Toxicity Assessment of PAHs and Metals to Bacteria and the Roles of Soil Bacteria in Phytoremediation of Petroleum Hydrocarbons

by

Wenxi Wang

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including

any required final revisions, as accepted by my examiners. I understand that my thesis may be

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Abstract

Petroleum hydrocarbons (PHCs) are a class of ubiquitous contaminants in the environment. PHCs impact to soil and water occur at well sites, refineries, service stations, and other facilities. Petroleum processing and consumption of petroleum products lead to the further release of other PHC pollutants such as Polycyclic Aromatic Hydrocarbons (PAHs) as well as metals. PHCs are causing serious environmental problems due to their widespread use. Hence, the central theme of this thesis addresses hydrocarbon pollutants and co-contaminating metals: their occurrence in environment, their mechanism of toxicity and their remediation via biological processes. This thesis is divided into two parts including seven chapters.

The first part includes chapters 2, 3 and 4. Using bioluminescent bacterium *Vibrio fischeri*, a widely used bioindacator in environmental toxicology, the individual and mixture toxicities of phenanthrenequinone (PHQ), an oxyPAH, combined with copper and cadmium were assessed. PHQ is a main photoproduct of phenanthrene (PHE), a dominant PAH in the environment. Results showed that PHQ is much more toxic than its parent PAH. PHQ, alone or as mixtures with Cu and Cd, damages bacterial cells via enhancing production of reactive oxygen species (ROS). The mixture toxicity of Cu/PHQ was found to be dependent on the ratio of each chemical in the mixture. Two up-regulated genes, protein translocase subunit SecY gene and putative polysaccharide export protein YccZ precursor, were identified to be possibly response to PHQ exposure. Both genes are related to the detoxification of ROS.

The second part of this thesis includes chapters 5, 6, and 7. Culture-dependent and -independent approaches were employed to evaluate the roles of bacteria as biodegraders or/and

plant growth promoters during phytoremediation at a petroleum land farm (PLF) with a PHC concentration of ~130 g Kg^{-1}. A plant growth promoting rhizobacteria (PGPR) enhanced phytoremediation system (PEP) was applied to remediate PLF soil. PEP promotes plant growth to establish dense vegetative cover. Results of both culturing and molecular methods showed that the enhanced populations and activities of soil microbes due to vigorous plant growth is a key factor in the success of PEP. Introduced PGPR could quickly establish significant populations by utilizing root exudates and dominate the PGPR population on seed coat and root surfaces at the early seedling stage of plant development, and thus modestly affected bacterial community structures at this time; thereafter, with plant growth, the effect of seed treatment on soil microbial community was masked by enhanced indigenous microbial population. Therefore, the introduced PGPR did not exert significant influence on the indigenous microbial ecosystem. It does dramatically improve plant growth and PHC remediation. Thus, the PEP should be considered as an environmentally safe and effective approach for removing PHCs from impacted soils.

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Abbreviation

BTEX: Benzene, toluene, ethylbenzene, and xylene

DGGE: Denaturing gradient gel electrophoresis

F1: Fraction 1 of petroleum hydrocarbons which contain C6-C10

F2: Fraction 2 of petroleum hydrocarbons which contain C10- C16

F3: Fraction 3 of petroleum hydrocarbons which contain C16-C34

F4: Fraction 4 of petroleum hydrocarbons which contain C34-C50

H_2O_2: Hydrogen peroxide

HO•: Hydroxyl radical

O_2^-: superoxide

PEP: Plant growth promoting rhizobacteria (PGPR) enhanced phytoremediation system

PAHs: Polycyclic aromatic hydrocarbons

PGPR: plant growth promoting rhizobacteria

PHCs: Petroleum Hydrocarbons

PHE: Phenanthrene

PLF: Petroleum land farm

PHQ: Phenanthrenequinone

qPCR: Quantitative polymerase chain reaction

ROS: Reactive oxygen species

SOD: Superoxide dismutase

UW4: one strain of plant growth promoting rhizobacteria *(Pseudomonas putida)*

Chapter 1 General Introduction

Environmental toxicology is a rapidly developing field. There is increased work in this area because many environmental issues have to be addressed. Research has focused on distribution and fate of pollutants within the environment and the harmful effects of pollutants on the constituents of ecosystems, including living organisms and man (Yu 2005). While the science of environmental toxicology is relatively new, it evolved from human or classical toxicology that has a longer history (Wright and Welbourn 2002). Perhaps the best-known pioneer in the field of toxicology is the Swiss physician Paracelsus (1499–1541) who proposed the idea of dose response, which has become a cornerstone of toxicological research.

The development of environmental toxicology began during the last century, when a large amount of chemicals intended to improve agriculture and daily life came into use. The most common chemical contaminants are pesticides, metals, and crude oil along with related petroleum products. Many of them have adverse effects on ecosystems and human health. As early as the 1870s, there were signs that arsenic from pesticides and lead from lead shot were causing wildlife deaths (Beyer et al. 1996). The first historical event that drew the world's attention to environmental hazards was the outbreak of itai-itai-byo or ouch-ouch disease caused by high concentrations of cadmium (Cd) in rice and drinking water in Japan in 1945 (Yu 2005). At that point, awareness that increasing industrialization was having adverse effects on species other than humans was becoming clear.

Public awareness of environmental toxicity was greatly increased by the publication of Rachel Carson's book 'Silent Spring' in 1962 (Carson 1962), in which she addressed the problems caused

by DDT and other anthropogenic chemicals. Intense and repeated applications of insecticides such as DDT exerted significantly adverse effects on ecosystems because of its very persistent, lipophilic properties that lead to their bioaccumulation and bio-amplification. Increasing human activities lead to continuous releases of pollutants from industry and personal use into the environment, which seriously threatened organisms in the environment. Large amounts of xenobiotic chemicals have been discharged into various environmental compartments, including soil, water and the atmosphere. Therefore, research on the toxicity of these xenobiotic chemicals and their eventual remediation are becoming critical for our environmental sustainability.

Petroleum hydrocarbons (PHCs) are one of the most prevalent contaminants in the environment. PHC impacts to soil and water occurs at well sites, refineries, service stations, and other facilities such as petroleum land farm sites. Petroleum processing and consumption of petroleum products leads to the further release of other hydrocarbon pollutants such as Polycyclic Aromatic Hydrocarbons (PAHs) as well as metals. Petroleum hydrocarbons are causing serious environmental problems due to their widespread use. Hence, the central theme of this thesis addresses hydrocarbon pollutants and co-contaminating metals: their occurrence in environment, their mechanism of toxicity and their remediation via biological processes.

1.1 Petroleum hydrocarbons

Petroleum is considered essential for our industrialized society. Crude oil was formed and deposited beneath the earth's crust millions of years ago. It is derived from marine animal and plant debris subjected to high temperature and pressure (Matar and Hatch 2001). These original deposits were slowly reduced under anaerobic conditions, forming crude oil through long and

complex chemical processes. Crude oil mainly consists of hydrocarbons mixed with variable amounts of sulfur, nitrogen, and oxygen (Matar and Hatch 2001).

PHCs are obtained from naturally occurring-reservoirs of crude petroleum (Merkl et al. 2006). They fall into three major groups: alkanes (paraffins), alkenes (olefins), and aromatics (Fig. 1.1) (Potter and Simmons 1998). Alkanes are one of the major constituents of crude oil and refined petroleum products such as gasoline, kerosene, diesel fuel, heating oil, etc. There are three major classes of alkanes (Potter and Simmons 1998), i.e. linear alkanes or n-alkanes, branched alkanes, and cycloalkanes. Linear alkanes are always present in large proportion in crude oil, except where biodegradation has occurred (Ollivier and Magot 2005). Branched alkanes have some branched carbons, thus creating many differing configurations. Cycloalkanes are molecules in which the carbon atoms are arranged in one or more rings, but do not contain aromatic rings. Alkenes are linear molecules with one or more double bonds. Most of alkenes are produced during the refining process of creating various petroleum products from crude oil (Ollivier and Magot 2005).

Aromatics are characterized by the presence of at least one benzene ring. The well-known examples of 1 ring (or mononuclear) aromatics are benzene, toluene, ethylbenzene, and xylene (BTEX), and they are important components of gasoline. Polycyclic aromatic hydrocarbons (PAHs) consist of multiple, fused aromatic rings. Examples of these are naphthalene, anthracene, pyrene, and many more.

Based on carbon chain length or molecular weight, PHCs were classified into four fractions by Canadian Council of Ministers of the Environment (CCME) as part of the Canada Wide Standards for Petroleum Hydrocarbons in soil (CWS-PHC) (CCME 2001). They are fraction 1 (F1) containing C6-C10, fraction 2 (F2) containing C10- C16, fraction 3 (F3) containing C16-C34 and

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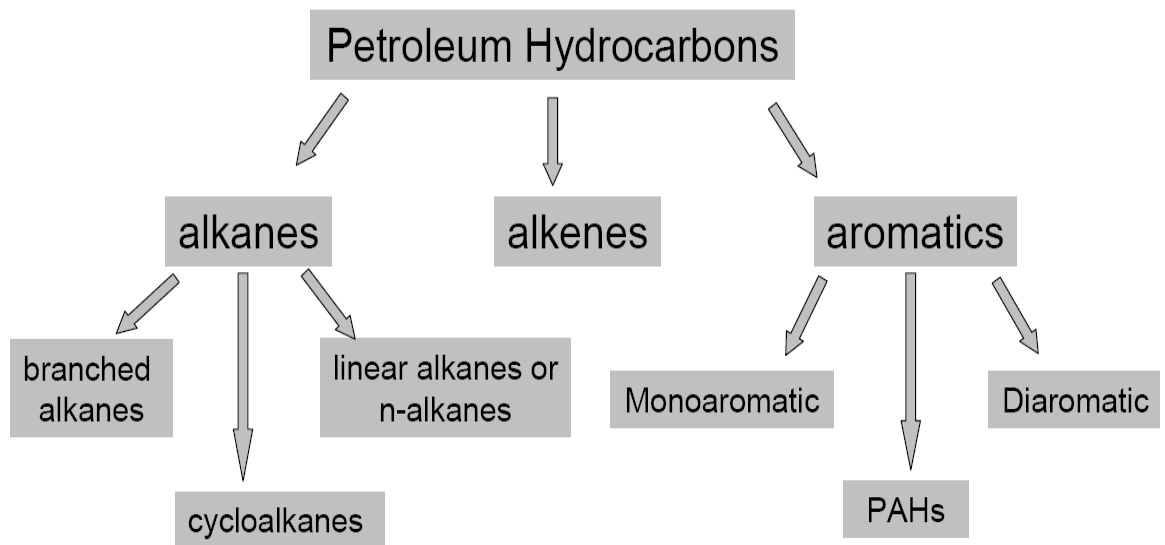
Figure 1. 1 Hydrocarbon structure relationships

fraction 4 (F4) containing C34-C50 (CCME 2001; USEPA 1994). This classification was established as an endpoint for analysis of PHC contaminants in environments. F1 – F4 are essentially identical to the respective VPH (volatile PHCs), LEPH (light extractable PHCs), HEPH (heavy extractable PHCs) fractions and EHEPH (extremely heavy extractable PHCs).

The mechanisms of toxicity associated with these fractions differ. The lighter fractions (F1-F2) have relatively high water solubility and bioactivity, thereby cause acute toxicity. F3 and F4 are a complex mixture of aromatic, aliphatic, heterocyclic and asphaltene hydrocarbons that are very hydrophobic and recalcitrant to breakdown. Some compounds from F3 such as PAHs are often highly toxic and are regulated due to their mutagenicity and carcinogenicity (CCME 2003).

1.2 Petroleum sludge

Most PHCs are consumed as fuels, 65% of these as gasoline (CEC 2004). Crude oil, the source material for nearly all petroleum products, contains a wide variety of elements combined in various forms (Matar and Hatch 2001). Crude oil needs to be refined through distillation to crack the large molecules into smaller ones, which leads to a variety of products, such as gasoline, diesel fuel, heating oil, etc. However, not everything in crude oil can be converted to useful products; the remainder from the refining process is referred to as sludge (Elektorowicz and Habibi 2005). It is estimated that at a typical refinery produces 30 000 tons of sludge each year (USEPA 1991).

Petroleum sludge consists of thousands of compounds of which about 250 have been identified to date (Hejazi et al. 2003; Weisman 1998). The nature of industrial sludge depends on the source of crude oil and the refinery process (Hutchinson et al. 2001). It could contain not only organic and inorganic matter, but also oil and grease, nutrients such as nitrogen and phosphorus, heavy metals

and organochlorine compounds (Asia et al. 2006; Lin and Hsiu 1997). Usually, petroleum sludge is comprised of around 10-50% hydrocarbons, 20-80% water, 5-20% solids, 0.5-1% sulfur, 0.1-1% metals and ash materials (Al-Futaisi et al. 2007; Asia et al. 2006). The main hydrocarbons are alkanes, alkenes, aromatics, asphaltics, phenols and PAHs (Shailubhai 1986). The principal metallic constituents of refinery waste are zinc, lead, copper, chromium, mercury, vanadium, and nickel (Shailubhai 1986). Among the components of sludge, hydrocarbons such as PAHs and BTEX (benzene, toluene, ethylbenzene, and xylenes) and metals are known to be as ubiquitous and carcinogenic pollutants in environment (Douben 2003). The discharge of sludge without further treatment leads to environmental contamination.

Sludge disposal is a worldwide problem and a wide variety of disposal routes have been adopted as dictated by local conditions (Asia et al. 2006). Petroleum land farming is one of major methods to treat the petroleum sludge produced by refineries (USEPA 1994). With this method, sludge is directly spread on soil where it is tilled regularly. Land farming is very effective at volatilizing water and small volatile molecules such as BTEX, but can result in high level of air pollution (USEPA 1994). After volatilization of small hydrocarbon molecules (i.e F1 and F2) through land farming, fraction F3 and F4 such as PAHs, metals, and aliphatic accumulate to a relatively high concentrations (Maila and Cloete 2004). Hence, the soil used in petroleum land farming for long period of time are characterized by relatively high concentrations of F3 and F4, as well as metals (Hutchinson et al. 2001).

1.3 Fate of petroleum hydrocarbons (PHCs) in soil

PHCs are a mixture of alkanes, alkenes, alkynes and aromatics (Potter and Simmons 1998). When petroleum products are released into soil, changes in product composition takes place based on weather and soil conditions. The main factors that affect the fate of PHCs are weathering processes and biodegradation. The main weathering processes are dissolution in water, volatilization, and photodegradation (Potter and Simmons 1998).

Different components of PHCs have different fates in soil. Increasing molecular weight leads to decreasing volatility and solubility, and relative persistence in soil (Ollivier and Magot 2005). More water-soluble and volatile chemicals such as fractions of F1 and F2 are lost rapidly by leaching, run off and volatilization (Dror et al. 2002). Hence, in PHC contaminated sites, the remaining components are mostly higher molecular weight compounds (i.e., F3 and F4).

Photochemical degradation of PHCs mediated by sunlight is also an important pathway for transformation of PHCs when they are in the environment, especially for oil rich in aromatics. Photodegradation of crude oil is caused by direct photochemical processes and heterogeneous photocatalysis. Aromatic compound are photodegraded faster than the other classes of hydrocarbons in oils (Ziolli and Jardim 2003).

Biodegradation is another significant factor that affects the fate of PHCs. In the case of PHC contaminated sites, biodegradation is the most important natural process for contaminant reduction (Khan and Husain 2003). Considering the immense variety of metabolic pathways of soil microorganisms, petroleum degrading microorganisms generally exist in PHC contaminated soils. Under the selective pressure of PHCs, populations of PHC-degrading microorganism gradually

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become dominant in response to these changing ecological niche with high PHC concentration (Maila et al. 2006). In the soil, the degradation rate depends on the molecular weight and structure of the contaminants. For PHCs, the trends in degradation rates according to structure are: (1) *n*-alkanes, especially in the C10 to C25 range which are degraded readily, (2) isoalkanes which are degraded more slowly, (3) BTEXs which are metabolized when present in concentrations that are not generally toxic to the microorganisms, (4) PAHs which degrade more slowly than monoaromatics, and (5) higher molecular weight cycloalkanes which are very slow to nbe degraded (Potter and Simmons 1998).

1.4 Polycyclic aromatic hydrocarbons (PAHs)

1.4.1 Occurrence in the environment

PAHs constitute a significant fraction of PHCs. They contain three or more fused benzene rings (Fig. 1.2). PAHs can be formed naturally by low-temperature, high-pressure reactions of natural organic matter. Steel productions, as well as incomplete combustion of petroleum, wood and coal are responsible for some of their formation (Eisler 1987). The largest emission of PAHs results from industrial processes and other human activities. The petroleum industry is an important source of PAHs, especially in the vicinity of petroleum refinery facilities. Waste water and soil in these areas are often characterized by PAH concentrations 100 times higher than in other areas (Douben 2003).

PAHs are found in air, soil, vegetation, water and ice (Holoubek et al. 2000). Due to their highly hydrophobic nature, PAHs are usually associated with particulate matter such as air borne
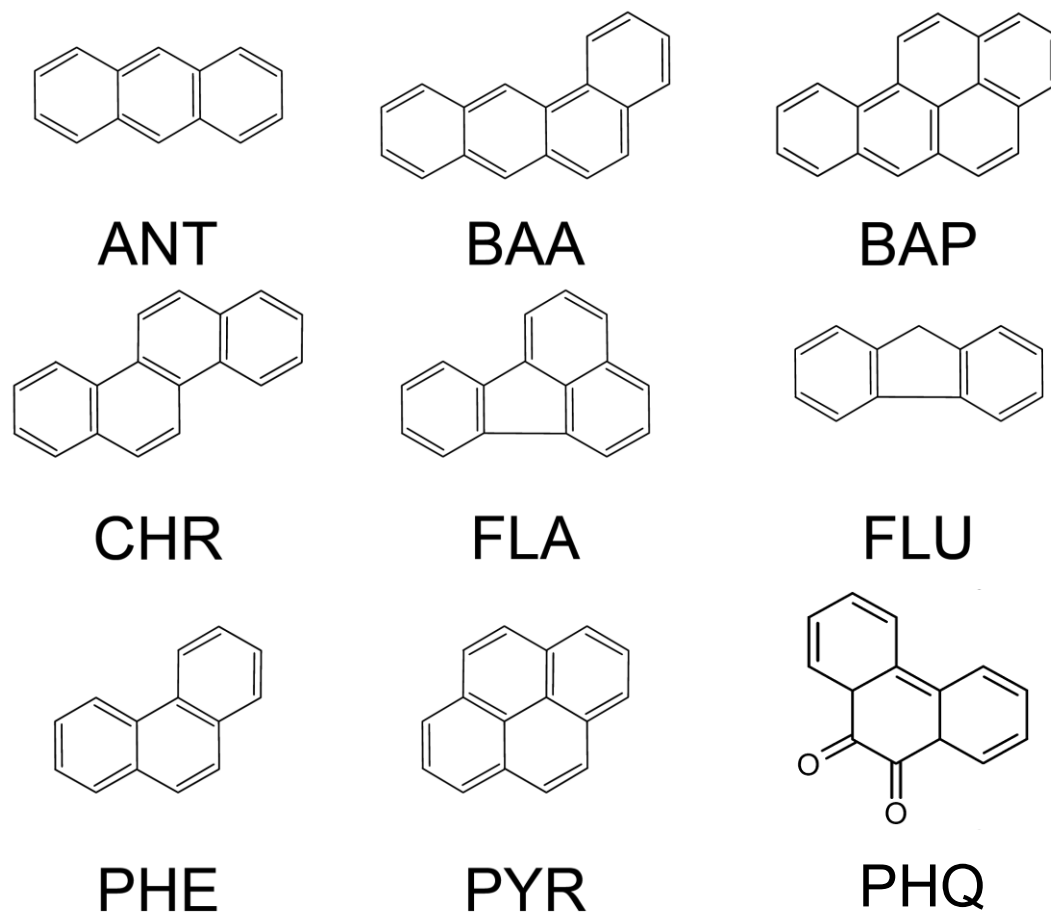
Figure 1. 2 Structure of several common PAHs and oxyPAH.

Abbreviations as follows: ANT, anthracene; BAA, benz[a]anthracene; BAP, benzo[a]pyrene; CHR, chrysene; FLA, fluoranthene; FLU, fluorene; PHE, phenanthrene; PYR, pyrene; PHQ, phenanthrenequinone.

particles, soil and sediments (Lampi et al. 2006). PAHs were also found in remote areas far from the emission sources (e.g. the arctic) (Sehili and Lammel 2007). Sediments in aquatic environments function as a reservoir for PAHs (Ankley et al. 1994). Owing to their widespread distribution, almost all organisms are exposed to PAHs. PAHs have been detected in marine (O'Connor 2002) and terrestrial organisms (De Maagd and Vethaak 1998). For example, fluorene, phenanthrene, anthracene, fluoranthene, benz(a)anthracene, benzo(b)fluoranthene and benzo(k)fluoranthene were detected at various concentrations in bivalves (*Mitylus galloprovincialis*), cephalopods (*Todarodes sagittatus*), crustaceans (*Nephrops norvegicus*), and fish (*Mullus barbatus, Scomber scombrus, Micromesistius poutassou, Merluccius merluccius*) in the Central Adriatic Sea (Perugini et al. 2007).

1.4.2 PAH risks to ecosystems

The United States Environmental Protection Agency (USEPA) has identified 16 priority PAHs due to their acute or chronic toxicity to mammals and other organisms (Lampi et al. 2006). Some PAHs have been classified as probable human carcinogens (Yu 2002) and show tumorigenic activity in mammals (Cavalieri and Rogan 1995) and fish (De Maagd and Vethaak 1998). Their toxicity, environmental persistence and widespread occurrence have made PAHs a pollutant class of global concern.

In addition to the parent PAHs, PAHs can undergo structural changes when they are discharged into the environment. The fate of PAHs in the environment and the routes of biological exposure are influenced by the environmental compartments (air, water, soil) in which the PAHs reside, and the light regimes to which PAHs are exposed. Although a number of possible routes of activation

exist (Krylov et al. 1997), one of the most important in the environment is the photoactivation by sunlight. The toxicity of PAHs to aquatic organisms can be enhanced by exposure to sunlight, especially ultraviolet radiation (Huang et al. 1993; Mallakin et al. 1999; McConkey et al. 1997; Newsted and Giesy 1987).

Photoinduced toxicity of PAHs is derived from two photochemical processes: photosensitization and photomodification (Krylov et al. 1997; McConkey et al. 1997; Newsted and Giesy 1987) . During photosensitization reactions, intracellular singlet-state oxygen ($^1O_2$) and other ROS are generated, which can cause oxidative damage in biological systems (Foote 1987). In the case of photomodification, PAHs are structurally altered to a variety of compounds, most of which are oxygenation products (oxyPAHs). Many of these photoproducts are more toxic than their parent PAHs (Huang et al. 1993; Huang et al. 1995; McConkey et al. 1997; Xie et al. 2006).

Photomodified PAHs are widely distributed in the environment. They have been found in PAH mixtures in sediments (Ankley et al. 1994; Ankley et al. 1995; Davenport and Spacie 1991; Douben 2003), air and water (Kosian et al. 1999; Lampi 2005; Marvin et al. 1999).

The bioavailability of PAHs is important when considering their toxic effects in the environment. Intact PAHs have low water solubility and they tend to adsorb to sediment particles in aqueous environments resulting in low accessibility to some aquatic plants and animals (Basu and Saxena 1978; Cook et al. 1983; Lampi et al. 2006). When PAHs are exposed to sunlight, however, they are rapidly photomodified to products that are generally more water soluble, and therefore more bioavailable to aquatic organisms. This is one reason oxyPAHs have greater toxicity. For instance, the EC50 of phenanthrenequinone (PHQ), the major photoproduct of phenanthrene (PHE), is well below its solubility limit and considerably more toxic than PHE (El-

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Alawi et al. 2001; McConkey et al. 1997; Xie et al. 2006). Sediment samples from PAH contaminated sites are more toxic under UV radiation than under just visible light (Ankley et al. 1994). Weathered crude oil exhibits photoactivated toxicity to a variety of species, probably due to the formation of oxyPAHs (Calfee et al. 1999; Cleveland et al. 2000; Little et al. 2000; Pelletier et al. 1997). Thus, PAHs and oxyPAHs pose a real risk to the environment.

PAHs are not evenly distributed in environmental samples. PAH composition patterns are dominated by the presence of PAHs with 3 rings (62%), followed by those with 4 rings (37%) and 5 rings (1%) in water samples from the Central Adriatic Sea (Perugini et al. 2007). PHE, a three-ring PAH, is one of the most common PAHs, accounting for 54% of total PAHs in some samples (Shimada et al. 2004). PHE is readily converted to PHQ under solar radiation (El-Alawi et al. 2001; El-Alawi et al. 2002b; McConkey et al. 1997). The half life of PHE is 48 h under low levels of simulated solar radiation, and it is converted mostly to PHQ (McConkey et al. 1997). It is thus believed that PHQ is widely distributed in the environment. PHQ is an important component of exhaust gas, comprising up to 12 % of total PAH quinones in the diesel exhaust particles (Xia et al. 2004; Xie et al. 2006).

The toxicity of oxyPAHs, such as the quinones, is due in large part to redox cycling between quinone and its associated semiquinone (Hasspieler and Digiulio 1994). Many quinones have the potential to induce production of reactive oxygen species (ROS) with PHQ and naphthoquinone ranking among the highest (Lemaire and Livingstone 1997; Xie et al. 2006). In the presence of molecular oxygen, the semiquinone may autooxidize and transfer electrons to oxygen, yielding the parent quinone and superoxide anion (an ROS). Superoxide may in turn lead to the formation of other toxic ROS, such as H_2O_2 and OH· (Hasspieler and Digiulio 1994; Lind et al. 1982). In

another example, 9,10-phenanthrenequinone (PHQ) has been demonstrated to stimulate oxyradical production in mammalian tissues and channel catfish (Chesis PL 1984; Hasspieler and Digiulio 1994; Nykamp 2007; Nykamp et al. 2001).

1.4.3 Biodegradation of PAHs

PAHs in different environments face different fates. Air-borne PAHs are present in the gaseous form or associated with particles. The major routes of PAH loss from the atmosphere are photolysis, deposition on soil, plant surfaces and desolved in water (Atkinson and Arey 2007). Soil is a major receptor of PAHs and biodegradation by microorganisms is the major pathway of PAH loss in the soil environment.

With PAHs occurring naturally in the environment, it is not surprising that many PAH degrading microorganisms inhabit the soil. PAH metabolizing microorganisms have been isolated from soils that can degrade PAHs from two rings (i.e., naphthalene) up to seven rings (i.e., Coronene) (Dean-Ross et al. 2002; Eaton 1997; Juhasz and Naidu 2000; Juhasz et al. 2002; Kanaly and Hur 2006; Meade et al. 2002; Takizawa et al. 1994; Vila et al. 2001). Most of the known, isolated bacteria only readily degrade 2 or 3 ring PAHs. The greater the number of PAH aromatic rings, the fewer the corresponding numbers of degrading microorganisms, and few microorganisms have been found that can degrade PAHs with more than 5 rings (Juhasz and Naidu 2000). One reason for this may be the low bioavailability of large PAHs due to their extreme hydrophobicity. Thus, PAHs of small molecular weight persist longer than smaller ones (Johnsen and Karlson 2005; Johnsen et al. 2005).

The initial step of PAH degradation in microorganisms is by diooxygenase or monooxygenase

enzymes, in which PAHs are oxidized, to form cis-dihydrodiols. These then undergo re-aromatization by dehydrogenases to form diphenolic intermediates. These in turn undergo ring cleavage to form tricarboxylic acid (TCA)-cycle intermediates (Zhang et al. 2006). For PAHs with more than 5 rings, such as BaP, their biotransformation can occur via co-metabolic pathways in which co-substrates are needed to induce the PAH biodegradation pathways (Kanaly and Bartha 1999; Kanaly et al. 2000).

Almost all the PAH biodegradation research has been done in the laboratory, where much higher than environmental concentrations of PAHs are used and PAHs are the sole carbon sources. For example, many laboratory-based screening experiments of PAH degrading microorganisms use 50 mg/L of PHE as the sole carbon source (Wong et al. 2004). This is more than 100 times higher than its environmental concentration even in heavily PAH contaminated soils. Furthermore the high PAH concentration make for an even more unrealistic scenario in soil as soil bacteria are generally carbon and energy-starved in the environment (Elsas et al. 2007). Soils have multiple carbon sources, microorganisms will choose the more favorable energy sources and suppress gene expression for other carbon sources via substrate suppression mechanisms, or they may co-metabolize a number of available carbon compounds (Phillips et al. 2006). In this case, bioavailability limits the degradation rates of PAHs. In soil, most of PAHs are absorbed to or are trapped inside soil particles (Burgess et al. 2003). Sizes of some fine particles such as silt and clay can be less than 0.02 mm and 0.0015 mm (Owabor and Ogunbor 2007), respectively. The pore sizes among these fine soil particles is often less than the size of bacterial cells and are therefore inaccessible to bacteria. Thus, the degradation of PAHs trapped inside pores depends on their diffusion to the bacteria. The PAH degrading bacterial number may fluctuate with the water

content, which can change the PAH bioavailability by solvating PAHs.

Bacterial mobility is also an important factor influencing PAH biodegradation. Bacteria with chemotaxis ability have an advantage for biodegradation. Various bacteria such as *Pseudomonas putida* have been shown to move toward higher concentrations of PAHs in soil, enhancing biodegradation rates (Grimm and Harwood 1999).

PAHs also can been accumulated and degraded by plants and other soil organisms, such as earthworms (Parrish et al. 2006). *Cucurbita pepo ssp. pepo* (zucchini), *Cucumis sativus* (cucumber), *Cucurbita pepo ssp. ovifera* (squash) and ryegrass (*Lolium perenne*) can take up significant quantities of PAHs up to the six rings in size. PAHs have been detected in plant roots, shoots and leaves, with the highest concentrations in roots (Parrish et al. 2006; Xu et al. 2005). The plant peroxidases and cytochrome P450s are thought to be responsible for the biotransformation of PAHs in plants (Chroma et al. 2002).

1.5 Metals

Metals are elements found in all parts of the environment and are released into environment from both natural and anthropogenic sources. Although metals are natural components of the environment, and can be ubiquitously present, anthropogenic activities are responsible for the highest levels of metals in environment (Nriagu and Pacyna 1988).

Some of these elements are essential for life, while others have no known biological function. Metabolic metals are part of many structural proteins and enzymes, and play a critical role in physiology (Gambling et al. 2004; Mehta et al. 2006). Some metals are toxic to microbes, plants and animals. All metals, even those that are metabolically essential (i.e., Cu), are toxic in excess.

Metals that are non-metabolic often interfere with the functions of physiologically required metals, accounting for some of their toxicity (Achard-Joris et al. 2007). The adverse effects of metals on human health, including lung diseases (cobalt and nickel), itai-itai disease (cadmium) and Minamata disease (methylmercury), are well documented (Ariza et al. 1999). Metals exert their toxic effects in biological systems through three key mechanisms: displacement of biologically functional metals, binding to specific cellular macromolecules and induction of oxidative stress in cells (Achard-Joris et al. 2007; Akhtar 2004).

1.5.1 Copper

Copper is an abundant element making up approximately 0.1 % of earth's crust. It is a reddish, ductile and malleable transition metal in group B of the periodic table. It has two oxidation states, Cu^+ (cuprous ion) and Cu^{2+} (cupric ion). Acting as an electron donor or acceptor, Cu is an essential metal in many enzymatic reactions (Gambling et al. 2004; Mehta et al. 2006). It is a co-factor in numerous enzymes, including alcohol dehydrogenase, phosphatases, catalases, cytochrome C oxidase, peroxidases and superoxide dismutase (Brazeau et al. 2004; MacPherson and Murphy 2007; Wilmot 2003). Copper has low toxicity to mammals and terrestrial vertebrates (Uriu-Adams et al. 2005). The recommended daily allowance of copper in humans is 2 mg (Ariza et al. 1999). However, excess copper is extremely toxic to invertebrates, plants and microorganisms. It can affect the physiological status of these organisms and can be acutely toxic at nanomolar concentrations (Atienzar et al. 2001; Babu et al. 2001).

Copper ions are usually bound in biological systems to ceruloplasmin and metallothionein (Achard-Joris et al. 2007; Mehta et al. 2006). Unbound copper ions are toxic largely due to its

redox cycling between two valence states, which induces oxidative stress (Halliwell and Gutteridge 1992). This redox cycling is a feature of transition metals that leads to oxidative stress, resulting in the formation of reactive oxygen species (ROS) (Carter 1995). Cu-induced ROS production is the basis of Cu toxicity that includes lipid peroxidation (Baryla et al. 2000; Xie et al. 2006), inhibition of photosynthesis (Babu et al. 2001), mitochondrial dysfunction (Arciello et al. 2005) and destabilization of lysosomal membrane (Pourahmad et al. 2001).

One of key toxicity mechanisms of Cu and other redox metals is to produce hydroxyl radical (HO•), the most toxic ROS, by the Haber-Weiss reaction (Haber and Weiss 1934). In most biosystems, Cu ions can bind cellular proteins such as thiol and carboxyl groups (Prohaska and Gybina 2004), being reduced by cellular antioxidants, such as ascorbic acid or glutathione, or by O_2^- (Suntres and Lui 2006). The reduced metal may react with H_2O_2 to produce HO• (Masad et al. 2007). Hence, hydrogen peroxide (H_2O_2) plays an important role in Cu toxicity (Kim and Metcalfe 2007).

$$ROOH + Cu^{2+} \longrightarrow ROO^. + Cu^+ + H^+$$

$$Cu^{2+} + O_2^- \longrightarrow Cu^+ + O_2$$

$$Cu^+ + H_2O_2 + H^+ \longrightarrow Cu^{2+} + H_2O + HO•$$
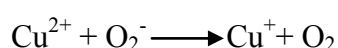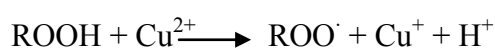
Hydroxyl radicals (HO•) produced by the Haber-Weiss reaction have a short half life (10^{-9} seconds). It is highly reactive and very harmful to biological organisms. The hydroxyl radical reacts nonselectively with other molecules that are close to its site of production in vivo at higher rate constants (10^8–10^9 M^{-1}/s^{-1}) (Horwell et al. 2007), leading to chain reaction of oxidation of lipids in

cell membranes (Chevion 1988). Due to the amplification effect of redox cycling, only trace amounts of metal are necessary for damage to occur via HO• (Ariza et al. 1999; Levine et al. 1998).

1.5.2 Cadmium

Cadmium is a hazardous environmental contaminant, widely distributed in the environment due to its widespread use in industries such as battery production and electroplating (Liu et al. 2007). It has been shown that Cd can be bioconcentrated owing to its long half-life in organisms (10-30 years) (Xie 2005). Due to industrialization, the frequency of biological exposure to Cd is on the rise (Li and Lim 2007). The toxicity of Cd to various organisms has been studied extensively. In animals, it is very damaging to many organs, such as kidneys, liver, lungs, testis, bones, the blood system and the central nervous system (Lopez et al. 2007; Lopez et al. 2006). In plants, Cd accumulation alters membrane function by affecting lipid composition (Leon et al. 2002), enzyme activity (Fodor et al. 1995), photosynthesis and chlorophyll stability (Burzynski and Zurek 2007; Kupper et al. 2007). For instance, uncoupling of electron transport in chloroplasts and perturbation of the carbon reduction cycle has been observed (Lees 2005). Inhibited plant shoot and root growth (Devi et al. 2007; Leon et al. 2002) and toxicity on microorganisms (Achard-Joris et al. 2007) have also been observed.

Some evidence suggests that the excessive production of reactive oxygen species (ROS) is the major toxicity mechanism of Cd in plants (Krantev et al. 2007), animals (Amara et al. 2007; Xie et al. 2006) and bacteria (Achard-Joris et al. 2007). The ability of cadmium to induce ROS formation has been described by many authors in different cells types (Amara et al. 2007; Hansen et al. 2006; Krantev et al. 2007; Lin et al. 2007; Liu et al. 2007). Unlike Cu, Cd is not thought to catalyze

Fenton-type reactions leading to ROS production because it does not readily cycle between redox states (Waisberg et al. 2003). Cd inhibits activity of antioxidative enzyme such as superoxide and catalase can lead to enhanced ROS production. Superoxide dismutase (SOD) and catalase are two of the most important antioxidative ROS scavengering enzymes. They are involved in the detoxification of O_2^-, and hydroxyl peroxide (H_2O_2), respectively, thereby preventing the formation of HO• radicals. Cd^{2+} can also decrease intracellular glutathione levels which play a critical role as cellular antioxidants by scavenging ROS (Elia et al. 2007). Therefore, Cd exposure may directly or indirectly result in ROS production by affecting SOD and catalase activity or reducing intracellular glutathione levels, respectively (Martelli and Moulis 2004).

1.6 Synergistic effects of oxyPAHs and metals

Metals and polycyclic aromatic hydrocarbons are co-contaminants in many environments (Babu et al. 2001; Lahr et al. 2003). In a study by Lahr et al. (Lahr et al. 2003), more than 80% of these toxicity data could not be explained by the toxicity of single compounds. Most samples analyzed in these experiments contained both metals and PAHs. Most petroleum hydrocarbon (PHCs) contaminated sites contain mixtures of metals and PAHs. Because of the rapid photomodification of PAHs such as PHE, modified PAHs are widely distributed in PAH contaminated sites (Lampi et al., 2006). Many samples with metals have higher toxicity than that predicted by their laboratory EC/LC50 data (Sarakinos et al. 2000) due to other environmental stressors such as UV radiation, temperature, pH and co-contaminants, (eg., PAHs). Thus, it is clear that metal/PAH/oxyPAH co-toxicity is a subject of environmental concern.

The mechanism of co-toxicity of metals and PAHs is believed to be ROS-mediated. It is hypothesized that the generation of ROS is involved in these co-toxicity due to the redox cycling properties of both chemicals. Several *in vitro* studies have shown that Cu can accelerate the oxidation of hydroquinone to benzoquinone (Li and Trush 1993) and catalyze redox cycling of PHQ (Yu et al. 2002), accompanied by the production of ROS (Xie et al. 2006). *In vivo* studies with several organisms such as *Lemna gibba* (Babu et al. 2001), *Daphnia magna* (Xie et al. 2006), and rat corpus luteal and human placental JEG-3 cells (Nykamp 2007; Nykamp et al. 2001), have shown concurrent increases in ROS production together with the synergistic toxicity on the target organisms. Therefore, ROS mediated synergistic toxicity might be a universal toxicity mechanism of Cu/oxyPAH mixtures. In biological systems, oxyPAHs could cause ROS production by redox cycyling (Nykamp 2007) and/or inhibited electron transport (Babu et al. 2001), while Cu could accept electrons from this compromised system and transfer the electrons to oxygen resulting in the production of ROS (Babu et al. 2001; Babu et al. 2005; Xie et al. 2006). Therefore, the presence of Cu and oxyPAHs could accelerate ROS production (Xie et al. 2006) and Cu-mediated Fenton-type reactions may occur to produce •OH (Achard-Joris et al. 2007; Babu et al. 2001). Thus, the synergistic toxicity of Cu/oxyPAHs may be due to generation of ROS, especially •OH radicals.

1.7 Use of bioluminescent bacteria to assay toxicity

Bioluminescence is defined as light emission by living organisms. Bioluminescence emitted by the bacteria has been extensively used in environmental science to monitor toxicity of many organic and inorganic chemical contaminants (Gellert et al. 1999). Methods based on

bioluminescent bacteria not only have the advantages common to all microbiologically based systems (small test organism size, large number of organisms, and convenient growth condition), but they also have the additional advantage of having an easily measured signal and very rapid response (Ren and Frymier 2003).

Eleven species bioluminescent of bacteria in four genera have been described (Meighen 1991). Most of them live in marine environments. The luminescent bacterium that has been most extensively used in environmental toxicity is *Vibrio fischeri*. *V. fischeri* is a free-living marine bacterium which lives symbiotically with squid and other marine organisms. They can colonize the light organ of squid and produce bioluminescence when they are at high cell density (Steinberg et al. 1995). In the laboratory, *V. fischeri* is cultured in media that is similar to seawater in salinity and mineral content. The growth of *V. fischeri* can be satisfied equally well by either Na^+ or K^+, whereas the development of luminescence is highly specific for Na^+ (Watanabe and Hastings 1986).

Light emission from *V. fischeri* results from the respiration electron transport chain.

$$FMNH_2 + O_2 + RCHO \xrightarrow[\text{Luciferase}]{Mg^{2+}} FMN + ROOH + H_2O + light$$
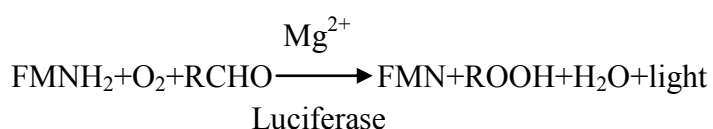
This bacterial bioluminescent system catalyzes a reaction between oxygen, a reduced flavin phosphate (luciferin), and an aldehyde (C8 to C16 straight chain) substrate which results in light and an inactive "oxyluciferin".

To monitor the toxicity to *V. fischeri*, the ability of contaminants to inhibit light emission is assayed. Light emission intensity changes with the concentration of most toxic chemicals. Thus, the EC50 is actually 50% inhibition of luminescence. Microbial bioluminescence involves

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respiration to produce substrates needed for the production of light. Because electron transport is connected to overall cell metabolism, any environmental condition that can hinder electron transport and change other related cellular processes should have an effect on bioluminescence (Steinberg et al. 1995). Factors other than the contaminant that can affect this toxicity assay are temperature, pH, the medium, and exposure time.

1.8 Phytoremediation of petroleum hydrocarbons (PHCs)

Study of remediation strategies of environmental contaminants is an important constituent of environmental toxicology. Toxicity bioassay with bacteria and other organisms serves and supports risk management decisions. The adverse effects including acute and mutagenic toxicity of PHCs have been observed in toxicological research using different organisms such as *V. fischeri*, earthworm (*Eisenia foetida*), corn (*Zea mays*), wheat (*Triticum aestivum*), oat (*Avena sativa*) (Salanitro et al. 1997a; Salanitro et al. 1997b), mummichog embryos (*Fundulus heteroclitus*) (Couillard 2002), and red blood cells (Couillard and Leighton 1993). Therefore, PHCs contamination in the environment poses clear risks, and regulations exist requiring remediation of PHCs.

Many techniques have been developed to remediate persistent organic contaminants from soils. These techniques include physical remediation, bioremediation and phytoremediation (Scullion 2006). Physical remediation includes incineration, air stripping, and thermal desorption (Singh and Ward 2004). Chemical remediation includes solvent extraction, oxidation-reduction, precipitation, neutralization, and encapsulation (Scullion 2006). Bioremediation includes bioreactors, biopiles, bioventing, bioaugmentation, landfarming, natural attenuation and biodegradation (Zhou and Hua

2004). Phytoremediation includes phytoextraction, phytostimulation, phytostabilization, phytotransformation, phytovolatilization, rhizofiltration and phytodegradation (Singh and Ward 2004). Of these methods, physical and chemical remediation is not only very expensive, but also requires the use of heavy equipment and substantial amount energy, making biologically based systems such as phytoremediation attractive.

Phytoremediation is defined as the use of plants to sequester, remove, or degrade hazardous substances from the soil (Fig. 1.3) (Glick 2003). Removal of pollutants by phytoremediation is the combination of a range of processes mediated by plants, including phytoextraction, phytostimulation, phytostabilization, phytotransformation, phytovolatilization, rhizofiltration and phytodegradation (Glick 2003; USEPA 2000). Contaminants can be extracted from soils, translocated, degraded, or concentrated in plant roots and shoots for recovery (Kirk et al. 2005a; Newman and Reynolds 2005).

Phytoextraction uses plant roots to take up pollutants from rhizosphere soil and accumulate them in the plants to higher concentration. Plants are harvested and the contaminants are removed (Kumar et al. 1995). Phytodegradation is defined as using plants grown in contaminated environments and associated microbes to degrade organic pollutants (Burken and Schnoor 1997). Rhizofiltration uses plant roots to absorb metals or other pollutants from waste streams (Dushenkov et al. 1995). Phytostabilisation lowers the mobility and bioavailability of contaminants in the environment either by immobilisation or by prevention of migration through binding of contaminants to plants or associated products in rhizosphere (Vangronsveld et al. 1995). Phytovolatilisation uses plants to volatilize pollutants into the atmosphere (Banuelos et al. 1997; Burken and Schnoor 1998; Pulford and Watson 2003). Rhizodegradation derived from
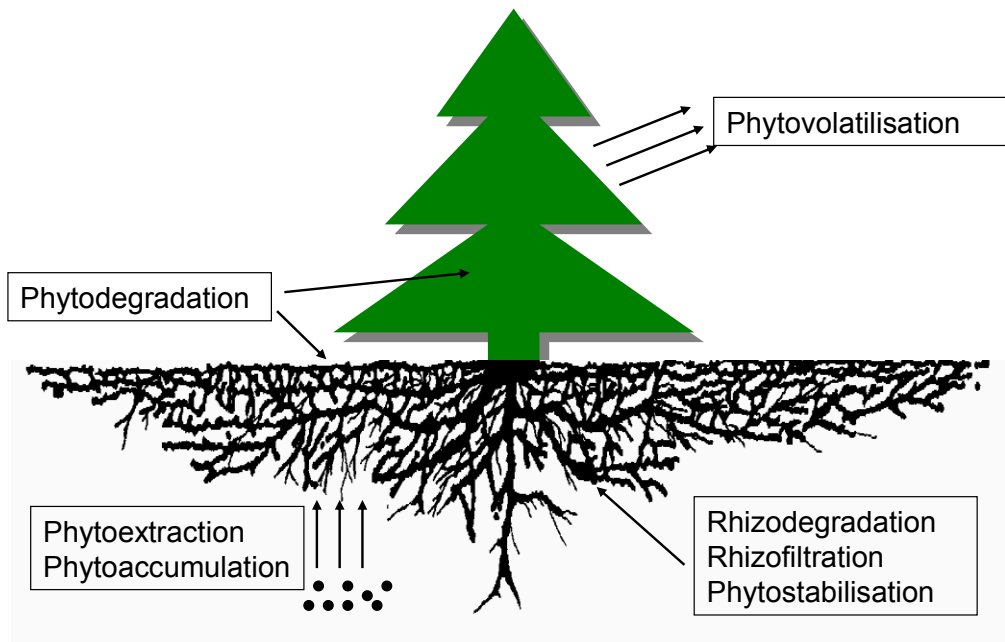
Figure 1. 3 The mechanisms of phytoremediation.

phytodegradation is the breakdown of organic contaminants in soil through microbial activity that is enhanced by the presence of the root zone (Euliss et al. 2008). These processes can work together or separately, depending on the target contaminants and condition of the site to be remediated. For example, in the case of phytoremediation of metals, phytoextraction and phytostablisation are the major processes by which metals are removed and/or sequestered to diminish exposure to other organisms (USEPA 2000).

In recent years, phytoremediation has received much attention and is expanding rapidly. It is gaining a significant amount of public attention owing to its advantages such as low cost, preserving the natural structure of soil and permanent *in situ* remediation (USEPA 2000). Extensive research has been performed on phytoremediation and there have been some notable successes, especially for the phytoremediation of PHCs (Bosma et al. 2002; Greenberg et al. 2006; Greenberg et al. 2007a; Huang et al. 2005; Huang et al. 2004a; Kaimi et al. 2006; Kirk et al. 2005a; Kramer 2005). However, concerns regarding phytoremediation include seasonal growth of plants, lowering the amount of time available for phytoremediation to occur as well as plant growth inhibition by contaminants which can hinder phytoremediation efficiency. Therefore, selection of plants highlighting long growing seasons and flourishing growth under stressed conditions is important for the optimal performance of phytoremediation (Belimov et al. 2005).

1.8.1 Plant growth

The first step in phytoremediation is to choose suitable contaminant-resistance plants with extensive root system that can explore large amounts of soil (USEPA 2000). Many plants have been studied in different phytoremediation systems (Huang et al. 2004b; Kirk et al. 2005b;

Kirkpatrick et al. 2006; Olson et al. 2007). Efficient phytoremediator plants must combine high biomass production and established cultivation methods with high tolerance to specific contaminants. Additionally, plants selected for phytoremediation should not be invasive or potentially invasive weeds or exotic plant species. Indigenous plants usually are the first choice for phytoremediation (Olson et al. 2007). Among the species tested, ryegrass, tall fescue and legumes were very effective and frequently used as phytoremediator species for petroleum contaminated soil (Greenberg et al. 2006; Greenberg et al. 2007a; Karthikeyan et al. 2004; Kirk et al. 2005a; Olson et al. 2007; Siciliano et al. 2003; Xu et al. 2005; Xu et al. 2006).

1.8.2 PGPR in phytoremediation

Phytoremediation is not always an efficient way to remove persistent organic contaminants because many plant species are sensitive to the contaminants (Huang et al. 2004b). Phytoremediation of contaminated soil and water environments is regulated and coordinated by the plant root system, yet root growth is often inhibited by contaminant-induced stress (Gerhardt et al. 2006). Prolific root growth is necessary for maximizing phytoremediation efficiency of inorganic or organic pollutants (Arshad et al. 2007). However, in heavily contaminated soils, roots grow slowly and do not accumulate sufficient biomass for effective phytoremediation (Huang et al. 2004b; Zhuang et al. 2007).

To improve phytoremediation in contaminated soil, plant growth promoting rhizobacteria (PGPR) have been used (Gerhardt et al. 2006; Glick 2003; Li et al. 2005; Nie et al. 2002; Reed et al. 2005). PGPR can stimulate plant root development, enhance root growth and relieve the stresses exerted on plant growing in contaminated soil. Some PGPR encoding 1-

aminocyclopropane-1-carboxylic acid **(**ACC)-deaminase have been used successfully for the phytoremediation of PHCs (Huang et al. 2005; Huang et al. 2004a), nickel (Nie et al. 2002), and copper contaminated soils (Gerhardt et al. 2006; Zhuang et al. 2007). Model of how PGPR with high ACC deaminase activity promote plant growth has been proposed (Gerhardt et al. 2006; Glick 2005; Glick et al. 2007a; Glick et al. 1998).

Plant responses to environmental stresses such as petroleum, metals or pathogen infection will results in production of higher than normal levels of ethylene. It is suggested that inhibited growth that occurs to plants is mostly due to ethylene action (as a stress hormone) and not from direct chemical or pathogen action (Glick et al. 2007a; vanLoon 1984). Hence, inhibitors of ethylene synthesis or ethylene action can significantly decrease the damage by pathogen infection or environmental stresses (Glick et al. 2007a). The biosynthesis of ethylene in plants is regulated by ACC, the immediate precursor of ethylene. The ACC is exuded to plant roots or seed surfaces. PGPR with ACC deaminase activity, attached to the surfaces of the seeds or roots, can convert the available ACC to ammonia and alpha-ketobutyrate, thereby lowering ACC concentration on root or seed surface. This leads to continuous ACC flow from inside the root or seed to the outside for maintaining the equilibrium, and thus lowering plant internal ACC concentration. By decreasing the level of ACC inside plant cells, less ethylene will be synthesized, and its inhibitory effect on root elongation will be alleviated (Glick 2005; Glick et al. 1998; Glick et al. 2007b).

1.8.3 Rhizodegradation of petroleum hydrocarbons

Rhizodegradation is defined as the breakdown of an organic contaminant in soil through microbial activity that is enhanced by the presence of root zone (USEPA 2000). Rhizodegradation

by microorganisms play a critical role in petroleum phytoremediation systems. The other removal mechanisms of phytoremediation, including phytostabilization and phytoextraction, are often not very effective for remediation of organic contaminants (Kramer 2005). For instance, direct plant-uptake of mixtures of PHCs, especially fractions F3 and F4, is generally extremely slow because these contaminants are too large to be taken up by roots and translocated inside the plants (Kaimi et al. 2007). However, in the presence of soil microbes, phytoremediation of PHCs can be actively accelerated by the catabolic potential of root-associated microorganisms (Huang et al. 2005; Ortega-Calvo et al. 2003). It is based on interactions between plants and their associated microorganisms in a process whereby plants draw pollutants into the rhizosphere via the transpiration stream; subsequently, microorganism-mediated degradation occurs in the rhizosphere (Barac et al. 2004). Thus, phytoremediation of petroleum is in reality the use of plants to stimulate the microbial community at and near the root–soil interface, to enhance the degradation of recalcitrant compounds by elevating microbial activity in the soil.

Rhizodegradation of petroleum hydrocarbons is the combined effect of plants and rhizosphere bacteria. They form a symbiotic relationship during phytoremediation. Plants exude organic compounds through their roots, which increase the density, diversity, and activity of specific microorganisms in the surrounding rhizosphere. The stimulated microbes in turn can degrade hydrocarbons (Cunningham et al. 1996; Siciliano et al. 1998). Plants can make contaminants in soil more bioavailable by releasing low molecular-weight organic acids, and root release of carbon and nitrogen compounds by plants can nourish microbes in the rhizosphere. In response to PHCs in the contaminated soil, plants can enhance degradation of soil contaminants by inducing

biochemical pathways within bacteria, leading to the shift of microbial community to that with

PHC degrading activities (Newman and Reynolds 2005).

1.9 Analysis of microbial diversity

Soil is considered as a microbial reservoir harboring novel genes due to their huge diversity

and population. It is estimated that in one gram dry soil there are approximately 1000-3000 types

of bacterial species, and populations up to 10^{11} cells per gram dry soil have been observed. The

diversity and populations of microbes in 2 cubic meters of soil exceed that in the oceans (Elsas et

al. 2007). Conventional methods of microbial analysis in soil or other environments are culture

dependent. Namely, culturing soil microorganisms in the laboratory using specially formulated

media to quantify and qualify microbes with certain functions. The cultured microorganisms can

be purified and identified, and different parameters such as total numbers or species can be

determined. Direct microcopy counts are also frequently used to determine the total microbial

number in soil samples. The advantage of culture dependant methods is that specific functions can

be connected to specific microbial species. Single living microorganisms can be cultured in the lab

for the further research on their morphology, biochemistry and genomic properties. However, this

method is biased by the fact that less than 1 % of all soil microorganism can be cultured under lab

conditions, and so it provides only limited information regarding the functional and polygenetic

diversity of microbial community (Cooper and Rao 2006). It is widely accepted that culture-based

techniques significantly underestimate the diversity of microbial species present in environmental

samples (Hugenholtz et al. 1998).

Over the past decade, culture-independent methods based on genomic and PCR technologies have been developed for the investigation of complex microbial communities (Muyzer and Smalla 1998). These methods enable the researcher to detect even single specific cells among thousands of others, and provide information about the population and diversity of entire microbial communities with very small samples and without the need to culture or even isolate a species. Many molecular techniques have been introduced to analyze microbial populations and diversity in the soil. These include fingerprint techniques, quantitative PCR (qPCR), also known as qPCR, and microarray metagenomics. Almost all these new techniques are based on PCR amplification of target DNA genes to increase detection sensitivity. Recognizing that both culture dependent and independent approaches have biases and limitations, combining these two methods broadens current understanding of microbial diversity in environmental samples (Cheng and Foght 2007).

1.9.1 Culture-based methods

Quantification and identification of microbes using selective media is the most common technique of microbial study. Various types of selective media have been developed to differentiate microbes of different groups with different biochemistry functions. For example, Gould's S1 agar was developed for recovering rhizosphere bacteria that belong to the genus *Pseudomonas* (Gould et al. 1985). In biodegradation studies of environmental contaminants, selective media were developed to isolate, enumerate, purify and identify microbes that can degrade the target contaminants. In these selective media, target chemicals or chemical mixtures which represent the dominant contaminants at a site were integrated into the medium as sole carbon or nitrogen sources. The microbes that can grow on it were considered to have the potential

to degrade these contaminants in the environment (Kirk et al. 2005a). For example, to recover and quantify the PAH degrading bacteria, PHE, the dominant PAH in PAH-contaminated sites, is added into medium as sole carbon source (Wong et al. 2004); to isolated the PGPR with ACC deaminase activity, ACC, the substrate of ACC deaminase, is integrated into medium as sole nitrogen source.

1.9.2 Denaturing gradient gel electrophoresis (DGGE)

A commonly used fingerprint technique is denaturing gradient gel electrophoresis (DGGE). DGGE allows the separation of the small DNA fragments with the same size but different sequences on denatured urea and formamide electrophoresis. This separation is based on their differing GC content and distribution that leads to different melting properties. Double stranded DNA are electrophoresed through an increasing linear denaturing gradient of urea and formamide at approximately 60°C. DNA fragments with different melting properties become partially melted at different points. Partial melting sharply decreases the mobility of the DNA fragments through the gel (Cooper and Rao 2006). The first DGGE application was analysis of 16s rDNA fragments amplified by PCR using bacterial 16s primers (Muyzer et al. 1993). After that, DGGE was widely used to analyze the diversity of different microbes such as bacteria (Corstjens and Muyzer 1993), fungi (Bastias et al. 2007), archea bacteria (Vieira et al. 2007), protozoa from various habitats including soil, water, plant, and animals (Muyzer and Smalla 1998). Specific primers were designed to target subunit 16s or 18s rRNA genes for PCR amplification using DNA extracted from environmental samples. The PCR products are subjected to DGGE analysis and fingerprints composed of the banding pattern were formed (Muyzer et al. 1993). The microbial diversity can be

calculated based on the band pattern in DGGE profile (Nocker et al. 2007). DNA bands can be sequenced for identification to yield taxonomic information through database searches and phylogenetic analysis (Ward and Roy 2005). This culture-independent method can detect microbial species not readily cultured in the lab. It is also a high throughput method that can analyze and compare lot of samples within short period of time (Cooper and Rao 2006).

DGGE also can be used to analyze the diversity of specific function gene. Specially designed primers are used to amplify certain gene sequences with PCR (Hong and Chen 2007). The PCR products are subjected to DGGE analysis to detect the diversity of this functional gene in environmental samples.

The disadvantage of DGGE analysis of 16s rRNA is that it cannot provide information about functional diversity. Many bacterial strains belong to the same bacterial species with common 16s rRNA sequences may have very different specific functions. For example, the bacteria *Pseudomonas putida* have many strains with different functions ranging from PGPR activity to petroleum hydrocarbon degradation (Hao et al. 2007; Ishaq et al. 2007).

Theoretically, DGGE can analyze the entire microbial community in environmental samples. However, this technique is limited by DNA extraction techniques and sampling strategies (Cooper and Rao 2006). Hence, DGGE can usually only detect the dominant microbial species in the samples. Furthermore, the inaccuracy in DGGE may result from microheterogeneity in the DNA sequence, leading to a situation where a single band may be composed of several species or several bands can be generated from a single species (Sekiguchi et al. 2001).

1.9.3 Quantitative PCR (qPCR)

qPCR is a technique used to simultaneously quantify and amplify a specific part of a given DNA molecule. It was first described by Holland et al. (Holland et al. 1991) and further developed and improved to include fluorescent dyes as labeled primers or probes that are used in the PCR reaction to emit a fluorescent signal (Asseline 2006; Rebrikov and Trofimov 2006). The fluorescence intensity is proportional to the amount of PCR products generated. The fluorescence increases exponentially as the PCR products accumulate until a reagent becomes limiting (Tse and Capeau 2003). A threshold fluorescence is defined within the exponential phase. The higher the amount of the starting template DNA, the earlier the fluorescence will cross the defined threshold. The copy number of the initial target DNA is thereby determined by comparison with a standard curve. It is used to determine whether or not a specific sequence and the number of the gene copies are present in the sample. The benefit of qPCR over other end-point PCR techniques is that it focuses on the logarithmic phase of product accumulation, resulting in a more accurate quantification of the starting template. It also avoids post PCR processing such as electrophoresis (Levin 2004).

qPCR has been widely used to detect and quantify specific bacteria and total bacteria (Castillo et al. 2006; Furukawa et al. 2006; Khan and Yadav 2004; Lacava et al. 2006; Wang et al. 2004b) from complex environmental samples. These assays are based on the knowledge of gene sequence of bacteria genome. Firstly, specific gene sequences correlating to specific microbes or microbial groups are identified. Then specific primers and probes are designed to target this unique gene sequence (Wang et al. 2004b). For the total bacterial assay, primers are designed to target the 16s

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rDNA shared by almost all bacteria (Castillo et al. 2006).

All qPCR systems rely upon the detection and quantification of a fluorescent reporter, the signal of which increases in direct proportion to the amount of PCR product in a reaction. Labeled-probes or dsDNA binding dyes, e.g. SYBR® Green, can be used to monitor amplicon synthesis (Santhosh et al. 2007). SYBR Green I is commonly used in qPCR applications as an intercalating dye that binds to the minor grooves of double stranded DNA. Binding of SYBR green to double-stranded DNA is non-specific (Giglio et al. 2003). It does not distinguish target DNA, non-target DNA and primer dimmers (Zipper et al. 2004). Therefore, additional testing such as melting curves is needed to confirm the target DNA as the sole amplicon. SYBR Green has been used to quantify the total bacterial population from different environmental samples (Castillo et al. 2006). Because of the complexity of environmental DNA samples especially soil DNA, it is almost impossible to design primers that only produce one single amplicon. Hence, SYBR Green is rarely used to quantify the specific bacterium from environmental sample.

TaqMan probes are most widely used in environmental assays to detect or quantify specific microbes or microbe groups from environment (Levin 2004; Pusterla et al. 2006; Wang et al. 2004b). It is a dual-labelled fluorogenic probe. The probes are fluorescently labeled with a reporter dye at the 5′ end and a quencher at 3′ end, which binds between the two primer sites to the target DNA (Heid et al. 1996). The fluorescence dye at 5′ end serves as a reporter, and its emission spectrum is quenced by the dye at the 3′ end of the probe. Ideally, the probe is located 50 bp upstream of the 3′ end of the amplicon, to achieve the highest possible sensitivity (Weller et al. 2000). When this probe bind to the target sequence, due to the 5′ nuclease activity of the Taq polymerase, the probe is dissolved from the target DNA and the reporter dye is released from the

molecule during DNA synthesis, which stops fluorescence resonance energy transfer and liberates a fluorescence signal. Therefore, only specific signals are detected by TaqMan assays. This method requires the design of a forward and a reverse primer, in addition to a probe targeting DNA sequence between two primers. The combination of primers and probe makes TaqMan assay very specific to target gene. It can distinguish the specific gene from thousand others in environmental samples.

1.10 objectives

It is clear that microbes play important roles in the study of environmental biology: both as bioindicators assays of toxicity assay and as biodegraders in the remediation of environmental contaminants. Some hydrocarbons, such as PAHs, show significant toxicity to the photobacteria *V. fischeri*. As well, some combinations of PAHs and metals show synergistic toxicity. For example, mixtures of the oxyPAH phenantherenequinone (PHQ) and Cu exhibit synergistic toxicity to *V. fischeri*. This is of concern since and they are co-contaminants at many sites due to their wide distribution in the environment. This leads to an interest to investigate the toxicity mechanisms of PHQ and metals, alone and in mixtures. Although studies have been done on the co-toxicty of PHQ and metals to *Daphnia* and *Lemna gibba*, no data is available for microbes. Therefore, the first part of this thesis addresses the individual and co-toxicity of PHQ and metals, and the exploration of a ROS-mediated toxicity mechanism induced by PHQ.

PHCs including PAHs are one class of ubiquitous contaminants. Phytoremediation of PHCs is an important research topic. However, most studies are done under controlled conditions in greenhouses or growth chambers. Phytoremediation is not effective at some PHC contaminated

sites, such as petroleum land farm, where the phytotoxicity of PHCs can inhibit plant growth and lead to unacceptably slow remediation rates. In this thesis, a field study was performed using PGPR to enhance plant growth under hydrocarbon stressed conditions, resulting in better phytoremediation efficiency. The main mechanism of phytoremediation of PHCs is considered to be the stimulation of microbial community at the root-soil interface. The microbes at this interface in turn degrade the PHCs in the soil. Therefore, another objective of this research is to explore the roles that microbes, including petroleum degraders and PGPR, play in phytoremediation.

This thesis has two objectives. The first objective includes individual and mixture toxicity of PHQ, and Cu or Cd to the photobacteria *V. fisheri*. Additional experiments include the exploration of the ROS-mediated toxicity mechanisms of individual and mixture of these chemicals. The second objective of this thesis addresses the roles of microbes (including introduced PGPR and indigenous soil microbes) during phytoremediation of PHC contaminated soil. This was done by kinetic analyses of soil microbial populations and microbial community structures at a PLF site in Sarnia, ON. Also, included in this thesis is a new rapid and sensitive method for tracking introduced PGPR (UW4) in field soil samples with qPCR.

Chapter 2

Assessment of Mixture Toxicity of Copper, Cadmium and Phenanthrenequinone to the

Marine Bacterium *Vibrio fischeri*

2.1 Introduction

Environments receiving contaminant discharges are generally exposed to toxicant mixtures, rather than single compounds (Mowat and Bundy 2002). Contaminants in mixtures are known to interact with biological systems in ways that can greatly alter the toxicity of the individual compounds. Some mixtures of contaminants have higher toxicity than that predicted by their Median Effective Concentration (EC50)/Median Lethal Concentration (LC50) values (Sarakinos et al. 2000; Tsiridis et al. 2006). Thus, even if the characteristics of individual chemicals are known, their behaviors in mixtures are not easily predicted. Transition metals and polycyclic aromatic hydrocarbons (PAHs) are co-contaminants at many sites (Irha et al. 2003). Both are generated in large quantities by industrial processes such as steel production and petroleum processing. This puts organisms in aquatic environments at risk from their integrated effects.

Mixture toxicity of contaminants can be traditionally classified as additive, synergistic and antagonistic (Norwood et al. 2003). The mechanisms of mixture toxicity in the environment depends on the chemistry of the individual compounds, environment-specific bioavailability, toxicologic modes of action, and possible interactions among contaminants once bioaccumulated (Gust 2006). Additivity often occurs when the components in mixtures affect the same target via the same mode of action. Antagonism may arise when one chemical affects either the delivery of another chemical to the site of toxic action or interferes with reactions at this site (McCarty and Borgert 2006). Synergism may occur due to accelerated bioaccumulation of contaminants when they exist as mixtures or if they mechanistically poise each other to be more toxic. For example, the synergism of

PHQ and Cu to *Daphnia magna* is due to the accelerated production of ROS and the generation of more toxic hydroxyl radicals when both chemicals are present (Xie et al. 2006).

Although a limited number of studies have shown that the toxicity of individual chemicals can be altered in chemical mixtures, and organisms are generally exposed in the environment to mixtures of contaminants, most ecotoxicological studies only focus on the exposure and effects of single chemicals. As well, most regulations for contaminant management are based on single-substance risk evaluations (De Zwart and Posthuma 2005). Therefore, evaluations of mixture toxicity, especially for contaminants that commonly co-exist in the environment, are urgently needed.

Copper (Cu) and cadmium (Cd) are among the most prevalent metals in the environment (Chang 1996; Manzl et al. 2004a). They are often present in the environment at concentrations that can be hazardous to organisms (Raskin and Ensley 2000). The mechanisms by which Cu and Cd are toxic have been studied extensively (Achard-Joris et al. 2007; Babu et al. 2003; Babu et al. 2001; Martelli and Moulis 2004; Mehta et al. 2006; Mowat and Bundy 2002; Padiyath et al. 1994; Villaescusa et al. 1996; Waisberg et al. 2003). The redox properties of Cu make it particularly useful, and yet harmful, in biological systems. This essential metal has distinct oxidation states and a redox potential of 200 - 800 mV when protein bound (Mehta et al. 2006; Stoyanov et al. 2003). Thus, Cu is a co-factor in many redox reactions via its ability to cycle between the oxidized (Cu^{2+}) and reduced (Cu^{+}) states (Lu et al. 2003). Although it is an essential trace element, excess Cu can be toxic due to its ability to generate free radicals by redox cycling in Fenton-like reactions. This leads to the production of reactive oxygen species (ROS), which can cause oxidative damage (Stoyanov et al. 2003; Xie et al. 2006).

Cadmium has few known physiological functions (Lane and Morel 2000), and is not thought to catalyze Fenton-type reactions leading to ROS production because it does not readily cycle between redox states (Waisberg et al. 2003). Increasing evidence in plants (Krantev et al. 2007), animals (Amara et al. 2007; Xie et al. 2006) and bacteria (Achard-Joris et al. 2007) suggest that excessive ROS production is a major toxicity mechanism of Cd. Cadmium can be sequestered by reduced glutathione and/or metallothionein to prevent its adverse interaction with biomolecules. However, either chronic exposure to low concentrations or acute exposure to toxic doses of Cd may overwhelm the cellular supply of GSH and metallothionein (Chin and Templeton 1993; Martelli and Moulis 2004; Waisberg et al. 2003). Reduced glutathione and other thiols play a critical role as cellular antioxidants by scavenging ROS (Elia et al. 2007). The depletion of glutathione and other thiols due to Cd exposure may indirectly result in elevated ROS levels (Martelli and Moulis 2004). Cadmium can also induce ROS production directly in mitochondria. It does so by binding cytochrome b of complex III between the semi-ubiquinone and cytochrome b_{566} of the Q_0 site, resulting in accumulation of semi-ubiquinones that can transfer one electron to molecular oxygen to form superoxide (Martelli and Moulis 2004). Thus, exposure to Cd can lead to cell damage or even apoptosis via both direct and indirect production of ROS (Risso-de Faverney et al. 2004).

Polycyclic aromatic hydrocarbons (PAHs) are a prevalent group of organic environmental contaminants (Douben 2003). Results of several studies have shown that when PAHs are exposed to ultraviolet radiation, enhanced toxicity is observed (Lampi et al. 2006; McConkey et al. 1997). Photoinduced toxicity of PAHs is derived from two photochemical processes: photosensitization and photomodification (Douben 2003; El-Alawi et al. 2002b; Lampi et al. 2006). During photosensitization reactions, intracellular singlet-state oxygen ($^1O_2$) and other ROS are generated,

which can cause oxidative damage in biological systems (Foote 1987). In the case of photomodification, PAHs are structurally altered to a variety of compounds, most of which are oxygenation products (oxyPAHs). Many of these photoproducts are more toxic than their parent PAHs (El-Alawi et al. 2002b; Lampi et al. 2006; McConkey et al. 1997). For instance, phenanthrenequinone (PHQ), the major photoproduct of phenanthrene (PHE), is considerably more toxic than its parent compound (McConkey et al. 1997).

The toxicity of quinones, such as PHQ, may be due in part to redox cycling (Jarabak et al. 1996; Jarabak et al. 1997). Phenanthrenequinone can be reduced by enzymes such as NADPH cytochrome P450 reductase, yielding the semiquinone radical (Hasspieler and Di Giulio 1994). In the presence of molecular oxygen, the semiquinone may be oxidized, yielding the parent quinone and superoxide ($O_2^{\bullet-}$). Superoxide is a ROS and it can be converted to other biologically damaging ROS such as hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\bullet OH$) (Hasspieler and Di Giulio 1994; Lind et al. 1982). PHQ is an efficient redox cycling agent and has been shown to stimulate ROS production in rat luteal cells (Nykamp et al. 2001), cell extract of flounder (*Platichthys flesus*) (Lemaire and Livingstone 1997) and catfish (*Ictalurus punctatus)* tissue (Hasspieler and Di Giulio 1994).

The mechanism of co-toxicity of metal and oxyPAHs is believed to be ROS-mediated. It is hypothesized that the generation of ROS is involved in the co-toxicity due to the redox cycling property of both chemicals. Several *in vitro* studies have shown that Cu can accelerate the oxidation of hydroquinone to benzoquinone (Li and Trush 1993) and catalyze the redox cycle of PHQ (Yu et al. 2002), accompanied by the production of ROS (Xie et al. 2006). *In vivo* studies with several organisms such as *Lemna gibba* (Babu et al. 2001), *Daphnia magna* (Xie et al. 2006), rat corpus luteus cells and human placental JEG-3 cells (Nykamp 2007; Nykamp et al. 2001), show concurrent

increases in ROS production and synergistic toxicity to target organisms. Therefore, ROS mediated synergistic toxicity might be a universal mechanism of Cu/oxyPAHs mixtures. In the current study, the photobacterium *Vibrio fischeri* was used as a test organism to characterize mixture interactions between Cu or Cd and an oxyPAH (PHQ). Mathematical algorithms were used to compute the predicted combined toxicity of the mixtures based on an additive toxicity mechanism (Calabrese 1991; Norwood et al. 2003). The predicted combined toxicity values assuming additive effects were compared with experimental toxicity data. Depending on the chemicals and their mixture ratios, additive, synergistic (greater than additive) or antagonistic (less than additive) toxicity was observed. The implication of ROS-mediated toxicity mechanisms of Cu, Cd, PHQ and their binary mixtures were investigated using the fluorogenic probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and Amplex red assay.

2.2 Materials and methods

2.2.1 Bacterial strain and growth medium

The marine Gram-negative luminescent bacterium *Vibrio fischeri* NRRL B-11177 was used. Bacteria were cultured in a medium of the following formulation: KH_2PO_4, 18.4 mM; NaCl, 0.5 M; $MgSO_4.7H_2O$, 4.1 mM; glycerol, 54.3 mM; yeast extract, 1 g/L; peptone, 5 g/L; bactopeptamin, 1 g/L. Agar (15 g/L) was added for preparation of agar plates. The final pH of the medium was adjusted to 7.2 ± 0.1 with 1.5 M NaOH. Fifty (50) ml of medium was distributed into 250-ml flasks and autoclaved. Twenty-four-hour stock cultures of *V. fischeri* grown on agar plates at 20°C were used for primary inoculation (El-Alawi et al. 2002a). Peptone and yeast extract were

obtained from BDH (Toronto, ON, Canada), and bactopeptamin was obtained from Difco Laboratories (Detroit, MI, USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2.2 Bacterial growth and toxicity tests

Bacterial cultures were harvested after growth for 24 hours at 20°C in the dark on a shaker at 50 rpm. Fifteen ml of the culture was centrifuged twice at 2300 x g for 15 minutes and the pellet was resuspended in 15 ml of a 2% (w/v) saline solution with 1 mM KH_2PO_4 (pH 7) (Newman and McCloskey 1996) immediately prior to toxicity testing. The bacterial suspension was adjusted to an absorbance of 0.2 at 650 nm using the saline solution. Twenty-four 500-µl aliquots of cells were added to a 48-well tissue culture plate (Costar Corporation, Cambridge, MA, USA). Toxicity assays were carried out at room temperature (20 ± 1°C). After 15 min acclimatization in the 48-well culture plates, the bioluminescence was measured at 495 nm (40 nm bandwidth) using a Cytofluor 2350 fluorescence measurement system (Millipore, Mississauga, ON, Canada). The excitation lamp of the detector was turned off to eliminate any background fluorescence. The bacteria were then dosed with the chemicals. Equal volumes (500 µl) of chemical(s) in 2% saline at twice the desired concentrations were added into wells containing the bacterial suspension in the 48-well plate. The final absorbance of bacterial suspension in wells was 0.1. A dilution series of each chemical or mixture was added to the wells in triplicate. A geometric series of seven concentrations was used for testing. For toxicity testing, stock solutions of PHQ (1 mg/ml) (Sigma) were prepared in DMSO and diluted with 2% NaCl saline plus 1 mM KH_2PO_4 (pH 7) to give the desired concentrations. For metals, a stock solution (1000 mg cation/L) of $CuSO_4 \cdot 5H_2O$ (Sigma)

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and CdCl$_2$ (Sigma) were prepared in reverse osmosis-purified water and also diluted with 2% NaCl to the required concentrations. Delivery of chemicals was periodically assayed to confirm accuracy. Bacterial luminescence was measured at 15, 30, 45 and 60 minutes after exposure to chemicals. Each experiment was repeated independently a minimum of three times.

Toxicity was measured as percent inhibition of light emission from a treated sample, corrected for loss of light in the control using Equation 1 (McConkey et al. 1997).

$$\% \text{ inhibition } (T_i) = 100 \big(1- (L_f \bullet C_i) / (L_i \bullet C_f) \big) \qquad \text{Equation 1}$$

where L_i is the initial luminescence of the bacteria prior to exposure to the toxicant, L_f is the luminescence of the bacteria following a given duration of exposure to chemicals, and C_i and C_f are the initial and final luminescence of the control bacteria. Calculation of median effective concentration (EC50) for inhibition of luminescence was based on a log function for continuous response data. The data for % inhibition vs. chemical concentration can be fit to Equation 2 (McConkey et al. 1997):

$$T_i = 100 / (1 + e^{\beta(\chi-\mu)}) \qquad \text{Equation 2}$$

where x is the log of the concentration, μ is the log of the EC50, and β is a measure of the slope of the concentration-response curve.

In the standard acute *V. fischeri* test with Microtox[®], the EC50 value is determined by measuring luminescence 5 to 15 minutes after exposure to a chemical (McConkey et al. 1997). The responses of this bacterium to metals have been found to be slow (Newman and McCloskey 1996). As well, uptake and toxicity of PAHs by many microbes (El-Alawi et al. 2001; Kallimanis et al. 2007) are highly time dependent. Thus, toxicity of metals and PAHs has been found to be limited by the kinetics of bioaccumulation. At 30 min exposure, the EC50s of three tested chemicals in

this study have a good agreement with those in the literature using Microtox® (El-Alawi et al. 2001; Gagan et al. 2007; McConkey et al. 1997; Newman and McCloskey 1996; Villaescusa et al. 1996). Therefore, exposures of 30 minutes or longer were used for toxicity assays of individual chemicals and mixtures of chemicals to obtain more reliable and thorough data.

2.2.3 Mixture toxicity assays: Experimental design

The toxicity of mixtures of Cu/PHQ and Cd/PHQ were determined using a fixed ratio design. In the present study, multiple ratios covering the possible interaction types were designed to investigate the joint toxicity effects. The fixed ratio method was chosen due to the established simple analysis methods (Stork et al. 2007). While keeping the mixture ratio constant, the total concentration of the mixture was varied so that a complete concentration-response relationship of the mixtures could be determined (Backhaus et al. 2000). Binary mixtures of Cu/PHQ and Cd/PHQ were combined using different ratios based on the EC50 of the individual compounds (30 min). The mixture ratios employed for Cu:PHQ were 1:4 [Cu:PHQ = (1×EC50 of Cu) /(4×EC50 of PHQ)], 2:3 [Cu:PHQ = (2×EC50 of Cu) /(3×EC50 of PHQ)], 3:2, and 4:1. The mixture ratios of Cd:PHQ were 1:3 [Cd:PHQ = (EC50 of Cd) /(3×EC50 of PHQ)], 1:1 (EC50 of Cu /EC50 of PHQ), and 3:1. Using EC50 mixtures is a typical experimental approach. It gives equitoxic mixtures, which has been widely used for assessing the joint action of chemicals in mixtures (Backhaus et al. 2000; Hermens et al. 1984).

2.2.4 Calculation of mixture effect

To analyze mixture toxicity, an additive interaction model was assumed (Calabrese 1991). An additive effect occurs when the toxicity of a mixture is equal to the sum of the toxicities of the individual components in the mixture. Toxicity is expressed as EC50s. Predicted EC50s of the binary mixtures were calculated for each mixture ratio used.

Two-sided effect isobole models were used to compute the effective toxicity of two component mixtures (Fig. 2.1) (Calabrese 1991). The isobolographic method has become recognized as both a simple and valuable technique for the study of chemical interactions (Gessner 1988). Despite this, there have only been a few efforts to use isoboles in environmental toxicological studies (Sorensen et al. 2007). It was used in this study because it clearly shows when mixture toxicity is additive, synergistic, or antagonistic. A straight line joining the EC50s of individual chemical A (e.g. Cu) and individual chemical B (e.g. PHQ) represents the expected EC50s of various Cu/PHQ ratios, assuming the interactions are due to simple concentration-additivity of the two chemicals. Toxicity of the mixtures was then assessed experimentally and EC50s of Cu and PHQ (at concentrations used in the mixtures) were calculated independently. The empirical EC50s of the mixtures are the comcemtrations of chemical A and chemical B in the mixture that result in 50% inhibition of luminescence. For example, the EC50 of a given mixture at point M in Fig.1 is the combination of 3 units of chemical B and 0.5 units of chemical A (Fig. 2.1). The empirical EC50s were compared with expected EC50s. For the hypothetical mixture in Fig. 2.1, synergistic toxicity is observed because the EC50 is lower than predicted additive toxicity. It should be remembered that EC50 varies as an inverse of toxicity (i.e., the lower the EC50 value, the higher the toxicity).

EC50 of chemical B individual

EC50 of chemical A individual

M

EC50 of mixture

antagonistic

additive

synergistic

[Chemical B] in the mixture at EC50

[Chemical A] in the mixture at EC50

\
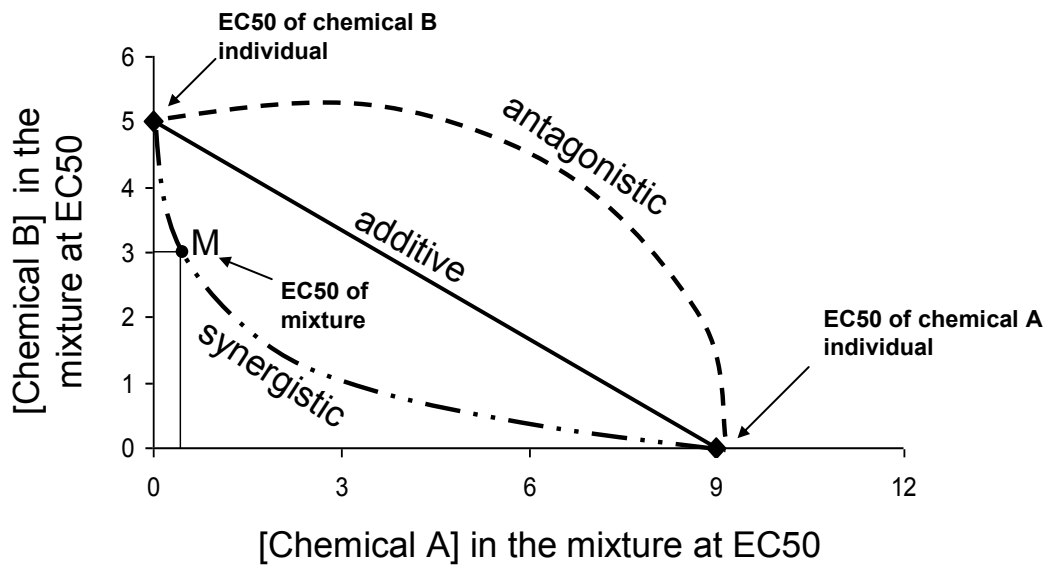
Figure 2. 1 Types of isoboles with two chemicals (Calabrese 1991).

Mixture interactions were identified as synergistic or antagonistic if the observed EC50s of the Cu/PHQ or Cd/PHQ mixtures were at least one standard deviation below, or above, the expected EC50s, respectively.

2.2.5 Determination of intracellular ROS

To evaluate the possible involvement of ROS stress in the toxicity of Cu, Cd, PHQ, and their mixtures, the effect of the chemicals on ROS production in *V. fischeri* was determined using the fluorescent dye, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Invitrogen, Burlington, ON, Canada). This dye diffuses across the cell membrane where it is deacetylated by intracellular esterases and reacts with ROS to form a highly fluorescent product, DCF (Royall and Ischiropoulos 1993). Fluorescence of this product is then detected with a fluorescence plate reader (Cytofluor 2350, Millipore). The fluorescence intensity was determined with excitation and emission wavelengths of 485 nm and 538 nm, respectively. Experimental protocols for ROS assays were identical to those of the toxicity assays, except that 10μM H_2DCFDA was added to each well in the culture plate. Measurements were taken every 15 minutes for 3 hours.

2.2.6 Fluorescent microassay of hydrogen peroxide (H_2O_2)

Hydrogen peroxide production was measured via horseradish peroxidase (HRP)-dependent oxidation of *N*-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent; Invitrogen) in 96-well tissue culture plates as described by Mohanty et al. (Mohanty et al. 1997) with some modifications. Briefly, 100 µl Amplex Red reagent solution (2% NaCl, 1 mM KH_2PO_4, 100 µM

Amplex Red, pH 7) containing 0.2 Unit/mL of HRP and different concentrations of the chemicals were added to the wells containing bacterial suspensions (OD = 0.2). The microplates were incubated at 20°C for 2 hr. The fluorescence intensity of each well was measured using a fluorescence plate reader (Cytofluor 2350, Millipore) with excitation and emission wavelengths of 530 nm and 570 nm, respectively. The concentration of H_2O_2 was determined based on a standard curve.

2.3 Results

2.3.1 Cu, Cd and PHQ toxicity to V. fischeri, assessed separately

In this study, toxicities of Cu, Cd, and PHQ were determined independently at 15, 30, 45 and 60 minutes (Fig. 2.2). The EC50s for Cu and PHQ were 146.88 μg/L and 65.49 μg/L at 30 minutes, respectively. Comparatively, Cd was much less toxic to *V. fischeri*. The EC50 of Cd was 9.96 mg/L at 30 minutes. The toxicity of all the chemicals increased with exposure time, probably due to the increased bioaccumulation of chemicals in bacterial cells over time. For Cu, the EC50 decreased from 201 μg/L at 15 minutes to 66.9 μg/L at 60 minutes. For PHQ and Cd, the EC50 decreased from 84.99 and 16.71 mg/L at 15 minutes to 46.07 μg/L and 4.60 mg/L at 60 minutes, respectively (Fig. 2.2). After 15 minutes of exposure, low concentrations of Cu (50 and 100 μg/L) were found to enhance luminescence (Fig. 2.2).

Figure 2. 2 The Concentration response curve of Cu, Cd and PHQ to V. fischeri.

V. fischeri (24 hour cultures, OD = 0.1) were exposed to concentration series of Cu, Cd, and PHQ using 48-well microplates. Each treatment was subdivided into 3 replicates in 3 wells. Light intensity was assessed at 0 (before dosing with chemicals), 15, 30, 45 and 60 minute exposure times. Vertical bars represent the mean ± SD of 6 replicates. Closed square: incubation 15 min; open circle: incubation 30 min; closed triangle: incubation 45 min; open triangle: incubation 60 min.
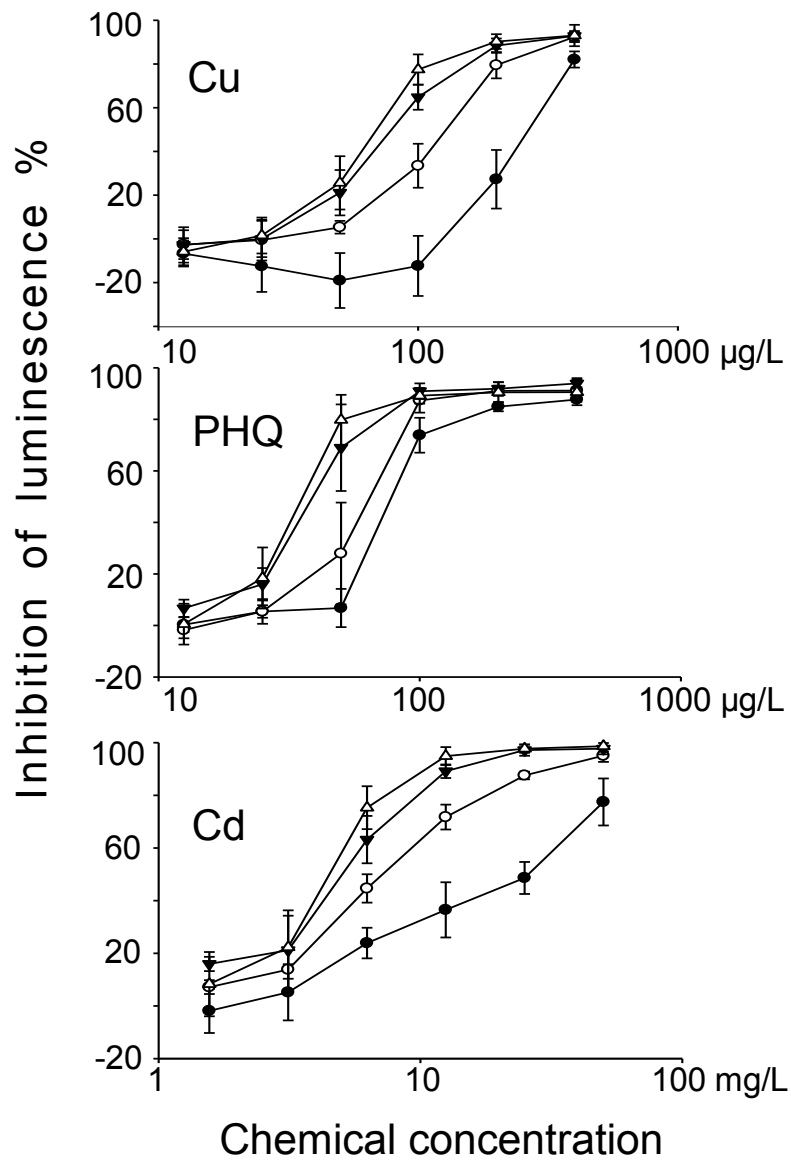
2.3.2 Toxicity of binary mixtures to V. fischeri

Mixture toxicities of Cu, or Cd, with PHQ were assessed at different ratios of Cu, or Cd, to PHQ. At four different ratios of Cu to PHQ (1:4, 2:3, 3:2, 4:1), three different mixture interaction types (antagonistic, additive, and synergistic) were observed (Fig. 2.3). At the lowest Cu to PHQ ratio (1:4), the experimental toxicities (EC50s) of the mixture were 19.4, 16.5, 15.5 µg/L for Cu and 68.1, 66.7, 61.9 µg/L for PHQ at the three exposure times of 30, 45, and 60 minutes, respectively. This is somewhat higher than the expected EC50s from the model (14.7, 11.5, 9.8 µg/L for Cu and 58.7, 46, 39.2 µg/L for PHQ) (Fig. 2.3), probably due to the fact that Cu acts, in part, as a luminescence enhancer. At the 45 and 60 min exposure times, the experimental EC50s of the the mixtures were also significantly greater than predicted by concentration addition (Fig. 2.3). Thus, mixture toxicity at this ratio was less than the sum of the individual toxicities for Cu and PHQ, suggesting an antagonistic interaction. At an intermediate Cu to PHQ ratio (2:3), the predicted EC50s of the mixture were not significantly different from the experimental EC50s at the three exposure times, indicating that the mixture interaction at this ratio was additive (Fig. 2.3). At Cu to PHQ ratios of 3:2 and 4:1, the experimental EC50s of the mixtures were significantly lower than the predicted EC50s at 30 and 45 minutes (Fig. 2.3). These mixtures showed enhanced toxicity to *V. fischer*i, suggesting a synergistic interaction.

When Cd and PHQ were combined, two types of mixture interactions were observed (Fig. 2.4). In contrast to the Cu/PHQ mixtures, the types of interaction did not change with different Cd to PHQ ratios. However, different interaction types occurred at various time points. A synergistic

Figure 2. 3 Isobologram for EC50s of binary mixtures of Cu2+ and PHQ at different exposure times. EC50 points are plotted with their 95% confidence limits. The predicted location of the EC50 points, given the occurrence of a simple additive effect, is given together with the 95% confidence limits by the solid and dotted straight line diagonals, respectively. Points on the solid curves give the actual EC50s observed for Cu and PHQ alone, and for different Cu/PHQ ratios (from left to right 4:1, 3:2, 2:3, 1:4). The points above, on and under the line indicate antagonistic, additive and synergistic effects, respectively. Significant differences from the predicted EC50 are marked with an asterisk (*). Vertical and horizon bars represent the mean ± SD of 4 replicates.

Y-axis (all three panels): [PHQ] in the mixture at EC50 (µg/L)

X-axis (bottom): [Cu] in the mixture at EC50 (µg/L)

Panel labels: 30 min, 45 min, 60 min
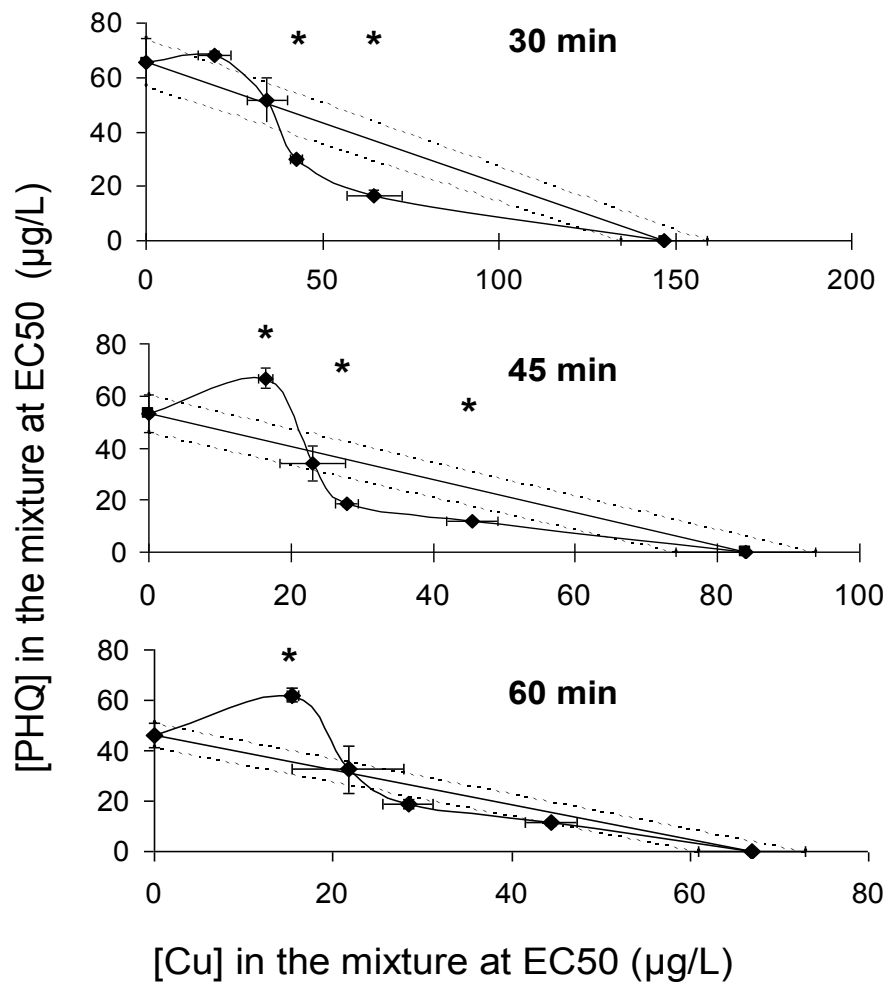
Figure 2. 4 Isobogram for EC50 of binary mixture Cd2+ and PHQ at different exposure times. EC50 points are plotted with their 95% confidence limits. The predicted location of the EC50 points, given the occurrence of a simple additive effect, is given together with the 95% confidence limits by the solid and dotted straight line diagonals, respectively. Points on the solid curves give the actual EC50s observed at different Cd/PHQ ratios (from left to right 1:3, 1:2, 3:1). The points above, on and under the line indicate antagonistic, additive and synergistic effects, respectively. Significant differences from the predicted EC50s are marked with an asterisk (*). Vertical and horizon bars represent the mean ± SD of 5 replicates.
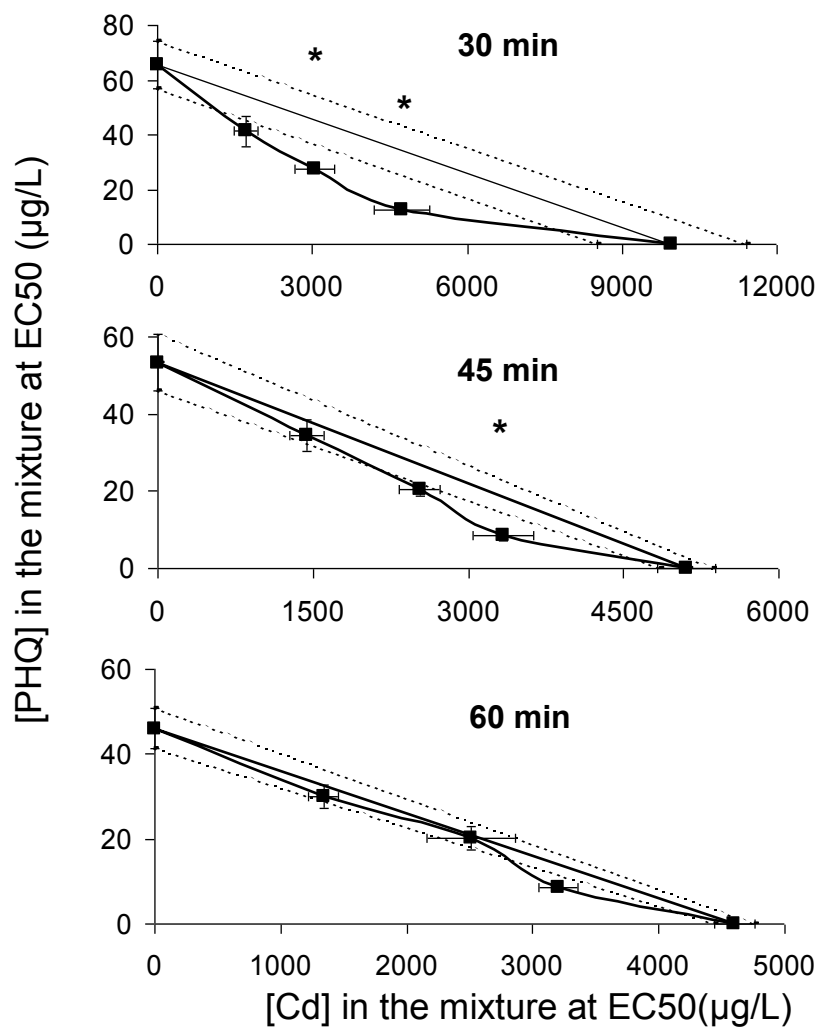
effect was observed at 1:3 and 1:1 Cd to PHQ ratios after 30 minutes of exposure, with the EC50 being significantly lower than predicted. That is, the experimental EC50s were significantly lower than predicted. However, after 45 and 60 min, the experimental EC50s were not significantly different from the predicted values, suggesting a mere additive interaction.

2.3.3 Intracellular ROS production induced by Cu, Cd and PHQ

Production of ROS was observed when *V. fischeri* were exposed to Cu, Cd and PHQ. A concentration (or dose) response relationship between ROS formation and chemical concentration was found (Fig. 2.5). A slight increase in ROS production was observed even under control conditions, reflecting the basal rate of ROS production putatively due to by-products of aerobic metabolism. In Cu-treated bacterial cells, ROS formation increased relative to untreated controls at Cu concentrations of 150 and 400 μg/L. In PHQ-treated cells, significantly enhanced ROS levels were detected at all applied concentrations. DCF oxidation in Cd-treated cells was not enhanced within 1 hour. Significant increases in ROS formation were detected after Cd treatments longer than 90 minutes.

2.3.4 Hydrogen peroxide production induced by Cd, Cu, PHQ, Cd/PHQ and Cu/PHQ

The production of H_2O_2 by *V. fischeri* cells treated with Cd, Cu, PHQ, Cd/PHQ and Cu/PHQ was assessed based on HRP-catalyzed oxidation of the Amplex Red reagent to yield a fluorescent compound. Using increasing concentrations of Cu or PHQ alone, there was a dose dependent H_2O_2 production in the treated cells (Fig. 2.6). For the binary mixture of Cu and PHQ, the concentration

Figure 2. 5 Time course DCF oxidation as measure of ROS production by V. fischeri treated with different concentrations of Cd2+, Cu2+ and PHQ.

Bacteria (24 hours old, OD = 0.1) were dosed with chemicals and H2DCFDA in 48-well microplates. DCF fluorescence was measured at 15 minute intervals for 3 hours. Excitation and emission wavelengths were 485 nm and 538 nm, respectively. Vertical bars represent the mean ± SD of 4 replicates.
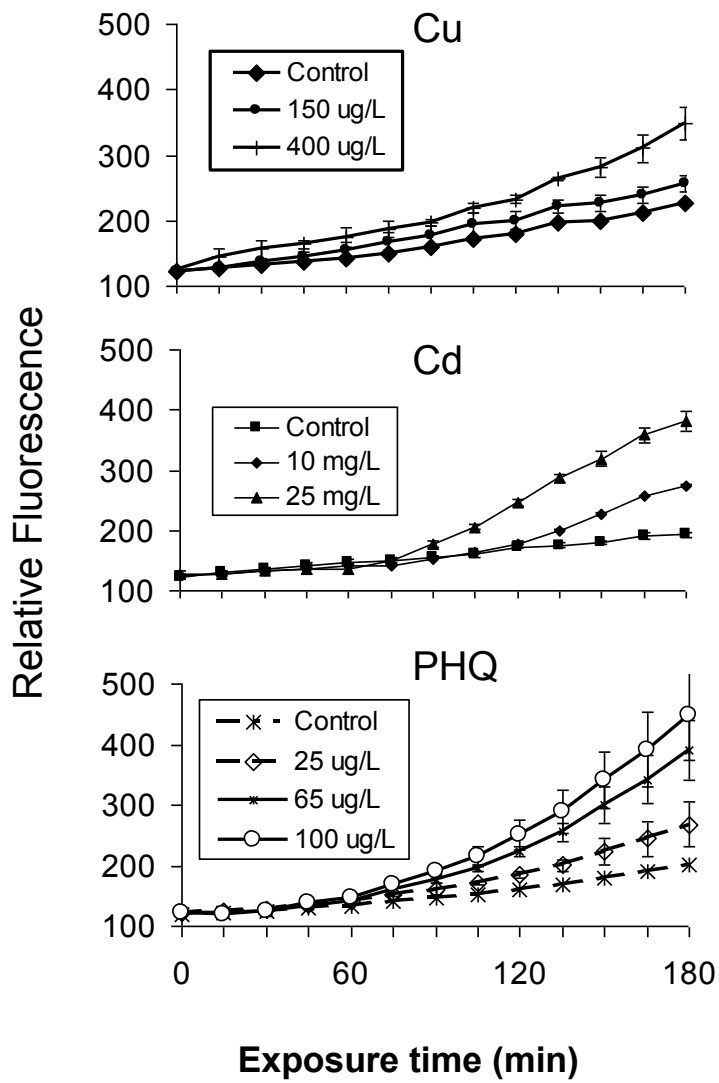
Figure 2. 6 H_2O_2 production of V. fischeri treated with Cu, Cd, PHQ, Cu/PHQ or Cd/PHQ. H_2O_2 production was detected with the Amplex Red assay. Bacterial fluorescence was measured in the presence of chemicals, Amplex Red reagent and horseradish peroxidase (HRP) in 96-well microplates. Bacterial cells (24 hours old, OD = 0.1) were exposed to a concentration series of Cu, PHQ, Cu/PHQ or Cd/PHQ. For Cu/PHQ mixtures, PHQ concentrations were held at 20 µg/L and different concentrations of Cu were added. For Cd/PHQ, Cd concentration was held constant at 3.125 mg/L and different concentrations of PHQ were added. Controls were set up as bacterial suspensions without toxicant. Fluorescence was measured at 20 minute intervals for 2 hours with excitation and emission wavelengths of 530 nm and 570 nm, respectively. Data for one hour are shown in this graph. Vertical bars represent the mean ± SD of 4 replicates.
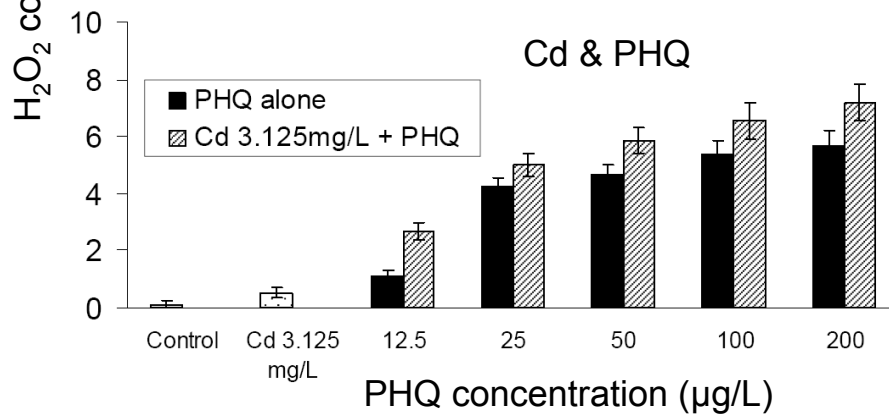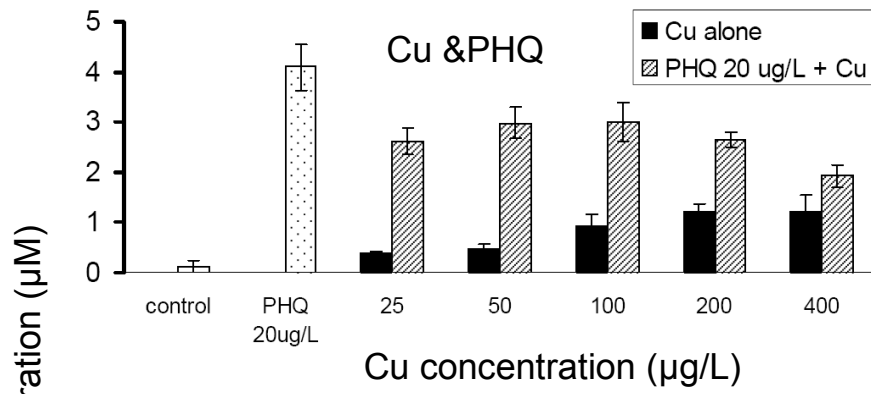
of PHQ was held constant at 20 µg/L and different concentrations of Cu were added (Fig. 2.6) to test the possible scavenging effect of Cu on H_2O_2. Hydrogen peroxide level in bacterial cells induced by PHQ (20 µg/L) alone was 4.1 ± 0.46 µM. Hydrogen peroxide levels in bacterial cells treated with a mixture of PHQ and Cu were 2.61 ± 0.26, 2.98 ± 0.31, 3.01 ± 0.38, 2.65 ± 0.16, and 1.92 ± 0.22 µM at Cu concentrations of 25, 50, 100, 200, and 400 µg/L, respectively. These values indicate that H_2O_2 levels in cells treated with a mixture of Cu and PHQ were lower than those in cells treated with PHQ alone. In cells treated with PHQ plus 25, 50, 100, and 200 µg/L Cu, H_2O_2 concentrations were not significantly different, but a decrease was observed with 400 µg/L Cu (Fig. 2.6).

For the binary mixtures of Cd and PHQ, the concentration of Cd was held constant at 3.125 mg/L and different concentrations of PHQ were added (Fig. 2.6) because the H_2O_2 production of *V. fischeri* treated with Cd individually did not change significantly at concentrations above 3.125 mg/L. Cadmium alone (3.125 mg/L) induced H_2O_2 levels of 0.52 ± 0.18 µM as compared to control levels of 0.12 ± 0.11µM (bacterial suspension in 2% saline, no toxicant was added). However, H_2O_2 levels in bacterial cells treated with mixtures of PHQ and Cd were 2.67 ± 0.30, 4.10 ± 0.41, 5.45 ± 0.45, 6.54 ± 0.61, and 7.18 ± 0.63 µM at PHQ concentrations of 12.5, 25, 50, 100, 200, and 400 µg/L, respectively. These values are higher than those in cells treated with PHQ alone (1.11 ± 0.22, 4.26 ± 0.27, 4.83 ± 0.38, 5.36 ± 0.48, 5.66 ± 0.55 µM) or Cd alone, and also higher than the additive values for Cd plus PHQ.

2.4 Discussion

Transition metals such as Cu and Cd, and oxyPAHs such as PHQ, frequently occur in the environment as co-contaminants (Mowat and Bundy 2002). Although there is information on the individual effects of these three chemicals on *V. fischeri* (Carlsonekvall and Morrison 1995; El-Alawi et al. 2002b; McConkey et al. 1997; Mowat and Bundy 2002; Villaescusa et al. 1996), studies that examine the effects of Cu/PHQ or Cd/PHQ mixtures on this bioluminescent bacterium have not been performed. In the present study, the co-toxicity of Cu/PHQ was found to be dependent on the mixture ratio. Generally, increasing the concentration of Cu in the mixture enhances the co-toxicity. Thus, at higher Cu to PHQ ratios (3:2 and 4:1), a synergistic toxicity effect was observed. For co-toxicity of the Cd/PHQ mixtures, as was previously observed in whole organism studies (Xie et al. 2006), the interaction did not change with varying ratios of Cd to PHQ. Mechanisms of ROS-mediated Cu, Cd and PHQ toxicity have been well established in several organisms using H_2DCFDA and Amplex Red fluorescence (Kudryasheva et al. 1999; Stoyanov et al. 2003; Xie et al. 2006). Copper, Cd and PHQ enhanced ROS production in *V. fischeri* cells (Fig. 2.5), implying the involvement of ROS in the individual and co-toxicity of these chemicals to *V. fischeri*.

Copper has been associated with both acute and chronic toxicity to bacteria (Utgikar et al. 2004). It strongly affects enzyme systems and essential cellular metabolism (Achard-Joris et al. 2007; Chang 1996). The EC50s of Cu in different studies with *V. fischeri* range from 100 to 170 µg/L after 30 minutes of exposure (Carlsonekvall and Morrison 1995; Newman and McCloskey 1996; Utgikar et al. 2004). The 30-minute EC50 of 146 µg/L, determined in this study, is in

agreement with those reports. Copper is an essential element, although it may be toxic at higher concentrations through its ability to form ROS and inhibit metabolism (Stoyanov et al. 2003). We found that Cu induces ROS production in *V. fischeri* as determined by DCF fluorescence (Fig. 2.5), indicating that ROS could be involved in Cu toxicity to *V. fischeri*. This supports previous whole organism studies that implicated enhanced ROS production in the mechanism of Cu toxicity to *D. magna* and *L. gibba* (Babu T. S. 2001; Xie et al. 2006).

The relatively low toxicity of Cd to *V. fischeri* compared to that of Cu and PHQ observed in this study, has also been demonstrated in previous studies (Carlsonekvall and Morrison 1995; Mowat and Bundy 2002; Villaescusa et al. 1996). The EC50s range from 3 mg/L to 19 mg/L at 30 minutes (Carlsonekvall and Morrison 1995; Newman and McCloskey 1996; Villaescusa et al. 1996). A potential reason for the relatively low toxicity is that organisms have several mechanisms of defense against Cd-induced toxicity. For instance, reduced glutathione (GSH) and other thiol containing proteins can scavenge ionic Cd^{2+} by binding it through the sulfhudryl group.

In this study, delayed ROS formation in *V. fischeri* was observed with prolonged exposure to Cd even though Cd is not a redox-active metal (Smeets et al. 2005; Waisberg et al. 2003) (Fig. 7). This result is in agreement with other studies, showing increasing ROS production in the presence of Cd (Manzl et al. 2004b). This is in contrast with the redox-active chemical Cu (Ariza et al. 1999), where ROS production was observed within 1 hour (Fig. 2.5). It is known that the redox cycling activity of Cd is less pronounced than that of Cu (Manzl et al. 2004b). It is possible that Cd-induced ROS production occurs via indirect mechanisms, possibly due to decreased activities of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (Manzl et al. 2004b). Long term exposure or exposure to toxic doses of Cd may overwhelm the supply of

GSH or other components that play a protective role in *V. fischeri*. This could be due to the production of ROS at a rate that exceeds *V. fischeri's* ability to regenerate GSH, resulting in ROS production and ROS-induced toxicity.

Phenanthrenequinone (PHQ), the major photoproduct of phenanthrenequinone (PHE), was the most toxic of the chemicals tested in this study. There is a narrow range of EC50s for PHQ reported in the literature: from 70 μg/L to 102 μg/L. As a result of photomodification, the toxicity of PHQ is greatly enhanced relative to the EC50 for PHE of 530 μg/L (McConkey et al. 1997). This is likely due to the properties of PHQ which, as an *o*-quinone, can promote toxicity by inducing ROS via redox cycling between the quinone and semiquinone (Hasspieler and Di Giulio 1994). At low concentrations, we found that PHQ can generate high levels of ROS in *V. fischeri* cells (Fig. 2.5). The concentration of PHQ that produces high levels of ROS corresponds to a level that is toxic to *V. fischeri* (25 μg/L) (Figs. 2.2 and 2.5). This indicates that ROS production is likely a key factor in the mechanism of toxicity of PHQ to *V. fischeri*. Kudryasheva et al. (Kudryasheva et al. 1999) reported that PHQ competes for electrons from NAD(P)H with the normal electron acceptor, FMN. This is followed by electron transfer from the PHQ semiquinone to molecular oxygen, forming $O_2^{\bullet-}$ and other ROS. These ROS could be toxic to *V. fischeri* (Fig. 2.7).

It is interesting to note that the co-toxicity of Cu/PHQ changes at different mixture ratios. This response has also been observed in mice exposed to ethanol and chloral hydrate mixtures (Calabrese 1991). In the case of Cu/PHQ, this response may be attributable to the ability of Cu to redox cycle. This characteristic makes Cu an essential element in several biological reactions including energy capture (cytochrome c oxidase), free radical defense (1-SOD and 3-SOD,
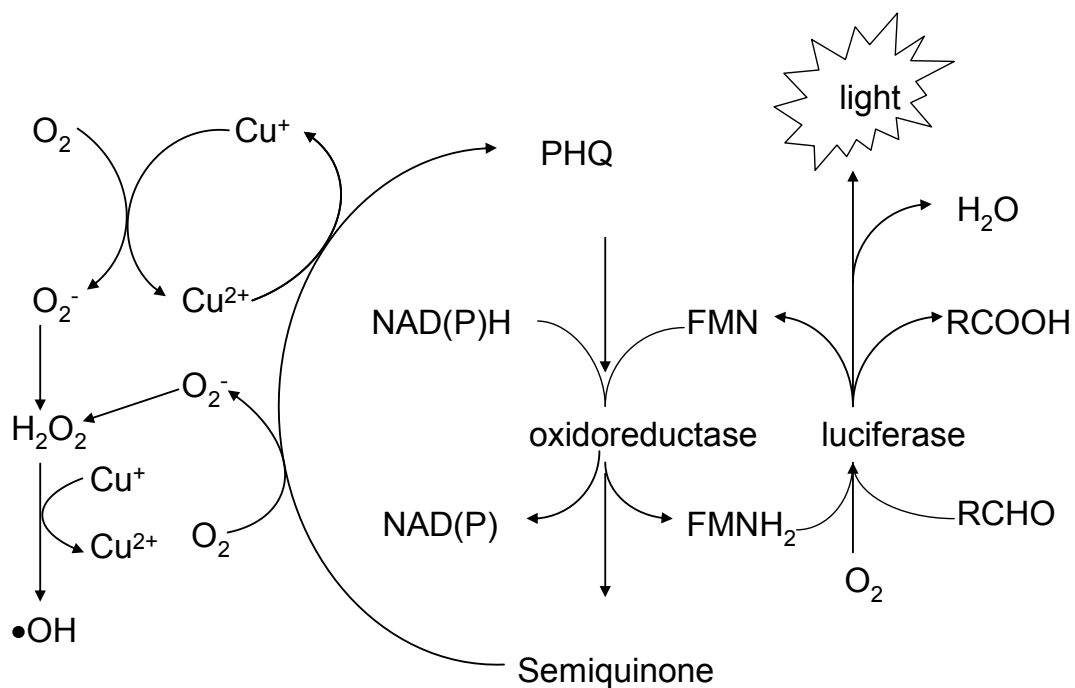
Figure 2. 7 The proposed pathway of Cu/PHQ mixtures to V. fischeri.

The electron transport chain from NADPH to light emission is outlined. PHQ can accept electrons

from NADPH, block electron transport to FMN and lead to formation of semiquinone. Quinone

and semiquinone cycling can result in the transfer of electrons to oxygen, generating superoxide

and other ROS. Cu^{2+} also can accept electrons to be reduced to Cu^+, via the Fenton reaction,

leading to peroxyl formation.

ceruloplasmin), and protection against light (tyrosinase) (Horn and Tumer 1999), but also contributes to its inherent toxicity (Harrison et al. 2000). Cells have a variety of mechanisms to maintain homeostatic balance of this essential, yet toxic, trace element. Protein-mediated homeostatic mechanisms for Cu (such as Cu chaperones and metallothioneins) have been reviewed extensively (Camakaris et al. 1999; Harrison et al. 2000; Mercer 2001). Toxicity of Cu depends on its free ion, which can redox cycle between Cu^+ and Cu^{2+}, and catalyzes the production of highly toxic hydroxyl radicals via Fenton-like reactions, with subsequent damage to lipids, proteins, DNA and other biomolecules (Camakaris et al. 1999).

In the mixture with the lowest Cu:PHQ ratio (1:4), Cu exists in the mixture at the concentration of a trace nutrient metal. Copper chaperones (eq. metallothionein) may combine with all Cu ions, transport them to the site of utilization by Cu-dependent proteins and prevent deleterious Cu interactions with other cellular components (Pufahl et al. 1997). In fact, Cu at low concentrations stimulates the growth and light emission of *V. fischeri* (data not shown). Copper at lower concentrations may detoxify ROS by enhancing SOD and other enzyme activity because it is an essential part of these biomolecules. Thus, Cu at low concentrations could lower the toxicity of PHQ. An antagonistic effect would therefore be expected in Cu/PHQ mixtures with a low ratio of Cu to PHQ, which was the observed effect in this study.

With increasing Cu to PHQ ratios, synergistic toxicity of Cu/PHQ mixtures on *V. fischeri* was observed. Enhanced co-toxicity of Cu plus quinones has been observed in other organisms (Babu et al. 2001; Das S. 1997; Jarabak et al. 1998; Xie et al. 2006). The mechanisms of Cu/quinone co-toxicity were found to involve ROS production. The present study shows that PHQ, even at low concentrations, can generate ROS (Fig. 2.5) and lead to toxicity in bacterial cells. Hydrogen

peroxide assays show that PHQ can generate large amounts of H_2O_2. PHQ may generate $O_2^{\bullet-}$ and H_2O_2 via redox cycling in bacterial cells through a NADH-dependent mechanism (Fig. 2.7). Toxicity of Cu depends on the concentration of free Cu ions in cells. At high Cu to PHQ ratios, excess Cu ions are present and protective mechanisms in the bacterial cells are probably overwhelmed. That is, chaperones and metallothioneins cannot bind all free Cu ions, thereby leaving free Cu ions in the cells. It is thought that Cu^{2+} bound to DNA in a site-specific manner is reduced to Cu^+ (Das and Mandal 1997) and/or reduced by redox cycling of PHQ (Xie et al. 2006) (Fig. 2.7). Through Fenton-like reactions, Cu^+ can react with H_2O_2, formed via normal cellular metabolism, or stimulated by PHQ. Hydrogen peroxide can be converted to hydroxyl radicals (●OH), a highly reactive and harmful ROS that can damage cellular components including DNA, enzymes and lipids (Hiraku and Kawanishi 1996). Hydrogen peroxide levels in *V. fischeri* cells treated with mixtures of Cu and PHQ were indeed lower than those treated with PHQ alone (Fig. 7). It is thus postulated that Cu is reacting H_2O_2 through Fenton-like reactions, thereby generating hydroxyl radicals (●OH) and leading to more serious damage in bacterial cells. Via this ROS mediated mechanism, Cu/PHQ mixtures at high Cu ratios exhibit synergistic toxicity to *V. fischeri*.

Unlike Cu, Cd does not have any known biological functions (Satarug et al. 2003). The interaction types of Cd/PHQ were additive (45 and 60 min) and synergistic (30 min). Both PHQ and Cd induce H_2O_2 production in the cells of *V. fischeri,* but apparently via different mechanisms. PHQ induces H_2O_2 by direct intracellular redox cycling (Fig. 2.7). Cadmium may induce H_2O_2 production by affecting the antioxidants that are responsible for degradation of H_2O_2 (Martelli and Moulis 2004). In the present study, we detected significantly higher H_2O_2 production in *V. fischeri* cells treated with Cd/PHQ mixtures than in those treated with individual chemicals (Fig. 2.6). This

suggests the involvement of H_2O_2 in the mechanism of toxicity for Cd/PHQ mixtures. Quinones can compete with FMN for electrons from NADH in the bioluminescent pathway of photobacteria (Kudryasheva et al. 1999). The electrons can subsequently be passed from the semiquinone to molecular oxygen to produce $O_2^{\bullet-}$ and H_2O_2 (Fig. 2.7). Notably, Cd has been shown to decrease the activity of some components of the antioxidant defence pathways (Manzl et al. 2004b) which could result in the accumulation of H_2O_2. Thus, mixtures of Cd/PHQ may damage *V. fischeri* cells via an H_2O_2-mediated mechanism.

The results presented in this report demonstrate that co-toxicity of Cu/PHQ to *V. fischeri* is dependent on the mixture ratio. Generally, increasing the Cu concentration relative to PHQ enhanced toxicity of the binary mixture. At high Cu:PHQ ratios (3:2 and 4:1), synergistic effects were observed. The interaction type changed to additive at an intermediate Cu to PHQ ratio (2:3), and antagonistic at a low Cu:PHQ ratio (1:4). The shift in interaction types may be due to the fact that Cu is a necessary trace element, and therefore beneficial at low concentrations, but toxic at higher concentrations. Conversely, the co-toxicity of Cd/PHQ was not dependent on mixture ratio. The mechanism of Cu, Cd, and PHQ toxicity to *V. fischeri*, alone and in mixtures, may involve ROS. Production of ROS was greatly enhanced in the presence of Cu, Cd or PHQ. Hydrogen peroxide production increased in cells exposed to Cd/PHQ mixtures relative to Cd or PHQ alone. This suggests the involvement of H_2O_2 in the mechanism of toxicity for Cd/PHQ mixtures. In contrast, H_2O_2 production in cells treated with PHQ was lower in the presence of Cu. In Cu/PHQ mixtures, a Cu-mediated conversion of H_2O_2 to ●OH may occur in *V. fischeri* cells through Fenton-like reactions, leading to further damage via this ROS. Data are in agreement with others that have shown that the presence of modified PAHs and their mixtures with metals in the

environment have enhanced toxicity via ROS formation, and that mixture ratio should be taken into account as a factor in mixture toxicity studies.

Chapter 3

Examination of the Mechanism of Phenanthrenequinone (PHQ) Toxicity to *Vibrio fischeri*:

Evidence for a ROS Mediated Toxicity Mechanism

3.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs), formed mainly as a result of incomplete combustion of fossil fuels, are a class of ubiquitous environmental contaminants (Tuominen et al. 1988). The toxicity of PAHs to many organisms has been shown to increase greatly upon exposure to light, especially ultraviolet radiation via such processes as photosensitization and photooxidation (McConkey et al. 1997) or metabolic activation by cellular biotransformation (Bolton et al. 2000). The generated products with oxygen-containing substituents, such as quinones, have been extensively studied (Babu et al. 2001; Jarabak et al. 1997; McConkey et al. 1997). Phenanthrenequinone (PHQ) is the most common photooxidation and biotransformation product of phenanthrene (PHE) (McConkey et al. 1997), one of the most prevalent PAHs in environment. PHQ has been demonstrated to have a greater toxicity than PHE to various organisms including *V. fischeri* (El-Alawi et al. 2001), *Lemna gibba* (McConkey et al. 1997), *Daphnia magna* (Xie et al. 2006) and rat luteal cells (Nykamp 2007).

The toxicity of quinones is generally thought to occur by two mechanisms: as Michael acceptors, damaging living organisms by binding with cellular nucleophiles, leading to depletion of cellular GSH levels and/or alkylation of protein and DNA (Bolton et al. 2000; Rodriguez et al. 2004); as a redox agent, undergoing enzymatic or nonenzymatic redox cycling with cellular electron donors to generate the superoxide anion (O_2^-) radicals and other reactive oxygen species (ROS) (Hasspieler et al. 1994; Jarabak et al. 1997). Lacking elctrophilic sites to attach, PHQ was considered to be mostly a redox cycling agent that will only exhibit oxygen-dependent toxicity (Rodriguez et al. 2004). This redox process can be catalyzed by NAD(P)H-dependant flavoprotein

reductases and proceeds via the univalent reduction of the quinone to the semiquinone anion radical (Monks et al. 1992; Nykamp 2007). The PHQ semiquinone can react with molecular oxygen forming O_2^- thus regenerating the parent quinone, and hence undergoes redox cycling with the consequent catalytic generation of ROS (Hasspieler and Digiulio 1994). PHQ-mediated O_2^- production has been demonstrated in *Daphnia magna* (Xie et al. 2007) rat luteal cells (Nykamp 2007), cell extract of flounder (*Platichthys flesus*) (Lemaire and Livingstone 1997), and cat fish (*Ictalurus punctatus*) tissue (Hasspieler and Digiulio 1994).

Although increasing evidence suggests that ROS is involved in PHQ toxicity, these data primarily come from studies using cell free systems and isolated subcellular fractions (Hasspieler et al. 1994; Jarabak et al. 1997). Limited numbers of *in vivo* studies on the role of ROS in PHQ toxicity are available (Nykamp 2007; Rodriguez et al. 2004; Xie et al. 2007). Under most cases, if a quinone was found to produce ROS via the redox cycling, it was considered that these ROS played a key role in the toxicity (Rodriguez et al. 2004; Xie et al. 2007). However, very little *in vivo* evidence is available for the direct relation between ROS production and toxicity.

As a model organism, *V. fischeri* is a widely researched bioluminescent bacterium, and has been used extensively in environmental toxicity testing and monitoring. The *lux* genes that encode bioluminescence expression were used as a reporter system in many toxicological studies (Kudryasheva et al. 1999). Considerable toxicity data (over 1700 chemicals) available for *V. fischeri* shows more than 70% correlation with that from other organisms (Kaiser 1998). Another important characteristic of *V. fischeri* is that it can grow both aerobically and anaerobically (Proctor and Gunsalus 2000). Therefore the toxicity of PHQ to *V. fischeri* under aerobic or anaerobic conditions and the ROS-mediated toxicity mechanism of PHQ can be examined.

Furthermore, as a single cell organism, ROS production and scavenging system can be studied *in vivo* (Wang et al. in press). In the present study, using *V. fischeri* as the test organism, the toxicity mechanism of PHQ *in vivo* system was investigated.

3.2 Material and method

3.2.1. Culture medium and growth conditions, bacteria growth and toxicity tests, ROS assay, fluorescence microassay of hydrogen peroxide

The methods used for these assays are same as those used in chapter 2.

3.2.2 SOD and catalase activity assays

Catalase and SOD assays were performed as previously described (Beauchamp and Fridovich 1971; Wayne and Diaz 1986). Preparation of cell lysates for enzyme activity gel analyses was according to Vattanaviboon et al. (Vattanaviboon et al. 2003). Briefly, bacterial cells were pelleted and washed once with 50 mM sodium phosphate buffer pH 7.0 containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and exposing them to intermittent sonication on ice until the suspensions became clear. The lysates were then centrifuged at 10,000 x g for 10 min and the supernatant were used for enzyme activity assays via native PAGE electrophoresis or a spectrophotometer. Total catalase activity was spectrophotometrically measured by the disappearance of H_2O_2 at 240 nm (Beers and Sizer 1952). Bacterial extracts (50 µl) were mixed with 1 ml 2% NaCl containing 30 µl 3% H_2O_2. Absorbance of the mixture was measured for a 100

sec period and catalase activity was calculated in units (U) per mg of protein (the activity of the catalase that decomposes 1 μmole of H_2O_2 per min is defined as 1 U of catalase activity) (Katsev et al. 2004). The protein concentration was measured using Bradford method.

To visualize SOD and catalase activity, non-denaturing electrophoresis was performed using a 10% polyacrylamide gel (pH 8.7) and a 5% stacking gel (pH 6.8). The electrophoresis was carried out at 100 V at 4 °C for 2 hours, followed by different staining procedures to visualize SOD and catalase, respectively. For SOD detection, the procedure was according to the method described by Babu et al. (Babu et al. 2003). Briefly, the polyacrylamide gels were first incubated in 80 ml reverse osmosis (RO) H_2O containing 0.016 g Nitroblue Tetrazolium (NBT) (Sigma, MO, USA) with gentle shaking under dark conditions for 20 minutes. Gels were transferred into 80 ml 0.05 M potassium phosphate buffer (pH 7.8) containing 1 mg riboflavin and 100 μl TEMED, and again shaken gently in the dark for 15 minutes. Gels were rinsed with dH_2O and put into 100 ml 0.05 M potassium phosphate buffer (pH 7.8) containing 15 mg EDTA. Gels were exposed to a 15 W luminescent light until SOD bands developed clearly. For the catalase activity stain, the ferricyanide staining method described by Wayne and Diaz (Wayne and Diaz 1986) was used. The plyacrylamide gels were washed 3 times for 15 minutes in RO water to remove interfering buffer salts, and transferred to 100 ml RO H_2O containing 0.01 ml 30% H_2O_2 and gently rocked for 10 min. The H_2O_2 was removed and the gel was rinsed immediately in RO H_2O. The rinsed gel was transferred to the fresh ferricyanide mixture containing 1% ferric chloride and 1% potassium ferricyanide both in H_2O. The catalase activity could be seen as a white band developed in contrast to the green background.

3.2.3 Growth of V. fischeri under aerobic and anaerobic conditions

V. fischeri was grown under aerobic and anaerobic conditions in 250-ml sealed air-proof glass bottles containing 15 ml of complex medium described previously (El-Alawi et al. 2001) with a series of concentrations of PHQ. The anaerobic conditions were generated by replacing O_2 in sealed bottles with nitrogen. For aerobic condition, the bottle had O_2 at the concentrations same as natural atmosphere. The gases inside bottles could not exchange with the outside air. All bottles were inoculated with same amount of *V. fischeri* cells. The bacteria were grown in a rotary shaker (50 rpm/min) at room temperature ($20 \pm 1˚C$) under dark conditions. The growth of *V. fischeri* was assayed by measuring OD at a 650 nm wavelength at 1 h intervals for 10 h.

3.3 Results

3.3.1 Toxicity of PHQ on V. fischeri

At the whole organism level, PHQ causes a decrease in the light emission of *V. fischeri* (Fig. 3.1). A typical concentration-response curve was observed. The EC50 at an exposure time of 30 min was 65.49 μg/L. The highest inhibition of light emission (91%) at was 200 μg/L and the least level of inhibition (5.4%) was observed at 25 μg/L.

3.3.2 In vivo production of H_2O_2 in exposure to PHQ

The production of H_2O_2 by *V. fischeri* cells treated with PHQ was measured using the HRP-catalyzed oxidation of the Amplex Red reagent. There was a concentration dependant induction of

76
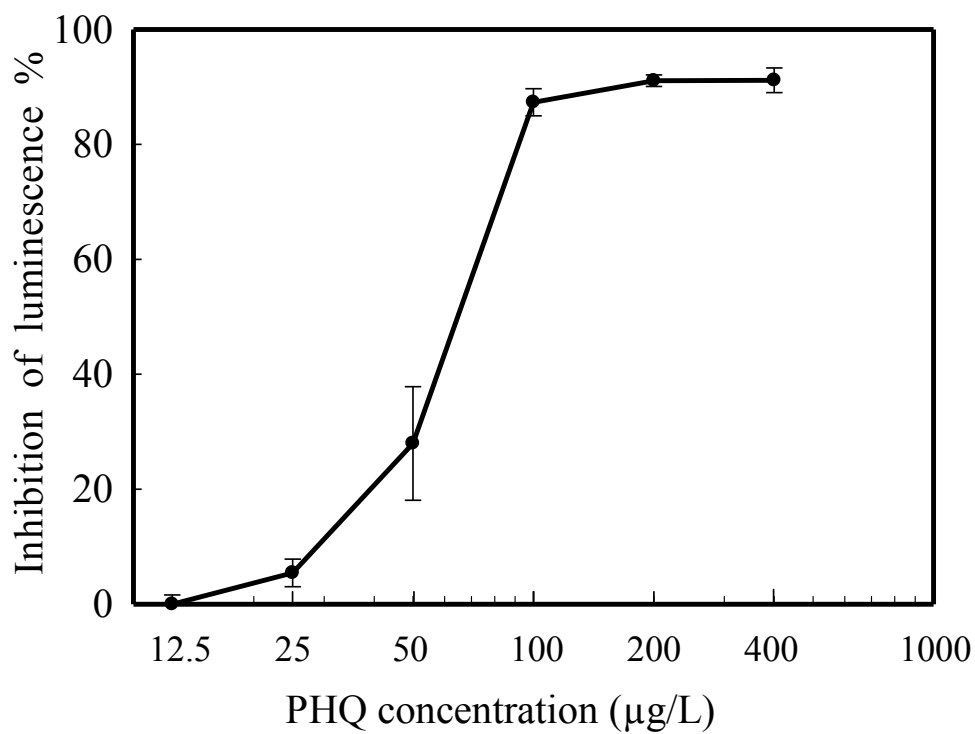
Figure 3. 1 Concentration response of PHQ to V. fischeri.

Bacteria (24-h culture, OD=0.1) were exposed to a concentration series of PHQ using 48-well microplates. Light emission was assessed immediately before PHQ exposure and after a 30 min exposure to PHQ. Inhibition of light emission was calculated from the data. Data are average ± SE. Each experiment was repeated independently 5 times.

H_2O_2 with PHQ (Fig. 3.2). Compared to H_2O_2 production in control cells, significantly greater amounts of H_2O_2 were produced with PHQ concentrations of 25 µg/L and higher ($p < 0.05$), indicating PHQ promotes the H_2O_2 production in bacterial cells.

3.3.3 In vivo ROS assay in response to PHQ

Total ROS generation in *V. fischeri* cells treated by PHQ was determined using 2',7'-dichlorodihydrofluorescein diacetate (Fig. 3.3). H_2DCFDA can be taken up by living bacteria cells, and oxidized by ROS in bacterial cells to form a highly fluorescent compound, 2',7'-dichlorofluorescein (DCF). The amount of DCF fluorescence is proportional to ROS production. Cells of *V. fischeri* exposed to different concentrations of PHQ (25, 50, 100 and 200 µg/L) exhibited higher DCF fluorescence than untreated control cells (Fig. 3.3). DCF fluorescence was elevated in a concentration dependent manner with production saturating at 100 µg/L PHQ. As well, DCF fluorescence increased with time of exposure to PHQ. This indicated that PHQ promoted ROS production inside bacterial cells.

3.3.4 SOD production in exposure to PHQ

Many organisms have several classes of superoxide dismutase (SOD). Eukaryotes have three types of SOD (CuZnSODs, MnSODs and FeSOD) localized to different cellular compartments (Drazkiewicz et al. 2007). Prokaryotes produce Mn-SOD or Fe-SOD in their cytoplasm
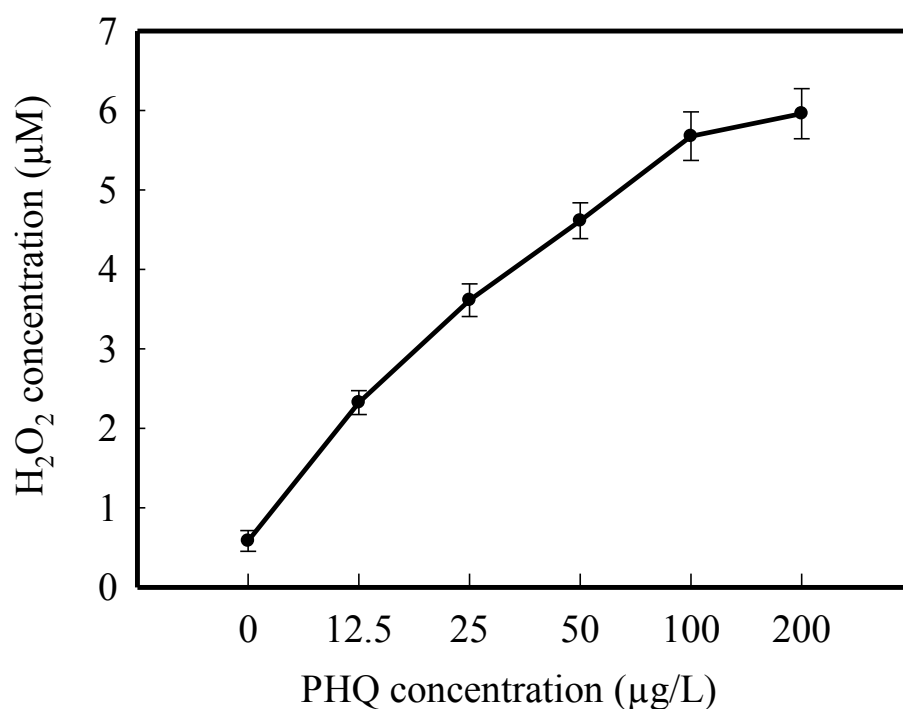
Figure 3. 2 H_2O_2 production of V. fischeri treated with PHQ using the Amplex Red assay.

Bacteria (24-h culture, OD=0.1) were exposed to PHQ in the presence of Amplex Red reagent and

horseradish peroxidase (HRP) in 96-well microplates. Fluorescence of oxidized Amplex Red was

measured every 10 minutes for 2 hours with excitation and emission wavelength set to 530 and

570 nm, respectively. The data at 30 min were shown in this graph. Data are the oxidized Amplex

Red fluorescence ± SE. Each experiment was repeated independently 5 times.
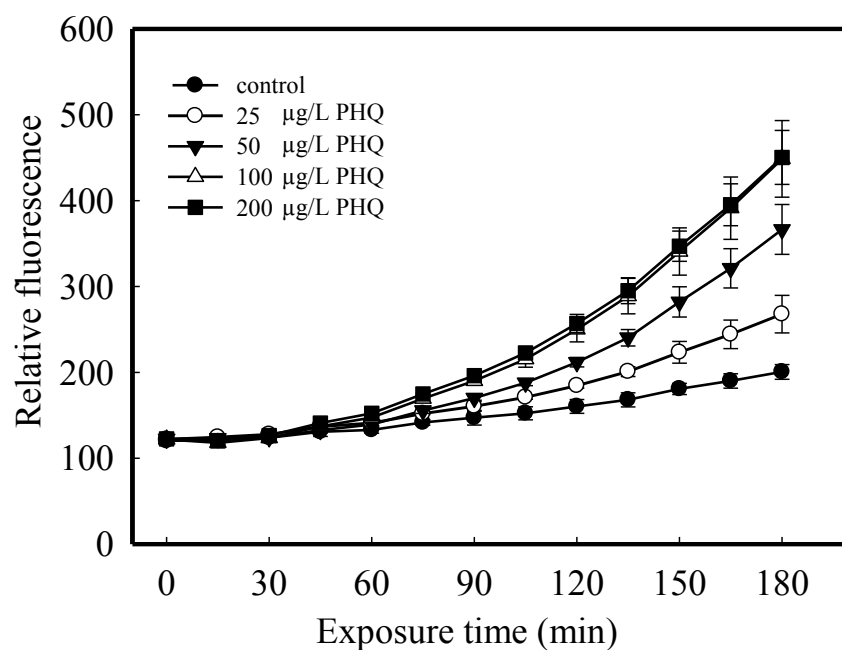
Figure 3. 3 Time course of DCF oxidation as a measure of the production of ROS by V. fischeri treated with different concentrations of PHQ.

Bacteria (24-h culture, OD=0.1) were treated simultaneously with PHQ and DCFH$_2$-DA in 48-well microplates. DCF fluorescence was measured every 15 minutes up to 3 hours with excitation and emission wavelength set to 485 and 538 nm, respectively. Each line represents a time course of fluorescence at different concentrations of PHQ. Error baas are SE (n = 5).

(Scott et al. 1987). However, for *V. fischeri*, only one SOD band was detected (Fig. 3.4). It was identified as Fe-SOD using specific inhibitors (data not shown). Treatment of *V. fischeri* for 30 min with increasing concentrations of PHQ resulted in elevated levels of the Fe-SOD as detected by native PAGE analyses (Fig. 3.4). This indicated that PHQ stimulated *in vivo* production of Fe-SOD. It is interesting to note that this trend is similar to the increase in ROS production in bacterial cells treated with PHQ (Figs 3.2, 3.3 and 3.4). The concentration of PHQ that resulted in elevated levels of ROS and Fe-SOD were on par with the concentration that cause whole organism toxicity (Figs. 3.1 to 3.4).

3.3.5 In vivo catalase activity in exposure to PHQ

Catalase activity of *V. fischeri* has been previously observed (Katsev et al. 2004). The activity level of catalase in bacteria treated with PHQ was measured spectrophotometrically using cell extracts. Generally, treatment with increasing concentrations of PHQ did not significantly increase catalase activity (Fig. 3.5).

One type of catalase enzyme was detected in native PAGE (Fig. 3.6). The catalase of *V. fischeri* is group III catalse encoded by *KatA* gene (Visick and Ruby 1998). As observed above with the spectral assay (Fig. 3.5), exposing bacterial cells to PHQ did not caused change in catalase activity levels as measured by catalase activity native PAGE (Fig. 3.6).

PHQ Concentrations (µg/L)
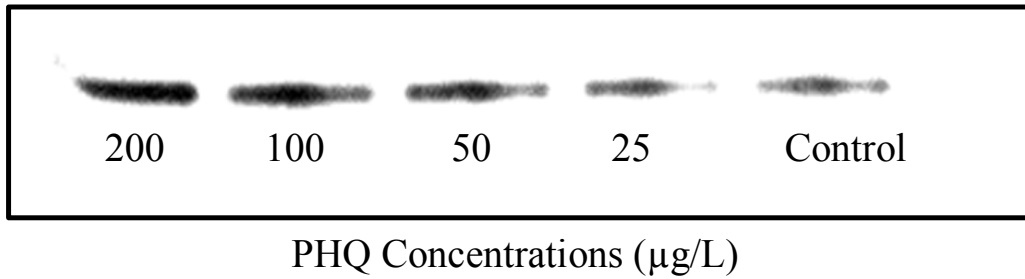
Figure 3. 4 The SOD activity of V. fischeri treated with series of concentrations of PHQ.

Bacteria (24 h culture) were harvested by centrifuge (2300 x g) and resuspended in 2% NaCl.

Bacteria (OD=0.1) were treated with 25-200 µg/L of PHQ for 30 min. The lysates was prepared

from treated bacteria for native SOD activity stains. Total protein of 5 µg were loaded each well of

polyacrylamide gel.
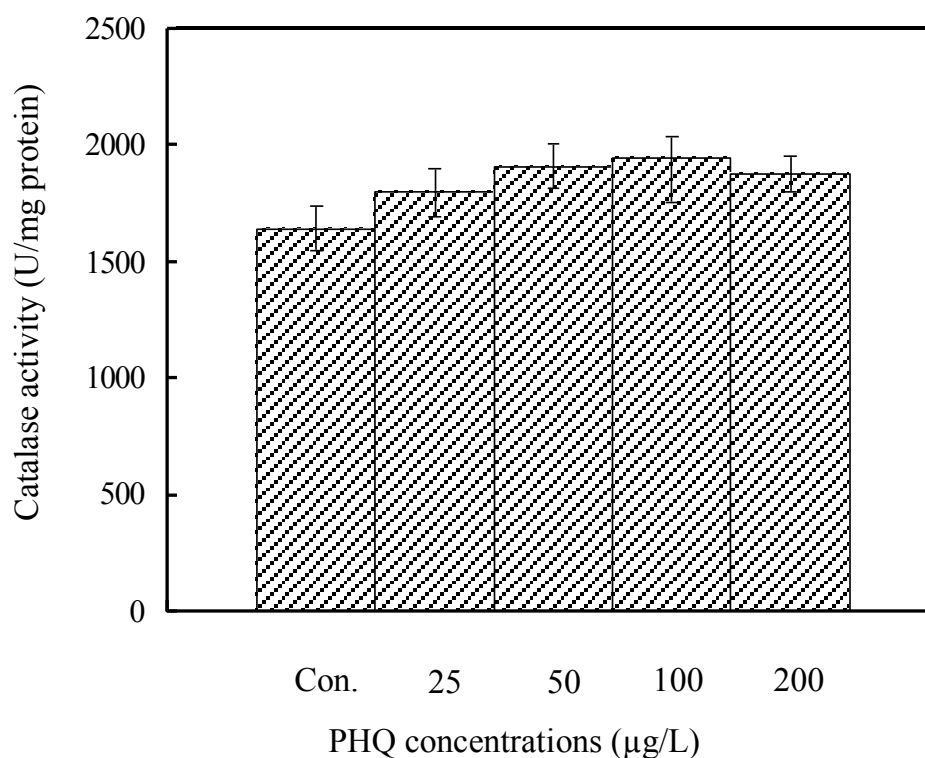
Figure 3. 5 Catalase activity of V. fischeri treated with a series of concentrations of PHQ.

Bacterial lysates were prepared using same method with SOD activity assay. Total catalase activity

was spectrophotometrically measured by the disappearance of H_2O_2 absorbance at 240 nm. Fifty μl

bacterial extracts were combined with 1 ml 2% NaCl containing 30 μl 3% H_2O_2. Absorbance of

the mixture was measured during a 100 sec period and catalase activities were calculated in U per

mg of protein. Data are U per mg of protein ± SE. Each experiment has been repeated

independently 5 times.
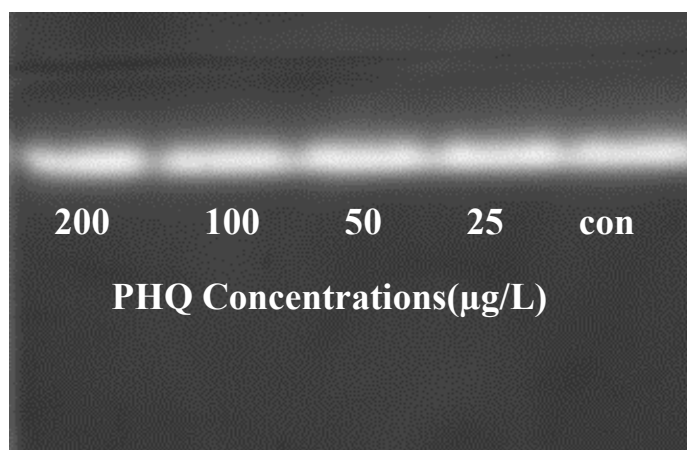
Figure 3. 6 The catalase activity native PAGE of V. fischeri treated with a series of concentrations of PHQ.

Bacteria (24 h culture) were harvested by centrifugation (2300 g) and resuspended in 2% NaCl. Bacteria (OD=0.1) were treated with 25-200 µg/L of PHQ for 30 min. The lysates prepared from bacteria of above treatments were used for catalase activity stains following native PAGE. Eight µg total protein was loaded each well of the polyacrylamide gel.

3.3.6 The effect of anaerobiosis on the toxicity of PHQ to V. fischeri

V. fischeri can grow under both aerobic and anaerobic conditions. This allowed me to examine the toxicity of PHQ to *V. fischeri* in the presence or absence of oxygen. Bacteria were exposed to PHQ under both aerobic and anaerobic conditions in a complex medium, and the effects of PHQ exposure on bacterial growth were followed by measuring absorbance at 650 nm for 10 h. As shown in Figure 3.7, under aerobic conditions, the growth of *V. fischeri* was significantly inhibited by PHQ at a concentration of 200 µg/L. This inhibitory effect was eliminated after 6 hours and growth rate recovered to that of controls (Fig 3.7A). PHQ completely eliminated growth of *V. fischeri* at 300 µg/L (Fig. 3.7A). However, under anaerobic conditions, PHQ did not show any toxicity to *V. fischeri* at the above concentrations. No significant inhibition of *V. fischeri* growth under anaerobic conditions was observed until a concentration of 2500 µg/L reached (Figs. 3.7B and 3.7C). These results show that the toxicity of PHQ to *V. fischeri* has a dependence on O_2.

3.4 Discussion

This study is the first to document the ability of PHQ to induce the ROS production in *V. fischeri* and to link this ROS production to toxicity. PHQ is a highly toxic compound to many organisms (El-Alawi et al. 2001; McConkey et al. 1997; Xie et al. 2006) . Reported EC50 of PHQ are 530 µg/L for *Lemna gibba* (McConkey et al. 1997), 357.6 µg/L for *Daphnia magna* (Xie et al. 2006), 308 µg/L for rat corpora lutea (Nykamp 2007), 416 µg/L for placental choriocarcinoma
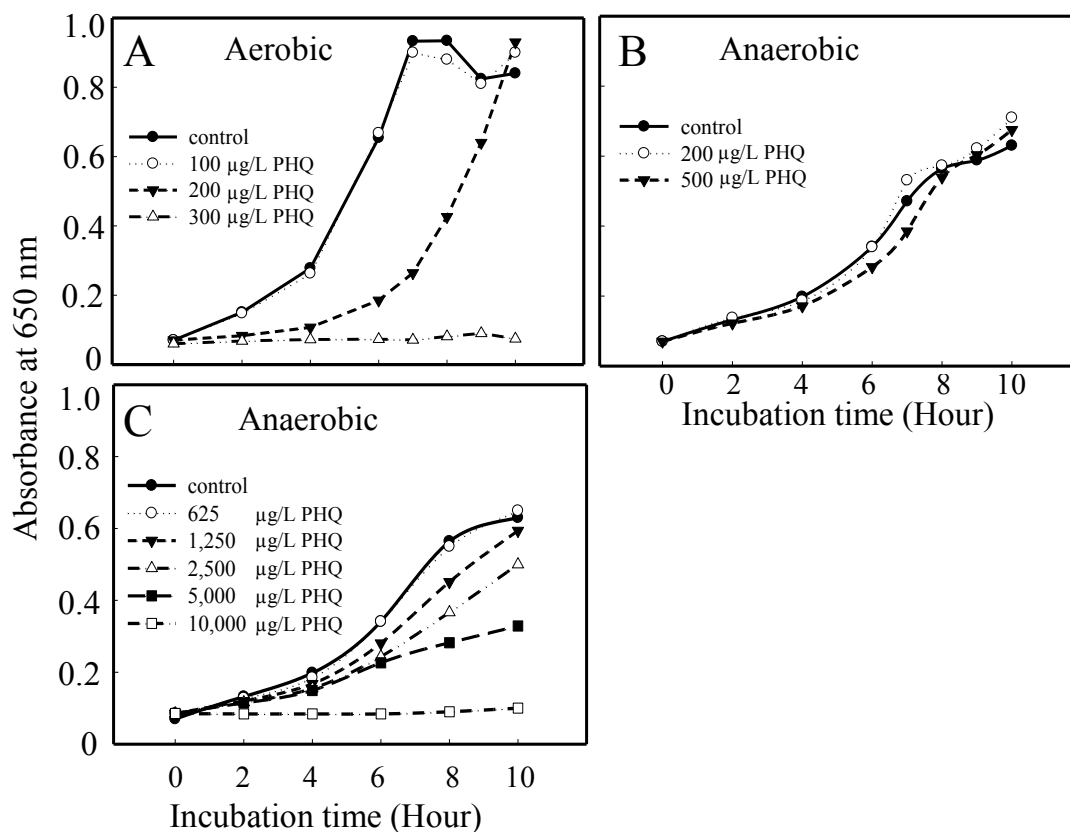
Figure 3. 7 Aerobic and anaerobic growth curves of V. fischeri in the presence of different

concentrations of PHQ.

The growth was monitored spectrophotometrically at 650 nm. Legend: control, no PHQ added;

100-10000 represent corresponding PHQ concentrations at μg/L.

cells JEG-3 (Nykamp 2007) and 64 μg/L for *V. fischeri* (30 min exposure) (Wang et al. in press). PHQ is a quinone with a strong redox cycling activity. It has been demonstrated that in vitro PHQ can redox cycle and transfer electrons to O_2 (Hasspieler et al. 1994; Monks et al. 1992; Nykamp 2007). In this study, PHQ enhanced the production of hydrogen peroxide and total ROS inside the bacteria cells (Figs. 3.2 and 3.3). It also induced higher activity of the antioxidant enzyme SOD, which likely detoxifies ROS in the cells (Fig. 3.4). The increased generation of reactive oxygen species (ROS) in bacterial cells implicated an ROS-mediated toxicity mechanism of PHQ. Furthermore, the toxicity of PHQ to *V. fischeri* was much lower under the anaerobic conditions (Fig. 3.7). Thus, by eliminating the ability of the cells to produce ROS, toxicity of PHQ to bacterial cells was diminished. This O_2-dependant toxicity of PHQ demonstrated, along with the connection of ROS production to PHQ exposure, provides direct evidence that ROS toxicity are directly involved in the toxicity for PHQ to *V. fischeri*.

The bioluminescence emitted by *V. fischeri* comes from the NAD(P)H-mediated respiratory electron transport chain (Fig. 3.8) (Bose et al. 2007). The light-emitting reaction in bacteria involves the oxidation of reduced riboflavin mono-phosphate ($FMNH_2$) and a long chain fatty aldehyde (RCHO) catalyzed by the luciferase resulting in the emission of blue-green light. Luciferase, composed of LuxA and LuxB, converts luciferin, $FMNH_2$, RCHO and O_2 to FMN, water and an fatty acid (Ziegler and Baldwin 1981) (Fig. 3.8). The $FMNH_2$ and RCHO can be regenerated with the electrons supplied by NAD(P)H, catalyzed with LuxCE and LuxG (NAD(P)H:FMN oxidoreductase), respectively (Bose et al. 2007). In the respiratory electron transport system, a dehydrogenase complex plays a crucial role to supply luciferase with electrons

Figure 3. 8 The possible pathways of toxicity of PHQ to V. fischeri.

This figure shows the electron transport processes used by *V. fischeri* for light emission. The primary path was from NADPH to light production is indicated (darker gray area). PHQ altered electron transport paths are shown in light gray area. PHQ can receive electrons from NADPH, lowers electron transport to FMN, and forming semiquinone. The quinone $\Leftrightarrow$ semiquinone cycle can transfer electrons to oxygen, generating superoxide (O_2^-) and other ROS. H_2O_2 generated from O_2^- can be consumed by catalase or luciferase (light gray area). H_2O_2 can react with luciferase, emitting faint light that is 280 times lower than the prevalent light emitting in normal reaction.
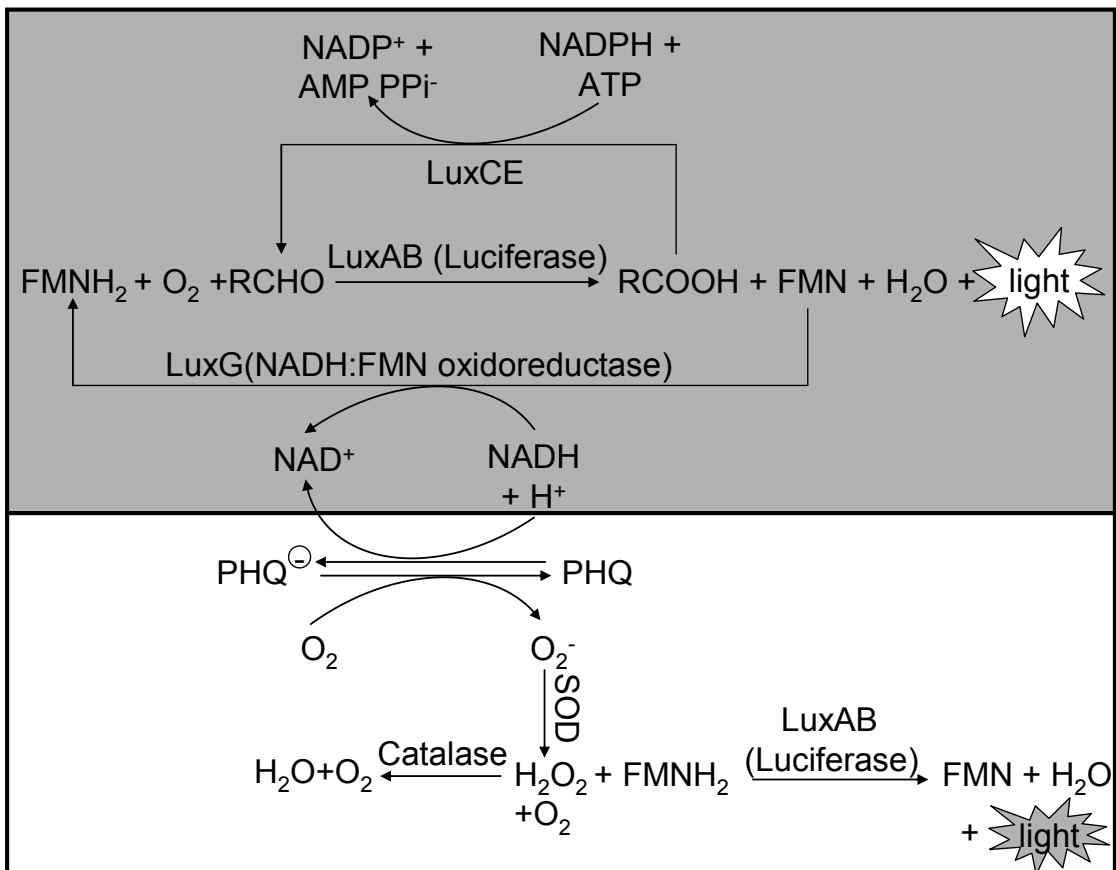
from $FMNH_2$. $FMNH_2$ can in turn be formed by the membrane bound NADH:FMN oxidoreducase encoded by the luxG gene (Lin et al. 1998; Ruby et al. 2005) using NADH and FMN (El-Alawi et al. 2001) (Fig. 3.8). PHQ can cause NAD(P)H-mediated toxicity via redox cycling between its quinone and semiquinone forms (Lemaire and Livingstone 1997). During this process, PHQ may acquire electrons from NAD(P)H and then transfer electrons to molecular oxygen to form O_2^- or other ROS (Fig. 3.8). Since both the ROS production induced by PHQ and bioluminescence of *V. fischeri* are NAD(P)H-mediated, it is likely that PHQ competes with FMN for electrons from NAD(P)H (Kudryasheva et al. 1999). When exposing *V. fischeri* to PHQ, the electron competition from NAD(P)H between PHQ and oxyluciferin (FMN) may inhibit respiratory electron flow from NAD(P)H to FMN, and thus the formation of $FMNH_2$, preventing the turnover of oxyluciferin (FMN) to luciferin ($FMNH_2$). This would result in decreased light emission from *V. fischeri* (Fig. 3.8).

PHQ can induce sufficient levels ROS to cause cellular damage. Most aerobic cells can express antioxidant mechanisms to detoxify the damaging effect of ROS. Two common redox-active enzymes that can diminish oxidative stress are superoxide dismutase (SOD) and catalase (Vattanaviboon et al. 2003). SOD catalyzes the dismutation of O_2^- to H_2O_2, which if unchecked will be readily converted to the very reactive ·OH via Fenton-type reactions (Xie et al. 2006). However, catalase catalyzes the conversion of H_2O_2 to harmless H_2O and O_2, preventing further ROS production.

Many organisms have more than one SOD (Mruk et al. 2002). However, in the native PAGE from extracts of *V. fischeri*, only one SOD, Fe-SOD, was observed (Fig. 3.4). As stated above, PHQ transfer electrons from NADH or NADPH to O_2, generating O_2^- in a redox cycling process

(Hasspieler and Digiulio 1994; Monks et al. 1992). Defending against the damage caused by O_2^-, which can give rise to more potent ROS, is performed primarily by SOD (Vattanaviboon et al. 2003). Higher SOD activity has been detected in green algae cells (*Scenedesmus armatus*) exposed to PHQ (Tukaj and Aksmann 2007). In this study, it was demonstrated that PHQ induced higher amounts ROS and higher SOD activity in bacterial cells, suggesting that PHQ may induce or trigger SOD expression of an SOD gene in *V. fischeri* via the production of O_2^-.

Catalase activity of *V. fischeri* did not change significantly when exposed to PHQ, regardless of the level of H_2O_2 induced by PHQ (Fig. 3.2). The catalase gene, *katA*, of *V. fischeri* has been sequenced (Visick and Ruby 1998). The predicted amino acid sequence of *katA* has a high degree of similarity to group III catalases. *In vivo*, PHQ gives rise to production of O_2^- that can be dismutated or chemically reduced to H_2O_2. Visick and Ruby (Visick and Ruby 1998) reported that *katA* is an H_2O_2-inducable gene. *V. fischeri* is a marine bacterium, and as such would be exposed to high concentrations of O_2^- and hydrogen peroxide formed photochemically in surface sea water by solar radiation (Rees et al. 1998). To defend from oxidative damage, *V. fischeri* likely has evolved very efficient mechanisms to detoxify ROS. The presence of a highly active catalase in the periplasm of *V. fischeri* cells would consume H_2O_2 before it could enter the cell (Ruby and McFall-Ngai 1999). Further, bioluminescence is thought to originally evolved for the detoxification of ROS (Rees et al. 1998; Rodriguez et al. 2004). The evolutionary origin of bacterial luciferase is proposed to relate to an original catalase role as a scavenger of ROS (Szpilewska et al. 2003; Watanabe et al. 1993). In the bioluminescent reaction of *V. fischeri*, luciferin (aldehyde) can be substituted by H_2O_2 to react with luciferase and emit light, but light yield is 280 times lower than the prevalent light emitting in normal reaction (Fig. 3.8) (Watanabe

et al. 1993). Luciferase constitutes up to 5% of the total soluble protein of *V. fischeri* (Ruby and McFall-Ngai 1999). Hence, *V. fischeri* could use catalase and luciferase to defend against H_2O_2 induced by PHQ. The H_2O_2 concentration induced by PHQ (Fig. 3.2) might have not been high enough to significantly increase the already high levels of catalase.

Because PHQ toxicity appears to occur via redox cycling of PHQ, leading to of ROS production, it was expected that growing organisms under anaerobic conditions would lower the toxicity of PHQ. As expected, PHQ showed much less toxicity under anaerobic conditions than that under aerobic conditions (Fig. 3.7). Rodriguez et al. (Rodriguez et al. 2004) reported that PHQ is more toxic in the presence of oxygen than in the absence of oxygen to yeast. Further research by Rodriguez (Rodriguez et al. 2007) demonstrated that exposure to PHQ leads to DNA deletions and point mutations only in the presence of oxygen, and this probably occurs via the ROS. The oxygen-mediated toxicity provides evidence that ROS production is critical to the toxicity mechanism of PHQ.

PHQ is toxic at high concentrations (>2000 µg/L) under the anaerobic conditions, suggesting a non-ROS mediated mechanism of PHQ toxicity. In this case, multiple toxicity mechanisms could be involved. Because PHQ may not readily redox cycle due to the lack of oxygen as the electron acceptor, the amplified damage caused by catalytic production of ROS would be hindered. Thus, PHQ might only exhibit toxicity due to marcosis. This could occur via direct damage to cell membranes. The toxicity at high concentration available PHQ was also observed in yeast (Rodriguez et al. 2005). In that case, the toxicity mechanism was proposed to be due to the selective inhibition of the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH is a key glycolytic enzyme involved in the central pathway of carbon metabolism. It is widespread

among bacteria of different phylogenetic groups (Fourrat et al. 2007), including *V. fischeri* (Ruby et al. 2005). GAPDH is a NADPH dependent enzyme (Fourrat et al. 2007). Under anaerobic condition, PHQ may compete with GAPDH for electrons from NADPH or other biomolecules, resulting in the inhibition of GAPDH activity.

It is interesting that under aerobic conditions at a concentration of 200 μg/L of PHQ, the growth of *V. fischeri* was inhibited at an early stage, but recovered to a growth rate similar with controls after 6 hours (Fig. 3.7). Because the experiments were performed in sealed containers, it is possible that the consumption of O_2 during bioluminescence resulted in partial anaerobic conditions, and hence lowered the toxicity of PHQ to bacterial cells. Bioluminescence is necessary for *V. fischeri* to compete with other bacteria for colonizing the light organs of squids, its original natural habitat (Visick et al. 2000). *V. fischeri* do so by lowering its surrounding ambient oxygen concentration below that required to support the activity of oxidases of other microbes inside light organ of squid (Visick et al. 2000). This is consistent with the fact that luminescent bacterial luciferases have an unusually high affinity for oxygen. They consume oxygen and lower its concentration quickly under conditions in which the supply of oxygen is limiting (Ruby and McFall-Ngai 1999). The diminished oxygen concentration can effectively lower the toxicity of PHQ. Hence, recovered bacterial growth might occurr in this study at 200 μg/L PHQ after sufficient amount of O_2 were consumed. Conversely, PHQ at 200 μg/L could be a threshold concentration for its toxicity. The PHQ-enhanced activity of antioxidant enzymes, such as SOD, that may in turn counter the effects of PHQ. As well, it is possible that PHQ degradation might also attribute to the recovered bacterial growth under these conditions.

In summary, PHQ is apparently toxic to *V. fischeri* via its redox cycle between quinone and

semiquinone forms, leading to ROS production. During this process, PHQ may compete with oxyluciferins for electrons which blocks the formation of luciferins, the substrate of bacterial light emission reactions. At the same time, PHQ may transfer the obtained electrons to molecular oxygen to form O_2^- and other ROS. These two processes result in blocking the formation of luciferins and generating ROS, leading to light emission inhibition of *V. fischeri* by PHQ at extremely low concentrations under aerobic conditions.

Chapter 4

Identification of Genes in *Vibrio fischeri* Response to 9,10-phenanthrenequinone Exposure

4.1 Introduction

The fate of PAHs released to the environment by accident or by industrial effluent is of great concern because of their acute and harmful chronic effects on ecological systems. These effects include direct toxicity (El-Alawi et al. 2001; Huang et al. 1993), phototoxicity (Lampi 2005; McConkey et al. 1997; Moza et al. 1999; Xie et al. 2006), and toxicity/mutagenicity via the metabolite intermediates of organisms (Kadry et al. 1995). PHQ is an oxyPAH that co-occurs with its parent PAH, PHE, in PAH contaminated sites (Xie et al. 2006). PHQ can be produced through car emission (Xia et al. 2004), photooxidation (McConkey et al. 1997), and biotransformation (Kadry et al. 1995; Meudec et al. 2006; Takizawa et al. 1994).

Organisms in PAH contaminated sites are often exposed to PHQ. PHQ has been shown to exert toxic effects via oxidative stress resulting from redox cycling of PHQ (Hasspieler and Digiulio 1994; Hasspieler and Digiulio 1995; Jarabak et al. 1996; Jarabak et al. 1997; Jarabak and Jarabak 1995). The redox ability of PHQ is very high (Jarabak et al. 1996). This involves (non)enzymatic reduction followed by autoxidation of the compound yielding reactive oxygen species (ROS), including superoxide anions (Hasspieler et al. 1996), hydrogen peroxide (H_2O_2) (Jarabak et al. 1996) and hydroxyl radicals (Sjolin and Livingstone 1997). These ROS lead to a variety of toxic processes including enzyme inactivation, lipid peroxidaton and DNA damage (Jarabak et al. 1996).

V. fischeri is a marine luminescent bacterium extensively used in toxicological studies. In its natural marine habitat, *V. fischeri* is exposed to high concentrations of superoxide anions and hydrogen peroxide formed photochemically in the upper sea water by solar radiation (Rees et al. 1998; Zika et al. 1985). As a symbiotic of squid, *V. fischeri* colonize its light organ where host

generated ROS as a part of its immune system to defend the invasion of bacteria (Visick et al. 2000). The competitive privileges of bacteria with bioluminescence for colonization of light organ are to reduce their exposure or increase their tolerance to host- and nature- generated ROS (Visick et al. 2000). Numerous defense mechanisms, such as increasing activity of antioxidase enzymes or excretion of polysaccharides, were developed in this bacterium to detoxify ROS (Visick et al. 2000) (Chapter 3).

The expression of genes in response to specific chemical exposure can be identified by comparing the mRNA between control and treated samples. Differential display PCR (ddPCR), developed by Liang and Pardee (1992), is a powerful tool that allows differentially expressed gene fragments to be isolated and identified from biological samples (Lees 2005; Liang and Pardee 1992). This technique has been applied in toxicological research to probe the gene expression alternation of organisms exposure to a variety of toxicants or stress conditions (Akhtar et al. 2005; Lees 2005; Nykamp 2007; Walters et al. 2001). However, only a few prokaryotic applications of ddPCR have been reported (Frias-Lopez et al. 2004; Walters et al. 2001). In the original description of ddPCR for eukaryote, anchored primers complementary to the poly(A) tail of mRNA were used for cDNA synthesis and the following PCR amplification (Liang and Pardee 1992). Because prokaryotic mRNA usually does not have polyadenylation (Fislage 1998), arbitrary primers are typically used for both reverse transcription and PCR steps in the application of ddPCR in bacteria (Chang et al. 2003).

Microorganisms like bacteria minimize their energy expenditure by expressing only the genes that they need under specific growth conditions (Walters et al. 2001). Differential display PCR of bacteria uses arbitrari oligonucleotide primers to create a unique cDNA fingerprint for bacteria

exposed to a particular physiological state (Bidle and Bartlett 2001), thus providing a powerful tool to assess differential gene expression in bacteria under stresses such as chemical toxicants. Although this technique has been applied to gene discovery in toxicology studies using various eukaryotes (Akhtar et al. 2005; Lees 2005; Nykamp 2007), it has not been applied to study toxicant induced gene examination in bacteria. In this chapter, ddPCR was used to identify the altered gene expression of *V. fischeri* exposure to the PHQ.

4.2 Material and methods

4.2.1 Bacterial, growth conditions and exposure to PHQ

V. fischeri was cultured on marine medium for 24 hours in the dark on a rotary shaker (50 rpm) at 20˚C (El-Alawi et al. 2001). Bacterial cells were harvested and washed twice with 3% saline by centrifuging at 2,300 x g and 4 ˚C. Washed cells were resuspended and adjusted to OD 0.2 with 3 % saline solution. The bacterial suspension was divided into six 50-ml sterilized Falcon centrifuge tubes. Each tube contained 40 ml bacterial suspension. PHQ dissolved in the DMSO was added into each tube to the final concentration of 0, 25, 50, 100, or 200 μg/L. Each tube was gently vortexed and allowed to settle under dark condition for 30 min. Treated cells were harvested by centrifuging at 2300 x g and 4 ˚C and the supernatant was carefully removed. The cell pellets were resuspended in 1 ml 3% saline for further analysis.

4.2.2 Isolation of total cellular RNA

PHQ treated bacterial cells were further treated with Bacterial RNA Protection Reagent (Qiagen Inc. Mississauga, ON, Canada) to freeze RNA (Hancock and Klemm 2007). Total RNA was stabilized before cell lyses by application of the RNAprotect bacterial reagent (Qiagen). RNeasy mini columns (Qiagen Inc) were used for RNA isolation following the manufacturer's instruction. Briefly, bacterial suspension (0.5 ml) was added into 2 ml microcentrifuge tubes containing 1 ml RNA protection reagents and stand for 5 min. Cells were collected by centrifuging at 5000 g for 10 min in a bench top microcentrifuge (Model 541C, Eppendorf-Brinkmann, Mississauga, ON, Canada) kept at 4 ℃. All subsequent steps were performed according to the manufacturer's instructions. The isolated RNA was finally dissolved in diethylpyrocarbonate (DEPC)-treated water. RNA concentration was determined spectrophotometrically at 260 nm using a Cary50 spectrophotometer (Varian, Palo Alto, CA). RNA concentrations of all samples were approximately 1000 μg mL^{-1}. RNA purity was estimated by calculating the RNA-DNA Abs260/protein Abs280 ratio. RNA integrity was verified prior to further processing using denaturing gel electrophoresis. RNA samples were stored at -80 ℃ until used for further analysis.

4.2.3 Reverse transcription of RNA

Reverse transcription was performed by using the First Strand cDNA synthesis kit (MBA Fermentas, Burlington, ON, Canada) following the manufacturer's instruction. RNA (3 μg) was used as template. The arbitrary primers used in this experiment are A3 (AAT CTA GAG CTC CAG CAG) and A4 (AAT CTA GAG CTC TCC TGG). There primers have been used with

several organisms and demonstrated as suitable for ddPCR (Lee 2005; Akhtar et al., 2005; Nykamp 2007). Reaction products were diluted 1:10 and 5 μL was used as a template for the following PCR.

4.2.4 Differential display PCR (ddPCR)

Differential display PCR (ddPCR) was carried out as previously described by Akhtar et al. (2005). Amplification of cDNA was performed using Ready-To-Go PCR Beads (Amersham Pharmacia Biotech Inc., Baie d'Urfe, QU, Canada) with the arbitrary primers (A3 and A4) (Table 5.1) in a 25 μL PCR reaction. The cycling conditions for the reactions were as follows: 94 ºC for 1 minute, 35 ºC for 5 minutes, 72 ºC for 5 minutes followed by 39 cycles of 94 ºC for 2 minutes, 50 ºC for 2 minutes, and 70 ºC for 2 minutes. All PCR reactions were performed in a thermocycler (MJ Research Products, Waltham, MA, USA). PCR products from control and PHQ-treated samples were fluorescently labeled with Cy 5.0 adapter primer (AAT CTA GAG CTC) (AGCT Inc., Irvine, CA, USA). Briefly, 1 μL of Cy 5.0 primer (0.2 μM mL^{-1}) was added to the entire PCR reaction and incubated for 2 minutes at 95 C. Reaction tubes were immediately cooled on ice and 1 μL of dNTPs (25 μM), 3 μL of 10X Klenow Reaction Buffer, and 1 μL of Klenow polymerase, exo- (5 U uL^{-1}) (MBI Fermentas) were added to the tube. The tube was gently mixed and incubated for 30 minutes at room temperature followed by 2 hours at 37 ºC. A portion (7 μL) of the fluorescently tagged PCR products were resolved on a 6 % acrylamide (19:1 acrylamide:*bis*-acrylamide; Bioshop, Burlington, On, Canada), 7 M urea denaturing polyarcylamide sequencing gel prepared in 1X Tris-borate-EDTA buffer and electrophoresed at 1600V, 55 W for 3 hours. PCR products were visualized using a phosphoimager (Typhoon Storm 860, Molecular Dynamics

Corp., Sunnyvale, CA, USA). Differentially displayed cDNA fragments of interest were excised from the gel and eluted using a polyacrylamide gel extraction kit (Qiagen Inc. Mississauga, ON, Canada) according to the manufacturer's instructions.

4.2.5 Isolation of ddPCR fragments

A full size print of the polyacrylamide gel was produced from the image provided by the Storm Imaging system. This print was carefully overlaid by the gel and the sites marked by loading buffer were aligned. The fragments of interest were excised from the gel using fresh sterile razor blades and the gel slice placed in a 1.5 ml microfuge tube. The gel was rescanned to confirm the removal of the desired bands. To each tube, 200 μl of diffusion buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% SDS pH 8.0) was added and incubated for 30 minutes at 50°C. DNA was recovered using the QIAGEN gel extraction kit (QIAGEN Inc.). The eluted cDNA was dissolved in 20 μl of sterile water and an aliquot was re-amplified using the same primers and conditions as previously stated. The re-amplified cDNA was purified using a 1% [w/v] agarose gel electrophoresis containing 0.7 mg ml^{-1} ethidium bromide. The products and GeneRuler 100bp DNA Ladder Plus (MBI Fermentas, Burlington, ON) were electrophoresed on the agarose gel for approximately 45 minutes at 100 V. Any DNA present in the gel was visualized under UV light. Bands corresponding in size to the ones excised from the polyacrylmide gel were cut out of the agarose gel using sterile razor blades and placed in 1.5 ml microfuge tubes. The DNA was extracted from the agarose using the QIAGEN gel extraction kit. The eluted DNA was dissolved in 20 μl of sterile water before the concentration was determined spectrophotometrically at 280 nm. The isolated DNA was stored at -20°C until further required.

4.2.6 Cloning of Recovered cDNAs

The reamplified PCR products of specific sizes eluted as described above were subsequently used for cloning and sequencing. An aliquot (3.5 μl) of the recovered DNA band was ligated into the pGEM T-easy vector (Promega, Madison, WI) in a 10 ml reaction containing 5 μl of 2X ligation buffer (100 mM Tris-HCl pH 7.5, 20 mM MgCl2, 20 mM DTT), 1 μl of vector (50 ng/μl), and 1 μl of T4 DNA ligase (2 Weiss units/ml). The samples were incubated at 4 °C for 24 hours. From the ligation reaction, an aliquot was used to transform *E. coli* JM109 high efficiency competent cells (Promega, Madison, WI). Briefly, 2 μl of ligation reaction product was transferred to a 1 ml microfuge tube whereby 50 μl of competent cells was added and incubated on ice for 20 minutes followed by a brief heat shock at 42 °C for 45 seconds. Tubes were immediately placed on ice for 3 minutes and 500 μl of LB broth (1 % [w/v] tryptone, 0.5 % [w/v] yeast extract, 1 % [w/v] NaCl) added. The samples were agitated at 150 rpm for 1.5 hours at 37 °C. From each, 100 μl was spread over the surface of LB agar plates containing ampicillin (100 μg ml-1) (MBI Fermentas, Burlington, ON), 0.5 mM IPTG, and 80 μg ml^{-1} X-Gal (Promega, Madison, WI). The transformed bacteria were allowed to adsorb to the agar surface for 20 minutes at room temperature prior to incubation at 37 °C for 16 hours.

4.2.7 Isolation of plasmid DNA

Bacteria containing the pGEM T-easy vector (Promega, Madison, WI) with an insert produced white colonies while those that did not contain the vector were blue. A single colony was selected and used to inoculate 5 ml of LB broth containing 100 mg ml^{-1} ampicillin. The culture tubes were

agitated at 200 rpm for 16 hours at 37 °C. From each tube, 1.5 ml of culture was transferred to a 1.5 ml microfuge tube and centrifuged at 13,000 x g for 1 minute at 4 °C. The supernatant was removed and another 1.5 ml of culture added to each corresponding tube for a second centrifugation as stated above. The plasmid were isolated and harwested with GeneElute Plasmid Miniprep Kit (Sigma, St Louis, MO, USA) and stored at -20 °C until required for use.

4.2.8 Restriction digest of purified plasmids

To release the inserts from the plasmids, the restriction enzyme *Eco* RI (Promega, Madison, WI) was used (Akhtar et al., 2005). A 1.5 μg aliquot of plasmid was added to a 0.5 ml microfuge tube and digested in a 20 μl reaction using 2 μl of 10 X reaction buffer H (90 mM Tris-HCl pH 7.5, 500 mM NaCl, 100 mM MgCl2), 0.5 μl bovine serum albumin (0.1 mg ml^{-1}), and 0.5 μl of Eco RI (10 U ml^{-1}). The mixture was incubated for 2 hours at 37 °C then analyzed on a 1 % (w/v) agarose gel containing 0.7 mg/ml ethidium bromide that had been electrophoresed for 1.5 hours at 95 volts. A GeneRuler 100 bp DNA Ladder Plus was added to the gel for size comparison.

4.2.9 Sequence analysis

Plasmids with inserts of confirmed size were sequenced using an Opengene DNA sequencer (Visible Genetics, Inc, Toronto, ON) at the Molecular Core Facility (Dept. of Biology, University of Waterloo, Waterloo, ON). Inserts were sequenced in both the forward and reverse directions (Table 5.1). Sequences were compared to data in Genbank using the NCBI (National Centre for Biotechnology Information) network BLAST.

4.3 Results and discussion

4.3.1 RNA isolation

Methods for isolating intact RNA from bacteria are problematic due to extremely short half life

of RNA in bacteria (Mangan et al. 1997). To ensure accurate analysis of gene expression in

bacteria, it is important to analyze RNA that truly represents *in vivo* gene expression. RNAprotect

Bacteria Reagent (Qiagen) was used in this study to stabilize RNA *in vivo* and ensure reliable gene

expression analysis. This Reagent provide immediate stabilization of the *in vivo* gene expression

profile in bacteria and prevents both degradation of RNA transcripts and induction of genes. (Fey

et al. 2004; Hancock and Klemm 2007) (Fig. 4.1). The general concentration of extracted RNA

was 1300-1800 μg/L.

4.3.2 PHQ–induced alterations in gene expression as detected by ddPCR

ddPCR analysis was used to test the effect of various concentrations of PHQ on this bacterial

gene expression. The resulting profile of ddPCR from polyacrylamide gel showed some distinct

changes between treatments (Fig. 4.2). Approximately 30-40 bands were observed in each lane of

Figure 4. 1 Total RNA extracted from bacterial cells exposure to different concentrations of PHQ. Bacterial cells of 40 ml (24 hour culture, OD=0.1) were treated with PHQ for 30 min. Treated cells were harvested, washed twice and resuspended in 2 ml 2 % NaCl. RNA protect reagent (4 ml) was added into bacterial suspension to stabilize RNA. Total RNA was extracted with RNeasy mini columns (Qiagen Inc) according to manufacturer's instruction.
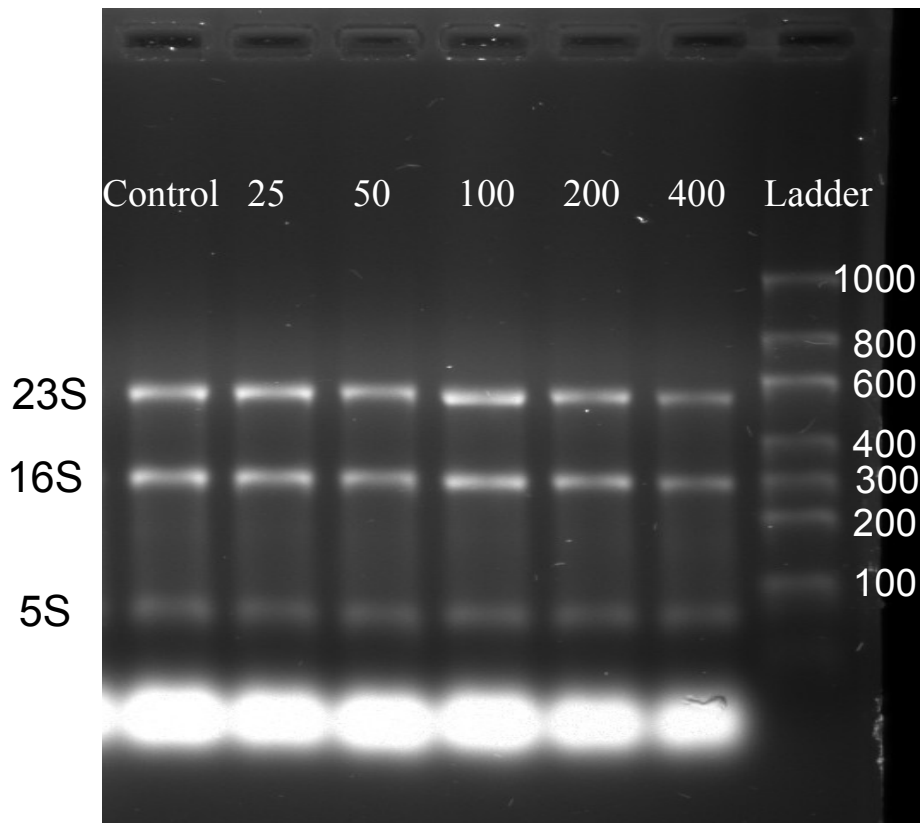
Figure 4. 2 Different display PCR profile generated from bacterial cells exposed to different concentrations of PHQ.

Bacterial cells were exposed to PHQ concentrations ranging from 0 to 200 for 30 min and subsequently harvested for ddPCR analysis (the EC50 of *V. fisheri* to PHQ is μg/L based on light emission inhibition). PCR products were labeled with Cy5 internal labeled primer. Labeled PCR products of cDNA of 4 ul amplified with A3, A4 primer were loaded in each lane. Electrophoresis was carried out at 1600V, 55w for 3 hours. Bands labeled B1, B2, and B3 appear to increase in intensity in response to PHQ exposure. These bands were excised from the gel, re-amplified, cloned and sequenced. A 500 bp DNA marker was included to indicate relative sizes of displayed cDNAs.
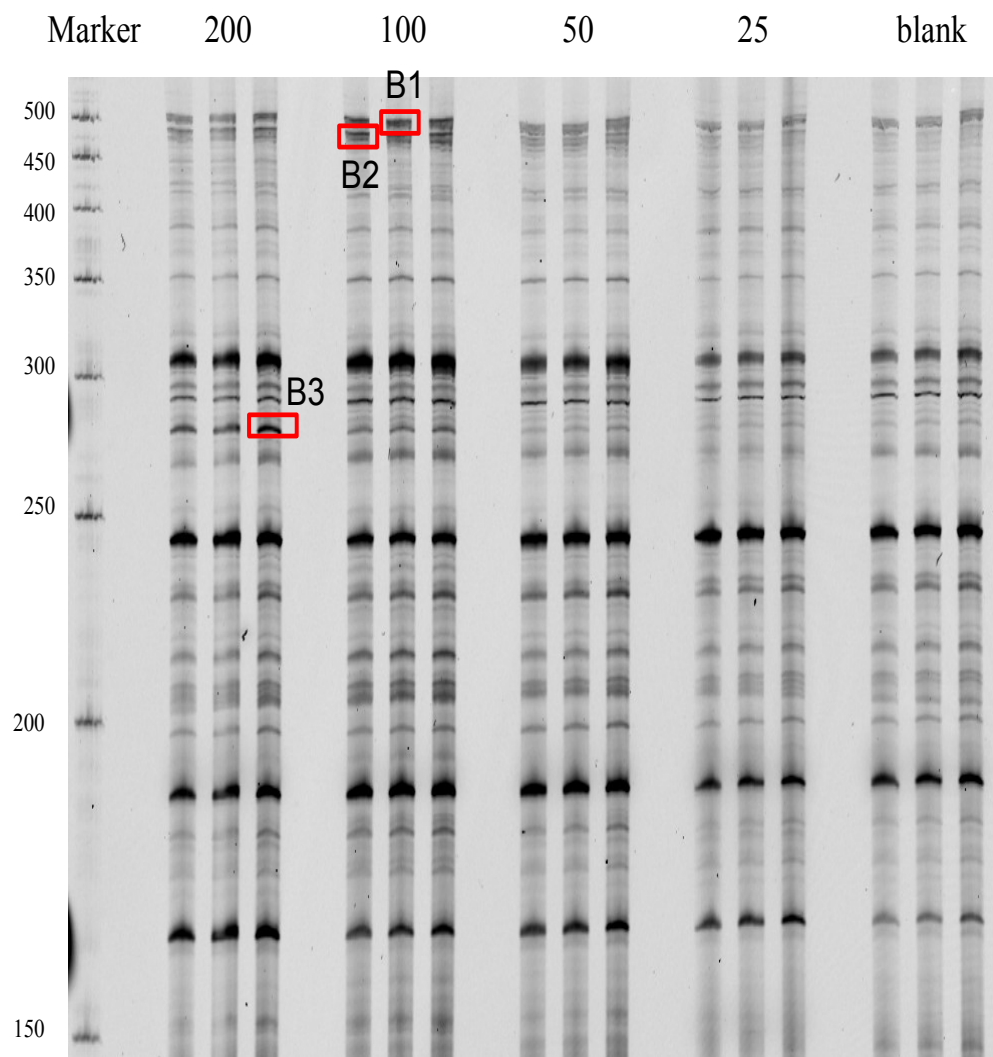
each independent ddPCR regardless of the treatments. It is similar to the results obtained from *Lemna gibba* (Akhtar et al. 2005; Lees 2005) and human placental cell line (Nykamp 2007) using the same primer set, where 40-50 bands were observed. From the gels, three cDNA fragments were excised for further analysis because they were consistently more intense than those in the control treatments in several independent experiments. Band intensity increased with increasing PHQ concentration. All these gene fragments were up-regulated due to PHQ exposure (Fig. 4.2). The sizes of these cDNA fragments range from 282 bp to 520 bp. The three gene fragments excised were subsequently re-amplified, cloned, and sequenced. Their identities, based on sequence similarity to known genes in the NCBI database, are given in Table 4.2. Because the genome of *V. fischeri* has been fully sequenced (Ruby et al. 2005), the exact genes from these bands were identified.

The genes fragments up-regulated by PHQ exposure can be grouped into two classes, the transcription/translation machinery genes (B1) (23s rRNA, DNA-directed RNA polymerase beta' chain) and protein transport genes (B2 and B3) (Protein translocase subunit SecY and putative polysaccharide export protein YccZ precursor) (Fig. 4.2; Table 4.1). rRNA comprises around 60% of total RNA and rRNA is generally 20 times more abundant than all other mRNA combined (Walters et al. 2001). rRNA commonly cause false positive in bacterial application of ddPCR (Nagel et al. 1999). Therefore, they are not discussed.

Expression of protein translocase subunit SecY gene (B2) appeared to be up-regulated with PHQ treatment (Fig. 4.2; Table 4.1). This gene encodes translocase subunit SecY protein (SecY protein) that is a component of Sec translocase (Mori and Ito 2001). Sec translocase are mostly responsible for the translocation and secretion of most extracellular proteins, such as

Table 4. 1 Identification of differentially displayed cDNA from V. fischeri exposed to PHQ.

Band	Fragment size (bps)	Sequence identification
B1	520	rRNA-large subunit ribosomal RNA; lsuRNA; 23S ribosomal RNA
B2	488	Protein translocase subunit SecY
B3	282	putative polysaccharide export protein YccZ precursor

polysaccharides, across the outer membrane of Gram-negative bacteria (Holland 2004; Natale et al. 2007). SecY binds two small integral membrane proteins, SecE and SecG, forming SecYEG complex as protein conducting channel, which has be found in every bacteria, archaeon, and eukaryote examined to date (Saier 2006). Protein conducting channels form a hydrophilic pore via which secretory proteins may pass membrane (Dalbey and Chen 2004). SecY are absolutely essential for protein export and insertion of the majority of tested membrane proteins (Mori and Ito 2001). The translocation event occurs at the cytoplasmic membrane in prokaryotes (Mori and Ito 2001). In the current study, up-regulation of protein translocase subunit SecY gene with PHQ treatment may signify an increase in synthesis of SecY protein by the cell as a response to transport function proteins for defending the toxicity.

Another up-regulated gene (B3) response to PHQ exposure is putative polysaccharide export protein YccZ precursor that encodes the biosynthesis of protein of putative polysaccharide export protein YccZ precursor (Fig. 4.2; Table 4.1). Putative polysaccharide export protein YccZ precursor is a protein involved in polysaccharide transport. Polysaccharide is the most important part of bacterial capsule that comprises the outmost layer of the cell wall of the Gram-negative bacteria (Leung et al. 2006). Many bacteria are capable of secrete polysaccharides, thus forming a capsule or biofilm to shield themselves from environmental stresses, host immune responses and phagocytosis (Snyder et al. 2006). Polysaccharides have been demonstrated to be a ROS scavenger (Trommer and Neubert 2005) in bacteria (Kishk and Al-Sayed 2007; Qian and Xu 2007), fungi (Cho et al. 1998; Sun et al. 2004a), and plant (Li et al. 2007b). Thus, both up-regulated genes in *V. fischeri* with PHQ exposure involve the proteins responsible for the transport of polysaccharide.

This may indicate that the PHQ exposure resulted in the increased translation of polysaccharide export protein and increased excretion of polysaccharide.

V. fischeri is a symbiont of squids, colonizing its light organ. This symbiont is highly specific, and only cells of *V. fischeri* are able to colonize the squid (Visick et al. 2000). *V. fischeri* excrete polysaccharides to defend host immune system including host generated ROS (Visick et al. 2000). The excretion of polysaccharides by *V. fischeri* is enhanced in the colonization period to defend host immune system or be ROS scavenger (Siegl 2004). PHQ exposure induces similar stress in bacteria with host colonization: the production of ROS. Thereby, this may results in the similar bacterial response: the more polysaccharide synthesis or excretion. This allows *V. fischeri* quickly build a protective barrier for detoxifying ROS against PHQ exposure.

Chapter 5

Microbial Analysis of Soil During Plant Growth Promoting Rhizobacteria Enhanced

Phytoremediation at a Petroleum Land Farm Site

5.1 Introduction

Petroleum Land farms (PLFs) are often located near well sites and refinery facilities. During land farming, conventional agricultural tilling of land farm soils is used to promote the volatilization and degradation of petroleum hydrocarbons (PHCs) (USEPA 1994). Because lighter (more volatile) fractions of PHCs such as fraction 1 (F1) containing C6-C10 and fraction 2 (F2) containing C10- C16 tend to be removed rapidly, soil at PLF sites maily consists of fraction 3 (F3) containing C16-C34 and fraction 4 (F4) containing C34-C50. F3 and F4 are a complex mixture of aromatic, aliphatic, hyterocyclic and asphaltene hydrocarbons that are very hydrophobic and recalcitrant to breakdown. Some compounds from F3 such as polycyclic aromatic hydrocarbons are very toxic to biological receptors (CCME 2001; USEPA 1994).

Typical remediation methods for PLF soil include *ex situ* and *in situ* treatments (Chaudhry et al. 2005). *Ex situ* treatments such as chemical inactivation or thermal degradation involve excavating and transporting large quantities of soils (Amatya et al. 2002). These treatments, though effective, are costly and disturb soil structure. Alternately, the soil may be remediated in situ by bioremediation or composting (Catalan et al. 2004). These *in situ* treatments are more cost effective. However, they are very slow at degrading PHCs from PLF soils due to low populations of PHC degrading microbes resulting from nutrient limitation and soil toxicity (Kirk et al. 2005a).

Phytoremediation, an *in situ* remediation technology, that is cost effective and environment-friendly, holds promise for decontamination of the PLF soils (Glick 2003; Glick 2004). Plant roots exude organic compounds, which will increase the density and activity of microbes in the rhizosphere. These microbes in turn can degrade PHCs, and also make them bioavailable for root

uptake and phytodegradtion (Cunningham et al. 1996; Siciliano et al. 2003). Although a few papers have reported that certain plants can take up, translocate, and degrade F3 and F4 inside plants (Meudec et al. 2006), plants alone are not effective for remediation of these high molecular weight PHCs (Kramer 2005). For instance, direct plant uptake of F3 and F4 of PHCs was very slow and minimal because of their high hydrophobicity and large size (Kaimi et al. 2007). Thus, the main mechanism of phytoremediation of PHCs is considered to be the stimulation of the microbial community at the root–soil interface. The microbes at this interface in turn degrade the recalcitrant PHCs in the soil.

One challenge that has hindered use of phytoremediation at PLFs is that PHCs at high concentrations can inhibit plant growth and lead to unacceptably slow remediation rates (Greenberg et al. 2007a; Huang et al. 2004a; Kirk et al. 2005a; Lin et al. 2002; Zhuang et al. 2007). One strategy for enhancing phytoremediation efficiency is to use plant growth promoting rhizosphere (PGPR) enhanced phytoremediation (PEP). The PGPR used in this research were UW4 *(Pseudomonas putida)* and UW3 *(Pseudomonas sp.)*. Both PGPR have high 1-amino-cyclopropane-1-carboxylic acid (ACC) deaminase activity that can consume ACC, the precursor of plant stress ethylene, consequently lowering the ethylene levels inside the associated plants (Glick 2005; Glick et al. 2007b). Environmental stresses such as petroleum, metals or pathogens inhibit plant growth often by inducing the production of higher than normal levels of stress ethylene inside plants (Glick et al. 2007a; vanLoon 1984). Hence, the inhibition of ethylene biosynthesis or ethylene action by PGPR can facilitate plant growth under environmental stress (Glick et al. 2007a). Greenhouse and field studies have demonstrated that seed inoculation with PGPR can significantly enhance phytoremediation efficiency of PLFs (Huang et al. 2005; Huang et al. 2004a).

As either petroleum degraders or plant growth promoters, microbes can play critical roles in the phytoremediation of PHCs. However, limited information is available on the microbial populations and diversities in PHC contaminated soil during phytoremediation (Kirk et al. 2005a; Maila et al. 2006; Phillips et al. 2006). Greenhouse studies revealed that plant growth enhances microbial growth in soil and alters the community structure in PHC contaminated soils (Kirk et al. 2005a; Phillips et al. 2006). However, no one has performed field kinetic analyses of microbial populations during phytoremediation. Although PEP can successfully remediate PLF soils, the mechanism of PEP is not very clear. Understanding phytoremediation mechanisms will help establish better remediation strategies in the subsequent remediation practices. The objective of this research is to explore the roles that microbes, including PHC degraders and plant-growth-promoting-rhizosphere (PGPR), play in PGPR enhanced phytoremediation (PEP) of a petroleum land farm (PLF). This was done by kinetic analyses of the populations of PHC degraders and PGPR in a PLF site with the concentrations of PHCs as high as 130 g Kg^{-1} dry soil.

5.2 Material and method

5.2.1 Experimental design:

Experiments were conducted at a PHC contaminated land farm site in Sarnia, ON owned by Imperial Oil Ltd. This site has been used as a land farm for more than 20 years. It is located at 43.00N, 082.18W, with a cold, snowy winter and a warm, humid summer. The annual average temperature is 8.25 °C, the annual precipitation averages 827 mm. Growth seasons are from the end of April to November for cold weather adapted grass such as rhygrass and fescue.

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Concentration of PHCs in the soil at this site was around 13% (130 g Kg^{-1} dry soil). There were three treatments in this field trial: The treatment seeded with PGPR treated seeds, the treatment seeded with non-PGPR treated seeds and unseeded treatment (without plant growth due to the phytotoxicity of extreme high PHC concentrations). Seeds that were and were not treated with PGPR were grown in a paired block design with unseeded control plots beside (without plants) at the PLF site (Fig. 5.1). A mixture of ryegrass (*Lolium multiflorum*), tall fescue (*Lolium arundinaceum*) and barley (*Hordeum vulgare*) were used based on previous results (Huang et al. 2004a). Seed planting density was approximately 300 seeds m^{-2}. No additional fertilizer and irrigation were supplied during 2 years of field trials. PGPR used in this experiment are the combination of UW4 *(Pseudomonas putida)* and UW3 *(Pseudomonas sp.)*.

5.2.2 Seeds and planting

Seeds of ryegrass, tall fescue and barley were purchased from Ontario Seed Ltd. (Waterloo, ON, Canada). Germination rates were measured before planting. Seeds were coated with a mixture of colorant, polymer (methyl cellulose) and bacterial suspension of UW3 and UW4 in a Hege II liquid seed treater (Winstersteiger, Saskatoon, Saskatchewan, Canada). Use of a colorant and polymer facilitates adhesion of PGPR to the seeds for prolonged periods and satisfies the safety regulations requiring all treated seeds to be visibly colored. Seeds were dried and stored for a maximum of 30 days prior to sowing (Greenberg et al. 2007a). UW3 or UW4 were prepared by growth in tryptic soy broth (30g L^{-1}, Fisher Scientific, Ottawa, Ontario, Canada) on a rotary shaker for 24 hours. Cells were harvested and washed three times with sterilized reverse osmosis (RO) H$_2$O. The cells were centrifuged at 2300 x g between each wash step. After the last wash step, cell

Figure 5. 1 Map of a field trial of phytoremediation of PHC contaminated soil at a petroleum land farm site in Sarnia, Ontario, Canada.

The area in green square was phytoremediation of petroleum land farm. The area within the red square was experimental site of this study. The labels within white area correspond to each block within the red square. Blank: unseeded treatment; +PGPR: Seeded with PGPR treated seed; -PGPR: Seeded with non-PGPR treated seed. Plants used here were a mixture of ryegrass, barley and tall fescue.

H_2O. The harvested pellets were resuspended in RO H_2O and adjusted to OD=2. Equal volumes of UW3 and UW4 suspension were combined and used for seed inoculation in the Hege II liquid seed treater as above. PGPR was coated to approximately 10^8 CFU/seed. Seeds were seededon April 27, 2006 and May 8, 2007 with drill seeder.

5.2.3 Sampling

First soil samples from experimental field were taken on Apr. 27, 2006, just before planting. Soils were sampled approximately each month throughout the growth seasons. Soil samples were taken using a 20-cm hand-held Edelman auger (Eijelkamp Agrisearch Equipment, Giesbeek, the Netherlands). Briefly, about 500 g of soil to a depth of 20 cm were randomly taken at 5 points in each plot. For seededtreatments, 5 points were chosen on the soils with plant growth. Soils from 5 points were mixed in a stainless steel bowl, and placed in a glass jar and sealed. The glass jars were immediately transported to the laboratory for analysis. Undisturbed plant samples with soils were taken by carefully digging 50 cm by 50 cm square of soil with 20 cm depth and transported to the laboratory for assessment of microbes in the rhizosphere and plant growth.

The soils in rhizosphere and soils in non-rhizosphere (soils taken at the middle of each plant rows, about 15 cm from plants) were also sampled at Sept. 21, 2006 for microbial analysis. The rhizosphere soils were taken by first vigorously shaking plant roots by hand to remove loosely attached soils. Then the rhizosphere soils were sampled by washing shaken roots in sterile RO H_2O. The increase of water weight was considered as the amount of rhizosphere soils. Non-rhizosphere soils were sampled in the middle of two row plants by auger in field near where plant samples were taken.

5.2.4 Quantification of different groups of microorganisms

To quantify the numbers of microbes in the soil, soil samples of 2 g were taken aseptically from each jar and placed in 20 ml sterilized 0.85 % NaCl (w/v) solution in 50-ml sterile Falcon tubes. The soil solutions were shaken for one hour on a Multi-Mixer (Melrose Pk, Illinois, US). The soil suspensions were allowed to settle for one hour. The supernatants from these soil extract suspensions were diluted serially to the dilution factors of 10^1, 10^2, 10^3, 10^4, and 10^5, respectively, for the microorganism quantification assays described below.

5.2.4.1 Total bacterial number

Culturable, aerobic heterotrophic bacterial cells were enumerated in triplicate using the plate count method (Kirk et al. 2005a). Soil extract suspensionsof 100 µl over the above range of serial dilutions were spread on tryptic soy agar (TSA) plates supplemented with 75 ppm cycloheximide to inhibit fungal growth. Plates were incubated at 20 ˚C for 48 h in the dark and colonies were counted.

5.2.4.2 Total fungi

Total fungal counts were performed in triplicate using 100 µl of each of the above extract dilutions plated on malt extract agar supplemented with 100 ppm of chloramphenicol and 50 ppm of Rose Bengal. Rose Bengal was used to slow the growth and spread of fast growing fungi, so that slower growing fungi could be enumerated. The malt extract agar contained, per litre, 20 g of malt extract, 15 g of agar and 1 g of yeast extract. Petri plates were incubated at 20 ˚C in the dark for 4 days and colonies were counted.

5.2.4.3 Petroleum-degrading bacterial

Petroleum degrading aerobic bacteria were enumerated in triplicate using the plate count method. One hundred μl of the above dilutions of the soil extracts were spread on a oil agar medium (Kirk et al. 2005a). Composition of this medium per liter was: Bushnell-Haas (BH) Agar 990 ml (15.0 g of agar, 1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 1.0 g NH_4NO_3, 0.2 g $MgSO_4.7H_2O$, 0.05 g $FeCl_3$ and 0.02 g $CaCl_2$, H_2O adjusted to pH 7.0). Ten ml of filter sterilized F2 Oil (Esso Heating Oil) was added to the sterile BH Agar which had been cooled to 60 ˚C. Plates were incubated at 20 ˚C for 4 days in the dark before enumerating petroleum degrading bacteria.

5.2.4.4 Petroleum-degrading fungi

Culturable petroleum degrading fungi were enumerated by spreading each soil extract dilution (100 μl) on plates that are selective for petroleum degrading fungi. The medium was supplemented with 100 ppm of chloramphenicol. Composition of this medium per liter was 250 mg KCl, 1 g of each of NaH_2PO_4 and NH_4NO_3, 0.5 g $MgSO_4$ and 20 g agar. Filter sterilized F2 Oil (Esso) (0.2 ml) was added to the 1000 ml medium once it cooled to 50 ˚C before pouring the plates. Plates were incubated at 20 ˚C for 4 days in the dark before enumerating petroleum degrading fungi.

5.2.4.5 Hexadecane-degrading bacteria

Hexadecane degrading aerobic bacteria were enumerated in triplicate using the plate count method. Soil extracts of 100 μl over the above range of dilutions were spread on oil agar medium. Composition of this agar medium per liter was: Bushnell-Haas (BH) Agar 990 ml. Ten ml of filter sterilized hexadecane (Sigma) was added to sterile BH Agar which had been cooled to 60 ˚C before pouring plate. Plates were incubated at 20 ˚C for 4 days in the dark before enumerating

hexadecane degrading bacteria.

5.2.4.6 PGPR

PGPR were enumerated in triplicate using the plate count method. The DF salt medium for PGPR count was prepared followed the method described by Penrose (Penrose and Glick 2003). The component of DF salt (per liter) was: 4 g KH_2PO_4, 6.0 g Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 2.0 g glucose, 2.0 g gluconic acid, 2.0 g citric acid, 0.1 ml $FeSO_4$ solution and 0.1 ml trace elemental solution. The elemental solution contains (per 100ml) 10 mg H_3BO_3, 11.19 mg $MnSO_4.H_2O$, 124.6 mg $ZnSO_4.7H_2O$, 78.22 mg $CuSO_4.5H_2O$, 10 mg MoO_3, pH7.2. $FeSO_4$ solution was prepared by dissolving 100mg $FeSO_4.7H_2O$ in 10 ml RO water. DF salt medium (without adding $FeSO_4$ solution and trace elemental solution) was autoclaved for 20 min and cool down to 60 ˚C, then filter sterilized $FeSO_4$ solution, trace elemental solution and ACC solution (0.5M in sterile RO H_2O) were added. The final concentration of ACC in DF salt medium was 3.0 mM. 100 µl serially diluted above soil extracts were spread on DF salt agar plates containing ACC as sole nitrogen source. Plates were incubated at 20 ˚C for 2 days in the dark before enumerating colonies.

5.2.5 Statistics

CFU were examined for overall treatment effects by analysis of variance, followed by a Tukey test (variances equal) or a Games Howell test (variances unequal) to determine whether significant differences occurred between treatments. CFU data were log transformed prior to analysis. Statistical tests were performed using SPSS software (SPSS 13.0, Chicago, IL).

5.3 Results

Overview of weather condition and remediation at the PLF .

Quantification of different groups of microbes at the Sarnia land farm was performed monthly during two full growth seasons (from April 2006 to September of 2007). During the same period, plant growth and soil PHC concentrations were monitored (Gurska et al. 2008). In 2006, the weather was optimal for phytoremediation, with moderate temperatures and sufficient rainfall (Fig. 5.2). In 2006, the average temperature in the growth season (from May to Sept.) was 18.2 ˚C with rain fall at 415 mm (Fig. 5.2). Plants grew well with a prominent PGPR effect. Higher biomass and better plant ground cover was observed with PGPR application (Gurska et al. 2008). Microbes including petroleum degraders and PGPR positively responded to plant growth and PGPR application. Significant remediation of PHCs was observed in the sites with PGPR application (~30 % PHC reduction), in contrast to ~20 % PHC reduction in sites seeded without PGPR treatment. In 2007, the weather was very dry in the first half of growth season with normal rainfall in the second half. The rain fall in the growth season (from May to Sept. of 2007) was 319 mm with an average temperature of 18.5 ˚C. July was especially dry in 2007 with only 40 mm precipitation. This was 1/3 of that observed in 2006 (Fig. 5.2). Plants showed drought stress in beginning of July, and recovered later in August due to sufficient rainfall (Fig. 5.2). Despite this, significant PHC remediation was observed in the sites where the PEP was applied (Gurska et al. 2008). This study presents a two-year kinetic analysis of microbial populations in this land farm site.
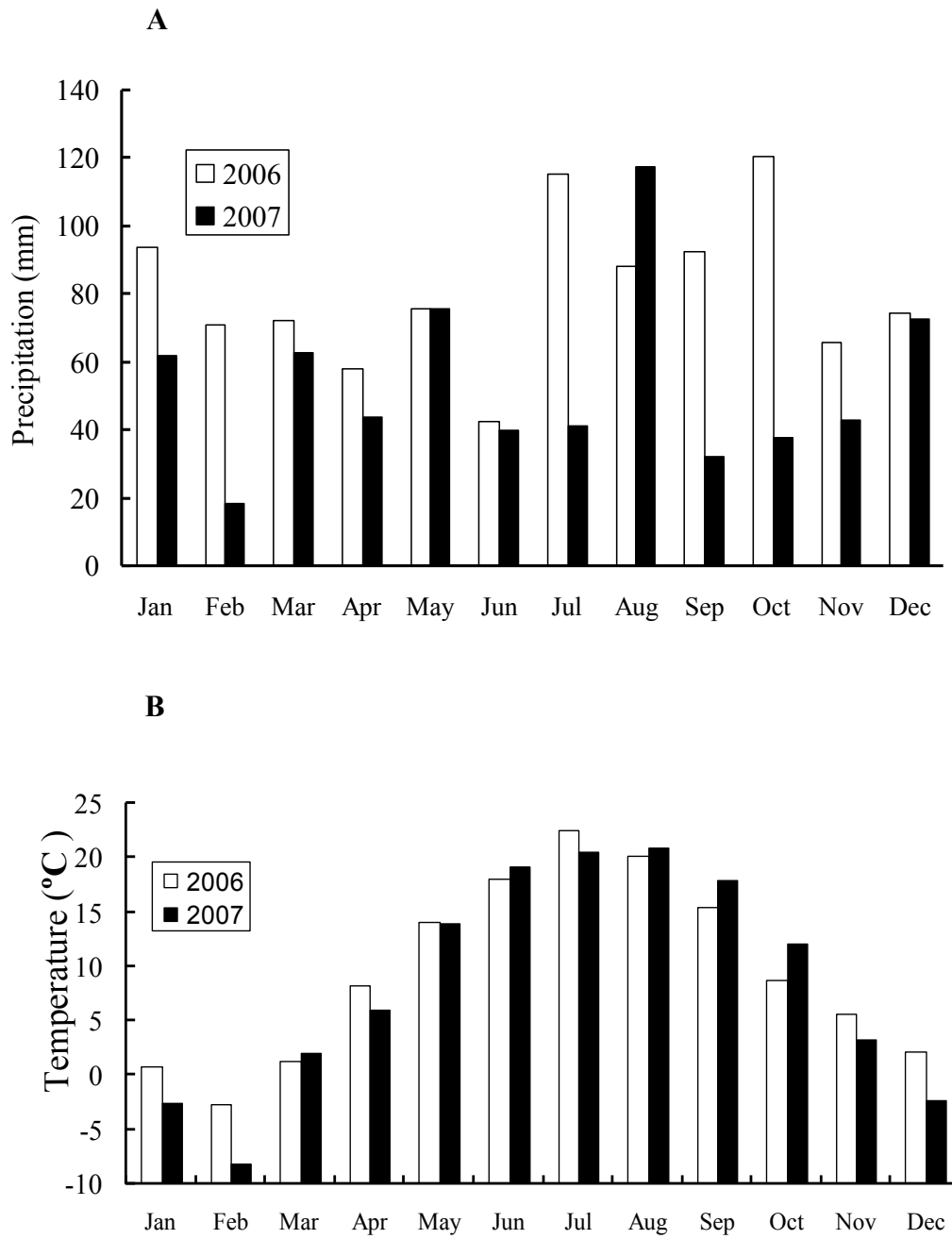
Figure 5. 2 Monthly precipitation (A) and average temperature (B) in Sarnia, ON for 2006 and 2007

5.3.1 Quantification of total heterotrophic bacteria

Over two year field trial, the averages of total heterotrophic bacteria numbers of three treatments were 7.1 x 10^5, 6.4 x 10^6 and 9.1 x 10^6 for unseeded soil, soil seeded with non-PGPR treated seed and soil seeded with PGPR treated seed, respectively (Fig. 5.3). The average of total bacterial numbers in soil seeded with PGPR treated seed was 29.6 % greater than soil seeded with non-PGPR treated seed ($P < 0.17$; n = 90). The averages of total bacteria numbers in two seeded treatments (seeded with PGPR and non-PGPR treated seed) were significantly greater than that in unseeded soil ($P < 0.05$).

In the first sample after planting at May 29, 2006, total bacterial numbers in soil seeded with PGPR treated seed was 3.35 x 10^7 CFU/g dry weight of soil, about 3 times higher than that in the soil seeded with non-PGPR treated seed (9.55 x 10^6 CFU/g dry weight of soil) (Fig. 5.3), suggesting that seed inoculation with PGPR may increase the total bacterial numbers in soil at the beginning of plant growth. Regardless of the sampling time, there was no significant difference between treatments seeded with PGPR and non-PGPR treated seed ($P < 0.05$).

Throughout the two years of the field study, the numbers of heterotrophic bacteria in the unseeded soil remained relatively constant (about 10^5 CFU/g dry weight of soil). However, both seeded treatment areas had greatly more increased heterotrophic bacterial numbers by the first sampling time (i.e. within the first growth month) (Fig. 5.3). After that the total bacteria numbers were relatively stable for the remaining time in this field study (Fig. 5.3). Total bacterial numbers in the seeded soils remained 1-2 orders of magnitudes higher than unseeded soils. This shows that having plants present at a certain site greatly increases the microbial activities of the soil.
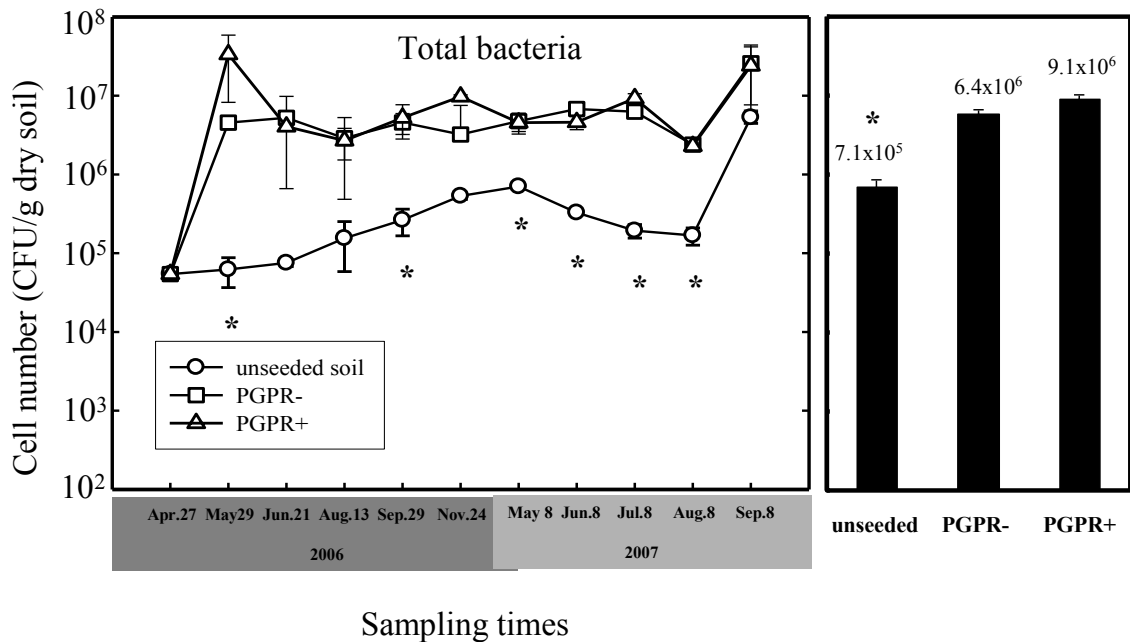
Figure 5. 3 Monthly precipitation (A) and average temperature (B) in Sarnia, ON for 2006 and 2007

Soil was from a land farm experimental site. The data represents two consecutive years of phytoremediation of this site. Samples were taken monthly. Total bacterial numbers were determined by the plate count method on tryptic soy agar (TSA) plates. The right graph shows average numbers of three treatments over two year field trials. PGPR-: soil seeded with non-PGPR treated seed; PGPR+: soil seeded with PGPR treated seed. * indicate statistically differences at a level of $P < 0.05$ between unseeded and seeded plots. Data are the means ± SE (n=9 for bacterial count on each time point and n=90 for two year average).

5.3.2 Quantification of petroleum and hexadecane -degrading bacteria

The changes of petroleum-degrading bacterial numbers and hexadecane-degrading bacterial numbers showed a similar trend with total bacterial numbers (Figs. 5.3, 5.4 and 5.5). The averages of two-year field trial in soils seeded with PGPR treated seed were 25.6% ($P < 0.16$) for petroleum-degrading bacteria and 22.8% ($P < 0.14$) for hexadecane-degrading bacteria greater than soils seeded with non-PGPR treated seed. The averages of both groups of bacteria numbers in two seeded treatments were significantly greater than unseeded one.

The growth of plants in the first two months of 2006 resulted in a rapid increase in soil bacterial numbers of both petroleum-degrading and hexadecane-degrading bacteria. The bacterial numbers in seeded soils remained high until the end of September of 2006 when the plants were mature and/or ceased to grow. After that there were declines in bacterial counts until May of 2007. For all of 2007, both groups of bacterial numbers in seeded soils remained stable. At most sampling times during the two-year field trial (8 times out of a total of 10 sampling points), the numbers of both bacterial types in the soils seeded with PGPR treated seed were higher. Although the differences were small, the consistent difference was observed (Figs. 5.4 and 5.5). Both groups of bacteria in seeded soils were approximately 1-2 orders of magnitude higher than those in unseeded soils at most sampling times ($P < 0.05$).

Figure 5. 4 Quantification of petroleum degrading bacteria from soil samples of two seeded treatments and unseeded soils.

Soil was from a land farm experimental site. The data represents two consecutive years of phytoremediation of this site. Samples were taken monthly. Petroleum-degrading bacterial numbers were determined by plate count method on Bushnell-Haas (BH) Agar medium with F2 oil as sole carbon source. The right graph shows average numbers of three treatments over two year field trials. PGPR-: soil seeded with non-PGPR treated seed; PGPR+: soil seeded with PGPR treated seed. * Statistical differences at a level of P < 0.05 between unseeded and seeded plots. Data are the means ± SE (n=9 for bacterial count on each time point and n=90 for two year average).
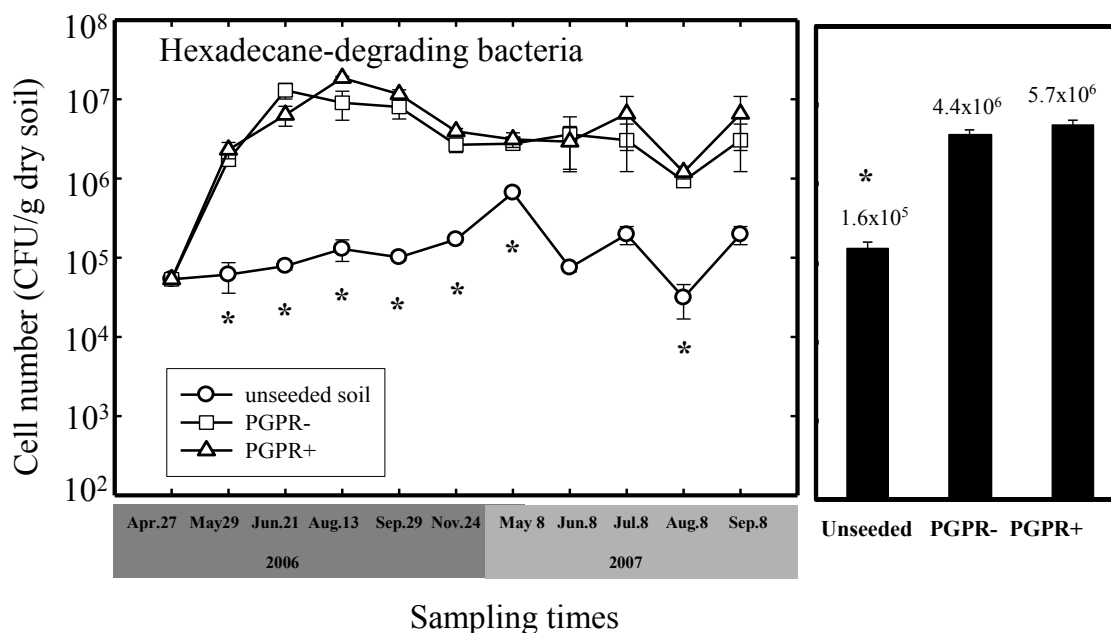
Figure 5. 5 Quantification of hexadecane degrading bacteria from soil samples of two seeded treatments and unseeded soils.

Soil was from a land farm experimental site. The data represents two consecutive years of phytoremediation of this site. Samples were taken monthly. Petroleum-degrading bacterial numbers were determined by plate count method on Bushnell-Haas (BH) Agar medium with hexadecane as sole carbon source. The right graph showed average numbers of three treatments over two year field trials. PGPR-: soil seeded with non-PGPR treated seed; PGPR+: soil seeded with PGPR treated seed. * Statistical differences at a level of $P < 0.05$ between unseeded soils with seeded ones. Data are the means $\pm$ SE (n=9 for bacterial count on each time point and n=90 for two year average).

5.3.3 Quantification of PGPR

Over the two-year field trial, the averages of PGPR numbers of the three treatments were 2.3 x 10^4, 7.2 x 10^5 and 9.6 x 10^5, respectively, for unseeded plot, plot seeded with PGPR treated seed and plot seedednon-PGPR treated seed (Fig. 5.6). The average PGPR numbers in soil seeded with PGPR treated seed was 25.0 % higher than soil seeded with non-PGPR treated seed ($P < 0.11$). The averages of PGPR numbers in two seeded soils were significantly higher than that in unseeded soil ($P < 0.05$).

The PGPR numbers in the seeded soils increased rapidly at the first two sampling times (May and June of 2006) (Fig. 5.6). They increased from 831 CFU/per gram dry weight of soil to 2.62 x 10^6 CFU/per gram dry weight of soil for treatment seeded with PGPR treated seed, 9.23 x 10^5 CFU/per gram dry weight of soil for treatment seeded with non-PGPR treated seed, and 3.23 x 10^4 CFU/per gram dry weight of soil for the unseeded soil (Fig. 5.6). At most of the sampling points (8 times out of 10 sampling points), the PGPR numbers in the soils seeded with PGPR treated seed were higher than those in soils seeded with non-PGPR treated seed. These may indicate that PGPR application enhances the PGPR numbers in soil. Throughout two-year field study, PGPR numbers in seeded samples were 1-2 orders of magnitude higher than those in unseeded soils, significantly at a level of $P < 0.05$ at most sampling times (Fig. 5.6).
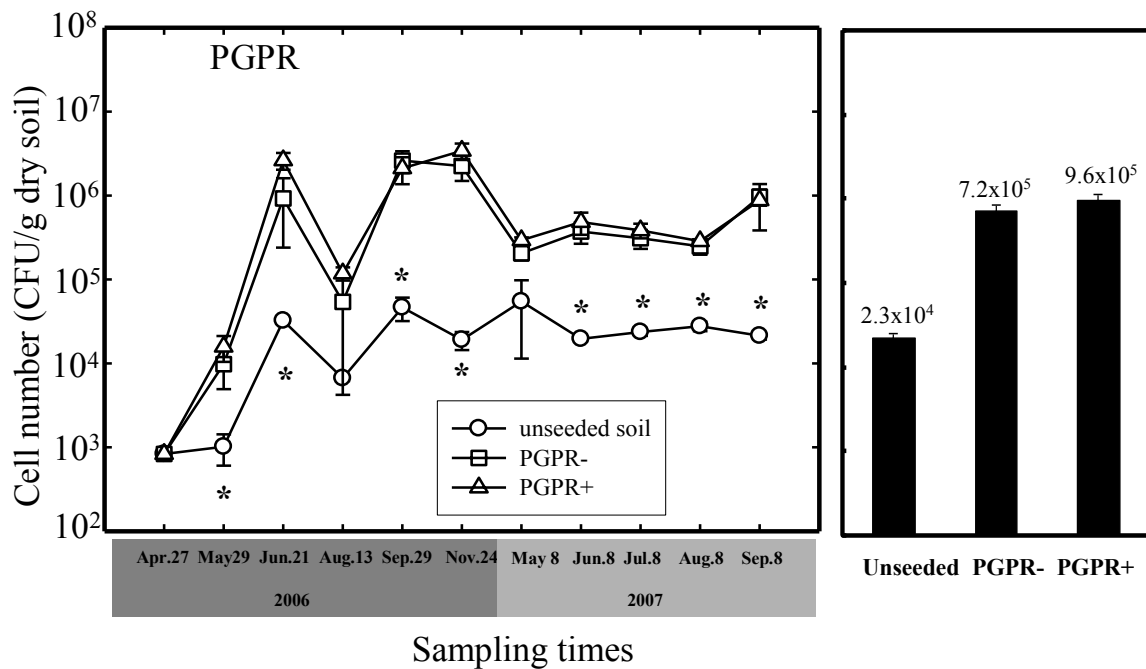
Figure 5. 6 PGPR counts from the soils of the two seeded treatments and unseeded soils from land

farm experimental site.

The data was from a two-year field trial. Samples were taken monthly. PGPR were determined by

the plate count method on DF salt agar media with ACC as sole carbon source. PGPR-: soil seeded

with non-PGPR treated seed; PGPR+: soil seeded with PGPR treated seed. * Statistically

differences at a level of $P < 0.05$ between unseeded soils and seeded ones. Data are the means $\pm$ SE

(n=9 for bacterial count on each time point and n=90 for two year average).

5.3.4 Quantification of total fungi and petroleum degrading fungi

Compared with total bacteria numbers (Fig. 5.3), fungal numbers were lower by 2 orders of magnitude (Figs. 5.7 and 5.8). For total fungi, the two seeded treatments had similar values (Fig. 5.6). However, petroleum-degrading fungi in the treatment seeded with PGPR treated seed was 25.0 % higher than treatment seeded with non-PGPR treated seed (P < 0.21) (Fig. 5.8). Seeded soils had significantly higher fungal numbers of both groups than unseeded soils (P < 0.05).

The kinetic of petroleum-degrading fungal numbers showed a very similar trend as the total fungal numbers. Plant growth led to greatly increased fungal numbers for both total fungi and petroleum-degrading fungi within two months of planting (increasing from approximately 10^3 to 10^5 CFU/per gram dry weight of soil). After that, the fungal numbers for the seeded sites were stable throughout the two years of the field trials. For the unseeded soils, fungal numbers of both groups fluctuated between 10^3 and 10^4 CFU/per gram dry weight of soil throughout the two years of field trial (Figs. 5.7 and 5.8). At most sampling times, both seeded treatments had significantly higher fungal numbers than the unseeded treatments (P < 0.05). The differences of two groups of fungal numbers between two seeded treatments are not significant (P < 0.05). However, at the most sample times (7 times out of 10 time points), the number of petroleum degrading fungi in the plots seeded with PGPR treated seed was greater than those in plots seeded with non-PGPR treated seed (Fig. 5.8).

5.3.5 Quantification of microbes in rhizosphere and nonrhizosphere soils

To further compare the microbial populations with respect to potential PGPR treatment effects,

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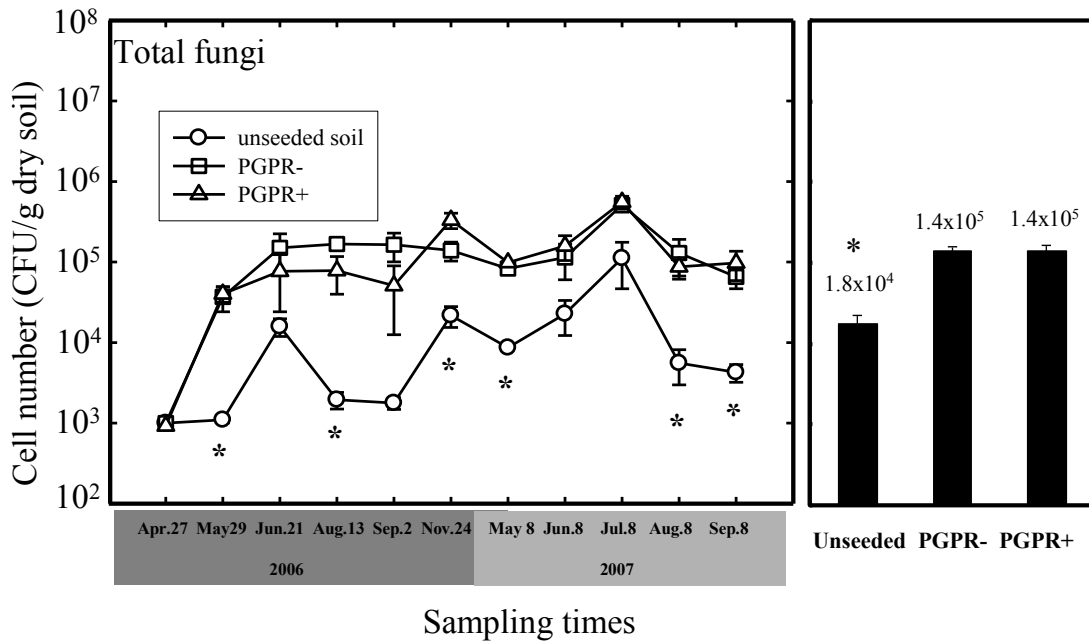
Figure 5. 7 Quantification of total culturable fungi from soil samples of two seeded treatments and unseeded soils.

Soil was from a land farm experimental site. The data represents two consecutive years of phytoremediation of this site. Samples were taken monthly. Total culturable fungi numbers were determined by plate count method on malt extract agar supplemented with 100 ppm of chloramphenicol and 50 ppm of Rose Bengal. PGPR-: soil seeded with non-PGPR treated seed; PGPR+: soil seeded with PGPR treated seed. * Statistically differences at a level of $P < 0.05$ between unseeded soils and seeded ones. Data are the means ± SE (n=9 for bacterial count on each time point and n=90 for two year average).
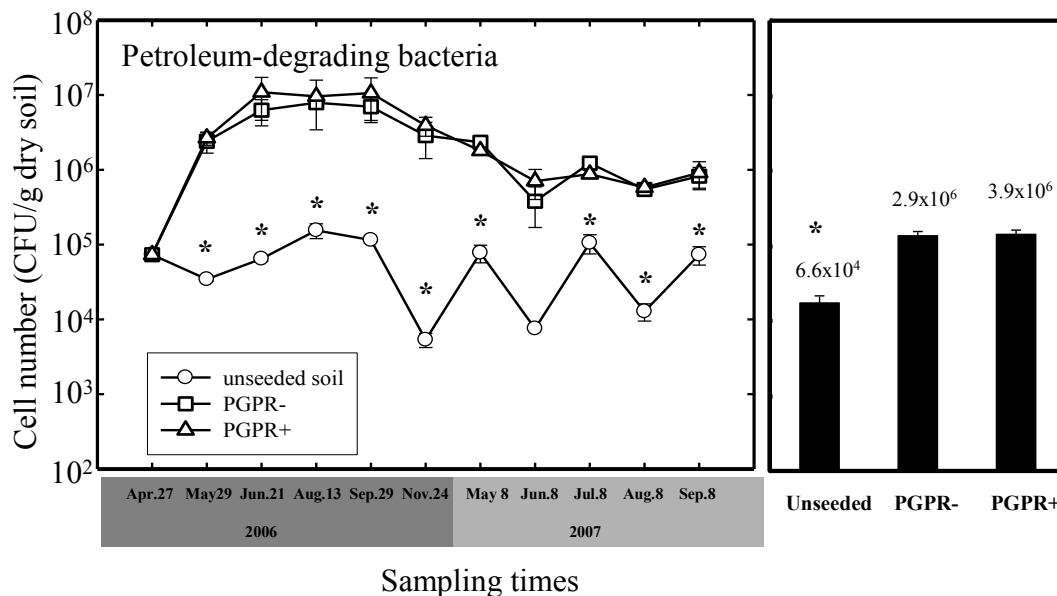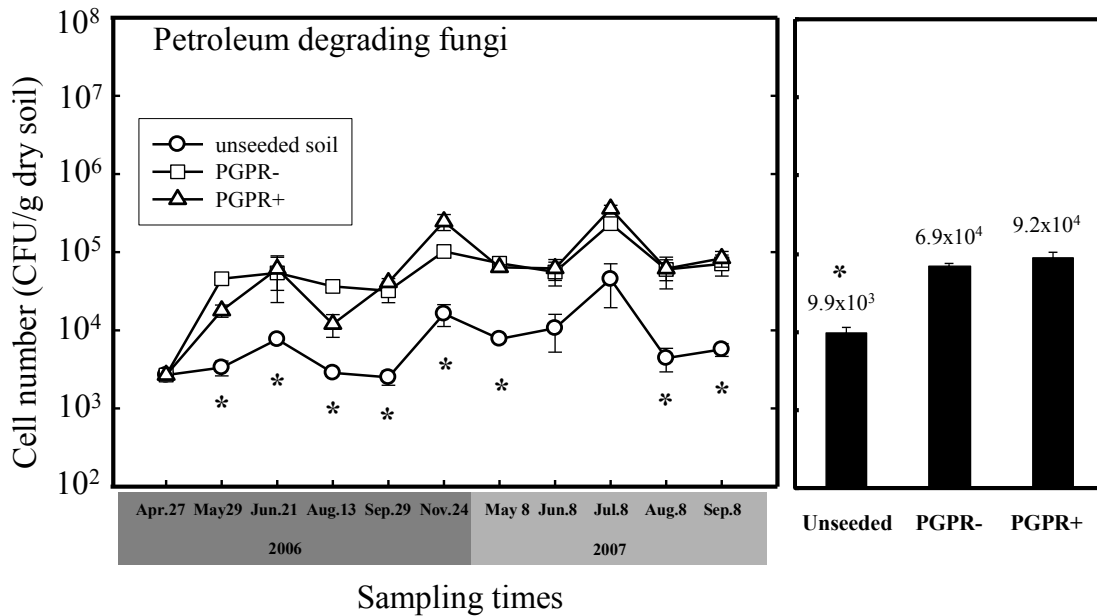
Figure 5. 8 Quantification of petroleum degrading fungi from soil samples of two seeded treatments and unseeded soils.

Soil was from a land farm experimental site. The data represents two consecutive years of phytoremediation of this site. Samples were taken monthly. Petroleum-degrading fungi numbers were determined by plate count method with F2 oil as sole carbon source. Media were supplemented with 100 ppm of chloramphenicol. PGPR-: soil seeded with non-PGPR treated seed; PGPR+: soil seeded with PGPR treated seed. * Statistically differences at a level of $P < 0.05$ between unseeded soils with seeded ones. Data are the means ± SE (n=9 for bacterial count on each time point and n=90 for two year average).

the rhizosphere (R) and nonrhizosphere (S) soils were compared. For this comparison an R:S ratio was used (Karthikeyan et al. 2007), which is ratio of microbial populations of rhizosphere soils vs non-Rhizosphere soils. Higher R:S ratio indicates greater enrichment effects of the rhizosphere on associated microbes. The R:S ratio is calculated by using the equation (*R:S Ratio = R/S*).

The microbial numbers in the rhizosphere soils, and nonrhizosphere soils were quantified using soil samples taken in September of 2006. At this time point, after one growth season, a new microbial ecological equilibrium would have been formed in rhizosphere by the interactions among the plants, soil and microbes.

The results of R:S ratio show that the numbers of all groups microbes in the rhizosphere soils were several times greater than nonrhizosphere soils (Table 5.1). The treatments seeded with PGPR treated seed had significantly higher R:S ratios (about 1.5-3 times higher) than those seeded with non-PGPR treated seed. This was the case for all microbes except for the total fungi. These data suggest that the rhizosphere treated with PGPR has a greater enrichment effect on microbes associated with petroleum degradation than was seen in plots where PGPR was not applied. Therefore, more petroleum degraders were found around the roots in the treatments seeded with PGPR treated seed than those seeded with non-PGPR treated seed, which may lead to the more rapid biodegradation of PHCs in PGPR treated rhizosphere than that without PGPR. It is interesting that R:S ratio of total fungi disagree with that of petroleum-degrading fungi. Treatment seeded with PGPR treated seed have higher R:S ratio for petroleum degrading fungi and lower ratio for total fungi. This indicates that application of PGPR may lead to the fungal community structure changes.

Table 5. 1 R:S ratio of different groups of microbes found in plots that were and were not treated with PGPR coated seed.

Microbial group	PGPR-	PGPR+
Total bacteria	2.3 ± 0.24	6.7 ± 0.84
Total fungi	3.9 ± 0.55	1.9 ± 0.31
PGPR	2.8 ± 0.25	4.6 ± 0.59
Petroleum degrading fungi	3.8 ± 0.39	7.1 ± 0.67
Hexedecane degrading bacteria	5.5 ± 0.78	8.2 ± 0.72
Petroleum degrading bacteria	5.4 ± 0.54	7.0 ± 0.63

* The R:S ratio was defined as the microbial populations ratio between rhizosphere microorganisms and non-Rhizosphere microorganisms using the following equation:

R:S Ratio = Rhizosphere microorganisms/Non-Rhizosphere microorganisms

Data were mean ± SE. All data were significantly different between treatments plant with PGPR treated seed and those with non-PGPR treated seed ($P < 0.05$).

5.4 Discussion

According to the law of Ontario, Canada, petroleum land farms (PLFs) will be phased out in 2008. However, these PLF sites will represent a unique remediation challenge for many years due to the accumulation of complex high molecular weight petroleum compounds (~13% TPHs) resulting from repeated applications of PHCs to soils. Phytoremediation, as a cost effective strategy, may be an excellent option to remediate these contaminated soils. However, no or poor plant growth often occurs on the PLF soils because of chemical toxicity, nutrient deficiency and/or water stress in the impacted soils (Kirk et al. 2005a). The PGPR enhanced phytoremediation (PEP) system utilizes plant/PGPR interactions to greatly increase plant biomass, particularly in the rhizosphere, and these significantly accelerate PHC remediation from PLF soil (Greenberg et al. 2006; Greenberg et al. 2007a; Huang et al. 2005; Huang et al. 2004a). The results of microbial analyses presented here provide important data on how the PEP works to remove PHCs from contaminated soils.

The results of microbial analyses show that populations of microbial groups including total bacteria and fungi, petroleum degrading bacteria and fungi, hexadecane degrading bacteria and PGPR had very similar patterns throughout the two-year field trial (Figs. 5.3-5.8). Specifically, the populations of all microbial groups in seeded soils showed rapid increases in titers within 2 months of planting. They then remained relatively constant in numbers throughout the rest of the two-year field trial. The microbial numbers in unseeded soils fluctuated in relatively narrow ranges throughout two-year field trial. Seeded soils had 1-2 orders of magnitude higher microbial numbers than unseeded soils ($P < 0.05$) at most sampling times and when averaged over the entire

two-year period (Figs. 5.3-5.8). These results suggest that plant growth significantly enhances soil microbial activities of total microbes and petroleum degraders; and, therefore, may be the key mechanism operating with regard to phytoremediation of PHC impacted soils.

Over the two-year field trial, the averages of the titers of PHC degraders, including total bacteria, petroleum degrading bacteria, hexadecane degrading bacteria, petroleum degrading fungi, and PGPR in soils seeded with PGPR treated seed were 22-30% greater than those in soils seeded with seed not treated with PGPR ($P < 0.11$ to 0.21) (Figs. 5.3-5.6 and 5.8). Because of the high variability of field data, these differences would be prominent. This agrees with R:S ratio results that the rhizosphere soil with PGPR treated plants had a higher R:S ratios in these microbial groups than those without PGPR (Table 5.1). Furthermore, PEP improved plant growth and removed PHC around 30% faster than treatment seeded with non-PGPR treated seed (Gurska et al. 2008). The PGPR used in this field trial, UW4 and UW3, were known to have active interactions with plants (Glick 2005). Vigorous plant growth as a result of PGPR application has been observed in many studies (Cheng et al. 2007; Glick et al. 2007b; Greenberg et al. 2007a; Huang et al. 2005). Actively growing plants exude more organic nutrients to the surrounding soils than weak plants (Elsas et al. 2007). Hence, the greater amounts of root exudates stimulated by the application of PGPR might provide more nutrients for microbial growth, resulting in the higher R:S ratios in PGPR treated rhizosphere. The higher R:S ratio, combined with the extensive roots in PGPR treated soils (Glick 2003; Glick et al. 2007b), could result in relatively high bacterial populations in soils seeded with PGPR treated seed. The high microbial populations, in turn, could lead to faster biodegradation of PHCs in PGPR treated rhizosphere than non-PGPR treated ones.

Although rhizodegradation by PHC-degrading microbes is considered to be a key mechanism

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of phytoremediation of PHC contaminated soils, the titers of PHC degraders determined in laboratory conditions are not always correlated with remediation effiency of PHCs. The relation between rhizosphere microbes and phytoremediation efficiency may vary with plant and contaminant types. In a field remediation study, Euliss et al. (2008) observed significantly greater PAH degrader numbers, but no corresponding significant diminishment in PHC or PAH concentrations. In a growth chamber phytoremediation study, Phillips et al. (2006) reported 100 times higher PAH degrader numbers and unchanged total bacteria numbers and hexadecane degrader numbers, but just 10 % PHC degradation. It thus appears that increased PAH degrading bacterial numbers do not result in active PHC remediation. This may be because in most PHC contaminated sites, aliphatic hydrocarbons comprise the most of contaminants. It has been reported that hexadecane (a C16 aliphatic hydrocarbon) degraders have a strong positive relation with the PHC degradation (Phillips et al. 2006). Hence, hexadecane degraders might be an indicator of activity of petroleum degrading bacteria in petroleum contaminated soil.

Another reason that leads to the disagreement between PHC degrader numbers and removal rates of PHCs may be that the conditions under which PHC degraders were determined in laboratory were different with those in field. PHC degrader numbers were determined in the laboratory by using PHCs as sole carbon source in the media. However, in the field soils, especially those with low PHC concentration, due to the availability of surrogate nutrition, catabolite repression and niche competition, PHC degraders determined in laboratory conditions using PHCs as sole carbon source may not use PHCs as substrates under field conditions. The catabolite repression, the repression of predominant carbon sources on the utilization of minor carbon sources, may lead to less efficient phytoremediation of soils with low concentrations of

PHCs. Conversely, in PLF soils with extreme high PHC concentration, the agreement between enhanced PHC degraders and increased PHC remediation would be most likely observed, such as in this study, because PHC were dominant chemicals in PLF. PHCs at high concentration in PLF may result in preference of PHC degraders targeting the dominant compounds with growth of remaining classes of microbes being suppressed (Euliss et al. 2008). Therefore, the dominant contaminants may be biodegraded faster than the minor contaminants. The catabolite repression could be a reason that phytoremediation may be more efficient in the first year and less effective with decreasing contaminant concentrations. This is most likely because of the decreasing bioavailability of contaminants as lowered contaminant concentration in soil and more available surrogate nutrients including root exudates and plant debris. This phytoremediation trend has been observed in our three-year field remediation study on this land farm soils (Gurska et al. 2008).

Compared with published greenhouse and growth chamber studies, this study reported lower total microbial and petroleum degrader numbers. The microbial numbers were one order of magnitude lower than a growth chamber study using weathered oiled soil (Phillips et al. 2006), two orders of magnitude less than in both a greenhouse study using artificial spiked soils (Kirk et al. 2005a) and a laboratory study in petroleum-refining wastewater irrigated agricultural soils (Li et al. 2007). These studies were conducted in the soils with PHC concentrations from 0.5 to 5%. Conversely, two research groups reported around 10^6-10^7 CFU/g soil total bacterial numbers in field (Euliss et al. 2008; Kaksonen et al. 2006) in a vegetated, 8% PHC contaminated soil (Euliss et al. 2008), falling in the range of this study. In all these studies, total bacterial numbers were quantified with plate count methods. The disagreements in total bacterial numbers between different studies may be attributed to various PHC concentrations and, to some extent, the culture

procedures. But the concentration and types of PHCs seem to be one critical factor that affects the microbial populations (Maila et al. 2006). In the Sarnia land farm soils, PHC at high concentrations could be toxic to many microbes. Only tolerant microbes can survive, and only those that can utilize PHCs as substrates can thrive. The extreme hydrophobicity of large PHC molecules and low water holding ability of oiled soil give PHC low bioavailability, limiting the growth of microbes, even if they can use PHC as substrates.

The rhizosphere supports more microbes than non-rhizosphere soils (Table 5.1). The diffusion of root exudates creates a radius of nutrition around the roots. This resulted in bacterial gradients (Corgie et al. 2006) and biodegradation gradients in the rhizosphere (Joner and Leyval 2003). Biodegradation gradients have been observed in phenanthrene (PHE)-contaminated soil (Joner and Leyval 2003). In this study, soil samples taken at two different distances from the roots show less total bacteria and PHC degraders in soils far from plants (Table 5.1), and soils taken at the middle site of the rhizospher and nonrhizosphere also showed medium numbers of total bacteria and PHC degraders (data not shown). This may implicate that a PHC gradient may exists around roots, and as a result PHCs closer to the roots would be consumed faster.

Seeded soil dramatically changes the fungal populations (Figs. 5.7 and 5.8). More than 40 fungal types or strains have been described to grow on crude oil (Davies and Westlake 1979). Several fungi have been successfully used in the bioremediation of PHCs (Yateem et al. 1998). In this study, total fungal populations and fungal petroleum degraders increased with plant growth. Fungi may interact synergistically with bacteria on degrading PHCs (Merkl et al. 2006). Different from bacteria that favor neutral conditions, fungi favor acidic conditions. During biodegradation, alkanes, the dominant components in petroleum hydrocarbons, are converted by monooxigenases

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or dioxigenases to alcohols, aldehydes, and fatty acids (Fig. 5.9), that can then be metabolized through the β-oxidation (van Beilen et al. 2001). The formation of the acids could lower the soil pH, resulting in the acidic conditions favored by fungi. As well, many root exudates are acidic, including CO_2 and amino acids, which can be dissolved in the rhizosphere (Tate 2000), lowering pH by 1–2 units compared to unseeded soil (Kaksonen et al. 2006). Although the number of petroleum fungal degraders are only 1 to 10 % of their bacterial partners, considering their much larger size than bacteria, fungi could play significant role in the degradation of PHCs. Furthermore, the higher R:S ratios of petroleum degrading fungi in PGPR treated rhizosphere than that in non-PGPR treated ones (Table 5.1) might also partly contribute to the faster removal of PHCs in PGPG treated soils.

In summary, PEP enhances phytoremediation efficiency of PHCs from soils. PGPR application resulted in better phytoremediation of land farm soil by increasing plant biomass especially roots and plant cover (Cheng et al. 2007; Farwell et al. 2007; Greenberg et al. 2006; Greenberg et al. 2007a; Huang et al. 2005; Huang et al. 2004b; Li et al. 2005; Nie et al. 2002; Reed et al. 2005). PGPR application leads to a greater rhizosphere, making roots reach larger volumes of soil. Furthermore, more petroleum degraders were around roots with PGPR than those without PGPR. This leads to the more rapid biodegradation of PHCs in PGPR treated rhizosphere than untreated one. Therefore, the key mechanisms of PEP of PHCs from petroleum land farm soil appear to be due to the PGPR eliciting a larger and more active rhizosphere that support higher numbers of petroleum degraders. The PEP, with the application of PGPR as a core procedure, is a promising approach to reduce toxic, persistent and recalcitrant contaminants from soil.

$$CH_3\text{-}(CH_2)_n\text{-}CH_3 \qquad \text{Alkane}$$

Alkane mono-
oxygenase $\downarrow$

$$CH_3\text{-}(CH_2)_n\text{-}CH_2OH \quad \text{Alcohol}$$

Alcohol
dehydrogenase $\downarrow$

$$CH_3\text{-}(CH_2)_n\overset{\overset{O}{\|}}{\text{-}CH} \qquad \text{Aldehyde}$$

Aldehyde
dehydrogenase $\downarrow$

$$CH_3\text{-}(CH_2)_n\overset{\overset{O}{\|}}{\text{-}C}\text{-}OH \qquad \text{Fatty acids}$$

$$\downarrow$$

$$\beta\text{-oxidation}$$
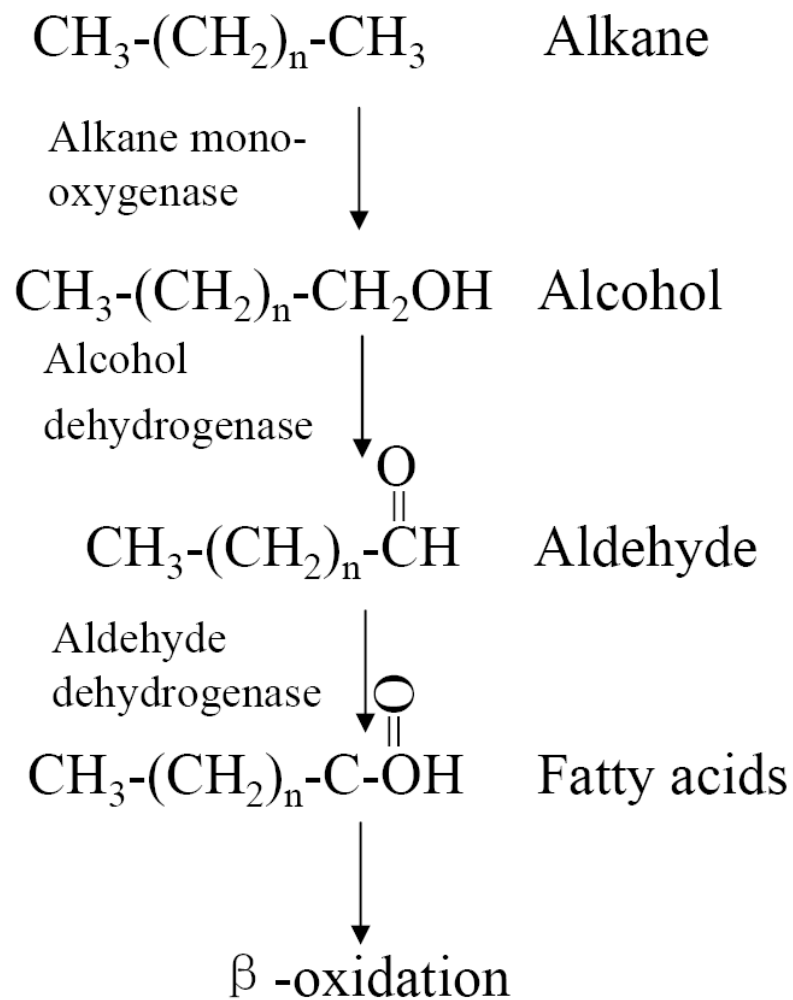
Figure 5. 9 One of major alkane biodegradation pathways. Fatty acid is formed as one of

intermediates. Fatty acid may lower pH in the soil, which favor the growth of fungi.

Chapter 6

Molecular Monitoring of Bacterial and Fungal Communities during Phytoremediation at a

Petroleum Land Farm Site

6.1 Introduction

The fate of petroleum hydrocarbons (PHCs) in the environment are primarily dependent on the nature and concentration of PHCs present and the interplay between chemical, geochemical, and biological factors (Bordenave et al. 2007). Biological factors have been manipulated for *in situ* remediation of PHCs to accelerate their degradation. The soil microbial population, microbial structure and microbial activity are key biological factors influencing bioremediation of PHCs. One approach for increasing bioremediation efficiency of PHCs from soil is phytoremediation by which root exudates (e.g. microbe nutrients) can enhance the population and activity of microbes including PHC degraders (Kirk et al. 2005a; Phillips et al. 2006)(Chapter 5).

Although phytoremediation is a promising strategy for remediation of recalcitrant organics, the challenge is that plant establishment is often limited by toxic contaminants especially when the chemicals are present in high concentration. This can be significantly improved by the application of plant growth promoting rhizobacteria (PGPR). PGPR promote plant growth under stress conditions and thus can help to build a better vegetative cover (Glick et al. 2007b; Greenberg et al. 2007a; Huang et al. 2005; Huang et al. 2004a). The established plants then provide a nutrient-rich environment that can stimulate microbial activity in the soil (Glick 2003). This can result in a rapid removal of contaminants such as PHCs from soil. Enhanced populations and activities of PHC degraders, and accelerated PHC remediation by PGPR enhanced phytoremediation (PEP) have been observed (Farwell et al. 2007; Greenberg et al. 2007a; Huang et al. 2005) (Chapter 5 of this thesis). Microbes play an important role in the success of PEP of PHCs by promoting plant growth and degrading contaminants. Thus, monitoring changes in the microbial community can aid in

assessing the success and mechanisms of different phytoremediation strategies.

Molecular methods have shown that the complexity of microbial communities is much greater than previously thought, and that the majority of soil bacteria are still unknown (Elsas et al. 2007; McDougald et al. 1998). This lack of knowledge is mostly attributed to the failure of many bacteria to grow in laboratory culture media, because nutritional requirements and the physicochemical conditions necessary for growth of a large number of microbes, as found in their natural environments, are not met in laboratory conditions (Escalante-Lozada et al. 2004). Therefore, soil microbial ecosystems cannot be well understood or characterized by culturing methods alone.

Quantitative molecular methods that can be used to examine soil DNA seem be more sensitive and unbiased than culturing methods, because they do not rely on the ability of bacteria to be cultured (Besser et al. 2008; Chang et al. 2008; Faveri et al. 2008; Nehme et al. 2008). The small subunit ribosome RNA genes (i.e. the 16s rRNA and 18s rRNA genes) are the basis of many phylogenetic studies, and are most frequently used as molecular indicators of microbial communities (Nakatsu 2007). In this chapter, denaturing gradient gel electrophoresis (DGGE) and quantitative polymerase chain reaction (qPCR), also known as qPCR, were employed to monitor bacterial communities based on 16s or 18s rDNA during a phytoremediation field study at a petroleum land farm (PLF). The study was performed at a PLF in Sarnia, ON with a PHC concentration of ~130 g Kg^{-1}. This particular site was chosen because it represents a specific ecosystem with very high levels of organic chemical contaminants. Soils from a PEP treated area were compared to soils with plant grown without PGPR and an unseededarea. The objective of this study is to better characterize the soil microbial community during PHC phytoremediation.

6.2 Material and methods

6.2.1 Experimental design, planting and sampling

The design, planting and sampling in this chapter were same as those used in chapter 5. There were three treatments in this field trial: unseeded soils, soils seeded with PGPR treated seed and soils seeded with non-PGPR treated seed.

6.2.2 Soil DNA extraction

Total DNA was extracted directly from soil samples of the three treatments using the Ultra Clean Soil DNA Kit (MoBio labratories Inc. Carlsbad, CA) following the manufacturer's instructions. Soils of 0.7 to 0.8 g were used for DNA extraction. The extracted DNA was visualized on agrose gel to assess the quality (Fig. 6.1). DNA samples were stored at -20 ˚C until required for further analysis.

6.2.3 Quantification of total bacterial numbers using qPCR

Quantification of total bacterial numbers using qPCR was performed as described by Castillo et al. (2006). The primers used to quantify total bacteria were F-tot (forward) 5'-GCAGGCCTAACACATGCAAGTC-3' and R-tot (reverse) 5'-CTGCTGCCTCCCGTAGGAGT-3' (Castillo et al. 2006) targeting 16s rDNA positions based on the *E. coli* 16s rDNA from nucleotide 63 to nucleotide 355. This region is highly homologous in most bacteria.
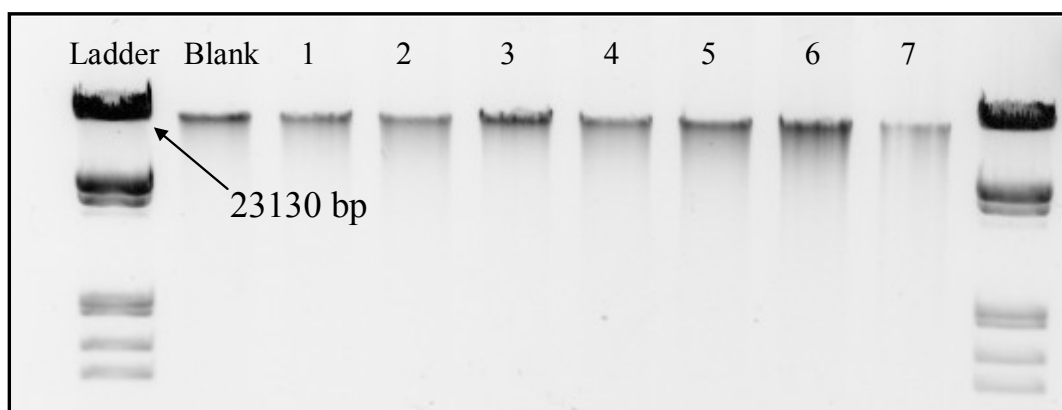
Figure 6. 1 Soil DNA extracted from petroleum land farm soils in Sarnia.

DNA were extracted from soils of the three treatments using Ultraclean soil DNA kit. DNA of 4 µl were loaded into each well of 1% agrose gel. Electrophoresis were performed for 1 hour. Blank: unseeded soil; Lanes 1, 2, 5, 6: soils seeded with PGPR treated seed; Lanes 3, 4, 7: soils seeded without non-PGPR treated seed.

A qPCR standard curve had to be constructed to quantify total bacterial numbers. *Pseudomonas putida* was used for standard curve construction, because *Pseudomonas* is a dominant bacterial genus in many non-impacted and PHC contaminated soils (Bordenave et al. 2007). A standard curve using likely dominant bacteria in the site soils should provide a more accurate measure of total bacterial numbers with qPCR (Nadkarni et al. 2002b). Soil used for standard curve construction was PLF soil taken from the experimental site. Soil was baked at 260 ˚C for 30 min to destroy all DNA of indigenous organisms in the soil. A bacterial suspension of 100 μl containing serially diluted known amounts of cells of *P. putida* ($9x10^5$—$9x10^9$ CFU) were added into 1 g of the baked soil. Soil water content was adjusted to 20%, which is similar to the PLF soil at the field site. Soils were gently shaken for 30 min to mix the bacteria with soil. DNA was extracted from the soil samples using the Ultraclean Soil DNA Kit (MoBio labratories Inc.). Extracted DNA contained a serial dilution of *P. putida* genomic DNA, and thus a serial dilution of 16s rDNA gene copies. These DNA extracts were used as templates in qPCR to construct a standard curve for quantification of total bacterial numbers in soils. In qPCR, the number of PCR cycles required to reach a threshold amount of PCR products is directly proportional to the DNA template concentration present at beginning of qPCR amplification. Thus, cell numbers are inversely proportion to the numbers of PCR cycles required to reach the threshold values (Ct). A standard curve can be constructed by plotting cell numbers against Ct. Total bacterial numbers in field soils were obtained by comparing the Ct values for the DNA extracted from the site soils with those of the standard Ct curve.

qPCR was performed with the MiniOption qPCR DNA engine (BioRad, Hercules, CA, US). SYBR Green was used as a fluorescent indicator of the PCR product concentrations. The PCR

reactions were performed on a total volume of 20 µl using the SYBR Green PCR Reagents Kit containing a premix of SYBR Green and other necessary qPCR reagents (Sigma). Each reaction included 10 µl 2×SYBR premix, 0.25 µl each primer (20 µM), 5 µl DNA sample (diluted 1/10). qPCR was initiated by 8 min at 94 °C, followed by 25 cycles of 94 °C for 20 seconds, 64 °C for 20 seconds, and 72 °C for 20 seconds. To determine the specificity of amplification, analysis of the product melting curve was performed after the last cycle of each amplification.

6.2.4 PCR amplification of 16s rDNA for DGGE

Oligonucleotide primers able to amplify a fragment spanning nucleotide positions of *E. coli* 16S rDNA from 968 to 1406 (Nubel et al. 1996), and full fungal 18s rRNA (Vainio and Hantula 2000) were purchased from Sigma (Sigma-Aldrich Canada Ltd. Oakville, ON, CA) (Table 5.1). These primers have been previously described for bacterial and fungal community analyses using DGGE (Vainio and Hantula 2000). A 40 bp DNA fragment consisting of only GC base pairs was attached as a clamp to the 5' end of one primer of each primer set. The GC clamp ensures that the PCR products were not completely separated into single strands during DGGE so that migration of PCR products in the polyacrylamide gel was based mainly on melting behavior of the DNA fragment and bands were separated based on their sequence differences.

DNA extracted from field soils from the three treatments (unseeded soils, soil seeded with and without PGPR) using UltraClean Soil DNA Kit were directly subjected to PCR amplification. PCR was conducted in a PTC-200 DNA Engine Cycler (MJ research) with the following program settings. PCR conditions for bacterial 16s rDNA amplification were 9 min at 94 °C, and 29 cycles

Table 6. 1 Primers used in the PCR amplification of soil DNA for DGGE analysis.

Primer	16s rDNA target	Primer sequence	Reference
PRBA968F	Bacteria V6 region (968-983)	5' #AA CGC GAA GAA CCT TAC 3'	(Nubel et al., 1996)
PRBA1406R	Bacteria V9 region (1406-1392)	5'ACG GGC GGT GTG TAC 3'	(Lan et al., 1988)
FR1	Fungal specific reverse primer	# AIC CAT TCA ATC GGT AIT	(Vainio et al. 2000)
NS1	Fungal specific reverse primer	GTA GTC ATA TGC TTG TCT C	(Vainio et al. 2000)

GC clamp added to the 5' end of the primer, 5'CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G 3'

of 94 ˚C for 30 s, 55 ˚C for 30 s, 72 ˚C for 30 s followed by 7 min at 72 ˚C and then held at 4 ˚C.

PCR conditions for fungal 18s rDNA were 8 min at 95 ˚C, followed by 35 cycles of 30 s at 95 ˚C,

45 s at 47 ˚C, 3 min at 72 ˚C, and final extension for 10 min at 72 ˚C.

The PCR products were a mixture of 16s rDNA of various soil bacteria with almost identical

sizes but with different nucleotide sequences. DGGE was performed to separate these 16s rDNA

PCR products based on their sequence difference. It is assumed that each band in DGGE profiles

represents a different bacterial species and the band intensities reflect the relative abundance of the

bacteria in soil bacterial community. Therefore, bacterial diversity and community structure of the

soil samples can be calculated and compared based on their band DGGE profiles.

6.2.5 DGGE

DGGE was performed on a Bio-Rad DeCode system (Bio-Rad, Mississauga, Ontario, Canada).

Electrophoresis was started with a relatively broad gradient (20-80 % denaturant) and then focused

to 35-75 % to achieve the best resolution of the bands. The gradient gels were prepared by mixing

100 % and 0 % denaturant solution at different ratios using a gradient maker. The 100 %

denaturant solution had 40 ml of formamide and 42 g of urea in 100 ml. The polyacrylymide gel

concentration and gradient were based on the size of the PCR products of interest. For 412 bps

bacterial 16s rDNA, 10 µl of the PCR products were loaded with 10 µl of 2 x loading dye (70 %

glycerol, 0.05 % Bromophenol Blue, 0.05 % Xylene Cyanole, 2 mM EDTA) into 8 %

polyacrylamide gel with 35–75 % denaturant gradient for DGGE. DGGE were performed for 16

hours at 80 V, 60 ˚C using 1 x TAE (50x TAE: 242 g Tris base, 57.1 ml Acetic acid, 100 ml 0.5M

EDTA, pH 8.5) as running buffer. Each DGGE experiment was performed three times on

independent soil extracts. For 1600-bps fungal 18s rDNA, 5 µl of the PCR products with 5 µl 2 x loading dye were loaded into a 6 % polyacrylamide gel with 10–43 % denaturant gradient. DGGE was performed for 16 hours at 190 V, 58 ˚C using 1 x TAE as running buffer. Gels were stained for 1 hour in 50 µg/ml solution of ethidium bromide in 1 x TAE buffer and destained for 15 min in deionized water and visualized under UV transmitter light (Fluorchem 8000, Alpha Innotech, San Leandro, CA). The gel images were digitized for analyses with the image processing software.

6.2.6 DGGE gel analysis

Gel images were analyzed using GelCompar II version 5.0 package (Applied Maths, Kortrijk, Belgium). First, the gel images were used to quantify the banding profiles within each DGGE gel lane by determining the total number of bands (S), the peak surface of each band (ni) and the sum of all the peak surfaces of all bands (N) (Sadet et al. 2007). This information was used to calculate the bacterial diversity using three indices: (i) The Shannon index (H) was calculated with the formula $H = -\sum (ni/N) \ln (ni/N)$. This index takes into account the numbers of bands and the band distribution in each lane of DGGE profiles. The index is increased either by having additional bands, or by having bands distributed more evenly in each lane of DGGE profiles (Krebs 1999). (ii) The dominance index (c) was calculated with the formula $c = \sum (ni/N)^2$. It is a measure of relative dominance status of bacterial species. The index is increased by having unusual intensive bands or by having bands distributed more unevenly in each lane of DGGE profiles. This index increases when diversity decreases. (iii) The evenness index (e) was calculated with the formula $e = H / \ln S$. This expresses how evenly the bands are distributed in each lane of the DGGE profiles.

GelCompar II software was used to normalize and compare all the DGGE profiles. To this end,

all the images of DGGE gels were matched using the internal control samples and the bands were quantified after a local background subtraction. A tolerance in the band position of 1% was applied. The similarities between two treatments were calculated by comparing the band numbers, intensities and positions in each lane of the DGGE profiles. Higher similarities mean higher homologies of bacterial community structures between two samples. Similarity of 100 % indicates the same banding pattern or the same community structure between two soil samples. Two treatments are considered to be similar if the similarity is higher than 70 % (Phillips et al. 2006). Clustering was done with the unweighted pair-group method using arithmetic averages (UPGMA) based on the similarities between treatments.

6.3 Results

6.3.1 The specificity of qPCR primers for total bacterial quantification

The specificity of qPCR primers to targeted 16s rDNA gene was tested first. SYBR Green, used in qPCR, non-selectively binds to double-stranded DNA, emitting fluorescence as an indicator of amount of PCR products. Thus, correct PCR products without any unexpected amplicons are essential for quantification of total bacteria. For standard curve construction of qPCR using DNA extracts from *P. putida*, the melting curve analysis of PCR products showed only one peak, indicating single amplification product (Fig. 6.2A). The melting temperature was 86 ˚C, which was the expected for PCR amplicon based on 16s rDNA sequence of *P. putida,* suggesting that the correct PCR amplification of 16s rDNA of *P. putida* was achieved. The melting curves

Figure 6. 2 Melting curves of qPCR products for bacterial 16s rDNA amplicons.

Panel A: melting curves of standard samples. $9\text{x}10^5$ to $9\text{x}10^9$ represent the melting curves using DNA template extracted from the respective serial dilution of *Pseudomonas putida* cells absorbed onto soil ($9\text{x}10^5$ to $9\text{x}10^9$ CFU/g dry soil). Panel B: Melting curves with one peak from field soil DNA extracts. Panel C: Melting curves with double peaks from field soil DNA extracts.
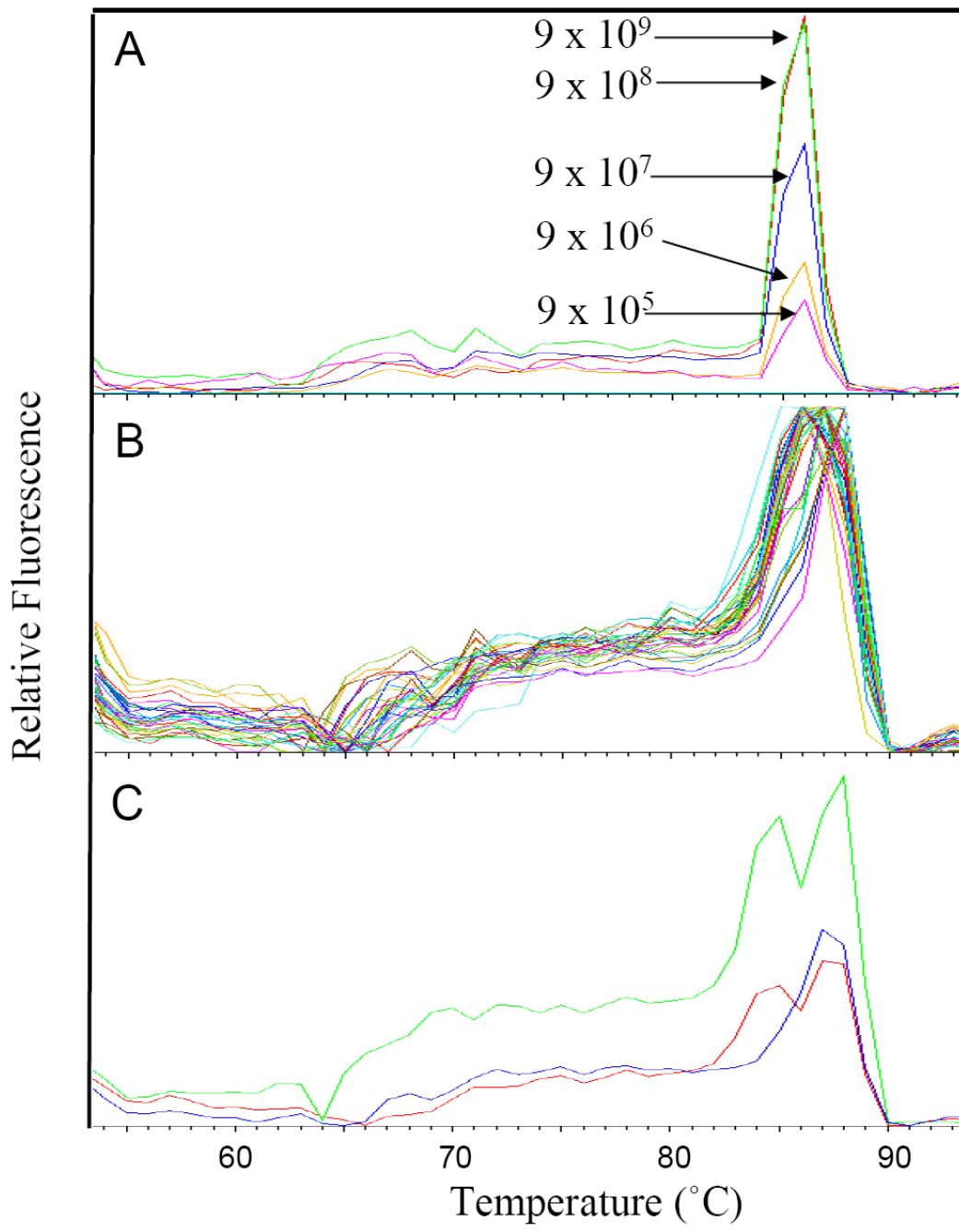
show sharp peaks with peak width (an index of peak sharpness) from 2.05-2.15, which is a typical peak width for a single PCR product (Fig. 6.2A).

For field samples, the melting curves of DNA extracts obtained at the end of most qPCR experiments had one peak with melting points similar to those of the standard samples (Fig. 6.2B). However, the peaks of melting curves were broader than those in the standard samples, with peak widths around 5 or more (Fig. 6.2B). The melting curves of a few samples had double peaks with similar melting points to the standard curve (Fig. 6.2C). The 16s rDNA from different bacteria often have high sequence similarity, and thus have close but not identical melting points, which would result in the broader and/or double peaks observed in melting curves of field samples. Despite this, it will not affect the accuracy of total bacteria quantification, because the design of this qPCR method is to target the 16s rDNA of all bacteria for accurate total bacterial count.

6.3.2 Quantification of total bacteria by the qPCR method

Quantification of total bacterial numbers was performed by comparing the Ct values of experimental soil samples with those of standard curves. The standard curve constructed using DNA extracts of *P. putida* was linear over 5 orders of magnitude (9×10^5-9×10^9 CFU per gram dry soil) (Fig. 6.3). Linear regression for the standard curve was $y = -2.405x + 41.84$, $r^2 = 0.995$.

Rhizosphere soils taken in September were used for total bacterial quantification. Results by qPCR and culturing methods are shown in Fig. 6.4. The total bacterial cell counts obtained by qPCR were 3.37×10^9, 9.53×10^9 and 9.75×10^9 per gram dry soil for unseeded soil, soil seeded with PGPR treated seed and soil seeded with non-PGPR treated seed, respectively. The values based on
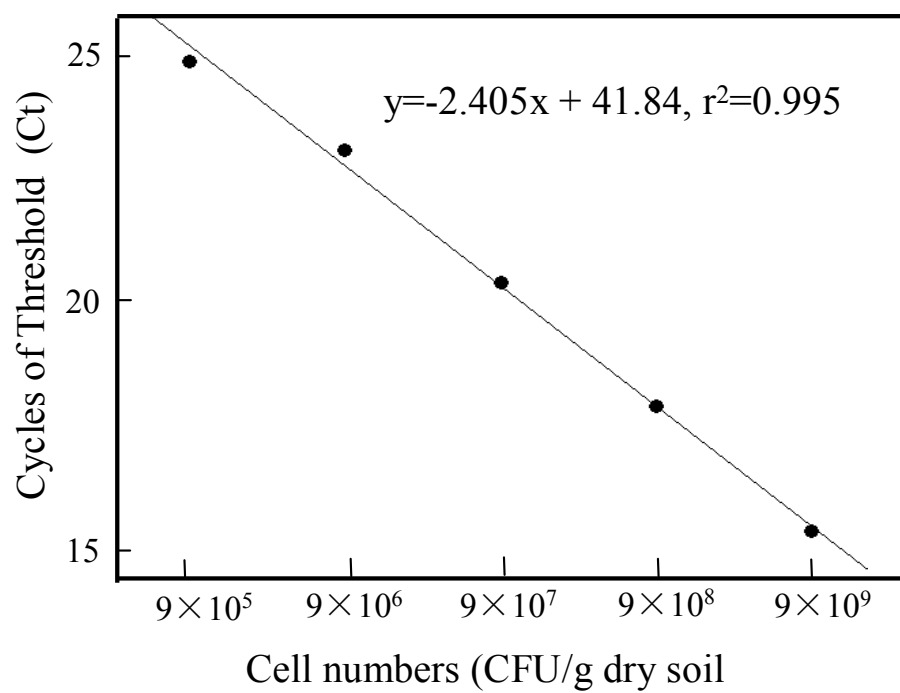
Figure 6. 3 Standard curve derived from qPCR using a bacterial 16s rDNA primer pair.

culturing methods were 2.64×10^5, 4.57×10^6 and 5.24×10^6 per gram dry soil for unseeded soil, soil seeded with PGPR treated seed and soil seeded with non-PGPR treated seed, respectively (Fig. 6.4). In all cases, total soil bacterial numbers quantified by qPCR gave higher values than culturing methods. The values obtained by the qPCR method were 3 orders of magnitude greater for seeded samples and 4 orders of magnitude greater for unseeded samples than for the culturing method. The ratios of total bacterial numbers by the qPCR method to total bacterial numbers by the culturing method is 2.09×10^3 for soil seeded with PGPR treated seed, 1.86×10^3 for soil seeded with non-PGPR treated seed, and 1.27×10^4 for unseeded soil (Fig. 6.4), showing that the culturing method greatly underestimates total bacteria in unseeded soil. The seeded soils with and without PGPR have very close total bacterial numbers by both methods.

6.3.3 DGGE analyses of soil samples from PLF

Bacterial diversities and community structures were monitored at the Sarnia PLF over one growth season by comparing the band pattern of each treatment in the DGGE profiles (Figs 6.5, 6.6 and 6.7). Generally, soil DNA extracts from the three treatments were similar in terms of bacterial diversity and community structure. DGGE analyses of the three treatments showed that they shared most of the dominant bands (Figs. 6.5 and 6.6). At the first sampling time after planting (May), comparing seeded and unseeded soils, some distinct bands (Bands 1, 3, 5, and 10) were present and intensities of some bands were noticeably changed (bands 2, 4, 6, 7, 8, 9, and 11) (Fig. 6.7A). At the second sampling time (July), only a few distinct (Bands 12 and 13) bands were present in unseeded soil relative to the seeded soils (Fig. 6.7B). After that, the three treatments shared almost all the same bands (Figs 6.7C and 6.7D). At all sampling times, the seeded soils with
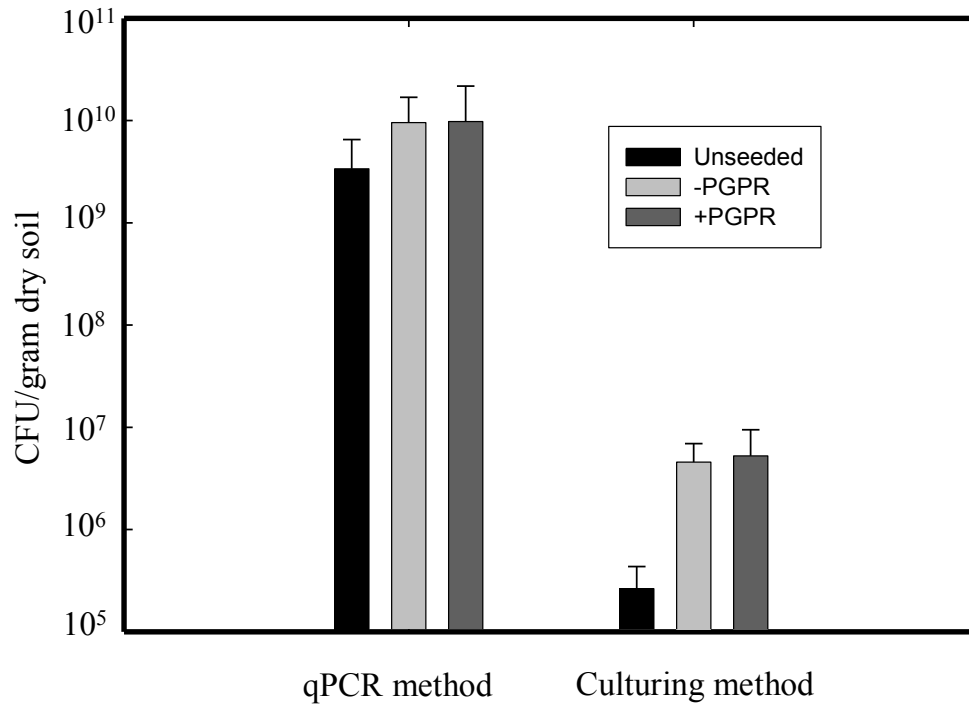
Figure 6. 4 Quantification of total bacteria with culturing method and qPCR method.

For culturing method, viable bacterial colonies were decided by growing bacteria on TSA plates.

+PGPR: soil seeded with PGPR treated seed; -PGPR, soil seeded with non-PGPR treated seed.

Data are the means ± SE. *: significantly different between seeded vs unseeded treatments.
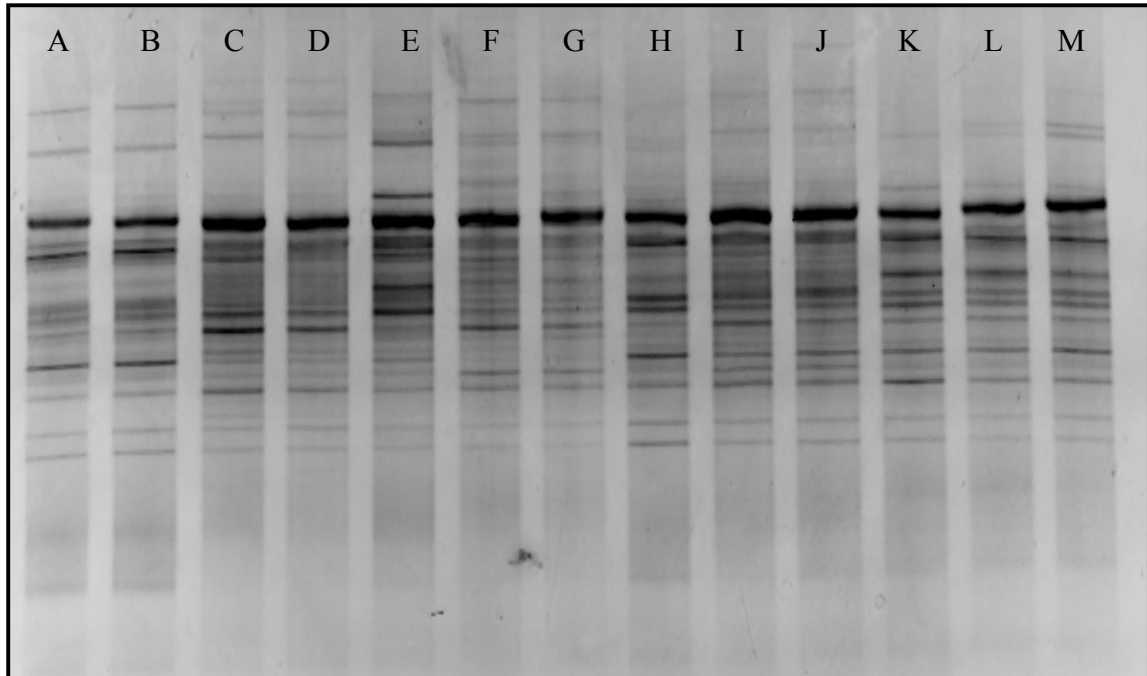
Figure 6. 5 DGGE profiles of the bacterial community from Sarnia soil samples.

Soil samples were taken at different times throughout the growth season. Bacterial 16s rDNA were

amplified by PCR and PCR products were subjected to the DGGE analysis. A: Unseeded, Apr. 27;

B: Unseeded, May 29; C: +PGPR, May 29; D: -PGPR, May 29; E: Unseeded, Jul. 21; F: +PGPR,

Jul. 21; G: -PGPR, Jul. 21; H: Unseeded, Aug. 17; I: +PGPR, Aug. 17; J: -PGPR, Aug. 17; K:

Unseeded, Sep. 21; L: +PGPR, Sep. 21; M: -PGPR, Sep. 21.
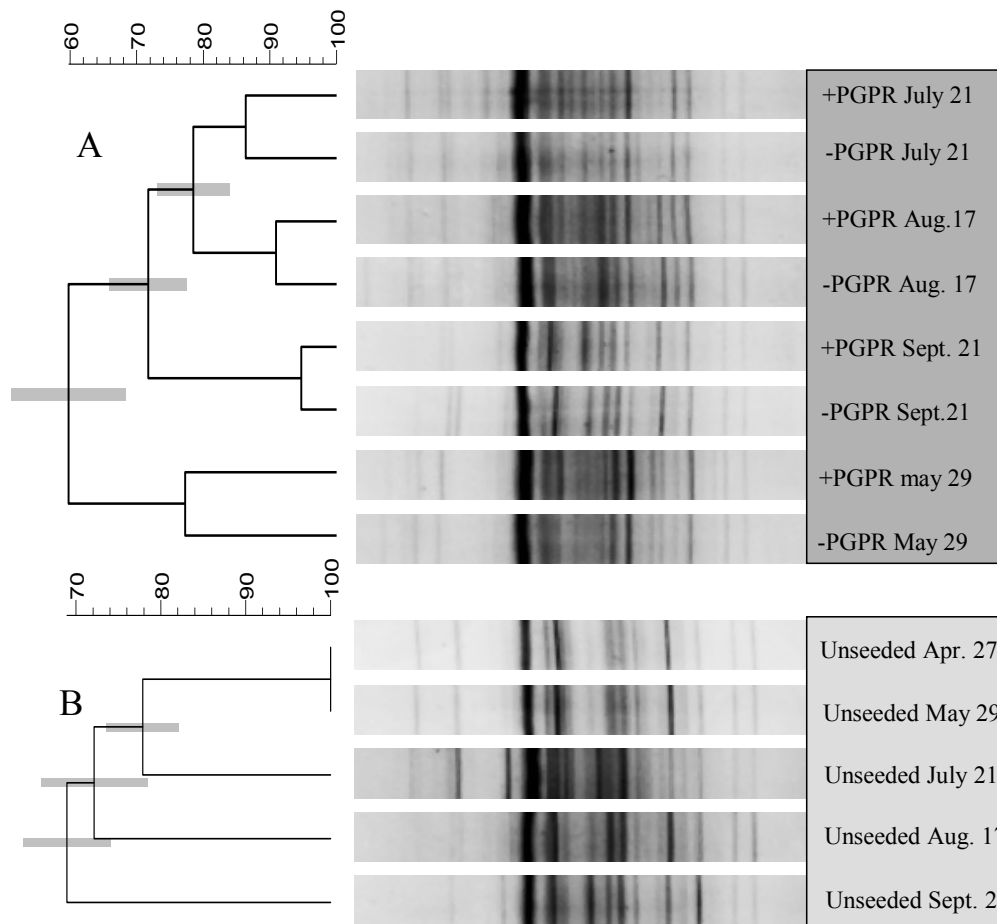
Figure 6. 6 Cluster analyses of bacterial DGGE profiles using UPGMA based on the similarities

of bacterial community structure between the treatments.

Soil samples were divided into seeded and unseeded groups and clustered respectively with

GelCompar software. The grey rectangles are standard deviation. A: Cluster analyses with seeded

soil samples. B: Cluster analyses with unseeded soil samples. The grey rectangles are standard

deviation.
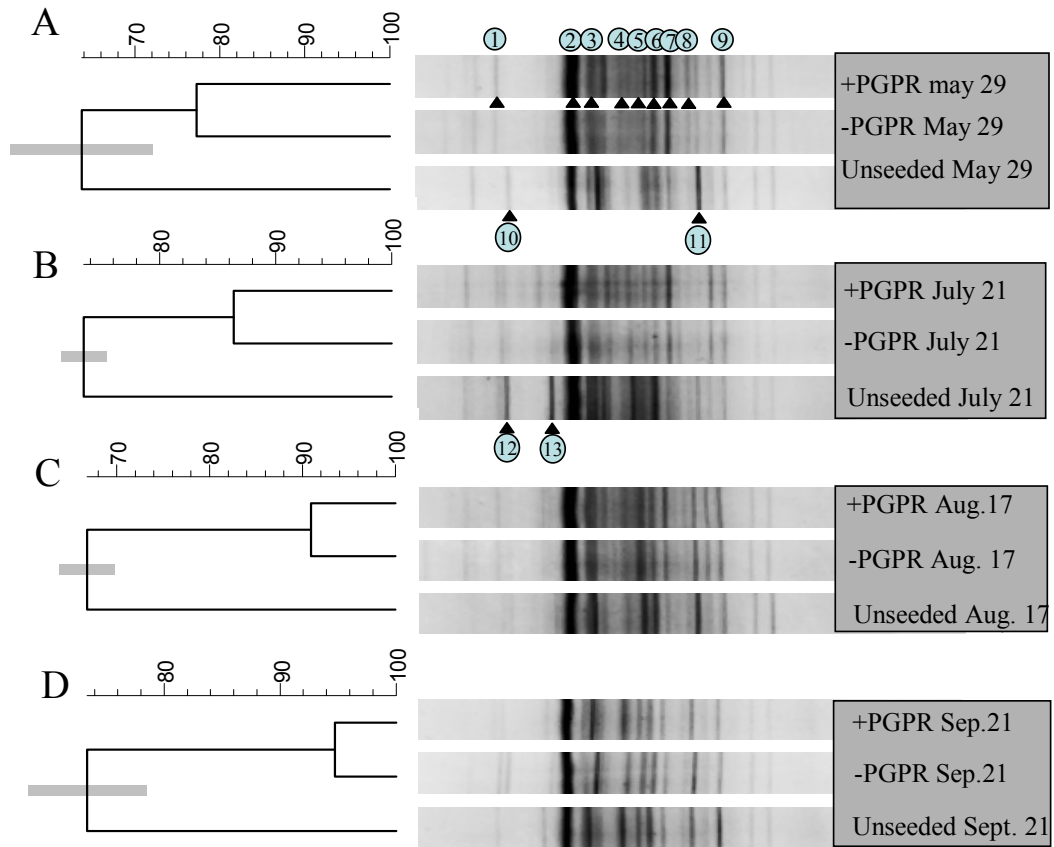
Figure 6. 7 Cluster analyses of bacterial DGGE profiles using UPGMA based on the similarities of bacterial community structure between the treatments.

Soil samples were regrouped and clustered based on sample times with GelCompar software. Filled cycles show bands of difference or with changed intensity. The rectangles are standard deviation. The grey rectangles are standard deviation.

and without PGPR have common bands, but some of bands did vary in intensity (Fig. 6.7).

6.3.3.1 Bacterial diversity at different sampling times during the phytoremediation

Throughout the field trials in 2006, the soil bacterial diversities in soils from the three treatments (unseeded, seeded with PGPR treated seed and seeded with non-PGPR treated seed) taken at different times were assessed by DGGE. The DGGE profiles showed that the numbers of bands in DGGE profiles of different samples ranged from 17 to 23, and most bands were common to all treatments (Table 6.2; Figs. 6.5 and 6.6), suggesting they share common bacterial species, likely due to microbes in these treatments originating from same soil bacterial pool. The bacterial diversities were assessed by the Shannon, Evenness and Dominance indices based on DGGE profiles (Table 6.2). There was no significantly different between any treatments as determined by all three indices. However, some treatments did very greatly in Dominance index. For example, in July sampling time, the Dominance indices were 0.070, 0.065, and 0.10 for unseeded soil, soil seeded with PGPR treated seed and seeded with non-PGPR treated seed, respectively. The highest Dominance index was attributed to soil seeded with non-PGPR treated seed because it had a distinct intense band (band 14) relative to other bands (Fig. 6.7). These results indicated that phytoremediation and PGPR application via seed treatment did not significantly alter the bacterial diversity of PLF soils.

6.3.3.2 Effect of phytoremediation on the bacterial community structure

The similarities in bacterial community structures between three treatments were calculated from the DGGE profiles using GelCompar II software. Results of this analysis showed that soils seeded with PGPR treated and non-PGPR treated seeds generally have similar bacterial

Table 6. 2 Biodiversity indices from DGGE profiles of soil bacterial communities

Treatments	Sampling date	Numbers of band	Shannon index	Dominance index	Evenness index
Blank	Apr. 27	17	2.63	0.085	0.93
Blank	May 29	18	2.69	0.081	0.93
+PGPR	May 29	20	2.59	0.098	0.86
-PGPR	May 29	17	2.51	0.105	0.89
Blank	July 21	22	2.77	0.070	0.89
+PGPR	July 21	21	2.80	0.065	0.92
-PGPR	July 21	17	2.57	0.102	0.91
Blank	Aug. 17	19	2.77	0.072	0.94
+PGPR	Aug. 17	20	2.87	0.076	0.96
-PGPR	Aug. 17	22	2.92	0.066	0.94
Blank	Sep. 21	17	2.57	0.092	0.91
+PGPR	Sep. 21	19	2.66	0.091	0.90
-PGPR	Sep. 21	18	2.72	0.080	0.94

* All values within each index were means of three replicates. All treatments within each index were not significantly different at p=0.05 level.

community structures when treatments were compared at each sampling time (Figs. 6.5, 6.6 and 6.7). The similarities between seeded soils with and without PGPR were 78.2 %, 86.0 %, 90.9 %, and 94.7 % at the May, July, August and September of 2006 sampling times, respectively. The similarities increased throughout growth season (Fig. 6.6A). There was no change in the population of bands present, but the relative band intensities changed. Therefore，most of difference are due to the changes in relative amount of the bacteria.

Bacterial community structures of the seeded soils differed to some degree with those of unseeded soils at all sampling times (Fig. 6.7). Cluster analyses based on similarity showed that seeded samples were distinctly grouped from the unseeded samples (Fig. 6.7). The similarities between unseeded samples and seeded samples with or without PGPR were 56.0 % and 71.4 %, 70.0 % and 76.2 %, 69.57 % and 68.1 %, 80.0 % and 86.7 % for the May, July, Aug. and Sep. sampling time (Fig. 6.6), respectively. The biggest difference in similarity (56.0% similarity) occurred between unseeded soil and soil seeded with PGPR treated seed at the first sampling time after planting (May 29). Different bands (Bands 1, 3, 5, 10) were present in the two soils and some band intensities (bands 2, 4, 6, 7, 8, 9, and 11) were noticeably changed (Fig. 6.7A). However, the DGGE profiles at other sampling times, regardless of being seeded or unseeded, had relatively high similarities of microbial community structures (70% or greater) (Fig. 6.7).

For unseeded samples taken at different times, microbial community structures gradually changed through growing season (Fig. 6.6B). The similarities between unseeded soils from April and unseeded soils taken at the other sampling times were 100 % for May, 73.9 % for July, 76.2 % for August, and 66.2 % for September. The decreased similarities might reflect the effects of

changing temperature and moisture that would occur during the growth season.

6.3.4 Fungal diversity and structure at different times during the phytoremediation

The fungal diversity and community structures were evaluated from DGGE profiles of fungal 18s rDNA using GelCompar II software (Figs. 6.8 and 6.9). Significantly enhanced fungal diversity and altered community structures were observed between seeded and unseeded soils. The similarity between seeded soils with or without PGPR was 73.9 %, 70.67 %, 80.0 % and 81.2 % for samples of May, July, August and September, respectively. Conversely, the similarities between unseeded soils and seeded soils without PGPR or seeded soils with PGPR were 0.0 % and 2.3 %, 31.4 % and 69.6 %, 26.5 % and 28.9 %, 53.4 % and 84.9 % for the May, July, August and September sampling times, respectively. Cluster analysis based on the similarity showed that seeded soils cluster distinctly from unseeded samples with one exception; this was the September sample, when all the three samples had very high similarity (70% or greater) (Fig. 6.9). Generally, seeded soils had very similar diversity and community structure. However, the seeded and unseeded soils were distinguishable with respect to fungal communities. These results suggest that plant growth during phytoremediation strongly changed the fungal community structure.
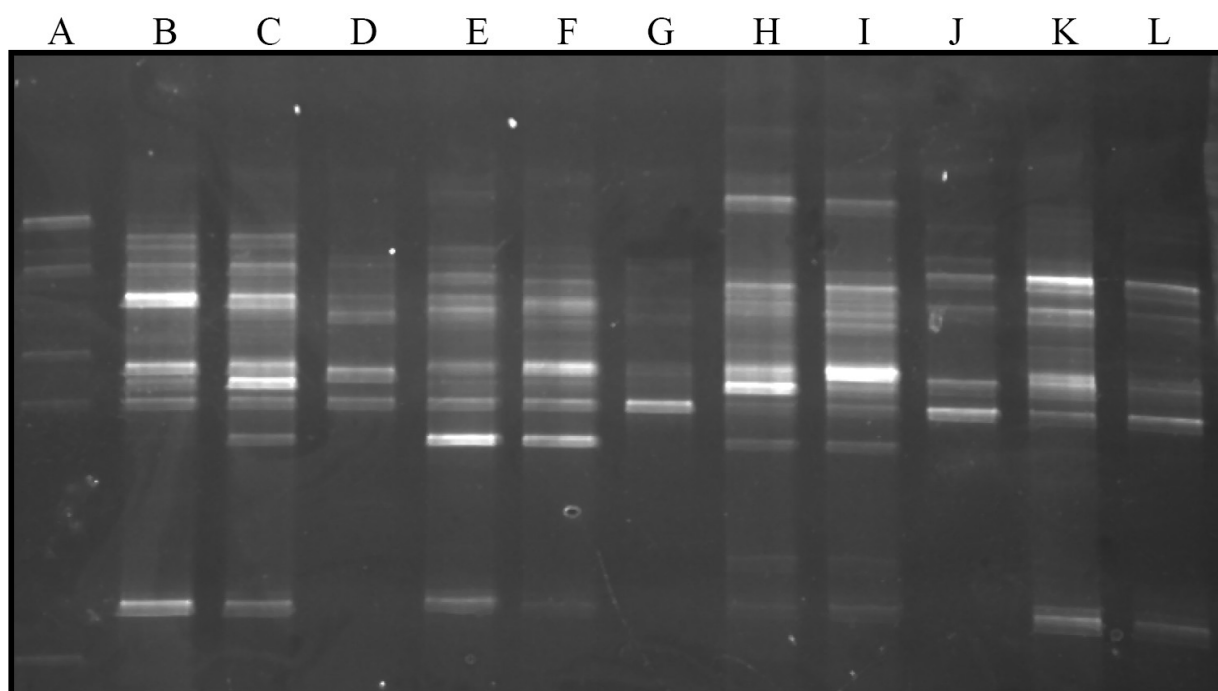
Figure 6. 8 DGGE profiles of the fungal community from Sarnia soil samples.

Soil samples were taken at different times throughout the growing season. Fungal 18s rDNA were amplified by PCR and subjected to the DGGE analysis. A: Unseeded, May 29; B: +PGPR, May 29; C: -PGPR, May 29; D: Unseeded, Jul. 21; E: +PGPR, Jul. 21; F: -PGPR, Jul. 21; G: Unseeded, Aug. 17; H: +PGPR, Aug. 17; I: -PGPR, Aug. 17; J: Unseeded, Sept. 21; K: +PGPR, Sept. 21; L: -PGPR, Sept. 21.
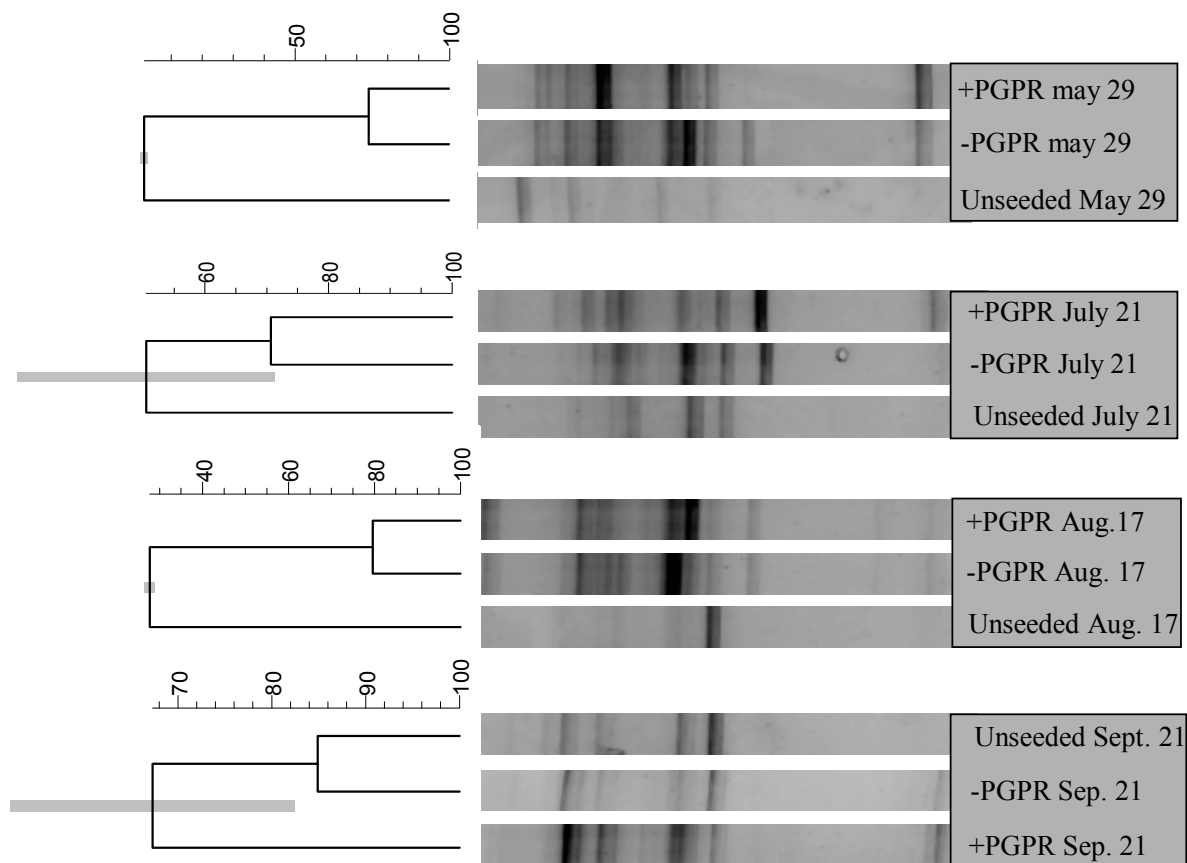
Figure 6. 9 Cluster analyses of fungal DGGE profiles using UPGMA based on the similarities of bacterial community structure between the treatments.

Soil samples were grouped and clustered based on sample time with GelCompar software. The grey rectangles are standard deviation. The grey rectangles are standard deviation.

6.4 Discussion

A limited numbers of studies have been performed to quantify total bacterial numbers in environmental samples using qPCR methods with primers targeting 16s rDNA sequences (Castillo et al. 2006; Huijsdens et al. 2002; Nadkarni et al. 2002b). Total soil bacterial number is an important indicator of microbial activity, especially for the bioremediation of degradable contaminants in the environment such as PHCs in a PLF. PHC degrading bacteria may constitute the most prevalent bacteria in PLFs (Kaksonen et al. 2006). Quantitative molecular methods are more sensitive, rapid and possibly reliable than culturing methods because they do not rely on the ability of bacteria to grow. Considering that it is thought that less than 1% of total soil bacteria and other bacteria can be cultured in laboratory conditions, it is not surprising that qPCR methods often report much higher total bacterial numbers regardless of which environmental samples are analyzed. In this study, it was found that total bacterial numbers were 3 to 4 orders of magnitude higher when measured by qPCR compared to culturing methods (Fig. 6.4). This is consistent with other studies (Castillo et al. 2006; Huijsdens et al. 2002) that used qPCR vs. culturing methods. The much greater total bacteria count by qPCR method has been attributed to the presence of free DNA, non-viable bacteria, or viable but not culturable bacteria aa of which are accounted for by the qPCR as opposed to the culturing method (Castillo et al. 2006). The overestimation of total soil bacteria due to the presence of free DNA is generally small (1-2%) (Lee and Levin 2007). The TSA plate count method used here for total bacterial counts usually favors only specific bacteria due to the lack of essential nutrients required for growth of many bacteria (Zengler et al. 2002). Furthermore, pre-treatment procedures such as serial dilution of soil samples used in culturing

methods overlooks the bacteria attached tightly to or living inside soil particles (Castillo et al. 2006). Hence, qPCR methods might provide a more sensitive measure of total bacterial numbers.

Although there were large differences in total bacterial numbers between the culturing methods and the qPCR methods, the ratios of total bacterial numbers obtained by the qPCR method to the total bacterial numbers by culturing methods are similar for the two treatments containing plants (2.09×10^3 for soil seeded with PGPR treated seed and 1.86×10^3 for soil seeded with non-PGPR treated seed) (Fig. 6.4). It is assumed that the total bacterial numbers measured by qPCR have a positive relation to the copy numbers of bacterial 16s rDNA on which qPCR method is based. The similar ratios between the two treatments containing plants with and without PGPR may be due to their highly homologous bacterial community structure and thus highly homologous 16s rDNA in their DNA extracts from the soils, as shown by the DGGE (Fig. 6.7).

For the unseeded soils, the ratio of total bacterial numbers measured by the qPCR method to total bacterial numbers measured by culturing method was 1.27×10^4, around 6 times higher than the ratio in seeded soil (Fig. 6.4). It is reported that each cell of different bacterial species or at different growth stages for the same bacteria can possess various numbers of copies of 16s rDNA genes. Ratios of up to 20:1 for the 16s rDNA copy numbers between two bacterial species has been documented (Nadkarni et al. 2002b). Thus, differences in bacterial community structure would interfere with the total bacterial numbers as measured by qPCR method. Therefore, the different ratios between seeded samples and unseeded one may be due to the heterogeneous nature of bacterial community structures between seeded soils and unseeded soils relative to that between seeded soils with and without PGPR, as shown by the DGGE results (Fig. 6.7). In addition, plants supply oxygen to root zone, which may result in higher ratio of aerobic bacteria in the seeded soil

than that in the unseeded soil. The aerobic culturing method used will not account for the anaerobic bacterial population, and thus might underestimate the total bacterial numbers in unseeded soils compared to the seeded soils, which may explain why large numbers of bacteria revealed by qPCR in unseeded soils did not result in significant PHC removal since anaerobic degradation of PHCs is known to be much slower than aerobic degradation (Bjorklof et al. 2008).

It is interesting that all three soil types (seeded with PGPR, seeded without PGPR, and unseeded soils) show highly similar bacterial diversity (Table 6.2). The similarities of microbial community structures among the three treatments were relatively high (70 % or greater) except for the first sampling time after planting (Figs. 6.6 and 6.7). Previous studies showed that microbial diversity can be smaller in rhizosphere soils than in unseeded soils and the microbial structures could be dramatically altered in the rhizosohere (Kaksonen et al. 2006; Killham 1994; Mavingui et al. 1992; Metzger et al. 1986). This could be due to the selective enriching effects of plant root exudates on certain bacterial groups, which would result in the suppression of other groups of microbes (Killham 1994). The inconsistency between this study reporting little change in microbial diversity and community structures and previous studies is probably due to site conditions for these experiments. PHCs of high concentration (~13%) in this PLF could be the dominant selective factor that affects soil bacterial populations. In unimpacted ecosystems, PHC utilizers generally constitute less than 0.1% of the microbial community; in oil-impacted ecosystems, they can constitute most of the viable microorganisms (Kaksonen et al. 2006). Hence, PHC degrading bacteria likely dominate the bacterial community in the PLF soil throughout this field trial. This was observed in chapter 4, where total bacterial numbers were similar as PHC degraders. In addition, before remediation, Sarnia PLF soil was frequently inoculated with a commercial

microbial product containing a mixture of PHC degraders. Thus, Sarnia land farm soils most likely have formed a unique microbial ecosystem dominated by specific groups of PHC degrading bacteria during the more than 20 years PHC land farming activities. Plant growth may increase the level of the existing bacterial populations. Together, these could lead to similar microbial diversity and generally high similarity of microbial structures in almost all soil samples no matter seeded or unseeded soils.

Despite the generally high similarity in microbial diversity and structure among three treatments, structure shifts induced by plant growth were still observed between soils with and without plantes (Fig. 6.7). It should be emphasized that soils with apparently similar bacterial community diversities are not necessarily composed of the same bacteria species (Metzger et al. 1986). The largest differences in bacterial community structure between soils with and without plants occurred at the first sampling time (May of 2006) (Fig. 6.7A). This agrees with the results obtained by the culturing method in chapter 4, where all groups of microorganisms in soil containing plants had the largest increase at the first sampling time after planting, and then remained relatively constant for the remainder of the season. Plant growth changes the environment in rhizosphere soils due to exudation of metabolic products from the root (Corgie et al. 2006). Thus, soil microorganisms in the rhizosphere of the PLF would be receiving nutrients from both of plant exudates and PHCs. The microorganisms that can utilize both would likely thrive. Preferential stimulation of bacteria could occur in the rhizosphere. Some bacteria might be enriched (Kaksonen et al. 2006), while others might be suppressed (Phillips et al. 2006), leading to changing in community structure.

The similarity between seeded soils with and without PGPR increased steadily with plant

growth, from 78.2 % at first sample after planting (May, 2006) to 86.37 in July, 90.91 in August and 94.7% in September (Fig. 6.6A). Therefore, the microbial community structures showed the largest difference in the first sampling time after planting (May) between soils seeded with PGPR treated seed and soils seeded with non-PGPR treated seed, as well as between soil seeded with PGPR treated seed and unseeded soil (56 % in similarity) (Fig. 6.7A). This suggests that seed inoculation with PGPR may affect soil bacterial community structure in the early stages of plant growth. This could be due to seed treatment with PGPR, such that these PGPR dominate the seed or root surfaces because they are able to efficiently utilize root exudates. However, introduced PGPR may only dominate the seed coat and the root surfaces in the early stages of plant growth and development (Ma et al. 2001). With root elongation, the indigenous microbial populations, including indigenous PGPR, that are the dominant microbes in the soil, may be able to compete with the PGPR inoculation on root surfaces. This would mask some of the effects of the introduced PGPR. As well, at this PLF, PHCs of high concentrations likely has a greater effect on the microbial community than the presence of a rhizosphere or seed coating with PGPR. This might also result in the increased similarity of microbial structure among three treatments over growth season.

In this study, phytoremediation dramatically changed the fungal community structure in the PLF soils, especially at the first sampling date after planting (May). Similarities of 0.0 % and 2.3 %, respectively, between unseeded soils and soils seeded with non-PGPR treated seed or soil seeded with PGPR treated seed were observed (Fig. 6.9). This suggests the rhizosphere may have a greater effect on fungal diversity than on bacterial diversity. One possible reason is due to the formation of acidic conditions by root exudates containing CO_2 and amino acids (Kaksonen et al.

2006), and PHC biodegradation intermediates such as fatty acids (Fig. 5.9 in chapter 5). This could result in fungal community structure changes because fungi prefer to grow under slightly acidic conditions.

In conclusion, as assessed by quantitative molecular methods, there were no significant differences between seeded soil with and without PGPR in total bacterial numbers, microbial diversity and community structure. PGPR application only modestly affected microbial community structures in the early growth stages. Most of the differences in bacterial community structure between seeded soils with or without PGPR treated seed were caused by the changes of relative abundance of the bacteria. These results agree with the results of the culturing methods in chapter 4, where numbers of different groups of microbes between seeded soils with and without PGPR treated seeds were similar. Therefore, the application of PGPR did not significantly affect microbial ecosystem. It does dramatically improve plant growth and PHC remediation (Gurska et al. 2008). Thus, the PEP should be considered as an environmentally safe and effective approach for removing PHCs from impacted soils.

Chapter 7

Specific Detection and qPCR Quantification of the Plant Growth Promoting Rhizobacteria

(PGPR) Pseudomonas putida (UW4) During PGPR Enhanced Phytoremediation of

Petroleum Land Farm Soils

7.1 Introduction

Petroleum hydrocarbons (PHCs) are one group of widespread organic contaminants. Many components of PHCs such as polycyclic aromatic hydrocarbons (PAHs) are toxic, mutagenic, carcinogenic and/or persistent, posing a threats to the environment and human health (Douben 2003; USEPA 1994). Thus, remediation technologies that can efficiently remove PHCs from soil in a cost-effective and environmentally responsible manner are needed.

One promising strategy for remediating PHCs from soil is phytoremediation (Bosma et al. 2002; Euliss et al. 2008; Gerhardt et al. 2006; Greenberg et al. 2007b; Huang et al. 2005; Huang et al. 2004a; Kaimi et al. 2007; Kirk et al. 2005a; Kramer 2005; Phillips et al. 2006). The extensive root systems of plants can reach large volumes of soil, thereby stimulating the biodegradation of PHCs. However, the growth inhibition induced by the PHCs often result in the failure or unacceptably slow rate of phytoremediation of impacted soil (Greenberg et al. 2007b; Huang et al. 2005; Huang et al. 2004a). One way for improving plant growth and phytoremediation efficiency is to treat seeds with plant growth promoting rhizobacteria (PGPR) (Belimov et al. 2005; Huang et al. 2005).

PGPR are a group of bacteria that can promote plant growth under stress conditions, such as PHC contaminated soils. In addition to the well characterized direct mechanisms of plant growth promotion, such as auxin production, siderophore synthesis, phosphate solubilization, and nitrogen fixation (Glick 1995; Grichko and Glick 2000), PGPR with 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity have been extensively studied (Glick 2005; Glick et al. 2007b). PGPR containing ACC deaminase can consume the plant ethylene precursor ACC, and thereby lower

ethylene concentrations in stressed plants. Lowering of plant ethylene concentrations by these bacteria enhances seedling survival and plant growth (Glick et al. 1994). This helps protect plants against the deleterious effects of stress ethylene, which is produced by plants as a consequence of exposure to various stressors such as PHCs (Huang et al. 2005; Huang et al. 2004a), salt (Cheng et al. 2007; Greenberg et al. 2007b; Mayak et al. 2004), or metals (Reed et al. 2005).

A commonly used strain of PGPR is *Pseudomonas putida* (UW4). It is an example of PGPR with high ACC deaminase activity. UW4 has been demonstrated to promote growth of a wide variety of plants in various harsh environments (Cheng et al. 2007; Farwell et al. 2007; Gerhardt et al. 2006; Nie et al. 2002; Stearns et al. 2005). It has been successfully applied in the phytoremediation of PHC, salt and metal contaminated soils, especially in the early stage of phytoremediation (Gerhardt et al. 2006; Huang et al. 2005; Huang et al. 2004a; Reed et al. 2005; Stearns et al. 2005)

For the better performance of PGPR, soil persistence and root colonization of PGPR are essential for their efficacy (Ma et al. 2001). It is necessary to track and quantify the introduced PGPR in the rhizosphere and surrounding soil to understand the interaction between PGPR and plants, as well to develop better phytoremediation strategies. Until now, monitoring and localization of selected bacteria in the environment, especially those with specific physiological functions in complex biological samples have mainly been performed by culture dependant techniques. These culture dependant techniques include determination of colony forming units (CFU) or most probable number (MPN) with selective media (Bach et al. 2000; Bach et al. 2002). Bacteria containing ACC deaminase enzyme activity can use ACC as sole nitrogen source (Li and Glick 2001; Penrose and Glick 2003). Thus, one quantification technique for these bacteria is to

use a selective media with ACC as sole nitrogen source. Because many bacteria in soil are able to use ACC as a sole nitrogen source, as they harbor ACC deaminase, it would be impossible to distinguish specific ACC deaminase containing PGPR from others with ACC deaminase activity using culture dependant methods. However, the difference in ACC deaminase gene sequences among these PGPR strains makes it possible to distinguish the specific strains with molecular based methods.

In recent years, qPCR technology, using fluorogenic probes such as TaqMan, has been demonstrated to be a rapid and sensitive method for quantifying genes and microorganisms in complex environmental media, including soils (Wang et al. 2004a). Primers and probes are designed to target specific genes from specific bacterial strains of interest to track the organisms in environmental samples. This technology has been successfully used for quantification of specific bacteria both in pure culture and complex environmental samples (Furukawa et al. 2006; Huijsdens et al. 2002; Khan and Yadav 2004; Nadkarni et al. 2002a; Sun et al. 2004). However, because of the interference of DNA from indigenous organisms, the detection sensitivity for a specific bacteria in complex environmental samples, such as soil, is much lower than that with its laboratory pure culture (Wang et al. 2004a). In this study, the first application of "nested plus qPCR method" for detecting a *Pseudomonas putida* (UW4) strain from field soils is presented. This method includes two rounds of PCR amplifications. Firstly, nested PCR was used to increase the specificity and sensitivity by first eliminating most non-target DNAs in the complex mixture of total soil DNA and amplifying the expected sequence. Then, the nested PCR products were subjected to qPCR for quantification of UW4. Primers and probes were designed to target the ACC deaminase gene of UW4, because it has a unique ACC deaminase sequences not present in all known PGPR. The

objectives of this study were to apply this method to quantify the amounts of UW4 in petroleum land farm (PLF) soils during phytoremediation. This allowed an assessment of ability of UW4 to colonize rhizosphere and promote plant growth in petroleum impacted soil during phytoremediation.

7.2 Material and methods

7.2.1 Experimental design and sampling

The design, planting and sampling in this chapter are same with those in the chapters 5 and 6. There were three treatments: the unseeded soil, soils seeded with PGPR treated seed (UW4 and UW3) and soil seeded with non-PGPR treated seed.

7.2.2 Quantification of ACC utilizing bacteria with culturing method

Colony forming units (CFU) of ACC utilizing bacteria were determined as visible colonies by using DF salt medium with ACC as sole nitrogen source (Penrose and Glick 2003). This method is same with that in chapter 5 used for PGPR number counting.

7.2.3 Soil DNA preparation

Total soil DNA was extracted by using the UltraClean Soil DNA Kit (MoBio Laboratories, Inc. Carlsbad, CA, US) following manufacture's instruction. Soils of 0.7-0.8g were used for DNA extraction. The extracted DNA were visualized by agrose gel electrophoresis to assess the quality.

DNA were stored at -20 ˚C for further analysis.

7.2.4 Standard curve preparation for qPCR

For a qPCR standard curve, a pure culture of UW4 was grown for 24 hours in tryptic soy broth (TSB) (30 g L^{-1}, Fisher Scientific, Ottawa, Ontario, Canada). UW4 cells were washed twice with sterile 0.85 % NaCl (w/v) and resuspended in 0.85 % NaCl. CFU of bacterial suspension was determined by plate count method on TSA (30 g TSB and 15 g agar L^{-1}) plates. UW4 cell suspensions were 10-fold serially diluted with 0.85 % NaCl. One hundred of each dilution containing UW4 cells (0, 4, 40, 4×10^2, 4×10^3, 4×10^4, 4×10^5, 4×10^6, 4×10^7, and 4×10^8) were centrifuged at 10,000 g for 2 min. The collected cell pellets were directly subjected to DNA extraction using Ultraclean Soil DNA Kit (MoBio Laboratories). The extracted DNA samples contained a serial dilution of pure UW4 genomic DNA (known as "pure UW4 DNA for standard curve" below). Alternatively, one hundred μl of each dilution were mixed evenly with 1 g of unsterilized soil from experimental PLF sites where UW4 was never applied. DNA was extracted from each dilution of the mixtures of UW4 and soil by using the UltraClean Soil DNA Kit (MoBio Laboratories). These DNA samples, containing the mixtures of a serial dilution of UW4 genomic DNA and DNA of indigenous soil organisms, were known as "soil DNA for standard curve" in the following text of this chapter. The soil DNA for standard curve were similar in complexity to DNA samples from experiment PLF soils. The standard curve constructed from the soil DNA for standard curve will provide an accurate measure of UW4 cells in soil.

The above two sets of DNA samples containing serial dilutions of UW4 genomic DNA were used to construct qPCR standard curves and to compare the sensitivity of qPCR using DNA templates from both pure culture and soil mixtures. The standard curves for quantification of UW4 were prepared using UW4 specific ACC deaminase gene-based primers and probe (Fig. 7.1). These DNA samples extracted from UW4 serial dilutions that correspond to varying UW4 numbers (0, 4,

4×10^1, 4×10^2, 4×10^3, 4×10^4, 4×10^5, 4×10^6, 4×10^7 and 4×10^8) were amplified by qPCR. The cycle of threshold value (Ct), the cycles of qPCR to reach threshold amount of PCR products, was plotted against the cell numbers (see below for detail).

7.2.5 Primers/probe design for nested PCR and qPCR

Primers and probe were designed to target the UW4 ACC deaminase gene because of the uniqueness of its sequence. UW4 ACC deaminase gene sequence was obtained from GeneBank (accession number AY823987) (Fig. 7.1) (Hontzeas et al. 2004). Primer sequences were developed using Primer 3 website server (Rozen and Skaletsky 2000). No more than 75% identical gene sequences with developed primers and probe were found by searching the gene database using BLAST. TaqMan probe sequence for qPCR was designed using primer express software and purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). The probe contained fluorescent 5-carboxyfluorescein (FAM) as a reporter fluorochrome on the 5' end and N,N,N',N' – tetramethyl-6-carboxy-rhodamine (TAMRA) as a quencher on the 3'-end of nucleotide sequence (Table 7.1). qPCR was optimized by testing different primers and probe concentrations to obtain the optimal cycle threshold value and get rid of noise fluorescent background.

7.2.6 UW4 quantification via the nested plus qPCR method

To distinguish and quantify low concentration of UW4 DNA from complex DNA from field soils with high indigenous microbial population, a method called "nested plus qPCR" was used. For regular qPCR method, DNA samples for standard curves and DNA from experimental sites were directly subject to qPCR quantification with qPCR primers and probe (Fig. 7.1). For the

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1    ATGAACCTGA ATCGTTTTGA ACGTTATCCG TTGACCTTCG GTCCATCCCC CATCACTCCC
61   TTGAAACGCC TCAGCGAGCA CCTGGGCGGC AAGGTGGAAC TGTATGCCAA GCGTGAAGAC
121  TGCAATAGCG GCCTGGCCTT CGGCGGGAAC AAAACGCGCA AGCTCGAATA TCTGATTCCC
181  GAGGCCATCG AGCAAGGCTG CGACACCTTG GTGTCCATCG GCGGTATCCA GTCGAACCAG
241  ACCCGCCAGG TCGCTGCGGT CGCCGCCCAC TTGGGTATGA AGTGTGTGCT TGTGCAGGAA
301  AACTGGGTGA ACTACTCCGA CGCTGTATAT GACCGCGTCG GCAACATCGA GATGTCGCGG
361  ATCATGGGAG CGGATGTGCG GCTTGATGCT GCAGGTTTCG ACATTGGAAT TCGGCCGAGC
421  TGGGAAAAGG CCATGAGCGA TGTCGTGGAG CGCGGCGGCA AACCGTTTCC AATTCCGGCG
481  GGCTGTTCCG AGCATCCCTA TGGAGGGCTC GGGTTTGTCG GCTTCGCTGA GGAAGTGCGG
541  CAGCAGGAAA AGGAGTTGGG CTTCAAGTTT GACTACATCG TGGTCTGCTC GGTGACCGGC
601  AGTACCCAGG CCGGCATGGT CGTCGGTTTC GCGGCTGACG GTCGCTCGAA AAACGTGATC
661  GGGGTCGATG CTTCGGCGAA ACCGGAGCAA ACCAAGGCGC AGATCCTGCG TATCGCTCGA
721  CATACCGCTG AACTGGTGGA GCTGGGGCGC GAAATCACTG AAGAGGATGT GGTGCTCGAT
781  ACGCGTTTCG CCTATCCGGA ATATGGCTTG CCCAACGAAG GGACGCTGGA AGCGATTCGC
841  CTGTGCGGCA GTCTTGAGGG GGTGTTGACC GATCCGGTCT ACGAGGGCAA ATCCATGCAC
901  GGCATGATTG AAATGGTACG CCGCGGGGAA TTCCCTGACG GCTCCAAAGT TCTTATGCC
961  CACCTGGGCG GCGCACCTGC GTTGAACGCC TACAGCTTCT TGTTTCGCAA CGGCTGA
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Figure 7. 1 Amplicon from the ACC deaminase gene of UW4 used to develop the PCR assay. The primers for nested PCR amplification and sequencing of the region are shown in italics. The sequences of the qPCR primers are shown in bold. Sequence of the TaqMan probe is underlined with dotted line. The probe contained fluorescent 5-carboxyfluorescein (FAM) as a reporter fluorochrome on the 5' end and N,N,N',N' – tetramethyl-6-carboxy-rhodamine (TAMRA) as a quencher on the 3'-end of nucleotide sequence. When this probe binds to target sequence, the reporter dye is released to emit a fluorescence signal.

Table 7. 1 Sequences of primers and TaqMan probes used for PCR amplification of ACC

deaminase gene of UW4

Primers/probe	Sequence (5'-3')	Tm(˚C)	Amplified fragment (bps)
Nested PCR			
Left primer	ACGTTATCCGTTGACCTTCG	63.2	
Right primer	CTGACGGCTCCAAAGTTCTT	63.7	934
qPCR			
Left primer	CTGTTCCGAGCATCCCTATG FAM-	60.6	
Probe	TCAGCGAAGCCGZCAAACCCGA-	66.0	89
Right primer	TAMRA CAAACTTGAAGCCCAACTCC	59.7	

FAM: 5-carboxyfluorescence; TAMRA: N,N,N',N'-tetramethyl-6-carboxy-rhodamine

nested plus qPCR method, quantification began from the nested PCR. The ACC deaminase gene in the DNA samples for both standard curves and experimental sites were amplified first with nested PCR using the nested PCR primers (Fig. 7.1). Then, qPCR was applied using products of nested PCR as templates with qPCR primers and probe. Numbers of UW4 cells in experimental site can be determined by comparing Ct values of standard curve with those of experimental soils.

qPCR reactions were performed in a single tube (20 μl) that consisted of 450 nM of each forward and reverse primer, 125 nM Taqman probe, 10 μl of 2×Taqman Master Mix Buffer (Sigma), and 1 μl templates. The qPCR was initiated with a 2 min cycles at 50 ˚C, followed by 95 ˚C for 10 min, and by 30 cycles of denaturing for 15 sec at 95 ˚C, annealing and extension for 1 min at 59 ˚C. qPCR was performed with MiniOpticon qPCR system (BioRad, Hercules, CA, US). The cycle of threshold values (Ct) were converted to CFU of UW4 in 1 gram dry soil using generated standard curves. The nested PCR condition is 95 ˚C for 5 min, 29 cycles of 95 ˚C for 40 seconds, 59 ˚C for 40 seconds, 72 ˚C for 1 min, followed by 7 min at 72 ˚C and held at 4 ˚C.

7.3 Results

7.3.1 DNA extraction from soil samples

The DNA samples extracted from the PLF soils with the Ultra-clean Soil DNA Kit (MoBio Laboratories) gave single bands following agrose gel electrophoresis (Fig. 7.2). Thus, high integrity total soil DNA samples were obtained. For soil DNA samples for the standard curves, although these DNA samples contain serial dilutions of UW4 genomic DNA, agrose gel electrophoresis of each sample shows similar band intensities (Fig. 7.2A). This indicates that UW4
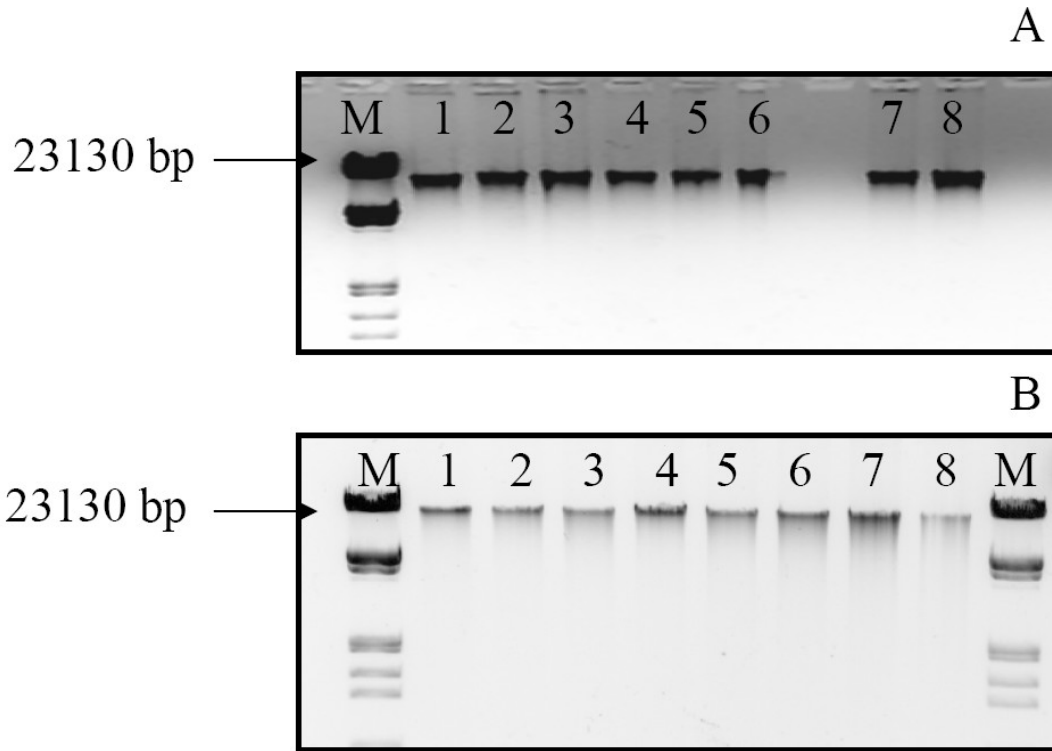
Figure 7. 2 DNA extracted from soil samples with the Ultraclean soil DNA kit.

Panel A: DNA samples extracted from soils used to creat qPCR standard curve. Extracted DNA samples of 4 μl were loaded on 0.7 % agrose gel. M: DNA ladder; Lane 1: soil not inoculated with UW4; Lanes 2-8: DNA samples extracted from soil samples inoculated with serially diluted UW4 cells (4×10^7-4×10^2 CFU/g dry soil). Panel B: DNA samples isolated from field soils taken on July 21, 2006. 2 μl extracted DNA samples were loaded on 0.7 % agrose gel. M: DNA ladder; Lane 1: unseeded soil; Lanes 3, 4, 7, 8: soils seeded with UW4 treated seed; Lanes 5, 6, 9: soils seeded with non-UW4 treated seed.

genomic DNA was only a minor part of the total DNA in the soil, probably due to the fact that the inoculated UW4 cells were only small fraction of microbial population in the soils. The DNA samples isolated from field soil (taken on July 21, 2006) corresponding with three treatments are shown in Fig. 7.2B. These data shows that high quality intact DNA was isolated from the PLF soils. Also, similar amounts of DNA were isolated from soils of three treatments (unseeded soils, seeded soils with PGPR and non-PGPR treated seed).

7.3.2 Specificity of primers and probe to UW4 cells

One pair of nested PCR and qPCR primers were designed on the basis of the UW4 ACC deaminase gene sequence (Fig. 7.1; Table 7.1). The efficiency and specificity of the qPCR primers were tested by using a melting curve and/or agrose gel electrophoresis. Using pure UW4 DNA samples containing a serial dilution of pure UW4 genomic DNA as templates, agrose gel electrophoresis of the qPCR products using qPCR primers confirmed the desired quality of the amplification as shown by the lack of formation of any primer dimers (Fig. 7.3 insert). A single amplification product was observed on agrose gel and the amount of PCR products increased with increasing template concentration as shown by increasing band intensity on agrose gel (Fig. 7.3 insert). A melting curve analysis of PCR products showed only one peak (Fig. 7.3). The melting temperature was 85 ˚C. For nested PCR using templates of soil DNAs for standard curve that contain mixture of a serial dilution of UW4 genomic DNA and DNA from indigenous soil organisms, agrose gel analysis showed a single band and this band intensity decreased with decreasing UW4 DNA template (Fig. 7.4). The melting curve analysis was not performed for these primers because the amplicon fragments (934 bps) were too long for qPCR. For the nested plus
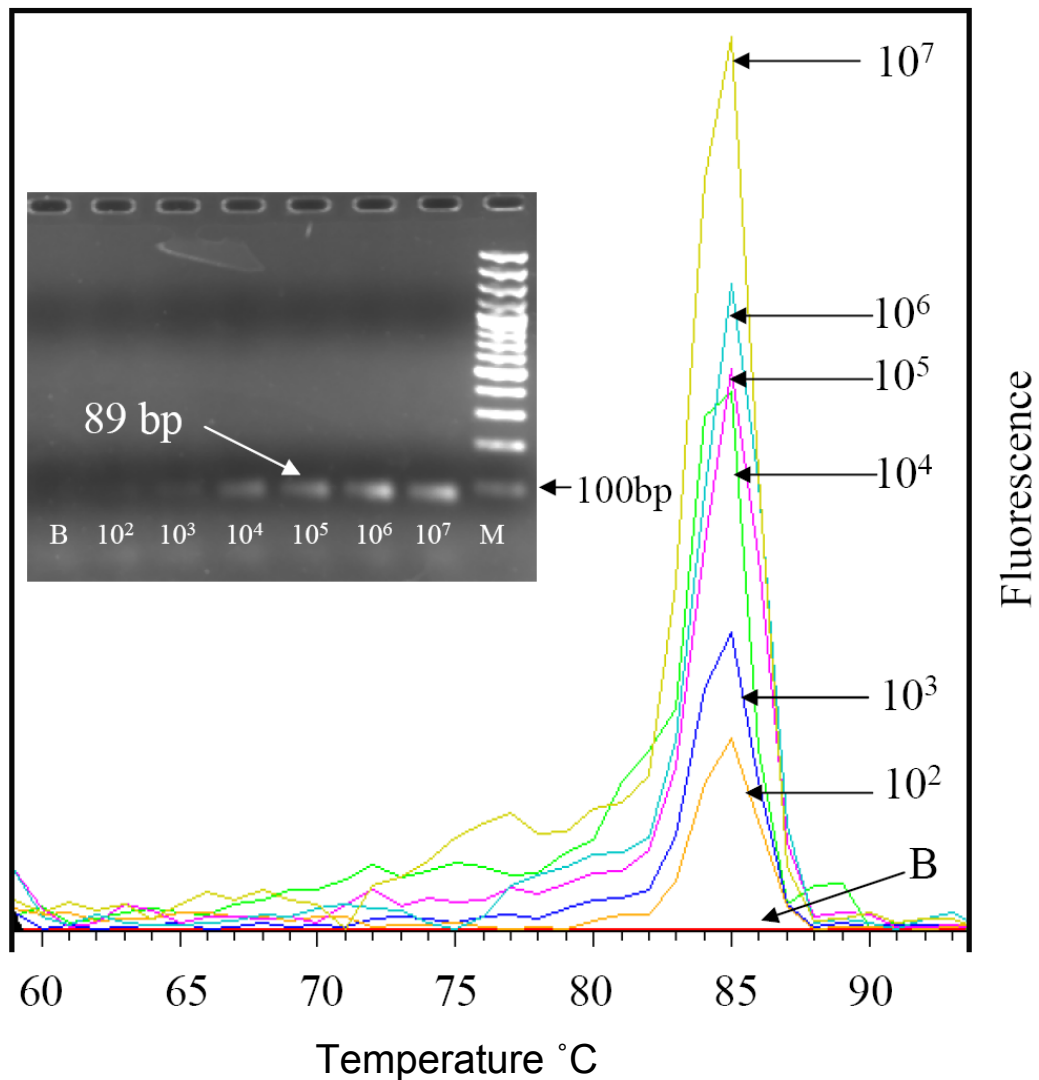
Figure 7. 3 Melting curve for ACC deaminase-specific amplicons during qPCR analysis using serially diluted pure UW4 genomic DNA.

Melting curves showing the same melting temperature (85 ˚C) for all amplicons. Insert: Agrose gel analysis of qPCR products. Increasing intensity of bands and enhancing fluorescence in melting curve corresponds to increasing template concentrations at the beginning of qPCR. M: ladder; B: No template added; 10^2-10^7: serially diluted 10-fold DNA template corresponds to UW4 from 4×10^2-4×10^7 CFU per gram dry soil.
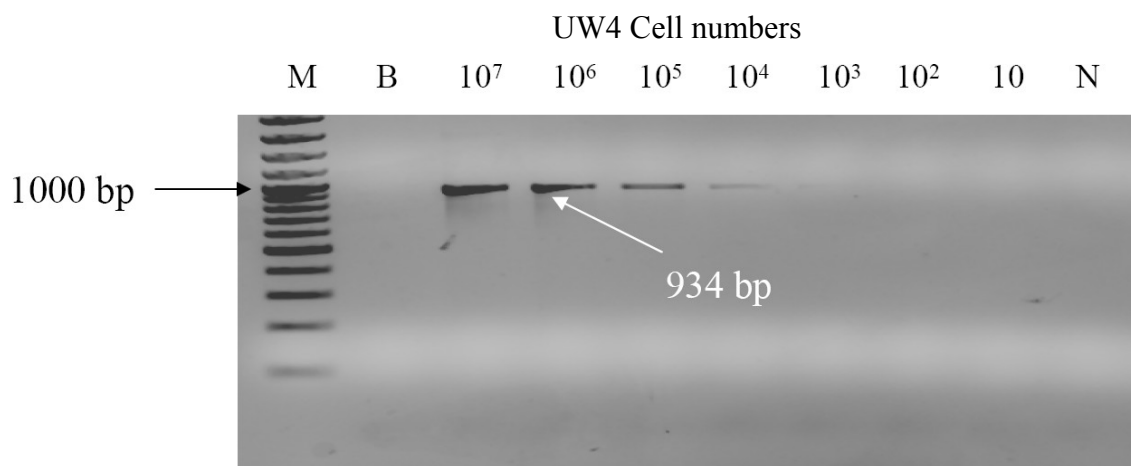
Figure 7. 4 Sensitivity of the nested PCR using the nested PCR primer set for the detection of the UW4 deaminase gene.

M: ladder; B: no template added; N: negative control (water); 10^7-10: serially diluted template corresponds to UW4 cell numbers from 4×10^7 to 4×10 CFU per gram dry soil.

Figure 7. 5 Amplification for qPCR showing detection sensitivity of qPCR alone and nested plus qPCR.

Curves 1-9: amplification charts of qPCR using serially diluted 10-fold DNA as template that corresponds to UW4 cell numbers from 4×10^8-4×10^0 CFU per gram dry soil (1, 4 x 10^8; 2, 4 x 10^7; 3, 4 x 10^6; 4, 4 x 10^5; 5, 4 x 10^4; 6, 4 x 10^3; 7, 4 x 10^2; 8, 4 x 10^1; 9, 4 x 10^0). **Panel A:** amplification plot of the real time alone method using pure UW4 genomic DNA, the detection limitation is 400 CFU / g dry soil. Blank, no DNA added. **Panel B:** amplification plot of qPCR alone method using soil mixture DNA, the detection limitation is 4×10^6 CFU / g dry soil. Blank, soil DNA without UW4. **Panel C:** amplification plot of nest plus qPCR method using soil mixture DNA, the detection limitation is 40 CFU / g soil. Blank, Soil DNA without UW4.

Threshold of cycles (Ct)
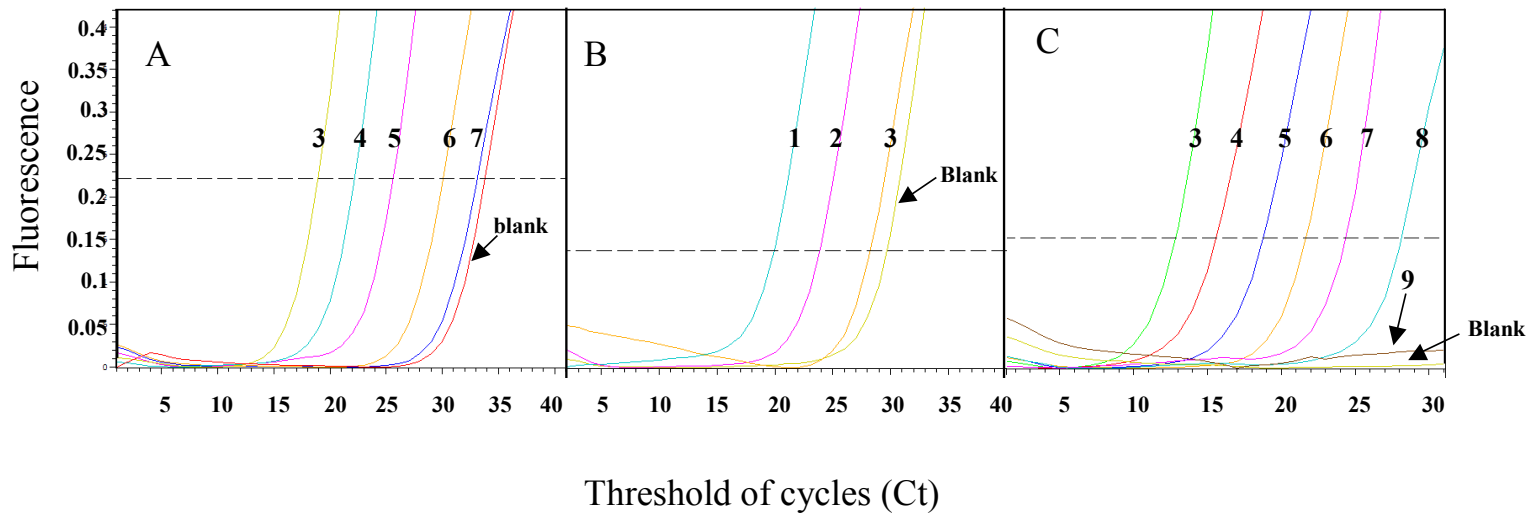
qPCR method, of primers and fluorescence probe specificity to UW4 was confirmed through monitoring fluorescence intensities. The fluorescence intensities increase with increasing concentration of UW4 DNA in soil DNA for the standard curve. No fluorescence was detected using template of soil DNA that did not contain UW4 (Fig. 7.5C). Therefore, the PCR primers and the probe designed for this study were found to be specific for UW4 cells under these experimental conditions.

7.3.3 Sensitivity of the qPCR alone and nested plus qPCR methods for UW4 detection

For the regular qPCR method, using pure UW4 genomic DNA to creat standard curve, the detection sensitivity was 400 CFU of UW4 per g dry soil (Figs. 7.5A and 6.6). However, when soil DNA spiked with UW4 cell was used to creat standard curve, the detection sensitivity was diminished due to the limitation of the DNA extraction efficiency from soils and the interference of DNA from indigenous soil organisms. The sensitivity was 4×10^6 CFU per g dry soil under these conditions (Figs. 7.5B and 7.6). For the nested plus qPCR method, using soil DNA for standard curve, the UW4 detection sensitivity was greatly increased due to the first round of amplification of target gene by nested PCR. The sensitivity increased to 40 CFU per gram dry soil, the most sensitive among three methods (Figs. 7.5C and 7.6).

The low fluorescence signal of the blank sample in the qPCR step of the nested plus qPCR method was observed (Fig. 7.5C). The qPCR step in the nested plus qPCR method used nested PCR products as templates. Indeed, no nested PCR products were produced in nested PCR for blank samples using soil DNA template without containing UW4 DNA. This resulted in the lower blank signal in qPCR step in nested plus qPCR method using template of nested PCR (no
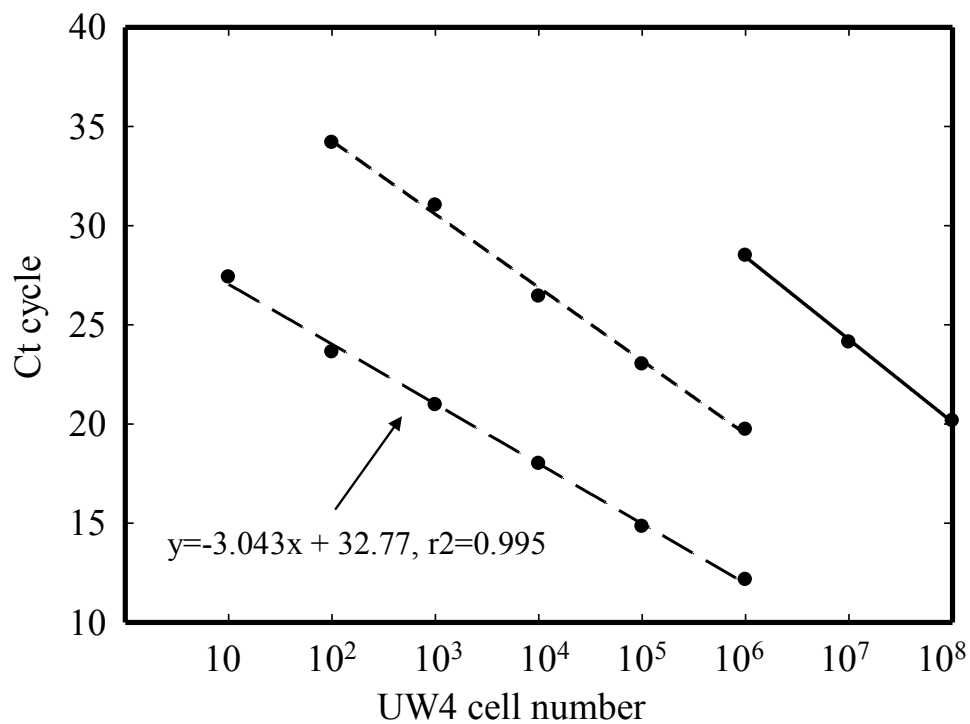
Figure 7. 6 Standard curve of qPCR for UW4 using primers and the probe targeting the ACC deaminase gene.

------: standard curve of qPCR using pure UW4 genomic DNA. $-----$: standard curve of nested plus qPCR using DNA prepared for standard curve from soil mixture. ———— : standard curve of qPCR alone using DNA prepared for the standard curve from a soil mixture.

product) relative to regular qPCR method using template of complex soil DNA (Fig. 7.5B). qPCR is not accurate if cycle numbers exceed 30 when noise fluorescene signal appear even using water as template. Thus, in nested plus qPCR method, we used less than 30 cycles of qPCR to avoid noise fluorescene and get accurate quantification (Fig. 7.5C).

7.3.4 Nested plus qPCR detection of UW4 cells in field samples

Using the nested plus qPCR method, it was possible to measure UW4 cells in the field during the course of phytoremediation due to the high sensitivity of this method. The UW4 numbers were determined by comparing Ct values of samples from experimental sites with those of standard curve. The standard curve for nested plus qPCR using soil DNA for the standard curve was linear over 6 orders of magnitude (4 x 10 - 4 x 10^6 CFU per gram dry soil). Linear regression value for the standard curve was: y=-3.043x + 32.77, r^2=0.995 (Fig. 7.6). Experiments for the standard curve were performed several times yielding similar results.

For the samples collected before planting on April 27, no UW4 cells were detected in all treatments (Fig. 7.7B). After that, the nested plus qPCR method detected UW4 cells of different amounts in the seeded soils inoculated with and without UW4 at the following 4 sampling times (May 29, July 21, Aug. 17 and Sep. 21). The unseeded soils had UW4 only at the last two sampling times (Aug. 17 and Sep. 21). The average cell numbers were 679 (ranged from 238 to 1130) CFU / g dry soil for seeded soil without UW4 application, 47189 (range from 2,147 to 82,713) CFU per g dry soil for seeded soil with UW4 application (Fig. 7.7B), and approximately 100 cells per gram dry soil for unseeded soils. UW4 numbers in the seeded soils inoculated with UW4 were approximately 1-2 orders of magnitude higher than those without UW4 inoculation, and three orders of magnitude higher than those in unseeded soils. These results suggest that seed
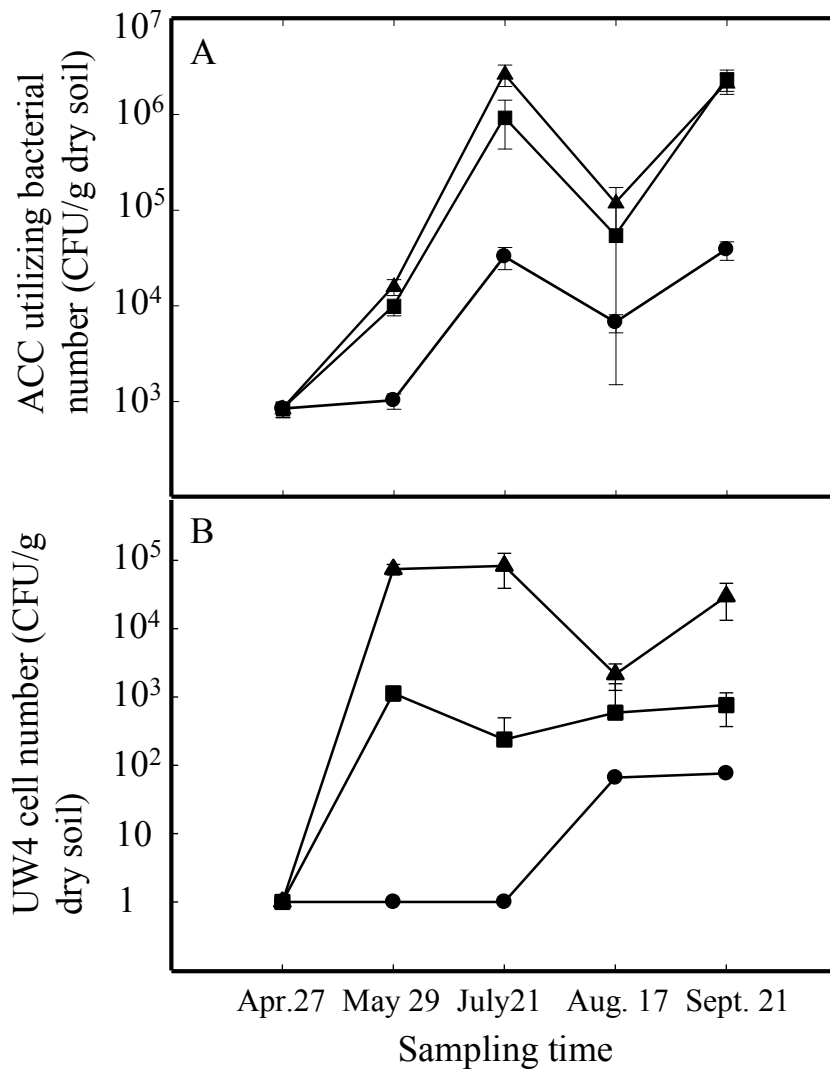
Figure 7. 7 Quantification of PGPR of field soil samples based on culturable plate count and

nested plus qPCR through the growth season.

Panel A: ACC-utilizing bacterial number counted on DF plate with ACC as sole nitrogen source.

Panel B: UW4 number measured by nested plus qPCR method. ▲ : soils seeded with UW4

treated seed. ■ : soils seeded with non-UW4 treated seed. ● : unseeded soils. Data are means ±

SE.

inoculation of UW4 enhances the UW4 population in the field soils. Since UW4 cells were found in unseeded soils. This may indicate that the UW4 ACC deaminase gene is present in native bacterial population in PLF, or migration and/or cross contamination between different treatments occurred because of the experimental activities and other natural factors such as wind and rain water running from the seeded areas.

There were slightly higher ACC utilizing bacteria in the soils with UW4 application than those without UW4 at the three sampling times after planting (May 29, July 21 and Aug. 17) (Fig. 7.7A). On the last sampling time (Sept. 21), two soils seeded with UW4 and without UW4 have almost identical ACC utilizing bacterial numbers.

7.3.5 Comparison of nested plus qPCR method with culturable plate count method

To estimate effect of UW4 inoculation on the field microbial ecosystem, a UW4 ratio was used. This is the ratio of UW4 number quantified by qPCR method to number of ACC utilizing bacteria by culturing method. Although the two numbers were quantified by different methods, it still provides valuable information reflecting the dynamics onf relative abundance of UW4 in soils (Table 7.2).

For the treatment with UW4 application, in the first sampling time after planting (May), the ratio in seeded soils with UW4 application was 469.6 % (Table 7.2; Fig. 7.7). UW4 numbers appear to be several times higher than ACC utilizing bacterial numbers. This is impossible because UW4 is included in the ACC utilizing bacteria. This could likely due to that bacterial numbers in complex environmental samples such as soil quantified by the qPCR method is 2 to 4 orders of magnitude higher than culturing methods (Castillo et al. 2006; Huijsdens et al. 2002; Nadkarni et al. 2002b) (Chapter 5). Even though this, the UW4 ratios in all other soil samples

Table 7. 2 The ratios of UW4 numbers by nested plus qPCR method to culturable ACC utilizing

bacteria in seeded soils inoculated with and without UW4

Sampling date	Ratio	
	UW4 +	UW4-
May 29	469.6	11.5
July 21	3.2	0.03
Aug. 17	1.8	1.1
Sept 21	1.4	0.03

UW4+: Treatment seeded with UW4 treated seed. UW4-: Treatment seeded with non-UW4

treated seed.

were < 3.2 %, showing that in later stages of phytoremediation, the introduced UW4 cell only comprise a small part of ACC deaminase bacteria, and by inference, the rest of microbial community. The UW4 ratios deceased more than 100 times after first sample after planting (Table 7.2). This was because the ACC utilizing bacteria were greatly increased with plant growth and UW4 remained relatively stable. Conversely, the ratios in seeded soils without UW4 application were much lower than those in the seeded soils with UW4 (Table 7.2), in agreement with the above results that UW4 inoculation increased UW4 population in soils (Fig. 7.7B).

7.3.6 Colonization of UW4 on root surfaces and soils

To assess the ability of UW4 to colonize root surfaces, UW4 cells in rhizosphere soils tightly attached to root surfaces were determined using samples taken at Sep. 21. It was presented as the ratios of rhizosphere soil samples with the presence of UW4 ACC gene to total soil samples. The detection ratios of UW4 cells on root surfaces were 100 % for plants with UW4 inoculation (n=32), 32% for plants without UW4 inoculation (n=31). These suggest that UW4 is able to colonize root surfaces.

7.4 Discussion

The plant promoting function of PGPR depends on their ability to colonize and persist on the root surfaces (Ma et al. 2001). Many PGPR have a better plant growth promoting effect in the lab and greenhouse experiments using sterilized soil, but failed to show plant growth promotion effects in field trials. One reason may be because they failed to compete with indigenous microorganisms in colonizing root surfaces. Hence, the effect of PGPR on promoting plant

growth will vary at different sites with different soil types and different soil microorganism communities. This variability is typical of PGPR due to their interactions with other organisms in soil, and many other variables that exist in field soil environment. In this chapter, enhanced UW4 populations were observed in the seeded PLF soils where UW4 was used to coat seeds. UW4 was able to colonize root surfaces and persist in the PLF. This, combined with its high ACC deaminase activity, makes UW4 promising PGPR for the enhanced phytoremediation of PLF soils. Indeed, the plant performance was improved and better remediation was obtained at the same time (Gurska et al. 2008). This indicates that UW4 application can be a key factor in promoting plant growth and improving phytoremediation efficiency of PHC contaminated soils. Thus, UW4 seed treatment appears to be a valuable technology in phytoremediation of contaminated soils.

It is interesting that the ratios of UW4 numbers to numbers of ACC utilizing bacteria decreased more than 100 times from the first sampling time after planting to the following sampling times (Table 7.2; Fig. 7.7). Seed coating allows UW4 to colonize the seed or root surfaces at the seedling stage. UW4 could quickly establish significant populations by utilizing root exudates and dominate the PGPR population on root surfaces at this stage. It might do this by suppressing other ACC utilizing bacteria probably due to catabolite repression. This could result in the highest UW4 ratio in seeded soil with UW4 application at the first sampling time after planting during the field trial (Table 7.2). This also makes UW4 application very helpful in the phytoremdiation of contaminants, because the seedling stage is highly susceptible to environment stresses, and thus is critical for success of phytoremediation. The introduced UW4 may be only present on both the seed coat and the upper of the root, and may not spread as fast as root elongation (Ma et al. 2001). The indigenous microbes, including indigenous PGPR, may

grow quickly to populate the root during elongation, and dominate these root surfaces and near rhizosphere. This would result in the lower UW4 ratios as growth season proceeds. This was in agreement with the results of chapter 6, that seed treatment with PGPR affected the microbial community structure at the first sampling after planting and the effect of seed treatment was masked with plant growth. Therefore, introduced UW4 does not come to dominate in the field and are not a threat to indigenous ecosystem.

Molecular methods such as qPCR are increasingly used for rapid and sensitive quantification of microorganisms with specific functional genes from the environmental samples (Furukawa et al. 2006; Huijsdens et al. 2002; Khan and Yadav 2004; Nadkarni et al. 2002a; Sun et al. 2004). This study describes the development of UW4 specific primers and fluorescence dual labeled probe targeting ACC deaminase genes of UW4. The method of nested plus qPCR was designed for detection and quantification of UW4 strains in field soils. This may be the first report of a qPCR detection method for UW4 by targeting its ACC deaminase gene, as well as the first report of using nested plus qPCR method to quantify specific bacteria from environmental samples. Nested PCR increases the detection specificity and sensitivity by first eliminating unspecific DNA from the complex mixture of soil DNA and amplifying the specific target sequence. The detection sensitivity was increased from 40 $\times 10^6$ CFU of UW4 per gram dry soil for regular qPCR to 40 CFU for nested plus qPCR method (Fig. 7.5). This sort of approach will be especially useful for the detection and quantification of scarce or rare microorganisms in the environment. The method report here is valuable for monitoring introduced UW4 population in field during phytoremediation.

In conclusion, several advantages make UW4 a promising PGPR in the phytoremediation of PHCs. They are the high plant growth promoting effect in field due to its high ACC deaminase

activity, the ability to colonize root surfaces and persist in soil, and the ability of quickly establish significant population which helps plant overcome the environmental stresses in the early susceptible seedling stage. The negligible number of UW4 relative to soil microbes in the late phytoremediation stage indicated UW4 application is safe to use in the environment. Together, these suggest that UW4 enhanced plant growth is an effective, environmentally responsible method of *in situ* phytoremediation strategy.

Chapter 8 General conclusions

Petroleum hydrocarbons (PHCs) including PAHs are a class of ubiquitous contaminants that are of concern due to their inherent toxicity, widespread distribution and high persistence in the environment. This concern is very high in areas sourrounding petroleum production and processing facilities. Thus, research on PHC toxicity and mechanisms of toxicity as well as development of remediation technologies are critical priorities. This thesis characterized the mechanism of one prevalent oxyPAH (PHQ) at the whole organism level and explored a biological method to remediate PHC contaminated sites.

In this research, I found that a key mechanism of toxicity for PHQ to *V. fischeri* is through generation of ROS. PHQ can redox cycle in bacteria, transfering electrons to O_2, enhancing the production of superoxide (O_2^-), hydrogen peroxide (H_2O_2), and other ROS. Furthermore, the mixture of PHQ and Cu or Cd also produce ROS and also is the likely a key mechanism of toxicity. .

Successful remediation of a petroleum land fram site was achieved by utilizing a plant growth promoting rhizobacterium (PGPR) enhanced phytoremediation (PEP) system. Significantly greater numbers of added PGPR were observed in the soils where PEP was applied. This system promoted plant growth, especially in roots, resulting in a very vigorous and extensive root system. PEP consequently created a very active rhizosphere that showed an enriching effect on indigenous microbes including PHC degraders. These microbes, in turn, can accelerate PHC degradation. The enhanced populations and activities of soil microbes due to vigorous root growth have been shown to be a key factor in the success of PEP. While the application of the PEP did increase the overall titer of soil microbes, PEP only modestly affected

bacterial community structures during plant growth. Thus, PEP is an effective and environmentally safe strategy for removing PHCs from petroleum impacted soils.

Phytoremediation clearly enhanced the microbial titers with altering microbial community structure. It would be interesting to know the dominant microbes or functional genes induced by the phytoremediation process. Through examining the abundance of specific functional genes, such as genes encoding the enzymes responsible for the PHC degradation, the mechanisms of phytormdiation may be further revealed and better remediation strategies could be designed.

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