soil (Simmons *et al*., 2005).

More information of Cd contamination around Mae Sot area is established in the year 2003-2004 by DPIM. They found that all soil samples collected at the upstream of Huai Mae Tao and Huai Mae Ku had Cd and Zn levels < 9 and 11-2,180 mg/kg soil, respectively. Cd amounts of samples around Zn deposit or mining area were 1-430 mg/kg soil while Zn quantities were 14-13,645 mg/kg soil which much higher than Cd and Zn concentrations at the alluvial plain or rice fields, 1-102 and 17-6,830 mg/kg soil in range (DPIM, 2006).

In addition, there is the evidence displayed that Cd pollution impacts on villagers who live within this area as measured the excessive of exposure from urinary excretion of Cd.

Witaya Swaddiwudhipong (2007) examined urine samples of 15 years old and older of 7,697 people from 12 villages and noticed that only 45.6% had Cd levels less than 2 µg/g creatinine, 4.9% were found 5-10 µg/g creatinine and 2.3% had Cd amounts over 10 µg/g creatinine which categorized upon World Health Organization (WHO) as dangerous level (Table 2.9).

Table 2.9 Classification of the exposure associated with urine Cd quantities
(Chantana Padungtod *et al*., 2006).

Concentrations (µg/g creatinine)	Exposure level
2	Environmental exposure
5	Occupational exposure
> 10	Renal damage

Furthermore, 88% of these citizens consumed locally grown rice that harvested from the contaminated areas and observed to have urinary Cd > 5 µg/g creatinine more than the residents who purchased rice from markets and other districts as shown in Table 2.10.

Table 2.10 Urinary Cd of the population surveyed classified by source of obtained rice (Witaya Swaddiwudhipong, 2007).

Rice-producing area	Number of surveyed	Urinary cadmium (µg/g creatinine)			
		< 2	2-4.9	5-10	> 10
Rice grown locally in contaminated areas	6,770	44.5%	47.7%	5.2%	2.6%
Rice purchased from Mae Sot markets[†]	858	52.6%	44.1%	3.0%	0.3%
Rice purchased from other districts	69	62.3%	37.7%	0.0%	0.0%
Total	7,697	45.6%	47.2%	4.9%	2.3%

* Expressed as percentage of the number surveyed; p-value < 0.01

[†] Containing rice from both contaminated and uncontaminated areas

CHAPTER III

RESEARCH METHODOLOGY

3.1 Equipments and Chemicals

3.1.1 Equipment

Analytical balance: BP211D (Sartorius, Germany)

Balance: BJ1000C (Precisa instruments, Switzerland)

Deep freezer -20°C: SF-C991 (Sanyo, Japan)

Denaturing gradient gel electrophoresis system: DCode universal mutation detection system (Bio-Rad laboratory, U.S.A.)

Desiccator cabinet: AD48 (F.G.E., Thailand)

Electrophoresis system: iMyRun (Helixx technologies, Canada)

Gel imaging system: GelDoc XR (Bio-Rad laboratory, U.S.A.)

Global positioning system (GPS) device: Nüvi 310 (Garmin Ltd., U.S.A.)

High speed refrigerated centrifuge: 6500 (Kubota, Japan)

Hot air oven: UNB400 (Memmert, Germany)

Incubator: INE700 (Memmert, Germany)

Inductively coupled plasma-optical emission spectrometry (ICP-OES): Optima 2001 DV (Perkin Elmer, U.S.A.)

Laboratory fume hood (Safety lab, Thailand)

Laminar flow cabinet (Safety lab, Thailand)

Mesh: wired 2 mm pore size

Microwave: R-215 (Sharp, Japan)

pH meter: SevenEasy (Mettler Toledo, Switzerland)

Power supply: PowerPac HC (Bio-Rad laboratory, U.S.A.)

PVC pipes: 55 mm diameter, 10 cm depth

Refrigerator 4°C: SJ-D52L (Sharp, Japan)

Spectrophotometer cells: Micro self masking (Quartz) 10mm path length (Starna, U.S.A.)

Thermo cycler: GeneQ (Bioer, China)

Topographical paper map, series L7018 sheet 4742III Amphoe Mae Sot scale 1:50,000 (Royal Thai Survey Department [RTSD], Thailand)

Ultra pure water purification system: Milli-Q Academic (Millipore, France)

Ultrasonic cleaner: E 30H (Elma, Germany)

UV transilluminator: ETX-20.M (Vilber Lourmat, France)

UV-visible spectrophotometer: Helios alpha (Thermo electron corporation, England)

Vertical autoclave: SS-325 (Tomy, Japan)

Vortex mixer: VX100 (Labnet, U.S.A.)

Water bath: WB 22 (Memmert, Germany)

Zip lock plastic bags

3.1.2 Chemicals

ϕX174 DNA-*Hae*III digest (New England Biolabs Inc., U.S.A.)

1 Kb DNA ladder (Vivantis, Malaysia)

Acetic acid, glacial (CH_3COOH) (BDH, England)

Agarose gel (Vivantis, Malaysia)

*Bca*Best^{TM} RNA PCR kit Ver.1.1 (Takara, Japan)

Cetyl trimethylammonium bromide (CTAB) (Biobasic, Canada)

Chloroform/isoamyl alcohol, 24:1 (Biobasic, Canada)

DCode electrophoresis reagent kit for DGGE contains 40% acrylamide/bis solution (37.5:1), urea, formamide (deionized), 50× TAE buffer, ethidium bromide (EtBr, 10 mg/ml), 2× gel loading dye, dye solution, tetramethylethylenediamine (TEMED) and ammonium persulfate (Bio-Rad laboratory, U.S.A.)

Diethylpyrocarbonate (DEPC) (Biobasic, Canada)

di-Potassium hydrogen orthophosphate (K_2HPO_4) (Ajax Finechem, New Zealand)

DNA Loading dye, 6x (Fermentas, Lithuania)

Ethanol (EtOH) (Merck, Germany)

Filter papers, no.1 (Whatman, England)

Glass beads, acid-washed, 150-212 μm diameter (Sigma-Aldrich, Germany)

Hydrogen peroxide, 3% (H_2O_2) (Vidhyasom, Thailand)

Hydrochloric acid, 37% (HCl) (J.T. Baker, U.S.A.)

Lamda DNA/*Hind*III marker (Fermentas, Lithuania)

Membrane filter, 0.2 μm GTBP (Millipore, Ireland)

NucleoSpin® RNA Clean-up kit (Macherey-Nagel, Germany)

Phenol/chloroform/isoamyl alcohol, 25:24:1, pH 8.0 (Sigma-Aldrich, Germany)

Polyethylene glycol, 6000 (PEG) (Ajax Finechem, New Zealand)

Potassium dihydrogen orthophosphate (KH_2PO_4) (Ajax Finechem, New Zealand)

QIAquick® PCR purification kit (Qiagen, Germany)

RQ1 RNase-free DNase (Promega, U.S.A.)

Sodium acetate (NaOAc; $NaC_2H_3O_2$) (Sigma-Aldrich, Germany)

Sodium chloride (NaCl) (Merck, Germany)

Sodium hydroxide anhydrous pellets (NaOH) (Carlo Erba Reagenti, Italy)

Takara Ex *Taq*™ (Takara, Japan)

Tris acetate EDTA (TAE) buffer, 10x (Promega, U.S.A.)

3.2 Experimental Methods

3.2.1 Sampling sites

Because Cd contamination problem occured around Mae Sot district, Tak province as shown in studies of IWMI and DPIM so that the sampling site of this research were determined within this area (Figure 3.1) according to the creeks located around Zn mine i.e. Huai Mae Tao Ngae Sai, Huai Mae Tao, Huai Nong Khiao and Huai Mae Ku by using the topographical paper map from RTSD and each collecting point was specified the universal transverse mercator (UTM) coordinate with global positioning system (GPS) and recorded via Garmin Nüvi 310.

The major sampling points were collected along Huai Mae Tao due to it is a creek that flows through Zn mining area. The others were sampled from 3 different creeks which irrelevant to the mine for using as references. These samples were riverbank soils since some interference might happen from anthropogenic effects such as the use of pesticide and superphosphate fertilizers in cultivated area.
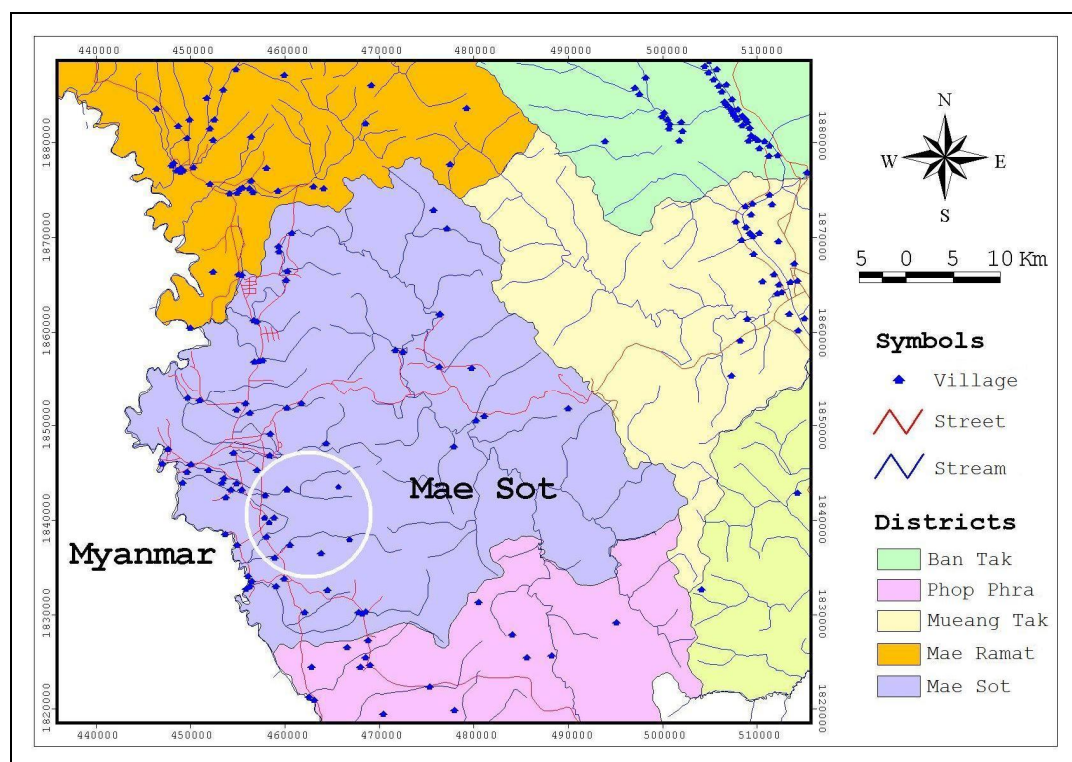
Figure 3.1 Map of study area at Mae Sot district, Tak province (modified from Department of Provincial Administration [DOPA], 2002).

3.2.2 Sample collection

The top layer soils (0-10 cm) were taken in May 2008 by 55 mm diameter soil cores (Roongnapa Tongarun *et al.*, 2008). Each sampling point, 1 kg-soil sample were collected in triplicate, tightly sealed in plastic bags and stored at -20°C until the experiment began (Wang *et al.*, 2007).

3.2.3 Sample preparation

The individual soil sample was weighed to 200 g and kept at -20°C for molecular analysis, the other was air dried in clean room then removed plant debris and gravel with a 2 mm wire mesh for soil properties testing (Roongnapa Tongarun *et al.*, 2008).

3.2.4 Soil characteristics investigation

The parameters and methods that used to study soil sample features are displayed in Table 3.1 (Department of Land Development [LDD], 2004).

Table 3.1 Parameters and techniques using for determining soil properties in samples.

Parameter	Analytical method
Soil texture	Hydrometer method
pH	Electrometric method (soil : water; 1:1)
Organic matter	Walkley and Black method
Cation exchange capacity	Ammonium saturation method

3.2.5 Total heavy metals examination

Cadmium (Cd) and zinc (Zn) were extracted from soil samples with boiling *aqua regia* [mixture solution of nitric acid (concentrated) and hydrochloric acid (concentrated)] and detected by ICP-OES (Amacher, 1996).

3.2.6 RNA extraction [adapted from methods described by Griffiths *et al*. (2004)]

Remove RNase enzyme by treating all solutions and plastic ware (except the EDTA and Tris-based solution that prepared with pre-treated water due to the reaction between DEPC and amines) with 0.1% DEPC at 37°C for 2 hours and autoclave at 125°C for 30 minutes to inactivate the DEPC residue. Bake glassware at 180°C, 8 hours, use RNase/DNase free filter tips, microcentrifuge tubes and clean lab space with 3% hydrogen peroxide solution (H_2O_2).

Nucleic acids were extracted from 0.5 g (wet weight) of soil sample using 2-ml tube containing 0.5 g glass beads (Brodie, 2008). Extractiond were performed by the addition of 0.5 ml 5% CTAB/phosphate buffer and 0.5 ml phenol/chloroform/isoamyl alcohol and tightly close lids. Samples were lysed for 10 minutes at a vortex maximum speed (Mo Bio Laboratories, Inc., 2006) by placing tubes horizontally on a flat-bed vortex pad with adhesive tape, and the aqueous phase containing DNA/RNA were separated by centrifugation at 16,000 × g for 5 minutes, 4°C. Then, this layer was transferred into a new microfuge tube and extracted twice by mixing with an equal volume of chloroform/isoamyl alcohol followed by centrifugation at 16,000 × g for 5 minutes, 4°C. The total nucleic acids were precipitated with 2 volumes of 30% (weight/volume) PEG 6000 solution at 4°C, overnight, followed by centrifugation at 16,000 × g for 10 minutes, 4°C. A half of supernatant was removed and 0.1 volumes of 3M NaOAc was added then nucleic acids were pelleted with 2.5 volumes of absolute chilled EtOH at -20°C for 30 minutes followed by centrifugation at 16,000 × g for 20 minutes, 4°C. The pellets were washed with 200 μl 70% (volume/volume) chilled EtOH, air dried in room temperature for 20 minutes and resuspended in 20 μl of RNase-free deionized water (heated at 65°C for 10 minutes may require to assist dissolving) then store at -20°C.

3.2.7 Agarose gel electrophoresis of nucleic acids

Total nucleic acid extracts of all soil samples were checked by using agarose gel electrophoresis method. First, electrophoresis set was cleaned with 3% H_2O_2 for 30 minutes and rinsed off with DEPC treated water. Then, 5 μl of crude extracts were mixed with 1 μl 6× loading dye, loaded into 1% agarose gel with 0.5× TAE buffer and run at 100V for 40 minutes. Gel was stained with 0.5 μg/ml EtBr for 20 minutes, destain in deionized water for 10 minutes and visualized upon UV transilluminator.

3.2.8 Purification of total RNA (Griffiths *et al*., 2004)

Nucleic acid of samples should be cleaned enough for further PCR based technique since contaminants or extraction chemicals may interfere this important step. DNA was removed by using RQ1 RNase-free DNase according to the manufacturer's instruction then total RNA was purified using NucleoSpin® RNA Clean-up kit as mentioned in user manual.

3.2.9 Reverse transcription PCR (RT-PCR) of partial 16S rRNA

Total RNA (0.5 μg) was reverse transcribed into complementary DNA (cDNA) using reverse transcriptase enzyme in *Bca*Best™ RNA PCR kit Ver.1.1 (Hoshino and Matsumoto, 2007). The cDNA was amplified with GC-341F (5'-<u>CgC CCg CCg CgC gCg gCg ggC ggg gCg ggg gCA Cgg ggg g</u>CC TAC ggg Agg CAg CAg-3') and 907R (5'-CCg TCA ATT CMT TTg AgT TT-3') primers (Muyzer *et al*., 2004) by using Takara Ex *Taq* kit. This pair of primers was specific for V3-V5 region of 16S rRNA for general Eubacteria and generated 566 bp PCR products.

Each PCR reaction of 50 μl final volume containing 5 μl of cDNA (0.1μg), 1 μl of 1.25 U/μl Takara Ex *Taq*, 5 μl of 10× Ex *Taq* buffer, 4 μl of dNTP mixture (2.5mM each), 1 μl of each primer (10 μM) and 33 μl of ultra pure water. PCR condition was run in accordance with modified method described by Muyzer *et al*. (2004) using touch-down and hot start technique as following :-

1 cycle of 94°C, 5 minutes; 80°C, 1 minute (add *Taq* enzyme); 65°C, 1 minute and 72°C, 3 minutes. 10 cycles of 94°C, 1 minute; 64°C, 1 minute (lowering 1°C every cycle) and 72°C, 3 minutes. 10 cycles of 94°C, 1 minute; 55°C, 1 minute and 72°C, 3 minutes. 1 cycle of 94°C, 1 minute; 55°C, 1 minute and 72°C, 10 minutes.

The amplified products were analyzed in 1.5% agarose gel electrophoresis (100V, 40 minutes) and used 1 Kb DNA ladder accompanied with Lamda DNA/*Hind*III marker to determine the product size.

3.2.10 DGGE determination of the partial 16S rRNA amplified from soil bacteria (Han, 2004; Muyzer *et al*., 2004; Green, 2005)

PCR product mixture from each sample was loaded into 6% polyacrylamide gradient gel (16 x 16 cm, 1 mm thick) with 35% to 70% denaturants. The gel was run at 100V for 18 hours in 1× TAE buffer at 60°C, then stained with EtBr and photographed with gel photo system.

3.2.11 Bacterial pattern analysis

The image of DGGE gel was manually defined and analyzed with Quantity One software version 4.6.1 (Bio-Rad laboratory, U.S.A.) by setting background subtraction at 10% by rolling disk method (Dilly *et al*., 2004) and specified the minimum band density > 0.05%, then position, intensity and number of bands were determined (Edenborn and Sexstone, 2007). Similarities of all DGGE lanes were generated using unweighted pair group method using arithmetic averages (UPGAMA) and expressed as a dendrogram.

3.2.12 Soil bacterial diversity investigation

Data derived from DGGE profile was used for calculating species richness (*R*), species evenness (*E*) and Shannon-Wiener index (*H*) of dominant bands by the amount of bands in each gel lane was determined as *R* (Ampe *et al*., 2000) while species evenness computed as follows (Dilly *et al*., 2004):

$$E = H/\log R$$

Shannon-Wiener index was calculated based on the equation:

$$H = -\Sigma(P_i)(\log P_i)$$

where $P_i = n_i/N$, n_i is the peak intensity of band *i* and N is the sum of peak intensities within DGGE gel (Briones *et al*., 2008).

3.2.13 Extraction of DGGE bands from gel

Dominant bands were excised from the DGGE gel with 70% EtOH-sterile razor blade. Each band was placed into a microfuge tube containing 25 μl of deionized water and incubated at 4°C, overnight (Eyers, Agathos and Fantroussi, 2004).

3.2.14 DGGE fragments sequencing

Diffused DNA that derived from the elution step as mentioned above was used as a template for re-amplifying 16S rRNA with the same primer pair as stated in 3.2.9 except forward primer (GC-341F) is applied without GC clamp.

PCR products were purified by using QIAquick® PCR purification kit and directly sequenced by Macrogen Inc., Korea (http://dna.macrogen.com). Sequencing results were compared to find related bacterial strains by searching in National Center for Biotechnology Information (NCBI) database with Basic local alignment search tool (Blastn) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and deposited in this database.

3.2.15 Phylogenetic tree determination

Alignment of similar sequences was done by using CLUSTAL X 1.83 (Thompson *et al.*, 1997) and phylogenetic tree was constructed by phylogeny inference package; Phylip program version 3.6 (Felsenstein, 2004) and molecular evolutionary genetics analysis (MEGA) software version 4.0 (Tamura, Dudley, Nei and Kumar, 2007) with the neighbor-joining (NJ) algorithm.

3.2.16 Statistical analysis

Total heavy metals concentrations, soil characteristics and Shannon-Wiener indices were analyzed by principle component analysis (PCA) with SPSS software for Windows version 17.0 and Pearson's correlation coefficients were achieved by R program for Windows version R-2.9.0 (R development core team, 2009).

CHAPTER **IV**

RESULTS AND DISCUSSION

4.1 Sampling Points

According to the topographical paper map, 4 interesting creeks in Mae Sot district were chosen.

4.1.1 Huai Mae Tao Ngae Sai locates in the north-eastern highland of Zn mine

4.1.2 Huai Mae Tao, a creek in the north-western part, flows through the mine and discharges to alluvial plain at Ban Mae Tao Mai

4.1.3 Huai Nong Khiao places in the south-western highland of Zn mine and irrelevant to mining because it is on the other side of the hill.

4.1.4 Huai Mae Ku situates along the south boundary of the mine and flows through Ban Mae Ku Nuea.

The study area was 36 km^2 which located in the area of Ban Pha Te on the north and Huai Nong Khiao on the south sides where the east and west parts was in Ban Tham Suea and Ban Mae Tao Mai, respectively.

Position of 8 soil sampling sites were specified with GPS and shown in Table 4.1 and Figure 4.1.

MT1 was collected from Huai Mae Tao Ngae Sai near Ban Tham Suea primary school operated by border patrol police where MT2 to MT5 were sampling along Huai Mae Tao. The control soil samples which irrelevant to Zn mining area were R1 taken from cornfield in Huai Nong Khiao, and R2-R3 collected from Huai Mae Ku near Ban Mae Ku Nuea and Ban Mae Ku Noi, respectively.

Table 4.1 GPS coordinates of all soil samples at 4 different creeks.

Creek	Sample name	UTM position	
		X UTM	Y UTM
Huai Mae Tao Ngae Sai	MT1	465447.89	1843916.34
Huai Mae Tao	MT2	464619.52	1842700.85
	MT3	461926.60	1842438.15
	MT4	460086.04	1843409.48
	MT5	457339.66	1843061.42
Huai Nong Khiao	R1	460993.37	1840712.88
Huai Mae Ku	R2	460276.82	1840885.67
	R3	457400.70	1841002.46

Figure 4.1 Map of MT1-MT5 and R1-R3 soil sampling points investigated in this research.

4.2 Soil Characteristics

Soil samples characteristics including pH, organic matter (OM), cation exchange capacity (CEC) and texture were shown in Table 4.2 and 4.3, respectively.

According to the standard mentioned in Department of Land Development [LDD] (2004), the MT1 to MT3 samples were slightly acid while MT4 to R3 samples were neutral. For organic matter content (OM), which were in range of 4.03 to 7.98%, could be categorized MT5 and R2 soils into high organic level while MT1 to MT4, R1 and R3 were very high organic level soils. About CEC, MT4, MT5 and R2 had slightly low whereas the rest were moderately high. In case of soil texture, only MT2 was medium-texture (loam) but the rest were coarse-texture soil (sandy loam).

Table 4.2 Chemical characteristics of soil samples.

Sample	pH	OM (%)	CEC (cmol/kg)
MT1	6.3	4.89	10.10
MT2	6.5	7.98	13.41
MT3	6.5	5.81	12.91
MT4	6.8	5.97	8.60
MT5	6.8	4.03	6.99
R1	6.7	6.32	14.71
R2	6.7	4.31	7.39
R3	6.8	5.39	14.57

Table 4.3 Physical properties of soil samples.

Sample	Sand (%)	Silt (%)	Clay (%)	Textural class
MT1	57.7	26.9	15.4	Sandy Loam
MT2	33.8	48.9	17.3	Loam
MT3	64.8	22.1	13.1	Sandy Loam
MT4	59.8	29.8	10.4	Sandy Loam
MT5	72.4	19.4	8.2	Sandy Loam
R1	52.8	30.6	16.6	Sandy Loam
R2	71.4	18.4	10.2	Sandy Loam
R3	54.2	30.8	15.0	Sandy Loam

From soil characteristics, it is shown that this area is a new soil with high organic matter content. This could be the result of intensively farming in this area.

4.3 Total Cadmium (Cd) and Zinc (Zn) Concentrations

The total Cd and Zn concentration in eight soil samples detected by ICP-OES were shown in Table 4.4.

Total Zn and Cd contents in all samples were in range of 27.05 to 1,998.00 and 1.10 to 34.95 mg/kg soil, respectively and the average Cd:Zn ratios from Huai Mae Tao Ngae Sai, Huai Mae Tao, Huai Nong Khiao and Huai Mae Ku are 0.03, 0.02, 0.04 and 0.04, respectively which were similar to the trend as stated by Parada Maneewong (2005). Since Zn and Cd can be found in general soil at the maximum amount of 770 and 0.5 mg/kg, respectively (Emsley, 2001; ICdA, 2009) then the maximum general ratio would be 6×10^{-4}. Therefore, the Cd:Zn ratio of all creeks were higher than the maximum normal ratio as stated by ICdA (2009) even in the area that supposed to be control sites. For this reason, it could be the nature of this area to have high Cd concentration.

In MT1 sample or Huai Mae Tao Ngae Sai site, total Zn amount was similar to the results investigated by the Department of Primary Industries and Mines [DPIM] (2006). The concentration was still in range of the maximum concentration defined by European Economic Community (EEC) for soils (300 mg/kg soil) (Simmons *et al.*, 2005). In comparison, almost every sample in Huai Mae Tao and Huai Mae Ku (except MT2) had total Zn quantities above the EEC standard level which this trend also found within the report as mentioned by DPIM (2006). The sample R1, collected from Huai Nong Khiao not only contained the lowest total Zn concentration but also lower than the Thai investigation level (70 mg/kg soil) (Zarcinas *et al.*, 2004). It might because the R1 sampling point was located in the area that not related to Doi Padaeng Zn deposit and suitable to be referred to as a control site.

Concentrate on Cd quantities, main factor for this research, the sample that collected from Huai Mae Tao Ngae Sai (MT1) had Cd amount almost equal to the EU MP safety level (3.0 mg/kg soil) as also reported by DPIM (2006) that soil sample collected from this creek had Cd quantity only 1 mg/kg soil which was not dangerous. It might due to the sampling site was not related to the mining area. The data was similar to the sample from Huai Nong Khiao (R1) which found Cd level of 1.10 mg/kg soil.

It was obviously noticed in Huai Mae Tao, the creek that runs through Zn mine, that the lower Cd amount was detected in the upstream soil sample (MT2) and increased in the downstream samples (MT3 to MT5) of lowland in the west. This expanded pattern was also noticed in the work of DPIM (2006). Cd accumulation ratio was calculated. MT3 had twice higher Cd than MT2 whereas the expansion rate of MT3 to MT5 was 13% per site. There should be some human activities that caused the high accumulation in MT3

Huai Mae Ku samples contained high Cd concentrations especially R2 soil which is collected beside a dyke for the community irrigation. This might suggest that the Cd accumulation was the result of water retention in the area as indicated by Simmons *et al*. (2005) who found the deposition of sediments through irrigation waters led to the loading of Cd. At R3 site or the downstream of this creek found Cd quantity around 5 times lower than the upstream.

From above data, considering in each creek, the total Cd levels along Huai Mae Tao and Huai Mae Ku were greater than those from Huai Mae Tao Ngae Sai and Huai Nong Khiao and also higher than the maximum EEC level for Cd but they were in the range of the soil quality standard for habitat and agriculture, Thailand (37 mg/kg soil) (Pollution Control Department [PCD], 2004). Therefore, the area is considered to be safe for agriculture and living.

Table 4.4 Total amounts of Cd and Zn in soil samples from different creeks.

Huai Mae Tao Ngae Sai		
Sample	Total Cd (mg/kg soil)	Total Zn (mg/kg soil)
MT1	3.05	97.45
Huai Mae Tao		
Sample	Total Cd (mg/kg soil)	Total Zn (mg/kg soil)
MT2	8.45	193.05
MT3	16.85	1,998.00
MT4	18.80	954.00
MT5	22.50	1,163.00
Huai Nong Khiao		
Sample	Total Cd (mg/kg soil)	Total Zn (mg/kg soil)
R1	1.10	27.05
Huai Mae Ku		
Sample	Total Cd (mg/kg soil)	Total Zn (mg/kg soil)
R2	34.95	789.00
R3	7.55	316.05

4.4 RNA Extraction from Soil Samples

The crude nucleic acids from soil samples were extracted with the method as previously described by Griffiths *et al*. (2004). Humic substance contaminated within samples was noticed in brown color as shown in Figure 4.2.
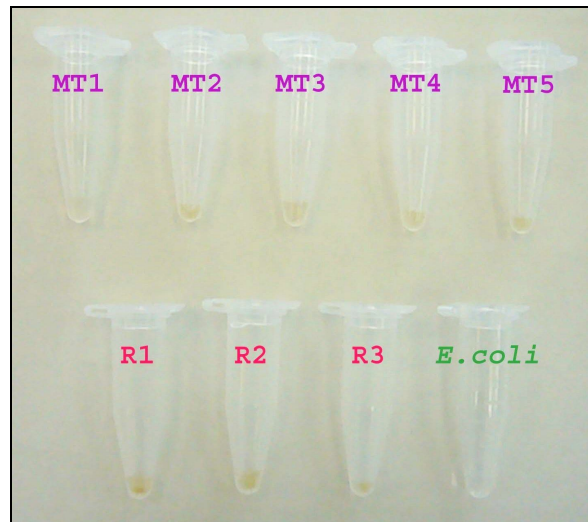
Figure 4.2 Crude extracts of nucleic acids derived from eight soil samples.

After staining the total nucleic extracts of all samples with ethidium bromide (EtBr), both DNA and RNA, 23S-16S rRNA, were observed in different band positions and intensities as shown in Figure 4.3.
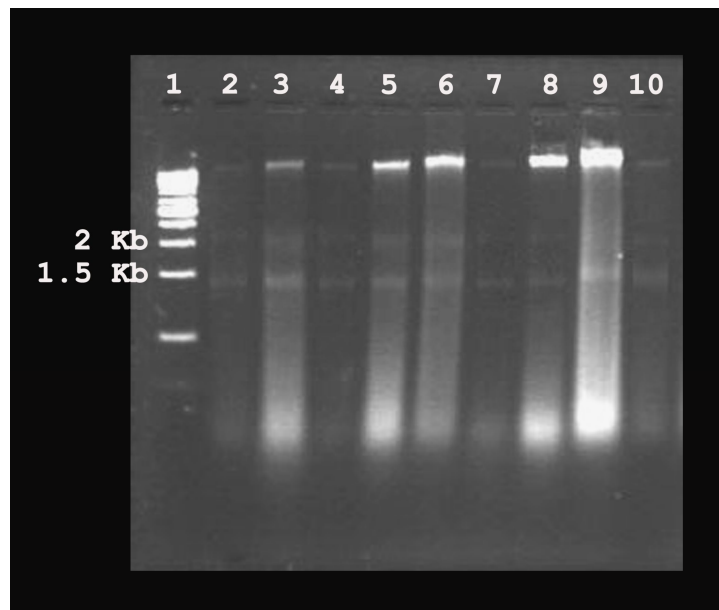
Figure 4.3 Total nucleic acids analyzed on 1% agarose gel and stained with EtBr.

Lane 1, 1 Kb DNA ladder; lane 2 to 9, total nucleic acids extracted from 0.5 g

of MT1 to R3 soil samples; lane 10, *E. coli* JM109 (positive control).

4.5 RT-PCR Amplification of 16S rRNA

After DNase treatment, to get rid of DNA, the reverse transcription reaction was performed to obtain cDNA from RNA in the sample. To confirm the absence of DNA contamination in RNA preparation step, the same reaction of RT-PCR without reverse transcriptase; PCR-RNA were done. The 16S rRNA amplification of V3-V5 region was carried out further to multiply the cDNA template with a GC-clamp primer, reverse primer and *Taq* enzyme, yielding the estimated 566 bp PCR product size as shown in Figure 4.4 and 4.5 (Muyzer *et al.*, 2004).
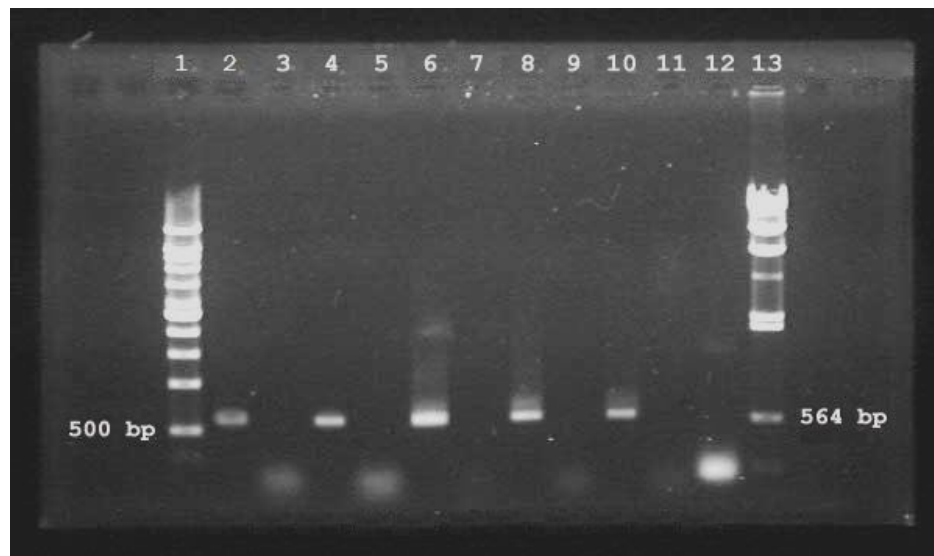
Figure 4.4 RT-PCR amplification products of 16S rRNA derived from MT1 to MT5

samples. Lane 1, 1 Kb DNA ladder; lane 2, MT1; lane 3, MT1 PCR-RNA; lane

4, MT2; lane 5, MT2 PCR-RNA; lane 6, MT3; lane 7, MT3 PCR-RNA; lane 8,

MT4; lane 9, MT4 PCR-RNA; lane 10, MT5; lane 11, MT5 PCR-RNA; lane 12,

no template (negative control); lane 13, Lamda DNA/HindIII marker.
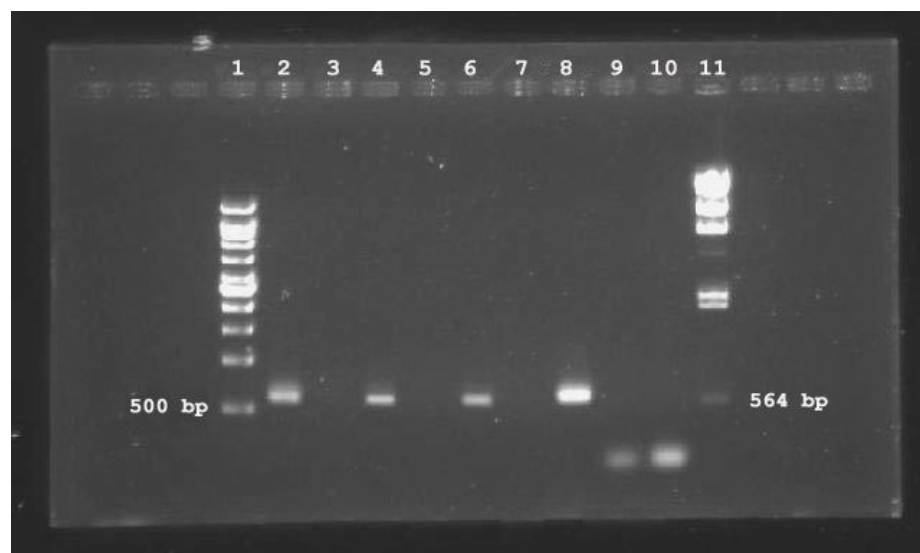
Figure 4.5 RT-PCR amplification products of 16S rRNA derived from R1 to R3 samples.

Lane 1, 1 Kb DNA ladder; lane 2, R1; lane 3, R1 PCR-RNA; lane 4, R2; lane 5,

R2 PCR-RNA; lane 6, R3; lane 7, R3 PCR-RNA; lane 8, E. coli JM109 (positive

control); lane 9,   E. coli JM109 PCR-RNA; lane 10, no template (negative

control); lane 11, Lamda DNA/HindIII marker.

4.6 Effect of Cd on the Soil Bacterial Communities Determined by DGGE

Amplification products from the eight soil samples were analyzed for the bacterial diversity by using DGGE technique. According to Figure 4.6, 36 different band positions were detected by Quantity One software. The DGGE patterns of each sample were differentiated by the presence/absence of bands as well as the changes in band intensity.

Since rRNA-based DGGE method provides more information of metabolically active bacterial members due to they transcribe more rRNA than the resting species (Teske *et al*., 1996), these generated DGGE patterns revealed the functioning bacterial composition in Cd contaminated soil samples.

It was apparently indicated that the highest Cd contained sample (34.95 mg/kg soil), R2, gave the most abundant bands with distinctive noticed intensity. Dominant bands assigned as A3, A4, A18, A19, A21, A29 and A31 were investigated further as active bacterial types. For other unique DGGE bands, A15 was found sharply in soil sample MT1 which contained Cd level 3.05 mg/kg soil and band A17 was discovered at the Cd concentration of 7.55 mg/kg soil (R3).

Considering the DGGE bands, A28 was noticed in the samples with Cd amounts 3.05 and 7.55 mg/kg soil in range (MT1 and R3) and faded away in the higher Cd levels. Band A23 and A24, the closest 2 bands, appeared strongly sharp in samples with Cd quantities 7.55 and 34.95 mg/kg (R3 and R2). In the soil contained Cd of 16.85 to 22.50 mg/kg soil (MT3, MT4 and MT5), A16 band was found in high intensity. Band A20 and A22 were detected in MT3 and MT5 which contained Cd at 16.85 and 22.50 mg/kg soil (Table 4.5).

It might conclude that band A15 could possibly be used as the indicator for Cd contamination in soil near the safety limit of 3.0 mg/kg soil and band A16 is the only dominant band that responded to Cd in the wide range of 16.85 to 22.50 mg/kg soil. These two bands could be developed further as the indicator for Cd contamination in soil.
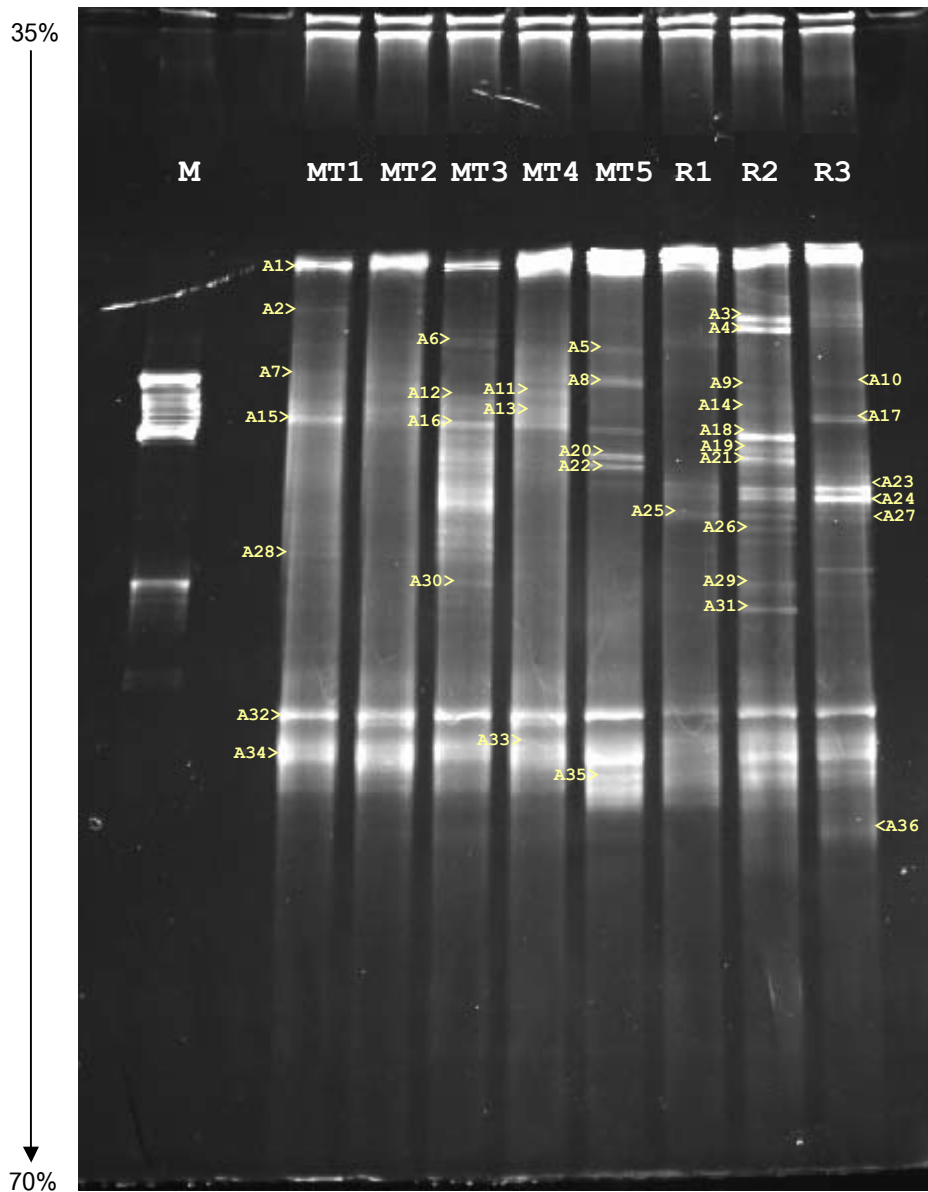
Figure 4.6 DGGE profiles of 16S rRNA derived from active bacterial communities in eight Cd-polluted soils, MT1 to R3. The sequenced bands were indicated with greater than sign and labeled with A1 to A36; M, ϕX174 DNA-*Hae*III digest.

Table 4.5 Presences of dominant DGGE bands within soil samples according to Cd
concentrations.

Sample	Total Cd (mg/kg soil)	DGGE band						
R1	1.10							A32
MT1	3.05	A15						A32
R3	7.55		A17			A23	A24	A32
MT2	8.45							A32
MT3	16.85	A16		A20	A22			A32
MT4	18.80	A16						A32
MT5	22.50	A16		A20	A22			A32
R2	34.95					A23	A24	A32

In accordance with DGGE profiles, the similarity of each lane was compared and represented in phylogenetic tree format which grouped by the cluster analysis method, unweighted pair group method using arithmetic averages; UPGAMA, shown in Figure 4.7.

From the dendrogram, bacterial populations were divided into 2 major clusters consisting of the group that contained Cd levels similar to the EU standard, MT1 and R1, and the other was the group that had greater Cd amounts which both clusters showed approximately 30% similarity.
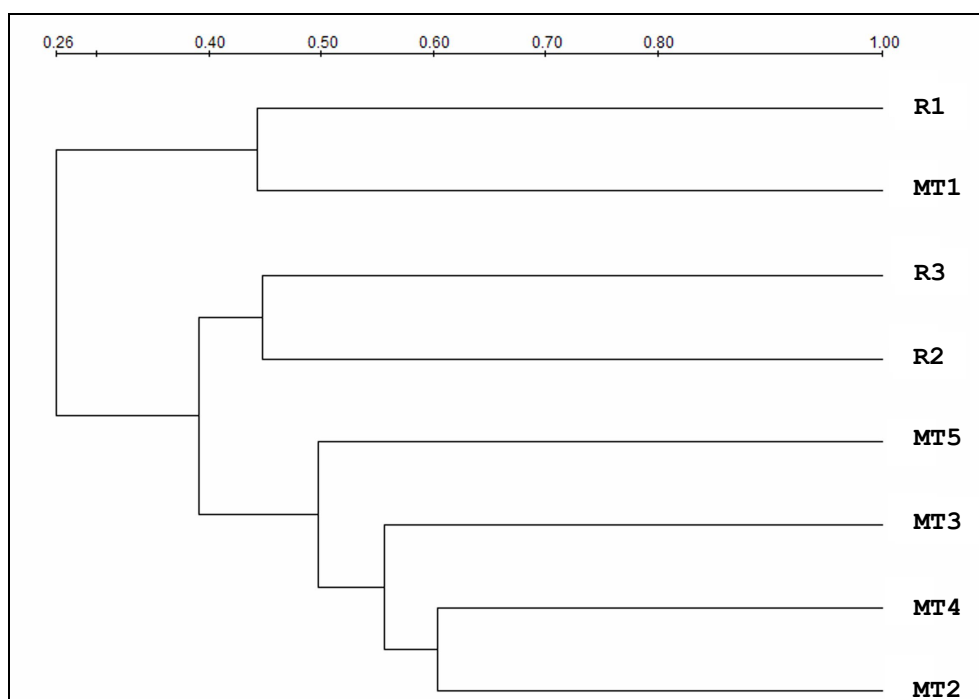
Figure 4.7 Similarity of DGGE patterns obtained from each soil sample.

Soil bacterial diversity of dominant DGGE bands was determined by using the Shannon-Wiener index as displayed in Table 4.6. It was found that R2 sample had the highest bacterial diversity and bacterial abundance whereas MT2 sample showed the lowest H index and species richness.

Table 4.6 Bacterial diversity in Cd contaminated soil samples calculated from DGGE patterns.

Sample	Total Cd (mg/kg soil)	Species richness (R)	Species evenness (E)	Shannon-Wiener index (H)
MT1	3.05	8	0.75	0.68
MT2	8.45	5	0.86	0.60
MT3	16.85	14	0.81	0.93
MT4	18.80	8	0.87	0.78
MT5	22.50	9	0.91	0.87
R1	1.10	8	0.70	0.64
R2	34.95	18	0.94	1.18
R3	7.55	16	0.82	0.99

4.7 Relationship between Total Cd, Zn Concentrations, Soil Properties and Bacterial Diversity of Samples

By using the statistical software, the possible relationships of all factors which are pH, OM, CEC, total Cd, total Zn, *R*, *E* and *H* were evaluated by PCA analysis based on the Kaiser normalization with Eigen value higher than 1 and used varimax as the rotation method. The outcome showed similarity and difference between variables as indicated that parameters located near each other in a component plot may be related (Kanlaya Vanichbuncha, 2009).

The Pearson correlation coefficient (*r*) was also calculated to expand the related information. This coefficient reflected linear relationship of two variables and the association depended on the number, ranged from +1 to -1, which displayed both direction and magnitude.

The direction of this coefficient expressed in positive or negative way and this explained how the relationship was. If the correlation was positive, when a variable increased the other also increased. If the correlation was negative, when one increased the other decreased. The strength of the correlation or magnitude was measured on the closer of +1 or -1, the stronger of the relation.

From PCA analysis of all factors, it was extracted into 2 principal components with 71.65% of total variation in the data matrix was found (Figure 4.8). The first component composed of pH, heavy metals and bacterial diversity (*R*, *E*, *H*) which had 57.82% of variance while OM and CEC were categorized into component 2 with 13.84% of variation.

Generally, soil pH had influence on heavy metal compounds in case of their availability. It was reported that adsorption and complexity between OM, humus, soil minerals and heavy metals occurred in alkaline soils whereas availability or solubility of heavy metals increased when decreasing soil pH. Moreover, form of heavy metals in soil also depended on soil alkalinity/acidity for example in high pH soil, high concentrations of Cd precipitated as hydroxides than carbonates (Hutchinson and

Meema, 1987; Vernet, 1991). Furthermore, it also affected on types, growth and activities of soil bacteria as mentioned by Arias *et al*. (2005), Zuberer and Wollum (2005) and Huebsch (2009). This information was corresponded to the PCA analysis that pH was situated in the same component and near total Zn, Cd and bacterial diversity but when considered the Pearson correlation (Table 4.7) found that pH was irrelevant to these parameters.

The relationship between Zn and Cd was found out in PCA which agreed with the fact that pure Cd didn't exist in nature but normally associated with other minerals especially Zn ores (Janya Sukornmuang, 2004) but the result that obtained from correlation test couldn't show this dependent pattern as similar to the research from Prayut Somboon (1999) who studied connection among these heavy metals in soil around Zn mining area.

According to the coordinate space in PCA plot, Cd was located at the same side and near both of species evenness and bacterial index this could be implied that Cd was the most important parameter that positively affected to diversity indices as the corresponding result also shown when considered to the Pearson correlation (r = 0.877 and 0.768, respectively) whereas there was no connection noticed between total Zn amounts in soil samples and any soil features. These might be concluded that total Cd concentrations could affect on the functioning soil bacterial communities with different interactions (Ron *et al*., 1992) because this sampling area contained domestic bacteria which might philic to this heavy metal.

Theoretically, CEC was related to the quantity of negative charged of clay particles and OM (Arias *et al*., 2005) since they provided the main adsorption parts in soil. This connection was expressed in the negative PCA plot with 13.84% of variation. Furthermore, according to the Pearson coefficient calculations found that there was the inverse (negative) relationship between total Cd concentrations and CEC in all soil samples (r = -0.749) that corresponded with the adsorption theory of clay particles to cations (Babich and Stotzky, 1978) then it was likely to suggest that this area was

already contained high Cd amounts and because of they were held in soil due to the CEC then the less leaching may occur (Nwosu *et al*., 1995) and some human activities might accelerate the Cd accumulation into the hazardous level.
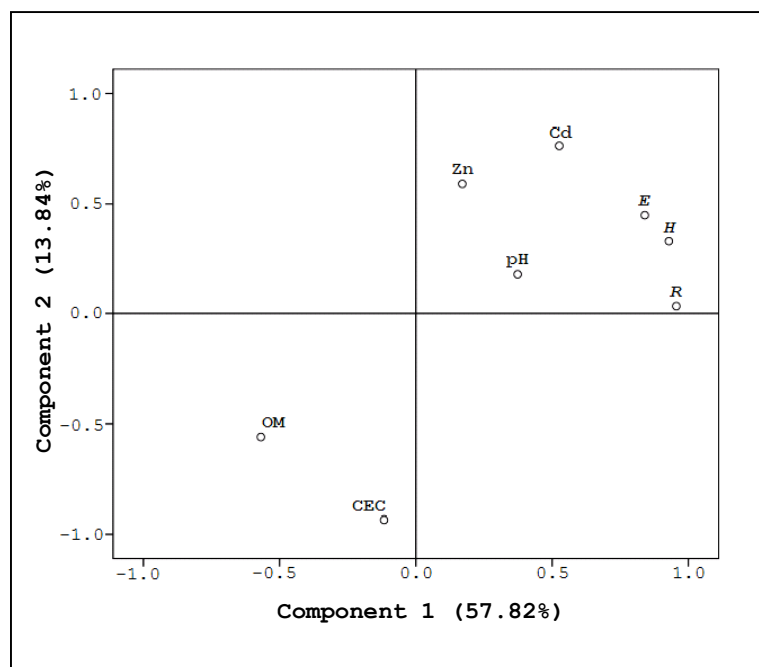
Figure 4.8 PCA plot derived from total heavy metals quantities, soil characterizations and bacterial diversity of 8 soil samples.

Table 4.7 Pearson correlation between soil properties, heavy metal concentrations and bacterial diversity in all soil samples.

	pH	OM	CEC	Cd	Zn	*R*	*E*	*H*
pH	1							
OM	-0.235	1						
CEC	-0.180	0.668	1					
Cd	0.388	-0.515	-0.749*	1				
Zn	0.129	-0.279	-0.321	0.584	1			
R	0.280	-0.535	-0.089	0.536	0.355	1		
E	0.409	-0.313	-0.686	0.877**	0.411	0.323	1	
H	0.403	-0.646	-0.377	0.768*	0.471	0.942**	0.595	1

* significant at 0.05 probability level (2-tailed).

** significant at 0.01 probability level (2-tailed).

The correspondent result of the correlation between Cd quantities and diversity index as stated above was clearly exhibited when the Cd concentrations were 8.45 to 34.95 mg/kg soil in range as illustrated in Figure 4.9. It might suggest that the active bacterial diversity increased when the Cd amounts were higher than or about 9 mg/kg soil. In case of lower Cd levels, the diversity might be affected by other soil factors especially nutrients and conditions of each sample apart from the heavy metal.
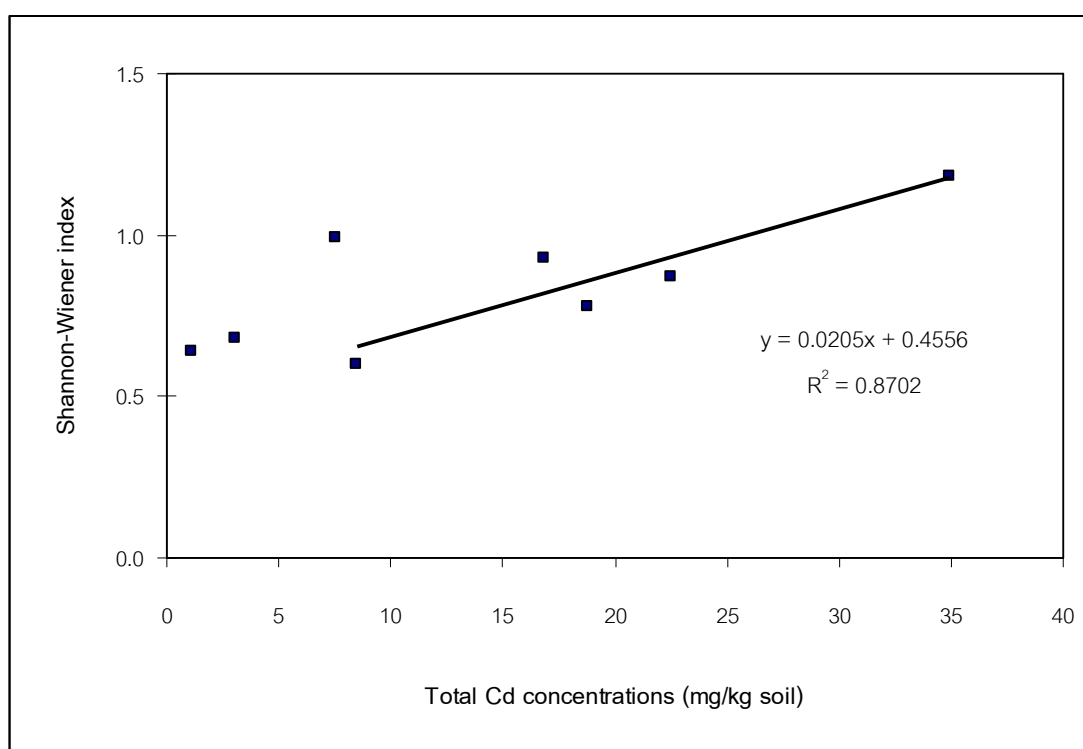
Figure 4.9 Trend of the active bacterial diversity index at Cd quantities 8.45 to 34.95 mg/kg soil.

4.8 Identification of Soil Bacteria by Sequencing

The dominant DGGE bands from the polyacrylamide gel profiles were excised, eluted, re-amplified and sequenced then the closest types of bacteria were specified by comparing the sequencing result with National Center for Biotechnology Information (NCBI) database (Table 4.8).

When aligned these sequences using CLUSTAL X and created the phylogenetic tree by Phylip program as shown in Figure 4.10 and 4.11 showed that the resulting sequences were the most similar to diverse range of bacterial classes which were the followings :-

- Alpha-proteobacteria (Lorenz *et al.*, 2006) i.e. Sphingomonas (Tangaromsuk *et al.*, 2002; Zimmer *et al.*, 2009).

- Beta-proteobacteria (Ellis *et al.*, 2003; Tsai *et al.*, 2005; Lazzaro *et al.*, 2008) i.e. Burkholderia (Macnaughton *et al.*, 1999; Lazzaro *et al.*, 2008; Zhang *et al.*, 2009), *Alcaligenes* (Ron *et al.*, 1992), *Azoarcus* (Krause *et al.*, 2006), *Denitrobacter* (Ellis *et al.*, 2003), *Ralstonia* (Goris *et al.*, 2001; Galperin, 2006; Zhang *et al.*, 2009), *Nitrosospira* (Mertoglu *et al.*, 2008) and *Variovorax* (Belimov *et al.*, 2005).

- Gamma-proteobacteria (Ellis *et al.*, 2003; Tsai *et al.*, 2005) i.e. *Klebsiella* (Ron *et al.*, 1992).

- Flavobacteria i.e. *Flavobacterium* (Ellis *et al.*, 2003; Lorenz *et al.*, 2006; Zhang *et al.*, 2008).

- Acidobacteria (Ros *et al.*, 2009)

- Firmicutes (Ellis *et al.*, 2003; Tsai *et al.*, 2005; Lorenz *et al.*, 2006).

- Actinobacteria (Ellis *et al.*, 2003; Lorenz *et al.*, 2006; Lazzaro *et al.*, 2008) i.e. *Streptomyces* (Babich and Stotzky, 1978; Bagot, Lebeau and Jezequel, 2006; Lazzaro *et al.*, 2008) and *Rhodococcus* (Desomer and Montagu, 1988; Ron *et al.*, 1992; Vig *et al.*, 2003).

According to the above data, major group of bacteria which detected within these Cd polluted soil samples was beta-proteobacteria as mentioned by Lazzaro *et al*. (2008) who examined dominant bacteria in Cd-treated forest soil by using T-RFLP. Moreover, *Rhodococcus yunnanensis* was dominant specie that found in all soil samples.

Band A15 and A16 which might be used as the alternative option for detection of Cd polluted soil as stated above, could be categorized by NCBI database to get the most closely related species as uncultured *Aquicella* sp. and *Xenophilus* sp., respectively with > 90% sequence similarity. There was not any evidence expressed that these bacterial types were detected within Cd contaminated soils but there were some researches reported that *Aquicella* was found in copper mine (Sun *et al*., 2010) and acid mine drainage (Yin *et al*., 2008) while *Acidovorax*, bacteria from family Comamonadaceae same as *Xenophilus*, was found in mercury polluted soil (Mera and Iwasaki, 2007).

Table 4.8 Closest bacterial species match of selected 16S rRNA DGGE bands in NCBI database.

Band number	Accession number	Most closely related species		Similarity (%)
		ID	Description	
A1	GQ463158	AJ519404	Uncultured *Flavobacterium* sp.	91
A2	GQ463159	AM935906	Uncultured Burkholderiaceae bacterium	98
A3	GQ463160	EU012271	Uncultured *Aquicella* sp.	94
A4	GQ463161	EU202753	Uncultured Acidobacteriales bacterium	90
A5	GQ463162	CU924477	Uncultured Alphaproteobacteria	93
A6	GQ463163	EF643435	Uncultured Acidobacteria bacterium	93
A7	GQ463164	AM936570	Uncultured Burkholderiaceae bacterium	91
A8	GQ463165	DQ337593	*Denitrobacter* sp.	99
A9	GQ463166	DQ337593	*Denitrobacter* sp.	99
A10	GQ463167	EU193049	Uncultured Coxiellaceae bacterium	94

Table 4.8 Closest bacterial species match of selected 16S rRNA DGGE bands in NCBI database (continue).

Band number	Accession number	Most closely related species		Similarity (%)
		ID	Description	
A11	GQ463168	DQ421393	*Alcaligenes* sp.	99
A12	GQ463169	FJ568934	Uncultured Acidobacteria bacterium	81
A13	GQ463170	AM936190	Uncultured *Sphingomonas* sp.	93
A14	GQ463171	AF408997	*Burkholderia* sp.	89
A15	GQ463172	EU440664	Uncultured *Aquicella* sp.	93
A16	GQ463173	AY566578	*Xenophilus* sp.	90
A17	GQ463174	CP001645	*Ralstonia pickettii*	94
A18	GQ463175	EF662862	Uncultured Firmicutes bacterium	85
A19	GQ463176	EF662862	Uncultured Firmicutes bacterium	93
A20	GQ463177	EU192991	Uncultured Acidobacteriales bacterium	93
A21	GQ463178	DQ462462	*Lysobacter niastensis*	91
A22	GQ463179	AM292622	Uncultured Actinobacterium	79
A23	GQ463180	AY161050	*Klebsiella* sp.	95
A24	GQ463181	FJ615552	*Klebsiella* sp.	90
A25	GQ463182	EU715956	Uncultured Actinobacterium	89
A26	GQ463183	EF073984	Uncultured Acidobacteria bacterium	94
A27	GQ463184	EU447637	Uncultured Gamma proteobacterium	80
A28	GQ463185	AM936094	Uncultured Alcaligenaceae bacterium	84
A29	GQ463186	EU440664	Uncultured *Aquicella* sp.	94
A30	GQ463187	EU043694	Uncultured Acidobacteria bacterium	84
A31	GQ463188	EU440664	Uncultured *Aquicella* sp.	94
A32	GQ463189	AY602219	*Rhodococcus yunnanensis*	92
A33	GQ463190	AM936044	Uncultured Nitrosomonadaceae bacterium	86
A34	GQ463191	AF011344	*Azoarcus* sp.	88
A35	GQ463192	AY192288	Uncultured Actinobacterium	95
A36	GQ463193	AJ746116	*Variovorax* sp.	97
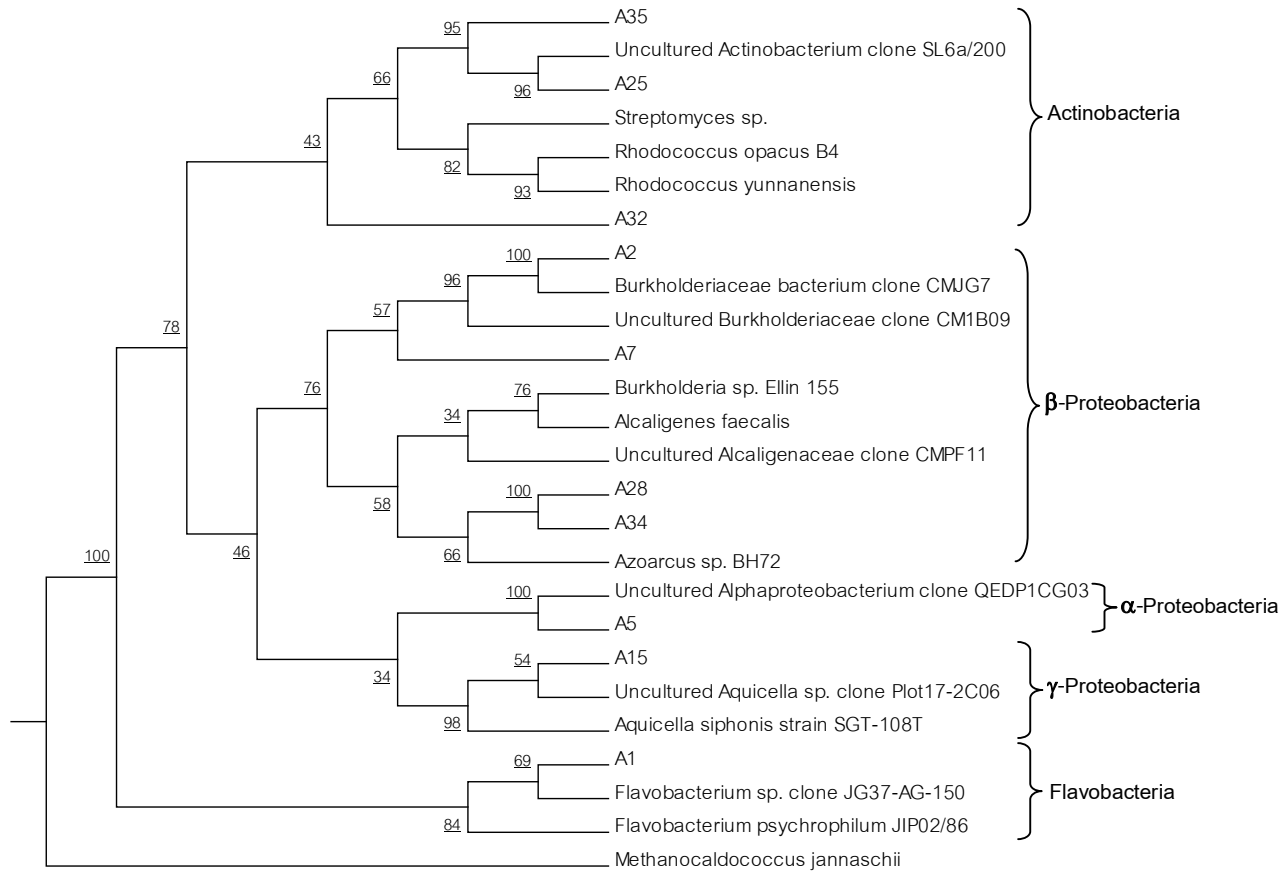
Figure 4.10 Phylogenetic tree of the dominant DGGE bands derived from MT1 and R1

soil samples generated by neighbor-joining (NJ) algorithm.
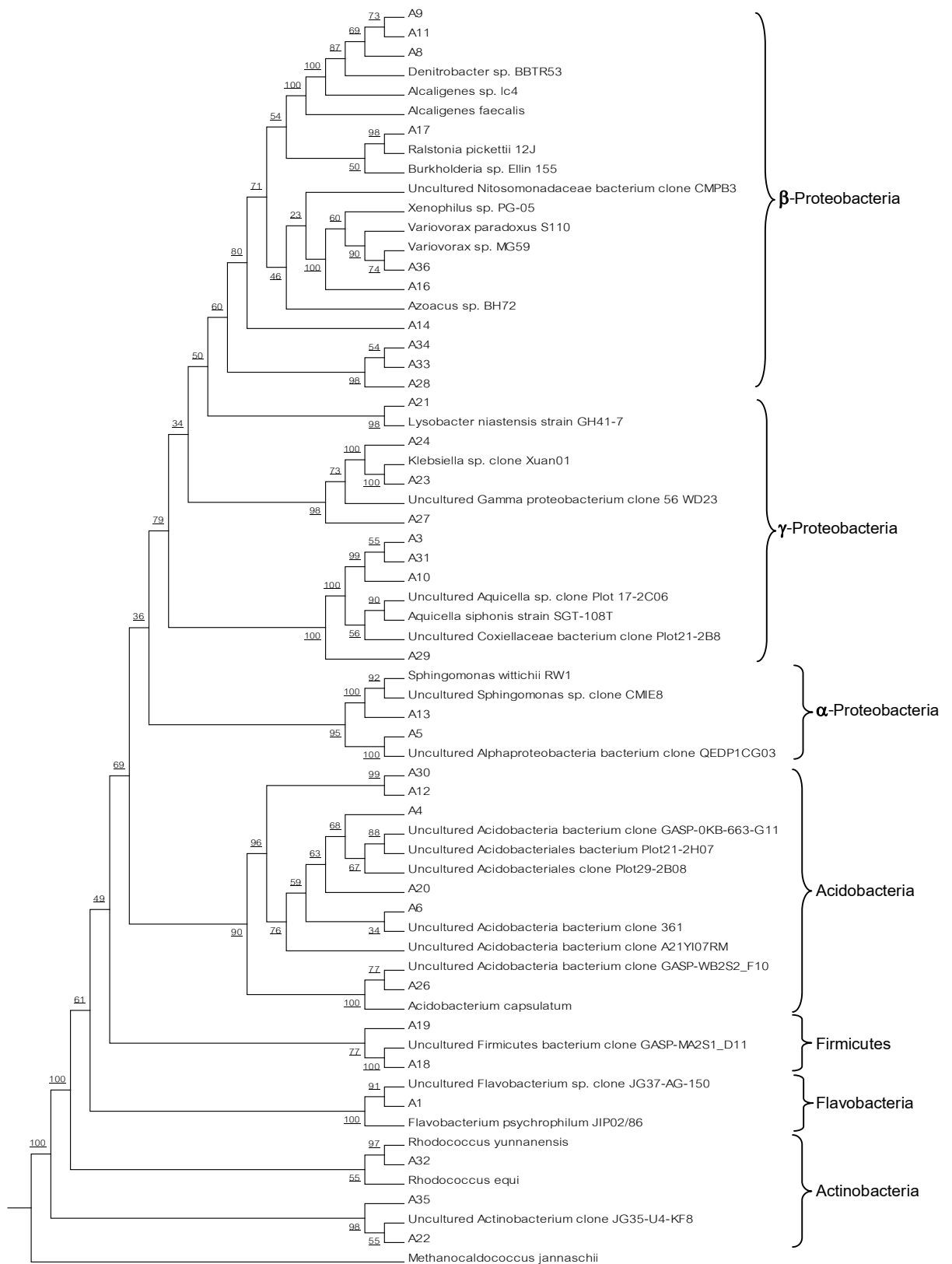
Figure 4.11 Phylogenetic tree of the dominant DGGE bands derived from MT2 to MT5 and R2 to R3 soil samples generated by NJ algorithm.

CHAPTER **V**

CONCLUSIONS AND RECOMMENDATION

5.1 Conclusions

The objective of this research was to investigate soil characteristics and determine bacterial diversity which had been influenced by Cd in a real situation of polluted soils using molecular method. From the results, it could be summarized as following

5.1.1 The soil properties of each sample were not distinctly different in both chemical and physical characteristics due to the fact that the sampling sites were in the same area or within 36 km^2 of Mae Sot district.

5.1.2 Considering heavy metals amounts, total Cd in all soil samples were not higher than the PCD soil quality standard for habitat and agriculture (the Zn standard was not stated) but when compared with the European standard, both Zn and Cd of the downstream samples in Huai Mae Tao and all samples of Huai Mae Ku were higher than the acceptable level.

5.1.3 From statistical analysis of all soil samples, total Cd and CEC had negative correlation (r = -0.749; at 0.05 level) while no relationship occur in other factors. It might because the adsorption theory between negative charged of OM and clay minerals with heavy metal. In case of total Zn, it was irrelevant to any other soil parameters.

5.1.4 The similarity of DGGE profiles in soil samples could be classified into 2 major groups composting of a group including the total Cd concentrations that were not exceeded of EEC level (MT1 and R1) and a group with the total Cd quantities that were above the standard.

5.1.5 The maximum number of Shannon-Wiener diversity index was found in the highest Cd contaminated sample and also showing that the active bacterial communities were influenced by total Cd amounts as displayed as the positive coefficient ($r = 0.768$; at 0.05 level).

5.1.6 After specified the 36 distinctive bands which detected in DGGE gel with NCBI database, the majority of bacteria belong to phylum Proteobacteria and the remainder were Bacteroidetes, Acidobacteria, Actinobacteria and Firmicutes. Additionally, *Rhodococcus yunnanensis* was a dominant bacterium that found in every soil sample.

5.1.7 There were 2 dominant DGGE bands that might change since the Cd quantities. Band A15, identified as uncultured *Aquicella* sp., appeared when Cd levels were not greater than 3.0 mg/kg soil and band A16, *Xenophilus* sp., occurred in the range of 16.85 to 22.50 mg/kg Cd-polluted soil samples.

5.2 Recommendation

5.2.1 Soil samples should be collected with various in duration to study the temporal analysis which the information might clearly show the alteration of bacterial profiles that responded with Cd contamination during time.

5.2.2 There are some parameters for example soil nutrients, mineral composition and available form of heavy metals were not detected in this research. These factors should provide basic data to understand more about the study area.

5.2.3 The results from this study displayed that there were many strains of bacteria which resistant to Cd pollution in high levels but the further study should focus on Cd-resistant genes for applying these knowledge to construct a bioindicator and use it to monitor Cd quantities within soil.

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APPENDICES

APPENDIX A

SOIL SAMPLING SITES

A-1 MT1 sampling site

A-2 MT2 sampling site

A-3 MT3 sampling site

A-4 MT4 sampling site

A-5 MT5 sampling site

A-6 R1 sampling site

A-7 R2 sampling site

A-8 R3 sampling site

APPENDIX B

SOLUTION PREPARATION

B-1 0.1% Diethylpyrocarbonate (DEPC); 1 L

Add 1 ml of DEPC to 1 L ultra pure water. Shake vigorously and place in a fume hood for 30 minutes.

B-2 5% CTAB/phosphate buffer; 200 ml

Mix 100 ml of 10% CTAB in 0.7M NaCl with 100 ml of 240 mM potassium phosphate buffer (pH 8.0). Autoclave at 121°C, 20 minutes.

B-3 30% Polyethylene glycol 6000 (PEG); 100 ml

Dissolve 30 g of PEG 6000 in 100 ml 1.6M NaCl. Filter through 0.2 μm membrane filter or autoclave at 121°C, 20 minutes.

B-4 70% Ethanol; 100 ml

Adjust 70 ml of EtOH to 100 ml with ultra pure water and chilled in -20°C.

B-5 3M Sodium acetate (NaOAc); 100 ml

Dissolve 24.609 g of NaOAc in 80 ml ultra pure water. Adjust pH to 5.2 with acetic acid and bring volume to 100 ml.

B-6 1% Agarose gel in 0.5x TAE buffer; 100 ml

Dissolve 1 g of agarose gel in 100 ml 0.5x TAE buffer and heat until it absolutely melt.

B-7 0.5 μg/ml Ethidium bromide (EtBr); 100 ml

Add 5 μl 10 mg/ml EtBr in 100 ml ultra pure water and mix. Keep in dark place.

B-8 1.5% Agarose gel in 0.5x TAE buffer; 100 ml

Dissolve 1.5 g of agarose gel in 100 ml 0.5x TAE buffer and heat until it absolutely melt.

B-9 10% Ammonium persulfate (APS); 1 ml

Dissolve 0.1 g of APS in 1 ml ultra pure water and mix. Aliquot and store at -20°C for a week.

B-10 6% polyacrylamide gel at 0% denaturant solution; 100 ml

Mix 15 ml 40% acrylamide/bis (37.5:1), 2 ml 50x TAE buffer and 83 ml ultra pure water. Filter through filter paper no.1 and degas for 15 minutes with ultrasonic machine. Store at 4°C in brown bottle for a month.

B-11 6% polyacrylamide gel at 80% denaturant solution

Mix 15 ml 40% acrylamide/bis (37.5:1), 2 ml 50x TAE buffer, 32 ml formamide (deionized), 33.6 ml urea and 17.4 ml ultra pure water. Filter through filter paper no.1 and degas for 15 minutes with ultrasonic machine. Store at 4°C in brown bottle for a month.

B-12 Polyacrylamide gel with 35% denaturants solution; 14 ml

Mix 7.875 ml of 6% polyacrylamide gel at 0% denaturant solution with 6.125 ml of 6% polyacrylamide gel at 80% denaturant solution. Then, add 10 μl TEMED and 100 μl of 10% APS.

B-13 Polyacrylamide gel with 70% denaturants solution; 14 ml

    Mix 1.49 ml of 6% polyacrylamide gel at 0% denaturant solution with 12.25 ml of 6% polyacrylamide gel at 80% denaturant solution. Then add 10 μl TEMED, 100 μl of 10% APS and 80 μl dye solution.

APPENDIX C

CORRELATION CURVE OF CADMIUM

C-1 Correlation between total Cd and Zn concentrations
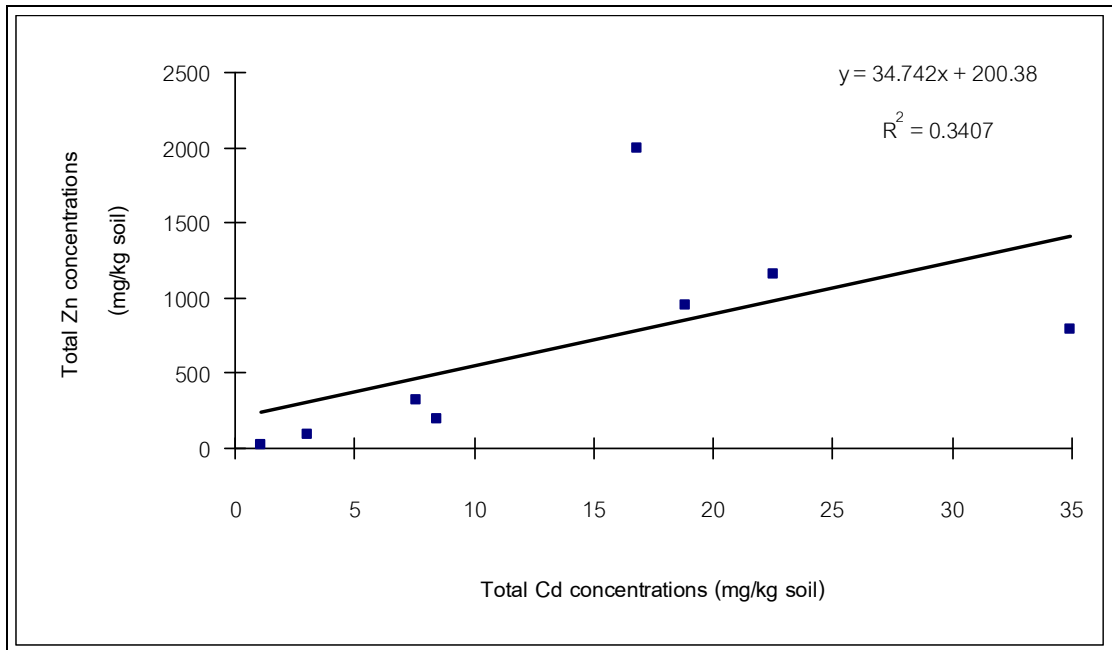
C-2 Correlation between total Cd concentrations and pH

C-3 Correlation between total Cd concentrations and organic matter

$y = -4.6512x + 40.145$

$R^2 = 0.2651$
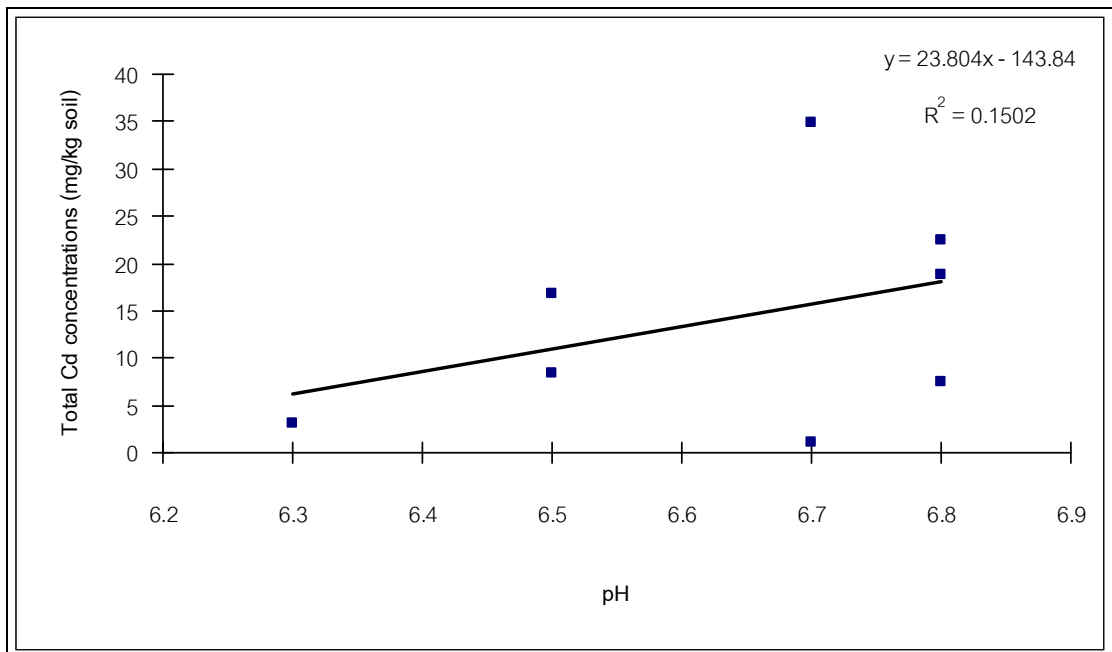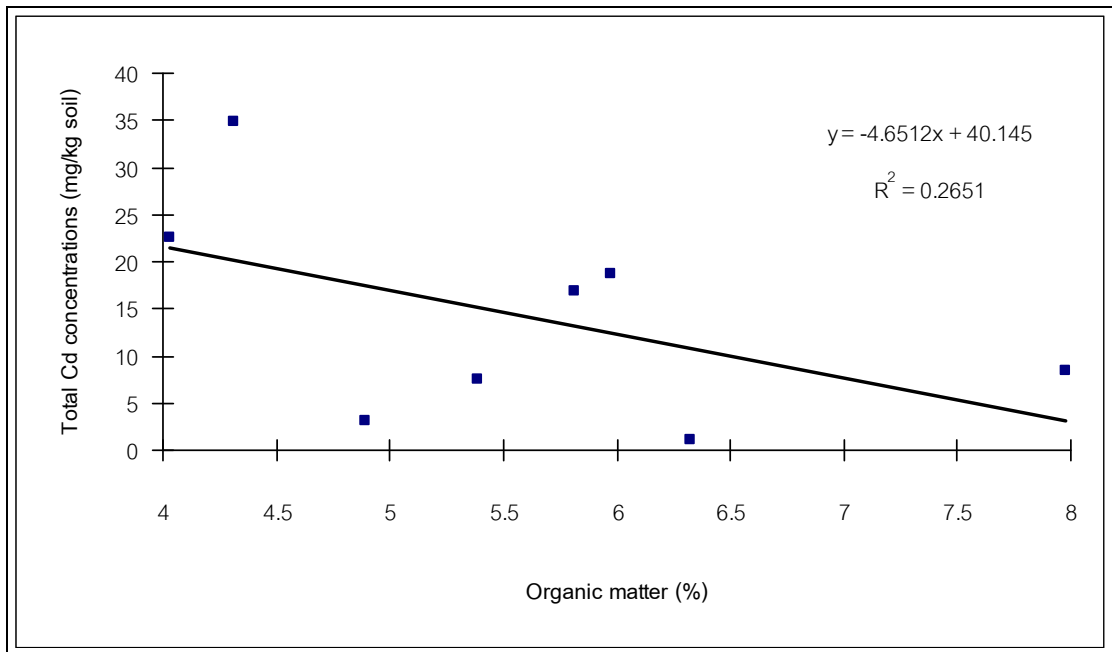
C-4 Correlation between total Cd concentrations and cation exchange capacity

$y = -2.6583x + 43.623$

$R^2 = 0.5616$
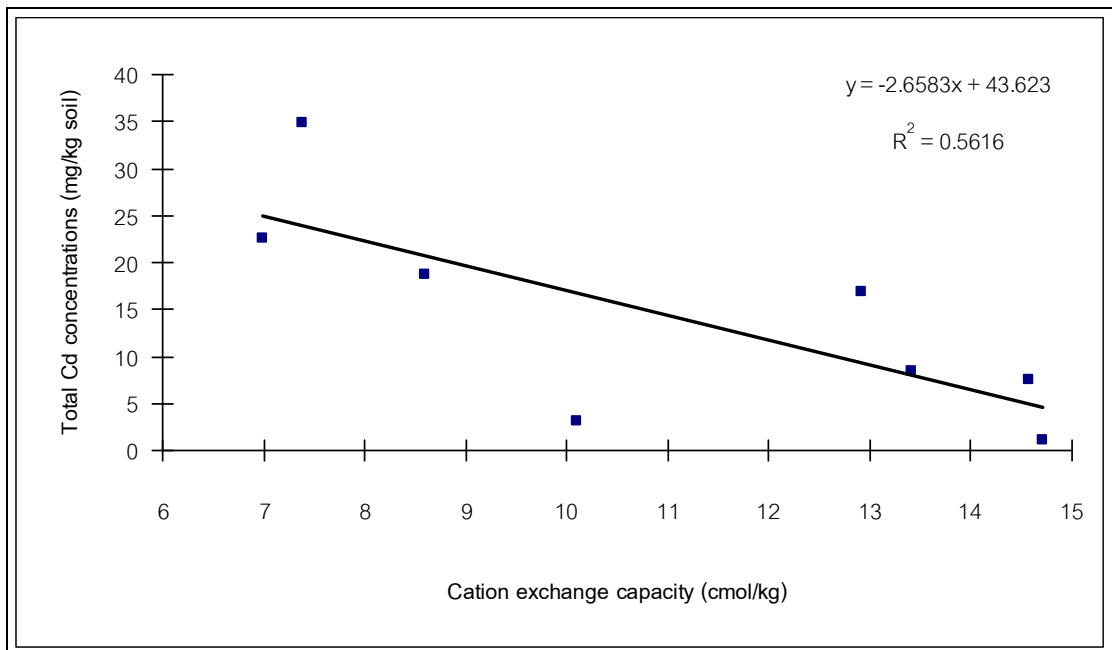
APPENDIX D

CORRELATION CURVE OF ZINC

D-1 Correlation between total Zn concentrations and pH
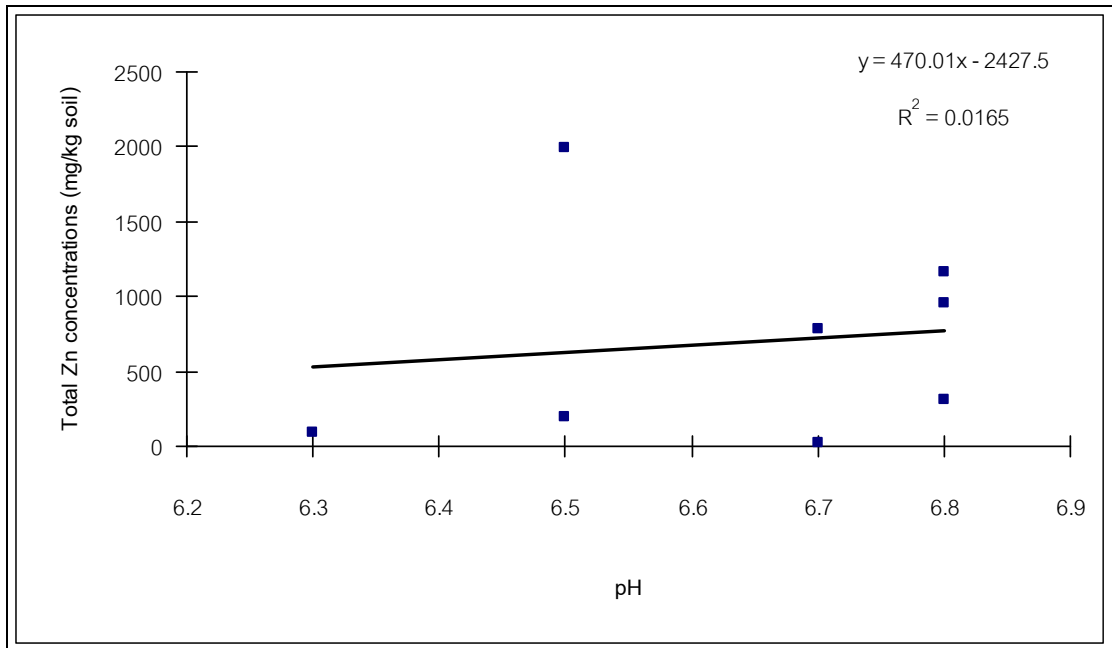
D-2 Correlation between total Zn concentrations and organic matter
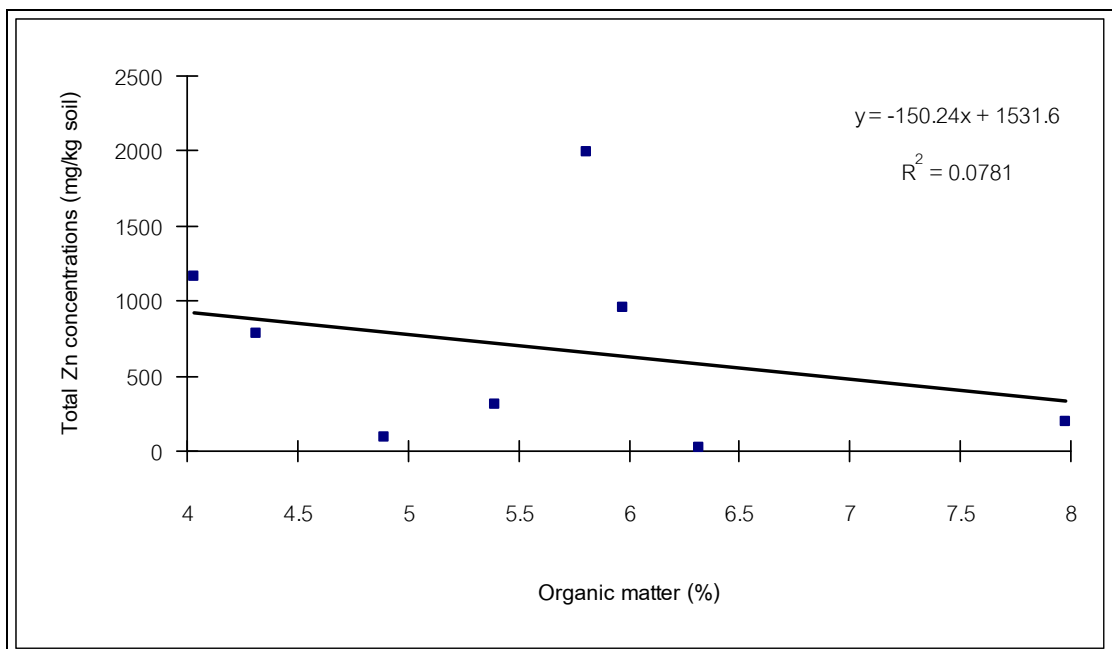
D-3 Correlation between total Zn concentrations and cation exchange capacity

$y = -67.745x + 1443.2$

$R^2 = 0.1029$
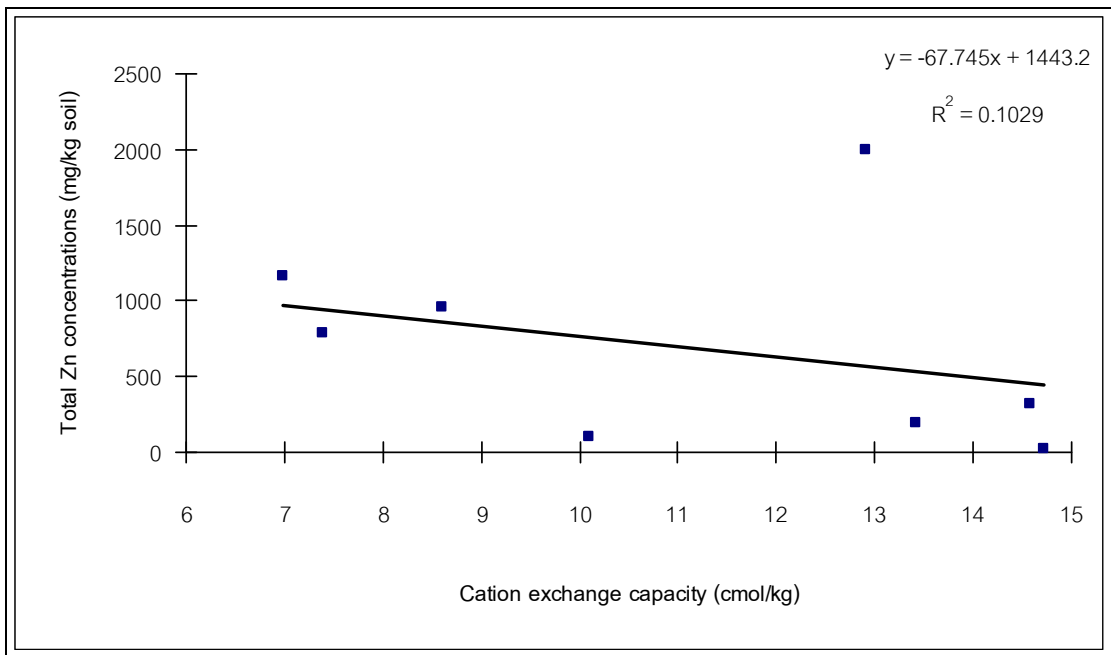
APPENDIX E

CORRELATION CURVE OF DIVERSITY INDEX

E-1 Correlation between diversity index and total Cd concentrations

$$y = 0.0134x + 0.6444$$
$$R^2 = 0.5892$$
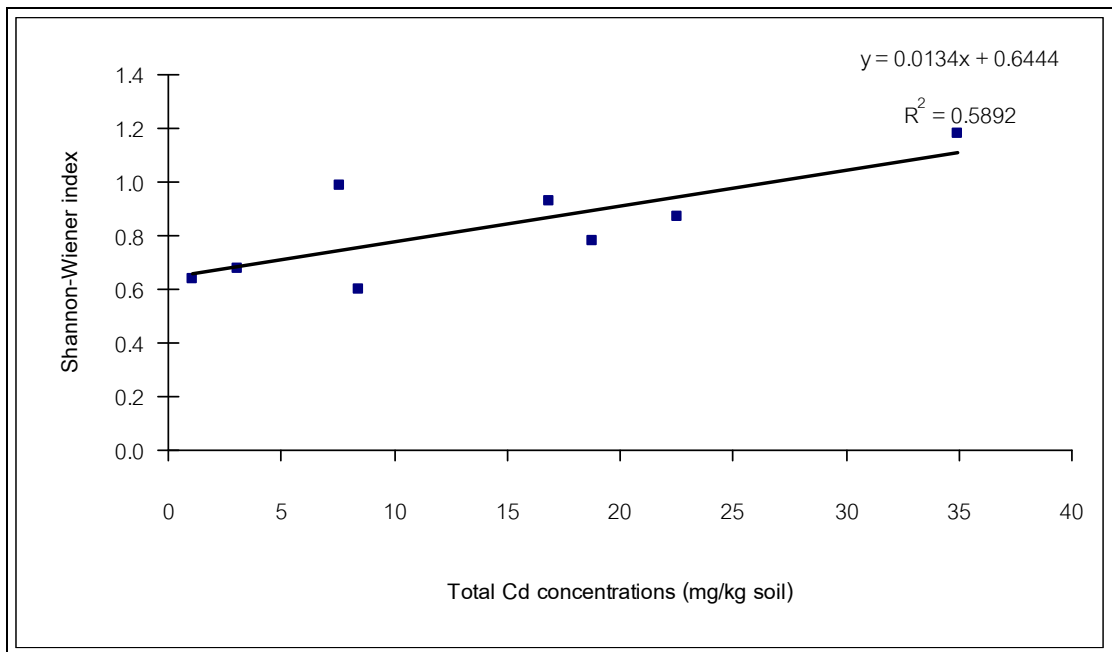
E-2 Correlation between diversity index and total Zn concentrations

$$y = 0.0001x + 0.7382$$
$$R^2 = 0.2221$$
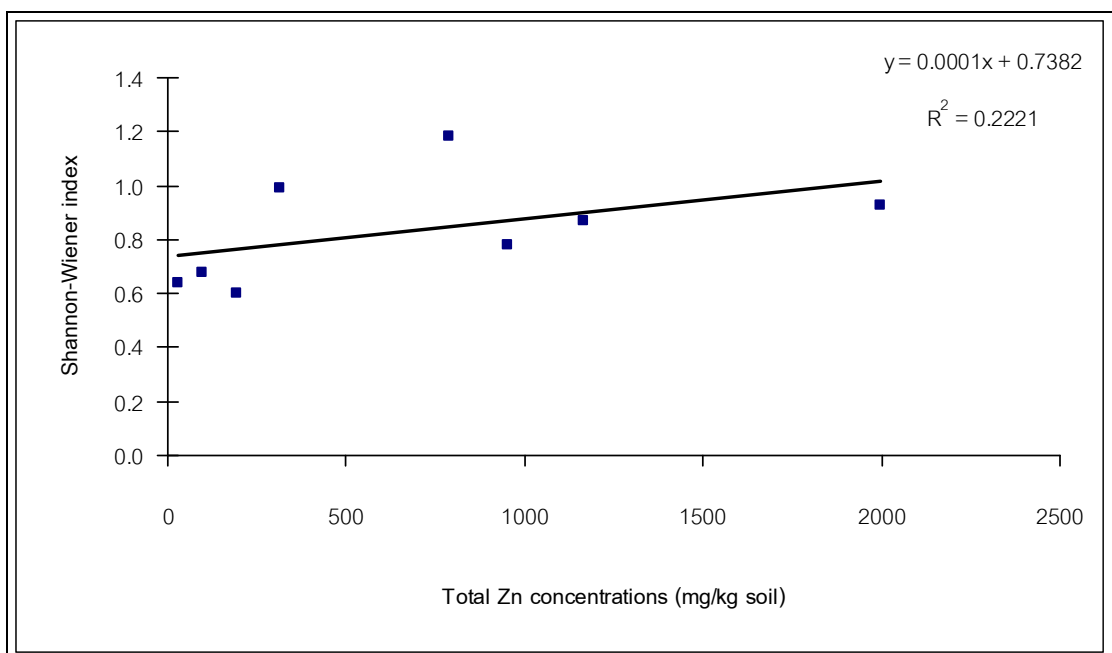
E-3 Correlation between diversity index and pH
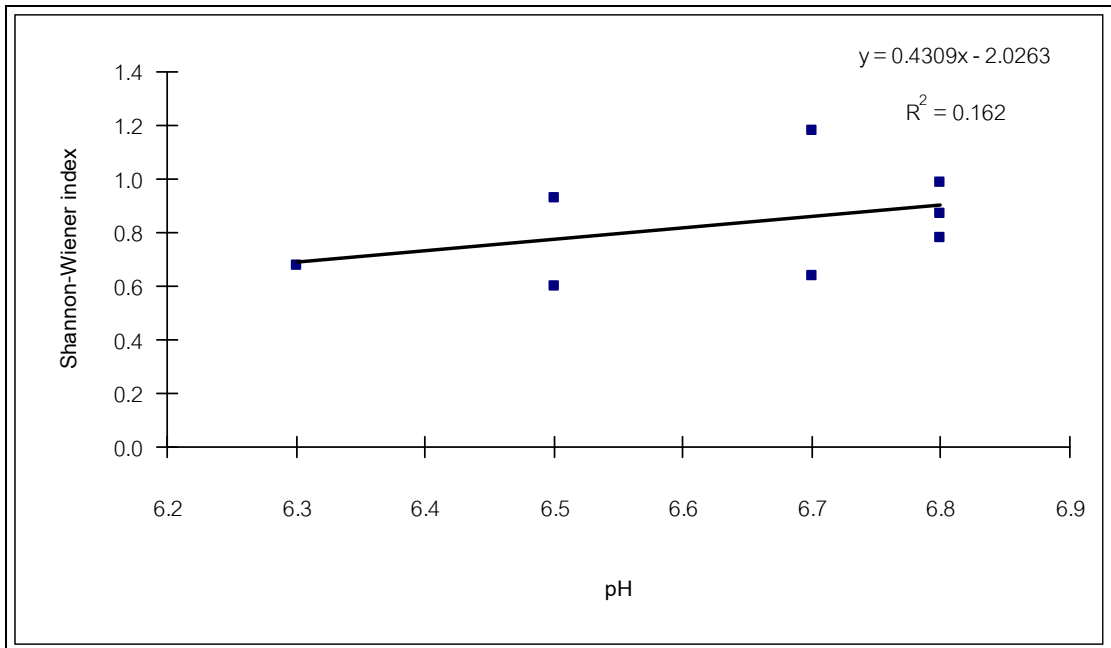
E-4 Correlation between diversity index and organic matter
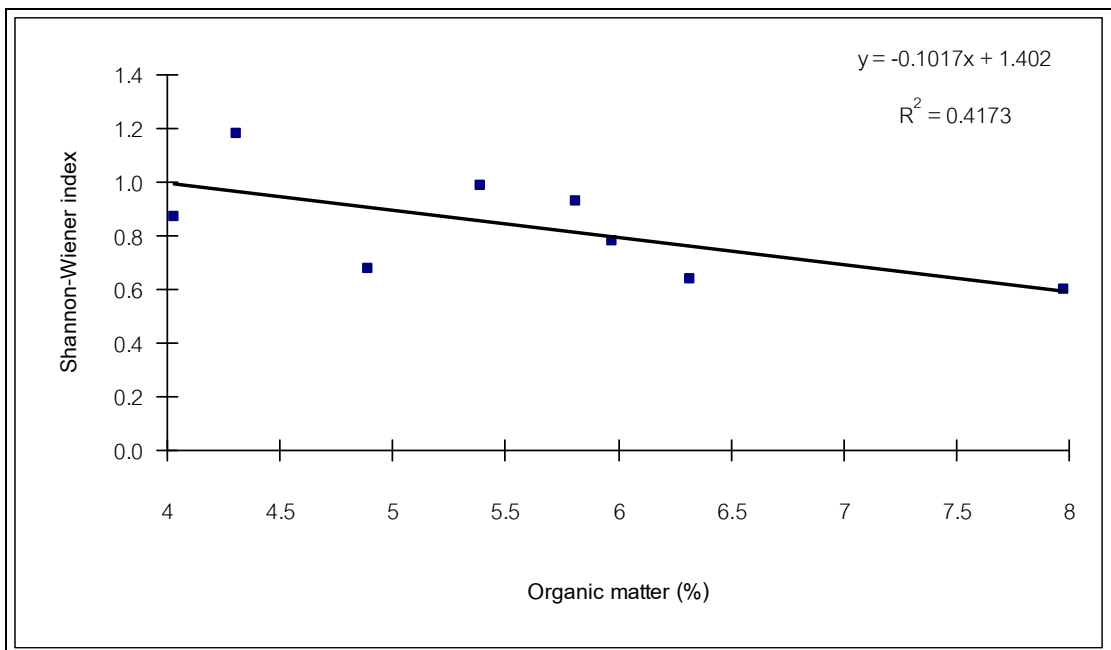
E-5 Correlation between diversity index and cation exchange capacity
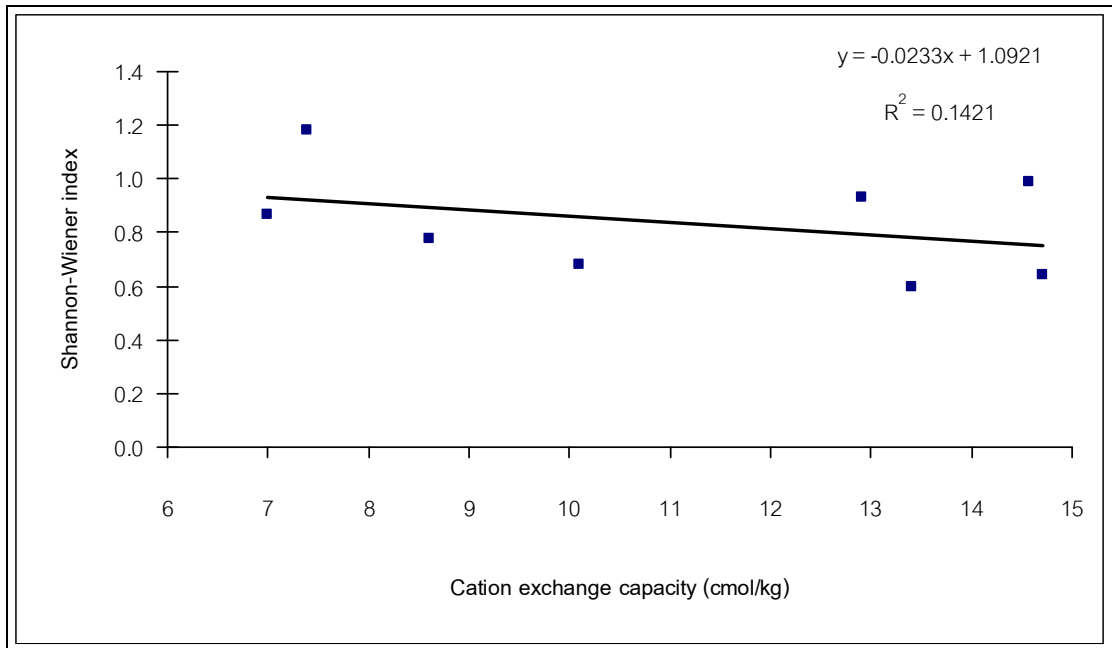
APPENDIX F

SEQUENCE INFORMATION OF 16S rRNA FROM SOIL BACTERIA

```
LOCUS       GQ463158           538 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A1 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 tgatcagcca tgccgcgtga gtgatgaatg ccctatgggt tgtaaacttc ttttgtacgg
       61 gagaaaattg tccggtcgtg accaaattga cagtactgta ccaataagca tcggctaact
      121 ccgtgccagc agccgcggta atacggagga tgcgagcgtt atccggattt attgggttta
      181 aagggtgcgt aggcggattt gtaagtcagt ggtgaaagcc tgtcgcttaa ctgtagcatt
      241 gccattgata ctggaattct tgagtatagt tgaggtaggc ggaatgtgta gtgtagcggt
      301 gaaatgctta gatattacac agaacaccga ttgcgaaggc agcttactaa gctgtaactg
      361 acgctgaggc acgaaagcgt ggggatcaaa caggattaga taccctggta gtccacgccg
      421 tacccgatga tcactggttg ttggcgatat actgtcagcg actgagcgaa gcattaagtg
      481 atccacctgg ggagtacgac cgcaacgatg aaactcaaat gaattgacgg agaacgct
//


LOCUS       GQ463159           534 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A2 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 agcctgatcc agccatgccg cgtgtgtgaa gaaggccttc gggttgtaaa gctctttcgg
       61 acggaacgaa atcgcgcggg ctaatatccc gcgtggatga cggtaccgta agaagaagca
      121 ccggctaact acgtgccagc agccgcggta atacgtaggg tgcgagcgtt aatcggaatt
      181 actgggcgta aagtgtgcgc aggcggccgc gcaagtcgag tgtgaaagcc ccgggcttaa
      241 cttgggaatt gcgctcgaaa ctacgtggct ggagtgtggc agaggaaggt ggaattccac
      301 gtgtagcggt gaaatgcgta gagatgtgga ggaacaccga tggcgaaggc agccttctgg
      361 gccaatactg acgctcatgc acgaaagcgt ggggagcaaa caggattaga taccctggta
      421 gtccacgccc taaacgatga tgactagttg ttggaggagt taaatccttt agtaacgcag
      481 ctaacgcgtg aagtcatccg cctggggagt acggtcgcaa gattaaaact caaa
//


LOCUS       GQ463160           534 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A3 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 tgatcagcga tgccgcgtgt gtgagaggcc ttcgggttgt aaagcccttt aggttgggaa
       61 gaagtgtatg tgatgataag tcttgtacat tgacggtact aacagaataa gcaccggcaa
      121 actctgtgcc agcagccgcg gtaatacaga gggtgcgagc gttaatcgga gttactgggc
      181 gtaaagggcg cgtaggcggt gatatgagtg tgatgtgaaa gccccgggct aacctggga
      241 agtgcatcgc aaacgatatg actggagtag atgagagggt ggcggaattt ccggtgtagc
      301 ggtgaaatgc gtagagatcg gaaggaacgt caatggcgaa ggcagccacc tggcatcata
      361 ctgacgctga ggcgcgaaag cgtggggagc gaacaggatt agataccctg gtagtccacg
      421 ctgtaaacga tgaggactag atgttgttag gggaacctag cagtatcgaa gctaacgtga
      481 taagtcttcc gcctgggaag tacggccgca aggttgaaac tcaaatgaat tgac
//
```

```
LOCUS       GQ463161              548 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A4 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 cgggaggcag cagaggggaa ttgttcgcaa tgggcgcagg cctgacgacg ccacgccgcg
       61 tggaggatga aggtcttcgg gttgtaaact ccttttgacc gggacgaata acctccgggt
      121 taacaccccg ggggtgggac ggacccgata gagaaggccc cggctacact ccgtgcccgc
      181 agccgcggta atacagggg ggcaagcgtt gttcgtaatt actgggcgta aagggcgcgt
      241 aggcggccgt ctaagtcaga cagtgaaatc ccccggctca accggggaac tgcgtctgat
      301 actggctggg cttgagtccg ggagagggat gtggaattcc agtgtgtagc ggtgaaagtg
      361 cgtgagatat ctggaggaac acccgtggcg aaggcggcct cctggaccgg cattgacgct
      421 gaggcgcgaa agccagggga gcaacacggg attagatacc ccggtagtcc tgtgcgccct
      481 aaacgatgac atgctctggt gtggtcgggt actcagatcc tgccgtgccg aagctaacgc
      541 attaagca
//


LOCUS       GQ463162              513 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A5 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 cctgatccag catgccgcgt gaatgatgaa ggccttcggg ttgtaaagtt ctttcacctg
       61 tgaagataat gacggtagca ggagaagaag ccccggctaa ctccgtgcca gcagccgcgg
      121 taagacggag ggggctagcg ttgttcggaa tgactgggcg taaagggcgc gtaggcggtt
      181 taataagtga ggcgtgaaag ccctgggctt aacccaggag gtgcgtttca tactgttaca
      241 cttgagtacg agagaggaaa gcggaattcc tagtgtagag gtgaaattca tatatattag
      301 gaagaacacc ataggcgaag gtggctttct ggctcgtagc tgacgcygac gcgcgaaagc
      361 gtggggagca aacaggatta aataccctgg tagtccacgc tgtaaactat gattgctaga
      421 cgttggggag aaatcttcgg tgtcgcgtct aacgcattaa gcactccgcc tggggagtac
      481 ggtcgcaaga ttaacactcg aaggaattga cgg
//


LOCUS       GQ463163              514 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A6 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 gcaatgggcg caagcctgac gaagcaacgc cgcgtggggg atgaaggtct tcgggttgta
       61 aacccctttc gaccgggacg aataagtcgc ggtttaacac accgggggat tgacggtacc
      121 gataaaggaa gccccggcta actctgggcc tgcacccgcg gtaatacaga gggggtcaag
      181 cgttgttcgg aattactggg cgtaaagggc gtgtaggcgg ccttataagt cagacgtgaa
      241 atccccggc ttaacctggg aaatgcgtct gatactggtg ggcttgagtg cgggagaggg
      301 atgtggaatt ccaggtgtag cggtgaaatg ggtagatatc tggaggaaca ccggtggcga
      361 aggcggcatc ctggaccgac actgacgctg aggcgcgaaa gccaggggag caaacgggat
      421 tagataccc ggtagtcccc gccctaaacg atgaatgctt ggtgtggcgg gtatcgatcc
      481 ctgccgtgcc gaagctaacg catttagacc tcct
//


LOCUS       GQ463164              528 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A7 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 tcttccgggg ggcaccagtg gggaatttta gccaatgggg gcaaccctga tccagccatg
       61 ccgcgtgtgt gagaaagccc ttcgggttgt aaagctcttt cggccggaac gaaatcgcgc
      121 gggttaatat cccgggtgga tgacggtacc ctaagaagaa gccccggcta actgcgtgcc
      181 agcagccgcg gtaatacgta gggagcgagc gttattcgga attattgggc gtaaagtgcg
      241 cgcaggtggc cttgcaagtc gcatgtgaaa tccccgggct caacccggga aatgcgcttg
      301 aaactacgcg gcttgagtgt ggcagaggga ggtggaattc cccgtgtagc ggtgaaatgt
      361 gtagagatat ggaggaacac ccatggcgaa ggcggccttc tgggcccaca ctgacgctca
      421 ggcgcgaaag cgtggggagc aaacacgatt agataccccg gtagtccacg ccctaaacga
      481 tgagtactag tgtgggggag taaatcctca gtaacgcagc taacgcgt
//
```

```
LOCUS       GQ463165              542 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A8 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 tgatgcagcc atgccgcgtg tgcgatgaag gccttcgggt tgtaaagcac ttttggcagg
       61 gaagaaacgg cctgagataa taccttgggc taatgacggt acctgcagaa taagcaccgg
      121 ctaactacgt gccagcagcc gcggtaatac gtagggtgca agcgttaatc ggaattactg
      181 ggcgtaaagc gtgcgcaggc ggttcggaaa gaaaggtgtg aaatcccagg cttaacctt
      241 ggaactgcac ttttaactac cgggctagag tacgtcagag gggggtagaa ttccacgtgt
      301 agcagtgaaa tgcgtagaga tgtggaggaa taccgatggc gaaggcagcc ccctgggatg
      361 atactgacgc tcatgcacga aagcgtgggg agcaaacagg attagatacc ctggtagtcc
      421 acgccctaaa cgatgtcaac tagctgttgg ggtttattaa ccttagtagc gcagctaacg
      481 cgtgaagttg accgcctggg gagtacggtc gcaagattaa aactcaaagg aattgacggg
      541 ga
//


LOCUS       GQ463166              531 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A9 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 tgatccagcc atcccgcgtg tgcgatgaag gccttcgggt tgtaaagcac ttttggcagg
       61 gaagaaacgg cctgagataa taccttgggc taatgacggt acctgcagaa taagcaccgg
      121 ctaactacgt gccagcagcc gcggtaatac gtagggtgca agcgttaatc ggaattactg
      181 ggcgtaaagc gtgcgcaggc ggttcggaaa gaaaggtgtg aaatcccagg cttaacctt
      241 ggaactgcac ttttaactac cgggctagag tacgtcagag gggggtagaa ttccacgtgt
      301 agcagtgaaa tgcgtagaga tgtggaggaa taccgatggc gaaggcagcc ccctgggatg
      361 atactgacgc tcatgcacga aagcgtgggg agcaaacagg attagatacc ctggtatcca
      421 cgccctaaac gatgtcaact agctgttggg gtttattaac cttagtagcg cagctaacgc
      481 gtgaagttga ccgcctgggg agtacggtcg caagattaag actcaaagga g
//


LOCUS       GQ463167              538 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A10 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 tgatcagcga tgccgcgtgg gtgaagaagg ccttcgggtt gtaaagccct ttaggttggg
       61 aagaagtgta tgtgatgata agtcatgtac attgacggta ctaacagaat aagcaccggc
      121 aaactctgtg ccagcagccg cggtaataca gagggtgcga gcgttaatcg gagttactgg
      181 gcgtaaaggg cgcgtaggcg gtgatatgag tgtgatgtga agcccccggg cttaacctgg
      241 gaagtgcatc gcaaacgata tgactggagt agatgagagg gtggcggaat ttccggtgta
      301 gcggtgaaat gcgtagagat cggaaggaac gtcaatggcg aaggcagcca cctggcatca
      361 tactgacgct gaggcgcgaa agcgtgggga gcgaacagga ttagataccc tggtagtcca
      421 cgctgtaaac gatgaggact agatgttgtt aggggaacct agcagtagcg aagctaacgc
      481 gataagtctt ccgcctggga agtacggccg caaggttgaa actcaaagga attgacgg
//


LOCUS       GQ463168              539 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A11 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 tgatcagcca tcccgcgtgt gcgatgaagg ccttcgggtt gtaaagcact tttggcaggg
       61 aagaaacggc ctgagataat accttgggct aatgacggta cctgcagaat aagcaccggc
      121 taactacgtg ccagcagccg cggtaatacg tagggtgcaa gcgttaatcg gaattactgg
      181 gcgtaaagcg tgcgcaggcg gttcggaaag aaaggtgtga atcccaggct taaccttg
      241 gaactgcact tttaactacc gggctagagt acgtcagagg gggtagaat tccacgtgta
      301 gcagtgaaat gcgtagagat gtggaggaat accgatggcg aaggcagccc cctgggatga
      361 tactgacgct catgcacgaa agcgtgggga gcaaacagga ttagataccc tggtagtcca
      421 cgccctaaac gatgtcaact agctgttggg gtttattaac cttagtagcg cagctaacgc
      481 gtgaagttga ccgcctgggg agtacggtcg caagattaaa actcaaagga attgacggg
//
```

```
LOCUS       GQ463169              543 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A12 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 tgacgccgca acgccgcgtg ggggatgaag gccttcgggt tgtaaactcc tttcgaccgg
       61 gacgaatggg tccgggttat accccggggg atggacggtc ccggtaaagg aacccccggt
      121 taactccggg ccagcagccg cggaaataca gggggggcaa gggttgttcg gatttactgg
      181 gcgtaaaggg gtggaaggcg gccttctaag tcggacgtga atccccgggg ttcacctggg
      241 gaactgcttc gaaaatgggg ggggttgggt gtggggaagg ggattggaat tccctgggga
      301 acagggaaat gggtaaatgt tcggaagaac acccgtggcg aaggggagcc cctggacctg
      361 aactggccct catggacgaa aaccctgggg gagcaaaggg gataacaccc ccgggtagtc
      421 ctggccctaa aaaatgaatg ctggtgtgtg ggcgttatct gatccctgcc gtgccgaagc
      481 taacgcatta ggacctcctg cctggggatg tacggtcgca aggctacaca ctcaaaggaa
      541 ttg
//


LOCUS       GQ463170              422 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A13 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 tgatgcagca atgccgcgtg agtgatgaag gccttagggt tgtaaagctc ttttataggg
       61 aggcgaatga cagtacctgg agaataagct ccggctaact ccgctgccag cagccgcggt
      121 aatacggagg gagctagcgt tgttggaatt actgggcgta aagcgctcgt aggtggctgc
      181 tcaagtcagg ggtgaaagcc cggggctcaa ccccggaact gccttcgaaa ctaaacagct
      241 agaatcttgg agaggtcagt ggaattccga gtgtagaggt gaaattcgta tatattcgga
      301 agaacaccat tggcgaaggc gactgactgg accagtattg acgctgatgt gcggaagcgt
      361 gaggagccaa caggattaaa taccctggta gtccacgccc atatactatg aaaactatct
      421 ct
//


LOCUS       GQ463171              531 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A14 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 tgatgcagcc atgccgcgtg tgtgaagaag gccttcgggt tgtaaagcac ttttgtccag
       61 gaagaaagcg gtgggttaat accttgggct catgacggta cccggacaat aagcaccggc
      121 taactccgtg ccagcagccg cggtaatacg gagggtgcaa gcgttaatcg gaattactgg
      181 gcgtaaagcg tgcgcaggcg gtttggtaag atcgatgtga atccccgggg cttaacctgg
      241 gaaccgcatt ggaaactgcc tggcttgagt gcgggagagg ggggtagaat tccacgtgta
      301 gcggtgaaat gcgtagagat gtggaggaat accggtggcg aaggcggccc cctggactga
      361 tactgacgct catgcacgaa agcgtgggga gcaaacagga ttagataccc tggtagtcca
      421 cgccctaaac gatgtctact aggtgttggg ggttttttatc ctttgtatct cacctaaacg
      481 tgaaattcac cccccgggga gtacggcccc agaattaaaa ctcaaggaat t
//


LOCUS       GQ463172              537 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A15 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 tgatcagcgc tgccgcgtga ggtgagaagc cttcgggttg taaagccttt tagcttggga
       61 agaagtgtgt gtggtgatca gcttgtagca ttgacggtac taacagaata agcaccggct
      121 aactctgtgc cagcagccgc ggtaatacag agggtgcgag cgttaatcgg aattactggg
      181 cgtaaagggc gcgtaggcgg tgatatgagt gtgatgtgaa agccccgggc ttaacctggg
      241 aagtgcatcg caaacgatag gactggagta tatgagaggg tggcggaatt tccggtgtag
      301 cggtgaaatg cgtatagatc ggaaggaacg tcaatggcga aggcagccac ctggcatcat
      361 actgacgctg aggcgcgaaa gcgtggggag cgaacaggat taaatacccct ggtagtccac
      421 gctgtaaacg atgaggacta gatgttggta ggggaaccta tcggtatcca aactaacgcg
      481 ataagtcttc cgcctgggaa gtacggccgc caggttgaaa ctcaaggaat ttgacgg
//
```

```
LOCUS       GQ463173              547 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A16 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 gggaggcagc agtgaaaatt ttcaacaagg ggagcaaccc tgatccagca atgccgcgtg
       61 gatgaagaag tccttcgggt tgtaaactcc ttttgtccgg gacgaaaagc tcctggttta
      121 ataccggggg gatatgacgg taccggaaga aaaacccccg gctacctacg tgccaacagc
      181 cgcggtaata cggagggcgc gagagtttat cgaaattacg gggcgtaaag cgtgcgcagg
      241 cggtgttgta agacagatgt gaaatccccg ggctcaacct gggaaatgca tttgagactg
      301 cacggcttga gtgtgggaga gggggttgga attccgcgtg tagcagtgaa atgcgtagat
      361 atacggagga acaccgatgg cgaaggcaat cccctgggcc tgcactgacg ctcatgcaca
      421 aaagcgtggg gagcaaacag gataagatac cctggtagtc cccgccctaa aagatgtcaa
      481 ctggttgtgg ggaatttgct ttctcagtaa cgaagctaac gcatgaagtt gaccgcctgg
      541 gtgagta
//


LOCUS       GQ463174              485 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A17 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 tgatccagca atgccgcgtg tgtgatgaag gccttcgggt tgtaaagcac ttttgtccgg
       61 aaagaaaggg cctgggttaa tacctggggt cgatgacggt acctgaagaa taaggaccgg
      121 ctaactacgt gccagcagcc gcggtaatac gtagggtgca agcgttaatc ggaattactg
      181 ggcgtaaagc gcgcgcaggc ggttgggaaa gaccgatgtg aaatccccgg gcttaacttg
      241 ggaattgcat ttgtgactgc cgggctagag tgtgtcagag gggggtagaa ttccacgtgt
      301 agcagtgaaa tgcgtagaga tgtggaggaa taccgatggc gaaggcagcc ccctgggata
      361 atactgacgc tcatgcacga aagcgttggg agccaacagg attaaatacc ctggtattcc
      421 acgccctaaa agatgtcaac tagttgttga ggattattat acttactttc gtagctcact
      481 cgtta
//


LOCUS       GQ463175              462 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A18 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 tgatgcagcg acgccgcgtg gacgatgaag gccttcgggt cgtaaagtcc tttcgggtgg
       61 gaagaaatgg cctggagctt atatctccag gccttgactg tcccaccgaa tgaagccccg
      121 gttaattcct ggccaccgcc cgcggtaatc cgaaggggGc cgcgtttgct cgaaattatg
      181 ggcgtaaagg gggcggtagg cgggatggta agtttgatgt gaaatcccgg ggttcacccg
      241 tggaattgct tctcatactg ccggattaaa gtctgggaga gggaagcgga tttccccggtg
      301 taccggggaa ttgcgtaaat atcggaggaa caccatgggc aaaggcggct tcctggacca
      361 gtactgacgc tgaggcgaaa agctagggga gcaaacggga ttaataccccc tggtgaccct
      421 taccttaaac aatgtgcttt gttgggggta acgacctctc ca
//


LOCUS       GQ463176              417 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A19 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 tgacgcagcg acgccgcgtg gacgatgaag gccttcgggt cgtaaagtcc tgtcgggtgg
       61 gaagaaatgg cctggagcta atatctccag gtcctgactg taccaccaaa ggaagccccg
      121 gctaactccg tgccagcacc cgcggtaata cggaggggGc cagcgttgct cggaattatt
      181 gggcgtaaag ggcgcgtagg cggtctggta agtttgatgt gaaatcccat ggctcacccg
      241 tggaactgca tctcatactg ccggactaga ttctgggaga gggaagcgga tttccccggtg
      301 tagcggtgaa atgcgtagat atcgggagga acaccagtgg cgaaggaggc ttcctggacc
      361 attactgacg ctgaggcgcg aaagctgggg gaggcaaacg gattaaatac cctggta
//
```

```
LOCUS       GQ463177              489 bp   DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A20 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 gtgggggatg aagatcttcg ggttgtaaac tcctttcgag cgggacgaaa gggtcccggg
       61 tgaatacccc gggggatgac ggtcccgata aagaaacccc cggctaactc cgggccacca
      121 gccgcggtaa tacgggggggg gcaagcgttg ttcggaatta ctgggcgtaa agggcgcgta
      181 ggcggccatc taagtcggac gtgaaatccc ccggctcaac ttgggaactg cgtctgatac
      241 tggatggctt gaattcggga gagggatgcg gaattccagg tgtagcggtg aaatgcgtag
      301 atatctggag gaacaccggt ggcgaaggcg gcatcctgga ccggcactga cgctgatgcg
      361 cgaaagccag gggagcaaac gggattagat accccggtag tcctcgccct aaacgatgaa
      421 tgcttggtgt ggcgggtatc gatccctgcc gtgccgaagc taacgcatta agcattcggc
      481 ttggggagt
//


LOCUS       GQ463178              455 bp   DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A21 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 gcctgatcca gccatgccgc gtgagtgatg aaggccttcg ggttgtaaag ctcttttggc
       61 cggaaagaag ggtagtcggc taataccctg ggggcatgac ggtaccggaa gaataagcac
      121 cggctaactt cgtgccagca gccgcggtaa tacggagggt gcaagcgtta atcggaatta
      181 ctgggcgtaa agcgtgcgca ggcggttcgg taagtctgat gtgaaagccc cgggctcaac
      241 ctgggaactg cattgcagac tggctaactg gagtgcggga gagggtggtg gaattcccgg
      301 tgtagcggtg aaatgcgtag atatcgggag gaacttcgat ggcgaaggca gctcctggac
      361 caatactgac gctgaggccc gaaagcgtgg ggagcgaaca ggattagata ccctggtagt
      421 ccccgcccta aacgatgtga actgtatgtt gggtg
//


LOCUS       GQ463179              522 bp   DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A22 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 agcctgatgc agccacgccg cgtgcgggat gaaggccttc gggttgtaaa ccgctttcag
       61 cagggacgaa gtgacggtac ctgtacatta aggcgcggac aactacgtgc cagcagccgc
      121 ggggacacgt aggggccaag cgttgtccgg atttattggg agttaacatt cgtaggcgct
      181 tgaggatgtc gcttgtgaaa acttggtgcg tcacccagat atggcatccc gatactgctg
      241 gtacttgctt tagctaaggt agcggggaat tcctagggta gaggggaatt ggaatttatt
      301 ttgtaaggaa cacctgcgga gaagtggcca ctctggcccg aggctgacgc taacccctga
      361 aatgatgctg agcctaaggg attaaatccc cgggagctcc acgccttaaa tattgtgcac
      421 tacgagtggg tttcaaccaa cgacttcccg tccgtcgcta agacattatt gcccccccct
      481 gggcagtacg gccccaccgc taggctcaaa gggatttcac gg
//


LOCUS       GQ463180              420 bp   DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A23 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 gcaagcctga tgcagccatg ccgcgtgtgt gaagaaggcc ttcgggttgt aaagcacttt
       61 cagcgcggag gaaggggggtg aggttaataa ccttgtcgat tgacgttacc cgcagaagaa
      121 gcaccggcta actccgtgcc agcagccgcg gtaatacgga gggtgcaaac gttaatcgga
      181 attactgggc gtaaagcgca cgcatgcgga ctgtcaagtt gaatgtgaaa tatcctggct
      241 caacgtggga accggatatg aggccatgtt tctattcttt gcagctggtg gtccctgtct
      301 ggtcaagcgt ttctgtactt ggagagatgg atcgaatacc gctggctatg taatcctacg
      361 acaagcctgc gccttcggtc agatacagcg gcgagaattc gatgtaggca tggactaatc
//
```

```
LOCUS       GQ463181            453 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A24 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 tgatgcagcc atgccgcgtg tgtgaagaag gccttcgggt tgtaaagcac tttcagtggt
       61 gaggaaggcc tgtagtttaa taccctcgtc gattgacgtt agccgcagaa gaagcaccgg
      121 ctaactccgt gccagcagcc gcggtaatac ggagggtgca agcgttaatc ggaattactg
      181 ggcgtaaagc gcacgtaggc ggcctgttaa gccagatgtg aaatccccgg gctcaacctg
      241 ggaactgcat ttcgaactgg cgggctagag tcttgtagag ggtggtagaa ttcccggtgt
      301 agcggtgaaa tgcgtaaaga tctggaggaa tacccgtggc caaaagcggc cccctggact
      361 aatactgacc ctcaagggcc aaagcgtggg gagcaaaaca ggaataaatt accctggtag
      421 ccaccactta aacaatgtca ctttgaaggt tgt
//


LOCUS       GQ463182            522 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A25 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 ctgatgcagc cacgccgcgt gcgggatgaa ggccttcggg ttgtaaaccg ctttcagcag
       61 ggacgaagct gacggtacct gcacaagaag gtgcggccaa ctacgtgcca gcagccgcgg
      121 tgacacgtag gcaccaagcg ttgtccggat ttattgggcg taagagctcg taggctgtcc
      181 gggcagtcgg gtgtgaaaac tctgggctca gcccagatgc ggcacccgat actgctgtga
      241 cttgagtacg gtaggggagt ggggaattcc tggtgtagcg gtgagatgaa ttgtagcgga
      301 ggaacgcctg tggggaaggc tcgacctggg cgtgatttac gctgacgacg aattgaacgc
      361 ctcgtaccga ttcttggaac cttgattatt ctgggtttag gcctaattca tttataggg
      421 ggctccatac cgaaatcgca ttctaaatct aagacccccca ctgcgaacct ctcggcatgg
      481 ggctcgcgtt ttggtttttgc ggcaatttca atgtatggct ta
//


LOCUS       GQ463183            517 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A26 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 tgatgcagcg acgccgcgtg agcgatgaag cccttcgggg tgtaaagctc tttcggccgg
       61 aacgatgatg acggtaccgg cagaagaagc tgcggctaac tacgtgccag cagccgcggt
      121 aatacgtagg cagcgagcgt tgttcggagt tactgggcgt aaagagtgcg taggcggtct
      181 gccaagtctg gtgtgaaatc tcccggctca accgggaggg tgcgccggaa actggtggac
      241 tcgagtgtgg gagaggcagg cggaattccc ggtgtagcgg tgaaatgcgt agatatcggg
      301 aggaacaccc gaggtgtaga cggcctgctg gaccacgact gacgctgagg cacgaaagcg
      361 tggggagcaa acaggattag ataccctggt agtccacgcc ctaaacgatg catacttggt
      421 gtgagccatt catttggttc gtgccggagc taacgcgtta agtatgccgc ctggggagta
      481 cggtcgcaag gctgaaactc aaaggaattt gaccgga
//


LOCUS       GQ463184            497 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A27 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 ctgatgagca tgccgcgtga gtgaagaagg ctcttcggat ctgaagctct ttgccttgga
       61 agaagccttg tggagaatct gccctgtagg ttgacggtac ttgcaaaata agccccggct
      121 aactctgtgc cagcagccgc ggtaatacgg agggtgcaag cgttaatcgg aattattggg
      181 cgtaaagggc gcgtaggcgg cgatttaagt ttgatgtgta atgccggggc tcaaccctgg
      241 atcgcacccca aaactgggct gcttgggtgc tggaaaagga gagcgtaatt ccccggtacc
      301 ggtgaatatg taagatatat gtgaggaaca ccggtggcga aggctgctct cttggctgtc
      361 actgacattc aggggtgagg aatgggtaaa tccgtggtta ggccgctcta taccgaaccg
      421 catagtggtt cgggtatccc cccctacgaa tctctcactc gggcaatcgt cctttccgtc
      481 tggcaacgac ttctata
//
```

```
LOCUS       GQ463185             456 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A28 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 ctgatccagc catgccgcgt gagtgatgaa ggccttcggg ttgtaaagct ctttcggccg
       61 ggacgaaagg gcccgggcta ataccccggg cgcttgacgg ttccctgcca agaagccccg
      121 gctaacttcg gggccgcagc cccggtaata cctaaggggc caacgtttgt cggaattact
      181 gggcgtaaag cgggcggagg cgggtttgta aaaccgatgt gaaatccccg ggcttaacct
      241 gggaactgcc tttgaaactg gggggcttga gtgcgggaaa gggaggtgga atttcccgtg
      301 taacagtgaa atgcgtaaaa atgcggagga acaccggtgg cgaaggcagc ccccgggccg
      361 gcactgaccc ttaagcccaa aacctgggga accaaccgga ttaaataccc tggtatcccc
      421 cccctaaacc aagtcaacta gtggttgggg gttttt
//


LOCUS       GQ463186             507 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A29 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 cctgatccag cgatgccgcg tgagtgaaga aggccttcgg gttgtaaagc tctttaggtt
       61 gggaagaagg gtatggggggg ataatcctgc tggcttgacg gtaccggcag aataagcacc
      121 ggctaactct gtgccagcag ccgcggtaat acagagggtg caagcgttaa tcggaattac
      181 tgggcgtaaa gggcgcgtag gcggtgtttt gagtgtgatg tgaaagcccc gggcttaacc
      241 tgggaagtgc atcgcaaact gtctgactgg agtacgtgag agggtggcgg aatttccggt
      301 gtagcggtga aatgcgtaga gatcggaagg aacgtcgatg gcgaaggcag ccacctggct
      361 tgatactgac gctgaggcgc gaaagcgtgg ggagcgaaca ggattagata ccctggtagt
      421 ccacgctgta aacgatgagg actagatgtt ggtaggggaa cctatcagta tcgaagctaa
      481 cgcggtaagt ctcccgcctg ggaatac
//


LOCUS       GQ463187             433 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A30 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 ctgacgcagc aacgccgcgt ggaggatgaa ggtcttcggg ttgtaaactc ctgtcgaccg
       61 ggacgaatgt gcccgggctg aataccccgg gggagggacg gtcccggtgg aggaaccccc
      121 ggctaactcc gtgccagcag ccgcggtaat acagaggggg caagcgttgt tcggaattat
      181 tgggcgtaaa gggcgcgtag gcggccgctt aagtcggaag tgaaatcccc tgctcaaccg
      241 gggaactgcg tcggaaactg cggggttcga gtccgggaga gggatgtgaa ttcctggtga
      301 agcggaggaa tgcctaaata ttgggagaag caccggggtc gaagtggtcc ctggacggat
      361 tctgaccctg aggacaaaaa cccaggggac caacgcgttt aaattccccg gtagccccgg
      421 ctgaaaacaa aga
//


LOCUS       GQ463188             531 bp    DNA     linear   ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A31 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 cctgatcagc gatgccgcgt gggtgaagaa ggccttcggg ttgtaaagcc ctttaggttg
       61 ggaagaagtg tatgtgatga taagtcatgt acattgacgg tactaacaga ataagcaccg
      121 gcaaactctg tgccagcagc cgcggtaata cagagggtgc gagcgttaat cggagttact
      181 gggcgtaaag ggcgcgtagg cggtgatatg agtgtgatgt gaaagccccg ggcttaacct
      241 gggaagtgca tcgcaaacga tatgactgga gtagatgaga gggtggcgga atttccggtg
      301 tagcggtgaa atgcgtagag atcggaagga acgtcaatgg cgaaggcagc cacctggcat
      361 catactgacg ctgaggcgcg aaagcgtggg gagcgaacag gattagatac cctggtagtc
      421 cacgctgtaa acgatgagga ctagatgttg ttaggggaac ctagcattat cgaagctaac
      481 gcgataagtc ttccgcctgg aagtacggcc gcacggttga tactcaaagg a
//
```

```
LOCUS       GQ463189              549 bp    DNA     linear  ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A32 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 gaccccgatc gtaacagtga cctgctttaa ggtatgcgat acacctcatg gtctcgcagc
       61 cctctggggt gggcattgta gcatgtgtga agccctgtac ataaggacca tgatgacttg
      121 atttggtcct caccttcctc tgagttgacc ccggcagtct cttacgagtc cccccccataa
      181 cgcgctggca acataagaca agggttgcgc tcggtgcggg acttaaccca acatctcacg
      241 actcgagctg aagacaccca tgcaccacct gtacaccgac cacaaggggg accgtatctc
      301 tacggatttc cggtgtatgt tataccctgg aaggttcttc gcgttgcatc gaattaatcc
      361 acatgctctg ccgcttgtgc gggcccccgt caattccctt tgagttttag ccttgcggcc
      421 gtactccca ggtggggcgc ttaatgcgtt agctacggca cagatcccgt ggaatgaacc
      481 tacatcttag cgcccacgtt ttacggcgtg cactactagg gtatctaatc ctgtttgcta
      541 ctcacgctt
//


LOCUS       GQ463190              476 bp    DNA     linear  ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A33 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 ctgatccagc catgccgcgt gagtgaagaa ggccttcggg ttgtaaagct ctttcggtcg
       61 gaacgaaagg gtccgggcta ataccccggg gggttgacgg taccctaaga agaagccccg
      121 gctaactacg tgccagcagc cgcggtaata cctagggtgc aagcgttaat cggaattact
      181 gggcgtaaag ggtgggcagg cggtttttta agtccgatgt gaaatccccg ggcttaacct
      241 gggaactgca tttgaaactg ggggacttga gtgcggaaaa gggagatgga attccctgtg
      301 gagcagtgaa atgcgtaaat attcggaggg ccacccgtgg ggaagggcat ccctggacc
      361 ggtactgaac ctcatgctcg aatccctggg gagccaaccg ggtttgatac cctggtaagc
      421 cccccctaa aatatggcgc ctgggtgtgg gggggatttg tcctcgttct cggagc
//


LOCUS       GQ463191              446 bp    DNA     linear  ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A34 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 agcctgatcc agccatgccg cgtgagtgaa gaaggccttc gggttgtaaa gctctttcgg
       61 acggaaagaa agggcgcggg ctaatatccc gggggggttga cggtacccta agaagaagcc
      121 ccggctaact acgtgccagc agccgcggta atacgtaggg tgcgagcgtt aatcggaatt
      181 actgggcgta aagcggggggc aggcggtttt gtaagactgg tgtgaaatcc ccgggcttaa
      241 cctgggaact gcctttgaaa ctggggggct tgagtgcggg aaaggggagt ggaattcccc
      301 gggtagcagt gaaatgcgta aaaatgcgga ggaacaccgg tggggaaggg ggccctctgg
      361 gccggtcctg cgctcatgg tcaaaaacct ggggaaaaaa caggatttga tacccgggca
      421 tttcccgccc taaacaaaat caacta
//


LOCUS       GQ463192              500 bp    DNA     linear  ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A35 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 cctgatgcag caacgccgcg tgcgggaaga aggccttcgg gttgtaaacc gctttcagca
       61 ggaacgaaac tgacggtacc tgcagaagaa ggtgcggcca actacgtgcc agcagccgcg
      121 gtgacacgta ggcaccaagc gttgtccgga tttattgggc gtaaagagct cgtaggcggt
      181 tcagtaagtc gggtgtgaaa actttgggct taacccagag cctgcatccg atactgctgt
      241 gacttgagtt cggtagggga cgggggaatt cctagtgtag cggtgaaatg cgcagatatt
      301 aggaggaaca ccggtggcga aggcgctgct ctgggccgaa actgacgctg aggagcgaaa
      361 gcgtgggtag caaacaggat taaaacccct ggtagtccac gccgtaaaca ttggggacta
      421 ggggtgggga tcaaccaacg gaatcggcgc cgtcgctaaa gcattaattg ccccgcctgg
      481 ggagtacggc ggcaaggtta
//
```

```
LOCUS       GQ463193               486 bp    DNA     linear  ENV 06-AUG-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band A36 16S ribosomal RNA
            gene, partial sequence.
ORIGIN
        1 aagcctgatc cagccatgcc gcgtgcagga tgaaggcctt cgggttgtaa actgcttttg
       61 tacggaacga aaaggtcctg gctaataccc ggggctgatg acggtaccgt aagaataagc
      121 accggctaac tacgtgccag cagccgcggt aatacgtagg gtgcaagcgt taatcggaat
      181 tactgggcgt aaagcgtgcg caggcggtgg tgtaagacag atgtgaaatc cccgggctca
      241 acctgggaac tgcatttgtg actgcattgc tggagtgcgg cagaggggga tggaattccg
      301 cgtgtagcag tgaaatgcgt agatatgcgg aggaacaccg atggcgaagg caatcccctg
      361 ggcctgcact gacgctcatg cacgaaagcg tggggagcaa acaggattag ataccctggt
      421 agtccgcgcc ctaaacgatg tcaactggtt gttgggcctt cactgactca gtaactaagc
      481 taacgc
//
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BIOGRAPHY

Miss Uratchwee Unhalekhaka was born in Bangkok, on March 28[th], 1976. After graduated from high school, she attended Chulalongkorn University and received B.Sc. degree in Microbiology in 1998. She furthered her M.Sc. study in Industrial Microbiology at the same university and graduated in 2002. During year 2003-2005, she worked as a food inspector at Overseas Merchandise Inspection Co., Ltd. and Laboratory Center for Food and Agricultural Products Co., Ltd. in microbiological analysis section. Thereafter, she studied further in Ph.D. at Chulalongkorn University and changed her major into Interdisciplinary program of Environmental Science in year 2005.

Research presentations and publications

Unhalekhaka, U. and Kositanont, C. 2008. Distribution of Cadmium in Soil around Zinc Mining Area. In *Proceedings of the First National Conference in Toxicology: World Perspective in Toxicology* by Toxicological Society of Thailand. 17-18 November, Twin Tower Hotel, Bangkok, Thailand. (Poster presentation)

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