The Interactions Between Xenobiotics And Soil Microbial Communities

by

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Dedication

I would like to dedicate my thesis to my beloved parents, Ashwani K Rayu and Vijayanti Matas, who have taught me to work hard for the things that I aspire to achieve. Thank you for your support along the way.

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Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. It contains no material previously published or written by another person. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institute.

Signed

Smriti Rayu

Date

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APPENDIX

List of Abbreviations

%- Percent

µ- Micro

µl- Microliter

µM- Micrometer

^{12}C-DNA- Unlabelled DNA

^{13}C- Labelled carbon

^{13}C-DNA- Labelled DNA

$^{13}CH_4$- Labelled Methane

16S rRNA- 16S ribosomal ribonucleic acid

AChE- Acetylcholine esterase

ANOSIM- Analysis of similarities

ANOVA-Analysis of variance

APVMA- Australian Pesticides and Veterinary Medicines Authority

ATP- Adenosine triphosphate

BI- Botany Industrial Park

BLAST- Basic local alignment search tool

bp- Base pair

BSA- Bovine serum albumin

C- Carbon

CH_4- Methane

ChE- Cholinesterase

CHN analyser- Carbon, Hydrogen, Nitrogen analyser

CO_2- Carbon dioxide

CP- Chlorpyrifos

CsCl- Cesium Chloride

DCE- Dichloroethylene

DGGE- Denaturing Gradient Gel Electrophoresis

Diss/SD- Dissimilarity/standard

DNA- Deoxyribonucleic acid

DNA-SIP- Deoxyribonucleic acid stable isotope probing

dNTPs- Deoxynucleoside triphosphate

EtOH- Ethyl alcohol

FID- Flame Ionization Detector

g- Gram

GC- Gas chromatograph

GTA- Gene transfer agents

HPLC- High-performance liquid chromatography

IC- Imidacloprid

k- Degradation rate constant

K2+G- Kimura-2-parameter model with Gamma distribution

kg- Kilogram

kg/ha- kilogram per hectare

LB agar- Luria broth agar

MDS analysis- Multidimensional scaling analysis

MEGA- Molecular Evolutionary Genetics Analysis

ml- Milliliter

mm- Millimeter

mM- Millimolar

MMO- Methane monooxygenase

MOPS- 3-(N-morpholino)propanesulfonic acid

MQ- Milli-Q

MSM- Mineral salt medium

N- Nitrogen

nACh- Nicotinic acethylcholine

NCBI- National Center for Biotechnology Information

nm- Nanometer

NMS- Nitrate mineral salt

^{o}C- Degree Celsius

OD_{600}- Optical density

OP- Organophosphorus

OTUs- Operational taxonomic units

OTUs- Operational Taxonomic Units

P- Phosphorus

PAHs- Poly aromatic hydrocarbons

PCB- Polychlorinated biphenyl

PCR- Polymerase chain reaction

PERMANOVA- Permutational multivariate ANOVA

pH- Potential of hydrogen

PLFA- Phospholipid fatty acid analysis

PLFA-SIP- phospholipid fatty acids stable isotope probing

pMMO- particulate MMO

pmoA- gene for alpha subunit of the pMMO

pmol- Picomoles

QC- Quality control

q-PCR- Quantitative polymerase chain reaction

RT – Room temperature

SEM- Soil extract medium

SIMPER- Similarity percentage

SIP- Stable isotope probing

SIR- Substrate induced respiration

sMMO- soluble MMO

SOM- Soil organic matter

STAMP- Statistical Analysis of Metagenomic Profiles

SU- Sydney University

$t_{1/2}$- Half-life

TBP- 2,4,6-tribromophenol

TCE- Trichloroethene

TCP- 3,5,6-trichloro-2-pyridinol

T-RFLP- Terminal Restriction Fragment Length Polymorphism

T-RFs- Terminal restriction fragments

US EPA- US Environmental Protection Agency

VP- Victoria Park

Abstract

Excessive use and lack of appropriate disposal technology for industrial xenobiotics have resulted in the contamination of ecosystems globally impacting the self-regulating capacity of the biosphere. This often results in irreversible alterations of ecosystem's structure and function, but the outcomes of these events on soil microbial communities (and their functional capabilities) are poorly understood. Assessing the impact of xenobiotics on soil microbial communities is of paramount importance as they play a vital role in ecosystem services and maintain soil health, which are key requirements for sustainable land use in terms of food security and environmental sustainability. Bacteria are the most abundant and diverse soil micro-flora and play a key role in the biogeochemical cycles of important elements including carbon (C), nitrogen (N), and phosphorus (P) and sulphur (S). The current work aimed to unravel the two-way interactions between xenobiotics and soil microbial communities; *i.e.*, how soil microbial communities modulate xenobiotic persistence through biodegradation and what impacts xenobiotic have on soil microbial community's structure and functions, with particular focus on widely used pesticides (chlorpyrifos and imidacloprid) and industrial solvents (trichloroethene)

In **Chapter 2**, characterisation of active methanotrophs involved in trichloroethene (TCE) degradation under different methane (CH_4) concentrations was evaluated. Methane (CH_4) enriched methanotrophic consortia from three Australian soils (Sydney University, Victoria Park and Botany Industrial Park) were examined for their effectiveness in TCE (50µM) degradation at 1%, 10% and 33% CH_4 concentration at 20^{o}C. Only the methanotrophic consortium from Sydney University (SU) soil was able to co-metabolically degrade TCE. The (SU) methanotrophic

growth and TCE degradation was accelerated under high CH_4 concentration degrading up to 30% (within 2 days) and 20% (within 5 days) TCE under 33% and 10% CH_4, respectively. No degradation of TCE was observed at 1% CH_4 concentration or in the absence of CH_4 suggesting the dependence on relatively high CH_4 availability for TCE degradation. *pmoA*-based stable isotope probing (SIP), terminal restriction fragment length polymorphism (T-RFLP), clone library construction and sequencing of TCE degrading SU methanotroph consortium revealed the dominance of novel uncultivable Type I methanotrophs (distantly related to *Methylovulum*-88%) belonging to TRF-53 in TCE degradation.

In **Chapter 3**, the effects of the pesticides chlorpyrifos (CP) and imidacloprid (IC) on soil microbial processes (*e.g*. biodegradation and respiration) and community structure were evaluated. Two soil treatments (from five sugarcane farms), one with no history of pesticide application (non-treated; 1H, 2H, 3H, 4H and 5H) and the other with ~20 years pesticide application (pesticide-treated; 1R, 2R, 3R, 4R and 5R), were used in this study. MicroResp^{TM}, q-PCR and T-RFLP analyses were combined to explore the relationship between pesticide degradation and soil microbial communities in soils spiked (3 times) with 10 mg/kg of CP or IC, under lab conditions. The results showed that the half-lives of CP decreased with application frequency and were 23-47, 8-20 and 3-17 days following the first, second and third application, respectively (for soils from five sugarcane farms). In particular, the soils from 4R, 4H and 5R showed enhanced CP degradation even when not exposed to CP for last 13 years due to legacy effect of the pesticide. Parallel analyses of IC degradation (10 mg/kg) showed high persistence of this pesticide in soil where repeated application increased half-lives from 30-60 days for the first treatment to 45-65 days for second treatment. The application of both pesticides (CP and IC)

reduced soil respiration (basal and substrate-induced) between 7-76% with the lowest respiration found in 5R and highest in 1R after the pesticides treatment, indicating that application of pesticides had an adverse impact on soil functional activity. The molecular analyses showed that both CP and IC significantly altered the soil bacterial community structure and reduced diversity, evenness and richness.

In **Chapter 4**, sequential soil and liquid culture enrichments enabled the isolation of six bacterial CP degraders with sequence homologies to *Xanthomonas* sp. (3), *Pseudomonas* sp. (1), *Rhizobium* sp. (1) and *Lysobacter* sp. (1). The efficacy of the isolated strains: *Xanthomonas* sp. 4R3-M1, *Pseudomonas* sp. 4H1-M3 and *Rhizobium* sp. 4H1-M1 were further investigated for biodegradation of CP and its primary metabolic product, TCP (3,5,6-trichloro-2-pyridinol). The results indicated that all three bacterial strains utilised CP (10 mg/l) and TCP (as CP degradation product) in mineral salt media (MSM) as a sole source of C and N. Bacterial strains *Xanthomonas* sp. 4R3-M1 and *Pseudomonas* sp. 4H1-M3 could also degrade 10 mg/l TCP as a sole C- and N-source, when provided externally. Thus, these bacterial strains promise to be effective in practical application of bioremediation of both CP and TCP.

In **Chapter 5**, using next-generation sequencing, the structure and potential functions of bacterial communities in pesticide-treated and non-treated reference sites was compared at finer levels. Across all soils, the functional beta diversity was correlated with taxonomic diversity indicating possible linkages between the structure and functioning of soil microbial communities. The pesticide-treated sites had higher relative abundance of Proteobacteria and Bacteroidetes, with Archaea exhibiting the opposite pattern. Metagenomic analysis revealed increases in the relative abundance of genes associated with key specialised functions (iron acquisition and metabolism,

motility, cell signalling, stress response) at pesticide-treated sites. The results suggested impacts of long-term pesticide application on soil microbial community composition and potential functions. Despite, a CP legacy effect, no marked difference was observed in abundance of genes related to P-metabolism between pesticide-treated and non-treated sites. Overall, the results supported taxonomic and functional adaptations in the soil microbial communities following pesticide treatment.

Overall, this study provides the novel insights into the interaction between xenobiotics and soil microbial communities both at structural (diversity, community structure) and functional (degradation) levels and should be considered in developing new bioremediation technologies and agronomic practices such as number and frequency of pesticide applications.

Chapter 1: General Introduction

1.1 Xenobiotics and soil microbial communities

A vast number of pollutants are disposed into the environment every year due to anthropogenic activities and the number is still increasing rapidly. Industrial and agricultural activities can require the intensive use of organic (nitro-aromatic, organochlorine, organophosphorus), inorganic (*e.g.* arsenic, mercury) and radioactive compounds (*e.g.* uranium) (Ecobichon, 2001, Bleise *et al.*, 2003, Pirrone *et al.*, 2010). A combination of extensive use and indiscriminate release of xenobiotics of different chemical structure and complexity into the environment cause substantial ecosystem stress (Sen and Chakrabarti, 2009). The presence of the compounds that are resistance to degradation are of concern because of their toxicity and biomagnification *via* the food web (Davies *et al.*, 2006, Sharma *et al.*, 2009, Takeuchi *et al.*, 2009), and the need for decontamination of such compounds has been therefore highlighted (Singh and Naidu, 2012).

Soils are increasingly under environmental pressure from pollution and intensified agricultural management practices, leading to the biotic and abiotic stress on the soil microbial communities (Creamer *et al.*, 2010). Understanding microbial ecology is important because soil microorganisms play a critical role in soil functioning, soil health restoration, maintenance of basic resources for food production and terrestrial biodiversity maintenance. In the context of soil contamination, they play a crucial role in metabolising recalcitrant xenobiotic compounds (*e.g.* pesticides) and thus are essential in global processes contributing to the health and life cycles of plants and animals. In addition to this, microorganisms also play essential roles in

biogeochemical cycles (*e.g.* carbon and nitrogen) and any changes in their community composition or activities may affect the availability of essential nutrients, thus affecting soil functions (Gianfreda and Rao, 2008, Grenni, 2011). For example, soil microorganisms make a valuable contribution to maintaining soil fertility through their catabolic role in degradation of plant and animal residues, recycling of soil nutrients and breakdown of a number of organic and inorganic molecules.

When xenobiotics are released into soils they can interact with natural environment (abiotic) or living organisms (biotic) in two ways:

a) Transfer of xenobiotics without alteration of their structure, by runoff, diffusion, adsorption *etc.* Of these, adsorption/desorption mechanisms are critically important as this interaction depends on the chemical properties of the xenobiotic, strongly influencing the movement of xenobiotics, their availability for uptake and transformation by microbial and impact on other soil processes.
b) Degradation of xenobiotics through abiotic (physical, photochemical and chemical) and biological transformation (plants and microorganisms), *via* enzymes, where the chemical structure of the compound is transformed.

Xenobiotic-induced stress is thought to affect the dynamics of the soil microbes (genomic stability, physical distribution or community composition and diversity) by: a) an extinction response where the sensitive microbial population is eliminated from the system and b) development of the microbial communities with innate capabilities to resist pollution and for direct utilisation of the compound for energy and nutrients (biodegradation) (Giller *et al.*, 1998, Gianfreda and Rao, 2008). Particular attention in this thesis is given to organophosphorus (OP) pesticides (*e.g.*

chlorpyrifos (CP)), neonicotinoids (*e.g*. imidacloprid (IC)) and OC compounds (*e.g.* TCE) because they are among the most widely used classes of chemicals in agriculture and/or chemical manufacturing industries (Whitacre and Gunther, 2012). Pesticides enter the soil system mainly through their application in agricultural and horticultural systems for management of pests (for increasing productivity) and disease vector control (*e.g.* mosquitoes), wash-off manufacturing waste and accidental spillage into the soil. Similarly, organochlorine (OC) compounds, such as, trichloroethene (TCE), enter the soil from chemical manufacturing industries, oil refineries and industrial-waste effluents.

1.1.1 Organochlorine compounds (TCE)

Organochlorines are a class of organic compounds in which at least one chlorine atom is covalently bound to a tetrahedral carbon (or tetravalent carbon). The chlorine substituent induces stronger interactions than hydrogen, modifying the physical and chemical properties of organic compounds in several ways. The carbon-chlorine bond is very stable to hydrolysis and results in prolonged persistence (Rossberg *et al.*, 2006) and hence, these chemicals are problematic once they enter the environment (Bradley, 2003, Fetzner, 2010). Although this property differ among the OC compounds, some of them may exhibit an ability to resist degradation, associate with sediments or other solids, and to accumulate in the tissue of invertebrates, fish, and mammals (Imo *et al.*, 2013, Ahmed *et al.*, 2015). Chlorinated organic compounds can be grouped into three classes (Figure 1-1):

i) Aliphatic; for example, trichloroethene (TCE)

ii) Polycyclic; for example, dichlorodiphenyltrichloroethane (DDT)

iii) Aromatic; for example, pentachlorophenol (PCP).

Their wide structural variety and divergent chemical properties has led to their manufacture for diverse applications, including industrial, military, agricultural and medical as solvents, degreasers, insecticides, plasticisers, defoliants and coolants (Field and Sierra-Alvarez, 2004).

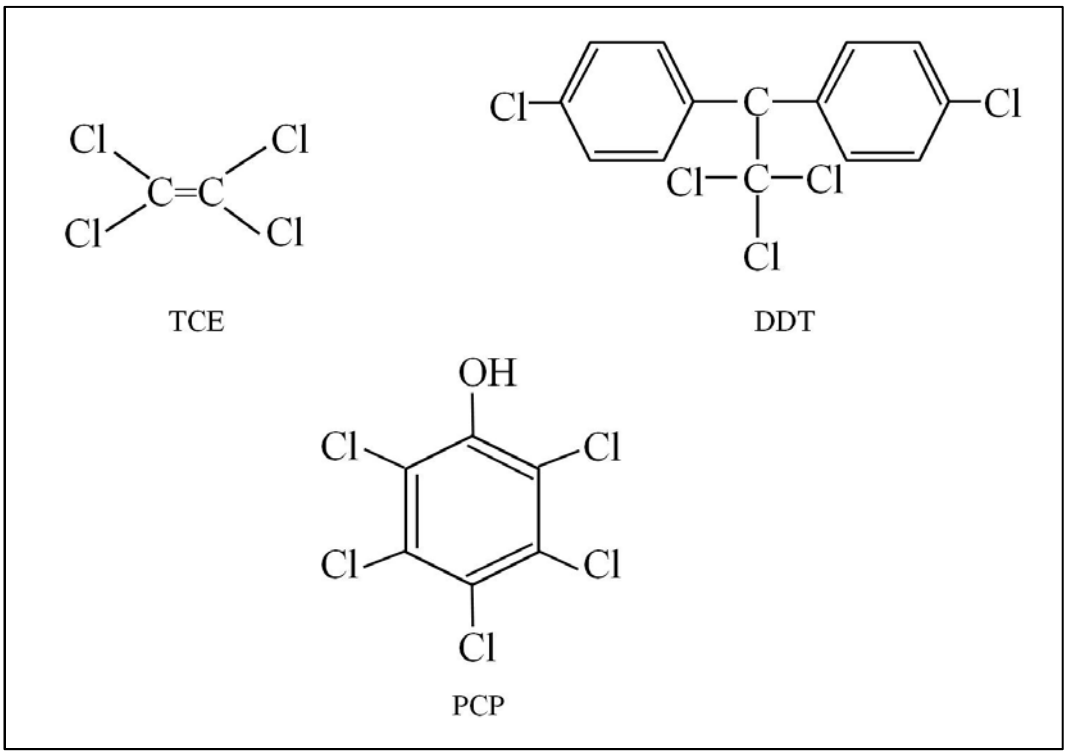


Figure 1-1 Chemical structure of a representative of the three classes of chlorinated organic compounds, where TCE, DDT and PCP belong to aliphatic, polycyclic class and aromatic classes, respectively. Abbreviations: TCE=trichloroethene, DDT= dichlorodiphenyltrichloroethane and PCP= pentachlorophenolate

Trichloroethene, a volatile, aliphatic chlorinated organic compound has two carbon (C) molecules joined by a double bond (ethenes) with three chlorine atoms (Figure 1.1). This compound has unique solvent properties making it suitable for various

industrial applications like dry cleaning, electroplating, printing, pulp and paper manufacturing, electronic industries and pharmaceutical chemical production (Ohlen *et al.*, 2005, Hazen *et al.*, 2009). The annual global consumption of TCE was $4.3x10^5$ metric tonnes versus global production capacity of $5.5x10^5$ metric tonnes in 2011 (www.dow.com). The physical and chemical properties of TCE are given in Table 1-1.

Table 1-1 Physical and chemical properties of TCE. Source: ATSDR (2014).

Properties	Value
Chemical formula	C_2HCl_3
Molecular weight	131.39 g/mole
Boiling point	87.2°C
Melting point	-84.7°C
Density at 20°C	1.4642 g/cm^3
Vapour pressure at 20°C	69 mm Hg at 25°C
Henry's law constant at 25°C	$9.85x10^{-3}$ atm-m^3 /mol
Solubility Water at 25°C Organic solvents	 1,280 mg/l Soluble in ethanol, diethyl ether, acetone, and chloroform

Trichloroethene is a toxic pollutant and enters into the biosphere through industrial effluents, disposal by consumers, emissions from landfills, leakage and accidental spills (Haest *et al.*, 2010). Owing to its environmental persistence and mobility , soil and groundwater contamination by TCE has become widespread (Riley and Zachara, 1992, Doucette *et al.*, 2007a). However, TCE also occurs naturally and is produced by some macro- and microalgae, making oceans a major source of TCE (Gribble, 2003). There is currently limited data available to estimate the production of TCE by the oceans which is correlated to temperature and seasonal variations (Yokouchi *et al.*, 1996, Abrahamsson *et al.*, 2003). On the other hand, according to the National

Pollutant Inventory (NPI, Australia) the anthropogenic release of TCE into the environment in Australia was 26 metric tonnes in 2012-2013 and therefore it is considered as one of the major pollutant emitted to the environment (http://www.npi.gov.au/).

The release of TCE to the environment poses a serious problem through soil and groundwater contamination where it remains persistent for decades. TCE has been identified in more than 700 hazardous waste sites (soil or groundwater) listed by the U.S. EPA (Chiu *et al.*, 2012). In addition to this, TCE is also known to have deleterious effects on food chains due to bioaccumulation and biomagnification (Rattner, 2009). An emerging concern is the uptake of TCE by tress grown in TCE contaminated area and the potential transfer into the plant edible tissue (Doucette *et al.*, 2007b).

Studies have shown that reproduction of aquatic invertebrates was adversely affected by TCE (Niederlehner *et al.*, 1998). The U.S. EPA has set the maximum permissible limit of TCE in the air and drinking water to 0.003 mg/m^3 and 0.005 mg/l, respectively. The Occupational Safety and Health Administration (OSHA) permissible exposure limit is a time-weighted average (TWA) of 100 ppm in two hour period. The epidemiologic studies have provided strong evidences that high TCE exposure increases the risk of many diseases (*e.g.* autoimmune disorder, systemic sclerosis and skin disorders) (Chiu *et al.*, 2013). In humans, high level TCE exposure is known as a central nervous system depressant (Bale *et al.*, 2011) and adversely affect the liver, kidney, immune-system and developing embryos (Bull, 2000, Lash *et al.*, 2000, Yauck *et al.*, 2004, Cooper *et al.*, 2009). Studies have also associated occupational TCE exposure to increased risk of liver and kidney cancer

(Rusyn *et al.*, 2014). Similarly, there is evidence that TCE can induce liver and lung tumours in mice (Maronpot, 2009)

1.1.2 Pesticides (chlorpyrifos and imidacloprid)

Pesticides are a diverse group of organic or inorganic synthetic chemicals that are purposely introduced and extensively used in agriculture as a part of control strategies to protect crops against weeds, insects, fungi and other pests and pathogens (Yang *et al.*, 2007). Pesticides are generally classified based on the type of organism they are designed to control and include herbicides, insecticides, nematicides, fungicides and soil fumigants (Imfeld and Vuilleumier, 2012). Each major group of pesticide can be further classified based on their structure (*e.g.* organochlorine and organophosphorus) and their biochemical targets (*e.g.* choline esterase inhibitors). Globally 4.6 million tons of chemical pesticides are annually sprayed into the environment (Zhang *et al.*, 2011b) with over 8000 pesticides registered for use in Australian agriculture alone (Immig, 2010). With the world's population expected to grow from 6.8 billion today to 9.1 billion by 2050 with limited croplands (Alexandratos and Bruinsma, 2012), further intensification of the use of pesticide to increase crop production in order to ensure food security is likely (Zhang et al., 2007, 2008c; Zhang, 2009). The use of pesticide is particularly high in countries with cash crops where pest pressures are high, including Australian sugarcane farms (total annual revenue of $2 billion).

Chlorpyrifos (CP) and imidacloprid (IC) have gained wide use in agriculture and currently there are 164 CP-containing and 37 IC-containing products registered in Australia (http://apvma.gov.au/). However, at present, there is no detailed and

publicly available information on Australian usage of individual pesticides. Between the years 2002-2006, the average annual global use of CP was 25 million kg active ingredients (Pengphol *et al.*, 2012). On the other hand, IC is gaining popularity due to its high insecticidal activity at low application rates, and comprises 41% of the market, becoming the second most widely used agrochemical (Goulson, 2013). The total consumption of pesticide world-wide is shown in Figure 1-2 (De *et al.*, 2014).

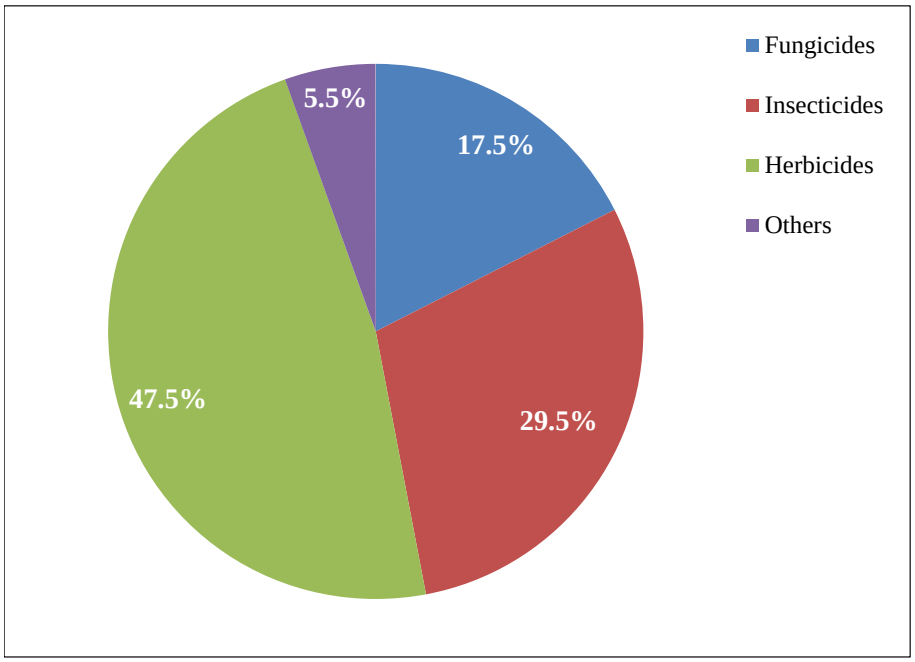


Figure 1-2 World-wide proportion of each class of pesticides used. Adapted from De *et al.* (2014)

Certainly, pesticides have improved crop production, but their potential to persist in the environment may cause adverse effects among different forms of life and ecosystems. Approximately 90% of agricultural pesticide application never reaches its target organisms with the remaining bulk contaminating Earth's ecosystems and affecting public health by entering the food chain (Carriger *et al.*, 2006). In addition to this, many pesticides are intentionally introduced into the soil system for the

control of soil borne pests and pathogen, which eventually results in the accumulation at unacceptably high levels (Redondo *et al.*, 1997, Singh, 2012). The fate of pesticides in the environment is influenced by many processes that determine their persistence and mobility. The interaction of pesticides with soil and/or water is complex and is usually controlled by various physio-chemical and biological factors.

Other concerns related to pesticide uses is run-off from the treated area into the water system (Larson *et al.*, 2010) and aerial volatilization causing atmospheric pollution (Armstrong *et al.*, 2013). Similar to TCE, studies have shown that pesticides affect the fertility and reproduction of aquatic invertebrates resulting in significant developmental changes (Boone *et al.*, 2007). Certain pesticides can accumulate in the tissue of the organisms (bioaccumulation) (Favari *et al.*, 2002) and the residues can travel up the food chain affecting non-targeted organisms in the ecosystem like earthworms (Pelosi *et al.*, 2014), pollinators (Gill *et al.*, 2012, Wu *et al.*, 2012) and birds (Guerrero *et al.*, 2012). In humans, some pesticides are known to be carcinogenic (Xu *et al.*, 2010, Band *et al.*, 2011, Cocco *et al.*, 2012), adversely affecting the nervous system (Hayden *et al.*, 2010), the cardiovascular system (Abdullah *et al.*, 2011), the respiratory system (Hoppin *et al.*, 2009) and the reproductive system impacting embryo development and causing birth defects (Greenlee *et al.*, 2003, Xavier *et al.*, 2004, Mesnage *et al.*, 2010). The severity of risk is normally associated with the chemical nature of the pesticide, level of toxicity, mode of action and level of exposure (Richter, 2002). Every year about three million acute pesticide poisoning are being reported worldwide resulting from accidental poisoning, occupational exposure, but also suicide attempts (Maiti *et al.*, 2011).

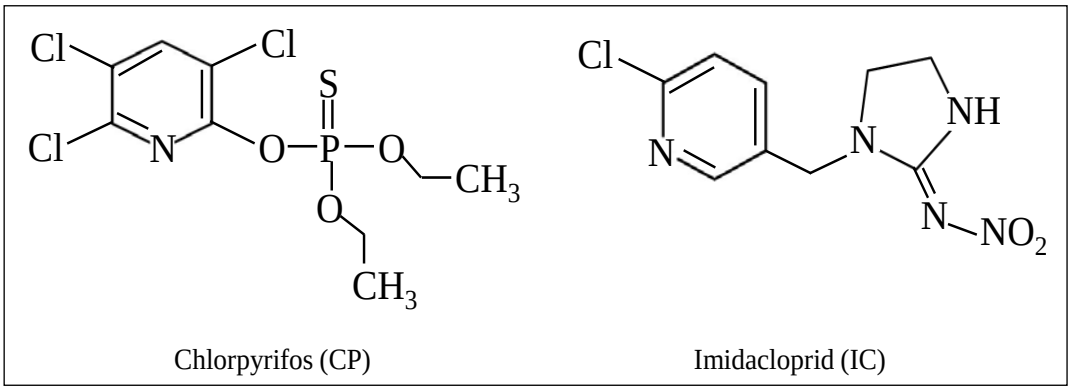
1.1.2.1 Chlorpyrifos (CP)

Chlorpyrifos [*O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate)], an OP pesticide is one of the most widely used pesticide in agriculture (Figure 1-3). The physical and chemical properties of CP are given in Table 1-2. Chlorpyrifos is a highly effective pesticide because it irreversibly inhibits acetylcholinesterase (AChE) enzymes leading to excess acetylcholine accumulation at nerve terminals resulting in agitation, hypersalivation, convulsion and ultimately death of insects and mammals (King and Aaron, 2015). Based on the results of animal testing, the U.S. EPA has set its health based standard for acceptable exposure to CP at 30 ng/kg of body weight (chronic) and 500 ng/kg of body weight (acute).

The Agriculture and Resource Management Council of Australia and New Zealand (ARMCANZ) have set a drinking water health guideline value of 0.01 mg/l. The National Occupational Health and Safety Commission (NOSCH) have set a permissible occupational exposure limit of 0.2 mg/m^3 (TWA) for an eight hour period. However, continuous and excessive use have shown to cause serious damage to humans that include developmental toxicity, liver damage, reproductive defects, endocrine disruptions, nervous system disorders and immune system abnormalities (Furlong *et al.*, 2006, Whyatt, 2011, Terry, 2012). Acute toxicity of CP has also been reported in other animals, aquatic invertebrates and fishes, arthropods and soil microorganisms (Michereff-Filho *et al.*, 2004, Palma *et al.*, 2008, Tuzmen *et al.*, 2008, Dutta *et al.*, 2010).

1.1.2.2 Imidacloprid (IC)

Imidacloprid 1-[(6-chloro-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimine, belongs to a relatively new class of synthetic organic pesticide (neonicotinoids, Figure 1-3), now widely used, that exhibit a novel mode of action by acting as an antagonist at the nicotinic acetylcholine receptors (nAChRs) leading to paralysis and death of pest organisms (Matsuda *et al.*, 2001, Matsuda *et al.*, 2009). Available data does not suggest that IC has either dermal or inhalation toxicity potential, therefore, there are no established health advisory levels for IC in drinking water or occupational/residential risk assessments (http://www.cdpr.ca.gov/). Although imidacloprid show low toxicity to some vertebrates, high exposures (300 mg/kg of body weight) have resulted in vomiting, disorientation, severe cardiac toxicity and chromosome abnormalities in humans (Agarwal and Srinivas, 2007, Demsia *et al.*, 2007). Imidacloprid is generally toxic to insects (*e.g.* termites) in minute quantities and the toxicity has been reported in various non-target organisms, for example, rats (140 mg/kg of body weight), birds (14 mg/kg of body weight), fish (16-177 ppm), pollinators (5 ng/insect) and soil microorganisms (Tišler *et al.*, 2009, Bhardwaj *et al.*, 2010, Cycoń *et al.*, 2013). A list of physical and chemical properties of IC is given in Table 1-2.



Chlorpyrifos (CP) Imidacloprid (IC)

Figure 1-3 Chemical structure of chlorpyrifos (CP) and imidacloprid (IC)

Table 1-2 Physical and chemical properties of chlorpyrifos (CP) and imidacloprid (IC). Source: http://pubchem.ncbi.nlm.nih.gov/

Properties	Chlorpyrifos	Imidacloprid
Molecular weight (g/mol)	350.57	255.70
Melting point (^{o}C)	42.00	144.00
Boiling point (^{o}C)	160.20	442.30
Density (g/cm^{3}) at 20^{o}C	1.44	1.54
Solubility in water (mg/l) at 20^{o}C	0.70	610.00
Partition co-efficient (log K_{ow})	4.82	0.57
Dissociation constant		
pK_a1	non-dissociative in nature	1.56
pK_a2	non-dissociative in nature	11.12

1.2 Influence of xenobiotics on soil microbial communities

Soil microorganisms are the key players of many soil functions such as nutrient cycling, organic matter decomposition, maintaining soil health and fertility (Wainwright, 1978, Lo, 2010). Xenobiotics may interact with the soil and its indigenous microorganisms, altering microbial diversity and community composition, and through this influence biochemical reactions and enzymatic activity

(Hussain *et al.*, 2009, Muñoz-Leoz *et al.*, 2011) and ultimately ecosystem functions and services; hence, it is important to elucidate the influence of xenobiotics on soil microbes to preserve soil's vital functions. Co-metabolism of an organochlorine, TCE (and toluene), in a lab-based study, was shown to cause a shift in microbial community composition where total culturable heterotrophs increased 500-fold during incubation with toluene and TCE, but only 3-fold during incubation with TCE alone (Hubert *et al.*, 2005). In a field based study, TCE (and phenol) has also been reported to alter the microbial community structure by increasing the population of *Variovorax* and *Burkholderia* in TCE-contaminated groundwater (Humphries *et al.*, 2005).

On the other hand, the impacts of pesticides on soil microbial communities (population) have been investigated in many studies with negative, positive and no effects observed (Jjemba, 2002, Pandey and Singh, 2004, Singh and Singh, 2005a). In one study, the application of exogenous endosulfan was reported to increase the bacterial biomass by 76% and reduce the fungal biomass by 47% (Xie *et al.*, 2011a). A recent study showed no effect of fenamiphos on urease and dehydrogenase activities (in Australian soils); however, soil microbial potential nitrification was highly sensitive to fenamiphos addition (10 mg/kg) clearly indicating a risk to soil health (Cáceres *et al.*, 2008). In addition to this, long-term application of pesticides, *e.g.* methyl parathion have also been shown to cause a loss of soil microbial diversity (Zhang *et al.*, 2006b). Inconsistent effects of pesticides on soil microbial communities were observed when the studies were conducted using repeated applications of one or more pesticides with different chemical properties (Gundi *et al.*, 2005, Wang *et al.*, 2007a, Chu *et al.*, 2008).

Intensive use of CP in agro-ecosystems has resulted in several studies of the effects of CP on soil microbial communities. The half-life of CP is generally between 10 and 120 days in soil but can be up to one year depending on soil types and climatic conditions (Singh and Walker, 2006). Persistence of CP residues in soils may affect the soil microbial community functions and stability (Kulkarni *et al.*, 2000). Microbial biomass, an important indicator of microbial activities and other ecological processes (Schultz and Urban, 2008) has been reported to respond negatively following CP treatment at a concentration of 10 mg/kg, the lowest dose used for agricultural purposes (Shan *et al.*, 2006, Vischetti *et al.*, 2008). In one of the studies it was found that the application of CP at concentrations from 10-300 µg/g in an agricultural loam significantly decreased aerobic dinitrogen fixing bacteria (*Azotobacter* sp.); however, total viable counts of fungi and denitrifying bacteria were not affected at this concentration (Martinez-Toledo *et al.*, 1992).

The application of CP has also been shown to affect the soil microbial functional diversity causing a significant decrease by 18.2%, 30.5% and 27.7% in soils treated with CP at concentration of 4, 8 and 12 mg/kg, respectively (Fang *et al.*, 2009). In a similar study, the diversity index of bacterial community significantly decreased when the soil was treated with 20 mg/kg of CP (Chen *et al.*, 2014). The impact of repeated application of CP has also been reported to decrease the soil microbial activity (Fang *et al.*, 2008). However, in all these cases, CP had a temporary or short-term inhibition effect on soil microbial communities and the diversity recovered gradually at the later stages of the experiment. While these studies showed an adverse effect of CP on soil microbial communities, some other studies have shown little or no effect of CP alone or in combination with other pesticides on soil microbial parameters (enzyme activity and total microbial biomass) (Singh *et al.*,

2002a, Singh *et al.*, 2002b). In addition to this, CP application has been shown to alter microbial activity (Wang *et al.*, 2010a), which in turn reduce the availability of nitrogen (N) and phosphorus (P) content in soils (Sardar and Kole, 2005), and reduction in plant productivity. A study by Menon *et al.* (2005) has also reported significant negative impacts of CP on N mineralisation in loamy sand and sandy loam soils.

While several studies have shown that CP impact soil microbial activities, very little is known about the environmental fate of IC and their impact on soil microbial community structure and function. Half-lives of this pesticide in soils appear to range from 40 days in unamended soil to 124 days in soils recently amended with organic fertilizers, and for the most common seed treatments, the half-life reported range from 28 to 1250 days in soils (Rouchaud *et al.*, 1994, Goulson, 2013).

Recently, studies have started to assess the effects of IC on soil microbial composition and activities (Cycoń *et al.*, 2013, Cycoń and Piotrowska-Seget, 2015a, Cycoń and Piotrowska-Seget, 2015b). These studies found that IC did not affect microbial soil functioning when applied at field rate (1 mg/kg). However, at the concentration of 10 mg/kg, IC induced significant changes in community composition. It is expected that the repeated applications of IC would lead to their accumulation in soils in successive years (Goulson, 2013), eventually altering microbial soil functioning. At high concentrations IC (10 mg/kg soil) had a negative effect on total soil microbial biomass and metabolic enzymatic activities and functions (respiration, phosphatase and urease). In addition to this, IC was also reported to negatively affect soil nitrification rates by altering the community structure of ammonia oxidising microorganisms, thus impacting the global N-cycle and compromising on the soil quality (Cycoń and Piotrowska-Seget, 2015b).

In other studies, IC has also been found to have short-term or no effects on soil enzymatic activities (urease, dehydrogenase and alkaline phosphomonoesterase) in groundnut fields and turfgrass management, respectively (Ingram *et al.*, 2005, Singh and Singh, 2005b); however, IC application had adverse effects on fungi and actinomycetes population composition at field rates of 2.8 g active ingredient/kg seed in another study (Singh and Singh, 2005a). Likewise, Tu (1995) reported inhibitory effect of IC on fungal population at 10µg active ingredient per gram of soil. In general, xenobiotics (OC and pesticides) have a negative impact on soil microbial populations and activities but the effect is moderated by environmental factors, such as soil properties, as well as the persistence, toxicity and concentration of the applied xenobiotics, and its bioavailability to the soil microorganisms (Hussain *et al.*, 2009).

1.3 Transformation of xenobiotics by soil microbial communities

Microorganisms are ubiquitous in soil, with a large biomass ranging from 2-806 g C m^{-2} (Curtis *et al.*, 2002, Fierer *et al.*, 2009), and represent a substantial proportion of Earth's diversity. For example, one gram of soil contains upto 10 billion microbial cells, representing more than 10,000 genomes (Torsvik and Øvreås, 2002). They possess highly specialized metabolic systems and catalytic mechanisms (Chen *et al.*, 1999, Paul *et al.*, 2005) to degrade and utilise various toxic compounds. These compounds can be used as a source of energy for their growth *via* aerobic respiration, anaerobic respiration, anoxic processes, fermentation, and co-metabolism in normal as well as more extreme conditions (Mishra and Lal, 2001, Watanabe, 2001). Biodegradation of xenobiotics can be broadly categorised into aerobic and anaerobic. In aerobic biodegradation, microorganisms use oxygen as the final electron acceptor in the degradation process. Anaerobic degradation, on the other hand, occurs in the

absence of oxygen. Under these conditions manganese and iron ions, and elements like sulphur, sulphate, nitrate and carbon dioxide, may function as an electron acceptor in biotransformation (Bosma *et al.*, 2001).

Xenobiotic compounds can be degraded in the environment as part of the natural biogeochemical processes. Growth-linked degradation processes occur when micro-organisms degrade organic compounds for energy and growth. Organochlorine compounds, however, tend to be resistant to biodegradation due to the presence of the highly electronegative halogen substituent, which provide stability to the molecule. Some of these persistent organic compounds are mainly transformed by a mechanism known as co-metabolism. Co-metabolism is the process in which a microorganism transforms a substance, without being able to utilise the energy derived from this transformation, in the obligate presence of a growth substrate. Figure 1-4 summarises different biodegradation approaches undertaken by the microorganisms (in the presence and absence of oxygen) (Singh *et al.*, 2009).

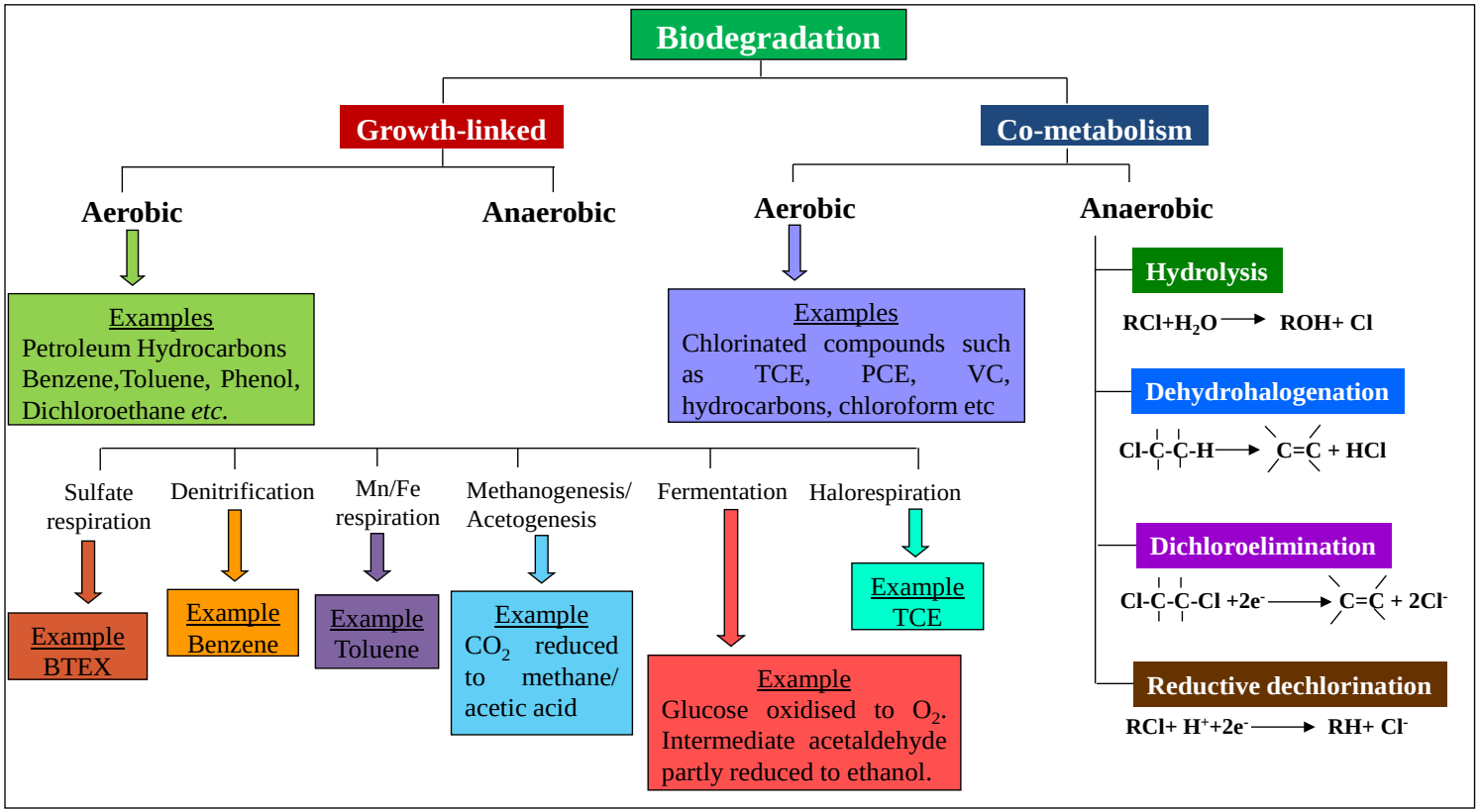


Figure 1-4 Flow-diagram outlining the main degradation mechanisms in microbe-assisted biodegradation.

Anthropogenic compounds can be degraded in the environment as part of the natural biogeochemical processes. *Growth-linked* degradation occurs when micro-organisms degrade organic compounds as a source of energy. Organochlorine compounds, however, tend to be resistant to biodegradation due to the presence of the highly electronegative halogen substituent, which provide stability to the molecule. Persistent organic compounds are, however, transformed by a mechanism known as co-metabolism. *Co-metabolism* is the process in which a microorganism transforms a substance, without being able to utilise the energy derived from this transformation, in the obligate presence of growth substrate.

Microbial degradation has proved to be versatile as it offers a natural, inexpensive and environmentally friendly strategy to detoxify xenobiotics like TCE and pesticides (Kosaric, 2001, Furukawa, 2003, Finley *et al.*, 2010). Most of the research regrading biodegradation of pesticide and TCE have primarily focused on bacteria because bacteria are easy to culture, generally grow faster than most of the other microbes and are more susceptible to genetic modification (Ortiz-Hernández *et al.*, 2011). Bacteria are considered to produce enzymes such as oxygenases, transferases, isomerases, hydrolases and ligases that efficiently catalyse the biodegradation of pesticides (Singh and Singh, 2005b, Sharma *et al.*, 2006, Pizzul *et al.*, 2009, Van Eerd *et al.*, 2009, Semrau *et al.*, 2010). Here we will focus on microbial degradation (transformation) of the two most widely used xenobiotics; OC compounds (TCE) and pesticides (CP and IC)

1.3.1 Biological transformation of organochlorine compound (TCE)

Concern about the environment and human health hazards have led to the development of new approaches for TCE remediation. Several physico-chemical remedial methods like air stripping (Rabideau *et al.*, 1999), active carbon adsorption (Miyake *et al.*, 2003), advanced oxidation (Yuan *et al.*, 2012) and the pump and treat process (Bazzazieh, 1996) have been developed and implemented for TCE decontamination but are relatively slow, costly and inefficient (Bankston *et al.*, 2002). By contrast, biological treatment or bioremediation has emerged as an effective tool because of its low cost and its potential for complete degradation of pollutant (Arp *et al.*, 2001). For example, biological remediation treatment generally costs between $7-250/tonne soils when compared to other physio-chemical

treatments ($20-700/tonne soils) (Juwarkar *et al.*, 2010). Biodegradation of TCE can take place under both aerobic and anaerobic conditions through different pathways.

1.3.1.1 Anaerobic degradation

The process of removing chlorines under anaerobic conditions, generally occurs through reductive dechlorination (Holliger *et al.*, 1998, Smidt and de Vos, 2004) where the chlorinated compound is used as an electron acceptor and dechlorinated stepwise (Figure 1-5). The reductive dechlorination of TCE primarily occurs through two processes: a) co-metabolism and b) halorespiration, where the chlorinated compound (TCE) is metabolised to ethylene through 1,2-dichloroethene (*cis*-1,2-DCE or *trans*-1,2-DCE) and vinyl chloride (VC) as intermediates (Mohn and Tiedje, 1992, Wohlfarth and Diekert, 1997, Ferguson and Pietari, 2000, Wang and Tseng, 2009, Tiehm and Schmidt, 2011). While co-metabolic dechlorination is a non-specific side reaction, halorespiration is a specific enzymatic reaction in which a chlorinated compound is used as a terminal electron acceptor for generation of metabolic energy (Middeldorp *et al.*, 1999). In recent times, biochemical basis for the halorespiration is becoming better understood with the purification of dehalogenases (Neumann *et al.*, 1995, Magnuson *et al.*, 1998, Holliger *et al.*, 2003) and it seems to be a major mechanism in the natural attenuation of TCE at many contaminated sites (Yong and Mulligan, 2003, Tiehm *et al.*, 2007). However, a serious concern of the application of anaerobic TCE degradation is the incomplete dechlorination of the compound resulting in the accumulation of undesirable by-products, for example VC, which are more toxic and carcinogenic than the parent compound (Carter and Jewell, 1993, Anguish and Maymo-Gatell, 1999, Byl and

Williams, 2000, Griffin *et al.*, 2004). Therefore, anaerobic degradation cannot be relied upon as a single treatment system for the complete dechlorination of chlorinated compounds in contaminated environments.

1.3.1.2 Aerobic degradation

Aerobic degradation of chlorinated compounds, like TCE, has been widely examined as an alternative to the anaerobic degradation because it is much faster and does not favour the production of undesirable metabolites, like VC. Aerobic degradation involves an oxygenation reaction catalysed by mono- or dioxygenase enzymes to yield epoxides or dihydrodiols of the chlorinated compound (Lee *et al.*, 2006, Van Beilen and Funhoff, 2007, Lee *et al.*, 2008). The heavily chlorinated compound, TCE, is considered resistant to biodegradation and co-metabolism is the only aerobic mode of degradation in which the microorganisms degrade the compound without gaining any energy or growth benefit (Ensley, 1991, Ryoo *et al.*, 2000, Futamata *et al.*, 2001). There have been several reports on co-metabolism of chloroethenes by bacterial communities using another primary substrate such as methane (CH_4) (Lee *et al.*, 2006, Shukla *et al.*, 2009), ethene (Coleman *et al.*, 2010, Le and Coleman, 2011), propane (Chang and Alvarez-Cohen, 1995, Frascari *et al.*, 2008), toluene (Cox *et al.*, 1998, Yeager *et al.*, 2004), butane (Kim *et al.*, 2000, Halsey *et al.*, 2007), ammonia (Yang *et al.*, 1999, Kocamemi and Cecen, 2010), methanol (Lee *et al.*, 2003, Shukla *et al.*, 2010a) or phenol (Bielefeldt and Stensel, 1999, Futamata *et al.*, 2005) to induce specific oxygenase for degradation. Although various microorganisms have shown promising results in co-metabolizing TCE, CH_4-assisted degradation of TCE by methanotrophs are of interest because they are non-pathogenic and exist in a

variety of habitats. They degrade a broad spectrum of highly toxic organic compounds and have higher TCE oxidation rates than other degraders. A list of microorganisms involved in aerobic and anaerobic degradation of TCE is given in Table 1-3

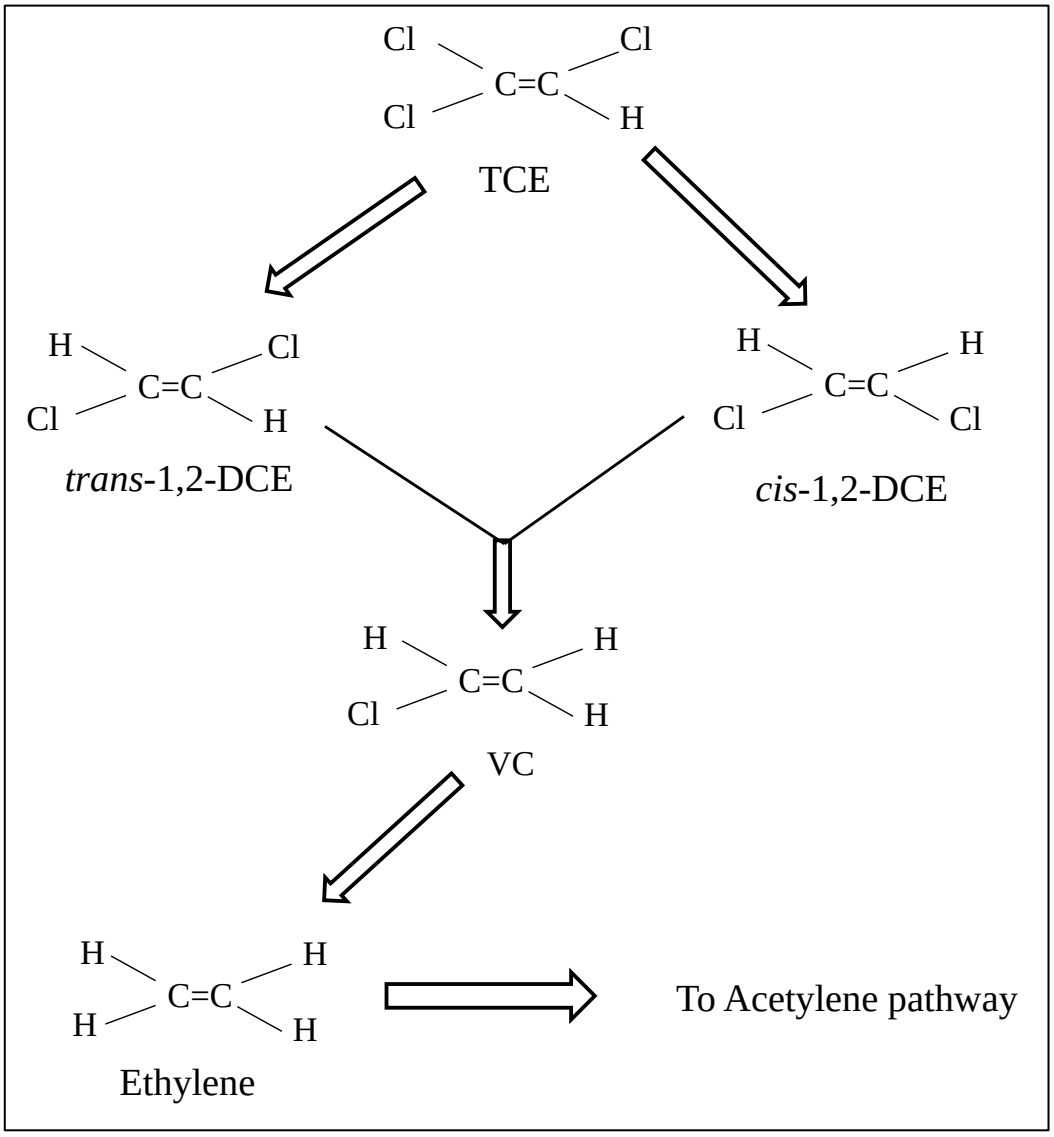


Figure 1-5 Anaerobic degradation pathways for trichloroethylene. Modified from Lorah and Olsen (1999) Anguish and Maymo-Gatell (1999) and Pant and Pant (2010). Abbreviations: TCE=trichloroethene, DCE= dichloroethene and VC=vinyl chloride.

Table 1-3 List of microorganisms involved in aerobic and anaerobic degradation of TCE

Microorganisms	Mode of degradation	Enzymes	References
Dehalospirullum sp.	Anaerobic	Dehalogenase	(Miller *et al.*, 1996)
Desulfuromonas sp.	Anaerobic	Dehalogenase	(Krumholz, 1997)
Sulfurospirillum sp.	Anaerobic	Dehalogenase	(Luijten *et al.*, 2003)
Desulfitobacterium sp.	Anaerobic	Dehalogenase	(Furukawa *et al.*, 2005)
Clostridium sp.	Anaerobic	Dehalogenase	(Kim *et al.*, 2006)
Dehalococcoides sp.	Anaerobic	Dehalogenase	(Zhang *et al.*, 2011c)
Propionibacterium sp.	Anaerobic	Dehalogenase	(Chang *et al.*, 2011)
Nitrosomonas sp.	Aerobic	Ammonia monooxygenase	(Arciero *et al.*, 1989)
Pseudomonas sp.	Aerobic	Toluene mono-dioxygenase	(Clingenpeel *et al.*, 2012)
Mycobacterium sp.	Aerobic	Propane monooxygenase	(Wackett *et al.*, 1989)
Diazotrophs	Aerobic	Nitrogenase	(Shukla *et al.*, 2010b)

1.3.1.3 Methanotrophic degradation of TCE (co-metabolism)

Methanotrophs or CH_4 oxidising Gram-negative bacteria (MOB) are a subset of a physiological group of bacteria known as methylotrophic bacteria (Hanson and Hanson, 1996) that utilise CH_4 for energy and a C-source (Semrau *et al.*, 2010) (Figure 1-6). All methanotrophs belong to the phyla Alpha- and Gamma - *proteobacteria* and oxidise CH_4 to carbon dioxide. Methanotrophs have been classified into three major groups based on morphological differences, their pathway of C assimilation, intra-cytoplamic membrane (ICM) structure and other physiological characteristics (Whittenbury *et al.*, 1970, Whittenbury and Dalton, 1981, Hanson and Hanson, 1996, Jiang *et al.*, 2010, Semrau *et al.*, 2010).

Gamma-proteobacteria or Type I methanotrophs utilise a ribulose monophosphate pathway (RuMP) for C-assimilation, has predominant 16-C phospholipid fatty acids (PLFAs) and the bundles of vesicular discs of membrane are distributed throughout the organism. *Alpha-proteobacteria* or Type II possess serine pathway for C-assimilation, contain a high level of 18-C PLFAs and paired membranes around the periphery of the cell. On the other hand, the genus *Methylococcus* and *Methylocadum* possess the characteristics of both Type I and Type II and belonged to Type X methanotrophs, but are now reclassified as a subset of Type I methanotrophs (Bowman *et al.*, 1993b). Further-more, filamentous methanotrophs have been discovered within the genera *Clonothrix* and *Crenothrix* (Stoecker *et al.*, 2006, Vigliotta *et al.*, 2007) but these are considered to be Type I methanotrophs. Another recently discovered acidophilic methanotroph of the *Verrucomicrobia* phylum represents a unique group of methanotrophs (Dunfield *et al.*, 2007, Islam *et al.*, 2008, Semrau *et al.*, 2008), although it shares many characteristics Type II methanotrophs. The general characteristics of the different types of methanotrophs are given in Table 1-4.

The biodegradation of TCE by methanotrophs was first demonstrated in 1985 (Wilson and Wilson, 1985) using a sand column inoculated with methanotrophs. Since then significant advancement has been made in the application of methanotrophic bacteria in bioremediation of TCE (Erwin *et al.*, 2005, Wymore *et al.*, 2007, Yu, 2008). Interest in methanotrophic bacteria as biocatalysts for synthetic chemistry and bioremediation stems almost exclusively from the unique catalytic properties of the two CH_4 monooxygenase (MMO) systems, most importantly their ability (a) to oxidize CH_4 to methanol and (b) to co-oxidize a wide range of organochlorine substrates (like TCE), where the enzyme induces the formation of

epoxides that are extremely unstable and spontaneously breaks down to simpler compounds (Figure 1-7). Methanotrophs possess two forms of MMO: the cytoplasmic soluble form (sMMO) and membrane bound particulate form (pMMO) (Burrows *et al.*, 1984, Murrell *et al.*, 2000b, Shukla *et al.*, 2014). All methanotrophs express pMMO, except for *Methylocella* and *Methyloferula* sp. (Dedysh *et al.*, 2005, Vorobev *et al.*, 2011); while sMMO is expressed mainly by Type II and Type X methanotrophs (Hanson and Hanson, 1996, McDonald *et al.*, 2006) in the absence of copper (Koh *et al.*, 1993, Nielsen *et al.*, 1997, Grosse *et al.*, 1999, Semrau *et al.*, 2010).

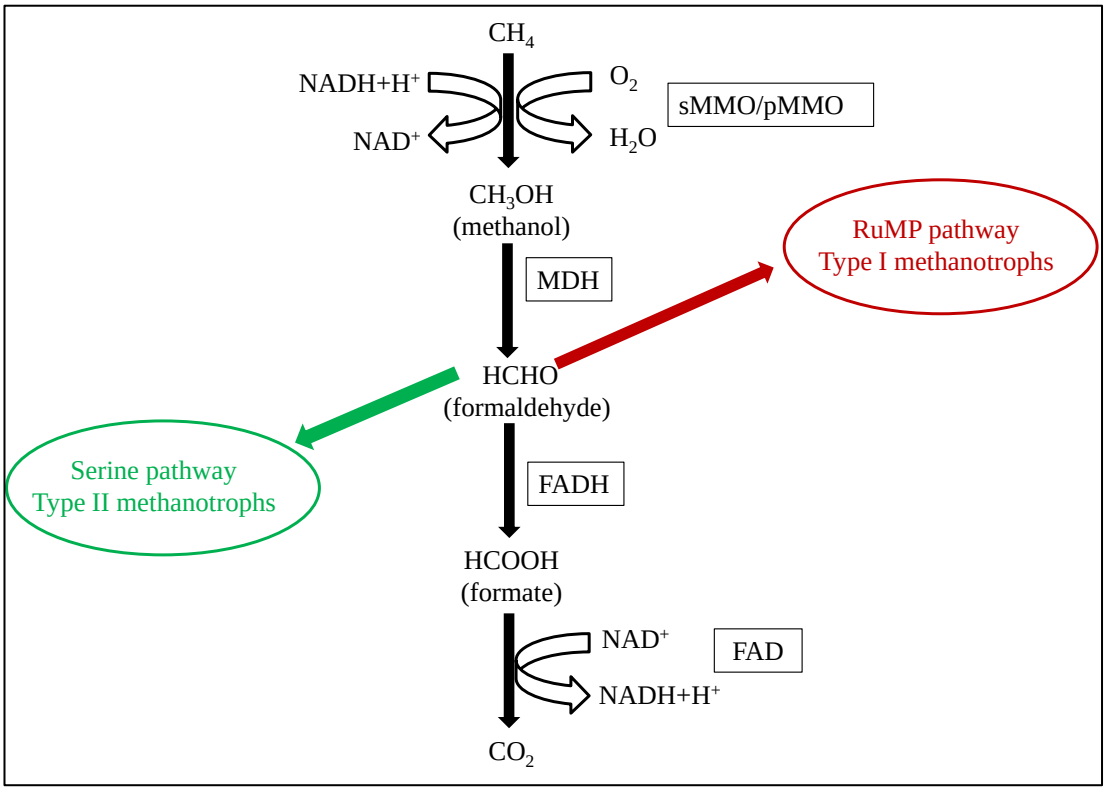


Figure 1-6 Aerobic oxidation of methane. Abbreviations: FAD- formate dehydrogenase, FADH- formaldehyde dehydrogenase, MDH- methanol dehydrogenase and MMO-methane monooxygenase (s=soluble, p=particulate). Adapted from Hanson and Hanson (1996)

Table 1-4 General characteristics of aerobic methanotrophic bacteria. Adapted from Semrau *et al.* (2010)

Characteristics	*Gamma-proteobacteria*[+] (Type I)	*Alpha-propteobacteria* (Type II)	*Verrucomicrobia*
Genera	*Methylobacter, Methylococcus*, Methylocadum*, Methylohalobius, Methylomicrobium, Methylomonas, Methylosoma, Methylosphaera, Methylosarcina, Methylothermus, Methylovulum*	*Methylocystis, Methylosinus, Methylocapsa, Methylocella, Methyloferula*	*Methylacidiphilum*
Cell morphology	cocci-rods, cocci and rods	pyriform, vibroid, curved cocci or rods	
C-assimilation pathway	RuMP	serine	serine
IMC	vesicular discs	parallel to cell periphery	vesicular discs
Cyst formation	varies between genera	varies between genera	absent
pMMO	Present	present, except *Methylocella, Methyloferula*	present
sMMO	absent, except *Methylococcus*	varies between genera	absent
Major PLFAs	14:0, 16:0, 16:1, 18:1	18:1	14:0, 15:0, 18:0
G+C content (mol %)	43-65	60-67	40.8-45.5

* Type X methanotrophic genera. They possess the characteristics of both Type I and Type II but are now reclassified as a subset of Type I methanotrophs

[+] with the exception of *Clonothrix* and *Crenothrix*

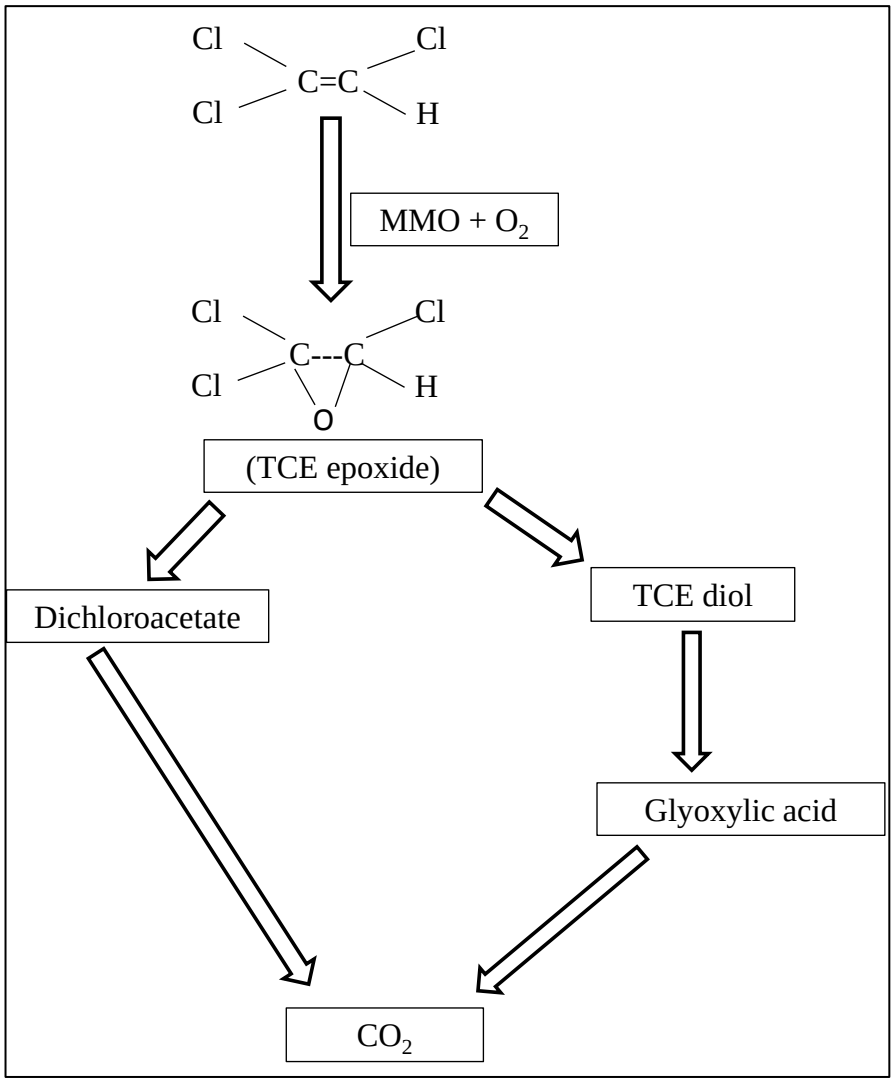


Figure 1-7 Proposed mechanism for TCE co-metabolism by methane monooxygenase (MMO). Adapted from Brigmon (2002)

Soluble methane monooxygenase (sMMO) is a non-heme, iron containing enzyme complex consisting of three components: hydroxylase (245 kDa), B component (15.8 kDa) and reductase (38.4 kDa). The most well characterised and highly conserved sMMO are purified from *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* (Lipscomb, 1994, Gassner and Lippard, 1999, Lee and Lipscomb, 1999). Soluble MMO possess a wide range of substrate specificity and can degrade a wider

variety of compounds, such as chlorinated aliphatic compounds and aromatics (Shigematsu *et al.*, 1999, Hesselsoe *et al.*, 2005). The detailed structure of the sMMO molecule is described by Murrell *et al.* (2000a) By comparison, information on the structure of pMMO was limited until the crystal structure of pMMO was obtained (Lieberman and Rosenzweig, 2005). Particulate methane monooxygenase (pMMO) consists of three polypeptide subunits with molecular weights; α~27 kDa, β~45 kDa and γ~45 kDa encoded by *pmoA, pmoB and pmoC* genes respectively. The active site of the enzyme is thought to lie within α and β subunit as specific inhibition of these subunits by acetylene, a suicide substrate for MMO, has been demonstrated (Semrau *et al.*, 2010).

Unlike sMMO, pMMO has a relatively narrower substrate specificity but is more suitable for *in situ* bioremediation because it is produced by all methanotrophs (Hakemian and Rosenzweig, 2007). An early study showed that the ability of methanotrophs to degrade TCE substantially increased with increasing copper availability (Smith *et al.*, 1997, Lontoh and Semrau, 1998). In methanotrophs, for example *Methylosinus trichosporium* OB3b, that possess both pMMO and sMMO, copper ion regulates the expression of the particular enzyme type (Nielsen *et al.*, 1997, Murrell *et al.*, 2000b, Semrau *et al.*, 2010). In copper starved condition (as low as 1 μM) only sMMO is expressed. Further-more, copper is thought to affect the MMO expression at the genetic level by activating *pmo* gene transcription cluster, with no detectable sMMO expression (Murrell *et al.*, 2000b). The model of copper-dependent transcription and regulation of pMMO/ sMMO is described in details elsewhere (Knapp *et al.*, 2007, Kenney and Rosenzweig, 2011)

1.3.2 Biological transformation of pesticides (CP and IC)

The metabolic fate of persistent toxic pesticides that are released into the environment is dependent on the abiotic environmental factors (temperature, pH, moisture, *etc*.), microbial community composition and chemical characteristic of the pesticide. Abiotic biotransformation of the pesticide occurs by processes such as photolysis (Tsogas *et al.*, 2006, Ramezani, 2008, Tajeddine *et al.*, 2010), hydrolysis (Katagi, 2002) and re-dox rearrangements (Chaplain *et al.*, 2011). However, enzymatic transformation mediated by indigenous soil microorganisms is by far the most common type of transformation (Ortiz-Hernández *et al.*, 2011).

Currently, among the various groups of pesticides, OP pesticides form the most widely used group of agrochemicals. The OP pesticides share a similar general structure consisting mainly of ester or thiol derivatives of phosphoric, phosphonic or phosphoramidic acids (Singh, 2009). Most of the OP pesticides are degraded by indigenous microbes, in the environment, as a sole energy and C- or P-source. Both co-metabolic and mineralisation of OP pesticides by mixed and isolated bacterial strains have however also been reported (Singh and Walker, 2006, Chishti and Arshad, 2013).

In general, degradation of OP compounds starts with hydrolysis of P-O alkyl or P-O aryl bonds followed by oxidation, alkylation and dealkylation (Singh *et al.*, 1999, DeFrank and White, 2002, Sogorb and Vilanova, 2002, Kanekar *et al.*, 2004, Theriot and Grunden, 2011). Hydrolases are a broad group of enzymes that catalyses the hydrolysis, a fundamental step for the complete degradation of the molecule. Several genes encoding for different hydrolase activity have been described earlier from a number of microorganisms, for example, organophosphate hydrolases (OPH;

encoded by *opd* gene) that was first isolated from *Brevundimonas diminuta* and later from *Flavobacterium* sp. (Serdar *et al.*, 1982, Siddavattam *et al.*, 2003). Organophosphate hydrolases have shown to degrade a wide range of toxic OP compounds (Cho *et al.*, 2002, Ortiz-Hernández *et al.*, 2003, Efremenko *et al.*, 2005) including chemical warfare nerve agents (Rastogi *et al.*, 1997, Chen *et al.*, 2000, Gopal *et al.*, 2000, Raushel, 2002). A similar enzyme, organophosphorus acid anhydrolase (OPAA; encoded by *opaA* gene) was isolated from *Alteromonas* sp. (Cheng *et al.*, 1993). Another OP degrading enzyme OpdA (encoded by *opdA* gene) with broad substrate specificity to cleave P-CN and P-F bonds in addition to P-O bonds (Scott *et al.*, 2008, Blatchford *et al.*, 2012).was isolated from *Agrobacterium radiobacter* (Horne *et al.*, 2002) In addition to this, the microbial enzyme, methyl parathion hydrolase (MPH; encoded by *mpd* gene), originally isolated from *Plesiomonas* sp. (Zhongli *et al.*, 2001) have also been found in several phylogenetically unrelated bacteria like *Achromobacter*, *Pseudaminobacter*, *Ochrobactrum* and *Brucella* (Zhang *et al.*, 2005).

Studies have shown the capacity of the microorganisms *Flavobacterium*, *Pseudomonas* and *Sphingomonas* to degrade OP pesticides, such as parathion, cadusafos and ethoprophos (Karpouzas and Walker, 2000, Karpouzas *et al.*, 2000b, Karpouzas *et al.*, 2004). Bacterial strains belonging to *Serratia* (Pakala *et al.*, 2007) and *Burkholderia* (Zhang *et al.*, 2006c) have moreover been reported to utilise methyl parathion and fenitrothion as a sole source of C and energy. Another bacterium with a capacity to transform parathion and methyl parathion into amino acid derivatives (reductive transformation) has also been identified (Yang *et al.*, 2007). Wang *et al.* (2010b) and Pravin *et al.* (2012) also reported isolation of *Hyphomicrobacterium* sp. and *Nacardiopsis* sp., respectively, with the capability to

degrade methamidophos and other OP and OC pesticides. For microorganisms, the advantage of degradation activities is to get access to the additional sources of nutrients (C, N and P) for growth; while the degrading enzymes have the potential in bioremediation (Russell *et al.*, 2011). The (bio) availability and (bio) degradation of a pesticide is interlinked and is mainly determined by its adsorption to and desorption from soil colloids. The binding of pesticides to soil matrix is often considered to reduce bioavailability and therefore limits potential biodegradation of the compound (Guerin and Boyd, 1992, Singh and Sharma, 2013). This pesticide-soil interaction is affected by various factors, such as soil properties, concentration and chemical characteristics of the pesticide. For example, pesticides are strongly adsorbed to soils that are high in clay or organic matter, are less readily released from such soils and therefore become more persistent (Dalla Valle *et al.*, 2005). Moreover, studies have shown that sites with a long history of pesticide contamination and constrained release of aging pesticide may increase the resistance of the compound to biodegradation in soils (Hatzinger and Alexander, 1995, Alexander, 2000). Ahmad *et al.* (2004) reported that the aging reduced availability of pesticide, carbaryl, in a long term contaminated (*i.e.*, aged) soil (>12 years). Similar reports concerning the effects of aging are available for other pesticides, such as DDT (49 years) (Morrison *et al.*, 2000), 1, 2-dibromoethane (3 years) (Steinberg *et al.*, 1987) and simazine (20 years) (Scribner *et al.*, 1992).

In addition to this, extensive and repeated use of OP pesticides has shown to increase microbial degradation of the pesticide, due to the development of enhanced degradation in soils under field conditions, resulting in the lack of efficacy of the pesticide against target pests. This phenomenon is known as accelerated or enhanced degradation, caused by an adaptation of one or more species of indigenous

microorganisms to metabolise the pesticide (Racke and Coats, 1990). For example, a lack of efficacy of the pesticide phorate in UK carrot fields was reported due to accelerated degradation (Suett *et al.,* 1996). The author also reported that 12 out of 13 pesticides that were available in the UK for soil application were susceptible to undergo enhanced microbial degradation. Previous literature has also reported enhanced degradation and failure of many other OP and carbamate pesticides (*e.g.* diazion and carbofuran) to provide adequate control against their target pests (Sethunathan, 1971, Williams *et al.*, 1976, Felsot *et al.*, 1981).

A range of biotic and abiotic factors has been shown to influence the stability of the pesticide and induction of enhanced degradation in soils (Felsot and Shelton, 1993, Bending *et al.,* 2003, Singh *et al.,* 2003a, Singh *et al.,* 2003b). The problem of enhanced degradation becomes more significant when the degradation of pesticide in a soil is induced by another pesticide from the same group of chemicals (cross-enhancement) (Singh *et al.,* 2005). Cross-enhancement of accelerated degradation of several OP pesticides and one nematicide was observed under lab conditions, in Australian soils that showed enhanced degradation of fenamiphos or CP in the fields (Singh *et al.*, 2005). This phenomenon has also been reported within many other groups of pesticides, for example, carbamates, thiocarbamates, dicarboximides and isothiocyanates (Wilson, 1984, Horng and Kaufman, 1987, Mitchell and Cain, 1996, Morel-Chevillet *et al.*, 1996, Warton *et al.*, 2003).

The degradation of CP (an OP pesticide) by soil microorganisms has been studied extensively with 3,5,6-trichloro-2-pyridinol (TCP) as the major degradation product (Xu *et al.*, 2007). Repeated applications of CP have shown inconsistent results from enhanced degradation by soil microbial communities (Fang *et al.,* 2008) to no effects (Singh *et al.,* 2002a) in lab controlled conditions. However, the first report on the

loss of efficacy of CP, after extensive and repeated application of the pesticide, came from Australian sugarcane farms due to the development of enhanced biodegradation (Robertson *et al.*, 1998, Singh *et al.*, 2003a).

Most of the studies on biodegradation of CP have reported aerobic transformation of CP by hydrolysis to produce TCP and diethylthiophosphoric acid (DETP) (Figure 1-8). Chlorpyrifos is reported to be degraded co-metabolically in a liquid media by *Flavobacterium* sp. (initially isolated from diazinon treated fields) and *Arthrobacter* sp. (Mallick *et al.*, 1999). The first report on CP mineralisation was reported for *Enterobacter* strain B-14 which was isolated from Australian agricultural soils with the ability to degrade CP to DETP and TCP and to utilise DETP as a source of C and P (Singh *et al.*, 2003a, Singh *et al.*, 2004).

Two other bacterial strains, *Stenotrophomonas* sp. YC-1 (Yang *et al.*, 2006), and *Sphingomonas* sp. Dsp-2 (Li *et al.*, 2007), isolated from a waste-water treatment facility of a pesticide manufacturer, degraded 100% CP (100 mg/kg) within 24 h as a sole source of C and P. *Synechocystis* sp. PUPCCC 64, isolated from agricultural waste-water, also have been shown to degrade 93% of 5 mg/l CP within 5d (Singh *et al.*, 2011a). Similarly, a number of bacterial strains capable of utilising both CP and its primary degradation product, TCP, in liquid culture as well as soil have been reported. These include *Alcaligenes faecalis* DSP3 (Yang *et al.*, 2005), *Paracoccus* sp. TRP (Xu *et al.*, 2008), *Bacillus* sp. (Anwar *et al.*, 2009, Liu *et al.*, 2012, Eissa *et al.*, 2014), *Pseudomonas* sp. (Lakshmi et al., 2009), *Ralstonia* sp. T6 (Li *et al.*, 2010) and *Mesorhizobium* sp. HN3 (Jabeen *et al.*, 2014) that have been isolated from different parts of the world. Many researchers have also successfully isolated bacterial strains from pesticide-treated agricultural soils with CP degrading capabilities (Kumar, 2011, Sasikala *et al.*, 2012).

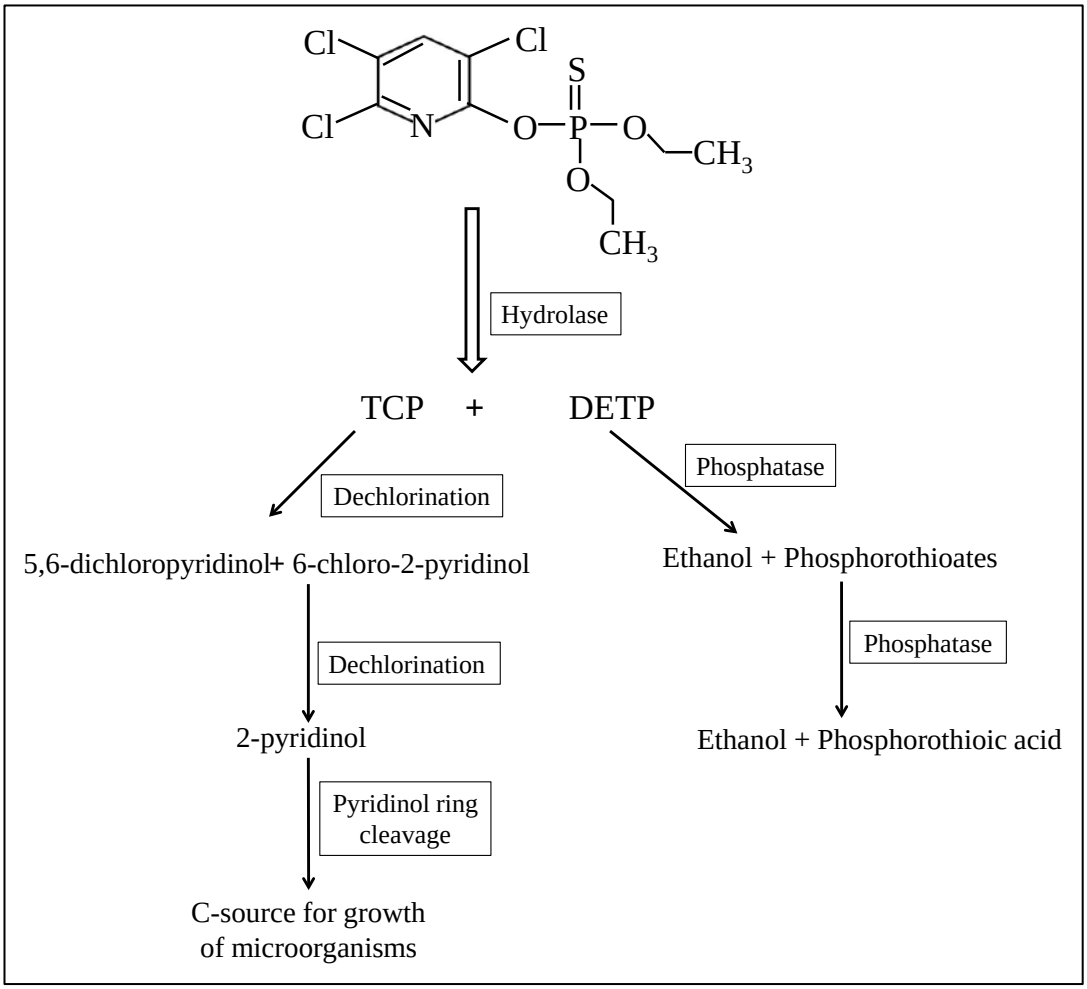


Figure 1-8 Proposed pathways for degradation of chlorpyrifos (CP) by microorganisms. The scheme is adapted from (Singh and Walker, 2006) and (Lu *et al.*, 2013). Abbreviations: TCP=3,5,6-trichloro-2-pyridinol, DETP=diethylthiophosphate.

On the other hand, while metabolism of IC has been extensively studied in mammalian systems (Tomizawa and Casida, 2005, Ford and Casida, 2006), the environmental fate of the compound is poorly understood. Recently a pathway for the transformation of IC in *Pseudomonas* sp. was proposed (Pandey *et al.*, 2009) where the pesticide was transformed to nitrosoguanidine (=N-NO), desnitro (=NH) and urea (=O) metabolites (Figure 1-9). Very little literature is available to date

describing mineralisation of IC by bacterial isolates which is limited to *Leifsonia* sp. (Anhalt *et al.*, 2007), *Stenotrophomonas maltophilia* (Dai et al., 2006) and *Pseudomonas* sp. (Pandey *et al.*, 2009). Other than these, bacterial strains belonging to genera *Ensifer, Stenotrophomonas* and *Pigmentiphaga* have been reported with the ability to degrade other neonicotinods, such as thiamethoxam, thiacloprid and acetamiprid, respectively (Zhao *et al.*, 2009, Yang *et al.*, 2013, Zhou *et al.*, 2013). However, the genes and enzymes responsible for the microbial degradation of IC (and other neonicotinods) are currently unknown.

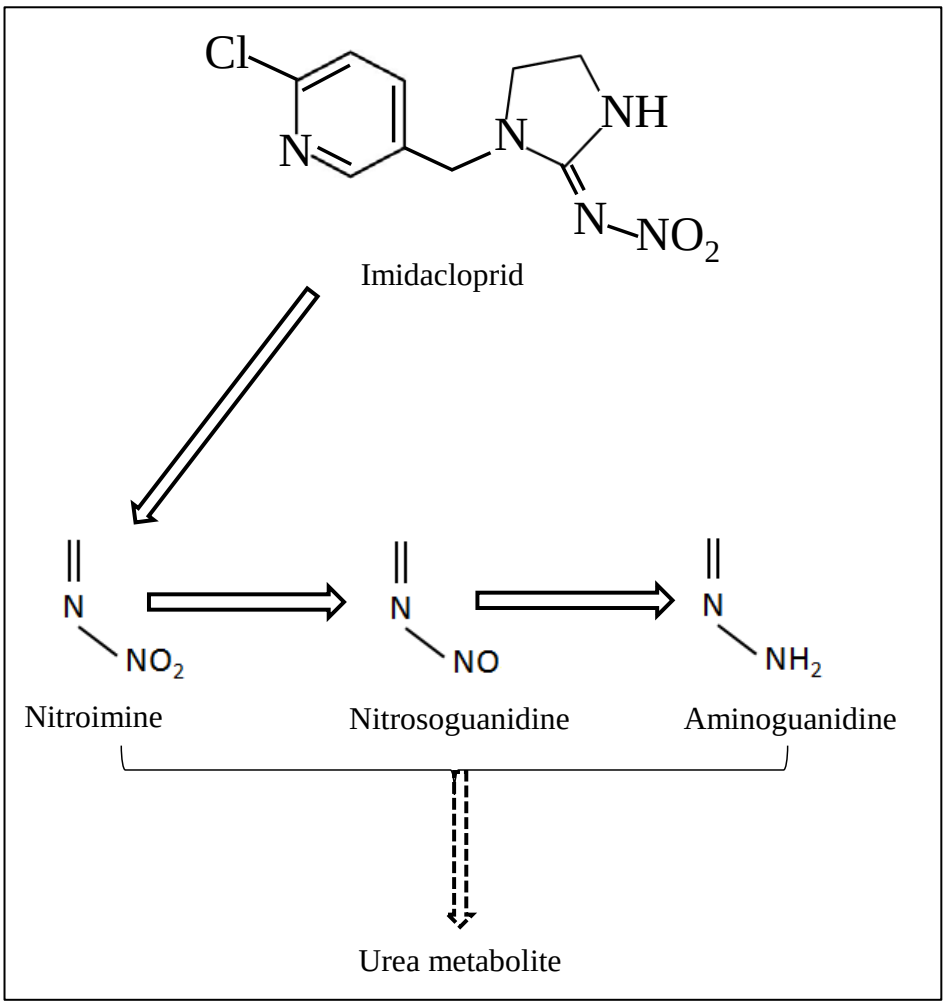


Figure 1-9 Proposed metabolic pathway for transformation of imidacloprid in *Pseudomonas* sp.1G. Adapted from Pandey *et al.* (2009). Dotted arrow indicates that the conversion occurs through a series of intermediates.

1.4 Methods to determine impacts of xenobiotics on soil microbial communities

Extensive amount of research have been conducted to understand the effects of xenobiotics on soil microbial communities and how these microbes respond to the anthropogenic stressors. This research has ranged from using the traditional cultivation dependent and independent analyses to state-of-art emerging metagenomics approaches. Some of the techniques used in this study include both broad (*e.g.* Microresp^{TM}) fine-scale (*e.g.* stable isotope probing) analytical methods and are briefly described below.

1.4.1 Community level physiological profiles (CLPPs)

Community level physiological profiles (CLPPs) approach is widely used to determine the activity of the soil microflora involved in carbon cycling (functional diversity). This is usually assessed by carbon substrate utilization and MicroResp^{TM} method (Campbell *et al.*, 2003) is widely used method as it was designed to be a 'whole soil' technique that increases the specificity and sensitivity of the assay. Microresp^{TM} consists of two micro-titre plates, one of which is a 96 deep-well plate for soil and carbon substrate; the other plate is designed for carbon dioxide (CO_2) detection. The detection plate contains a gel-based bicarbonate buffer with indicator dye (Rowell, 1995) that responds to a change in pH when evolved CO_2 from substrate (carbon) utilization gets adsorbed onto the gel; also known as colorimetric method of detection. The change in colour is quantified after six hours incubation using the plate reader (Campbell *et al.*, 2003). This method has been widely applied to monitor the microbial soil functions in response to pesticide contamination (Wang

et al., 2006, Wakelin *et al.*, 2008, Dutta *et al.*, 2010, Cycoń and Piotrowska-Seget, 2015a), metal contamination (Bérard *et al.*, 2014, Singh *et al.*, 2014, Wakelin *et al.*, 2014) and hydrocarbon contamination (Kaufmann *et al.*, 2006).

1.4.2 Terminal restriction fragment length polymorphism (T-RFLP)

Terminal restriction fragment length polymorphism (T-RFLP) is an automated, sensitive and popular high-throughput fingerprinting technique used to assess changes in the microbial community structure (Liu *et al.*, 1997, Schütte *et al.*, 2008). The technique involves the restriction digestion of fluorescently end labelled PCR products to produce terminal restriction fragments (T-RFs). The digested fragments (T-RFs) are then separated out, based on size, using either gel or capillary based systems and detected by an automated analyser (Osborn *et al.*, 2000). Since the differences in the T-RFs reflect phylogenetically distinct populations, this analyses method has been used to determine the impacts of xenobiotics on microbial community structure.

Studies by Dai *et al.* (2015) and (Luo *et al.*, 2009a) compared the abundances of individual T-RFs (T-RFLP analysis) and illustrated the remarkable increase in the proportion of a particular bacterial population in 2,4-dichlorophenoxyacetic acid (2,4-D) and another population in toluene contaminated sites, respectively. Zhang *et al.* (2008) reported that the pyrethroid insecticide cypermethrin significantly altered the bacterial community structure within the cucumber phyllosphere. In another similar study, Vázquez *et al.* (2009) utilised T-RFLP to interpret temporal microbial community dynamics during bioremediation of diesel oil-contaminated Antarctic soil and reported the differences in microbial community composition in treated and

control plots. Despite providing high throughput analysis of highly complex microbial communities (Dunbar *et al.*, 2000, Lukow *et al.*, 2000, Pesaro *et al.*, 2004), T-RFLP approach suffers from a major drawback as each T-RF can be a representative of multiple organisms or different strains of the same microbial species can produce different sized T-RFs (Kaplan and Kitts, 2003, Schmitt-Wagner *et al.*, 2003).

1.4.3 Stable isotope probing (SIP)

Stable isotope probing is a widely used technique that relies on the incorporation of a highly enriched substrate in a stable isotope, such as ^{13}C or ^{15}N (Paterson *et al.*, 2009), and the identification of active microorganisms by analysis of isotope enriched cellular components (DNA, RNA, PLFA, protein) (Dumont and Murrell, 2005). Thus, SIP can be used to monitor the specific biological function of microorganisms in a particular environment or conditions; for example, to monitor the effects of pesticides on soil microbes or to understand the chemical/ biological (active degraders) aspects of pesticide fate within the environmental conditions. This approach has been successfully used to characterise Beta-proteobacteria where the use of ^{13}C labelled 2,4-D (DNA-SIP) facilitated the characterisation and subsequent amplification of soil degrading community (Cupples and Sims, 2007). Building on this, Lerch *et al.* (2009) used SIP coupled with fatty acid methyl esters (FAME-SIP) and confirmed Proteobacteria as the principal degraders of 2,4-D in soil during a 6 month incubation study. In addition to these studies, SIP has been also applied to identify aerobic and/ or anaerobic naphthalene, anthracene (Zhang *et al.*, 2012a),

MTBE/ TBA (Key *et al.*, 2013), RDX (Cho *et al.*, 2013), and toluene degraders (Fowler *et al.*, 2014).

1.4.4 Next generation sequencing

Next generation sequencing has seen a rapid advancement in recent years. The considerably increased amount of data (at finer levels) has improved the ability to assess the overall structure, function and dynamics of the microbial population in environmental samples (Christen, 2008, Wooley and Ye, 2010). The procedure involves isolation of DNA from environmental samples followed by sequencing of the whole metagenome. Data generated provides key information not only on community composition and diversity but also the relative abundance of functional genes; and therefore the potential capability of ecosystem, from where samples are obtained (Rastogi and Sani, 2011). This approach has already been applied to a range of soil systems including contaminated sites (Ono *et al.*, 2007, Lu *et al.*, 2011). However, studies applying next generation sequencing technique to explore pesticide impacts on agri-ecosystems are limited. (Sangwan *et al.*, 2012, Smith *et al.*, 2013)

1.5 Knowledge gaps

While previous literature has provided some insight into the impact of xenobiotics on soil microbial communities, there is still limited knowledge on the impact of the three widely used compounds (TCE, CP and IC) on soil microbial communities, particularly in Australian ecosystems. Australia is a geographically isolated and biologically diverse continent. It is home to more than one million endemic species, which is shared by no other country in the world. Several characteristics of soils here is different from the rest of the world (Paton *et al.*, 1995) which mainly consist of weathered rocks with poor in nutrient levels. Thus, a more global view of soils is needed, with focus on soil microbial communities, which will enhance the ecological knowledge of the continent and contribute to land management strategies. Details on knowledge gaps in particular areas (highlighted here) are described in appropriate experimental chapters.

- Trichloroethene has been known to adversely impact the structure and functions of the soil communities; however, no such study has been conducted on soil microbial communities in Australian systems. While some studies have examined the distribution and diversity of aerobic TCE dechlorinating bacteria (Coleman *et al.*, 2010, Le and Coleman, 2011), there is no information on whether TCE would impact the presence or abundance of potential TCE degraders (methanotrophs) that can be used for bioremediation. Given that the degradation of TCE by methanotrophs is co-metabolic in nature, evidence on linking active methanotrophs to degradation of TCE is limited. Thus, DNA-SIP approach can be applied to achieve this.

- Most of studies on the interaction between the pesticides CP and IC and soil microbial communities have been short-term and carried out on single application of the particular pesticide thus, limited information is available about the effects of repeated application of the pesticides, which is the general agricultural practice. While previous studies have mainly used crude DNA fingerprinting techniques (*e.g*. T-RFLP), very limited information on the community structure of the environmental micro-biota could be obtained. Next generation sequencing approach can offer a more global view of the soil microbial communities, allowing the better assessment (at finer resolutions) of phylogenetic diversity and potential metabolic pathways.

- Repeated applications of some pesticides have been known to influence the rate of degradation, but there is still a lack of information on the legacy effects of CP and IC. For example, after the loss of efficacy due to development of enhanced degradation in soils, no CP was applied to Australian sugarcane farms (about 13 years ago); we have no knowledge of whether the microbial community with CP degrading abilities still survive in absence of selection pressure from CP.

- Moreover, it has been observed, that enhanced degradation generally develops in response to the repeated application of pesticide; information about the duration of the phenomenon, and for soils to return to normal state, vary widely among pesticides, soils and climatic conditions (Anderson *et al.*, 1992), yet, no such study has been conducted on CP and IC globally. This information is critical for development of the pest management strategy. Since pesticides' efficacy could be compromised due to enhanced degradation it would cause adverse effect on the production of any crop and significant economic loss of pesticide companies.

1.6 Aims and objectives

The overarching aim of the thesis was to determine the two-way interactions between the xenobiotics and soil microbial communities in Australian soils using organochlorine (TCE) and pesticides (CP, IC) as model compounds. A schematic diagram of the approaches to achieve aims of this project related to pesticides (Chapter 3-5) is given in Figure 1-10. On the other hand, the impact of TCE was determined only on a specific group of soil microorganisms, namely methanotrophs and thus a different approach was undertaken (described in Chapter 2). The work presented here is split into four experimental chapters each with their own defined aims, methods, results and discussion.

Chapter 2 aimed to determine the effects of CH_4 concentration on CH_4-assisted TCE degradation as well as to characterise the active methanotrophs involved in the biodegradation of TCE. This chapter tested the hypothesis that the high CH_4 concentrations are required to maximise the production of MMO for efficient TCE removal. This was accomplished using a modified stable isotope probing (SIP) technique coupled with cloning and sequencing. The objectives of this chapter were:

a) To identify and characterise novel methanotrophs that promote TCE degradation in Australian soils.
b) To determine that a positive relationship exists between CH_4 concentration and the rate of TCE biodegradation.

Chapter 3 aimed to determine the impacts of repeated pesticide (CP, IC) applications on soil bacterial community composition and the rate of biodegradation, in pesticide-treated and non-treated soils. This study tested the hypothesis that the soils which have shown enhanced CP degradation previously,

will maintain their pesticide degrading capability due to legacy effect of the pesticide. Secondly, repeated application of pesticides will have limited impact on soil microbial communities due to rapid loss of pesticide *via* enhanced biodegradation. The objectives of this chapter were:

a) To determine the response of soil microbial communities to CP and IC treatments.
b) To identify potential long-term effects (legacy) of pesticides application on metabolic capabilities of microbial communities

Chapter 4 aimed to enrich pesticide degraders (only CP) from Australian soils that showed enhanced CP degradation under lab conditions (Chapter 3), followed by the isolation and identification of the potential degraders. This chapter tested the hypothesis that the enhanced CP degrading soils will enrich/select for bacterial strains adapted to utilise CP and its primary metabolite, TCP, as a source of C and nutrients. The objectives of this chapter were:

a) To identify the physiological characteristics of pesticide (CP) degraders.
b) To assess the potential of the isolated pesticide degraders for the degradation of CP and its primary by-product (TCP) as a source of C, N and/or P.

Chapter 5 aimed to investigate, at high resolution, the impact of OP pesticide (CP) on the soil microbial community and functional capabilities using next generation sequencing technique approach. This chapter tested the hypothesis that legacy effect of OP pesticide would negatively impact bacterial community structure and metabolic functions in pesticide-treated sites. In light of this, the P-metabolism genes will be over-expressed, relative to other genes, in pesticide-treated sites as many

bacteria are reported for their ability to utilise OP pesticide as P-source. The objectives of this chapter were:

a) To determine the impact of pesticides on soil microbial diversity and community composition
b) To identify the legacy impact of CP application on functional capabilities with a focus on phosphate metabolisms.

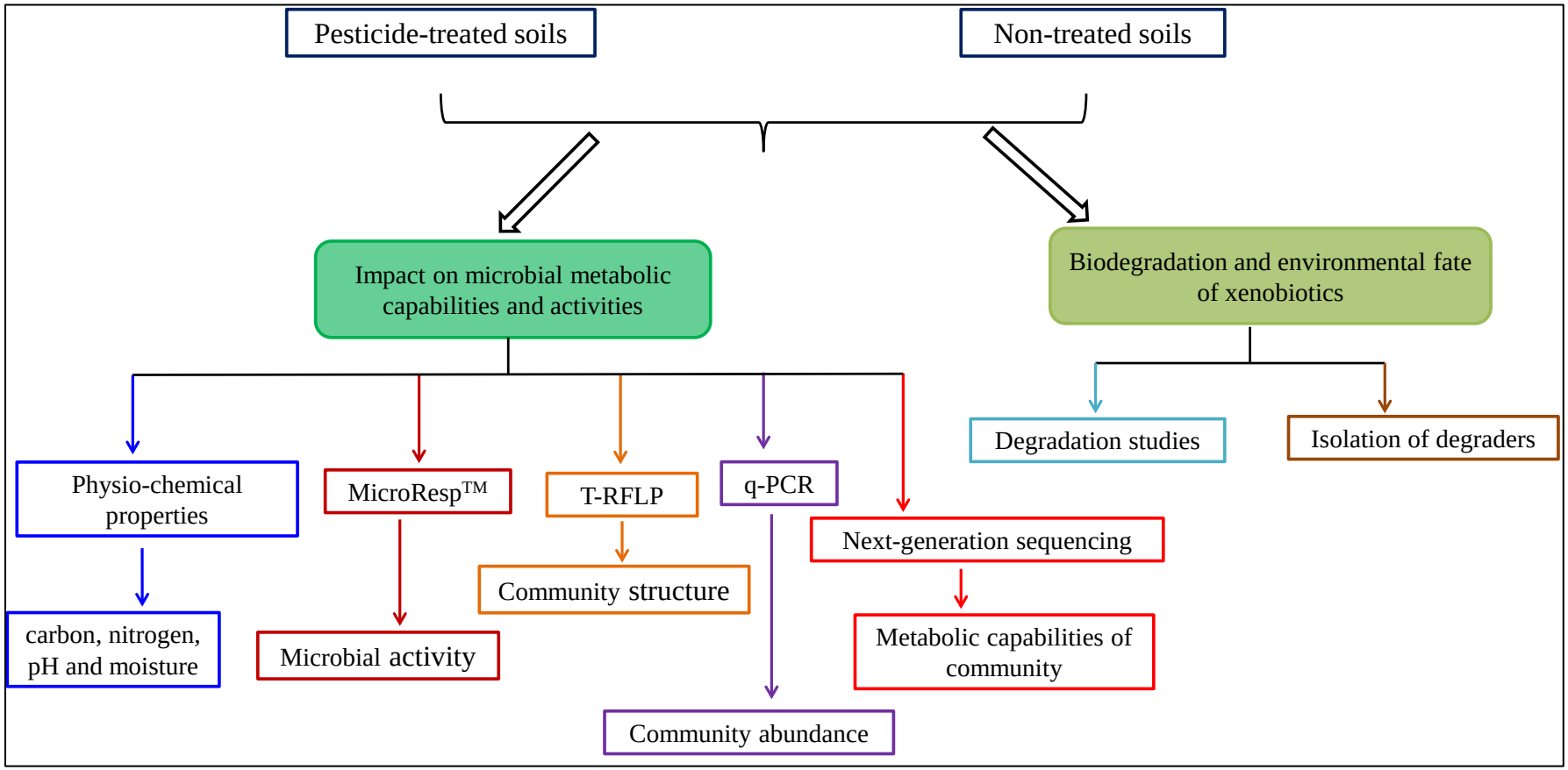


Figure 1-10 Schematic diagram of approaches used to achieve aims of the project (Chapter 3-5). Xenobiotic-induced stress (when released into the environment) is thought to affect the dynamics of the soil microbial community by: a) an extinction response where the sensitive microbial population gets eliminated from the system or b) development of the microbial communities with innate capabilities to resist pollution and proliferate by direct utilisation of the compound for energy and nutrients (biodegradation).A separate approach (described in Chapter 2) was undertaken to determine impact of TCE on soil microbial communities.

Chapter 2: Characterisation of active methanotrophs involved in the methane-assisted biodegradation of trichloroethene (TCE) in three Australian soils

2.1 Introduction

Trichloroethene (TCE) is one of the most abundantly used organochlorine (OC) solvents used as a degreaser in vapour degreasing, for cold cleaning metals and various other industrial applications (Hazen *et al.*, 2009). The primary sources of TCE emissions are the industries that manufacture it or use it in production such as chemical industry, rubber manufactures, pharmaceutical industries etc. As a result of improper disposal, TCE enters the environment posing a serious concern of air, water and soil contamination. Based on assessments by the National Industrial Chemical Notification and Assessments Scheme (NICNAS, 2000), any product containing more than 0.1% TCE is classified as a hazardous substance. The International Agency for Research on Cancer (IARC, WHO) has also classified TCE as a 'probable human carcinogen'.

In recent years there has been an increased interest in the use of microorganisms for removal of chlorinated compounds, including TCE from the environment. One such class of organisms, methanotrophic bacteria (also called methanotrophs), are particularly well suited to facilitate the degradation of halogenated organic compounds. Trichloroethene (TCE) bioremediation using methanotrophs is considered one of the most efficient approaches because of their higher oxidation rates of TCE than other degraders (Moran and Hickey, 1997). These bacteria utilise methane (CH_4) as their sole carbon (C) and energy source and have long been shown to co-metabolise TCE by methane monooxygenase (MMO) enzymatic systems, both

the soluble (sMMO) and membrane bound particulate (pMMO) enzyme (Uchiyama *et al.*, 1995, Hanson and Hanson, 1996, Erwin *et al.*, 2005, Wymore *et al.*, 2007, Semrau *et al.*, 2010). This has drawn significant attention and various laboratories (Lee *et al.*, 2006, Shukla *et al.*, 2009, Shukla *et al.*, 2010a) and field studies (Conrad *et al.*, 2010) have established the effectiveness of the oxidative reactions mediated by MMO in degradation of halogenated compounds (McCue *et al.*, 2003). However, the ability of the methanotrophs to sustain TCE degradation is critical. The degradative enzyme MMO prefers its physiological substrate (*i.e.*, CH_4) and presence of CH_4 may inhibit the TCE degradation by competitive inhibition (for active enzyme sites) (Hanson and Hanson, 1996). This competitive relationship between TCE and CH_4 as the substrate thus plays a major role in the efficiency of biodegradation of TCE (Strandberg *et al.*, 1989, Oldenhuis *et al.*, 1991). In this context, the effect of enzyme competitive inhibition on TCE degradation has been observed previously (Mu and Scow, 1994, Shih *et al.*, 1996, Mars *et al.*, 1998, Futamata *et al.*, 2003); however, there still remains limited knowledge on CH_4 feeding operation strategies on the competitive behaviour of methanotrophs that would eventually influence TCE degradation.

Remediation processes using methanotrophs have been applied both *in situ* and *ex situ*. *In situ* methods involve the bio-stimulation of indigenous methanotrophic communities by adding methane and other nutrients to enhance the co-metabolic degradation of halogenated organic compounds (Sutfin and Ramey, 1997, Eguchi *et al.*, 2001, Wymore *et al.*, 2007). On the other hand, Shukla *et al.* (2010a) enriched and isolated a mixed methanotrophic culture from uncontaminated soil to use for the degradation of TCE in batch and continuous modes for *ex situ* bioremediation. The

authors reported that the methanotroph *Methylocystis* sp. was highly effective in TCE degradation.

Most of the earlier studies on degradation of TCE were conducted with pure methanotrophic cultures belonging to the genera *Methylocystis*, *Methylomonas, Methylomicrobium* and *Methylosinus* (Oldenhuis *et al.*, 1989, Koh *et al.*, 1993, Im and Semrau, 2011). For example, in a bioreactor-based study, pure cultures of *Methylosinus trichosporium* and *Methylocystic* sp. were observed to degrade TCE (Aziz *et al.*, 1995). However, most of the methanotrophs are uncultivable under lab conditions and the role of uncultivable methanotrophs to degrade halogenated organic compounds remains limited. In order to combat the problems associated with culture-dependent analyses, culture-independent molecular techniques allows studying the uncultivable methanotrophs and their role in degradation.

Various functional genes, *pmoA* (encodes for pMMO), *mmoX* (encodes for sMMO hydroxylase) and *mxaF* (encodes for methanol dehydrogenase), are available for targeting methanotrophs. However, the *pmoA* gene has been found to be an excellent functional marker as all the methanotrophs possess this gene except for the genera *Methylocella* (Dedysh *et al.*, 2000) and *Methyloferula* (Vorobev *et al.*, 2011). Methanotrophic diversity has been assessed in various environments using culture-independent techniques like cloning, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), phospholipid-derived fatty acids (PLFA) and DNA microarrays (Newby *et al.*, 2004, Tresse *et al.*, 2005, Qiu *et al.*, 2008, Hazen *et al.*, 2009, Vishwakarma and Dubey, 2010, Chiang *et al.*, 2012).

Stable isotope linked to molecular and biochemical approaches have increasingly gained attention as a novel tool for tracing the fate of labelled substrates (^{13}C) and linking function with taxonomic identification for complex environmental communities (known as stable isotope probing, SIP) (Chen and Murrell, 2010). For example, DNA-SIP has been widely employed to identify active CH_4 oxidising microorganisms in freshwater, marine sediments, terrestrial caves, soils and artic lakes (Radajewski *et al.*, 2000, Hutchens *et al.*, 2004, Friedrich, 2006, Cébron *et al.*, 2007, Dumont *et al.*, 2011, He *et al.*, 2012, Kumaresan *et al.*, 2014). For example, one study where a DNA-SIP based technique was employed to detect the response of methanotrophs to different temperatures showed that the identity and relative abundance of active methanotrophs differed with incubation temperature (He *et al.*, 2012). Similarly, SIP of phospholipid fatty acids (PLFA-SIP) has been used to study the activity and composition of methanotrophic communities in various ecosystems (Singh and Tate, 2007, Chen *et al.*, 2008b, Qiu *et al.*, 2008, Murase *et al.*, 2011, Nazaries *et al.*, 2011). SIP has been also applied to identify aerobic and/or anaerobic naphthalene, anthracene (Zhang *et al.*, 2012a), MTBE/TBA (Key *et al.*, 2013), RDX (Cho *et al.*, 2013), and toluene degraders (Fowler *et al.*, 2014). However, DNA-SIP has not been applied to study TCE degradation by aerobic methanotrophs.

Hence, this study aims to identify and characterise novel uncultivable methanotrophic consortia in Australian soils using SIP that promote biodegradation of TCE in mixed microbial communities under different CH_4 concentrations. Although isolating pure methanotrophic TCE degraders from contaminated sites provides an insight into linking functions to the biodegradation, the general framework of this study was to assess the ability of whole communities (both isolated and enriched cortium) to degrade TCE that would provide stronger evidence

for this relationship. For example, a potential TCE degrader in the lab does not necessarily reveal the actual degradation reactions or the key microbial TCE degrader in the field. Additionally, expanded knowledge on mode of TCE biodegradation mechanisms would be useful for the development of strategies for efficient bioremediation of the contaminated sites. In addition to this, CH_4 concentration would also effect TCE transformation, and thus, I hypothesised, in studies with longer incubation periods (*e.g.* field trials, bioreactors), high CH_4 concentrations are needed to maximise MMO production and minimise competitive inhibition for higher TCE removal efficiencies.

Mixed methanotrophic cultures were enriched from three Australian soils using nitrate minimal salt (NMS) media, and their capacity for CH_4 and TCE oxidation was evaluated under different CH_4 concentrations (1%, 10% and 33%). DNA-SIP was used together with *pmoA*-based cloning and sequencing to better understand the link between TCE degradation and the methanotrophic community. Labelled CH_4 ($^{13}CH_4$) was used as a substrate to track the activity and community structure of the active methanotrophs (in microcosm) in response to TCE.

2.2 Materials and methods

2.2.1 Site description and sample collection

For this work several field sites were initially chosen in NSW (total=7). Three out of seven sites were located at the Hawkesbury Forest Experiment (HFE), representing forest, non-grazing pasture and grassland (latitude -33.61111^{o}, longitude 150.74069^{o}). The other four sites consisted of grassland and were located at EucFACE driftway (latitude -33.61781^{o}, longitude 150.74057^{o}), Sydney University ground (latitude -33.887551^{o}, longitude 151.18734^{o}), Victoria Park (latitude -33.88611^{o}, longitude 151.19274^{o}) and the Botany Industrial Park (latitude -33.95596°, longitude 151.21652°), respectively. All the sites, except for Botany Industrial Park, were under vegetation and had never been exposed to high concentration of halogenated organic compounds, particularly TCE. The site at Botany Industrial Park, on the other hand, has a long history of chlorinated hydrocarbon (hexachlorobenzene, chloroform, TCE) contamination. Contamination of groundwater at Botany Industrial Park was first reported in 1990 (http://www.orica.com/).

At each site, four replicate soil samples were randomly collected. Each replicate was composed of three soil cores taken by a soil corer by digging a hole about 10 cm deep. The soil corer was sterilized with 70% ethanol each time a new replicate/sample was collected within the same or different sites. Soil samples from Botany Industrial Park were provided by School of Molecular Biosciences, the University of Sydney, Australia. Fresh soil samples were wet-sieved through a 2mm sieve to remove vegetation and other coarse particles from the soil.

2.2.2 Methane oxidation activity of soils

A microcosm study for CH_4 consumption was performed on all the soils collected in 120 ml serum glass bottles. Five gram of each soil was added to 30 ml of NMS media (Section 2.2.3) in serum glass bottles. Initial CH_4 concentration in the headspace was adjusted to 1% v/v of air headspace by injecting the appropriate volume of pure CH_4 (purity ≥ 99.0%; Sigma Aldrich, USA). The microcosms were then incubated at 20^{o}C in the dark and continuously shaken at 150 rpm. Gas samples (250 µl) were collected periodically using a 250 µl gas-tight syringe. CH_4 oxidation activity was determined from the change in initial concentration of CH_4. Soils showing significant CH_4 consumption (Sydney University, Victoria Park and Botany Industrial Park) were further used in microcosm studies of TCE degradation.

Soil properties were also determined for the soils that showed CH_4 oxidation activity. The soils were analysed for total C and nitrogen (N) content, pH and moisture. For determining soils total C and N, the samples were milled into a fine powder by using a Retsch mill at frequency of 20 hertz for 2 mins. Total C and N content was then quantified with a CHN analyser according to the manufacturer's instructions (Leco TruSpec Micro, USA). pH was determined in 1:2.5 (wt/vol.) diluted water suspension and measured with a pH meter (Mettler Toledo, Australia). Soil moisture content was assessed by measuring the difference of weight of soil samples before and after oven drying at 105°C for 24 hours, and expressed as percentage of soil weight.

2.2.3 Preparation of media

Unless otherwise specified, all media and buffers prepared were sterilised *via* wet cycle autoclaving at 120^{o}C, and 15 psi for 15 mins. Milli-Q (MQ) water was used for preparation of all media, buffers and other molecular biology reagents. A nitrate mineral salt (NMS) medium adapted from Whittenbury *et al.* (1970), was used in both the enrichment and degradation studies. The NMS medium is mineral-based with added N and contained (g/l):

Solution I: 1.00 g KNO_3, 1.00 g $MgSO_4.6H_2O$, 0.2 g $CaCl_2$ (anhydrous);

Iron EDTA: 3.8 g FeEDTA; Sodium molybdate: 0.26 g $Na_2MoO4._2H_2O$;

Trace elements solution: 0.2 g $CuSO_4.5H_2O$, 0.5 g $FeSO_4.7H_2O$, 0.4 g $ZnSO_4.7H_2O$, 0.015 g H_3BO_3, 0.05 g $CoCl_2.6H_2O$, 0.25 g EDTA di sodium salt, 0.02 g $MnCl_2.4H_2O$, 0.01 g $NiCl_2.6H_2O$ (solution stored in dark); Phosphate buffer: $Na_2HPO_4.12H_2O$, KH_2PO_4.

During the preparation of 1X NMS medium pH was adjusted to 6.8 with H_2SO_4. Sodium molybdate (0.1% v/v), trace elements (0.1% v/v), Fe EDTA (0.01% v/v) and phosphate buffer (1% v/v) were added aseptically after autoclaving to avoid precipitation of salts. The C-source was provided in the form of gaseous hydrocarbon (CH_4 1% or 33% v/v of air headspace); filtered through 0.22 µm filters (Millipore, USA). For the preparation of 5 mM TCE stock solution, 45 µl of TCE (purity ≥ 99.5%; Sigma Aldrich, USA) was added to 100 ml Milli-Q (MQ) water in a 120 ml serum glass bottle containing a magnetic flea. Following this, serum bottles were sealed with a PTFE-lined butyl septum and aluminium crimp-top cap (Sigma, U.S.A) and the stock solution was stirred until TCE globules were completely dissolved and equilibrated (Cohen and Mccarty, 1991). The stock solution was wrapped in

aluminium foil and stored in darkness upside down. The TCE–saturated solution was removed with a gas-tight syringe through the septum, avoiding bubbles, and added to NMS media to get a final concentration of 50 µM along with CH_4. Each serum bottle (NMS+CH_4/TCE) was shaken at 150 rpm for 30 minutes at room temperature (RT) to equilibrate CH_4 and TCE before analysis on the gas chromatograph (GC).

2.2.4 Headspace analysis

Methane and TCE concentrations in the microcosm headspace were measured on a gas chromatograph (GC; Agilent Technologies, USA) using an analytical capillary column HP-PLOT/Q (30 m x 0.53 mm ID x 40 µm; Agilent technologies), equipped with a flame ionisation detector (FID), and run in splitless mode. Two hundred and fifty µl headspace samples were injected onto the column using a Precision Pressure-Lok® gas-tight syringe (VICI Precision Sampling, Inc.) with a stainless steel needle cone tip, side port, point style 5 (Sigma, U.S.A) and analysed using a five minute isocratic run. Operating temperatures for GC runs were set at 180^{o}C for the oven, 200^{o}C for the injector and 250^{o}C for the detector at constant pressure (60 kPa). Ultra-high purity nitrogen was used as a carrier gas, while the FID was supplied with high-purity hydrogen, nitrogen and instrumental grade air. The retention times of CH_4 and TCE were 0.64 and 3.4 mins, respectively. Quantification of the analytes was done *via* peak area, by referring to the external standard curves constructed from five data points, using triplicates at each point.

2.2.5 Enrichment of methanotrophic consortia

The soils from the three sites Sydney University (SU), Victoria Park (VP) and Botany Industrial Park (BI) actively oxidised CH_4 (Section 2.3.1) and thus were used for enrichment of mixed methanotrophic communities. Soil microcosms were set up in triplicates similarly as described in Section 2.2.2 and enriched with CH_4 as a source of C (33% v/v of air headspace). The mixed methanotrophic cultures were initially grown to the late exponential phase (OD_{600}~ 1.2, nanodrop) and headspace CH_4 concentrations were determined regularly by GC. After >90% of CH_4 was consumed, the microcosm was flushed and re-equilibrated with air to ensure that the microcosm remained aerobic. Further CH_4 was added (33% v/v of air headspace) and incubated until an OD_{600}~ 1.2 was reached (Choi *et al.*, 2013). The enriched methanotrophic culture was also used to isolate cells by plating on NMS agar plates and incubating under 33% CH_4 concentration in a glass desiccator.

2.2.6 Effect of different CH_4 concentrations on TCE degradation by methanotrophic consortia

To determine the effect of CH_4 concentrations on TCE degradation, two different CH_4 concentrations (1% and 33% v/v of air headspace) with TCE was added to each microcosm (in NMS) to achieve a final concentration of 50 µM of TCE with the desired CH_4 concentration. The microcosms were incubated on a rotary shaker (150 rpm, 20^{o}C) for 30 mins to equilibrate. Pre-grown cultures were then aseptically transferred to equilibrating microcosms to a get a final OD_{600}~ 0.2 (Lontoh and Semrau, 1998). The microcosms were shaken vigorously for 5-10 seconds. Immediately after that 250 µl headspace samples were taken for confirmation of

initial concentration of TCE and CH_4 at 0 hr for all soil types (Section 2.2.4). The degradation of substrates was monitored by taking samples at different time points followed by GC analysis. Controls with and without CH_4/TCE and abiotic control (without culture) were also maintained for each TCE degradation assay set up. Growth was also monitored *via* OD_{600} over the period of the degradation assay.

Stable isotope probing (SIP) incubations for the characterisation of enriched TCE-degrading methanotrophic consortia were done as described in Section 2.2.2 and 2.2.4 except for that the microcosms were incubated with labelled CH_4 ($^{13}CH_4$) (10% v/v air headspace; Cambridge isotope laboratories, USA). A non-labelled $^{12}CH_4$ exposed microcosm was also set up as a control for SIP. The microcosms were incubated until all the CH_4 had been consumed by each microcosm (two injections of 10% v/v air headspace).

2.2.7 Characterisation of enriched TCE degrading methanotrophic consortia by molecular methods

2.2.7.1 DNA extraction and SIP fractionation

DNA was extracted from 30 ml of enriched culture (^{13}C and ^{12}C) in a sterile environment. The enriched media was filtered via 0.22 µm pore size membrane filter units (Stericup filter units, Millipore) by vacuum. The filters were removed in sterile conditions from the disposable filter unit and DNA was extracted using PowerWater® DNA isolation kit (Mobio, USA) following the manufacturer's protocol. The DNA was eluted in 50 µl and not in 100 µl as suggested by the manufacturer. After DNA isolation, the concentration and purity of DNA was

confirmed by NanoDrop™ 2000C spectrophotometer (ThermoScientific) and DNA was stored at -20^{o}C until further analysis.

Cesium Chloride (CsCl) density gradient centrifugation and fractionation were modified from a previously described method described (Zhang *et al.*, 2011a). Extracted DNA (~1 µg) from both labelled and control samples were added to CsCl gradients with an initial density of 1.72 g/ml. Ultracentrifugation was done at 140,000 g_{av} (70,000 rpm) in a S120VT vertical rotor (Hitachi, Japan) at 20^{o}C with vacuum for 60 h. Centrifuged gradients were fractionated from bottom to top into 18 equal volumes (~100 µl per fraction) by displacing the CsCl medium with sterile water, using a hand-made fraction recovery system and a LSP02-1B syringe infusion pump at a flow rate of 100 µl min^{-1} (Baoding Longer Precision Pump Co. Ltd., China). The buoyant density of each collected fraction was measured by weighing the fractions from the blank gradient on a digital microbalance. DNA was precipitated from CsCl overnight by adding two volumes of polyethylene glycol (PEG) 6000 in 1.6M NaCl, washed with 70% ethanol and eluted in 30 µl of nuclease-free water (Griffiths *et al.*, 2000, Sharp *et al.*, 2012). The primer sets used in this study for the characterisation of methanotrophs are listed in Table 2-1.

Table 2-1 PCR and sequencing primers used in this study

Target Gene	Fluorescent label[d]	Primer set	Sequence (5' to 3')[e]	References
pmoA (All methanotrophs)[a]	none 6-FAM™ none	A189F A189F Mb661R	GGNGACTGGGACTTCTGG GGNGACTGGGACTTCTGG CCGGMGCAACGTCYTTACC	(Paszczynski *et al.*, 2011)
16S rRNA (TypeII methanotrophs)	none none VIC®	TypeII F TypeII R TypeII R	GGGAMGATAATGACGGTACCWGGA GTCAARAGCTGGTAAGGTTC GTCAARAGCTGGTAAGGTTC	(Chen *et al.*, 2007)
pGEM®-T Easy[b] +cloned product[c]	none none	T7 SP6	TAATACGACTCACTATAGGG TATTTAGGTGACACTATAG	(Zhou *et al.*, 2008)

[a]Except *Methylocella* and *Methyloferula* sp.
[b]Vector kit (Promega Corporation, USA)
[c]Amplified pmoA, cloned into the vector.
[d]6-FAM™: 6-carboxyfluorescein; VIC®: registered trademark of Life Technologies Corporation
[e]N represents A, C, T or G; M represents A or C; Y represents C or T; W represents A or T; R represents A or G

2.2.7.2 q-PCR of *pmoA* genes

The abundance of *pmoA* was determined on a rotor-type thermocycler (Corbett Research; Splex) using the primer pairs A189F and Mb661R (Martineau *et al.*, 2010, Paszczynski *et al.*, 2011). These primers can amplify the *pmoA* gene of most known methanotrophs, with some exceptions (Dunfield *et al.*, 2003, Stralis-Pavese *et al.*, 2004). Each reaction was performed in a 20 µl volume using SensiFAST™ SYBR® No-ROX Kit (Bioline Reagents Ltd). Reactions were set up as per the manufacturer's instruction (except 0.75 mg ml^{-1} bovine serum albumin was added to each reaction) with 0.4 µM of each primer and 2 µl of fractioned DNA (heavy and light). Standards were made from a 10-fold dilution of DNA template extracted from a pure culture *Methylosinus trichosporium*, obtained from NCIMB following the manufacturer's instruction (Wizard® Genomic DNA Purification Kit, Promega).

After the initial denaturation at 95^{o}C for 3 mins, PCR amplification conditions were as follows: 40 cycles of 10 sec at 95^{o}C, 10 sec at 53^{o}C and 20 sec at 72^{o}C. An additional 15 sec reading step at 83^{o}C was added at the end of each cycle. Melting curve analysis was performed at the end of real-time PCR to check the specificity of each reaction. After the range of fractions containing ^{13}C-labeled DNA or unlabelled (^{12}C) DNA were identified by quantitative PCR (q-PCR), the fractions were combined to compose a 'heavy' fraction and 'light' fraction, from each sample for further molecular analysis (*e.g.* PCR, T-RFLP).

2.2.7.3 PCR amplification and T-RFLP analysis

Polymerase chain reaction (PCR) amplification of *pmoA* and 16S rRNA was achieved using the primers A189F/ Mb661R and TypeII F/Type II R respectively (Table 2.1). To minimise bias when amplifying genes from 'light' and 'heavy' fractions of the gradients, the sub-fractions in each gradient fraction from ^{13}C-DNA and ^{12}C-DNA were pooled. CsCl gradient sub-fractions with densities between 1.65-1.67 g ml^{-1} were considered light fractions and those between 1.70-1.73 g ml^{-1} were considered heavy fractions (Dumont *et al.*, 2011).

pmoA-based PCR reaction mixture contained (final concentration) 1X NH_4^+ reaction buffer, 6 mM $MgCl_2$, 200 μM of deoxynucleotide mix, 0.02U μl^{-1} BioTaq™ DNA polymerase (all reagents from Bioline, UK), 0.3 μM of each primer (Sigma-Aldrich), 0.3 mg ml^{-1} bovine serum albumin (New England Biolabs, USA) and 2μl of DNA template. For 16S rRNA-based PCR reaction (final concentration) 1X NH_4^+ reaction buffer, 3 mM $MgCl_2$, 200 μM of deoxynucleotide mix, 0.5 U μl^{-1} BioTaq™ DNA polymerase (all reagents from Bioline, UK), 0.2 μM of each primer (Sigma-Aldrich), 0.3 mg ml^{-1} bovine serum albumin (New England Biolabs, USA) and 2 μl of DNA template were used (Chen *et al.*, 2007).

Both *pmoA* and Type II 16S rRNA genes (to cover genera that does not contain *pmoA* gene) were amplified using PCR. For 16S rRNA the annealing temperature was set at 60^{o}C and 35 cycles were performed. For *pmoA* the annealing temperature was set at 56^{o}C and 30 cycles were performed. After initial denaturation at 96ºC for 5 min (95^{o}C in case of Type II 16S rRNA), each PCR cycle consisted of 1 min at 94ºC, 1 min at the annealing temperature, 1 min at 72^{o}C and a final extension at 72^{o}C for 10 mins (Bodrossy *et al.*, 2003). Amplification was performed in a total volume of

50 µl, for both target genes, in a DNA Engine Dyad® Peltier Thermal Cycler (Bio-Rad, USA). Aliquots of the amplicons (3 µl) were checked by electrophoresis on 1% (w/v) agarose gel stained with SYBR® Safe (Life Technologies, USA) and observed using a Gel Doc XR+ system.

For profiling of the *pmoA* and 16S rRNA genes using T-RFLP analysis, labelled primers were used (Table 2-1) and target genes were amplified by PCR as described above. PCR products were purified using Wizard® SV Gel and PCR clean-up system as per the recommendation by the supplier (Promega Corporation, USA). Purified PCR products were then quantified with NanoDrop 2000C spectrophotometer (Thermo Scientific) and approximately 200 ng of purified target gene was digested in a 20 µl volume containing 1X NEB buffer with 0.2 mg ml^{-1} bovine serum albumin and 10 U of restriction endonucleases: *Hha*I (*pmoA*) and *Mbo*I (16S rRNA) (all reagents from New England Biolabs, USA). The reactions were incubated at 37^{o}C for three hours in a DNA Engine Dyad® Peltier Thermal Cycler (Bio-Rad, USA) and the enzymatic reaction was stopped by incubation at 95^{o}C for 10 mins.

Aliquots of digested PCR products (2 µl) were mixed with 7.7 µl of Hi-Di™ formamide and 0.3 µl of GeneScan™-600 LIZ® internal size standards (Applied Biosystems, UK). The mixtures were denatured at 95°C for 3 min and then chilled on ice. T-RFLP analysis was performed on an automated genetic analyzer ABI3500 (Applied Biosystems, UK). Terminal restriction fragments (T-RFs) generated by the sequencer were analysed using the size-calling software GeneMapper™ 4.0 (Applied Biosystems, UK) and quantified by advanced mode using second order algorithm. T-RFs in a T-RFLP profile were selected by the software if their minimum peak height was above 25 relative fluorescence units. Only TRFs between 25-600 bp were considered in order to avoid peaks caused by primer-dimers and to obtain fragments

within the linear range of the internal size standard (Singh *et al.*, 2007). T-RFs with peak height comprising less than 0.5% were removed before downstream analysis and the peaks that differed by less than 1 bp were binned into the same fragment. The relative abundance of a T-RF in a profile was calculated as a proportion of the total peak height of all the T-RFs in a profile for further analysis.

2.2.7.4 *pmoA*-based cloning, sequencing and phylogenetic analysis

The phylogenetic assignments of individual *pmoA* T-RF was confirmed using a cloning and sequencing approach. This was performed only on the ^{13}C-DNA fragments from the SU soils to identify the methanotrophic community involved in the TCE degradation. The *pmoA* gene was amplified, purified (as described in Section 2.2.7.3), cloned using the pGEM®-T Easy Vector System (Promega Corporation, USA) and transformed into *E. coli* JM109 competent cells (Promega). To minimise PCR bias and sample variation, replicates from SU soil were pooled prior to cloning. About 25 clones were selected from SU soil cloned library and screened for *pmoA* gene inserts with the vector-specific SP6/T7 primers. PCR amplicons were purified as described in Section 2.2.7.3.

Sequencing was performed using the BigDye Terminator cycle sequencing reaction kit (v3.1 kit, Applied Biosystems). The quality of sequences were checked (edited, assembled and aligned) using Sequencher 4.10.1 (Gene Codes Corporation), excluding the primer regions that were present in all sequences. The good-quality sequences thus obtained were then exported to ExPASy (http://web.expasy.org) to translate nucleotide sequences of *pmoA* gene to confirm the presence of functional proteins and no stop codons. Sequences were analysed for phylogenetic similarity in

the NCBI database (http://www.ncbi.nlm.nih.gov) using BLAST and related sequences were included in alignments and compared with MUSCLE (MEGA 6). A phylogenetic tree was constructed using MEGA 6 (Molecular Evolutionary Genetics Analysis) (Tamura *et al.*, 2013) by performing maximum likelihood tree analysis with 1000 bootstrap replicates using Kimura-2 with the gamma distribution algorithm (K2+G).

Furthermore, sequences obtained from individual clones were virtually digested (*Hha*I recognition sequence) to predict T-RF size in Microsoft Office® Word. The size of the *in silico* T-RFs was confirmed experimentally to match individual clones with specific T-RFs in the ^{13}C SU soil mixed cultures T-RFLP profiles. To do so, DNA from the selected clones was amplified using labelled *pmoA* primers (Table 2-1) and digested with *Hha*I as described previously in Section 2.2.7.3

2.2.8 Statistical analysis

Statistical data analysis was carried out with the software package IBM SPSS Statistics 17 (SPSS, Inc.). To investigate the differences in the methane oxidation and TCE degradation between different types of soils, an independent samples *t*-test was carried out. Level of significance (*p* value) was set to at 0.05. The Univariate test (SPSS, Inc.) was also used to determine the main soil properties (pH, moisture, total C and total N) that would affect the methanotrophic community structure. Bacterial community analysis was conducted using PRIMER v.6 (PRIMER-E Ltd, UK). Relative T-RFLP abundance data (*pmoA* and 16S rRNA) that had been square root transformed prior to analysis was used to generate a Bray-Curtis similarity matrix. Multivariate analysis (PERMANOVA) was used to determine the influence of soil

type and methane type ($^{12}CH_4/^{13}CH_4$) on the methanotrophic community structure. A non-metric multidimensional scaling (MDS) analysis was carried out using the default programme settings to obtain an ordination diagram.

2.3 Results

2.3.1 Methane oxidation activity of soils

During 15 days of incubation, CH_4 oxidation was rapid in three soil samples, namely SU, VP and BI (Figure 2-1). A significant decrease in CH_4 concentration was observed within 5 days (VP soil) and 7 days (SU soil, BI soil) incubation at 20^{o}C (p <0.001). By contrast, no CH_4 consumption was observed in the soils from HFE forest, non-grazing pasture, grassland and EucFACE driftway even after 15 days of incubation (p>0.05). Therefore, all further degradation experiments were carried out on SU, VP and BI soils that showed active oxidation of CH_4. Selected soil properties of the methane-oxidising soils are given in Table 2-2.

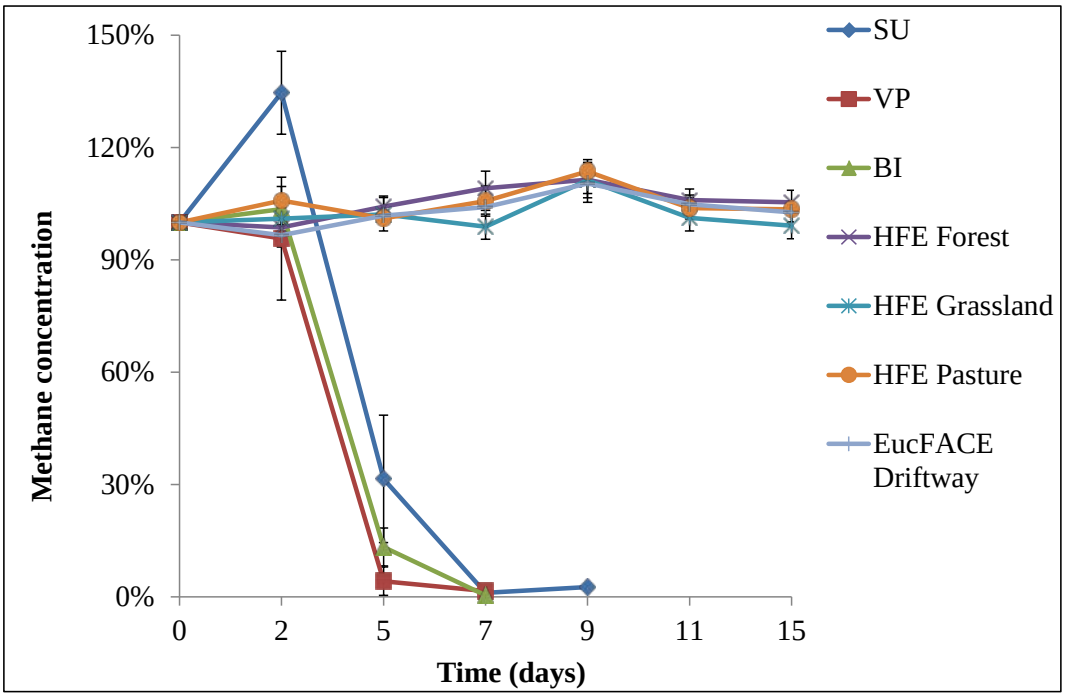


Figure 2-1 Methane (CH_4) oxidation by enriched methanotrophic consortia from different soils in nitrate mineral salt (NMS) liquid media over time. Error bars represent standard error of the mean (n=3). Legends: SU=Sydney university, VP=Victoria Park, BI= Botany Industrial Park, HFE= Hawkesbury forest experiment

Table 2-2 Selected soil properties of the soils from Sydney University (SU), Victoria Park (VP) and Botany Industrial Park (BI) that showed methane oxidation activity.

Soil Properties	pH	Carbon (%)	Nitrogen (%)	Moisture (%)
SU	7.1±0.2	3.3±0.1	0.2±0.0	1.4±0.1
VP	6.6±0.2	5.5±0.6	0.3±0.0	2.4±0.1
BI	7.2±0.0	1.3±0.1	0.02±0.0	5.8±0.0

2.3.2 Effect of different methane concentration on TCE degradation by a methanotrophic consortium

Only the soils that showed CH_4 oxidation activity (Figure 2-1) were selected for the assessment of the effect of CH_4 concentration on TCE degradation. When incubated with 33% CH_4 concentration, pre-grown cultures from all three selected soils oxidised CH_4 at a similar rate ($p<0.001$) (Figure 2-2a); however, only the SU methanotrophic consortium was able to degrade (up to 30%) the added TCE ($p<0.05$) (Figure 2-2b). Victoria park (VP) soil appeared to have some TCE degrading capabilities but this was not statistically significant from the control ($p>0.05$), whereas BI soils appeared unable to degrade TCE. The degradation of CH_4 and TCE started simultaneously in SU soil consortium (Figure 2.2a, b). The maximum degradation of TCE by the SU soil methanotrophic consortium occurred during the active phase of cell growth (Figure 2-2c) and stopped after two days, and no further depletion of CH_4 or TCE occurred (Figure 2-2 a, b) during the 8 day incubation period.

Since TCE degradation stopped after two days, the experiment was repeated on degrading SU soils with frequent sampling (hourly) to observe the TCE degradation trend. The results suggested that the degradation of TCE started after 24 hours of the incubation under 33% methane and the maximum degradation was observed at 36^{th} hour (Figure A-1a, b). The TCE concentration after this remained constant and no further decrease was seen till the end the of incubation period. The degradation trend was similar to the one when sampling was done every two days (Figure 2-2b).

All the enriched cultures were accompanied with an increase in biomass and SU cultures show a sharp decrease in OD_{600} after 10 days of incubation (Figure 2.2c). No decrease in the OD_{600} was observed in SU soil control where no TCE was added to the microcosm (data not shown). When CH_4 wasn't added to the headspace gas of the degrading soil microcosm (SU), neither TCE degradation (Figure 2-2d) nor cell growth (data not shown) was observed.

On the other hand, although the pre-grown cultures from all the three soil types completely oxidised 1% CH_4 ($p<0.001$) (spiked 2 times) at the same rate, no degradation of TCE ($p>0.05$) was observed, in this case, during 10 days of incubation period (Figure 2-3a, b). 1% CH_4 was completely consumed by the growing cultures within two days of incubation (Figure 2.3a). Due to this rapid decrease in CH_4 concentration the microcosm were spiked with 1% CH_4 another two times. To maintain aerobic conditions, the microcosms were flushed with air before injecting CH_4, which also resulted in spiking the microcosm with TCE too (in total three injections). No degradation of TCE can be ascribed to the low CH_4 concentration resulting in decrease in biomass (Figure 2-3c) and operational stability to degrade TCE.

Multiple injections of TCE resulted in declining CH_4 consumption rates of the consortium. Between days 0 and 6 CH_4 was consumed within two days, but after the second TCE spike on the 6th day the CH_4 uptake rate decreased (Figure 2-3a). The additive toxic effect of TCE might have caused this decline. Degradation of TCE and no cell growth was also observed with any of the soil types in absence of CH_4 (data not shown). No loss of TCE and CH_4 was observed in the abiotic controls (33% and 1% experiments, Figure 2-2, 2-3, respectively) suggesting the degradation occurred due to microbial activity

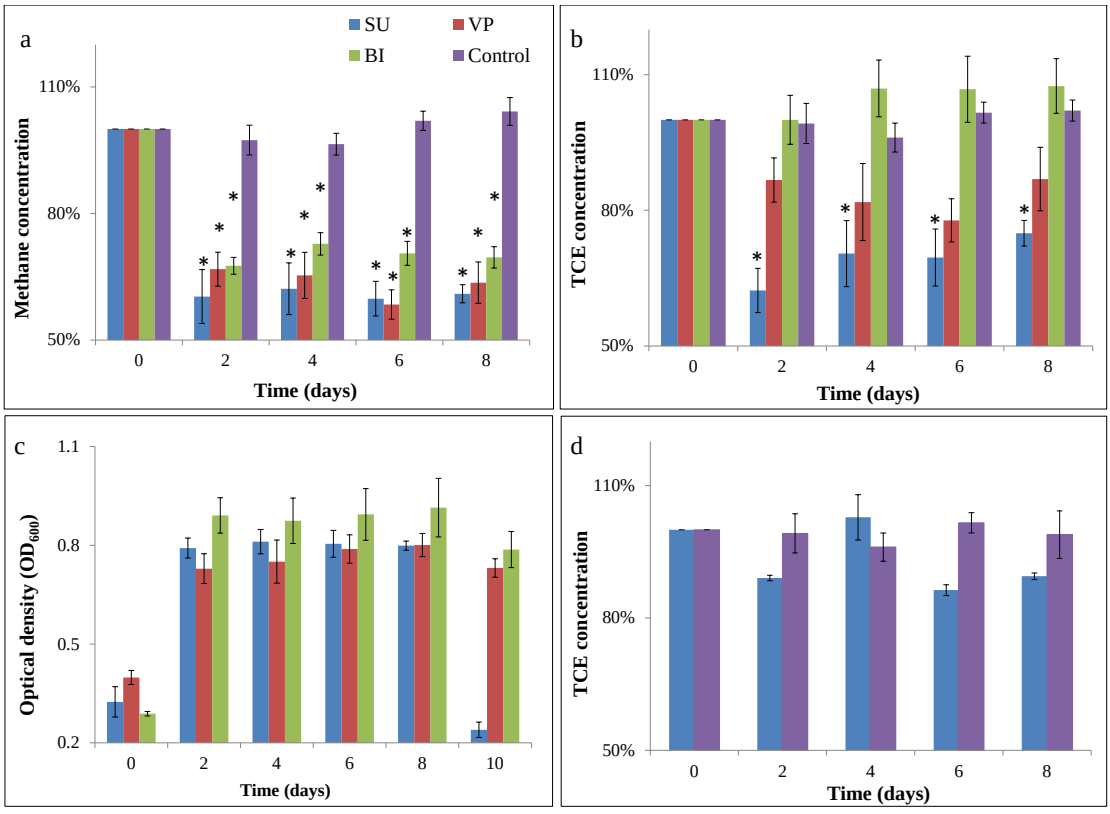


Figure 2-2 (a-c) Methane oxidation (33%), TCE degradation and corresponding growth of mixed cultures (control not shown) from three Australian soils; d) TCE degradation in SU cultures in absence of CH_4. Error bars represent standard error of the mean (n=3). The symbol (*) represents statistically significant *p* values (when compared with controls). Legends: SU=Sydney University, VP=Victoria Park, BI= Botany Industrial Park.

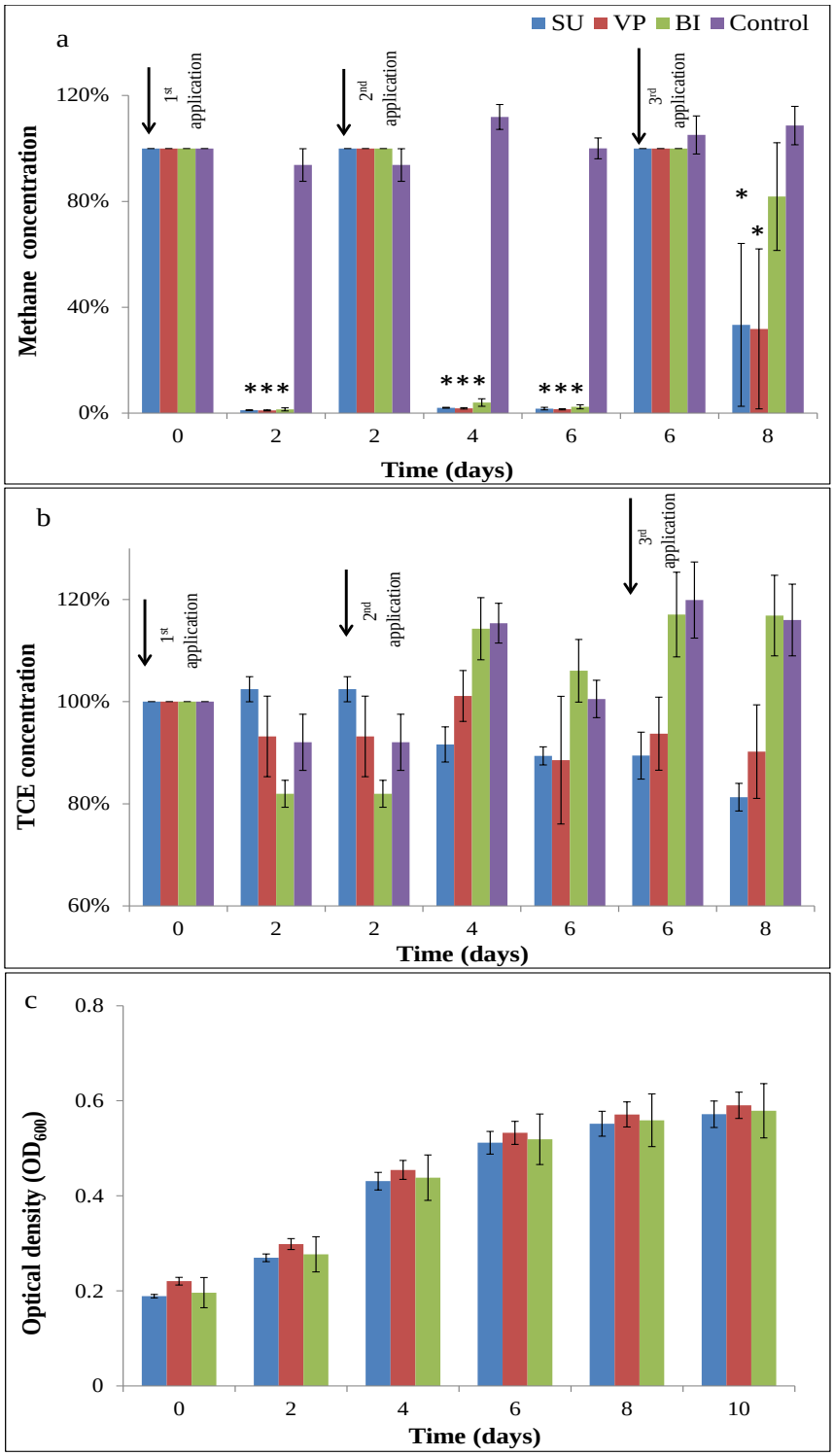


Figure 2-3 (a-c) Methane (CH_4) oxidation (1%), TCE degradation and corresponding growth of mixed cultures (control not shown) from three Australian soils where arrow represents re-spiking of the cultures with CH_4 and TCE. Error bars represent standard error of the mean (n=3). The symbol (*) represents statistically significant *p* values (when compared with controls). Legends: SU=Sydney University, VP=Victoria Park, BI= Botany Industrial Park.

The experiment was repeated with SU soil at 10% CH_4 headspace concentration based on the results of the TCE degradation experiments under 33% and 1% CH_4 concentration. The addition of 10% CH_4 promoted TCE degradation (up to 20%) within 5 days of the incubation period (Figure 2-4). The degradation of TCE at 10% CH_4 was, however, lesser than in 33% CH_4 (Figure 2-2b) further indicating that an optimal CH_4 concentration is required for high-density biomass production of methanotrophs resulting in increased TCE degradation.

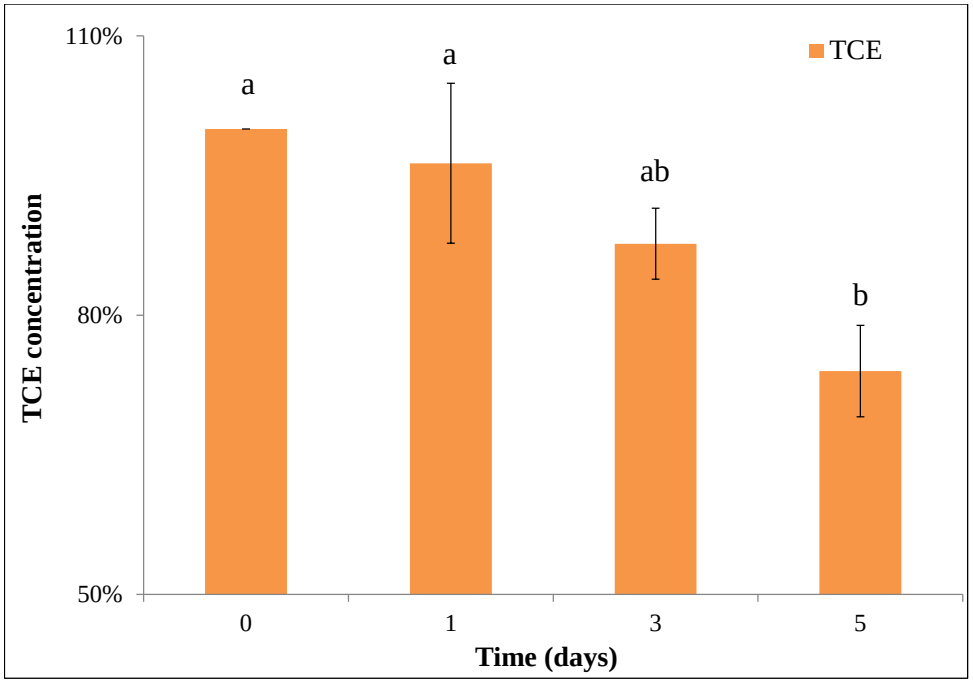


Figure 2-4 TCE degradation by the methanotrophic consortium from Sydney University (SU) soil at 10% methane (CH_4) concentration in nitrate mineral salt (NMS) liquid media over time. Error bars represent standard errors (n=3). Different letters indicate values that are significantly different ($p < 0.05$)

2.3.3 Characterisation of enriched TCE degrading methanotrophic consortia

2.3.3.1 ^{13}C-labelling and q-PCR

The incorporation of ^{13}C into DNA of the pre-grown cultures (SU, VP and BI soil, Section 2.2.7.1) was measured by quantification of the relative abundance of *pmoA* gene copies in each SIP fraction (Figure 2-5). The mixed cultures, from all soil types grown in presence of ^{13}CH4+TCE, showed an increase in DNA density and a clear shift in the distribution of *pmoA* genes towards the heavy fraction (^{13}C-DNA) when compared with unlabelled DNA (^{12}C-DNA) suggesting an incorporation of labelled CH_4 as a C-source. However, one of the replicate from SU soil was lost during SIP incubation and further molecular analysis on SU soil was done using two replicates.

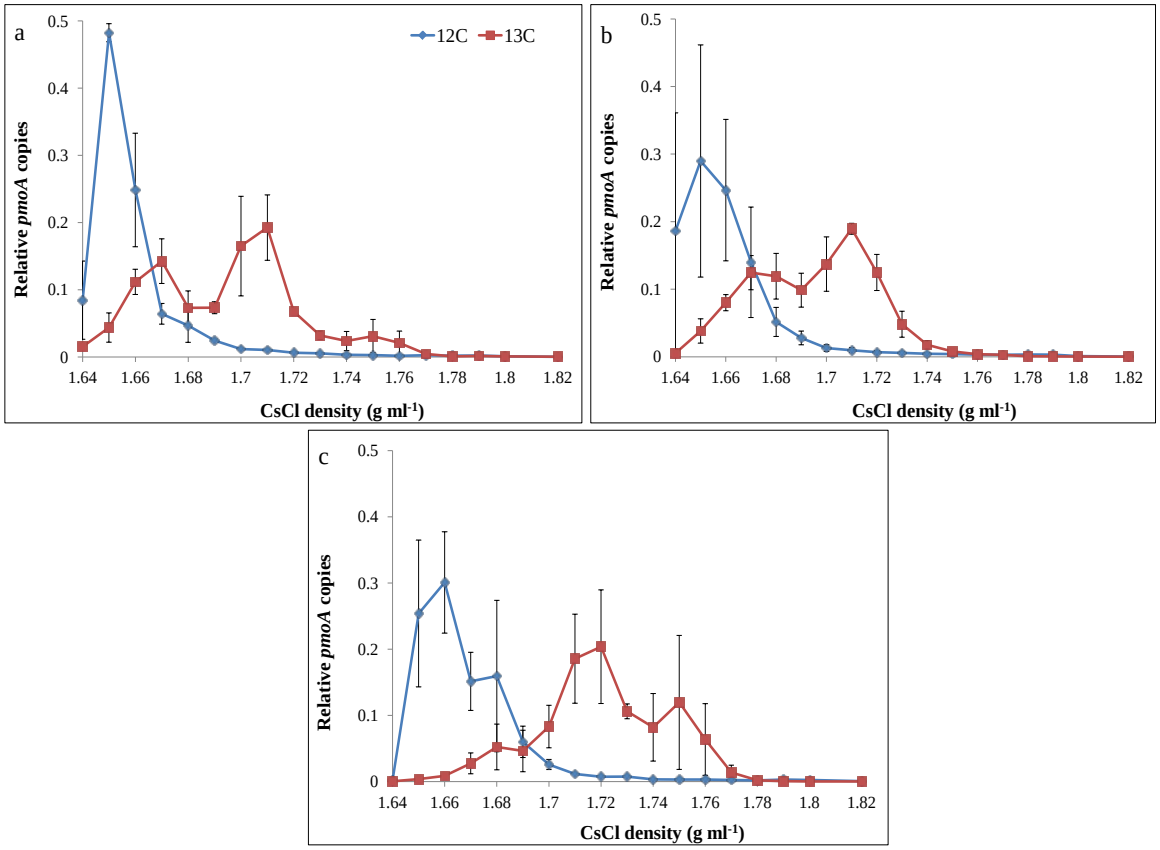


Figure 2-5 Distribution of the relative abundance of *pmoA* gene in CsCl gradient for $^{13}CH_4$ and $^{12}CH_4$ treatments during DNA-SIP microcosm of mixed cultures from three selected Australian soils: a) Sydney University (SU) b) Victoria Park and (VP) and c) Botany Industrial Park (BI). A total of eighteen fractions were recovered after CsCl ultracentrifugation of the ^{13}C- and ^{12}C-DNA. Relative abundances rather than absolute *pmoA* gene counts were used to facilitate comparison. The y-axis indicates relative abundance at each gradient fraction, with the total quantity detected from a gradient (^{12}C-DNA or ^{13}C-DNA) equal to 1.0. Error bars represent standard errors (n=3 for VP, BI and n=2 for SU soils)

2.3.3.2 T-RFLP analysis

Most of the replicates produced similar *pmoA*-based T-RFLP profiles of the methanotrophic community, but there was a marked difference between the profiles from BI, VP and SU soils on the whole. The most dominant *pmoA* T-RF that was present in all the soil types was of size 124 bp, which dominated VP soil (82%) and was well represented in SU soil (50%) and BI soil (35%) (Table 2-3). Another *pmoA* T-RF of size 53 bp was abundant in SU soil (40%). The *pmoA* T-RF 53 was present in VP soil but accounted for only 0.8% and it was not found in BI soil. On the other hand, 16S rRNA-based T-RFLP profiles showed that one dominant T-RF of size 402-bp was present in BI soil (30%) and VP soil (32%) (Table 2-3). A list of other dominant T-RFs associated with *pmoA* and 16S rRNA-based T-RFLP profiles in the particular soils is given in Table 2.3.

PERMANOVA analysis revealed that the methanotrophic community structure differed between soil samples from the three sites (Table 2-4). There was no significant effect of methane type (total $^{12}CH_4$ and active $^{13}CH_4$) on the methanotrophic populations. A univariate test (SPSS, Inc.) was also performed to examine the effect of main environmental factors affecting the methanotrophic community. It was observed that *pmoA*-based methanotrophic community differ based on soil type where total C ($p<0.05$), total nitrogen ($p<0.01$) and moisture ($p<0.001$) were found to be significant. However, for 16S rRNA-based, only soil pH ($p<0.05$) was found to be significant factor.

Table 2-3 Relative abundances of T-RFs (%) produced after digestion of the *pmoA* and 16S rRNA genes with the restriction enzymes *Hha*I and *Mbo*I respectively for Sydney University (SU), Victoria Park (VP) and Botany Industrial (BI) park soils. The sign (-) indicates absence or less than 1% dominance of the T-RF in the sample.

Soil type	*pmoA* (*Hha*I)				16S rRNA (*Mbo*I)		
	TRF-35	TRF-53	TRF-77	TRF-124	TRF-402	TRF-522	TRF-524
Phylogenetic assignment	USC α related	*Methylococcaceae*	*Methylococcaceae/ Methylocystaceae*	*Methylococcaceae/ Methylocystaceae*	*Methylocystaceae*	Unknown	Unknown
SU	-	40%	-	50%	-	35%	50%
VP	-	-	7%	82%	32%	35%	-
BI	34%	-	16%	35%	30%	40%	25%

Table 2-4 PERMANOVA table of results for the T-RFLP data *versus* different factors and their interactions. (* 95% significance)

Factors	*p* value	
	pmoA	16S rRNA
Soil type	0.007*	0.011*
Methane type ($^{12}CH_4$/ $^{13}CH_4$)	0.939	0.967
Soil type*Methane type ($^{12}CH_4$/ $^{13}CH_4$)	0.986	0.988

The results of the PERMANOVA analyses were supported by MDS biplot, which showed that despite a significant impact of soil type, no noticeable influence of methane type was observed for any of the site for both pmoA and 16S rRNA T-RFLP data (Table 2-4). There were notable differences in the methanotrophic communities (*pmoA*-based T-RFLP) associated with SU and BI soils, however, methanotrophic communities from VP soil clustered with both SU and BI on the MDS biplot (Figure 2-6). On the other hand, for 16S rRNA-based T-RFLP all the three soil types (SU, VP, and BI) clustered separately from each other (Figure 2-7)

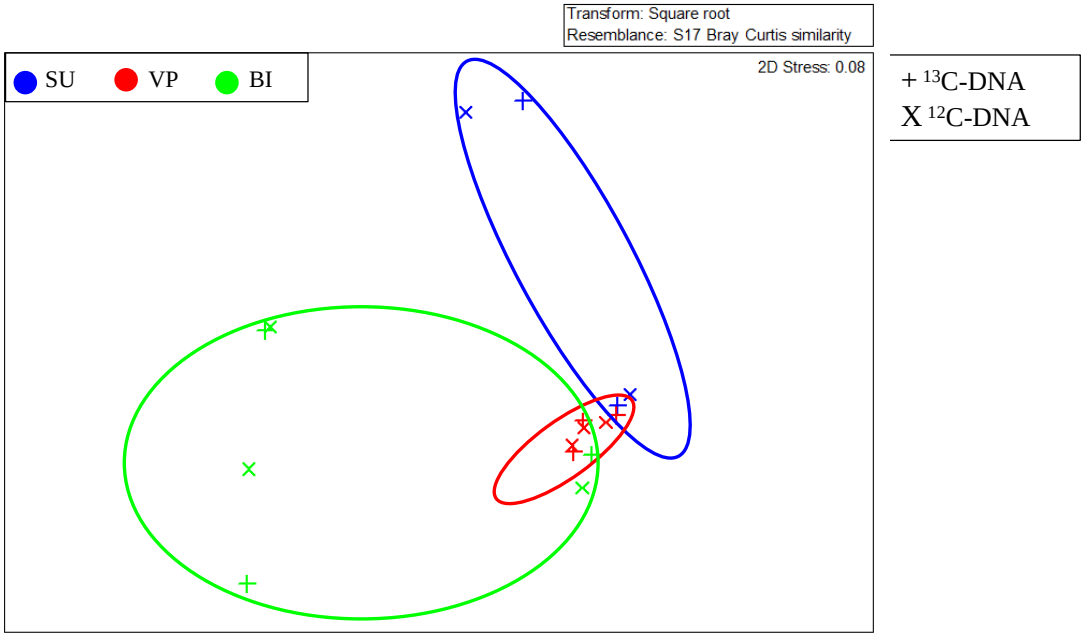


Figure 2-6 2-D MDS ordination of *pmoA*-based methanotrophic community structure obtained for different site-enrichment combinations based on the √-transformed gene abundance and Bray-Curtis index (stress=0.08). The methanotrophic community for each site (both ^{12}C and ^{13}C) is grouped together, represented here by a circle. T-RFLP was performed on the pooled light (unlabelled) and heavy fractions (labelled) for each replicate from three mixed cultures from the three selected Australian soils. Abbreviations: SU=Sydney university, VP=Victoria Park and BI= Botany Industrial Park.

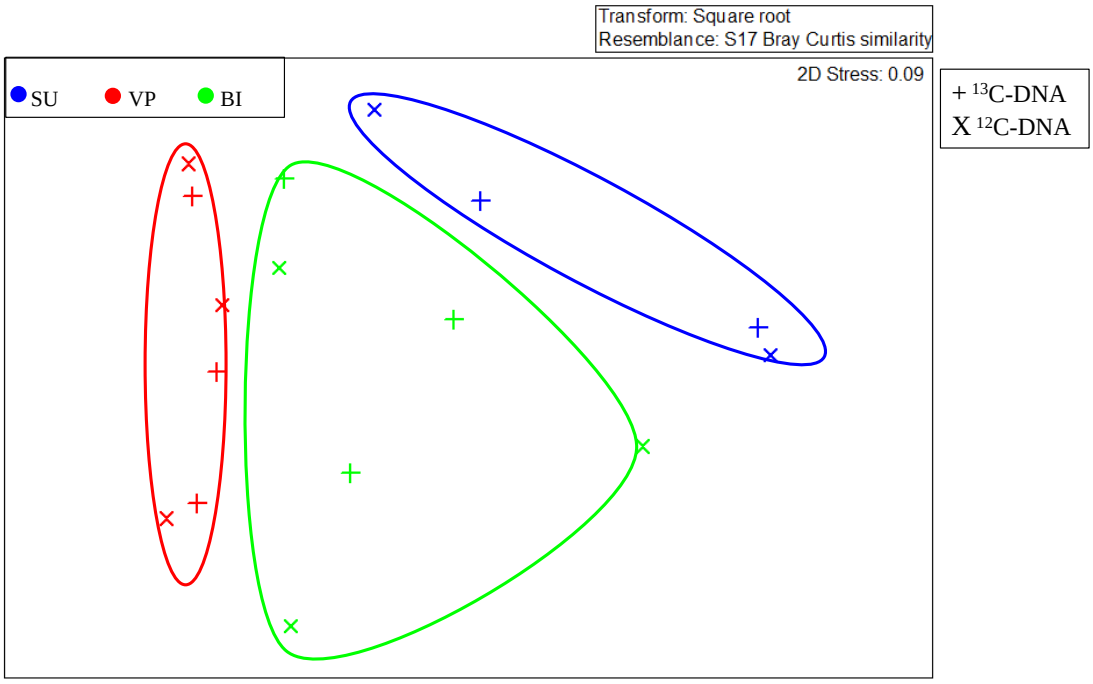


Figure 2-7 2-D MDS ordination of 16S rRNA-based methanotrophic community structure obtained for different site-enrichment combinations based on the √-transformed gene abundance and Bray-Curtis index (stress=0.09). The methanotrophic community of each site (both ^{12}C and ^{13}C) is represented here by a circle. T-RFLP was performed on the pooled light (unlabelled) and heavy fractions (labelled) for each replicate from three mixed cultures from the three selected Australian soils. Abbreviations: SU=Sydney university, VP=Victoria Park and BI= Botany Industrial Park

2.3.3.3 *pmoA*-based cloning, sequencing and phylogenetic analysis

A total of 25 clones (numbered from 1 to 25) from ^{13}C SU soil were selected for further analyses, but 3 clones (13, 18, and 25) failed to produce the expected PCR product and hence were excluded from further analysis. The remaining 22 clones were all identified as *pmoA* gene sequences. Alignment of the *pmoA* sequences from all 22 clones with the GenBank database showed that they were all related to *pmoA* from Type I methanotrophs (Figure 2-8). Three major clone groups were identified; thirteen clones (groups G1 and G2 with T-RFs 130 and 57 respectively) clustered

within the genus *Methylovulum* showing 88% identity with *Methylovulum miyakonense* HT12 (GenBank Accession no. AB501285) as the most closely related cultured methanotroph. Seven clones (group G3 with T-RF 130) and one clone (clone 14 with T-RF 130) clustered with the genus *Methylobacter* with 89% similarity (GenBank Accession no. AF016982). Clone 4 showed 92% similarity with *Methylomicrobium album* (GenBank Accession no. FJ713039). Different clustering groups (G1, G2 and G3) were seen but they all belonged to the family *Methylococcaceae* (Type I methanotrophs) (Figure 2-8). Although the clones showed similarity to methanotrophic species from soil, sediments and TCE oxidation studies, they were not closely related to any pure isolated culture. In contrast, most of the *pmoA* sequences were closely related to uncultured methanotrophs/clones.

Clonal group G1 was 99% similar to an uncultured methanotroph from liquid manure crusts (GenBank Accession no. JN790620), group G2 showed 99% similarity with uncultured methanotrophs obtained from grassland soil (GenBank Accession no. JF798628) and 98% similarity from landfill cover soil (GenBank Accession no. EU292155) and group G3 was closely related (97%) to uncultured methanotrophs from rice rhizosphere soil (GenBank Accession no. JQ671236). Likewise, clone 4 showed 99% similarity with uncultured methanotrophs from wetland soil (GenBank Accession no. JQ038171), landfill soil (GenBank Accession no. EU275104), and rice paddy soil (GenBank Accession no. JX113066), and clone 14 showed 96% similarity with uncultured methanotrophs from landfill soil (GenBank Accession no. EF472939) and lake sediments (GenBank Accession no. HQ383760). Thus, it appeared that all the *pmoA* clones represented unknown/uncultured methanotrophs.

The *in silico* digestion of the clonal *pmoA* sequences with *Hha*I enzyme showed the presence of two dominant T-RFs of 57 and 130 bp present among the different clonal

groups; only clone 14 did not contain a *Hha*I restriction site (Figure 2-8). Theoretical T-RFs of the clones (T-RFs 57 and 130) were experimentally verified and showed slight differences in size (T-RFs 53 and 124, respectively). This was considered to be a normal drift due to capillary migration during electrophoresis. The slight difference between the measured and predicted fragment lengths data has also been previously reported (Xie *et al.*, 2010b, Xie *et al.*, 2011b). The T-RFs profile of the clones (*in silico* and experimentally) confirmed the identity of T-RFs 53 and 124 as being two distinct operational taxonomic units (OTUs) and distantly related to cultured methanotrophs *Methylomonas* sp. as shown in Figure 2-8. Only the dominant T-RFs were identified from the T-RFLP profile as the number of clones sequenced was small (25) indicating the dominance of Type I methanotrophs in SU soil samples.

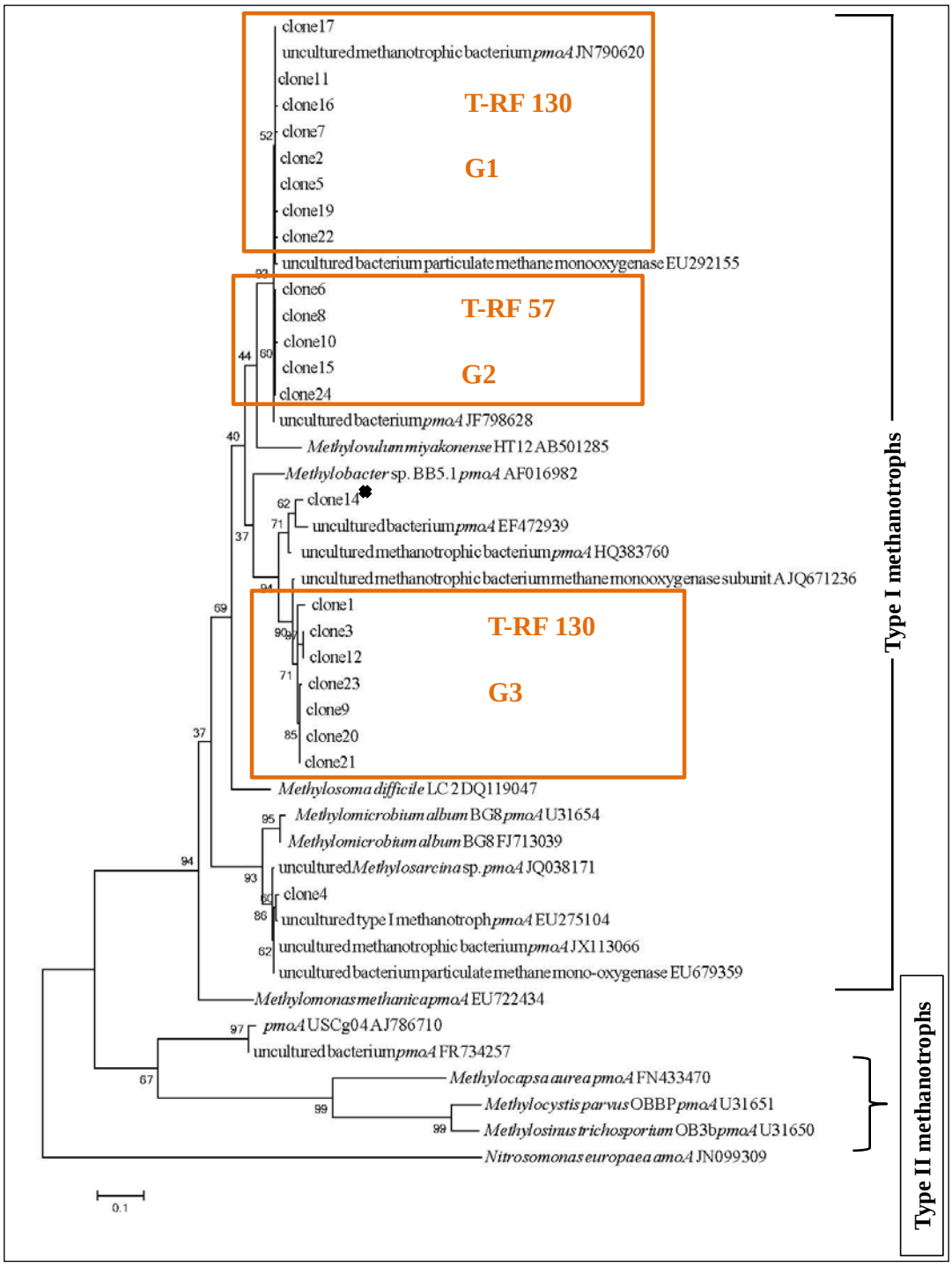


Figure 2-8 Maximum likelihood phylogenetic tree representing *pmoA* gene sequences derived from the ^{13}C fractions of mixed methanotrophic cultures from the SU soil that received $^{13}CH_4$ and related species based on the *pmoA* gene sequences retrieved from NCBI. The tree was rooted with *Nitrosomonas europaea.* The Bootstrap values expressed as randomisation of 1000. The scale bar represents the evolutionary distance of 0.1. The symbol (✖) indicates that the clone did not possess a *Hha*I restriction site. Boxes represent the three different clonal groups (G1, G2 and G3) along with their respective *in silico*-determined T-RF size.

2.4 Discussion

2.4.1 Methane oxidation activity of soil

The immediate and complete oxidation of 1% v/v CH_4 in the SU, VP and BI soils indicated the presence of microorganisms in soils that were actively consuming CH_4 as a C-source, which resulting in the selective enrichment of low affinity methanotrophic communities. The other four soils used in this study (HFE forest, pasture, grassland and EucFACE driftway) did not show any signs of CH_4 oxidation during the 15 days of incubation presumably due to the absence of active methanotrophs, slower rates of developing methanotrophic capacities and/or the high concentrations of CH_4 that the soils were exposed to. Methanotrophic communities and activities are greatly affected by soil properties and environmental conditions (Reeve *et al.*, 2010), but as the environmental conditions were similar for all soil microcosms (soil+NMS) the differences in the CH_4 oxidation could be attributed to the differences in composition of the microbial communities due to different soil properties between sites (field conditions).

Previous studies have also reported the influence of soil organic matter (SOM) content on CH_4 oxidation rates, along with other parameters (Czepiel *et al.*, 1995). Since the methanotrophic communities were enriched from soil types that varied in SOM content [0.7% in HFE soils (Barton *et al.*, 2010); 0.5% in EucFACE], and the soils with higher OM content (Table 2.2) showed accelerated CH_4 oxidation, it could thus suggest that there could be a link between the SOM content and maximal CH_4 oxidation activity of soils (Börjesson and Svensson, 1997, Christophersen *et al.*, 2000, Merino *et al.*, 2004). On the other hand, in the present study the BI soil had a low SOM content like that of HFE and EucFACE soils, but showed similar CH_4

oxidation activity to SU and VP soils. This could be due to the presence of an active indigenous methanotrophic community in this soil (as those present in SU soil and VP soil). BI is one of the many contaminated sites in the Sydney region and has a long history of chlorinated hydrocarbon contamination. Studies on the microbial community structure in hydrocarbon-contaminated areas have also shown greater presence of methanotrophs compared with non-contaminated soils (Bowman *et al.*, 1993a, Yan *et al.*, 2006, Allen *et al.*, 2007). Thus it can be concluded that despite of low soil OM content, BI soil showed an accelerated CH_4 oxidation capacity due to the presence of active methanotrophic communities.

2.4.2 Effect of methane concentration on TCE degradation by a methanotrophic consortia

Mixed methanotrophic consortia were obtained from three different Australian soils, grown under similar environmental conditions and different CH_4 concentrations (33% v/v and 1% v/v). The decrease in CH_4 concentration (at both 33% and 1% initial CH_4 concentration) and an increase in cell growth (OD_{600}), in all three mixed consortia, suggested that CH_4 was utilised as a source of C and energy. A time course experiment established to determine the TCE degradation under 33% CH_4 concentration showed degradation only with methanotrophic consortium in SU soils. The degradation was fast enough in those soils to result in significant removal within the set incubation time. Since all three consortia were grown under the same growth conditions and could utilize CH_4, the difference in TCE degradation could be attributed to different inherent abilities of the particular methanotrophic populations in degrading TCE. Similar results were reported previously where only three out of

eight mixed cultures of CH_4-oxidising bacteria, from seven different water systems, were able to degrade TCE in the presence of CH_4 or methanol as a growth substrate (Broholm *et al.*, 1993).

The methanotrophic consortium from SU could not consume TCE as a source of energy and growth, suggesting a co-metabolic mechanism of TCE degradation. This was supported by incubating the methanotrophic consortium from SU under 1% CH_4 or no added CH_4 (control) where TCE was not consumed, while growth, as indicated by turbidity (OD_{600}), took place only in CH_4 amended microcosm. Co-metabolic degradation of TCE and other chlorinated organic compounds, with any organisms having active oxygenases, has been widely reported (Ward, 1997, Suttinun *et al.*, 2013). For example, *Methylosinus trichosporium* OB3b expressing MMO is reported to be involved in TCE co-metabolism (Oldenhuis *et al.*, 1989, Fox *et al.*, 1990, Chu and Alvarez-Cohen, 1998). Other studies on TCE degradation by *Pseudomonas putida* (Guo *et al.*, 2001), degradation of chlorinated aliphatic hydrocarbons by methanotrophs (Powell *et al.*, 2014), and a field study on TCE removal from groundwater (Conrad *et al.*, 2010) also reported the co-metabolic degradation of TCE.

CH_4 oxidation and TCE degradation was accompanied by an increase in initial cell OD_{600} but stopped after two days and remained constant through-out the incubation period. This could be because of non-availability of oxygen as the system was already saturated with a high concentration of CH_4 (initial concentration of 33%) and increased cell density (2 days) exhausted the oxygen supply more quickly. This was also supported by incubating the methanotrophic consortium from SU under 10% CH_4 concentration where a gradual decrease in the TCE concentration was seen over a period of 5 days (Figure 2.4). Since TCE is a volatile substrate, to avoid the loss by

evaporation the experiment was designed in a closed system and no oxygen loss could be replaced. The oxygenase enzymes generally requires oxygen molecules during the oxidation of both energy generating and co-metabolic substrates (Arp *et al.*, 2001) and thus lack of oxygen would have caused the inhibition of CH_4 or TCE degradation. This also resulted in the reduction in microbial growth, and eventually death, in all three methanotrophic consortia. These results were in agreement with a study where the increase in *Pseudomonas fluorescens* cell density resulted in exhaustion of oxygen and leading to the reduction in micro-organism activity and even death (Li *et al.*, 2014).

A steep decrease in OD_{600} seen only for the SU consortium could be the result of product toxicity resulting from TCE co-metabolism. Previous studies have reported toxicity resulting from co-metabolic oxidation of chlorinated solvents (Alvarez-Cohen and McCarty, 1991, Rasche *et al.*, 1991, Ensign *et al.*, 1992, Heald and Jenkins, 1994, Field and Sierra-Alvarez, 2004, Jiang *et al.*, 2010). This toxicity could result in damage to the oxidative enzyme itself or damaging the cellular respiration and viability of the bacterial community (Halsey *et al.*, 2005). In another study Heald and Jenkins (1994) showed similar results where TCE oxidation by *Pseudomonas putida* containing toluene dioxygenase caused a decrease in the growth rate of cultures and rapid cell death. A number of studies with methanotrophs have reported either linear (Anderson and McCarty, 1994, Ely *et al.*, 1995, Chang and Criddle, 1997) or exponential (Van Hylckama *et al.*, 1996) inactivation of cells due to chlorinated solvent oxidation. Tompson *et al.* (1994) showed that during a two-week experiment, both linear and exponential inactivation models adequately fitted their experimental data describing the degradation of TCE by resting cells of *Methylosinus trichosporium* OB3b. The author also observed that cell inactivation was not solely

due to toxicity, but also due to starvation, deprivation of reductant and endogenous cell decay.

It is worth pointing out that the degradation of both CH_4 and TCE, in this study, began simultaneously indicating a) the degrading enzyme(s) were co-ordinately regulated or b) substrate competition for binding at the active site of the enzyme. Substrate competition between primary and co-substrate has been reported earlier where the rate of primary substrate utilisation was compared to the rate of TCE co-metabolism. It has been shown that for CH_4 degrading bacteria the rates of TCE transformation are comparable to CH_4 utilisation (Semprini, 1997). Some studies reported that low TCE and high CH_4 concentration have resulted in the inhibition of TCE transformation as all the active sites on the MMO are occupied with CH_4 (Strandberg *et al.*, 1989, Alvarez-Cohen and McCarty, 1991, Landa *et al.*, 1994, Kang *et al.*, 2001, Suttinun *et al.*, 2013).

A previous study that tested the effect of CH_4 on the degradation of TCE by *Methylosinus trichosporium* OB3b found that the TCE degradation rate decreased in the presence of CH_4 (Oldenhuis *et al.*, 1991). Similar results were observed with the degradation of TCE and *trans*-1,2 dichloroethylene (DCE) by a methanotrophic consortium in a fixed-film packed-bed bioreactor (Strandberg *et al.*, 1989). The authors also suggested that if there was a substrate competition between CH_4 and TCE/DCE for the MMO enzyme, then the restriction of the CH_4 supply might have improved the TCE degradation rate. However, it should be noted that the duration of experiments in those studies ranged from few minutes to hours. In experiments with longer incubations (*e.g.* in reactors), it should be noted that the rate of degradation depends greatly on the amount of biomass present and thus increasing TCE concentration would not help as it will decrease the growth of the active cells and

eventually leading to no degradation. This is also supported by a study in which a model incorporated cell growth and decay to understand the mechanism of co-metabolic degradation of TCE and phenol by *Pseudomonas putida* (Chen *et al.*, 2008a). The study reported that growth of cells decreased under higher TCE and declined to zero in low phenol concentration. Similar observations were made in a study that evaluated a model for co-metabolism of TCE and CH_4 by methanotrophic mixed cultures (Chang and Criddle, 1997). The model suggested that the amount of CH_4 needed to sustain transformation of TCE was related to the incubation time in a system and microorganisms could not sustain TCE transformation when TCE concentration was too high relative to CH_4. Another study also reported an increase in the rate of TCE degradation when the initial OD_{600} of the culture increased (Li *et al.*, 2014). Our results demonstrated that SU consortium under high CH_4 concentration resulted in higher cell density of the methanotrophic community and degraded more TCE when compared to 10% and 1% CH_4 concentration. Thus for maximum TCE degradation to occur optimal biomass, CH_4/TCE ratio and oxygen is required. These findings provide additional information about the interaction between CH_4 and TCE degradation (based on the competitive inhibition), which often limits the efficacy of the *in situ* bioremediation process, and thus could be useful in engineering models or tools for the effective *in situ* bioremediation of TCE contaminated areas.

2.4.3 Characterisation of TCE degrading methanotrophic communities

The effect of microbial community structure on TCE degradation was tested by fingerprinting of the *pmoA* and 16s rRNA genes using T-RFLP. 16S rRNA genes T-

RFLP was performed to identify the relative abundance of methanotrophs that do not possess *pmoA* genes (*Methylocella* and *Methyloferula*). The analyses of T-RFLP profiles of *pmoA* and 16S rRNA genes using PERMANOVA showed that the TCE degrading methanotrophic community from SU soil was different from that of VP and BI soils. T-RFLP result of SU replicates (*pmoA* and 16S rRNA) showed some differences in their profiles, which might have resulted from an inherent spatial variability. This was also evident from the difference in TCE degradation by the replicates (data not shown). Soil is one of the most complex and species-rich environments on Earth and there is often significant variability in community composition even at very fine scales within sites (Nielsen *et al.*, 2012). In a recent a recent study, Ramirez *et al.* (2014) showed that soil samples collected in Central Park (New York City, USA) per unit sampling effort harboured nearly as many distinct soil microbial phylotypes and types of communities as found in biomes across the globe further indicating substantial within-site heterogeneity.

Comparison of the *pmoA*-based T-RFLP profiles, confirmed the dominance of T-RF 53 (distantly related to Type I methanotrophs) only well-represented in TCE degrading SU soil. On the other hand, the 16S rRNA results showed the complete absence of T-RF 402 in the SU soil, suggesting the enrichment of Type I methanotrophs in SU soil in presence of TCE. The *pmoA*-based cloning data together with T-RFLP data supported that Type I methanotrophs were potential degraders of TCE in SU soils.

TCE can be degraded by a diversity of bacteria such as methanotrophs and species of *Pseudomonas* (Landa *et al.*, 1994, Cox *et al.*, 1998). In this study, TCE was likely degraded by a methanotrophic consortium from SU soil as evident from no degradation of TCE in absence or low concentration of CH_4. However, all attempts

to culture methanotrophs from SU soil were unsuccessful. Therefore, ^{13}C *pmoA*-based molecular analyses coupled with SIP approach was applied to identify the active TCE degraders (Radajewski *et al.*, 2000). From the *pmoA*-based cloning and sequencing data two dominant T-RFs (57 and 130 bp) were identified, indicating the dominance of two distinct types of methanotrophs. The *pmoA* data (though limited) showed that the sequences of the unclassified bacteria obtained in this study were not closely related to any cultured methanotrophs. Most of the sequences only showed 88-89% similarity with any other cultured sequence in the GenBank database. The clones also showed a low similarity with a cultured sequence obtained from estuary sediment (AF016982) capable of TCE oxidation (Smith *et al.*, 1997). The most closely related sequences came from several uncultured and unclassified species. Therefore, the microorganisms represented by fragments 57 and 130 bp (*Hha*I digest) could be novel candidate degraders of TCE. No *pmoA* sequences affiliated with Type II methanotrophs were found.

Although the clones were not closely related to any of cultured methanotrophic species, they clustered together within the family *Methylococcaceae* indicating the dominance of Type I methanotrophs in the SU soil. A similar observation was reported where Type I methanotrophs dominated in a nutrient rich soil and played an important role in the TCE degradation (Baker *et al.*, 2001, Knief *et al.*, 2006, Kong *et al.*, 2014). Thus in this study, the abundance of Type I methanotrophs might be attributed to the high CH_4 mixing ratio and nutrient condition such as organic content (3.3%) of the soil from which the communities were isolated/enriched. In addition to this, the higher abundance of T-RF 53 (SU soil),belonging to Type I methanotroph, exposed to TCE suggested that this particular TRF might be tolerant to TCE and play an important role in TCE degradation. No previous work has reported the role of this

methanotrophic strain (T-RF 53) in TCE degradation. Although uncultivable, the enrichment for this strain from the SU soil can be used for mesocosm and field studies to confirm its capability to remove TCE from the polluted environment.

2.5 Conclusion

This study aimed to characterise the novel methanotrophs in CH_4-assisted degradation of TCE. Activity and community structure of methanotrophs varied in the three soils (SU, VP and BI) exposed to TCE. Type I methanotrophs, in particular *pmoA* T-RF 53 dominated the SU soil and seemed tolerant to TCE, and is likely to play an important role in TCE degradation. Further assessments of uncultivable (while enriched) microbes related to this T-RF (53) would provide better understanding of the activity and community structure of methanotrophs in TCE contaminated soils and their degrading ability can be harnessed for successful removal of the compound. It was observed that maximum degradation of TCE was achieved at high CH_4 concentrations (33%). The higher methane concentration resulted in high-density biomass production of degrading methanotroph and thus, the feeding strategies can be used to manipulate indigenous methanotrophs in natural environment to improve the efficiency of *in situ* bioremediation. Furthermore, an additional insight on the microbial ecology of aerobic TCE degradation in mixed culture consortia was obtained and an important link between metabolic activity and phylogeny, which is often lacking in mixed culture studies, was illustrated.

Chapter 3: Effects of chlorpyrifos (CP) and imidacloprid (IC) on soil bacterial community composition

3.1 Introduction

Synthetic pesticides are purposely introduced and extensively used in agriculture as part of control strategies to protect crops against weeds, insects, fungi and other pests (Yang *et al.*, 2007). According to the guidelines for the approval of pesticides, they should be used in a way that their residues don't build-up in the environment and have minimal effect on the non-target organisms (Lo, 2010). However, the amount of pesticides applied that reaches the target organism is only about 0.1%, with the remaining bulk contaminating Earth's ecosystems and affecting public health by entering the food chain (Carriger *et al.*, 2006). On the other hand, some of the important cash crops like sugarcane require repeated application of different types of pesticide (Singh *et al.*, 2002b), raising the concern on its possible long term negative side-effect on the soil microbial communities.

Soil microorganisms play a vital role in important nutrient cycling and organic matter decomposition, contaminant removal and overall soil health (Wainwright, 1978, Lo, 2010). As pesticides are designed to be biologically active (Schuster and Schröder, 1990), extensive and excessive application often harm and influence the activities of the soil microbial communities (Omar and Abdel-Sater, 2001, Singh and Singh, 2005a), causing soil pollution (Antonious, 2003) and effecting the sustainability of agro-ecosystems. A lot of research have been conducted that focused on the use of pesticides alone (Getenga *et al.*, 2000, Xie *et al.*, 2004, Gundi

et al., 2005) or in combination (Singh *et al.*, 2002b, Wang *et al.*, 2007a, Chu *et al.*, 2008) to study the adverse impacts on soil microbial diversity and activities (Ingram *et al.*, 2005, Wang *et al.*, 2006, Littlefield-Wyer *et al.*, 2008). However, studies often report idiosyncratic effects of pesticides on soil microorganisms and soil enzymes (Sannino and Gianfreda, 2001, Jjemba, 2002, Singh and Singh, 2005b). Certain pesticides stimulate the growth of microorganisms, but others suppress or have no effects when applied at normal rates. Hence, it is difficult to predict the relationship between pesticide and its impact on various groups of soil microorganisms. Likewise, some microbial groups are capable of utilising pesticides as a source of energy, whereas the pesticide might be toxic to other groups (Johnsen *et al.*, 2001). Detailed research has also shown that repeated application of pesticides results in the increased rate of microbial degradation leading to the loss of the efficacy of the pesticide against target pests (Roeth *et al.*, 1990, Arbeli and Fuentes, 2007).

Chlorpyrifos (CP) is one of the most widely used organophosphorus (OP) pesticide against a broad spectrum of insect pests of economically important crops, like cotton and sugarcane (Kale *et al.*, 1999, Mallick *et al.*, 1999, Fang *et al.*, 2006, Singh and Walker, 2006, Wang *et al.*, 2007b). The half-life of CP is generally between 10 and 120 days in soil but can be up to one year depending on soil type, soil moisture, pH, climate and other environmental factors (Howard, 1991, Singh and Walker, 2006) with 3,5,6-trichloro-2-pyridinol (TCP) as the major degradation product. Between the years 2002-2006, the average annual global use of CP was 25 million kg active ingredients (AI) (Pengphol *et al.*, 2012). In Australia itself, CP has gained wide usage in agriculture dating from mid 1960s and currently there are 164 CP-containing products registered in Australia (http://apvma.gov.au/).

Unlike other OP pesticides (*e.g.* diazinon) that failed to provide adequate control against target pests due to the enhanced degradation in the soil (Sethunathan, 1971), CPs stability and effectiveness against a wide range of pests has resulted in large scale use of CP across the globe. It was suggested that the accumulation of TCP, the major degradation product of CP, has antimicrobial properties preventing the proliferation of CP degrading microorganism resulting in lack of development of enhanced CP degradation (Racke *et al.*, 1990). However, following the repeated use of CP, an increased number of crop failures have been linked to CP inadequacy to control pests in Australian sugarcane farms since 1992. The failure of CP to control pests in those soils, as a result of enhanced degradation, was first reported by Robertson *et al.* (1998). The report found that CP was abiotically hydrolysed to TCP in the alkaline soils and the enhanced microbial degradation of TCP was associated with the loss of the efficacy of CP in the soils. Although soil pH has been shown to be associated to the hydrolysis of CP, no consistent relationship between high pH and chemical lysis of CP has been confirmed (Racke *et al.*, 1996, Singh *et al.*, 2003a). It was not until later in 2003 that the role of microorganisms in the enhanced degradation of CP was first reported from Australian soils with a history of CP use (Singh *et al.*, 2003a). This study highlighted the adaptation of soil microorganisms to the repeated exposure of CP (for more than 14 years) and development of capabilities to degrade CP as a source of energy.

Following the enhanced degradation and loss of efficacy in most of the Australian sugarcane farm soils (about 13 years ago) CP was replaced by imidacloprid (IC) as a choice of pesticide and IC is now widely used for the control of insects including termites, white grubs and beetles. Due to its high insecticidal activity at low application rates and long residual activity, IC is regarded as a promising broad-

spectrum pesticide. Half-lives of this pesticide in soils usually ranges from 40 days in an unamended soil to 124 days for soils recently amended with organic fertilizers (Rouchaud *et al.*, 1994), and for the most common seed treatments the half-life reported range from 28 to 1250 days in soils (Goulson, 2013). However, there have been no reports about the impacts of repeated IC applications on their persistence (enhanced degradation) and soil microbial communities. While studies have shown the influence of the repeated application on degradation rates of CP and impact on soil microbial activities, legacy effects of CP on the soils that showed enhanced degradation has not been investigated. There is also no knowledge on whether the previously CP-treated soils (with enhanced degradation capability) has restored to their original state (in terms of functional capabilities) after they were not exposed to CP post loss in efficacy (13 years ago).

The aim of this study was to determine the effects of two selected pesticides (CP and IC) on soil microbial community and activity in two soil treatments; one without previous history of pesticide application and the one that has been repeatedly exposed to pesticides (both CP and IC, showed enhanced CP degradation). These pesticides were selected to reflect current commercial applications, for they are widely used (in rotation) to curb grubs in the Australian sugarcane farms. Although previous literature has provided some insight into the interaction between pesticides and soil microbial communities, there is a lack of complete information on the effect of long-term CP and IC exposure on adaptation responses associated with functional stability and structural composition of soil microorganisms. Long-term studies on agro-ecosystem are important as they provide opportunities to study the impacts of various biological interactions on soil quality, which is important in projecting long term productivity of soils (Rasmussen *et al.*, 1998).

In this study, I hypothesised that soils which have previously shown enhanced degradation of CP, will maintain their capability to degrade pesticides even in the absence of any application of the compounds for 13 years (legacy effect). In addition to this, in soils with enhanced degradation, repeated application of CP will have limited impact on soil microbial community due to rapid loss of pesticides. It can be argued that in the absence of selection pressure or nutritional advantage (*e.g.* in absence of CP application), microbes could lose their genetic ability to degrade pesticides, and hence enhanced (CP) degradation capability would be lost; however, no experimental evidence is available for any legacy impacts of enhanced degradation of any pesticide. Such knowledge is not only important for advancement of ecological science but also for economic reasons. For example, developing and manufacturing of chemical pesticides need significant investment and loss of efficacy can lead to a significant economic burden on the manufacturer.

Particular emphasis was placed on bacteria as they are the most abundant and important part of soil micro-flora because of their key role in the biogeochemical cycles of important elements like carbon (C), nitrogen (N), and sulphur (S). The soil bacterial diversity was analysed before and after repeated applications of CP and IC. As it has been estimated that only 0.1% of bacteria can be cultured (Amann *et al.*, 1995), a cultivation-independent approach was taken to understand the interrelationship between bacteria and their pesticide environments. The response of bacterial respiration rate to pesticides was measured and the effect on the bacterial community composition evaluated using soils from five sugarcane farms. Soil bacterial communities were compared by q-PCR and T-RFLP analysis of 16S rRNA gene, amplified from total community DNA to evaluate the impact of pesticides on the bacterial soil community.

3.2 Materials and methods

3.2.1 Site description and sample collection

Soil was retrieved from five sugarcane farms in the Mackay, Burdekin and Tully areas in Queensland, Australia with a history of pesticide use to control sugarcane grub. Three out of five sites were located in Burdekin district, which consisted of site 1, site 2 and site 4. Site 3 was located in the Mackay district and site 5 in the Tully region. All the sites differed from each other in terms of the application of pesticides (number of times pesticide applied, amount applied and years applied). Soils from the sites Burdekin and Tully that received annual field application of CP developed an enhanced rate of CP degradation 13 years ago. The use of CP was discontinued and IC has been used since then. On the other hand, CP is still successfully used to control pests at Mackay. Soil type and sugarcane grub pesticide history for each site is given in Table 3-1.

Sampling was undertaken within and from outside (headland, no farming) of each designated farm at each site. Sampling from within the farm was taken from the row at least 8 meters in from the headland. Samples from the outside were taken from the headlands and surrounding areas approximately 6–8 m from the farm of sugarcane. Sampling was avoided downhill from the fields to avoid contamination of active ingredients with runoff. Headlands were expected to be free from direct exposure to pesticides and therefore, used as control for the respective sites. In each site, three replicate soil samples were randomly collected from within the farm (designated R) and headland (designated H). Each replicate was composed of three soil cores taken by a digging a hole about 30cm deep by the shovel. The shovel was cleaned with 70% ethanol (EtOH) during sampling. These three sub-samples were mixed together and a composite 2 kg sample was taken for each replicate. The soils were received by

post and kept at 4^{o}C in the dark until use in degrading experiments. Fresh soil samples were sieved through 2 mm sieve to separate vegetation and other coarse particles from the soil. Figure 3-1 illustrates an example of the sampling pattern for each of the five sites.

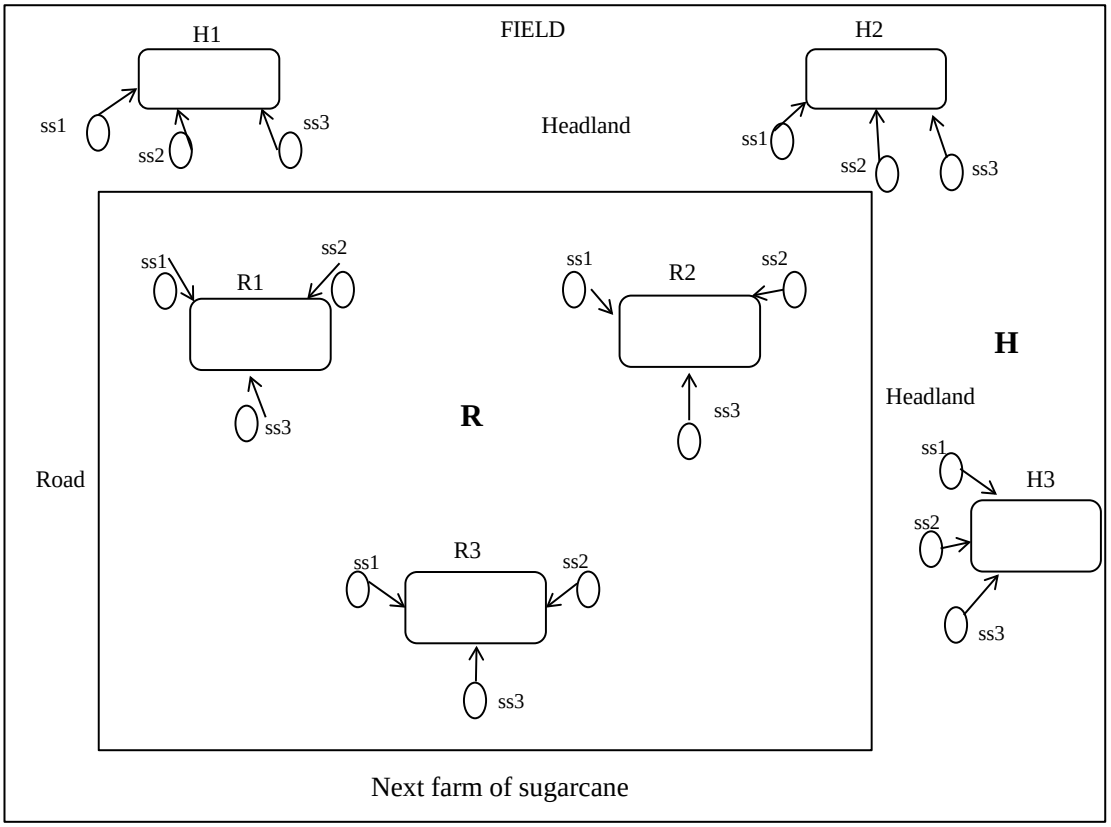


Figure 3-1 A schematic diagram of sampling pattern for each sugarcane farm site. R represents the samples collected from within the farm with previous pesticide history. H represents the samples collected from headland with no previous pesticide history. ss1, ss2 and ss3 represent the three sub-samples of each replicate.

Table 3-1 Pesticide history of five sugarcane farms in Queensland used in this study**.**

Site	Region	History of use of sugarcane grub control products* (past pesticide treatment)
1	Burdekin (latitude -19.59683°, longitude 147.38606°)	Clay loam soil that had rapid degradation/greyback grub failures following use of suSCon Blue[a] in 1990's. suSCon Maxi[c] (15 kg/ha) subsequently used 3 times per year since permitted in 2002
2	Burdekin (latitude -19.86424°, longitude 147.24409°)	River loam soil that had rapid degradation/greyback grub failures following use of suSCon Plus[b] in 1990's. suSCon Maxi (14 kg/ha) subsequently used 2 times per year since permitted in 2002
3	Mackay (latitude -21.12813°, longitude 149.06880°)	Red clay soil where suSCon Blue is still successfully used for French's sugarcane grub control and has been used at least 2-3 times per year in the last 12 years.
4	Burdekin (latitude -19.86424°, longitude 147.24409°)	River loam where there has been use of suSCon Blue in 1990's. suSCon Maxi (15 kg/ha) subsequently used 2 times per year since permitted.
5	Tully (latitude -19.59683°, longitude 145.94784°)	Sandy loam soil with history of suSCon Blue treatment in 1998 and 2001. suSCon Maxi (15 kg/ha) subsequently used 3 times per year since permitted.

(*) data available till the year 2012

suSCon is the commercial product name of Crop Care (Australia)

[a]active ingredient chlorpyrifos (140 g/kg)

[b]active ingredient chlorpyrifos (75 g/kg)

[c]ingredient imidacloprid (50 g/kg).

3.2.2 Soil properties

All the soils were analysed for total C and N content pH and moisture. All soil parameters measured are given in Table 3-2. Details of the methods of analysis are described in Section 2.2.2.

3.2.3 Experimental design and pesticide treatment

Commercial formulation of CP 500 EC (500 g/L, Nufarm) and IC 350 SC (350 g/L, Apparent Pty Ltd) were used in this study. About 250 g of soil (both R & H type) from all the five sites were placed into plastic jars and mixed with solutions of CP or IC to a final concentration of 10 mg/kg. Soils treated with the pesticide were left for 3 to 4 hours in a fume hood for drying. The water holding capacity of the soil was adjusted to 40% and was maintained by regular addition of Milli Q water. Each treatment including the control was performed in triplicate.

The screw cap plastic jars containing the treated soil were incubated and maintained under aerobic conditions, in the dark at room temperature. All the soil-pesticide combinations were sampled periodically upto 105d for CP/ 102d for IC to determine the microbial properties and degradation of pesticides. After 45d CP/ 43d IC, or when more than 75% of the initial concentration of the pesticides disappeared, another spike of the appropriate pesticide was applied to the soil at final concentration of 10 mg/kg. The soils were retreated with the third application of pesticide (10 mg/kg) on 50d CP/ 55d IC after the second treatment, when maximum degradation of pesticide took place. In addition to this, soil samples (25 g) treated with antibacterial and antifungal agents, chloramphenicol and cycloheximide (1 ml

each; 5 mg/ml in water), respectively (Singh *et al.*, 2003a) were also maintained. All the soil-pesticide combinations described above, with their untreated controls, were investigated to determine the effect of pesticides on soil bacterial communities.

3.2.4 Determination of CP and IC

Pesticides and their metabolites were extracted from soil (2.5 g) by mixing with acetonitrile:water (90:10, 5 ml) in McCartney glass vials. The vials were vortexed and the pesticide extraction was done by shaking the mix for one hour on a shaker (130 rpm). The samples were centrifuged for five minutes at 15000 rpm and the supernatant was filter sterilized through a 0.22 µm nylon syringe filter for HPLC analysis using Agilent 1260 Infinity HPLC system. CP and IC were separated on Agilent Poroshell 120 column (4.6 x 50 mm, 2.7 µm) with Agilent ZORBAX Eclipse Plus-C18 guard column (4.6 x 12.5 mm, 5 µm). The injection volume was 10 µl and the mobile phase was acetonitrile:water (75:25), acidified with 1% phosphoric acid. The analytes were eluted at 40 °C with isocratic mobile phase flow rate of 0.8 ml/min for 4.5 min. The pesticides were detected spectrophotometrically at 230 nm (CP) or 270 nm (IC).

3.2.5 Community respiration and substrate induced respiration (SIR)

Functional activities (basal respiration and SIR) of two pesticides (CP and IC) treated soils were measured using MicroResp^{TM} (Macaulay Scientific Consulting, UK), as described by Campbell *et al.* (2003) and compared with their untreated controls after first pesticide application (45d CP/ 43d IC). Approximately 0.3 g of the soil samples

were delivered to each well in the 96- deep well microplates. The microplates were incubated for 24 hours at room temperature (RT) in the dark before the assay was carried out. To measure the SIR, filter sterilized glucose (Sigma Aldrich, USA) was supplied at 30 mg ml^{-1} of water contained by the soils, to the wells. Sterile deionised water was delivered to the wells to measure the basal respiration. All the additions, for both basal and SIR, were done in replicates (n=3). Prior to addition of glucose/deionised water, CO_2 detection plates were read (T0). The deep-well plates were sealed with the pre-read CO_2 detection plates and incubated at 25°C for 6 hours in the dark, as per the manufacturer's protocol. The change in optical density was then measured on a spectrophotometer microplate reader (EnSpire® 2300 Multilabel Reader, Perkin Elmer, USA) at a wavelength of 570 nm (T6). The rate of CO_2 respiration expressed per gram of soil per well was determined using the formula provided in the MicroResp™ manual:

$$\frac{\left(\%\frac{CO_2}{100}\right) \text{x vol x} \left(\frac{44}{22.4}\right) \text{x} \left(\frac{12}{44}\right) \text{x} \left(\frac{273}{273+\text{T}}\right)}{\frac{\text{soil fwt x (soil \% dwt/100)}}{\text{incubation time}}}$$

where, vol is the headspace volume, soil fwt and dwt is the soil fresh weight and dry weight respectively and T is the room temperature (25^{o}C).

The SIR responses were calculated by subtracting the basal response (water) from glucose response.

3.2.6 Total community DNA extraction and quantitative PCR (q-PCR) of bacterial 16S rRNA gene

To determine the abundance (copy number) of 16S rRNA gene during three applications of pesticides (sampling days; 0, 45d CP/ 43d IC and 105d CP/ 103d IC), total community DNA was extracted from 0.25 g of soil sample after a harsh lysis step (Retsch Tissue lyser, Qiagen) using PowerSoil®- 96 well soil DNA Isolation Kit (MO BIO laboratories, USA) following the manufacturer's protocol. After total community DNA isolation, the concentration and purity was confirmed by NanoDrop™ 2000C spectrophotometer (ThermoScientific, USA) and DNA was stored at -20^{o}C until further analysis. Abundance of bacterial community was determined by amplifying 16S rRNA gene on a rotor-type thermocycler (Corbett Research; Splex) using primer pair Eub338F (5'ACTCCTACGGGAGGCAGCAG 3') and Eub518R (5'ATTACCGCGGCTGCTGG 3') (Fierer *et al.*, 2005). This primer set targets and amplifies the 16S rRNA gene present in all the soil bacterial groups. Reactions were performed in 20µl volumes using ™ SYBR® No-ROX Kit (Bioline Reagents Ltd) as described in Section 2.2.6 with changes in PCR conditions. After initial denaturation at 95^{o}C for 3 mins, PCR conditions were as follows: 40 cycles of 10 sec at 95^{o}C, 20 sec at 53^{o}C and 20 sec at 72^{o}C. An additional 15 sec reading step at 83^{o}C was added at the end of each cycle.

3.2.7 PCR amplification of 16S rRNA gene and T-RFLP analysis

Soils were sub-sampled at time zero, 45d CP/ 43d IC and 105d CP/ 102d IC for both pesticides and controls, and analysed for community composition changes by T-RFLP. For profiling of 16S rRNA gene composition using T-RFLP analysis, a

similar protocol as given in section 2.2.7.3 was followed. For 16S rRNA gene -based PCR reaction (final concentration) 1X $NH4^+$ reaction buffer, 2 mM $MgCl_2$, 400 µM of deoxynucleotide mix, 2.5 U $µl^{-1}$ BioTaq™ DNA polymerase (all reagents from Bioline, UK), 0.2 mg ml^{-1} bovine serum albumin (New England Biolabs, USA) and 1 µl of DNA template were used. Forward bacterial primer labelled 63F-VIC® (5' AGGCCTAACACATGCAAGTC 3') and 1087R (5'CTCGTTGCGGGACTTAACCC 3') (Marchesi *et al.*, 1998) were used at a concentration of 0.2 µM of each primer (Sigma-Aldrich, USA) and the target gene was amplified by PCR in triplicate; initial denaturation at 95^{o}C for 5 mins, followed by 30 cycles of 30 sec at 94^{o}C, 30 sec at 55^{o}C and 1 min at 72^{o}C, and a last cycle of 10 mins extension period at 72^{o}C.

The PCR products were purified (section 2.2.7.3) and digestion of the purified PCR products was carried out with 10 U of restriction endonucleases, *HhaI* (section 2.2.73). Aliquots of digested PCR products (2 µl) were mixed with 7.7 µl of Hi-Di™ formamide (Applied Biosystems, UK) and 0.3 µl of GeneScan™-600 LIZ® internal size standard (Applied Biosystems, UK). The mixtures were denatured at 95°C for 3 min and then chilled on ice. T-RFLP analysis was performed on an automated genetic analyser ABI3500 (Applied Biosystems, UK). Terminal restriction fragments (T-RFs) generated by the sequencer were analysed using the size-calling software GeneMapper™ 4.0 (Applied Biosystems, UK) and quantified by advanced mode using second order algorithm. T-RFs in a T-RFLP profile were selected using similar protocol as given in section 2.2.7.3 except for T-RFs present in between 30-600 bp were considered in order to avoid T-RFs caused by primer-dimers and to obtain fragments within the linear range of the internal size standard.

3.2.8 Diversity Measures

The T-RFLP data was used to calculate the richness, evenness and diversity of the bacterial communities after three applications of CP/ IC. Richness (S) represented the number of unique taxa present in the sample (represented here by number of peaks in T-RFLP profile). Diversity and evenness was calculated using T-RFLP relative abundance data. The Simpson's index (D) was calculated as a measure of diversity that combines information about richness and evenness (Simpson, 1949). The diversity index was computed as:

$$D = \frac{\sum n\,(n-1)}{N\,(N-1)}$$

where, n is the total number of organisms of a particular species (here TRFs) and N is the total number of organisms of all the species (total TRFs). To represent the diversity of the assemblage this value was expressed as the reciprocal (1/D). The evenness (E) of the community structure was calculated as $E_{1/D}$= (1/D)/S, where S represented the richness of the community.

3.2.9 Statistical analysis

To determine a degradation rate constant (k), individual decay curves for the parent compound (and sites) were fitted to a first order kinetics model by linear regression analysis of the logarithm of the residual concentration against incubation time. The half-life ($t_{1/2}$) of CP/IC was calculated using a first order function expressed as $t_{1/2}$=ln2/k. Bacterial community analysis was conducted using PRIMER v.6 (PRIMER-E Ltd, UK). Relative T-RFLP abundance data that had been square root

transformed prior to analysis was used to generate a Bray-Curtis similarity matrix. PERMANOVA was used to determine the influence of site and pesticide treatment (both in fields and lab) on the bacterial community structure. A non-metric multidimensional scaling (MDS) analysis was carried out using the default programme settings and a scatter plot was produced to display the relationship between bacterial community structure with different factors, e.g. sites and field and/or lab pesticide treatments. To analyse the relationship between bacterial community structure and respiration (function), the MDS axes coordinates (MDS1 and MDS2) were exported and linear regression (SPSS, Inc.) was performed (along MDS axis 1). Similarly, to analyse the relationship between bacterial diversity indices (diversity, richness and evenness), bacterial abundances and respiration (function) diversity, richness and evenness were exported and linear regression was performed. The bacterial abundance, SIR and basal respiration values were normalised by taking the natural log before analysis. Univariate analysis was performed to analyse the lab pesticide treatment effect (CP/ IC) in shaping the bacterial community attribute when compared with their untreated controls.

A Univariate test (general linear model; IBM SPSS 17, Inc.) seemed to be the most appropriate approach for this data. Environmental factors (soil properties) and a history of pesticide use (past pesticide) that would affect the bacterial abundance, community structure (diversity, evenness and richness) and function (respiration) were determined. Initially all the environmental factors were selected in the analysis but later on the factors found to be significant were used for further analysis. This analysis method was performed separately on the two pesticides (CP and IC), at a given incubation time, and compared with their untreated controls to determine the

lab pesticide treatment effect (CP/ IC) in shaping the bacterial community and function. Level of significance (*p* value) was set at 0.05.

3.3 Results

3.3.1 Soil properties

Soil pH for all the five sites ranged from slightly acidic to neutral with soil moisture ranging mostly within 1-2%. Nutrient parameter values also differed among sites. Table 3-2 shows the physical and chemical properties of all the five sites.

Table 3-2 Soil properties measured for all the five sites (sugarcane farms). Carbon (%) and Nitrogen (%) represents the total carbon and nitrogen of the soil sample. Each data point represents the mean of three experimental replicates (n=3).

Site No.	Site Name	Soil type	pH	% Carbon	% Nitrogen	%Moisture
1	Burdekin I	R	6.8±0.1	1.12±0.2	0.05±0.0	1.88±0.2
		H	7.0±0.1	1.27±0.1	0.02±0.0	1.95±0.1
2	Burdekin II	R	6.5±0.0	1.49±0.1	0.08±0.0	1.96±0.0
		H	6.6±0.0	0.88±0.04	0.06±0.0	1.53±0.0
3	Mackay	R	5.1±0.1	1.85±0.1	0.08±0.0	2.24±0.1
		H	5.7±0.0	2.36±0.0	0.02±0.0	2.35±0.0
4	Burdekin III	R	6.51±0.1	1.18±0.1	0.06±0.0	1.85±0.1
		H	6.9±0.1	1.05±0.1	0.06±0.0	2.26±0.3
5	Tully	R	6.4±0.1	0.96±0.0	0.04±0.0	1.04±0.0
		H	5.0±0.2	0.87±0.0	0.05±0.0	1.09±0.0

3.3.2 Degradation of CP/IC in soil

The degradation patterns of CP/ IC after repeated lab treatments with CP/IC in soils are shown in Figure 3.2 and 3.3. The half-life of CP/IC, calculated by first order function, differed between test sites. The kinetic data of CP/ IC degradation of all the sites are given in Table 3-3 and 3-4. The results indicated an overall decrease in the half-life of CP during sequential treatment, therefore suggesting enhanced degradation of the compound in all the test sites except for site 3 (Mackay) where

half-life of CP remained similar after second and third treatment. Among the different sites, the disappearance of CP after three applications was particularly rapid in 4H, 4R and 5R (Figure 3-2; Table 3-3 and 3-4). On the other hand, repeated application of IC was associated with reduced rates of degradation (Figure 3-3; Table 3-3 and 3-4). Depending on the test site, the overall half-life of IC was about 30-65 days for the first treatment and this was extended to 45-65 days for the second dose. Also, there was a very little change in the IC concentration following the third treatment (Figure 3-3) further suggesting that the sequential treatment of IC considerably slowed down the degradation of IC. In addition to this, there was no significant influence of soil types on the degradation rates of pesticides (Table 3-3 and 3-4). However, addition of chloramphenicol and cycloheximide to soils inhibited CP/IC degradation (>90 %, data not shown)

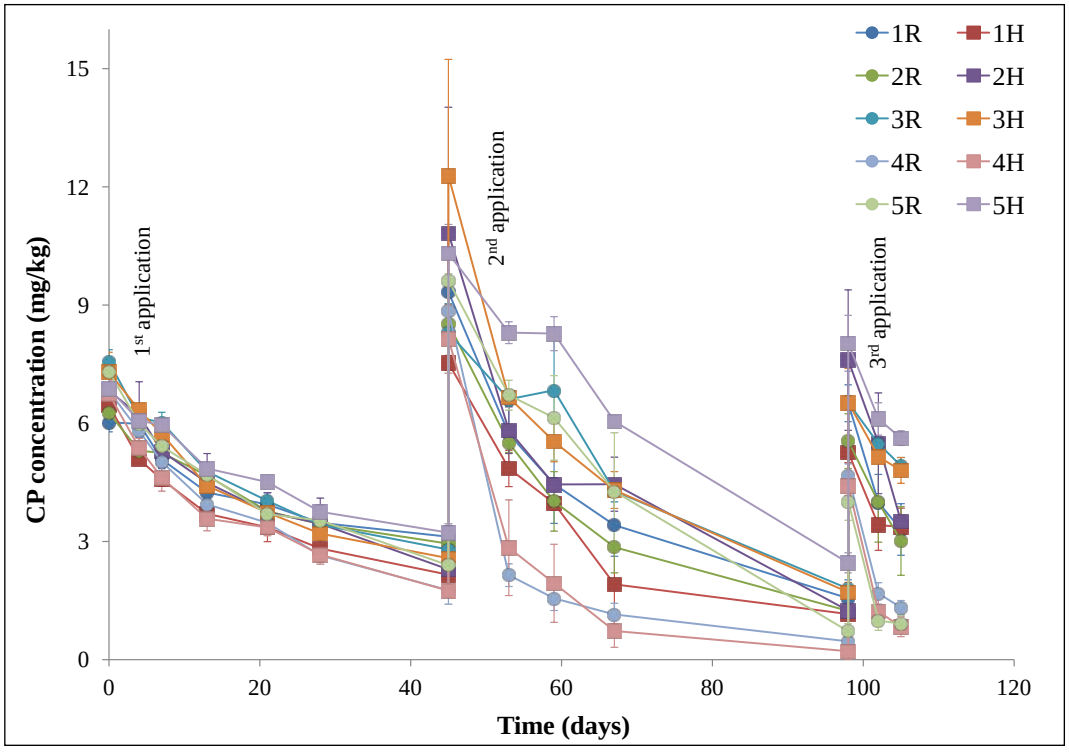


Figure 3-2 Degradation of chlorpyrifos (CP) in the different sites (soils) after three repeated applications (10 mg/kg). R represents the sites treated with pesticides in the fields and H represents headland not treated with pesticides. Error bars represent standard error of the mean (n=3).

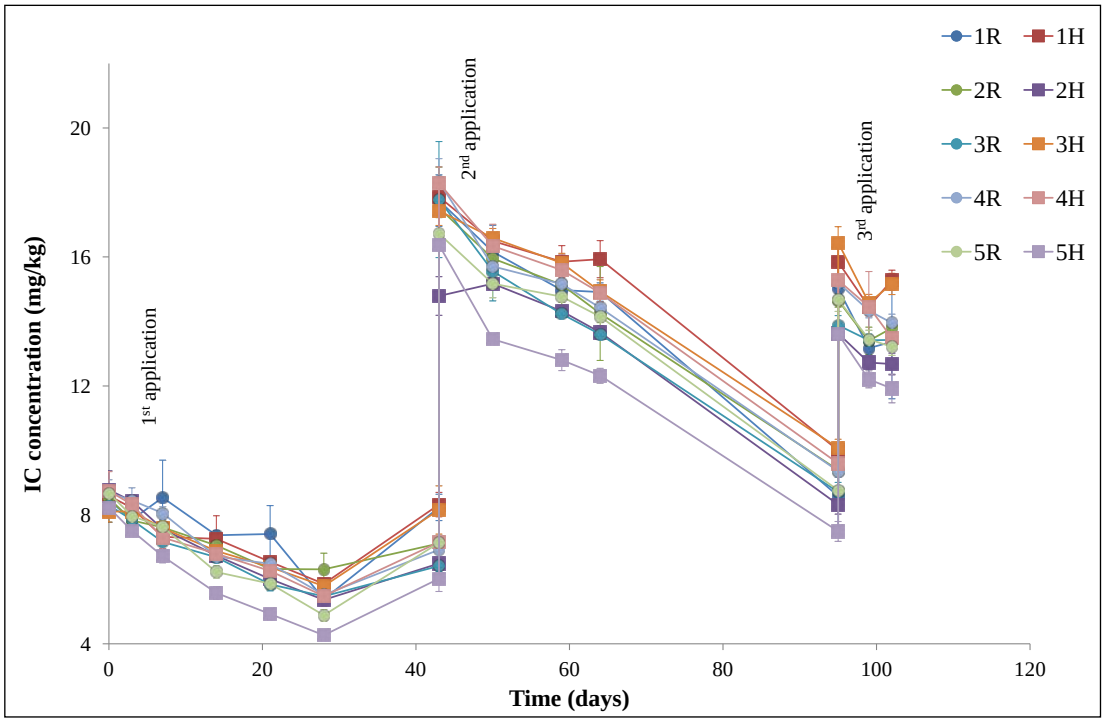


Figure 3-3 Degradation of imidacloprid (IC) in the different sites (soils) after three repeated applications (10 mg/kg). R represents the sites treated with pesticides in the fields and H represents headland not treated with pesticides. Error bars represent standard error of the mean (n=3).

3.3.3 Community respiration and SIR

Basal respiration rates in untreated controls (Figure 3.4a) ranged from 1.39 to 2.12 µg g^{-1} h^{-1} CO_2-C, whereas pesticide-treated soils (lab treatment) displayed basal respiration between 0.95 to 1.34 and 0.88 to 1.28 µg g^{-1} h^{-1} CO_2-C for CP and IC, respectively (Figure 3-4a). The effects of site and pesticide (lab treatment) were significant for both CP (both p<0.001) and IC (both p<0.001) when incubated for 45d CP and 43d IC. A significant interaction of pesticide (lab treatment) and site was also observed (p<0.001 and p=0.014 for CP and IC, respectively). A significant interaction of IC lab treatment, site and past pesticide (in fields) was also observed

(p<0.001), whereas past pesticide (in field) treatment alone was not significant (p=0.431 and p=0.515 in CP and IC lab-treated soils, respectively). The response of SIR within untreated controls was highly variable between different soils (Figure 3-4b). Overall, untreated controls displayed higher respiration rates (between 1.48 and 14.86 µg g^{-1} h^{-1} CO_2-C) than CP lab-treated samples (between 0.34 and 1.73 µg g^{-1} h^{-1} CO_2-C) and IC lab-treated samples (between 0.21 and 1.94 µg g^{-1} h^{-1} CO_2-C). In all soil samples, both CP and IC significantly decreased SIR when applied (45d CP, 43d IC; p<0.001) to different degrees in the various soil types. Suppression of soil respiration was between 7-76% with the lowest respiration found in 5R and highest in 1R after the pesticides treatment. Univariate analysis also showed a significant effect of site and past pesticide (under field conditions) treatment on SIR response when soils were lab-treated with CP (p<0.001 and p<0.027). On the other hand, a significant effect of site (p<0.001) and but no past pesticide (in fields; p=0.144) treatment on SIR response were observed when soils were lab-treated with IC. There was a significant interaction between pesticide lab treatment (CP/IC) and sites (CP/IC lab treatment*sites, p<0.001); and a three-way interaction between pesticide lab treatment, site and past pesticide (in fields) treatment (CP lab treatment*site*past pesticide, p=0.021; IC lab treatment*site*past pesticide, p=0.003). However, there was no significant interaction between sites and past pesticide (in fields) treatments (p>0.05) (Table 3-5).

Table 3-3 Kinetics data of the two pesticides (CP/ IC) in five different sites. The degradation of the pesticides (from Figure 3-2 and 3-3) was ascribed by the first-order function ($C_t=C_o$ x e^{-kt}). The half-lives of the pesticides were obtained by function $t_{1/2}$ = ln2/k. Each value is a mean of three replicates (n=3). Here, H represents the headland area with no past history of pesticide application.

Site	Applications	R^2		Rate constant, k (day^{-1})		Half-life, $t_{1/2}$ (days)	
		CP	IC	CP	IC	CP	IC
1H	1	0.848	0.949	0.024	0.013	28.4	50.7
	2	0.750	0.961	0.043	0.011	15.8	62.0
	3	0.832		0.063		10.9	
2H	1	0.924	0.991	0.024	0.017	28.5	39.4
	2	0.750	0.928	0.050	0.011	13.7	62.5
	3	0.995		0.110		6.2	
3H	1	0.873	0.989	0.023	0.012	29.9	57.7
	2	0.578	0.990	0.045	0.010	15.1	65.7
	3	0.939		0.043		15.8	
4H	1	0.864	0.960	0.029	0.013	23.1	41.5
	2	0.546	0.989	0.084	0.012	8.1	55.8
	3	0.887		0.237		2.9	
5H	1	0.915	0.969	0.016	0.023	41.2	29.5
	2	0.971	0.957	0.033	0.015	20.7	46.0
	3	0.940		0.051		13.5	

Table 3-4 Kinetics data of the two pesticides (CP/ IC) in five different sites. The degradation of the pesticides (from Figure 3-2 and 3-3) was ascribed by the first-order function ($C_t=C_o$ x e^{-kt}). The half-lives of the pesticides were obtained by function $t_{1/2}$ = ln2/k. Each value is a mean of three replicates (n=3). Here, R represents the farm site with past history of pesticide application.

Site	Applications	R^2		Rate constant, k (day^{-1})		Half-life, $t_{1/2}$ (days)	
		CP	IC	CP	IC	CP	IC
1R	1	0.852	0.767	0.014	0.016	47.3	43.0
	2	0.772	0.983	0.041	0.013	16.6	49.5
	3	0.944		0.096		7.1	
2R	1	0.905	0.931	0.016	0.010	41.3	64.7
	2	0.783	0.994	0.044	0.011	15.4	57.7
	3	0.997		0.087		7.9	
3R	1	0.881	0.975	0.022	0.014	31.3	46.6
	2	0.935	0.984	0.035	0.013	19.4	50.4
	3	0.991		0.040		17.2	
4R	1	0.907	0.972	0.030	0.016	23.0	42.7
	2	0.442	0.972	0.068	0.012	10.0	53.5
	3	0.886		0.180		3.8	
5R	1	0.898	0.975	0.024	0.020	27.9	33.8
	2	0.943	0.978	0.060	0.012	11.4	55.7
	3	0.834		0.211		3.2	

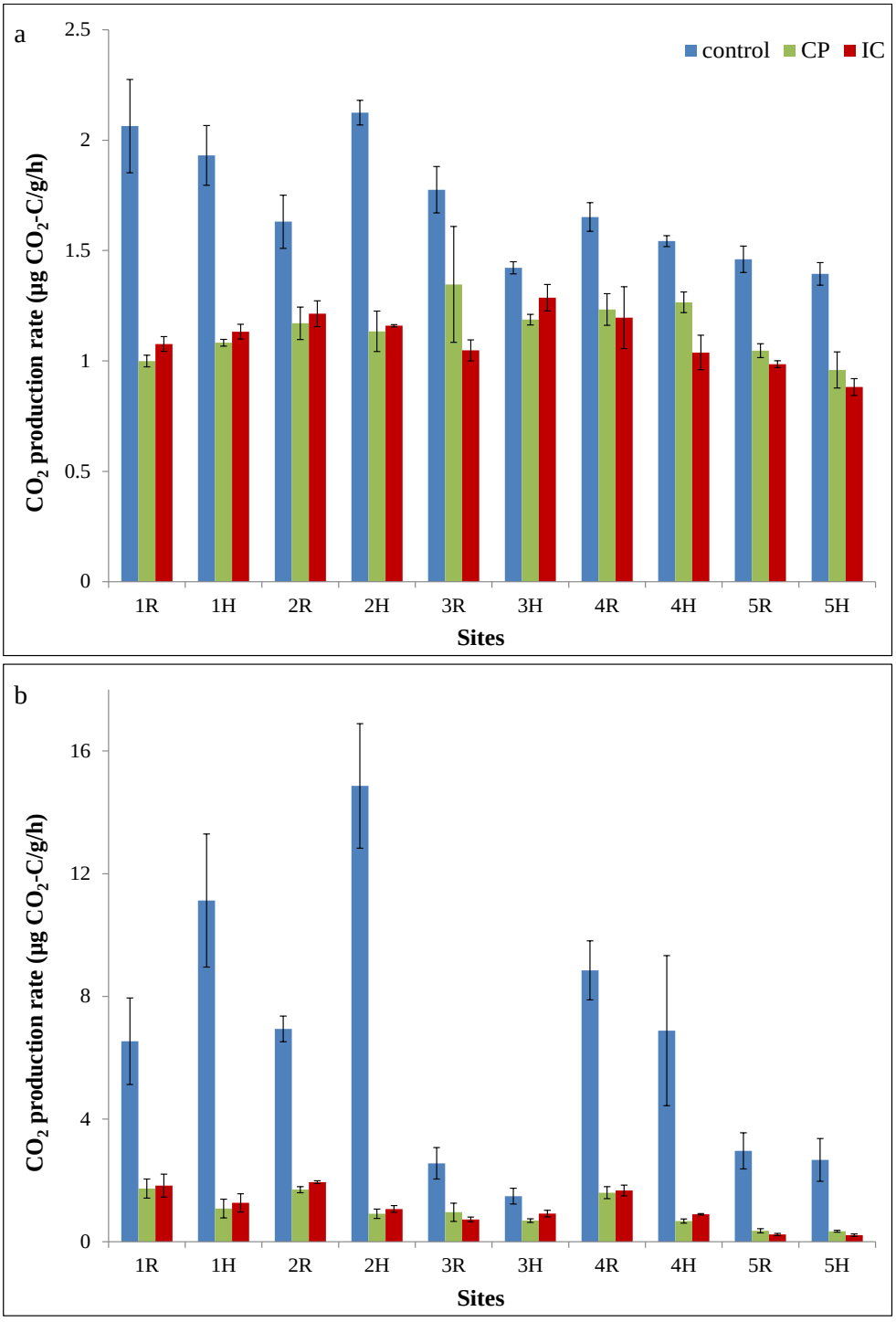


Figure 3-4 Microbial respiration in soil samples after lab pesticide treatment (45d CP/ 43d IC) with their untreated controls a) Basal respiration and b) Substrate Induced respiration (SIR). Error bars represent standard error of the mean (n=3). Legends: CP=chlorpyrifos and IC=imidacloprid.

Table 3-5 Results of the univariate analysis for basal and substrate induced respiration after 45d CP and 43d IC treatment. The values in bold shows significant effect of treatment and/or interactions ($p \leq 0.05$). Abbreviations: CP=chlorpyrifos, IC=imidacloprid

Variables	Basal_CP (*p* values)	SIR_CP (*p* values)	Basal_IC (*p* values)	SIR_IC (*p* values)
CP_lab treatment	**0.000**	**0.000**	n/a	n/a
Site	**0.001**	**0.000**	n/a	n/a
Past_pesticide	0.431	**0.027**	n/a	n/a
CP_lab treatment*Site	**0.000**	**0.001**	n/a	n/a
CP_lab treatment*Past_Pesticide	0.963	**0.009**	n/a	n/a
Site*Past_pesticide	0.073	0.091	n/a	n/a
CP_lab treatment*Site*Past_Pesticide	0.113	**0.021**	n/a	n/a
IC_lab treatment	n/a	n/a	**0.000**	**0.000**
Site	n/a	n/a	**0.000**	**0.000**
Past_pesticide	n/a	n/a	0.515	0.144
IC_lab treatment*Site	n/a	n/a	**0.014**	**0.000**
IC_lab treatment*Pesticide	n/a	n/a	0.711	0.053
Site*Past_pesticide	n/a	n/a	0.066	0.188
IC_lab treatment*Site*Past_Pesticide	n/a	n/a	**0.000**	**0.003**

3.3.4 Quantitative PCR (q-PCR) of bacterial 16S rRNA genes

Bacterial communities in the soils were quantified by means of q-PCR. The average bacterial abundances for CP- or IC-treated samples was similar to that of control samples for 0d (Figure 3-5a, 3-6a), 45d CP/ 43d IC (data not shown), 105d CP/ 102d IC (Figure 3-5b, 3-6b) and ranged between 10^7 to 10^8 16S rRNA gene copies g^{-1} soil. The q-PCR of the bacterial 16S rRNA gene copies showed that there was no significant impact of CP (p=0.188) or IC (p=0.092) on the bacterial abundance, as determined by univariate analysis. However, a strong three-way interaction between site*past pesticide*CP lab treatment was seen on 105d (p=0.024) and the responses were related to site characteristics (p=0.029) (Table 3-6). In addition to this, a weak but significant interaction effect between site*past pesticide*IC lab treatment (p=0.050) was observed on 102d, suggesting that the bacterial communities responded differently depending on the site, past pesticide and IC lab treatment (Table 3-7).

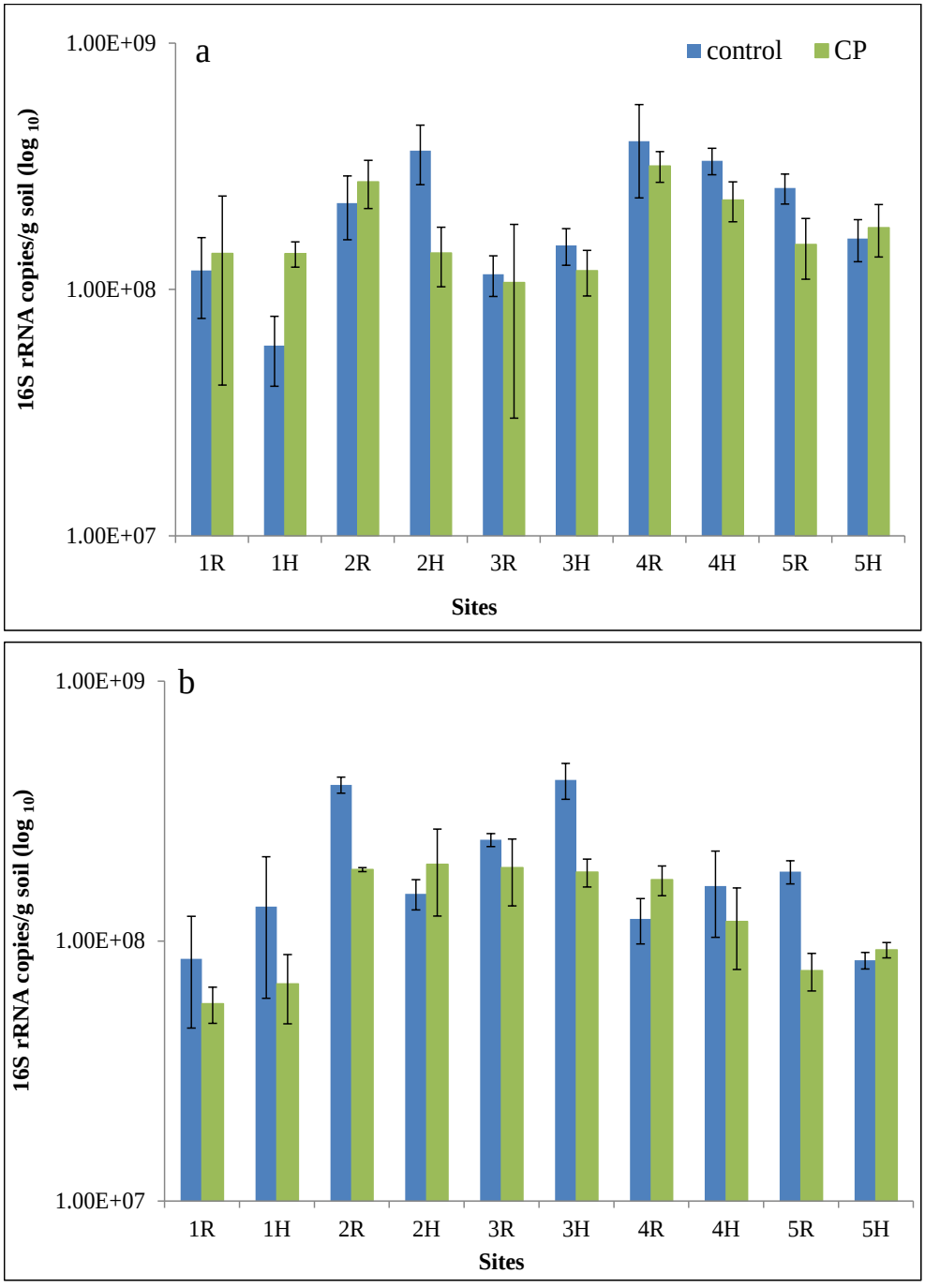


Figure 3-5 Bacterial 16S rRNA gene copies g^{-1} soils amended with pesticides CP in the lab a) 0d and b) 105d recorded as a $\log_{10}$ scale for each site. R represents the sites treated with pesticides in the fields and H represents headland not treated with pesticides (in fields). Error bars represent standard error of the mean (n=3). Legends: CP=chlorpyrifos.

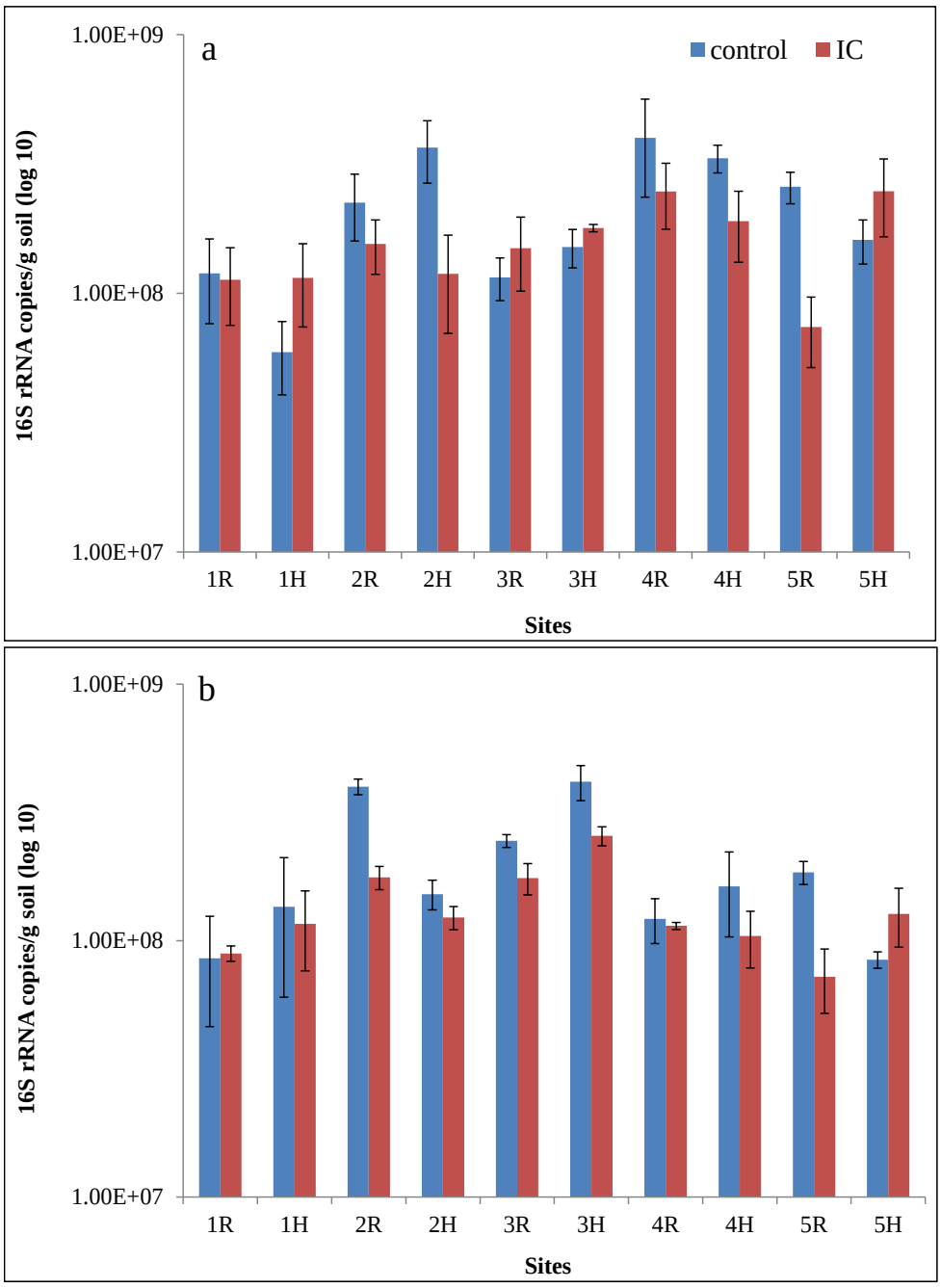


Figure 3-6 Bacterial 16S rRNA gene copies g^{-1} soils amended with pesticides IC in the lab a) 0d and b) 102d recorded as a $\log_{10}$ scale for each site. R represents the sites treated with pesticides in the fields and H represents headland not treated with pesticides. Error bars represent standard error of the mean (n=3). Legends: IC=imidacloprid.

3.3.5 T-RFLP analysis and diversity measures

Univariate analyses demonstrated that there was no difference in diversity, evenness and richness between CP lab-treated and control samples at the start of the experiment (0d). However, the addition of CP (in lab) had a significant negative impact on bacterial diversity ($p<0.001$), richness ($p=0.037$) and evenness ($p<0.001$). At time 0 the diversity, richness and evenness were 35.28±2.03, 59.37±2.5 and 0.61±0.02, respectively (Figure 3-7a, b and c, respectively). After the 105d incubation period there was a loss in diversity, evenness and richness in the treated samples, which were then 22.51±1.7, 54.43±1.8 and 0.41±0.02 respectively (Figure 3-7a, b and c, respectively). In the case of Simpson's diversity, a strong 2-way interaction site*CP lab treatment ($p<0.001$) and a 3-way interaction of site*CP lab treatment*past pesticide (in fields) ($p=0.005$) was also significant and the responses here were related to both site characteristics and lab treatment effect (Table 3-6, Appendix Figure A-2). However, a weak 3-way interaction of site*CP lab treatment*field treatment on bacterial richness and evenness was observed on 105d, suggesting that the bacterial communities responded differently depending on the sites (Appendix Figure A-3 and A-4, respectively).

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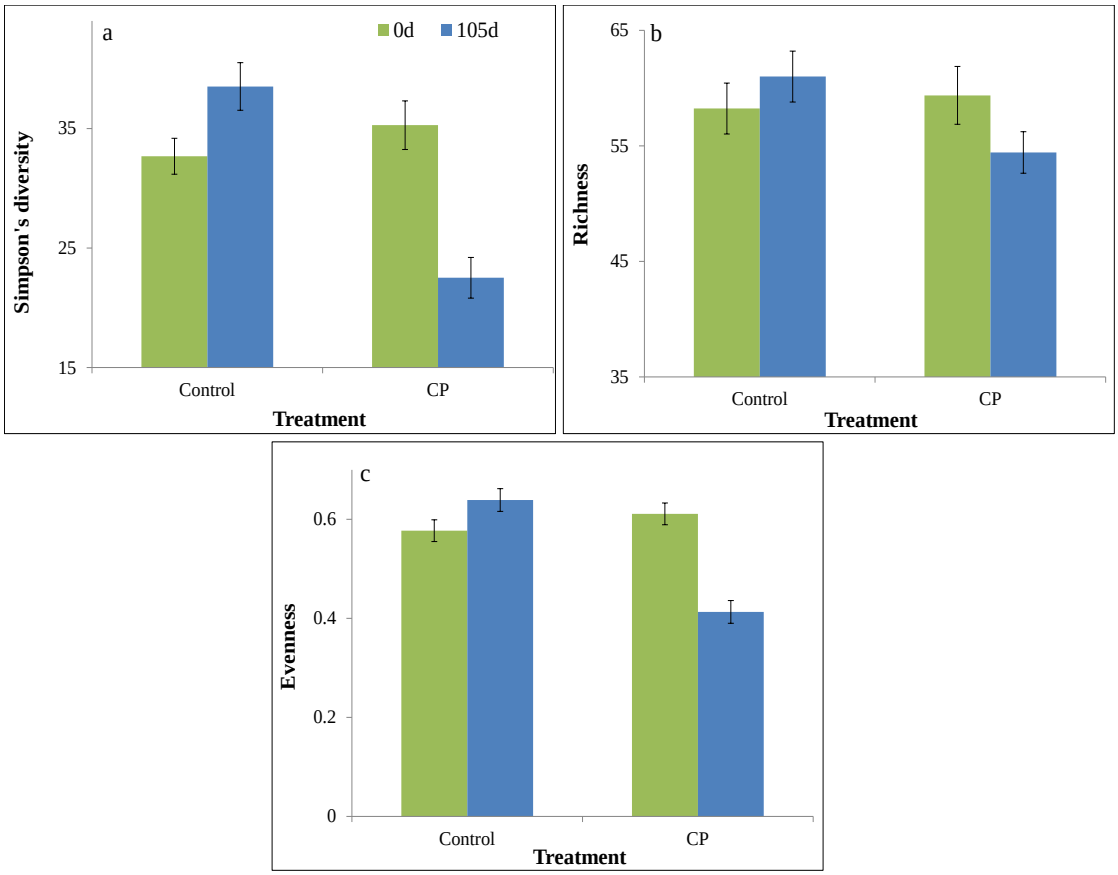

Figure 3-7 Results of the T-RFLP analysis showing effect of chlorpyrifos (CP) on a) Simpson's diversity, b) Richness and c) Evenness for soil treated with CP at 0d and 105d with their respective untreated controls. Error bars represent standard error of the mean (n=3).

Table 3-6 Results of the univariate analysis for diversity indices and abundances after CP treatment for 105 days. The values in bold shows significant effect of treatment and/or interactions ($p \leq 0.05$).

Variables	q-PCR (*p* values)	Diversity (*p* values)	Evenness (*p* values)	Richness (*p* values)
Site	**0.029**	0.297	0.107	0.870
Past_pesticide	0.965	0.127	0.661	0.263
CP_lab treatment	0.188	**0.000**	**0.000**	**0.037**
Site*Past_pesticide	0.094	0.200	0.242	0.806
Site*CP_lab treatment	0.885	**0.000**	0.091	0.153
Past_pesticide*CP_labTreatment	0.600	0.957	0.835	0.768
Site*past_pesticide*CP_lab treatment	**0.024**	**0.005**	0.191	0.550

PERMANOVA analysis showed that there was a significant site (p=0.001) and past pesticide application (in fields; p=0.001) effect on the bacterial community on 0 day. No effect of CP lab treatment (p=0.441) on the structure of bacterial communities was observed on 0 day. However, after 105d there was a significant CP lab treatment effect on the bacterial community (p=0.001). These analyses also showed a significant site*CP lab treatment (p=0.033) and a site*past pesticide*CP lab treatment effect (p=0.023) on bacterial community at 105d when compared to 0 day, indicating that the bacterial communities in the CP-treated soils differed from the control communities on 105d. Simpson's diversity measures and PERMANOVA analysis were only measured for 0d and 105d CP/ 103d IC since at days 45d CP/ 43d IC the bacterial community structure was similar to 105d CP/ 103d IC on MDS biplot (3-8b and c; Figure 3-9b and c, respectively).

The results of the PERMANOVA were also backed up by MDS analysis, which showed CP addition changed the bacterial community composition at 45d in comparison with 0d (Figure 3-8a and b, respectively). No recovery was observed during the experimental period (105d) (Figure 3-8c), indicating that CP application caused a long-term change in the structure of the bacterial communities. Despite a significant impact of past application of pesticide (in fields) on the bacterial community structure (p=0.001), no noticeable influence of past pesticide application on CP lab treatment was observed within a particular site (between R and H) for both 0d (p=0.619) and 105d (p=0.094). However, a noticeable change in the grouping of bacterial community representing the 45d and 105d site*past pesticide*CP lab treatment indicate a significant difference in bacterial community composition (p<0.05, Figure 3-8b and c, respectively) when compared to 0d, (Figure 3-8a). This was most notable in the bacterial communities associated with site 3 and 5, which

clustered with site 1 on 0d of the MDS biplot (Figure 3-8a), while clustering separately on 45d and 105d CP treatment (Figure 3-8b and c, respectively). Similarly, site 2 and 4 clustered separately from sites 1, 3 and 5 on 0d (Figure 3-8a), but showed a similar bacterial community profile with site 1 at the 45d and 105d of CP treatment (Figure 3-8b and c, respectively).

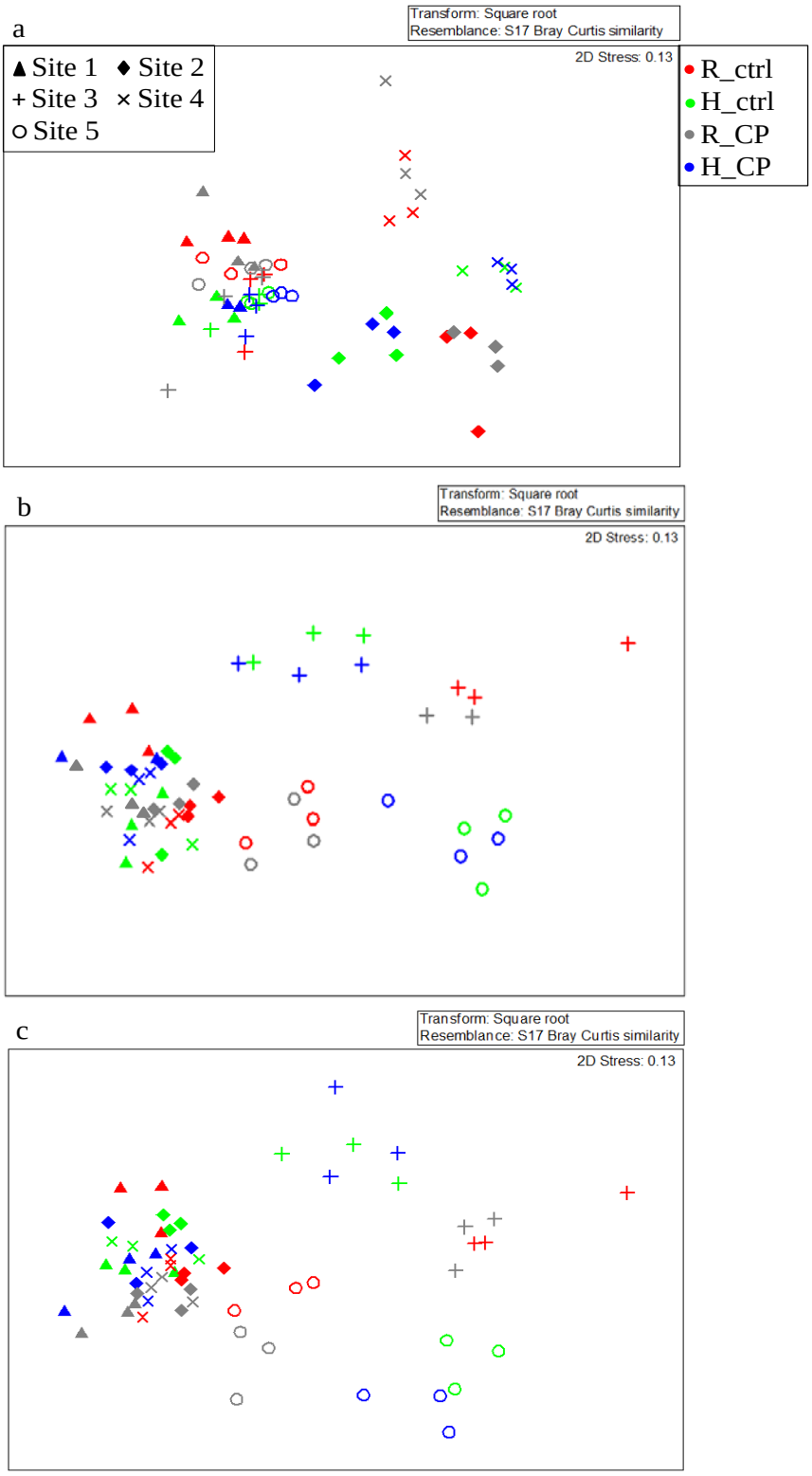

Figure 3-8 MDS ordination of bacterial community structure grouped on site, past pesticide and chlorpyrifos (CP) lab treatment a) 0d, b) 45d and c) 105d based on the √-transformed T-RF peak abundance and Bray-Curtis index (stress=0.13). Legends: R_ctrl=past pesticide treatment (in fields) and no CP lab treatment (control), H_CP=no past pesticide treatment (in fields) but CP lab treatment, H_ctrl=no past pesticide treatment (in fields) and no CP lab treatment (control), R_CP=past pesticide treatment (in fields) and CP lab treatment.

On the other hand, although no main effect of IC lab treatment was found significant (p>0.05), a general trend of decreasing microbial diversity indices (diversity, richness and evenness) was observed (Figure 3-9a, b and c, respectively). Moreover, the interaction between the incubation time and IC lab treatment significantly affected the diversity (p<0.001), richness (p<0.01) and evenness (p<0.001) index value. The analysis also showed an interaction of site and IC lab treatment had an effect on microbial diversity (p<0.001) and evenness (p<0.001) but richness wasn't affected by the interaction (p=0.525) (Table 3-7). Effect of IC on diversity indices was also measured for each site which differed among sites, suggesting that the bacterial communities responded differently depending on the sites (Appendix Figure A-5, A-6 and A-7)

Table 3-7 Results of the univariate analysis for diversity indices and abundances after IC treatment for 102 days. The values in bold shows significant effect of treatment and/or interactions (p≤0.05).

Variables	q-PCR (p values)	Diversity (p values)	Evenness (p values)	Richness (p values)
Site	**0.000**	0.230	0.953	0.118
Time	**0.008**	**0.000**	**0.000**	0.174
Past_pesticide	0.518	0.067	**0.047**	0.879
IC_lab treatment	0.445	0.094	0.057	0.932
Site*Time	**0.000**	**0.001**	**0.000**	**0.024**
Site*Past_pesticide	0.525	**0.003**	**0.018**	0.730
Site*IC_lab treatment	0.211	**0.000**	**0.001**	0.525
Time*Past_pesticide	0.226	**0.000**	**0.002**	0.108
Time*IC_lab treatment	0.094	**0.000**	**0.000**	**0.009**
Past_pesticide*IC_labTreatment	0.666	0.997	0.811	0.607
Site*Time*Past_pesticide	**0.005**	0.150	**0.004**	0.333
Site*Time*IC_lab treatment	0.273	0.910	0.221	0.571
Site*past_pesticide*IC_lab treatment	**0.000**	0.468	0.096	0.889
Time*past_pesticide*IC_lab treatment	0.797	0.509	0.427	0.895
Site*Time*past_peaticide*IC_lab treatment	**0.000**	**0.006**	0.163	0.219

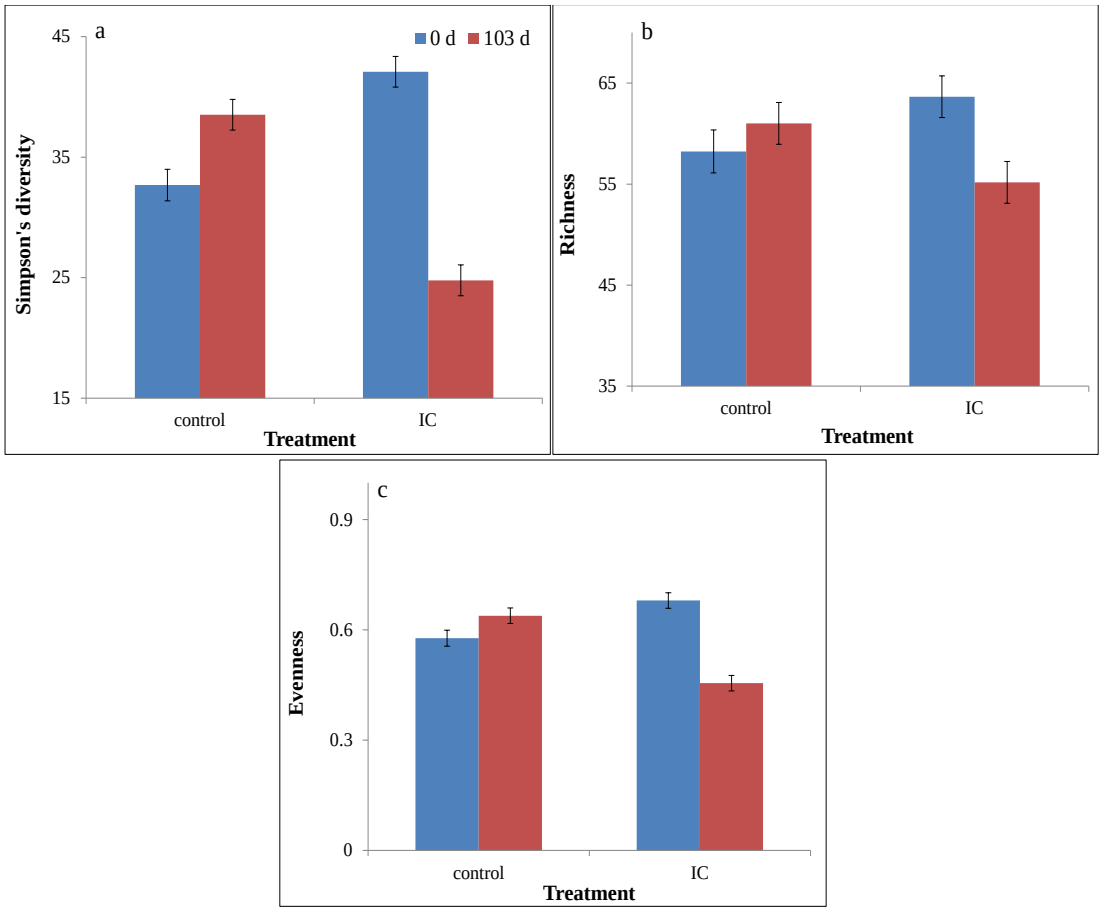

Figure 3-9 Results of the T-RFLP analysis showing effect of imidacloprid (IC) on a) Simpson's diversity, b) Richness and c) Evenness for soil treated with imidacloprid at 0d and 103d with their respective untreated controls. Error bars represent standard error of the mean (n=3).

In addition to this, T-RFLP patterns assessed using a MDS biplot revealed that bacterial communities were affected by IC treatment during 103d experimental period (Figure 3-10a, b and c). Similar to CP lab treatment, an overall change in the bacterial community was observed at 43d in comparison with 0d (Figure 3-10a and b, respectively). The community remained altered throughout the experiment (103d) (Figure 3-10c), indicating that IC application caused a long-term changes in the structure of the bacterial communities.

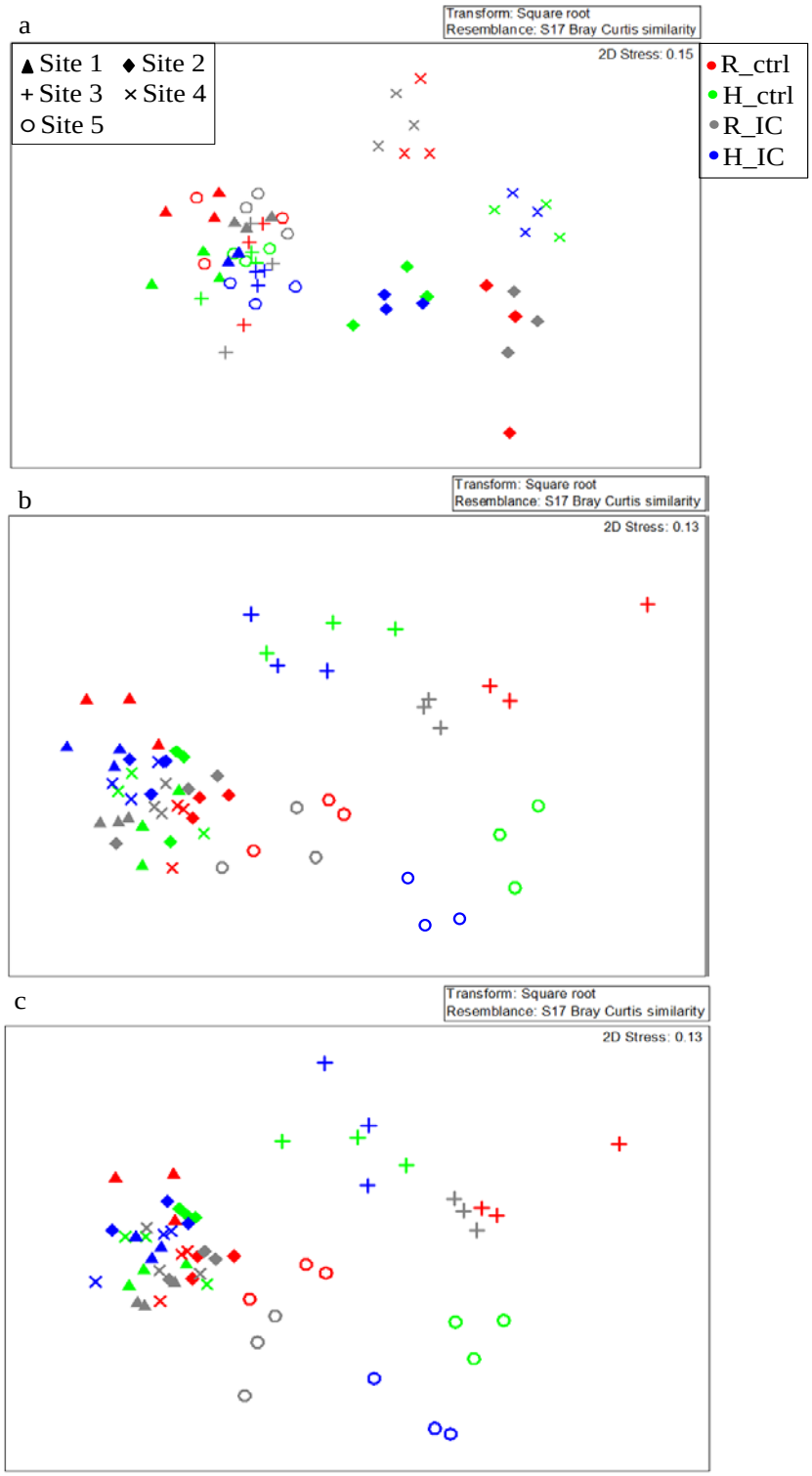

Figure 3-10 MDS ordination of bacterial community structure grouped on site, past pesticide and imidacloprid (IC) lab treatment a) 0d, b) 45d and c) 105d based on the √-transformed T-RF peak abundance and Bray-Curtis index. Legends: R_ctrl=past pesticide treatment (in fields) and no IC lab treatment (control), H_IC=no past pesticide treatment (in fields) but IC lab treatment, H_ctrl=no past pesticide treatment (in fields) and no IC lab treatment (control), R_IC=past pesticide treatment (in fields) and IC lab treatment.

3.3.6 Linking microbial community structure to functions

Persistence of the residues of the pesticides in soil may have a significant impact on soil microbial communities and their functions. Microbial respiration (basal and SIR) is a useful indicator to determine the impact of pesticides on soil microbial activity, which in turn is considered linked to soil health and fertility. The changes in the microbial community structure (measured as MDS1 axis of T-RFLP data) were correlated with the differences in the microbial activity (measured as microbial respiration). The relationships between soil microbial respiration (basal and SIR) and microbial community structure in pesticide lab-treated (CP/IC) and control soils are shown in (Figure 3-11) which indicate that the community functioning was highly correlated with the bacterial community structure (CP_{basal}, R^2=0.016; IC_{basal}, R^2=0.004 and CP_{SIR}, R^2=0.233; IC_{SIR}, R^2=0.21). On the other hand, no relationship between bacterial abundances and bacterial community indices (diversity, richness and evenness) of pesticide (CP/IC) treated soils' with soil respiration was observed (data not shown), indicating that the differences in the bacterial microbial activity were mainly driven by change in the community structure. However, there seemed to be some general trend in the respiration data such that the pesticide lab-treated soil (CP/ IC) were associated with lower SIR indicating that the community function was adversely affected by the application of CP/ IC. Both the pesticides (CP/IC) were shown to alter microbial community structure and functions.

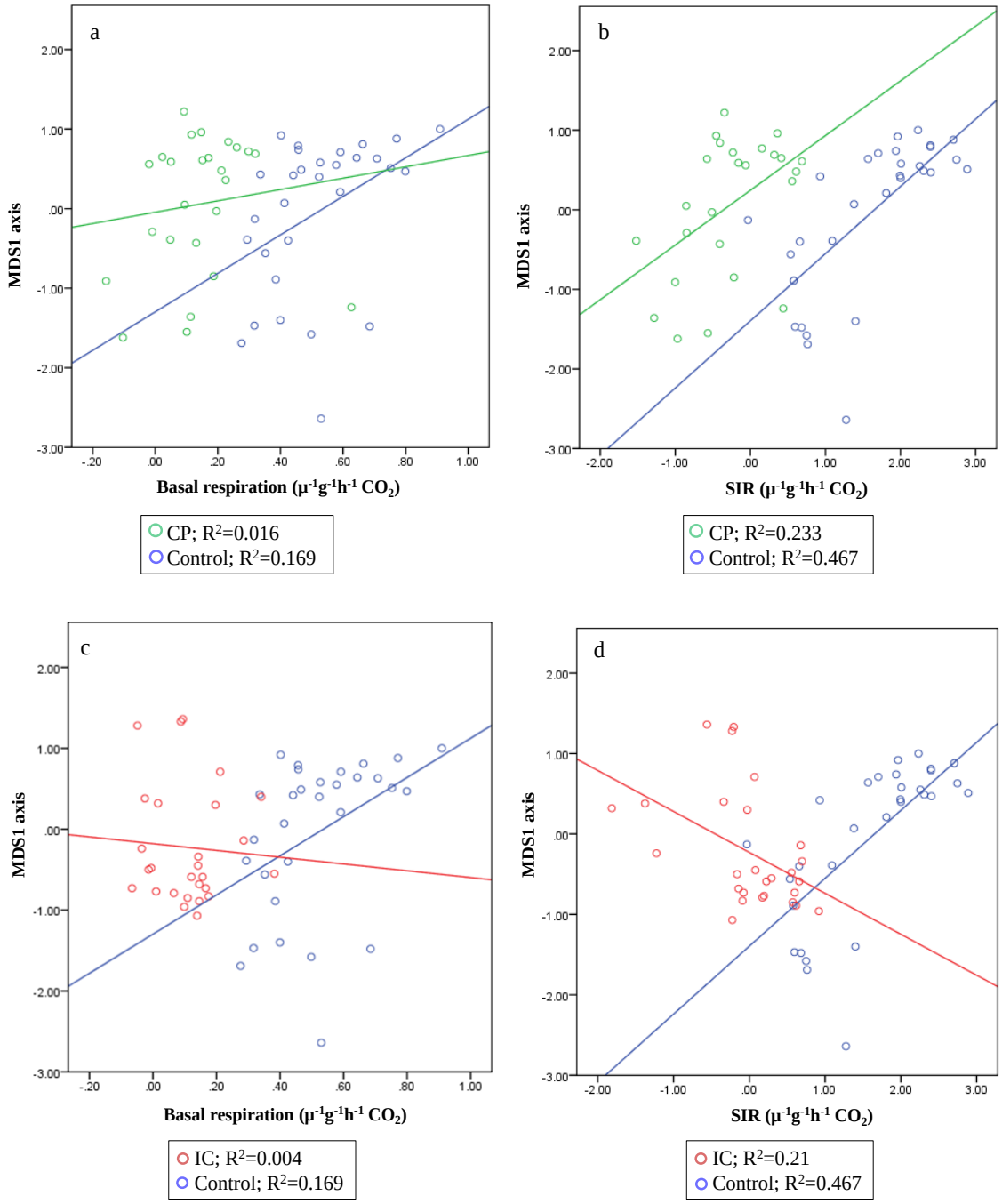

Figure 3-11 Linear regression of natural log transformed microbial respiration (basal and SIR) and community structure (MDS axis 1) split by pesticide treatment a) CP-treated basal respiration, b) CP-treated SIR, c) IC-treated basal respiration and d) IC-treated SIR. The regression line (R^2) shows the relationship between soil respirations and bacterial community structure after treatment with pesticides (45d CP/ 43d IC). Legends: CP=chlorpyrifos, IC=imidacloprid.

3.4 Discussion

3.4.1 Degradation of pesticides (CP and IC) by soil bacterial communities

Assessing the degradation of pesticides (CP and IC) in soils would help to improve understanding of the impact of pesticides on target and non-target organisms. In this study, the half-lives of CP were in between 23-47, 8-20 and 3-17 days for the first, second and third application respectively, which is consistent with the half-lives reported earlier (Singh *et al.*, 2002a, Fang *et al.*, 2006, Chu *et al.*, 2008) indicating less persistence of CP in the soil with repeated applications. Moreover, the results suggested that repeated application of CP resulted in an enhanced degradation phenomenon (except for site 3), where an increased soil microbial population could utilise CP as a readily available source of C and energy. The limited ability of site 3 to develop enhanced degradation (in lab) is consistent with the fact that this site have not shown enhanced CP degradation under field conditions and the pesticide is still in use at this site. Previous literature showed similar results where repeated application of pesticides significantly accelerated its degradation in the some field but not others (Robertson *et al.*, 1998, Baxter and Cummings, 2008, Fang *et al.*, 2008), suggesting a role of intrinsic soil biotic and abiotic properties in enhanced degradation (Singh *et al.*, 2002a)

The rapid development of enhanced degradation of CP (under lab conditions) could be attributed to the potential legacy effect of CP on soil microbial communities, allowing for the survival of CP degraders in pesticide-treated soils-even after the sites (site 1R, site 2R, site 4R and site 5R) were not exposed to CP, following its efficacy loss due to development of enhanced degradation in the soils (about 13 years ago). The results suggested that the degradation capability of soil microbial

communities were preserved even in the absence of CP and the microbial population quickly recover the ability to degrade CP as this became readily available. This supported the hypothesis that a legacy of CP application would allow soil microbes to maintain or at least recover enhanced degradation even in the absence of CP availability. In addition to this, soils from site 1H, site 2H, site 4H and site 5H also showed enhanced CP degradation irrespective of no previous direct CP applications (in fields). The enhanced degradation of CP in those soils could possibly be due to the transport of potential CP degraders from pesticide-treated sites (1R, 2R, 4R and 5R) to non-pesticide treated sites (1H, 2H, 4H and 5H) through for example, flooding as these sites were located just outside the pesticide applied fields. This might have later led to the enrichment of CP degraders during lab treatments eventually resulting in CP degradation. Moreover, pesticide biodegradation is a ubiquitous environmental process. The responsible microorganisms are present or evolve in most ecosystems (*e.g.* soils, sediments, water and sewage sludge) where the contaminants (here, CP) may serve as a source of C and energy resulting in enrichment of the degrading microbial communities soon after they are exposed to the contaminant (Margesin *et al.*, 2000, Karunya and Saranraj, 2014). Six bacterial strains capable of utilising CP were isolated from the CP-treated soils (lab) after three successive treatments with CP (Chapter 4), resulting in CP degradation, which confirmed the finding of this chapter.

Once a soil developed enhanced degradation for CP, it persisted for many years as was demonstrated with the soils from site 1, site 2, site 4 and site 5. For example, the degradation of CP was still accelerated 13 years after the last applications in the fields. Similar observations for short-term effects were reported earlier for thiocarbamate where the accelerated degradation persisted for 1-2 years (Roeth,

1986), and for aldicarb, and oxamyl where persistent accelerated degradation was found for more than 5 years (Suett *et al.*, 1993, Smelt *et al.*, 1996). However, in the last 10 years studies on accelerated degradation of pesticides in soils has declined considerably and there is limited knowledge on the duration of the phenomenon (Arbeli and Fuentes, 2007) and this is the first study to examine the long-term legacy impact of CP.

On the other hand, IC exhibited high persistence in soil where repeated application was associated with reduced rates of degradation. The half-life of IC was between 30-60 days for the first for the first treatment and 45-65 days for second treatment. The half-lives of IC were in accordance with those reported earlier (Sarkar *et al.*, 2001, Liu *et al.*, 2011, Cycoń *et al.*, 2013) and confirmed that the repeated application of IC exhibited even longer persistence in soils. This observation was in accordance with the impact of IC on bacterial community (section 3.4.2) where persistence and accumulation over time influenced the bacterial community composition. To the best of my knowledge no study has been done so far to determine the response of the soil microorganisms on repeated application of IC and this is important because IC are currently repeatedly used in agriculture globally. Moreover, there was no significant difference between the degradation kinetics of different sites (except for site 3), despite different soil types, indicating that soil type did not influence degradation of pesticides in this study. However, further in-depth study on the nature of soil would provide more realistic approach on pesticides degradation. Further, experiments with chloramphenicol and cycloheximide indicated considerable impact on degradation of both CP and IC. This may be explained by the fact that these agents can inhibit microorganisms that degrade

CP/IC, indicating a direct role of soil microbial community in degradation (Singh *et al.*, 2003a).

Although previous studies have reported the fate and ecotoxicology of CP and IC on soil microbial processes, they have been mostly carried out using single application of pesticides (Wang *et al.*, 2010a, Maya *et al.*, 2011, Cycoń *et al.*, 2013). In practice, however, these pesticides may be applied repeatedly over a growing season. Thus, the results from this study are more realistic and have provided strong insight into the interaction between the pesticides (CP and IC) and soil microbial communities in field conditions.

3.4.2 Impact of pesticides (CP and IC) on soil bacterial communities

The impacts of CP and IC were determined on soil bacterial abundances and community structure over a 105d CP and 103d IC incubation period (after three successive applications). A q-PCR analysis was performed to determine the effects of the two pesticides on the abundance of 16S rRNA gene copy number in pesticide-treated and untreated (control) soils. In general, no detectable differences in the bacterial abundances (both 0 and 105d CP/ 102d IC) were observed among the pesticide-treated and control soils. The maintenance of 16S rRNA gene copies indicated no reduction of bacterial population and/or adaption of the bacteria in the pesticide-treated soils forming stable bacterial communities.

These findings were consistent with previous reports where no change in the abundance of bacterial population, expressed as 16S rRNA gene, was observed in OP pesticide-treated and control soils (Kwak *et al.*, 2012, Tortella *et al.*, 2014). Similar observations were also reported on the impact of a paenimyxin (a biopesticide) on

the soil bacterial communities where despite of significant reduction in the count of viable bacteria, the numbers of 16S rDNA sequences did not differ significantly in control and paenimyxin-treated soils (Selim *et al.*, 2007). Thus, this study provides evidence that bacterial abundances were not impacted by either CP or IC repeated lab treatments.

Although q-PCR indicated that there was no effect on bacterial abundances, the structure of the soil bacterial community was remarkably affected by three successive applications of CP or IC. The diversity indices (diversity, richness and evenness) were used to evaluate the dominance, richness and homogeneity of the bacterial population present in the soil. Compared to the control, the diversity and evenness indices in the soil treated with CP (three repeated applications) decreased significantly. The decrease in the bacterial diversity community indices could be attributed to an inhibition effect of CP on the soil bacterial communities at this application frequency. This might have caused the change in the relative abundance of certain species, including dominance by a certain groups of organisms, eventually reducing the bacterial community evenness. The loss of diversity over the 105d experimental period suggested that the bacterial community had a low level of resistance to CP. Similar findings have been demonstrated by Zhang *et al.* (2006b) who found a decrease in microbial diversity in soils that has been contaminated with methyl parathion for more than 20 years. A decrease in microbial diversity and evenness was also observed in CP-treated soil where the diversity indices were temporarily reduced during the initial days after CP application (Fang *et al.*, 2008, Wang *et al.*, 2009, Chen *et al.*, 2014). However, contrasting results were reported Singh *et al.* (2002a) where the overall soil microbial diversity and evenness were little affected by CP application at the concentration of 10 mg/kg (lab incubation).

A significant decrease in richness was also observed following the application of CP, suggesting that some bacterial species were not tolerant to pesticide application and failed to adapt. In contrast to this result, little or no fluctuation in soil microbial richness was observed by Singh *et al.* (2002a) and Fang *et al.* (2008). Moreover, other lab studies with pesticides such as glyphosate and atrazine concluded that the application of those pesticides had significant positive effects on the richness of soil microbial communities (Mijangos *et al.*, 2009, Tortella *et al.*, 2013).

By contrast, although statistical analysis did not reveal significant effects of IC application on the microbial community indices, a general pattern of decrease in diversity, richness and evenness of bacterial indices were observed. Moreover, incubation time and a two-way interaction between incubation time and IC lab treatment explained most of the differences. This indicated that addition of IC influences the soil microbial diversity over time. In a recent study, similar results were demonstrated where the microbial diversity indices were significantly affected not only by incubation time but also directly by IC treatment at 10 mg/kg concentration (Cycoń *et al.*, 2013) however, there was no direct influence of IC on microbial diversity when added at the concentration of 1 mg/kg. It is worth mentioning here that the impact of pesticides on soil microbial communities not only depends on environmental variables (*e.g.* soil properties) but also on the pesticide types, mode of action and the concentration of the particular pesticide.

MDS analyses of the bacterial community structure (based on T-RFLP) post 105d CP/ 103d IC application further supported that pesticide application caused a significant shift in bacterial community composition. A comparison of the community profiles between lab treatments (CP or IC) over time indicated that repeated pesticide application could affect the soil bacterial community rapidly and

persistently. Overall, my results showed a lack of resilience and robustness of bacterial communities in soil towards the repeated application of the pesticides (CP or IC). In addition to the results above, both pesticides markedly influenced the microbial functional activity by reducing basal respiration and SIR (45d CP/ 43d IC). The changes in the microbial community composition along MDS axis 1 following CP and IC treatment suggested that broad microbial functions (microbial respiration) were compromised in stressed conditions. The low respiration (basal and SIR) observed in the soils that showed the enhanced degradation of CP, suggested that the microbial community functions were compromised even at the shorter exposure time. This is contrary to the hypothesis that lab treatment with pesticide would have less or no effect on soil microbial activities in soils with pesticide enhanced degradation history (in fields).

Assessing microbial functions are important because they are critical to crop production, environmental sustainability, and soil quality (Topp, 2003). The decrease in soil respiration activity with pesticides use may be associated with high toxicity of the parent compound or their intermediate metabolites after degradation negatively affecting the metabolic activity and integrity of microbial cells. Similar results have been reported from other studies where pesticides negatively affected SIR (Taiwo and Oso, 1997, Das *et al.*, 2005, Cycoń and Piotrowska-Seget, 2009, Cycoń and Piotrowska-Seget, 2015a). In turn, stimulatory effect (Wang *et al.*, 2008, Wu *et al.*, 2015) or no effect (Lupwayi *et al.*, 2009, Dutta *et al.*, 2010) on soil respiration treated with pesticides has also been reported. The variable effect of pesticides on the cellular metabolism of soil microbial community is largely because of the nature of the agro-chemical and the soil type. Some microorganisms could utilise the pesticide as a source of C and energy resulting in the stimulating effect of the microbial

respiration, while others cannot utilise pesticide resulting in decrease or no effect on the broad metabolic functions (Cycoń and Piotrowska-Seget, 2015a).

The responses of soil microbial communities (resistance and resilience) to various pesticides have been documented earlier. However, to my knowledge this study provides novel evidence that pesticide impact on soil function is mainly mediated by the shift in microbial community structure but not abundance. This is also a first report that demonstrates the magnitude of long-term CP and IC contamination on soil microbial diversity and functions in Australian agricultural soils. There is lack of information on the toxicology assessment of pesticides on below-ground organisms; the findings of this study provide a quantitative time-response relationship regarding pesticide application in the agricultural practice and more accurate assessments of both short and long-term pesticide impacts. However further studies are required to assess the impact of CP and IC in other soils of different characteristics because the trend and patterns of pesticides impact on microbial diversity also depends on soil properties (Sigler and Turco, 2002).

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3.5 Conclusion

The main aim of this work was to ascertain the interactions of widely used broad-spectrum pesticides (CP and IC) and bacterial community composition, biomass and activity. In summary, the results supports the hypothesis that despite no exposure to CP for 13 years, the enhanced degradation capacities of soil microbial community were retained due to the legacy effect of previous use of the compound. However, repeated application of CP and IC (in lab) was associated with the reduction in microbial respiration, even in the soils that had developed enhanced degradation capacity (in fields), indicating the adverse impact of the pesticides on broad metabolic functions even when exposed to short period of time. The study also demonstrated that the repeated application of 10 mg/kg of CP or IC reduced the bacterial diversity, richness and evenness. The inhibitory effects were maintained throughout the experiment and the bacterial population did not recover. Further research using isolation and meta-genomic approaches would greatly improve our understanding of changes in microbial community structure or expression of genes involved in stress response in response to the CP/ IC application.

Chapter 4: Isolation and molecular characterisation of chlorpyrifos (CP) and 3,5,6-trichloro-2-pyridinol degrading (TCP) degrading bacteria from Australian sugarcane farm soils

4.1 Introduction

Pesticides are applied in agro-ecosystems to reduce the impact of plant pests and pathogens on crop yield in an effort to meet the increased demand of food for Earth's growing population. Among the various groups of pesticides applied, organophosphorus (OP) pesticides are the most commonly used agrochemicals. The OP pesticides have been extensively used for its high effectiveness against target pests and being relatively less persistent than organochlorine and carbamate pesticides in the environment (Ortiz-Hernández *et al.*, 2011).

Chlorpyrifos (CP) is one of the most widely used OP pesticides against a broad spectrum of insect pests of economically important crops (Kale *et al.*, 1999, Mallick *et al.*, 1999, Fang *et al.*, 2006, Singh and Walker, 2006, Wang *et al.*, 2007b). CP is regarded as less persistent and of more moderate toxicity to mammals, than other organochlorine (OC) and OP pesticides; however, its extensive use in the agricultural sector have resulted in contamination of soil, air and ground- and surface-water (Sapozhnikova *et al.*, 2004, Yang *et al.*, 2005, Yu *et al.*, 2006). The half-life of CP generally ranges between 10 and 120 days in soil but can be up to one year depending on the abiotic factors (Howard, 1991, Singh and Walker, 2006). There is also a growing concern of widespread contamination of the environment leading to potential risks to non-target organism because of its entry into the food chain (Aysal *et al.*, 2004, Chandra *et al.*, 2010, Muhammad *et al.*, 2010) and undesirable health issues (Ragnarsdottir, 2000, Furlong *et al.*, 2006, Rauh *et al.*, 2011, Alavanja *et al.*,

2013). In humans, CP does not build up or persist in body tissues; however, studies involving continuous CP exposure do show a significant degree of bioaccumulation in the tissues of a number of aquatic organisms (Tilak *et al.*, 2004, Jantunen *et al.*, 2008, Singh and Singh, 2008)

In the environment, CP is degraded to 3,5,6-trichloro-2-pyridinol (TCP), which is the primary and major degradation product (Singh *et al.*, 2003a, Yang *et al.*, 2005, Singh *et al.*, 2006, Kim and Ahn, 2009, Li *et al.*, 2010). TCP is classified as a persistent metabolite by the US Environmental Protection Agency (US EPA) with a half-life ranging from 65 to 360 days in soil (Armbrust, 2001). The presence of three chloride atoms on the N-aromatic ring contributes to increased resistance of this metabolite to degradation (Singh *et al.*, 2003a, Chishti and Arshad, 2013, Jabeen *et al.*, 2014). When accumulating in the environment, TCP that has antimicrobial activity, prevents the proliferation of CP-degrading bacteria (Racke, 1993).

Owing to TCPs' greater persistence and water solubility compared to its parent compound, it leaches into the ground and water bodies causing widespread contamination of soils and aquatic environments (Feng *et al.*, 1997, Vogel *et al.*, 2008, Xu *et al.*, 2008, Grzelak *et al.*, 2012, Watts, 2012). The Australian Pesticides and Veterinary Medicines Authority (APVMA) has identified CP and its metabolites to be assessed for spray drift risks due to human health and environmental concerns (Immig, 2010). Other sources of contamination are industrial effluents, disposal by consumers, leakages and accidental spills that require large-scale decontamination. Therefore, developing technologies to remove CP and TCP from contaminated areas or disposal of manufacturing wastes are needed in order to minimise their impact on human health, non-target organisms and the environment.

Among proposed remediation techniques, bioremediation is considered one of the most promising approaches. It is a relatively low cost, easy to use and environmentally-friendly technique (Rayu *et al.,* 2012). The use of microorganisms having the right metabolic pathways is one of the most viable options for the remediation of chlorpyrifos and TCP in soil and water (Li *et al.*, 2010, Thengodkar and Sivakami, 2010, Singh *et al.*, 2011b). Previously CP was reported to be resistant to degradation (Racke *et al.*, 1990, Mallick *et al.*, 1999), but later studies identified bacteria from the genera *Enterobacter* (Singh *et al.*, 2004), *Pseudomonas* (Lakshmi *et al.*, 2009, Farhan *et al.*, 2012, Chawla *et al.*, 2013), *Bacillus* (Liu *et al.*, 2012, El-Helow *et al.*, 2013) and *Klebsiella* (Ghanem *et al.*, 2007) that were able to degrade CP efficiently.

In other recent studies, it was further determined that some CP degrading bacterial strains from the genera *Bacillus* (Anwar *et al.*, 2009), *Alcaligenes* (Yang *et al.*, 2005), *Paracoccus* (Xu *et al.*, 2008), *Gordonia* (Abraham *et al.*, 2013), *Sphingobacterium* (Abraham and Silambarasan, 2013) and *Mesorhizobium* (Jabeen *et al.*, 2014) could utilize CP as a sole source of carbon (C) and also degrade TCP. The first ever bacteria (*Pseudomonas* sp.) capable of utilising TCP as a sole source of C and energy was reported in 1997 (Feng *et al.*, 1997) and a recent study by Li *et al.* (2010) reported *Ralstonia* sp. to degrade concentrations of TCP as a sole source of C and energy. However, very little literature is available on the microbial metabolism of TCP. Although a significant phylogenetic diversity of microorganisms capable of degrading OP pesticides and their metabolites have been isolated, the potential for degradation is highly influenced by biotic and abiotic environmental factors (Mrozik and Piotrowska-Seget, 2010, Niti *et al.*, 2013). Therefore, it has been highlighted that characterisation of diverse groups of biodegrading isolates will expand knowledge on

the different metabolic routes of degradation of these compounds. It will also give flexibility to use efficient bioremedial agents (isolate) for the development of effective bioremediation technologies based on the environmental conditions (*e.g.* pH, nutrients, moisture level) (Singh, 2010).

The present study aimed to isolate bacterial strains, from Australian sugarcane farm soil, that were able to rapidly degrade not only CP but also TCP. In addition to this, the study also aimed to identify the mode of biodegradation (mineralisation or co-metabolism) and nutritional requirements of the isolates, in order to provide comprehensive knowledge for future use in bioremediation. To achieve this aim, isolation was done from sugarcane farm soils that showed an enhanced degradation of CP in a lab-based study due to the legacy effect of the compound (Chapter 3). The driving hypothesis of this study was that the enhanced CP degrading soils would select for bacterial strains with specialised functions *via* positive selective pressure that utilise CP and its metabolite (TCP) as a source of C, nitrogen (N) or phosphorus (P); thus promoting their efficiency for bioremediation of contaminated sites . In addition to this, isolating and utilising indigenous bacterial strains capable of metabolising OP compounds, for *in situ* bioremediation, are favourable given that they are well adapted to the local conditions. This also avoids the risk of introducing foreign microflora for decontamination, which can have unintended consequences on the environment.

Through repetitive enrichment and successive culturing indigenous bacterial strains were examined for their potential to degrade CP and TCP (alone and together) in liquid media under various nutritional conditions. Bacterial strains capable of degrading both CP and TCP (Australian soils) can be exploited to enhance the

efficiency of bioremediation as they will be able to completely mineralise CP for source energy.

4.2 Material and methods

4.2.1 Site description and sample collection

Pesticide degrading bacteria were isolated from soils collected at five different sugarcane farms in the Mackay, Burdekin and Tully areas in Queensland that had almost 20 year history of pesticide use to control sugarcane grub. The history of pesticide application differed between sites (Figure 3-1). The description of soil sample collection, preparation and soil properties (Table 3-2) is given in section 3.2.1 and 2.2.2.

4.2.2 Preparation of media

Two different media, a mineral salt medium (MSM) and a soil extract medium (SEM) were used for liquid enrichments and isolation of CP and TCP degraders. MSM was mineral based media with no added C but supplemented with nitrogen (MSM+N) and contained g/l Milli-Q (MQ) water:

Solution-1: 2.27 g KH_2PO_4, 5.97 g Na_2HPO_4, 1.00 g NaCl, 1.00 g NH_4Cl;

Solution-2: 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2 \cdot 2H_2O$, 0.02 g $MnSO_4 \cdot 4H_2O$;

Solution-3: 0.025 g $FeSO_4$.

During the preparation of media, solution 1 and solution 2 were autoclaved separately for 15 minutes at 121^{o}C and combined when cool, to avoid the precipitation of metal ions as insoluble phosphate compounds. Solution 3 was filter-sterilized (0.22 µm pore size, Millipore) prior to adding to the cool medium. The C-source was provided in the form of formulated CP (500 mg/ml, Nufarm; Australia)

or TCP (Sigma Aldrich, USA) dissolved in an organic solvent (20 mg/ml in methanol; Sigma Aldrich, USA) and was added aseptically to MSM+N and SEM after autoclaving to achieve a concentration of 20 mg/l (Cullington and Walker, 1999). A commercial grade CP was used throughout the experimental studies because it may closely resemble the compounds (active agents and formulation) that microorganisms were likely to be exposed to in the soil environment. TCP was used for the screening process to isolate bacteria that assist in the complete mineralisation of CP and TCP (in liquid or soil).

Soil extract media (SEM) was prepared using soils from the field sites in Sydney (latitude -33.887551^{o}, longitude 151.18734^{o}). Soil and MQ water were mixed in a 1:2 (w/v) ratio and sterilised for 15 minutes at 121^{o}C. The slurry was centrifuged at 4000 rpm for 10 minutes to remove soil particles and the supernatant was recovered and autoclaved for 15 mins at 121^{o}C. SEM was allowed to cool and CP and TCP solutions were added aseptically at a final concentration of 20 mg/l (Karpouzas *et al.*, 2000b, Singh *et al.*, 2004). The pH of both the media (MSM+N and SEM) was maintained at 7.0±0.2 to allow the growth of indigenous degraders of pesticides present in the pesticide-treated soils. MSM+N agar and SE media agar containing CP or TCP were prepared in a similar way as above, except that agar was added at the concentration of 15 g/l. LB plates (Sigma) were also prepared according to the manufacturer's instruction.

4.2.3 Enrichment of CP and TCP degraders

Enrichment of the potential CP and TCP degraders was done from the soils 4H, 4R and 5R that showed enhanced degradation of CP in lab-based degradation study, post

three successive CP applications (Figure 3-2). Out of these soils (4H, 4R and 5R); one soil (5R) was further treated separately with TCP (10 mg/kg) to enrich for TCP degraders. The experimental set up for the pesticide treatment is described in section 3.2.3. The enhanced degrading soils (4R, 4H and 5R) were used to set up the liquid enrichment cultures to select CP and/or TCP degraders (section 4.2.5) and degradation of the pesticide was monitored every two days. Pesticides and their metabolites were extracted from liquid culture (1 ml) by mixing with 100% acetonitrile (5 ml). The tubes were vortexed and the pesticide extraction was done by shaking the mix for one hour on a shaker (130 rpm). The samples were centrifuged for ten minutes at 15000 rpm, after which a sub-samples of the clear supernatant were analysed directly by HPLC. The HPLC conditions used were the same as those described in section 3.2.4. Both CP and TCP were detected spectrophotometrically at 230 nm.

4.2.4 Isolation of CP and TCP degraders

The isolation of CP and TCP degraders was attempted from soils that showed the greatest degradation rates in previous study (Figure 3-2): CP degraders from 4R and 4H (only CP-spiked soil samples); TCP degraders from 4R and 4H (only CP-spiked soil samples) and from 5R (CP- and TCP-spiked soil samples). These variations were set up to screen for strains that could degrade CP and/or TCP alone or together. Approximately 1.5 g soil (wet weight) from each soil replicate was added to 10 ml of MSM+N with sterile glass beads and vortexed for 1 minute. About 2 ml of the supernatant was then used to inoculate separate bottles containing 40 ml of MSM+N and 20 mg/l of pesticide (CP or TCP). In MSM+N, CP or TCP were the only sources

of C. All the replicates from a particular soil sample were treated separately for isolation purposes. Un-inoculated sterile controls were also prepared in triplicate along with the inoculated samples. Pesticide degraders were enriched in the dark on a shaker at 180 rpm for four days at 28°C. A second enrichment was done by transferring 1 ml of pre-grown culture from each replicate to respective media (40 ml) and the same incubation conditions were followed for another four days. Appropriate un-inoculated sterile controls were also maintained throughout the experiment (Singh *et al.*, 2004).

Following the second enrichment, 10-fold dilution series were prepared and 0.1 ml of these dilutions were spread in triplicate on MSM+N agar plates (with 20 mg/l CP or TCP concentration) and incubated for 2 days at 28^{o}C. Purity streaking for isolated colonies was done on SE media and LB agar plates. To check the ability to degrade CP and TCP in their purified state, the isolates were inoculated separately in SE and MSM+N liquid media supplemented with 20 mg/l of CP or TCP. The bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}) based on the turbidity of the cell suspension.

4.2.5 Identification of isolated bacteria by DNA based methods

4.2.5.1 Colony DNA extraction and amplification by polymerase chain reaction (PCR)

Well-isolated bacterial cells (from soil cultures) were picked with sterile tooth picks and carefully resuspended in 10 µl of nuclease-free water. DNA was extracted by boiling the cells at 95^{o}C for three minutes and then cooling on ice for one minute. The resuspended mix was then centrifuged for five minutes at 15000 rpm. 1 µl of the

supernatant was directly used for PCR amplification, targeting the bacterial 16S rRNA gene. The PCR conditions used were 0.5 µl of each primer (20 pmol), 1 µl of poly deoxynucleoside triphosphate mix (dNTPs, 20 mM), 5 µl 10X NH^{+}_{4} PCR buffer, 2 µl of $MgCl_2$ (50mM), 1 µl BSA (20mg/ml), 0.5 µl of Taq polymerase (all reagents from Bioline, USA) and 38.5 µl of sterile nuclease-free water to make the reaction volume 50 µl. Negative controls, with 1 µl of molecular grade water as a template, were included in all sets of PCR reactions to provide a contamination check. The primer set used for the amplification of 16S rRNA gene was 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') (Lane, 1991, Dees and Ghiorse, 2001, Martin-Laurent *et al.*, 2001). This primer set amplifies a >1400 bp region. All amplifications were carried out in a DNA Engine Dyad® Peltier Thermal Cycler (Bio-Rad, USA). The PCR protocol began with a denaturation step at 95^{o}C for 4 min followed by 29 cycles of denaturation at 94^{o}C for 30 s, annealing at 55^{o}C for 1 min and extension at 72^{o}C for 1 min. The protocol was concluded with an additional final extension at 72^{o}C for 10 mins. After amplification, 3 µl of PCR products were electrophoretically separated and visualised in 1X TBE, 1% (w/v) agarose gel stained with SYBR® Safe (Life technologies, USA) to confirm the size (HyperLadder™ 100 bp, Bioline; USA) and purity of DNA. The DNA was then purified (described in section 2.2.7.3) and quantified with NanoDrop 2000C spectrophotometer (Thermo Fisher, USA) before submitting samples for sequencing.

4.2.5.2 Sequencing

Purified DNA samples were sequenced using the BigDye Terminator v3.1 kit (Applied Biosystems). About 15 ng of DNA as template and 3 pmol of both forward (27f) and reverse primer (1492r) were used per 20 µl reaction mixture in separate reactions. 16S rRNA gene was amplified with the above primer set resulting in product length of ~1400 bp. The amplified products were collected, purified and sequenced. The quality of the sequence was checked using Sequencher 4.10.1 (Gene Codes Corporation) where both forward and reverse strands were edited, assembled and aligned together to generate contig for further analyses

All 16S rRNA gene partial sequences (>1200 bp) were compared with those available on BLAST search of the GenBank database (blast.ncbi.nlm.nih.gov/). Sequences with the greatest similarity were extracted and compared with MUSCLE (MEGA 6). Those aligned DNA sequences were then used to construct a phylogenetic tree using MEGA 6 (Molecular Evolutionary Genetics Analysis) (Tamura *et al.*, 2013) by performing the maximum likelihood tree analysis with 1000 bootstrap replicates using Kimura-2-parameter model with Gamma distribution (K2+G).

4.2.6 Degradation of CP and TCP as a source of carbon (C), nitrogen (N) and phosphorus (P)

The capacity of the isolates to degrade CP and TCP as C-, N- and P-source was determined. For this purpose, MSM (described in section 4.2.2) was modified and adapted from Coleman *et al.* (2002) recipe. The media was supplemented with CP or

TCP (20 mg/l). The following solutions/components were used to make up the media (g/l MQ water):

Solution-1: 2.27 g K_2HPO_4, 0.95 g KH_2PO_4, 0.67 g $(NH_4)_2SO_4$;

Vitamin Supplement (ATCC®, USA);

Trace salt solution: This consisted of 6.37 g $Na_2EDTA.2H_2O$, 1.0 g $ZnSO_4.7H_2O$, 0.5 g $CaCl_2.2H_2O$, 2.5 g $FeSO_4.7H_2O$, 0.1 g $NaMoO_4.2H_2O$, 0.1 g $CuSO_4.5H_2O$, 0.2 g $CoCl_2.6H_2O$, 0.52 g $MnSO_4.H_2O$, and 29.30 g $MgSO_4$. pH of the trace salt solution was adjusted to 6.4±0.2, which is very critical for all the trace salts to dissolve and form homogenous solution. The solution was filter-sterilized (0.22 µm pore size, Millipore) and stored at 4^oC in a foil-wrapped bottle.

For preparation of MSM, vitamin supplement (0.5% v/v) and trace salt solution (0.2% v/v) were added aseptically to solution-1 after autoclaving, to avoid precipitation of salts. Four variations of MSM were set up: CP/TCP as a sole source of C (+N +P –C); CP/TCP as a sole source of N (–N+P+C); CP/TCP as a source of P (+N-P+C) and CP/TCP as sole C and N source (-N+P-C). Bulk media was autoclaved and 10mM glucose ($C_6H_{12}O_6$) was added as an alternative C-source where required. The MSM without N was prepared simply by omitting ammonium sulfate [$(NH_4)2SO_4$] and for P-free media K_2HPO_4 and KH_2PO_4 were replaced with 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS). No glucose or ammonium sulphate was added to prepare a C- and N-free media. The final pH of the media was adjusted to 7.0±0.2.

For all the further degradation studies, the isolates from different bacterial families that could degrade CP and/or TCP were selected. Those bacterial isolates were first grown in SEM supplemented with CP or TCP (20 mg/l) to get an increased active

biomass of the degrading culture before inoculating them into MSM with different nutrient compositions. The cultures were centrifuged at 14,000 rpm for five minutes. The supernatant was discarded and the pellet was then washed three times and resuspended in autoclaved tap water before inoculating in different media. Media with different compositions (in terms of C, N and P) were used to understand the ability, physiology and kinetics of degrading bacterium. Triplicate samples (20 ml) of each media were inoculated with actively growing isolates of CP or TCP degraders (1 ml). The bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}) based on the turbidity of the cell suspension. Triplicate sets of each media combination without inoculation were maintained in all the experiments as controls.

4.2.7 Statistical analyses

To investigate the differences in the pesticide degradation between different isolates, an independent samples *t*-test was carried out. Level of significance (*p* value) was set at 0.05. Statistical analyses were carried out with the software package IBM SPSS Statistics 17 (SPSS, Inc.).

4.3 Results

4.3.1 Isolation of CP and TCP degraders

Degradation of CP in MSM+N (liquid enrichment media) supplemented with soil microbial inoculums from 4R (4R1, 4R2, and 4R3) and 4H (4H1, 4H2 and 4H3) soils are shown in Figure 4-1a. For the first CP application (enrichment), CP degradation rate occurred more rapidly in 4R3 and in 4H1 as complete degradation of CP took place in just 3 and 4 days, respectively. After 4 days of incubation, soil samples 4R1 and 4R2 showed only 20% and 38% CP degradation, respectively, whereas no degradation was seen with microbial inoculum from 4H2 and 4H3. The primary metabolite of CP, TCP, was found in all samples displaying CP degradation. (Figure 4-1b).

A second application of CP further increased the degradation rate for both 4R3 and 4H1, with most CP degraded in two days. Degradation rates also increased/started earlier for other soil samples (4R1, 4R2). The second enrichment also led to the degradation of CP in 4H2 and 4H1, degrading 20% and 30% CP respectively. TCP was also produced following a second application of CP due to CP degradation (Figure 4-1c). However, no degradation of accumulated TCP was seen in any of the soil samples. No significant degradation of CP was seen in any of the un-inoculated controls as shown in Figure 4-1a. It was surprising to see that one of the replicates from both soil types showed an accelerated degradation during first application, which could be attributed to the biological variations. However, all replicates (both 4R and 4H) showed an increased rate of degradation after the second application, likely due to the increase in the degrading microbial population size (Figure 4-1a). As this study's primary aim was to isolate potential CP and/or TCP degraders, the

replicates that showed enhanced degradation (4R3 and 4H1) in MSM+N liquid media were used for isolation purpose.

Various attempts to isolate single colonies with CP-degrading bacterial colonies from enriched cultures were made. A total of six potential degraders were obtained after successive sub-culturing from 4R3 and 4H1 soils and named 4H1-M1, 4H1-M2, 4H1-M3, 4H1-M4, 4R3-M1 and 4R3-M2. The isolates 4R3-M1, 4R3-M2, 4H1-M2 and 4H1-M4 were medium sized circular colonies whereas isolates 4H1-M3 and 4H1-M1 were small sized circular and medium sized irregular colonies, respectively. No fungal growth was seen in any of the plate types (data not shown).

On the other hand, no detectable degradation of TCP was seen with any of the soil types (4H, 4R and 5R) when added externally in MSM+N (data not shown). There was no effect of repeated transfer on TCP degradation rates. Despite no degradation of added TCP in liquid MSM+N media, the microbial inoculum from soils enrichments of 4H, 4R and 5R (section 4.2.3) were plated on MSM+N agar plates to check for their ability to grow in presence of added TCP on solid media. However, microbial inoculum failed to grow from any of the enriched soils (4H, 4R and 5R) on MSM+N agar when TCP was added externally, during the set incubation time. Thus, they were dropped from further analyses and only the CP-degrading isolated bacterial strains (4H1-M1, 4H1-M2, 4H1-M3, 4H1-M4, 4R3-M1 and 4R3-M2) were further analysed for their ability to degrade a) CP, b) TCP, as an accumulated metabolite, and c) TCP, when added externally.

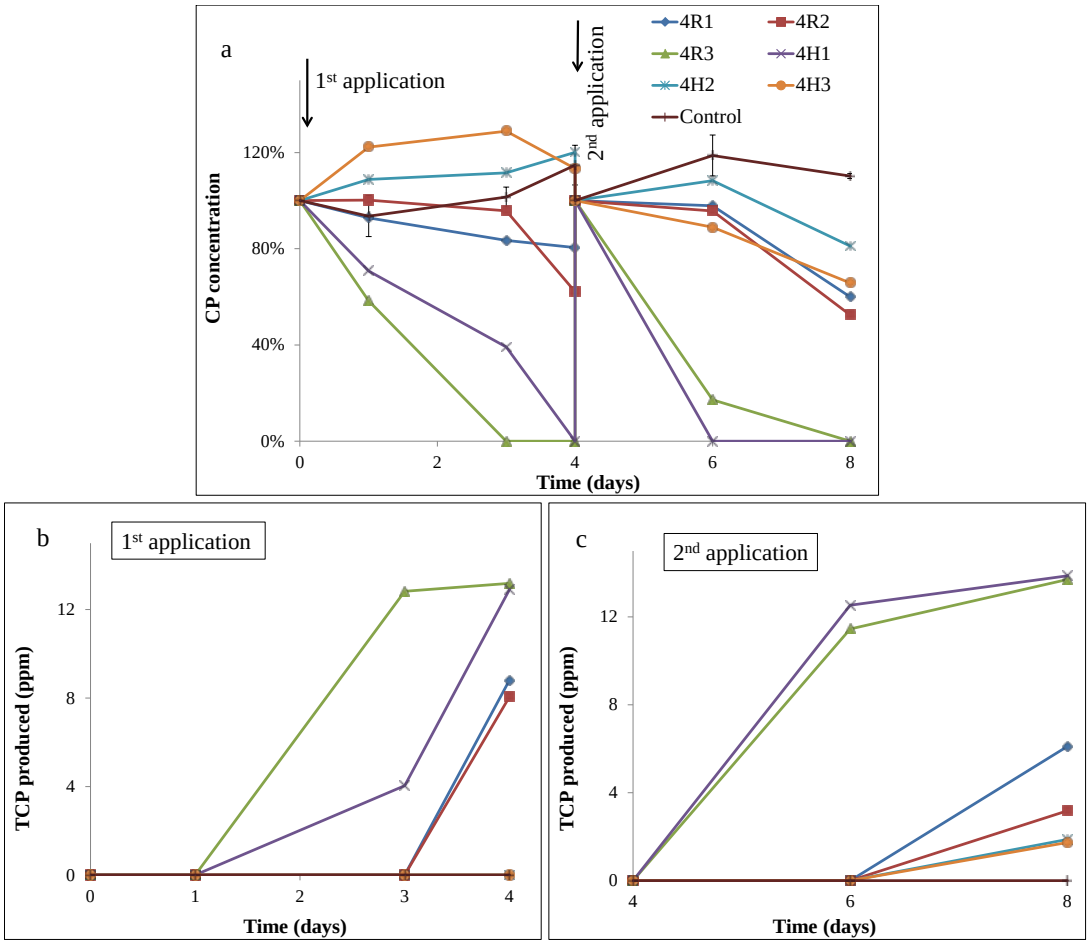


Figure 4-1 Degradation of chlorpyrifos (CP) and production of TCP following two successive applications of CP in MSM+N inoculated with bacterial communities (4R and 4H) and control samples; a) CP degradation, b) TCP production after the first CP application (enrichment), and c) TCP production after second CP application (enrichment). For isolation purpose, all the replicates of 4R and 4H were plotted individually here. Error bars on control (un-inoculated treatment) represents standard error of the mean (n=3). Abbreviation: TCP= 3,5,6-trichloro-2-pyridinol

A quick preliminary experiment to check the ability of pure strains in degradation of CP and TCP was conducted. Minimal degradation of CP were seen when the six isolates were inoculated in MSM+N supplemented with CP (20 mg/l) (Appendix Figure A-8) when compared to SEM (Appendix Figure A-9a-f). In SEM all isolates completely degraded CP within two days except for isolate 4H1-M1, which degraded 50% of CP in two days and complete degradation taking place by day six (Appendix

Figure A-9c). During the degradation of CP by the isolates, TCP, as the primary metabolite, accumulated in MSM+N (data not shown) but it was mostly degraded in SEM (Appendix Figure A-9a-f). Following the successful degradation of CP and its primary metabolite, TCP in SEM, the six isolates were then re-inoculated separately into fresh SEM supplemented externally with TCP (20 mg/l). All the isolates, except for 4H1-M1 (Appendix Figure A-10c) efficiently degraded almost 80% of externally added TCP in SEM (Appendix Figure A-10a-f). No biotic or abiotic degradation of TCP was observed in un-inoculated controls (data not shown)

4.3.2 Molecular characterisation of isolated CP and TCP degraders

All six isolated bacteria were found capable of degrading chlorpyrifos and TCP (as accumulated metabolite or added externally) in SEM, except for 4H1-M1which did not degrade TCP when added externally. To assess the phylogeny of the isolates, a BLASTN analysis (16S rRNA sequence) was carried out through GenBank (http://www.ncbi.nlm.nih.gov). This showed that all the six isolates clustered into the Proteobacteria group but belonged to four different genera. The 16S rRNA gene sequences of the isolates 4R3-M1, 4R3-M2 and 4H1-M4 illustrated a high similarity to the reference sequence from members of the genus *Xanthomonas*, showing 99% similarity with *Xanthomonas* sp. R9-741 (GenBank Accession no. JQ660016), *Xanthomonas* sp. RP-B14 (GenBank Accession no. FM997990), *Xanthomonas* sp. 33DCP (GenBank Accession no. HQ891021), *Xanthomonas campestris* (GenBank Accession no. NR074936) and *Xanthomonas* sp. Y4 (GenBank Accession no. KC708559).

Strain 4H1-M3 clustered with genus *Pseudomonas* showing 100% similarity with *Pseudomonas putida* (GenBank Accession no. KF815695, KC189961, GQ200822), *Pseudomonas plecoglossicida* BF-1 (GenBank Accession no. FJ592171) and other *Pseudomonas* species (GenBank Accession no. KJ733977, HM748053, EU851056, EU375660, DQ079062).

Isolate 4H1-M2 clustered within the clade of genus *Lysobacter* showing 99% similarity with uncultured bacteria (GenBank Accession no. DQ404715, JX236701, AB234277) and *Lysobacter* sp. ITP09 (GenBank Accession no. FR667176). This strain also showed 98% similarity with *Lysobacter* sp. BUT-8 (GenBank Accession no. KJ008918) and other uncultured bacteria (GenBank Accession no. HM438549, JQ426704).

Isolate 4H1-M1 showed 99% similarity with *Rhizobium gallicum S*109 (GenBank Accession no. AY509211), *Rhizobium alamii* (GenBank Accession no. NR042687), *Rhizobium* sp. CHNTR53 (GenBank Accession no. DQ337578), *Rhizobium* sp. 1NP2 (GenBank Accession no. KJ000027), *Rhizobium* sp. CC-RB302 (GenBank Accession no. GQ161992) and *Rhizobium* sp. I29 (GenBank Accession no. HM008945). A phylogenetic tree that depicts the position of all the six isolates and their related species is presented in Figure 4-2. Based on these observations, the strains were designated as *Xanthomonas* sp. 4R3-M1, *Xanthomonas* sp. 4R3-M2, *Xanthomonas* sp. 4H1-M4, *Lysobacter* sp. 4H1-M2, *Pseudomonas* sp. 4H1-M3 and *Rhizobium* sp. 4H1-M1.

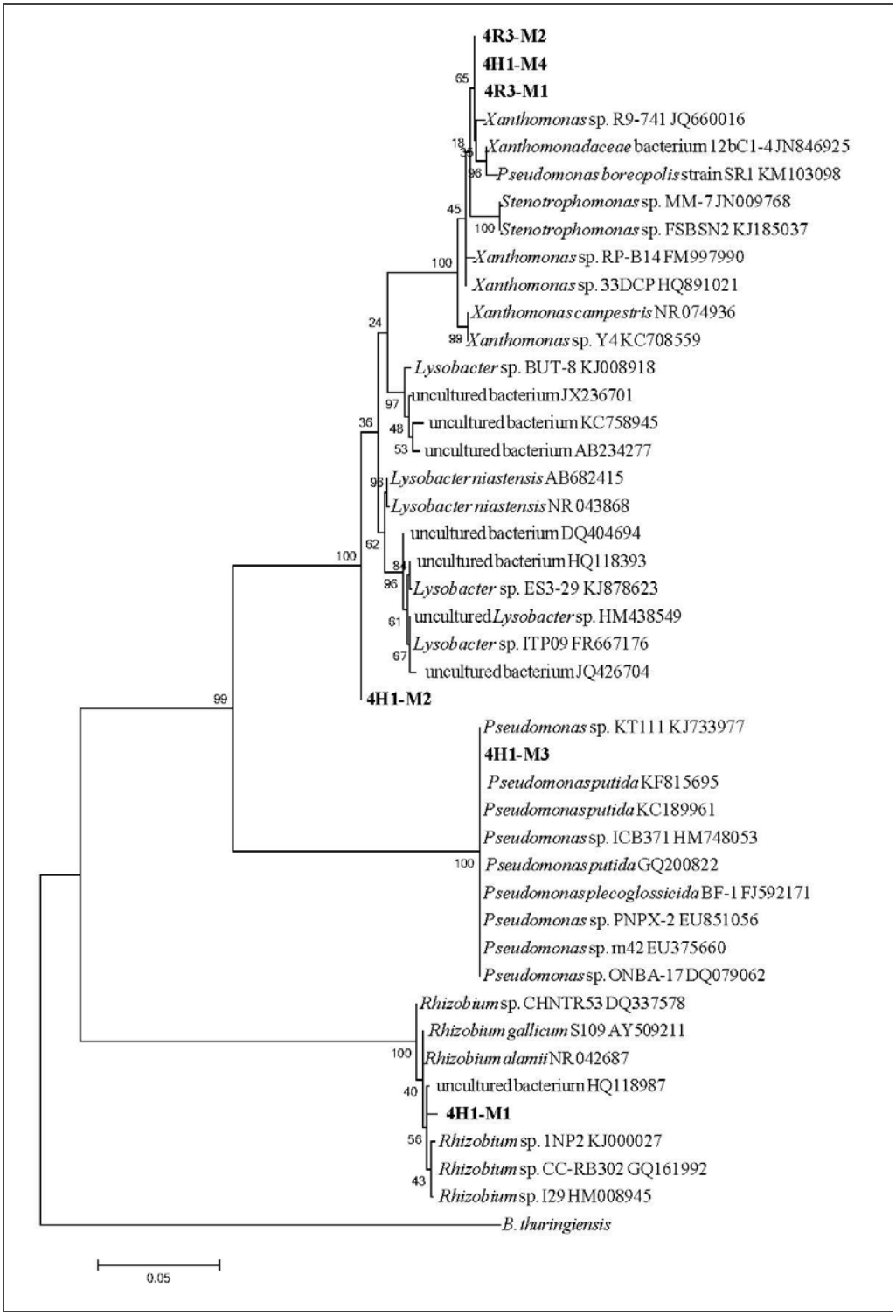


Figure 4-2 Maximum likelihood tree showing the phylogenetic relationship between all six isolates (in bold) and related species based on the 16S rRNA gene sequences retrieved from NCBI. The tree was rooted with *Bacillus thuringiensis.* Bootstrap values are expressed as randomisation of 1000. The scale bar represents the evolutionary distance of 0.05.

4.3.3 Degradation of CP and TCP as a source of carbon (C), nitrogen (N) and phosphorus (P)

4.3.3.1 CP degradation

Isolates representing three bacterial families were selected for further CP and TCP degradation study. (*Xanthomonas* sp. 4R3-M1, *Pseudomonas* sp. 4H1-M3 and *Rhizobium* sp. 4H1-M1), and were screened to determine their ability to degrade CP in SEM and MSM with different nutrient compositions (+N+P–C, –N+P+C, +N-P+C and –N+P-C) (Figure 4-3a-d, 4-4). The CP-degrading ability of all the strains was greater in the media MSM without C (+N+P-C; $p<0.05$) (Figure 4-3a), MSM without N (–N+P+C; $p<0.05$) (Figure 4-3b) and SEM ($p<0.05$) (Figure 4-3d) when compared to MSM without P (+N-P+C; $p>0.05$) (Figure 4-3c).

The strains *Xanthomonas* sp. 4R3-M1, *Pseudomonas* sp. 4H1-M3 and *Rhizobium* sp. 4H1-M1 utilised 80%, 90% and 75% of CP in MSM (+N+P-C), respectively. About 90% CP degradation was observed in SEM when inoculated with *Xanthomonas* sp. 4R3-M1 and *Pseudomonas* sp. 4H1-M3 (Figure 4.3d). By contrast, *Rhizobium* sp. 4H1-M1 utilised only 60% of the CP within 6 days of incubation. These results were consistent with the previous SEM inoculations (preliminary results) where degradation rates for 4H1-M1 were lower than the other strains (Appendix Figure A-9c).

Degradation rates of CP were lower in MSM (–N+P+C) (Figure 4-3b) for all bacterial strains (*Xanthomonas* sp. 4R3-M1-60%, *Pseudomonas* sp. 4H1-M3-70% and *Rhizobium* sp. 4H1-M1-50%) compared to MSM (+N+P-C) and SEM (Figure 4-3a, d respectively). TCP accumulated at approximately the same rate as that of decrease in CP in a particular media (Figure 4-3a-d). TCP yields for *Xanthomonas*

sp. 4R3-M1 and *Pseudomonas* sp. 4H1-M3 were highest in SEM (Figure 4-3d) followed by MSM (+N+P-C) (Figure 4-3a) and MSM (–N+P+C) (Figure 4-3b). No TCP degradation was observed during initial accumulation, which may be considered a lag phase required to induce the production of TCP degrading enzymes. However, CP degradation by *Rhizobium* sp. 4H1-M1 did not result in the production/accumulation of TCP in any of the media, which wasn't consistent with the other two strains. Conversely, TCP produced as a result of CP degradation by *Xanthomonas* sp. 4R3-M1 and *Pseudomonas* sp. 4H1-M3 was also degraded in all media types (Figure 4-3). It was difficult to conclude TCP utilisation in MSM (+N-P+C), because CP degradation was reduced in this media resulting in lower TCP production (Figure 4-3c).

In this experiment, bacterial growth could not be determined accurately due to the turbidity caused in the media by adding commercially available CP (data not shown). No degradation of CP or production of TCP was seen in any of the un-inoculated controls (Figure 4-3).

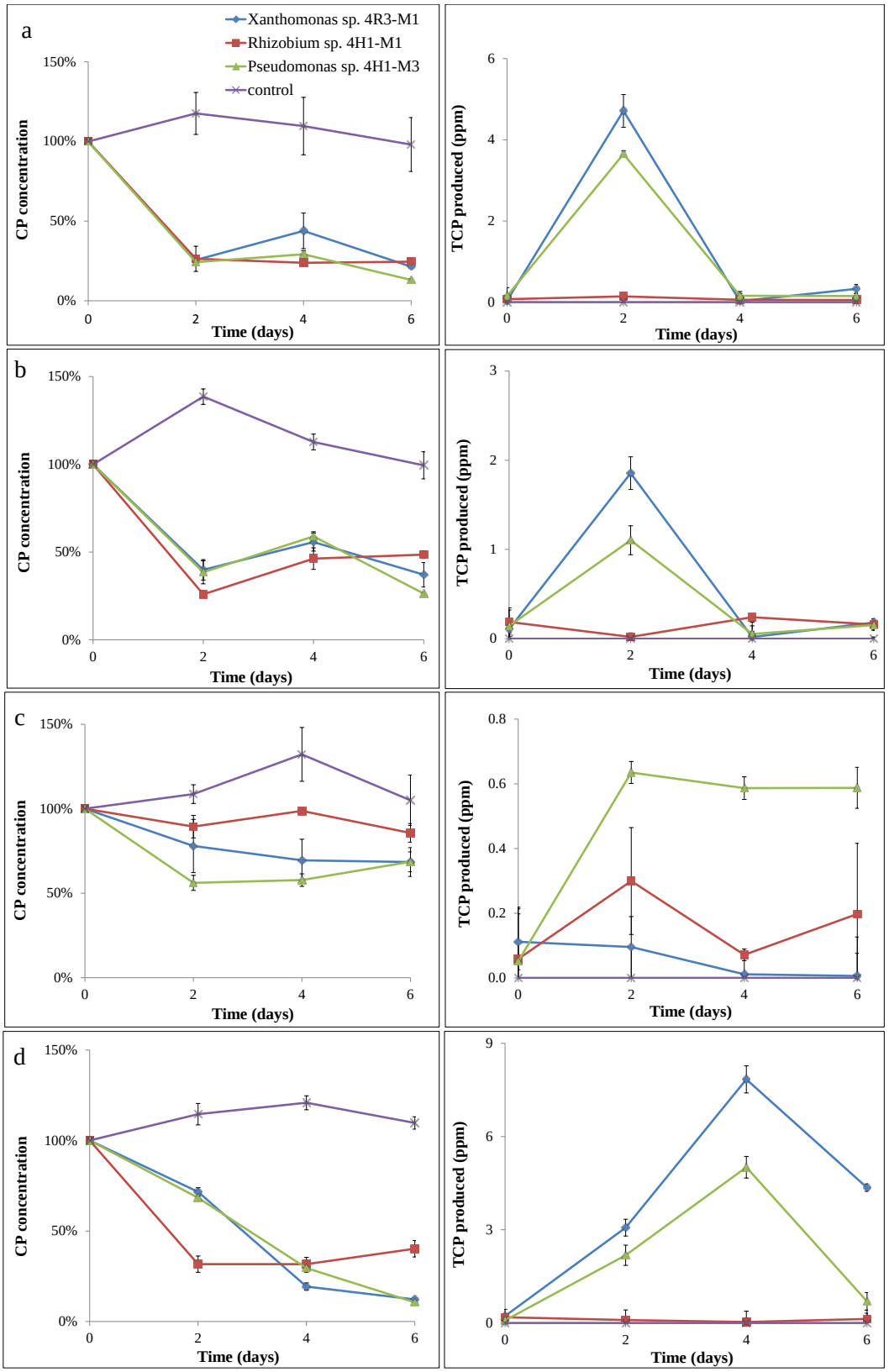


Figure 4-3 Degradation of CP (%) and production of TCP (ppm) by *Xanthomonas* sp. 4R3-M1, *Rhizobium* sp. 4H1-M1 and *Pseudomonas* sp. 4H1-M3 in different media: a) MSM (+N+P-C), b) MSM (-N+P+C), c) MSM (+N-P+C) and d) SEM. Error bars represent standard error of the mean (n=3).

From the above results, *Xanthomonas* sp. 4R3-M1 and *Pseudomonas* sp. 4H1-M3 seemed to be versatile CP degraders and were used to study the effect on CP degradation in MSM (-N+P-C; *i.e.*, without carbon and nitrogen). *Xanthomonas* sp. 4R3-M1 and *Pseudomonas* sp. 4H1-M3 showed similar degradation patterns utilising 70% and 80% of CP, respectively. TCP was also consumed in the process (Figure 4-4).

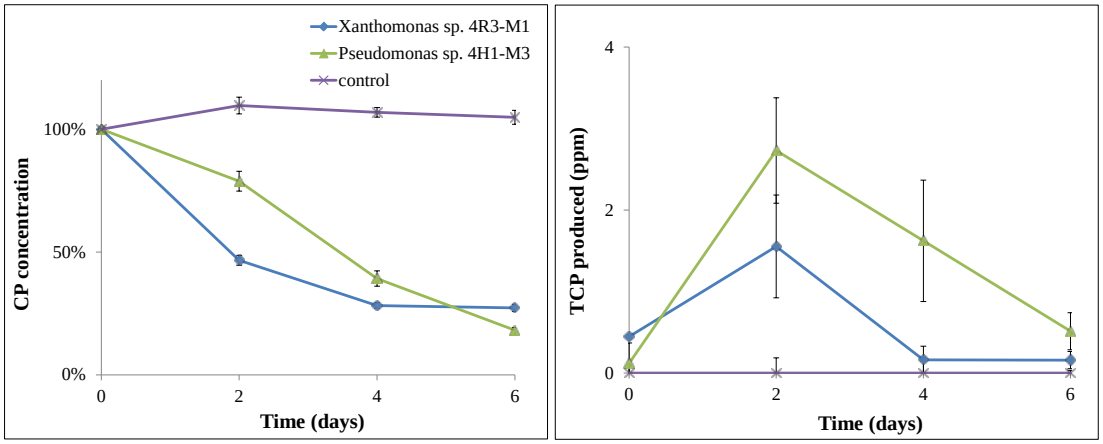


Figure 4-4 Degradation of CP (%) and production of TCP (ppm) by *Xanthomonas* sp. 4R3-M1and *Pseudomonas* sp. 4H1-M3 in MSM (-N+P-C). Error bars represent standard error of the mean (n=3).

4.3.3.2 TCP degradation

The TCP degradation pattern by all three strains in different media composition is shown in Figure 4-5. The degradation dynamics of TCP, in different media compositions, were almost similar for *Xanthomonas* sp. 4R3-M1 and *Pseudomonas* sp. 4H1-M3. Both strains could degrade 100% TCP in MSM (+N+P-C; $p<0.001$) as C-source within 4 days and MSM (-N+P+C; $p<0.001$) as N-source within 6 days (Figure 4-5a and 4-5b, respectively). After 8 days of incubation, TCP utilisation for *Xanthomonas* sp. 4R3-M1 were 20% (not statistically significant from control)

(MSMP+N-P+C; $p>0.05$), 54% (SEM; $p<0.01$) and that of *Pseudomonas* sp. 4H1-M3 were 67% (MSM+N-P+C; $p<0.05$) and 40% (SEM; $p<0.01$) (Figure 4-5c and 4-5d, respectively). The degradation of TCP was slower in MSM (+N-P+C) and SEM showing an effect of media on degradation. The degraded amount (%) of TCP in SEM was relatively less than the previous SEM inoculations (Appendix Figure A-10). In all cases, TCP degradation was accompanied by an increase in bacterial OD_{600}. In contrast, *Rhizobium* sp. 4H1-M1 did not degrade TCP in any of the media assessed ($p>0.05$) and no degradation was observed in un-inoculated controls (Figure 4-5).

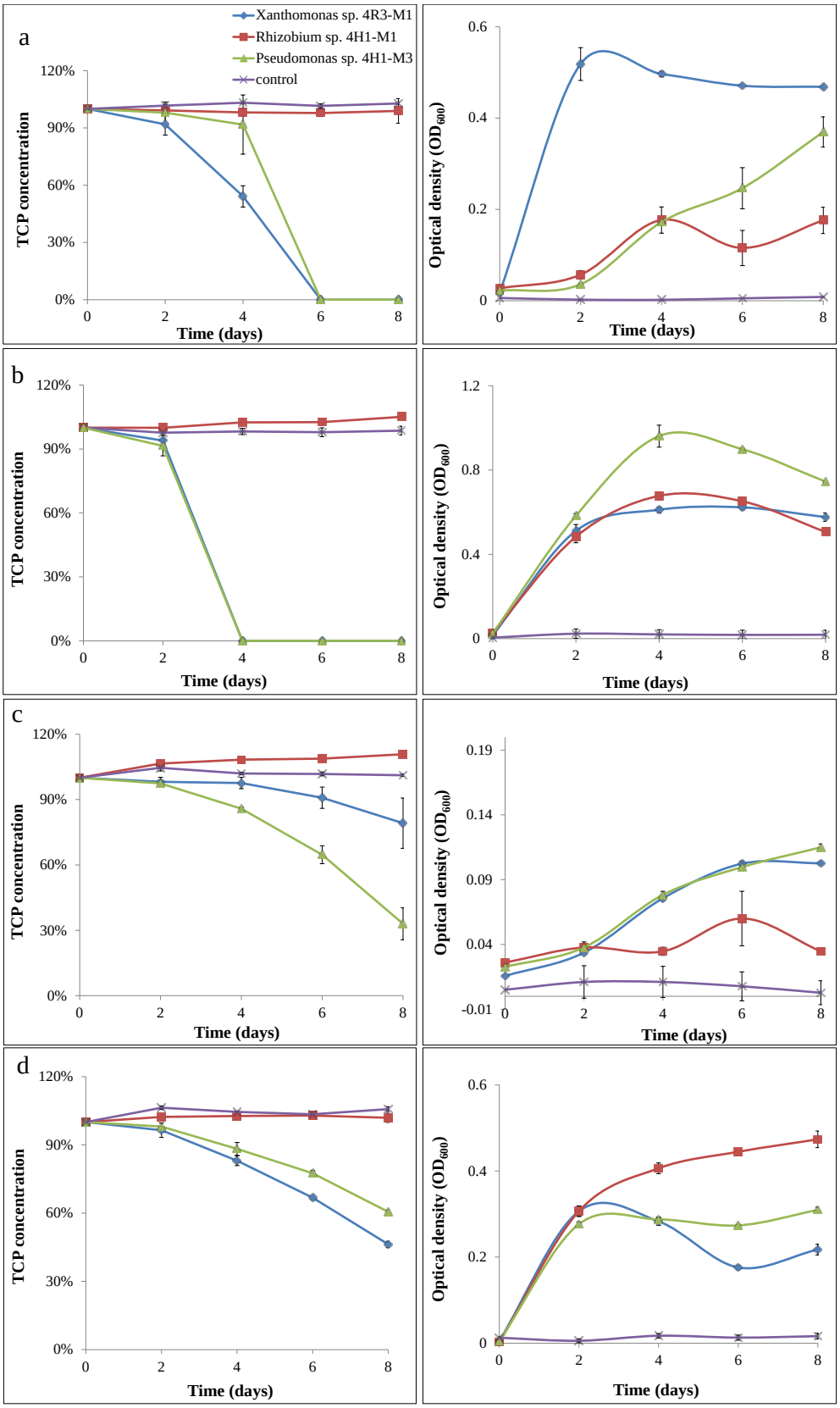


Figure 4-5 Degradation of TCP (%) and corresponding growth (OD_{600}) of *Xanthomonas* sp. 4R3-M1, *Rhizobium* sp. 4H1-M1and *Pseudomonas* sp. 4H1-M3 in different media: a) MSM (+N+P-C), b) MSM (-N+P+C), c) MSM (+N-P+C), and d) SEM. Error bars represent standard error of the mean (n=3).

Since *Rhizobium* sp. 4H1-M1 did not show any TCP degradation when added externally, *Xanthomonas* sp. 4R3-M1 and *Pseudomonas* sp. 4H1-M3 were further studied for their role in TCP degradation in C and N deficient media. Both strains showed similar degradation patterns consuming 90% of TCP in 6 days ($p<0.01$); however, degradation of TCP by *Xanthomonas* sp. 4R3-M1 was accompanied by a higher bacterial growth (Figure 4-6).

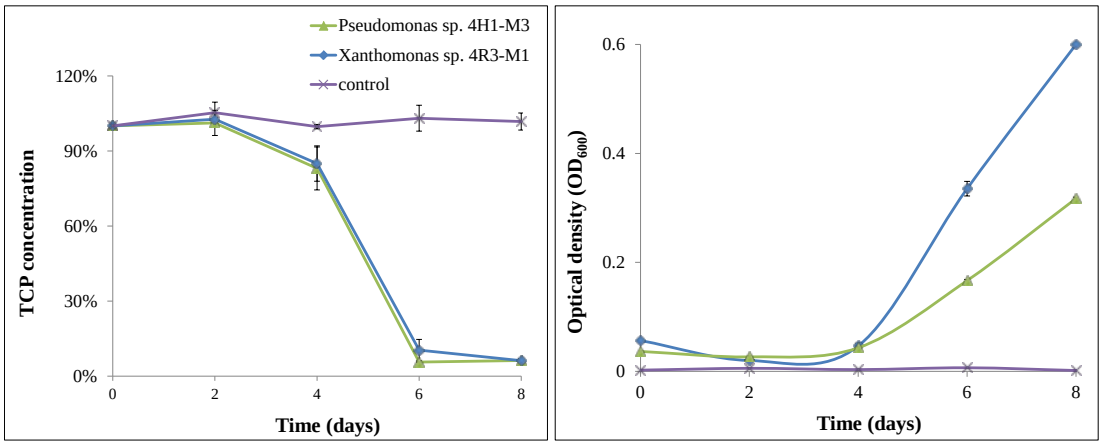


Figure 4-6 Degradation of TCP (%) and corresponding OD_{600} of *Xanthomonas* sp. 4R3-M1and *Pseudomonas* sp. 4H1-M3 in MSM (-N+P-C). Error bars represent standard error of the mean (n=3).

4.4 Discussion

4.4.1 Isolation of CP and TCP degraders

In order to isolate potential microorganisms that degrade CP and TCP, bacterial consortia were established from sugarcane farm soils using a selective enrichment technique, providing CP and/or TCP as a sole source of C. The ability of bacterial cultures to efficiently degrade CP in consortia with MSM+N, and not individually in pure cultures, may be attributed to the synergistic effect of various bacterial isolates in the consortium (Sasikala *et al.*, 2012). However, the microbial consortia stopped degrading CP in MSM+N after 4 days of incubation. This could be due to the accumulation of TCP in the media that prevented the proliferation of CP degrading bacteria (Racke *et al.*, 1990) or due to the limitation of growth factors, like vitamins.

Degradation of both CP and TCP (the primary metabolite of CP degradation) was observed in SEM suggesting that an alternative C-source was utilised by degrading bacteria resulting in an active cell biomass that overall led to the degradation of those compounds. On the other hand, bacterial consortium also degraded TCP (when added externally) only in the SEM but not in MSM+N providing evidence that the soil organic material might contain some soluble growth factors that would have stimulated TCP degradation. A study on biodegradation of 2,4,6-tribromophenol (TBP) by *Achromobacter piechaudii* strain TBPZ showed similar results where soil extracts assisted in TBP degradation in comparison with the mineral medium control (Ronen *et al.*, 2005). In addition to this, when isolated bacterial strains were inoculated in MSM (+N-P+C, +N+P-C, -N+P+C) supplemented with vitamins it resulted in the degradation of TCP as a source of energy. This suggested that growth factors, like vitamins, are essential substances that were required by the bacterial

cells to fulfil their biosynthetic role. Certain bacteria cannot synthesize vitamins on their own and require such growth factors to be added in culture media in order to grow. Although, at a low concentration in media (0.5% v/v here), vitamins are not metabolised as a direct source of C and energy, they are assimilated by cells to carry out specific metabolism (Todar, 2012). Thus, the addition of vitamins in this degradation study, not only promoted the growth but also the activity of the bacterial strains responsible for the degradation (He *et al.*, 2007).

Previous studies have reported dehalogenation of chlorine to be an important microbial mechanism for degrading TCP (Feng *et al.*, 1998, Van Pee and Unversucht, 2003, Math *et al.*, 2010, Cao *et al.*, 2013). However, there is still limited information about the pathway involved in the microbial metabolism of TCP. On the basis of what is available in the literature and the results from the current study, it can be suggested that the bacterial strains attacked the TCP ring structure for C and N only in vitamin-supplemented media. Oxygenases involved in the dehalogenation of the aromatic compounds generally require a reducing co-factor (NADH or NADPH) in addition to Fe (II) and aerobic environment (Mason and Cammack, 1992). Thus, it can be hypothesized that vitamin solution, which contained nicotinic acid might be involved in the activity of oxygenases/or other basic enzymes responsible for TCP degradation by bacterial isolates in MSM (+N-P+C, +N+P-C, -N+P+C) media. Hence no degradation was seen in MSM media without vitamins. These findings provide additional insight to the limited information about microbial TCP degradation mechanism and could be utilised for maximum degradation of both CP and TCP.

4.4.2 Molecular characterisation of isolated CP and TCP degraders

In the present study, six indigenous CP- and TCP-degrading isolates were obtained from Australian sugarcane farm soils. The phylogenetic relationships of the isolated CP-degrading bacteria was analysed by sequencing the 16S rRNA gene and their degrading abilities. Among them four isolates (4R3-M1, 4R3-M2, 4H1-M2 and 4H1-M4) showed similarities to members of the *Xanthomonadaceae* family and the other two isolates (4H1-M3 and 4H1-M1) showed similarities to the members of the families *Pseudomonadaceae* and *Rhizobiacea,* respectively. This indicated that the genus *Xanthomonas* dominated the soil samples and were easily culturable in the set lab conditions. Results of phylogenetic diversity revealed that the isolated degrading strains were closely related to species previously isolated from polluted or contaminated sites. *Xanthomonas* sp. 4R3-M1, *Xanthomonas* sp. 4R3-M2 and *Xanthomonas* sp. 4H1-M4 showed 99% similarity with a degrader of chorophenoxyalconoic acid (GenBank Accession no. HQ891021) that was isolated from contaminated soils in an industrial area.

Pseudomonas sp. 4H1-M3 showed 100% similarities to *P. putida* (GenBank Accession no. KF815695, KC189961) and *P. plecoglossicida* BF-1 (GenBank Accession no. FJ592171) that have been identified as degraders of organophosphates, such as paraoxon, and aromatic compounds like phenols and nitro-phenols in soils. *Rhizobium* sp. 4H1-M1 showed 99% similarity with exopolysaccharide producing *Rhizobium* species (GenBank Accession no. NR042687, KJ000027 and GQ161992) and with those harbouring antibiotic resistance genes isolated from swine effluent impacted environments (GenBank Accession no. DQ337578 and AY509211). A 99% similarity was also seen with *Rhizobium* sp. I29 (GenBank Accession no. HM008945) suggesting that the isolated

strain might have phosphate solubilising properties and could be used as bio-inoculant to facilitate the nutrient supply to plants and preventing negative side-effects such as eutrophication.

Different species of *Xanthomonas, Pseudomonas* and *Rhizobium* have been reported to degrade OP compounds catabolically as C-, N- or P-sources or co-metabolically (Rosenberg and Alexander, 1979, Liu *et al.*, 1991, Bano and Musarrat, 2003, Cycoń *et al.*, 2009, Jabeen *et al.*, 2014). However, this is the first time bacteria from the genus *Rhizobium* have been shown to degrade CP and its primary metabolite, TCP. Munnecke et al. (1974) and Tchelet et al. (1993) reported isolates of *Pseudomonas sp.* and *Xanthomonas sp.* that were adapted to grow on parathion by hydrolysing it. In another recent study, *Xanthomonas* and *Pseudomonas* species were isolated from leaf surfaces in the rape phyllosphere with activity for the biodegradation of dichlorvos, an OP pesticide (Ning *et al.*, 2010). Several *Pseudomonas* species alone or in consortia have also shown to degrade chlorpyrifos (Lakshmi *et al.*, 2009, Sasikala *et al.*, 2012) and its primary metabolite TCP (Feng *et al.*, 1997, Lakshmi *et al.*, 2009) in soil and liquid media. Recently Chawla *et al.* (2013) isolated *Xanthomonas* sp. that degraded chlorpyrifos. In this study, (isolated) bacterial strains that belonged to the genus *Xanthomonas* degraded both CP and its primary metabolite TCP simultaneously, a finding that was not reported before.

Lysobacter sp. 4H1-M2, family *Xanthomonadaceae,* showed similarities of 98%-99% to cultured and uncultured strains previously isolated from various contaminated environments; for example, contaminated sediments (GenBank Accession no. DQ404715), dioxins contaminated soils (GenBank Accession no. JX236701) and other polychlorinated contaminated soil (GenBank Accession no. AB234277). *Lysobacter* sp. 4H1-M2 showed 98% similarity with *Lysobacter sp.*

BUT-8 strain (GenBank Accession no. KJ008918) isolated from the sludge of the pesticide manufacturing factory and *Lysobacter* sp. ITP09 strain (99%), which is a potential chlorophenol degrader isolated from an agricultural Mediterranean calcareous soil (GenBank Accession no. FR667176). The strain isolated in this study also showed 98% similarity to an uncultured *Lysobacter* identified from anthracene contaminated soils (GenBank Accession no. HM438549, JQ426704) thus making this *Lysobacter* sp. 4H1-M2 a probable degrader of a wide range of contaminants. Not many members of genus *Lysobacter* with the ability to degrade xenobiotics had been reported before Wang *et al.* (2011a) and Caliz *et al.* (2011) isolated *Lysobacter* strains that could degrade chlorothalonil and chlorophenols respectively. However, to the best of our knowledge, no chlorpyrifos and TCP degrading bacterium has been reported from the genus *Lysobacter*.

4.4.3 Degradation of CP and TCP as a source of carbon (C), nitrogen (N) and phosphorus (P)

4.4.3.1 CP degradation

One of the important factors that influence the ability of microorganisms to degrade pesticide is the availability of C and nutrients. This study revealed that *Xanthomonas* sp. 4R3-M1, *Rhizobium* sp. 4H1-M1 and *Pseudomonas* sp. 4H1-M3 were able to degrade CP over a wide variation of media composition, including MSM (+N+P-C), MSM (-N+P+C) and MSM (-N+P-C), suggesting that these strains could utilize CP as a source of both C and N. Bacterial species have been reported earlier to utilise OP compounds as a source of C or N (Singh and Walker, 2006). Initial slower rate of CP degradation observed in SEM might be related to soil properties but once the isolates

acclimated, the degradation was greater in the SEM than MSM (+N-P+C, +N+P-C, -N+P+C and -N+P-C). This can prove to be important features of these strains for application in bioremediation because SEM is more similar to real field conditions in terms of nutrient types. These strains utilised CP as a sole source of C and N indicating that the pyridinyl ring of CP had been cleaved and utilised as a source of C for growth and cellular activities.

The degrading capability of all of the three strains was not markedly influenced by the presence of an alternative C-source (glucose) in the MSM (-N+P+C). Karpouzas and Walker (2000) found similar results with ethoprophos degradation by *Pseudomonas putida*, in which the presence of other C-sources had no effect on the degrading ability of the bacteria. The strains in this study preferred to utilise CP even in nutrient rich media potentially due to the constitutive expression of CP degrading enzymes even in the presence of readily available C-sources (Anwar *et al.*, 2009, Farhan *et al.*, 2012). This result contrasts with previous findings of Singh *et al.* (2004) who reported that with addition of C-sources, an *Enterobacter* strain stopped degrading CP and only after 3 days of incubation started utilising CP again. Further, supplementation of MSM (+N+P-C) with N improved the degradation of CP when compared to MSM (-N+P+C). Similar results have been reported earlier by Li *et al.* (2007) and Chawla *et al.* (2013) where addition of N in the media resulted in higher biomass density eventually resulting in higher CP utilisation.

There was visual evidence of a slight reduction of CP concentration in MSM without P (+N-P+C) suggesting a capacity of the bacterial strains to degrade CP as a source of P but this was not statistically significant, despite the fact that the same enzyme phosphotriesterase catalyses the hydrolysis of P-O-C linkage of OP compounds (as evident from TCP accumulation). In one study, Shelton (1988) isolated a bacterial

consortium that could use diethylthiophosphoric acid as a C-source only and was unable to degrade it as a P- or sulphur (S)-source. A possible explanation is that most often a particular compound is used to supply only a single element and the way in which the metabolism (degradation) is regulated depends strongly on the organism and OP compound studied (Kertesz *et al.*, 1994). The results suggested that the bacterial isolates could utilise CP and its primary metabolite (TCP) as both C- and N-sources but not as a source of P. This is the first time species of *Xanthomonas*, *Pseudomonas* and *Rhizobium* is shown to be able to utilise CP as a source of both C and N.

Previous research on CP degradation reported that the removal of CP resulted in the formation of metabolites like CP-oxon, 3, 5, 6-trichloro-2-methoxypyridine, 2-chloro-6-hydroxypyridine (Singh *et al.*, 2006, Yu *et al.*, 2006). Although *Enterobacter* strain B-14 degraded 40% of 25 mg/l CP within 48 h (Singh *et al.*, 2004), *Stenotrophomonas* sp. YC-1 degraded 100% of 100 mg/l within 24 h (Yang *et al.*, 2006) and *Synechocystis* sp. PUPCCC 64 degraded 93.8% of 5 mg/l CP within 5 d (Singh *et al.*, 2011a), these strains failed to utilise TCP for growth and energy. However, previous literature has reported certain bacterial strains capable of also degrading TCP produced as a primary CP degradation product. *Alcaligenes faecalis* DSP3 degraded 100% and 93.5% of 100 mg/l CP and TCP within 12 d (Yang *et al.*, 2005), *Paracoccus* sp. TRP degraded 50 mg/l CP and TCP in 4 d (Xu *et al.*, 2008), *Bacillus pumilus* C2A1 degraded 89% of 1000 mg/l CP within 15 d and 90% of 300 mg/l TCP within 8 d (Anwar *et al.*, 2009), *Mesorhizobium* sp. HN3 degraded 100% of 50 mg/l CP and TCP (Jabeen *et al.*, 2014). In the present study, only transient accumulation of TCP was observed by *Xanthomonas* sp. 4R3-M1 and *Pseudomonas* sp. indicating degradation of both CP (20 mg/l) and TCP were achieved by the same

isolate within 6 d of incubation. This was further confirmed by the degradation and growth on TCP, when added externally.

In contrast, CP degradation by *Rhizobium* sp. 4H1-M1 did not show formation of TCP in any of the media types. It suggests that this bacterial strain might be utilising some alternative mechanism for CP degradation for intracellular pathways, with the help of endo-enzymes. Similar results were obtained in a microcosm study where no TCP formation occurred during CP degradation by *Bacillus* species (Lakshmi *et al.*, 2008). The ability of *Rhizobium* strains to produce exo- and endo-cellular phosphodiesterase and phosphotriesterase, and to participate in the hydrolytic detoxification of OP pesticide, was first studied in 1994 (Abd-Alla, 1994). Considering the magnitude of toxicity of TCP, an organism that uses a pathway for degrading TCP intracellular would be well-suited for bioremediation of contaminated sites. It is worth mentioning here that *Rhizobium* bacteria are known for their ability to colonise roots of legumes and assist in the growth of host plant by fixing atmospheric nitrogen. Thus, there exists a potential for application of CP and TCP degrading *Rhizobium* strains to clean-up contaminated system and maintain soil health.

4.4.3.2 TCP degradation

Degradation of TCP, when added externally, occurred with *Xanthomonas* sp. 4R3-M1, and *Pseudomonas* sp. 4H1-M3 when inoculated in various liquid media. The finding revealed that both *Xanthomonas* sp. 4R3-M1 and *Pseudomonas* sp. 4H1-M3 utilised TCP as a source of C while they were grown solely on TCP; however, supplementation with an alternate C-source stimulated the rate of degradation. A

similar result was observed for an isolate of *Pseudomonas* sp. capable of TCP degradation (Feng *et al.*, 1997). The disappearance of TCP occurred in all MSM media except for MSM without phosphorus (+N-P+C) with increases in OD_{600} indicating that TCP was utilised as sources of N and C for growth by *Xanthomonas* sp. 4R3-M1 and *Pseudomonas* sp. 4H1-M3 (discussed above), even when added externally to the media. However, the degraded amount (%) of TCP in SEM was relatively less than the preliminary SEM inoculations. This might be due to the soil properties (a different soil was used here; HFE soil-latitude -33.61111^{o}, longitude 150.74069^{o}) that may play a major role in the degradation of OP compounds (Baskaran *et al.*, 2003).

The strains showed reduced degradation of TCP in MSM (+N-P+C) accompanied by poor microbial growth. These results suggested that although the strains were utilising TCP for C and N, the limitation of phosphorus in the media caused the biomass to decrease (Todar, 2012). *Rhizobium* sp. 4H1-M1 appeared to be a potential degrader of CP (and its primary metabolite) but failed to utilise TCP for growth and energy when TCP was added externally into the liquid media. These observations were consistent with the preliminary CP and TCP degradation study in SEM (section 4.3.1) where this particular isolate showed TCP degradation only when present as a primary metabolite of CP but failed to degrade it when added externally (Appendix Figure A-9c and A-10c, respectively). Since very little literature is available about TCP metabolism it is difficult to conclude anything. However, it can be hypothesised that in this particular strain, CP enters the cell and is intracellularly degraded as a C and N-source along with its primary metabolite, TCP. It is possible that this strain does not possess mechanisms or enzyme-linked receptors to transport externally added TCP for intracellular degradation and thus no degradation of externally added

TCP was observed. However, further work is required to confirm this. Similar to CP degradation, this is the first study to report bacterial strains from genera *Xanthomonas* and *Pseudomonas* to utilise TCP as source of both C and N.

4.5 Conclusion

In the present work, novel CP and TCP degrading bacterial strains were isolated from Australian sugarcane farm soils that showed enhanced degradation of CP in a lab-based study. All strains isolated were able to grow in the presence of CP and/or TCP and were able to degrade CP and/or TCP in a broad range of media with different C, N and P additions. The use of such efficient indigenous bacterial strains promises to be effective in the practical application of bioremediation of both CP and TCP since the microbes have already adapted to the localised habitat conditions. The isolated strains can also be added to other soils as microbial inoculants (bio-augmentation) for their potential to degrade pesticides to improve soil quality in order to create a more sustainable agriculture and environment. Although this study provide significant insights and promising organisms for bioremediation, the application of such organisms require further research and understanding to be implemented. Characterisation and identification of the OP degrading genes, enzymes and pathways are other areas for further investigation.

Chapter 5: Impact of pesticides on soil microbial community structure and functional capabilities

5.1 Introduction

Soil microbial communities are highly diverse, dominate belowground biomass and play critical roles in ecosystem functions by maintaining biogeochemical cycles and plant productivity (Nannipieri *et al.*, 2003, Fierer *et al.*, 2012). Intensive land-use practices may directly or indirectly alter soil biological properties (Johnsen *et al.*, 2001, Lo, 2010) and in turn influence ecosystem processes. It is, therefore, important to evaluate the soil microbial diversity and the metabolic processes performed by them in response to anthropogenic stressors (*e.g.* pesticide) for better understanding of ecosystem functioning. Pesticides represent a diverse group of organic and inorganic chemicals used in agriculture to protect crops against pests in order to improve crop yield and quality. Ecosystems across the globe are receiving pesticides at an increasing rate to meet the rising demand for food with the ongoing global rise in population size (Popp *et al.*, 2013). Among all the chemical pesticides used chlorpyrifos (CP), an organophosphorus (OP) pesticide, is the most widely used pesticide worldwide.

The impact of pesticides (*e.g.* chlorpyrifos (CP)) on soil microorganisms has become a serious concern as microbial community structure, diversity and functional characteristics moderate crop production, environmental sustainability and soil quality (Topp, 2003). Although, it is well established that the application of pesticides effect soil microbial community structure and diversity (Zhang *et al.*, 2006b, Fang *et al.*, 2008, Chen *et al.*, 2014), far less is known about how long-term pesticide input (under typical field condition) influence the structure and potential

function of the below-ground microbial community. In addition to this, there is a large knowledge gap of whether changes in the microbial community structure and diversity translate to functional characteristics of the community, especially the specialised functions that may be impacted even with a moderate loss of diversity (Singh *et al.*, 2014). In recent years, various studies have provided important insights in predicting the relationship between pesticides and its effect on soil microorganisms, particularly in the context of exposure to pesticide concentrations and mixtures (Bünemann *et al.*, 2006, Imfeld and Vuilleumier, 2012). However, the consequences of long-term repeated pesticide use on key metabolic functions and microbial taxa remains poorly understood. In addition to this, the observations from previous studies to understand the effects of pesticide input on soil ecological parameters, such as microbial community shifts and pesticide resistance/degradation, microbial, polymerase chain reaction (PCR)-based molecular and statistical tools were applied. However, such indicators are increasingly criticised for being biased (e.g. PCR) and difficult to interpret. For example, a study by Hong *et al.* (2006) has highlighted the limitation of available statistical tools to estimate species diversity indices, therefore, resulting in uncertainties in the calculated diversity indices.

Advances in metagenomic technologies has allowed for the direct sequencing of collective genomes found in a given environmental sample (Simon and Daniel, 2011) increasing the potential to understand and monitor the dynamics of overall and active microbial communities under different conditions, which are often very difficult to measure using traditional biogeochemical or culture-based approaches (Tringe and Rubin, 2005). Metagenomics has already been applied to a range of soil systems (Ginolhac *et al.*, 2004, Demaneche *et al.*, 2008, Rajendhran and Gunasekaran, 2008) including contaminated sites (Ono *et al.*, 2007, Lu *et al.*, 2011). For example, recent

studies have used metagenomic sequencing technologies to characterise the structure and metabolic functions of an active soil microbial community in a hydrocarbon contaminated environment (Sangwan *et al.*, 2012, Yergeau *et al.*, 2012, Smith *et al.*, 2013). However, to my knowledge such tools have not yet been used to directly compare microbial metagenomes across different soil types to determine pesticide impacts on agro-ecosystems. To accomplish this goal, shotgun sequencing was used on soil samples (in replicates) from sugarcane farms with a long-term history (Table 3-1) of pesticide application (CP and imidacloprid) along with non-treated counterparts as controls. However, the use of CP was discontinued after it showed efficacy loss 13 years ago. My working hypothesis was that long-term pesticide application (legacy effect), which was established in Chapter 3, would negatively impact bacterial diversity as well as functional capabilities at pesticide-treated sites. I also hypothesised that functional genes involved in the phosphorus (P)-metabolism will be over-represented relative to other genes in pesticide-treated samples because OP pesticides were reported to be used as P-source by many bacteria (Singh and Walker, 2006).

The main aim of the study was to identify the impact of pesticide on soil microbial diversity and potential functional capabilities. Additionally, having replicates in the analysis would provide deeper and more realistic information to model the changes in the functional diversity affected by the presence of stressor (*e.g.* pesticide) in the soil, which are often lacking in metagenomic studies (Prosser, 2010). Thus this study, presents a highly desirable option for the future as it provides far more comprehensive identification of defining metabolic profiles and microbial taxa. It would allow a better understanding of how the microbial community are adapting and responding to the environmental changes, (Gianoulis *et al.*, 2009) by eliminating

the inherent biases associated with any PCR-dependent method (*e.g.* T-RFLP, cloning).

5.2 Materials and method

5.2.1 Site description and sampling

Soil was collected from five sugarcane farms in the Mackay, Burdekin and Tully areas in Queensland, Australia. A description of the sites, soil type and sugarcane curb pesticide application history can be found in the method section of Chapter 3 (Table 3-1). Soils were collected and prepared as described in Section 3.2.1. Soil samples (from sites) that had previously shown enhanced pesticide degradation capabilities, in sugarcane farms (Table 3-1) or lab-based degradation (Table 3-3 and 3-4), were selected for sequencing. The selected soils were 1R, 2R (enhanced CP degradation in fields), 4R, 5R (enhanced degradation in fields and lab-based study) and 4H, 5H (enhanced degradation in lab-based study). For sequencing, DNA was extracted directly from field soils (where R represented pesticide-treated sites and H represented non-treated sites, Figure 3-1), without any lab amendments with pesticides, of the selected soil samples

5.2.2 DNA extraction and sequencing

DNA was extracted from 10 g homogenised soil using bead beating and chemical lysis (PowerMax® soil DNA Isolation Kit Mobio, USA) following the manufacturer's protocol. Genomic DNA concentration was quantified using a Qubit 2.0 fluorometer (Invitrogen). A shotgun metagenomic library was generated and sequenced using Illumina® HiSeq™ at the Hawkesbury Institute for Environment NGS research centre. Sequencing utilized the TruSeq DNA PCR-free HT (Illumina®) library preparation. The process involves fragmentation of genomic

dsDNA with 3' or 5' overhangs. The fragmentation process was optimised to obtain final libraries with the average insert size of 550 bp (DNA input per sample=2 µg). The overhangs resulting from fragmentation were repaired by removing 3' overhangs. Following the end repair, the appropriate library size was selected for sample (DNA) purification. The purified DNA was then adenylated at 3' ends to prevent ligating to each other during the adapter ligation reaction. A corresponding 'T' nucleotide on the adaptor 3' end (multiple indexing adaptors) provided a complementary overhang for ligation (adaptor and fragment), preparing them for hybridisation onto a flow cell. The libraries were then validated and quantified by using Eco Real-Time PCR system (Illumina®, USA) and KAPA library quantification kit (Kapa Biosystems, USA) and were used for Illumina® sequencing platform (for three runs). The libraries were multiplexed during sequencing and analysed individually. The details of each step followed can be found on http://supportres.illumina.com/

5.2.3 Bioinformatics and statistical analysis

To determine the functionality of microbial communities inhabiting the pesticide-treated and non-treated environments, unassembled DNA sequences from each site were annotated using the Meta Genome Rapid Annotation using Subsystems Technology (MG-RAST, Version 3) pipeline (Meyer *et al.*, 2008). Within MG-RAST, metabolic assignments were annotated to the SEED subsystems database (Overbeek *et al.*, 2005) and taxonomic identification was based on the top BLAST hits to the m5nr taxonomy (Wilke *et al.*, 2012). The SEED subsystems database contains enzymes grouped into metabolic pathways and organised into three

hierarchical levels for metabolic profile organization and six for taxonomy and allows the data to be exported at each level.

Metabolic and taxonomic reconstruction generated using MG-RAST with an E-value cut off of $1x10^{-5}$ and a 50 bp minimum alignment length were imported into the STatistical Analysis of Metagenomic Profiles (STAMP) package to test for statistically significant abundance differences in taxonomic and metabolic groupings (Parks and Beiko, 2010). These were investigated at the first (level 1), second (level 2) and third level (level 3) of the MG-RAST metabolic hierarchy and the genus level of the MG-RAST taxonomic hierarchy.

The statistical hypothesis tests were performed using the Welch's t test (Bluman, 2007) while confidence intervals were calculated using the Welch's inverted method with a Storey's FDR multiple test correction applied (Agresti, 1999, Storey and Tibshirani, 2003), as recommended by the STAMP user manual. Hence, all quoted q-values represent corrected values (equating to q) with results filtered to display only categories with a q-value of <0.05. Non-metric multi-dimensional scaling (MDS, PRIMER) of Bray-Curtis similarities was performed as an unconstrained ordination method to graphically visualise the patterns in the taxonomy and metabolic processes associated with pesticide-treated and non-treated sites metagenomes.

To further elucidate the relative enrichment of functional genes, characteristics of metagenomic profile within site were investigated to remove the effect of site variation. For this purpose Site 4 and Site 5 were chosen (R and H). Analysis of similarities (ANOSIM) was used to test the significant differences between pesticide-treated and non-treated groups both at taxonomical and level 2 of metabolic functions.

An additional focus was put on phosphorus (P) metabolism (at all three hierarchical levels) to enhance the understanding/role of this particular pathway since the sites used in this experiment had a previous history of OP pesticide (CP) application. The percent contribution of genes associated with P-metabolism between pesticide-treated and non-treated were assessed using similarity percentage (SIMPER) analysis to determine the shifts between the two groups (Clarke, 1993, Smith *et al.*, 2013). A dissimilarity/standard (Diss/SD) deviation ratio of 1.4 and higher indicated the key distinguishing genes (of P-metabolism) (Clarke and Warwick, 2001).

5.3 Results

5.3.1 Metagenomic sequences

The average number of sequences, derived from shotgun dataset, varied among different samples and ranged in between $2.3x10^6$- $5.9x10^9$, totalling $5.6x10^8$-$1.4x10^9$ base-pairs (bps), with an average length of 212-239 bps. The average sequences that passed quality control (QC) test ranged in between $2.3x10^6$-$5.8x10^6$, totalling $5.2x10^8$-$1.3x10^9$ bps with an average of 202-227 bps among different sites. Of the sequences that passed a QC test, total predicted protein features ranged in between $2.2x10^6$-$5.5x10^6$ and the sequences $6.7x10^5$-$1.7x10^6$ were assigned to functional categories. A summary of the obtained metagenomes (replicates, n=3) from pesticide-treated and non-treated sites is given in Table 5-1. In addition to this, the predicted protein features sequences were annotated with similarity to a protein of known functions and functional hierarchy. Table 5-2 summaries the number of features that were annotated by the different databases across pesticide-treated and non-treated sites (n=3).

The average sequences obtained and annotated for each replicate is given in Appendix Table A-1 and Appendix Table A-2, respectively.

Table 5-1 Summary of metagenomes (bp) obtained across sites, where 4H and 5H are non-treated sites and 4R, 5R, 1R and 2R are pesticide-treated sites. ± indicate standard deviation (n=3).

Sample Name	4H	5H	4R	5R	1R	2R
Upload: bp Count	8.2E+08 ± 3.48E+08	1E+09 ± 9.42E+08	7.7E+08 ± 1.63E+08	1.4E+09 ± 1.79E+08	5.6E+08 ± 1.42E+08	7.8E+08 ± 1.52E+08
Upload: Sequences Count	4.02E+06 ± 2.26E+06	5.84E+06 ± 6.61E+06	3.27E+06 ± 6.74E+05	5.94E+06 ± 7.50E+05	2.35E+06 ± 6.04E+05	3.24E+06 ± 6.24E+05
Upload: Mean Sequence Length	213 ± 43	212 ± 48	237 ± 43	231 ± 42	236 ± 43	239 ± 42
Upload: Mean GC percent	63 ± 9%	61 ± 9%	62 ± 9%	62 ± 9 %	62 ± 9%	62 ± 9%
Artificial Duplicate Reads: Sequence Count	3.15E+04 ± 5.07E+04	3.98E+04 ± 6.36E+04	4.65E+03 ± 1.52E+03	4.53E+03 ± 6.50E+02	3.04E+03 ± 5.94E+02	3.12E+03 ± 6.63E+02
Post QC: bp Count	7.63E+08 ± 3.13E+08	9.6E+08 ± 8.71E+08	7.3E+08 ± 1.53E+08	1.3E+09 ± 1.69E+08	5.2E+08 ± 1.34E+08	7.3E+08 ± 1.42E+08
Post QC: Sequences Count	3.90E+06 ± 2.12E+06	5.66E+06 ± 6.35E+06	3.22E+06 ± 6.63E+05	5.86E+06 ± 7.40E+05	2.31E+06 ± 5.95E+05	3.19E+06 ± 6.15E+05
Post QC: Mean Sequence Length	204.66 ± 50	202.66 ± 45	227 ± 51	223 ± 49	225 ± 52	224 ± 51
Post QC: Mean GC percent	63 ± 9 %	61 ± 9%	63 ± 9%	62 ± 9%	62 ± 9%	62 ± 9%
Processed: Predicted Protein Features	3.64E+06 ± 1.91E+06	5.14E+06 ± 5.64E+06	2.95E+06 ± 8.45E+05	5.53E+06 ± 7.12E+05	2.21E+06 ± 5.69E+05	3.05E+06 ± 5.79E+05
Processed: Predicted rRNA Features	3.43E+04 ± 2.34E+04	4.63E+04 ± 5.62E+04	2.54E+04 ± 5.68E+03	4.33E+04 ± 5.11E+03	1.79E+04 ± 4.80E+03	2.45E+04 ± 4.71E+03
Alignment: Identified Protein Features	1.32E+06 ± 5.79E+05	1.77E+06 ± 1.75E+06	1.23E+06 ± 2.66E+05	2.21E+06 ± 2.97E+05	8.75E+05 ± 2.25E+05	1.22E+06 ± 2.29E+05
Alignment: Identified rRNA Features	808.33 ± 375.79	909.33 ± 797.50	833.33 ± 214.43	1399 ± 194.31	621 ± 194.73	828 ± 181.34
Annotation: Identified functional categories	1.02E+06 ± 4.49E+05	1.36E+06 ± 1.34E+06	9.56E+05 ± 2.09E+05	1.74E+06 ± 2.45E+05	6.75E+05 ± 1.75E+05	9.42E+05 ± 1.75E+05

Table 5-2 Summary of the features that were annotated by the different databases across sites, for non-treated sites (4H and 5H) and pesticide-treated sites (4R, 5R, 1R and 2R). These include protein databases, protein databases with functional hierarchy information, and ribosomal RNA databases. ± indicate standard deviation (n=3)

Sample Name	4H (bp)	5H (bp)	4R (bp)	5R (bp)	1R (bp)	2R (bp)
GenBank	1.15E+06 ± 2.76E+05	1.92E+06 ± 1.92E+06	1.30E+06 ± 2.83E+05	2.34E+06 ± 3.16E+05	9.20E+05 ± 2.32E+05	1.28E+06 ± 2.42E+05
IMG	1.13E+06 ± 2.68E+05	1.85E+06 ± 1.85E+06	1.28E+06 ± 2.82E+05	2.32E+06 ± 3.17E+05	9.08E+05 ± 2.32E+05	1.27E+06 ± 2.39E+05
KEGG	1.04E+06 ± 2.45E+05	1.70E+06 ± 1.69E+06	1.18E+06 ± 2.59E+05	2.14E+06 ± 2.93E+05	8.36E+05 ± 2.16E+05	1.17E+06 ± 2.20E+05
PATRIC	1.12E+06 ± 2.65E+05	1.86E+06 ± 1.88E+06	1.28E+06 ± 2.80E+05	2.31E+06 ± 3.12E+05	9.00E+05 ± 2.31E+05	1.26E+06 ± 2.39E+05
RefSeq	1.17E+06 ± 2.76E+05	1.95E+06 ± 1.96E+06	1.33E+06 ± 2.92E+05	2.40E+06 ± 3.21E+05	9.36E+05 ± 2.39E+05	1.31E+06 ± 2.48E+05
SEED	9.94E+05 ± 2.36E+05	1.63E+06 ± 1.61E+06	1.13E+06 ± 2.48E+05	2.04E+06 ± 2.84E+05	7.99E+05 ± 2.03E+05	1.12E+06 ± 2.09E+05
SwissProt	4.82E+05 ± 5.62E+05	2.81E+05 ± 2.86E+05	2.48E+05 ± 4.34E+04	3.62E+05 ± 5.72E+04	1.36E+05 ± 3.46E+04	1.88E+05 ± 3.50E+04
TrEMBL	1.16E+06 ± 2.76E+05	1.92E+06 ± 1.92E+06	1.31E+06 ± 2.86E+05	2.36E+06 ± 3.18E+05	9.27E+05 ± 2.35E+05	1.29E+06 ± 2.44E+05
Eggnog	9.28E+05 ± 2.22E+05	1.52E+06 ± 1.50E+06	1.06E+06 ± 2.29E+05	1.94E+06 ± 2.74E+05	7.42E+05 ± 1.91E+05	1.04E+06 ± 1.94E+05
COG	7.91E+05 ± 1.88E+05	1.28E+06 ± 1.27E+06	9.03E+05 ± 1.96E+05	1.66E+06 ± 2.35E+05	6.33E+05 ± 1.64E+05	8.85E+05 ± 1.65E+05
KO	4.07E+05 ± 9.37E+04	6.71E+05 ± 6.84E+05	4.63E+05 ± 1.01E+05	8.53E+05 ± 1.23E+05	3.23E+05 ± 8.39E+04	4.52E+05 ± 8.43E+04
NOG	4.61E+04 ± 1.23E+04	7.68E+04 ± 7.07E+04	5.15E+04 ± 1.09E+04	9.49E+04 ± 1.28E+04	3.71E+04 ± 9.50E+03	5.21E+04 ± 9.74E+03
Subsystems	1.39E+06 ± 3.24E+05	2.29E+06 ± 2.33E+06	1.59E+06 ± 3.49E+05	2.93E+06 ± 4.38E+05	1.11E+06 ± 2.86E+05	1.54E+06 ± 2.84E+05
Greengenes	282.6 ± 78.5	479.6 ± 516.6	358.3 ± 97.2	665.6 ± 82.9	279 ± 92.7	361.3 ± 86.5
SILVA LSU	390.3 ± 70.4	682 ± 627.2	617.6 ± 109.0	1004.6 ± 88.3	392.6 ± 149.1	578.3 ± 118.4
RDP	303.3 ± 89.4	527.6 ± 566.8	375 ± 103.1	705.6 ± 88.5	296 ± 93.6	391 ± 89.3
SILVA SSU	393 ± 93.5	765 ± 733.5	569.6 ± 195.5	1003.6 ± 71.5	383.3 ± 120.1	537 ± 106.6

5.3.2 Taxonomic profiling of pesticide-treated and non-treated sites

The taxonomic profiles of soils from pesticide-treated and non-treated sites were investigated to determine the influence of pesticide application on sites. One of the replicates from site 1 (1R3) failed the quality control of MG-RAST at the genus level and hence was not included in any analyses of microbial community composition. Substantial treatment specific differences between the pesticide-treated and non-treated sites were evident from the taxonomic data at the genus level (Figure 5-1). Pesticide-treated sites harboured communities that were distinct and clustered apart from non-treated when community differences were measured by ANOSIM (R=0.55, p=0.005).

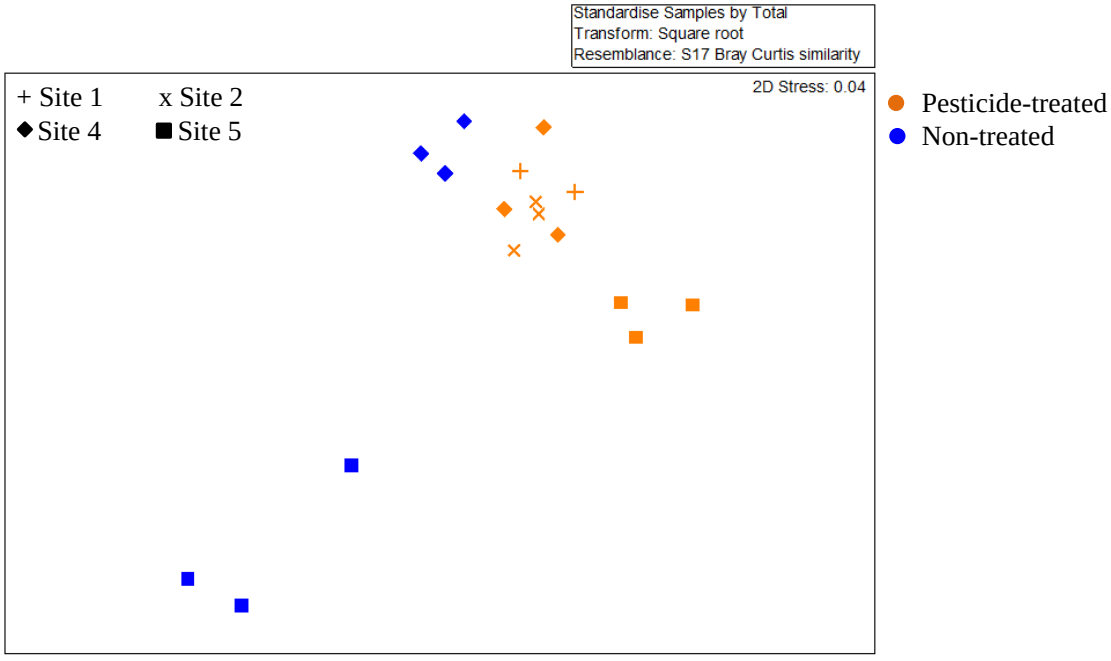


Figure 5-1 MDS ordination of bacterial community structure, based on the relative gene abundances of metagenomic taxonomic data, in pesticide-treated and non-treated sites derived from Bray-Curtis distance matrix.

In addition to this, alpha (α) diversity estimations (calculated by MG-RAST) based on OTUs showed that there was a statistically significant albeit weak increase in the microbial diversity in the pesticide-treated sites (p=0.05) when compared to the non-treated ones (Figure 5-2a) indicating that the presence of pesticide affected microbial diversity. Site also significantly influenced α-diversity (p<0.001) (Figure 5-2b) as did the interaction between the sites and treatment (p=0.002). *Post-hoc* tests between sites indicated that the microbial diversity at site 4 (R and H), site 1R and site 2R were significantly different from site 5 (R and H, p<0.001). Therefore, differences between pesticide-treated and non-treated samples seemed to be mainly driven by 5H and 5R samples.

To gain insight into the microbial community associated with pesticide-treated and non-treated sites, the taxonomic identity of microbial genes were investigated using STAMP and taxonomic groups that were statistically different in abundance were determined. *Candidatus solibacter* was found to be the most abundant bacterial group in all samples (3-4%). Other bacterial genera that were abundant in pesticide-treated and non-treated soils and accounted for top 25% of the total abundance are included in Table 5-3. The distribution of sequences at class level and the relative distribution of top 25% dominant genus across all sites (both pesticide-treated and non-treated) are given in Appendix (Figure A-11a and b, respectively).

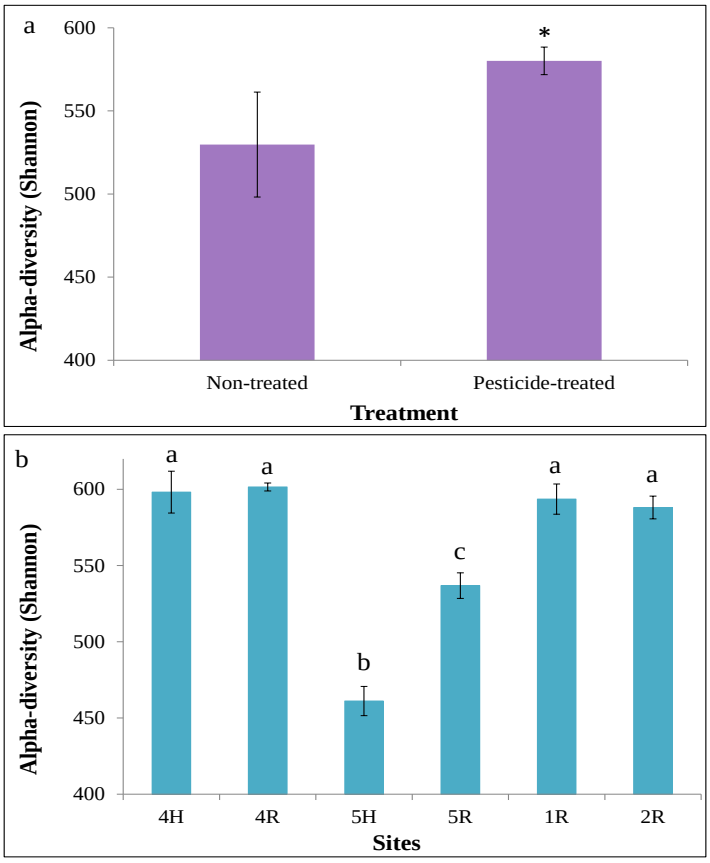


Figure 5-2 Difference in alpha diversity levels (Shannon diversity index calculated by MG-RAST), between pesticide-treated and non-treated sites based on the relative gene abundances of metagenomic taxonomic data a) Treatment and b) Sites. Bars indicate standard deviation (s.d.), n=6 for non-treated sites (a), n=11 for pesticide-treated sites (a) and n=3 for sites (b). Different letters and ***** indicate values that are significantly different ($p < 0.05$).

Despite their predominance across all samples (pesticide-treated and non-treated), analysis of relative abundances revealed that the dominant genera were not significant drivers (q>0.05) of variation in the community composition between pesticide-treated and non-treated soils. On the contrary, several 'rare' genera with comparatively lower abundances (0.03-0.50%) accounted for notable differences between pesticide-treated and non-treated sites. For example, *Pedobacter* (q=0.017), *Methylibium* (q=7.34e^{-3}), *Sphingomonas* (q=0.02), *Parabacteroides* (q=0.048),

Novosphingobium (q=0.020), *Sphingobium* (q=0.032), *Marivirga* (q=0.029), *Phenylobacterium* (q=0.032), *Maricaulis* (q=0.032), *Paludibacter* (q=0.040), *Rubrivivax* (q=0.010) and *Leptothrix* (q=0.020) were a few of the genera statistically more abundant in the pesticide-treated sites (Figure 5.3). Out of 33 genera that contributed significantly to differences in community composition between the metagenomes in pesticide-treated and non-treated sites, only five of them were statistically predominant in non-treated sites (Figure 5-3) and included *Methanococcus* ($q=7.34e^{-3}$), *Methanothermobacter* (q=0.016), *Methanopyrus* (q=0.032), *Archaeoglobus* (q=0.033) and *Thermococcus* (q=0.039).

Table 5-3 Average relative frequency of top 25% bacterial genera in pesticide-treated and non-treated sites. ± indicates standard deviation (s.d) where n=6 for non-treated sites and n=11 for pesticide-treated sites.

Bacterial genus	Non-treated sites	Pesticide-treated sites
	Mean relative frequency (%)	Mean relative frequency (%)
Candidatus solibacter	4.68±1.29	3.41±0.38
Streptomyces	3.18±0.40	2.38±0.40
Candidatus koribacter	2.98±1.45	2.05±0.89
Burkholderia	2.26±0.25	2.26±0.18
Bradyrhizobium	2.11±0.58	1.81±0.24
Mycobacterium	1.59±0.18	1.27±0.12
Anaeromyxobacter	1.52±0.45	1.73±0.17
Sorangium	1.43±0.29	1.95±0.14
Frankia	1.41±0.22	1.02±0.12
Geobacter	1.34±0.35	1.49±0.26
Rhodopseudomonas	1.31±0.32	1.14±0.15
Methylobacterium	1.21±0.14	1.12±0.07
Conexibacter	1.14±0.42	0.93±0.32
Acidobacterium	1.11±0.56	0.74±0.26
Myxococcus	1.10±0.18	1.37±0.08
Pseudomonas	1.07±0.20	1.15±0.05

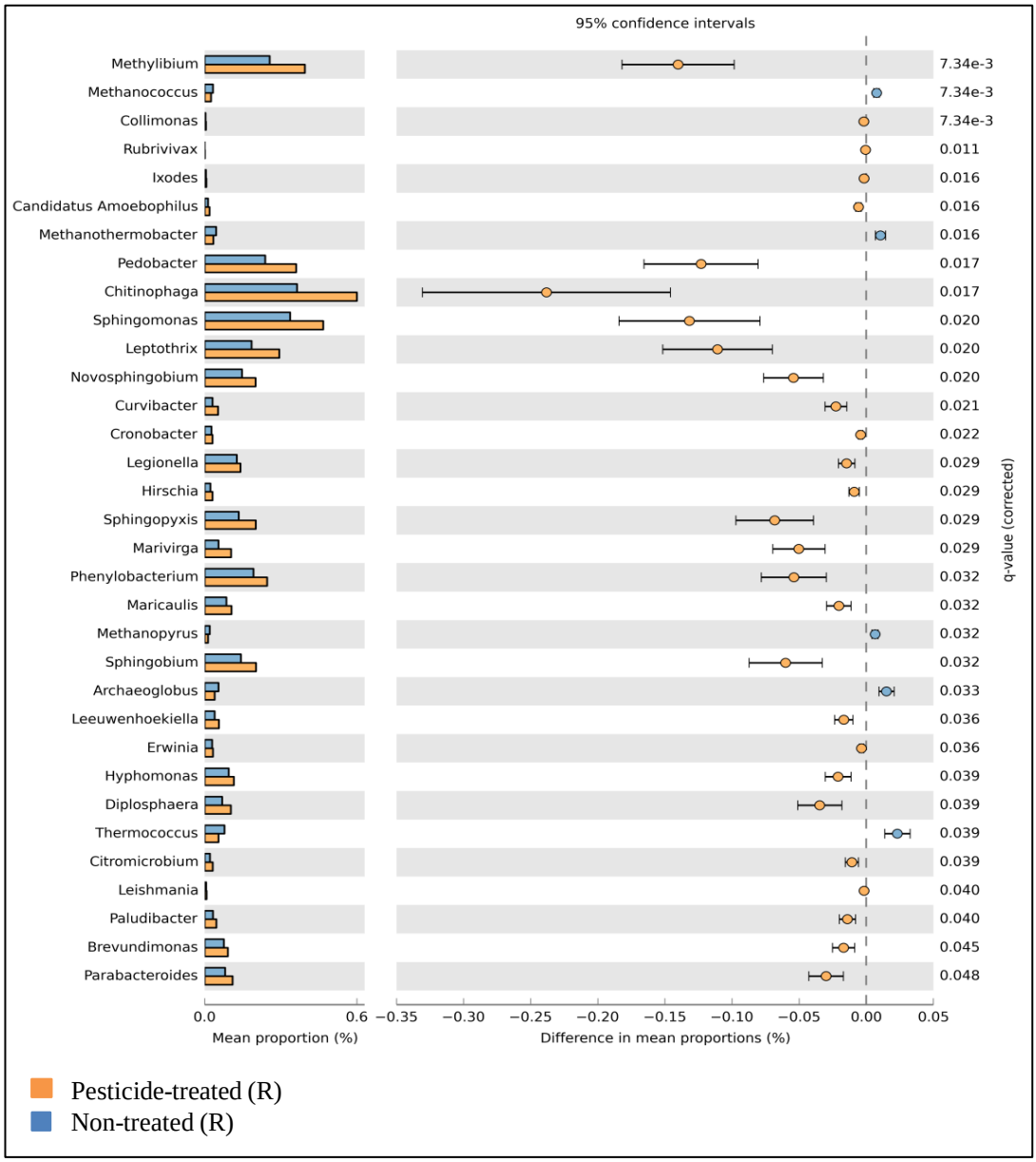


Figure 5-3 Taxa enriched or depleted in pesticide-treated (R) and non-treated (H) sites at genus level of MG-RAST taxonomic hierarchy. Corrected q-values were calculated using Storey's FDR approach.

5.3.3 Overall shift in soil functional and metagenomic profiles

The metabolic gene abundance profile was investigated to determine the influence of pesticide application on potential functionality of sites. MDS analysis (at hierarchical level 2) revealed a clear separation of the data between pesticide-treated and non-treated sites metagenomes (Figure 5-4). Pesticide-treated sites harboured metabolic gene profiles that were distinct, and clustered apart, from non-treated soils when measured by ANOSIM (R=0.61, p=0.002). Metagenomes derived from pesticide-treated sites showed a higher degree of similarity to each other than to the non-treated sites for function identity.

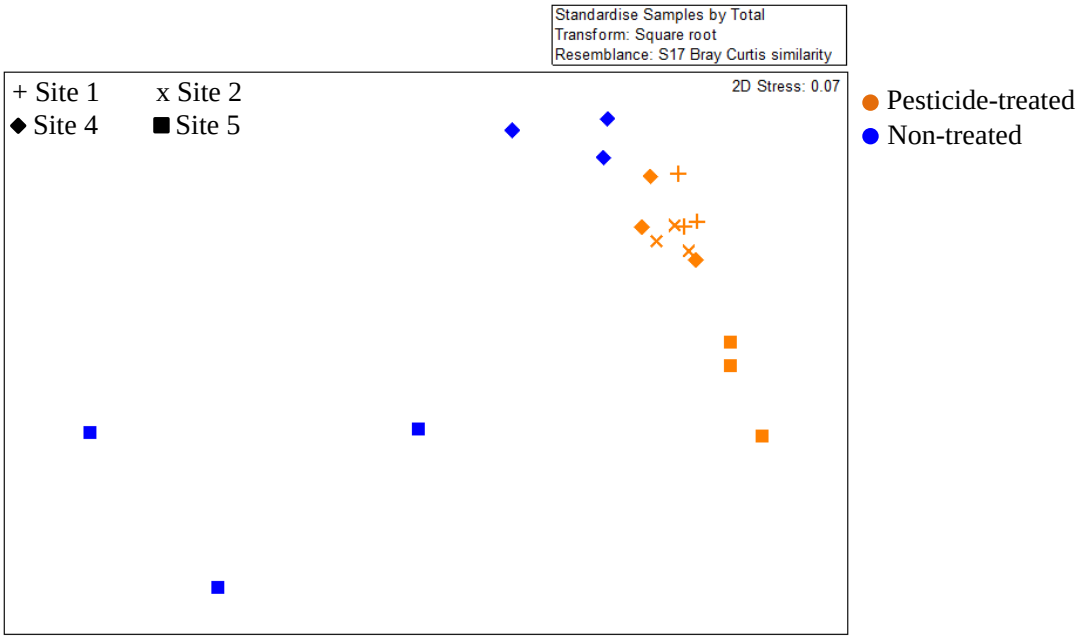


Figure 5-4 MDS ordination of the metabolic gene abundance profile, based on metagenomic metabolic processes data (at hierarchical level 2), in pesticide-treated and non-treated sites derived from Bray-Curtis distance matrix.

Sequences involved in basic metabolic processes comprised the most abundant hits in both pesticide-treated and non-treated metagenomes. These genes, often referred as house-keeping genes, were associated with respiration and carbohydrate, amino acid, protein, RNA and DNA metabolism. Despite their predominance in pesticide-treated and non-treated sites, analysis of differential abundances (at hierarchical level 1) revealed that the variation across the house-keeping genes did not contribute to the significant differences between the metagenomes (data not shown).

Analysis at a finer sub-system level resolution (hierarchical level 2; Figure 5-5) indicated that non-treated sites had an over-representation of house-keeping genes associated with carbohydrate metabolism (one-carbon metabolism, CO_2 fixation and central carbohydrate metabolism), respiration (sodium ion-coupled energetic), DNA metabolism (CRISPs) and protein metabolism (selenoproteins, proteosomes related genes) when compared with pesticide-treated metagenomes. By comparison, RNA metabolism genes, especially genes associated with RNA processing and modification was higher in the pesticide-treated sites than in non-treated sites.

Further analysis of metagenomic data indicated that microbial communities that were well adapted to the pesticide-treated sites had metabolic categories that were typically related to more specialised function and were over-represented in the pesticide-treated sites (Figure 5-5). These included genes responsible for the bacterial motility and chemotaxis (flagella protein, chemotaxis response regulators), pathways for iron acquisition and metabolism (siderophores), membrane transport (protein secretion system III) and cell signalling. Phage-associated genes (in particular genes affiliated with gene transfer agent), stress response genes (oxidative, periplasmic) were also significantly more abundant in contaminated sites. These metabolic categories were comparatively in lower abundances than house-keeping genes but

were also responsible for the variation in functional potential between pesticide-treated and non-treated metagenomes.

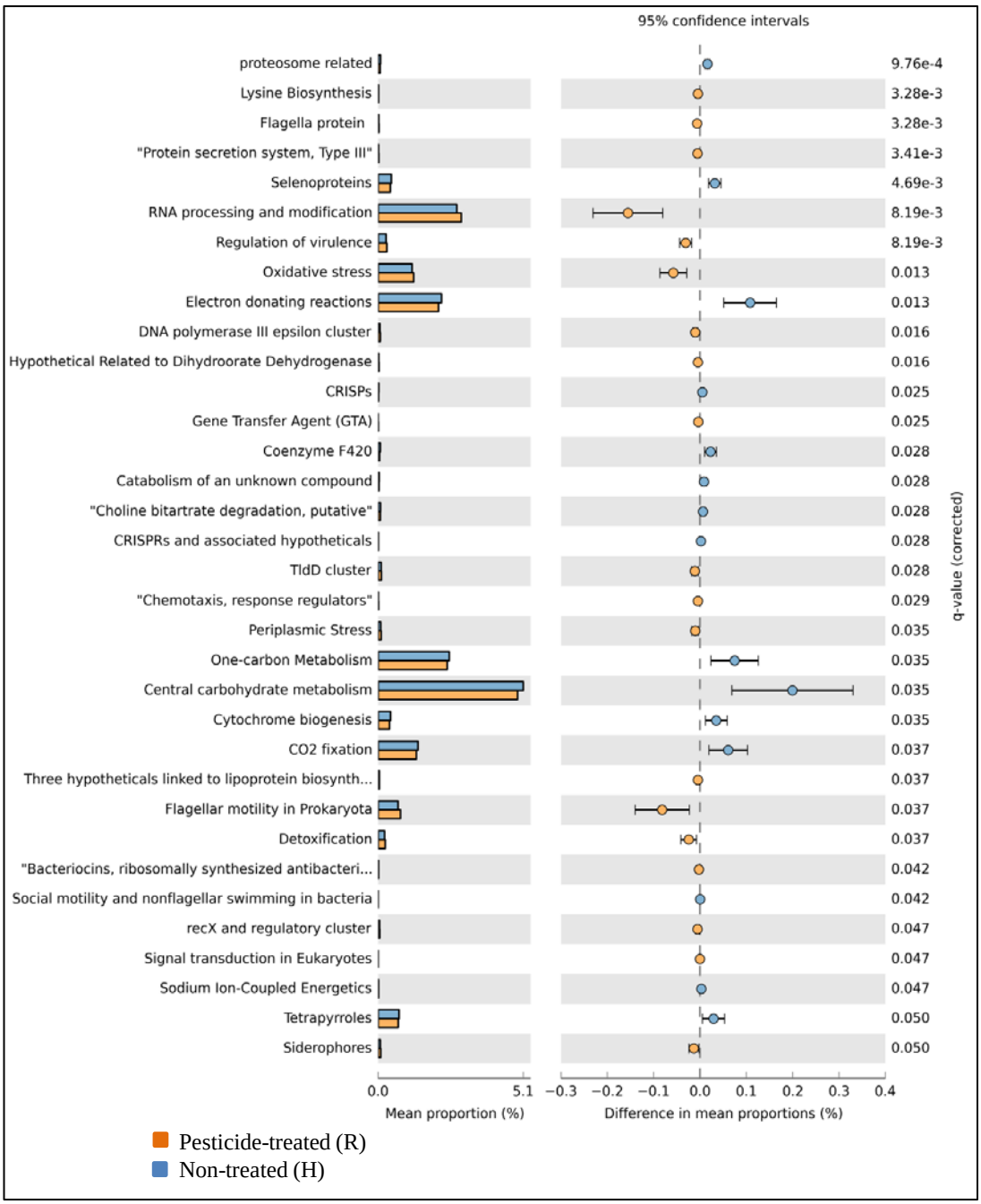


Figure 5-5 Metabolic processes over-and under-represented in pesticide-treated (R) metagenomes relative to the non-treated metagenomes (H) at metabolic hierarchical level 2 of MG-RAST. Corrected q-values were calculated using Storey's FDR approach.

Interestingly, analysis of metagenomes between pesticide-treated and non-treated (across sites) also revealed sequences responsible for P-metabolism and were found to be abundant in pesticide-treated sites (Figure 5-6). Phosphorus metabolism genes were significantly higher in pesticide-treated sites, at level 1 (p=0.02, Figure 5-6a), and ranged in between 0.847% to 0.872% when compared to non-treated sites (0.830% to 0.8585%). Similarly, at level 3, P-metabolism genes were significantly higher (p=0.052, Figure 5-6c) and ranged in between 0.565% to 0.584% when compared to the non-treated sites (0.538% to 0.574%) However, no significant differences between the relative abundances of the P-metabolism genes were observed between metagenomes of pesticide-treated and non-treated sites (p=0.055, Figure 5-6b) at level 2 of metabolic hierarchy. Similarity percent (SIMPER) analysis revealed that the main genes associated with P-metabolism (at level 3) contributing to the differences between the pesticide-treated and non-treated sites were alkaline phosphatase, inorganic phosphate transporter, inorganic pyrophosphatase, phosphate ABC transporter and phosphate transport ATP-binding proteins, together accounting for 45% of the overall differences (Table 5-4). Average dissimilarity between the pesticide-treated and non-treated sites was 5.25%

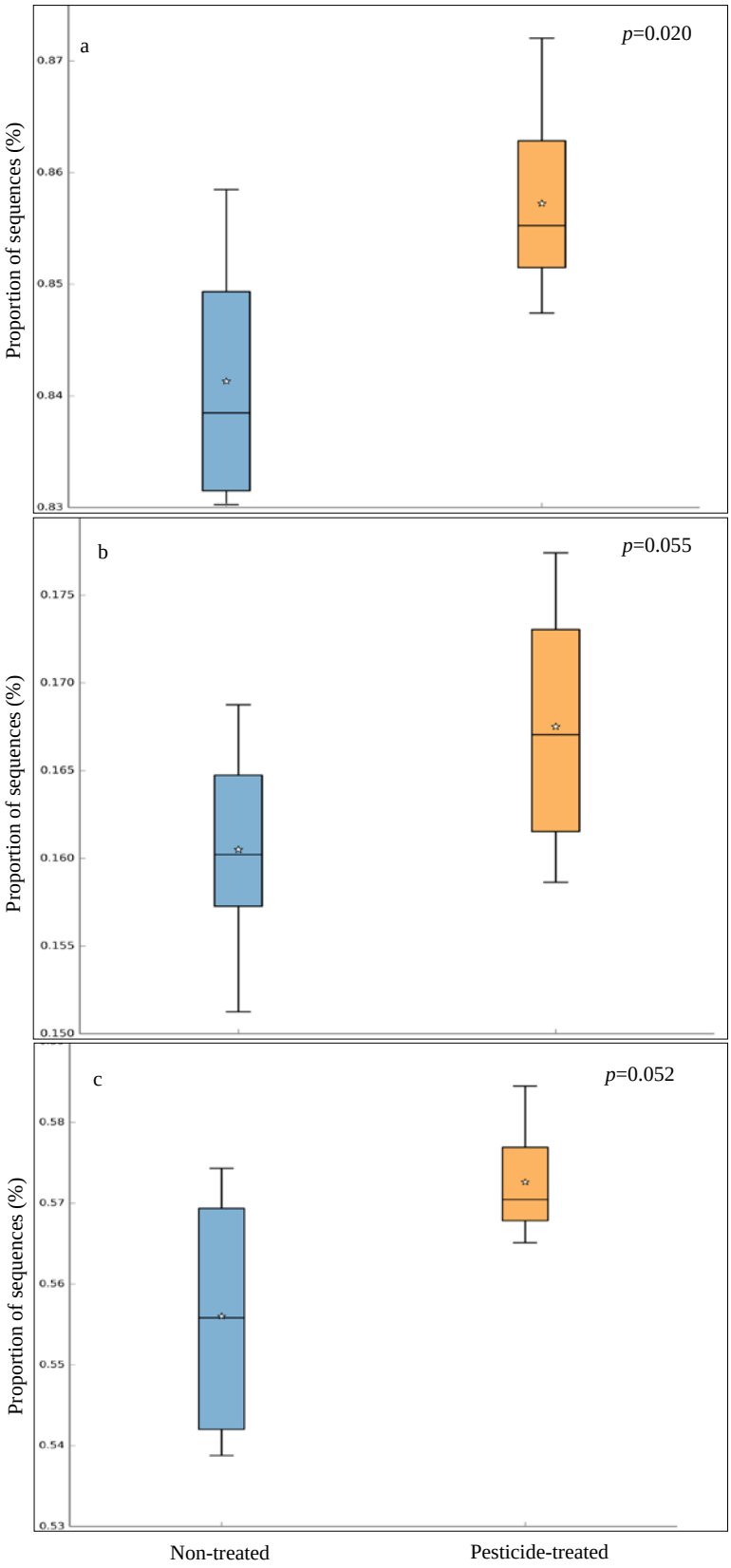


Figure 5-6 Box plot showing the distribution in the proportion of phosphorus metabolism sequences assigned to samples from pesticide-treated and non-treated sites a) at metabolic hierarchical level 1 b) at metabolic hierarchical level 2 and c) at metabolic hierarchical level 3. The median value is shown as a line within the box and the mean value as a star.

Table 5-4 Phosphorus metabolism associated genes (at level 3) contributing to the dissimilarity of pesticide-treated and non-treated sites metagenomes. The larger value in each case is shown in bold. * indicates the genes consistent with Diss/SD >1.4. Abundance values are the square root transformed data. 'Others' indicate genes contributing < 2% cumulative percentage of overall dissimilarity with Diss/SD <1.4. Abbreviations: Diss=dissimilarity; SD=standard deviation.

Genes	Average abundance (non-treated)	Average abundance (pesticide-treated)	Diss/SD	Cumulative (%)
Alkaline phosphatase	1.48	**1.9**	1.33	9.02
Secreted alkaline phosphatase	0.92	**1.34**	2.07*	17
Probable low-affinity inorganic phosphate transporter	2.07	**2.26**	1.24	22.07
Low-affinity inorganic phosphate transporter	**0.67**	0.56	1.62*	27.02
Inorganic pyrophosphatase	**2.23**	2.11	1.43*	31.49
PhoQ	**1.21**	1.06	1.05	35.65
Phosphate ABC transporter, periplasmic phosphate-binding protein PstS	**2.42**	2.28	1.97*	39.03
Response regulator in two-component regulatory system with PhoQ	**1.14**	0.97	0.85	42.29
Phosphate transport ATP-binding protein PstB	**2.62**	2.47	1.85*	45.28
Exopolyphosphatase	1.82	**1.93**	1.27	48.23
Polyphosphate kinase	**3.00**	2.99	1.35	51.1
Soluble pyridine nucleotide transhydrogenase	**1.63**	1.62	0.97	53.94
Phosphate transport system permease protein PstA	**2.16**	2.05	1.34	56.73
Phosphate transport regulator (distant homolog of PhoU)	1.28	**1.41**	0.75	59.42
Predicted ATPase related to phosphate starvation-inducible protein PhoH	1.28	**1.41**	0.75	62.11
NAD(P) transhydrogenase alpha subunit	**2.35**	2.21	1.13	64.78
Phosphate starvation-inducible ATPase PhoH with RNA binding motif	**0.93**	0.88	0.56	67.44
Pyrophosphate-energized proton pump	**3.06**	2.99	1.20	70.05
Phosphate regulon transcriptional regulatory protein PhoB	2.80	**2.86**	1.25	72.61
Alkaline phosphatase synthesis transcriptional regulatory protein PhoP	1.06	**1.07**	0.69	75.00
NAD(P) transhydrogenase subunit beta	**2.35**	2.26	1.14	77.27
Phosphate regulon sensor protein PhoR (SphS)	2.38	**2.47**	0.95	79.26
Others	**4.30**	4.29	<1.4	90.81

5.3.4 Shifts in functional potential in pesticide-treated and non-treated soils within site

Within site 4 and site 5, MDS analysis at metabolic profile hierarchical level 2, revealed a separation between pesticide-treated (4R and 5R) and non-treated (4H and 5H) metagenomes (Figure 5-4). STAMP analysis of the metabolic profile (hierarchical level 2) of site 4 (4R and 4H) showed that the metagenomes derived from 4R and 4H differed statistically only in protein secretion system, Type III (data not shown).

By contrast, the metagenomes derived from 5R were distinct from 5H, and the sub-set of specific gene categories that was found abundant in pesticide-treated sites (Section 5.3.3) was also found to be higher in 5R. These included genes associated with chemotaxis and flagellar motility, iron acquisition, stress responses, RNA modification and phage-associated genes (Figure 5-7) suggesting that some of the soil metagenomic profile responses to the pesticide addition are similar across or within sites.

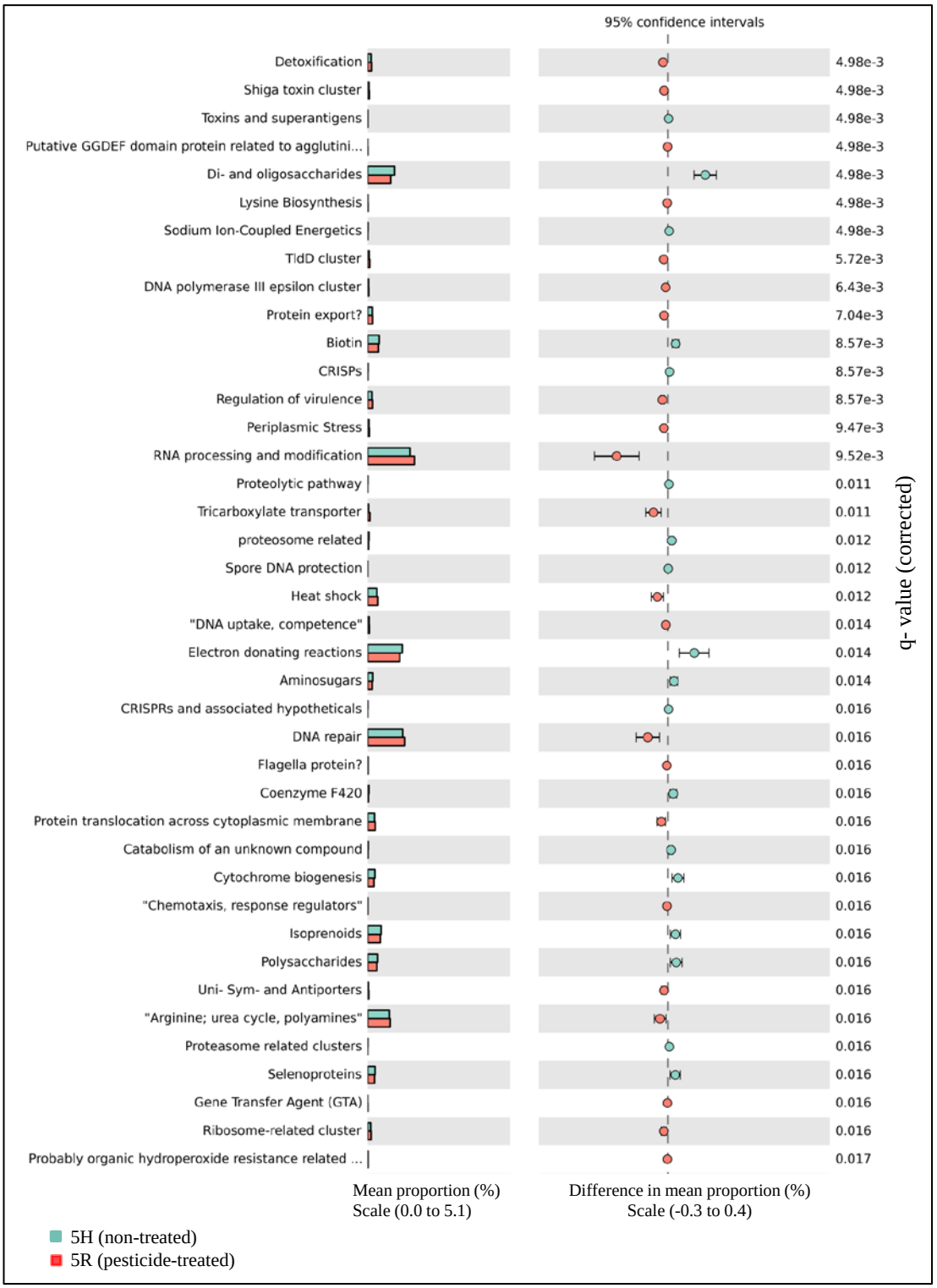


Figure 5-7 Metabolic processes over- and under-represented within site 5 in pesticide-treated (5R) metagenomes relative to the non-treated metagenomes (5H) at metabolic hierarchical level 2 of MG-RAST. The top 40 functional genes are presented here out of total 89 significantly different functional genes. Corrected q-values were calculated using Storey's FDR approach.

Analysis for P-metabolism genes was also performed within site 4 (4H and 4R) and site 5 (5H and 5R). Phosphorus (P) metabolism genes were significantly higher in 5R (0.850% to 0.851%), than 5H (0.830%-0.831%) at level 1 (p<0.001, Figure 5-8a); however, the P-metabolism genes contributed less to the observed difference between metagenomes of 5H and 5R sites (p=0.069, Figure 5-8b) at level 2. In addition to this, P-metabolism genes were also significantly higher in 5R than 5H (5R=0.565% to 0.567%; 5H=0.538%-0.545%; p=0.028, Figure 5-8c) at level 3 of metabolic hierarchy.

Although no significant difference was observed, the abundance of P-metabolism genes appeared slightly higher in 4R than 4H at level 1 and level 3 (Figure 5-9a and c, respectively). Similarity percent (SIMPER) analysis revealed that the main genes associated with P-metabolism (at level 3) contributing to the differences within site 4 and site 5 was similar to the ones found across all sites (Table 5-4) (data not shown).

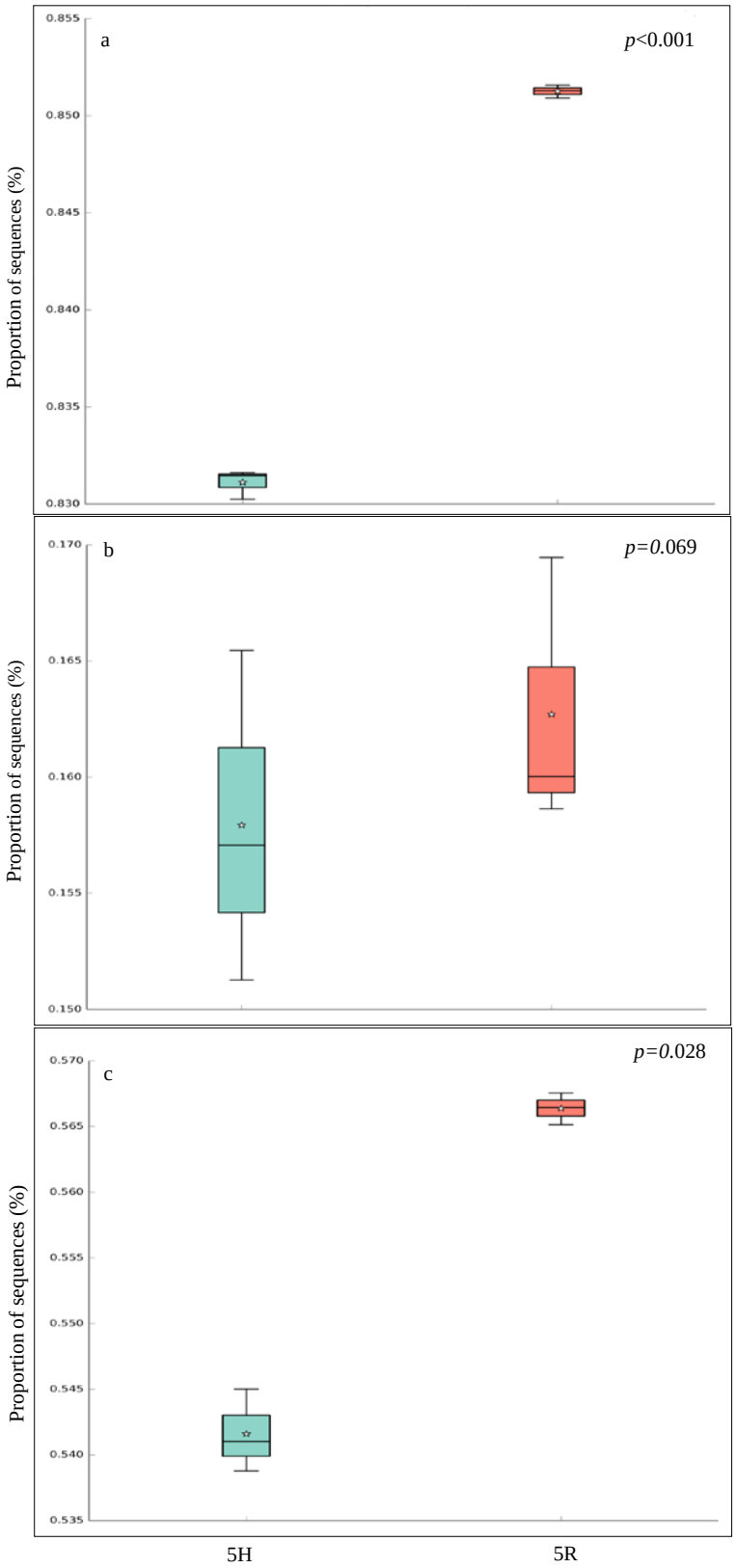


Figure 5-8 Box plot showing the distribution in the proportion of phosphorus metabolism sequences assigned to samples from 5H and 5R a) at metabolic hierarchical level 1 b) at metabolic hierarchical level 2 and c) at metabolic hierarchical level 3. The median value is shown as a line within the box and the mean value as a star.

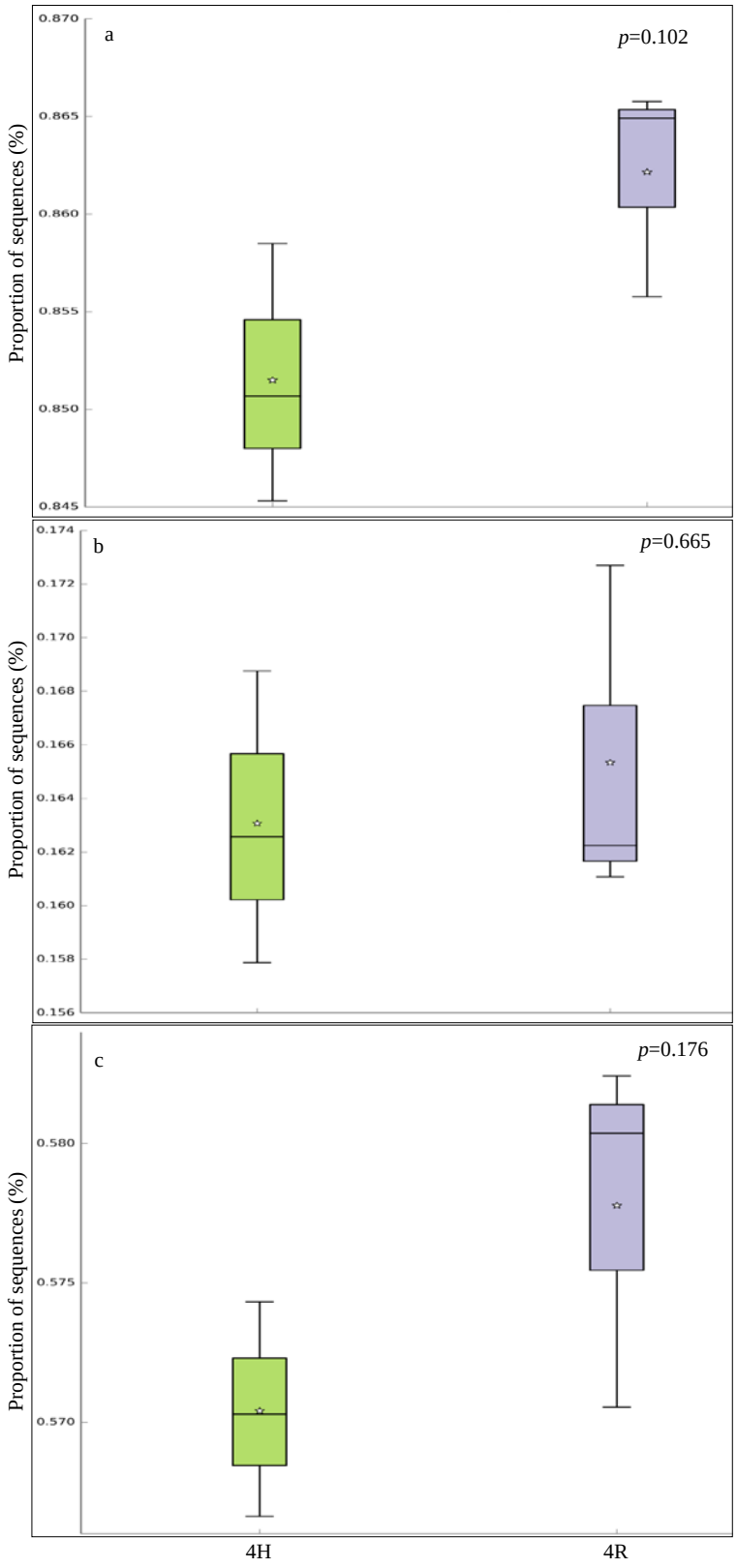


Figure 5-9 Box plot showing the distribution in the proportion of phosphorus metabolism sequences assigned to samples from 4H and 4R a) at metabolic hierarchical level 1 b) at metabolic hierarchical level 2 and c) at metabolic hierarchical level 3. The median value is shown as a line within the box and the mean value as a star.

5.4 Discussion

5.4.1 Taxonomy

Shifts in microbial communities were observed between pesticide-treated and non-treated sites. Pesticide-treated sites harboured a slightly higher α-diversity than the non-treated sites indicating a marginal effect of past pesticide treatment (in fields). These results were contrary to findings in previous chapter (Chapter 3), where past pesticide application (in fields) did not have significant effect on the bacterial diversity. This difference might have occurred because of the higher resolution approach used here (shotgun sequencing) and also the number of soil samples that were not same. However, the general trend of diversity remained the same. Contrasting to the general pattern of reduction in diversity in stressed/contaminated environments (Sutton *et al.*, 2013), the higher diversity might indicate that pesticide treatment suppress or otherwise dominant species relative to the rare species to maintain the stability of soil function during the stress (pesticide-treatment). However, it should be noted that microbial diversity is also influenced by the soil chemistry (Adamidis *et al.*, 2014). The contrary diversity pattern observed here might be because the soils varied in a variety of ways and especially because the pesticide driven increase in diversity was mainly contributed by site 5.

In addition to this, the dominant bacterial genera were similar across sites but statistically significant differences in the relative abundance of the less abundant genera (rare) were observed. The results obtained, showed a strong response of 'rare' but not dominant bacteria to pesticide treatment which has also been observed elsewhere (Sangwan *et al.*, 2012). Based on the relative abundances, at the pesticide-treated sites there was an increase in the abundance of Proteobacteria (*Methylibium*,

Sphingomonas, *Novosphingobium*, *Sphingobium*, *Phenylobacterium*, *Maricaulis*, *Rubrivivax* and *Leptothrix*) and Bacteroidetes (*Pedobacter*, *Parabacteroides*, *Marivirga* and *Paludibacter*). Dominance of Proteobacteria is consistent with the phylogenetic diversity observed in contaminated environments (Reddy *et al.*, 2011, Beazley *et al.*, 2012, Fang *et al.*, 2014) and many Proteobacteria have been noted for their ability to degrade and transform poly aromatic hydrocarbons (PAHs) (Ramana *et al.*, 2006, Kane *et al.*, 2007, Kertesz and Kawasaki, 2010, Reddy *et al.*, 2014), pesticides (Li *et al.*, 2007, Yan *et al.*, 2007, Li *et al.*, 2008, Hussain *et al.*, 2011, Chu *et al.*, 2014) and organochlorine compounds (Böltner *et al.*, 2005, Neufeld *et al.*, 2006, Nagata *et al.*, 2011). Although Bacteroidetes are not known to degrade xenobiotics, several related genera inhabit contaminated environments (Chanika *et al.*, 2011) and some have been reported to degrade pesticides. For example, *Pedobacter* is able to degrade carbofuran (Karpouzas *et al.*, 2000a), *Marivirga* and *Paludibacter* can degrade the pesticides endosulfan, chlorpyrifos and cypermethrin (Naphade *et al.*, 2013) and fluazifop-P-butyl (herbicide) (Darine *et al.*, 2015), respectively. Another genus, *Parabacteroides*, has been isolated from ecosystems with a history of polyaromatic hydrocarbon (PAHs) and polychlorinated biphenyl (PCB) (Braun *et al.*, 2015) pollution, and thus their significance in the degradation of xenobiotic compounds cannot be ruled out.

By contrast, the Archaeal-related genera (*Methanococcus*, *Methanothermobacter*, *Methanopyrus*, *Archaeoglobus* and *Thermococcus*) were enriched in the non-treated sites relative to pesticide-treated sites, indicating that these genera are sensitive to pesticide (OP) application. These results suggest that these microorganisms might be used as potential bio-indicators for monitoring pesticide-contamination of soils. This is in accordance with the study of taxonomical diversity in pesticide-treated soils

(*e.g.* imidacloprid, dazomet and mancozeb) where the ammonia-oxidising archaeal community was negatively affected by the pesticide-treatment (Cycoń and Piotrowska-Seget, 2015b, Feld *et al.*, 2015) implying that this domain might be vulnerable to pesticide-stress. This study provides the first metagenomic analysis focusing on the impact of OP pesticide application on the soil microbial communities and the relationship between the pesticide treatment and the distribution of the microbial taxa. Thus, these finding provide novel insight into predicting the responses of the soil bacterial diversity to long-term pesticide applications.

5.4.2 Predicted metabolic functions

Unconstrained MDS ordination showed a clear separation of samples from pesticide-treated and non-treated sites (Figure 5.4) suggesting consistent effect of pesticide application across sites on metabolic gene profiles. The composition of the metabolic profile (hierarchical level 3) responded similarly to the structural community composition and there was a significant pesticide impact on functional gene profiles. Several other studies have likewise shown that anthropogenic disturbances (contamination) have caused a shift in microbial community functionality (Hemme *et al.*, 2010, Smith *et al.*, 2012). For example, studies on soil microbial communities and functional gene diversity in a hydrocarbon-based study similarly showed differences in microbial community structure in contaminated versus non-contaminated sites (Liang *et al.*, 2011, Sangwan *et al.*, 2012, Smith *et al.*, 2013).

A comparison of metagenomic data between pesticide-treated and non-treated sites (both across and within) showed that genes affiliated with core metabolic functions were the most abundant across all the metagenomes analysed. These included

general metabolic functions like respiration, carbohydrate, amino acid, protein, RNA and DNA metabolism, which are essential for the survival of microbes and are maintained across all habitats. These observations were in line with metagenomic studies in the marine environment (Dinsdale *et al.*, 2008, Hewson *et al.*, 2009, Smith *et al.*, 2012, Tout *et al.*, 2014). However, less abundant gene categories associated with more specialised metabolic processes (iron acquisition and metabolism, motility, cell signalling, stress response) accounted for greater relative gene abundances in pesticide-treated sites than in non-treated sites. Similar patterns have been documented in other stressed or contaminated environments (Ford, 2000, Paul *et al.*, 2005, Ahmed and Holmström, 2014).

Microbial communities should adapt to the sudden loss of the key micro-nutrients like P and iron occurring due to pesticide addition or unintentional contamination. The increase in genes associated with iron acquisition (siderophores) and phosphorus metabolism in pesticide-treated soils indicated the potential of the indigenous microbial communities to adapt and survive in nutrient limited environments by allowing for an effective scavenging mechanism. Furthermore, pathways associated with carbohydrate and protein metabolism were reduced in the pesticide-treated sites indicating that the microbial communities are allocating more of their energy to acquisition of key nutrients, particularly iron and phosphorus, needed for biodegradation of the chemical pesticides. This eventually leads to a decrease in the complex carbohydrates and proteins pathways for growth. Siderophores related genes were relatively abundant indicating greater siderophores production by the bacterial community. The role of siderophores in several other sectors, like agriculture, bioremediation, bio-sensing and medicine is also documented (Saha *et al.*, 2015). The observations that toxic environments induce the production of

siderophores potentially allows siderophore-producing bacteria to assist in the detoxification of various heavy metals (Nair *et al.*, 2007, Rajkumar *et al.*, 2010, Wang *et al.*, 2011b, O'Brien *et al.*, 2014), mobilising metals from mine wastes (Edberg *et al.*, 2010), removal of OP compounds (Rani and Juwarkar, 2012) and hydrocarbons from contaminated sites (Barbeau *et al.*, 2002).

The genes related to bacterial motility and chemotaxis were relatively abundant in pesticide-treated sites and contributed to consistent differences in bacterial community composition within and across sites. The success of the microbial degradation of chemical pollutants is often limited by the inability of the bacteria to come in direct contact with the pollutants (Fernández-Luqueño *et al.*, 2011, Niti *et al.*, 2013). Therefore, bacterial chemotaxis provides a distinct advantage to the motile bacteria in finding their substrate and degrade them at higher rates (Pandey and Jain, 2002). Several studies on the isolation and characterisation of chemotactic microorganisms capable of catabolising a variety of hazardous environmental pollutants including nitroaromatic compounds, OP pesticides, hydrocarbons and atrazine have reported that flagellum dependent motility and chemotaxis are crucial factors in the process of degradation of xenobiotics (Parales *et al.*, 2000, Parales and Harwood, 2002, Law and Aitken, 2003, Paul *et al.*, 2006, Guo *et al.*, 2009, Pandey *et al.*, 2012). It is likely that motility and chemotaxis are particularly important for microbial communities in contaminated sites where the chemical products are often strong chemo-attractants (Samanta *et al.*, 2002, Parales, 2004, Kato *et al.*, 2008, Ahemad and Khan, 2011). It is also suggested that the degradation ability of bacteria is enhanced by initiation of biofilm formation that requires flagellar attachment (Ortega-Calvo *et al.*, 2003, Gordillo *et al.*, 2007, Banat *et al.*, 2010), which increase the availability of the chemical pollutant in the soil (Mohan *et al.*, 2006). Thus, the

higher relative abundance of flagellar genes suggests that the microbial communities in pesticide-treated sites have metabolic pathways that will allow for direct contact with pesticides for degradation.

Genes associated with phage-like gene transfer agents (GTA) were also more abundant in pesticide-treated site suggesting an increase in genetic mobility due to pollution. Studies have shown that phages move between environments and carry 'mobile genes' to manipulate host metabolisms (Canchaya *et al.*, 2003, Edgell *et al.*, 2010) allowing microbial communities to rapidly adapt to the presence of xenobiotics, such as OP compounds (Zhang *et al.*, 2006a, Singh, 2009), organochlorine compounds (Dogra *et al.*, 2004), and herbicides (Devers *et al.*, 2005) through horizontal gene transfer (Top *et al.*, 2002, Top and Springael, 2003). Pesticide-treated sites were also found to have a high abundance of genes related to stress responses indicating that the microbial communities possessed mechanisms to tolerate and overcome oxidative stress. Pollution degrading microbial communities often experience oxidative stress that is generated during biodegradation (Park *et al.*, 2004), but could adapt and adjust their physiology with the stress response (Chávez *et al.*, 2006, Zhang *et al.*, 2012b).

The data indicated an over-representation of genes associated with P-metabolism in pesticide-treated sites (both across and within) suggesting that the microbial communities degraded OP compound as an alternative source of inorganic P to survive P limiting conditions at that site. This indicates the presence of uncultivable microorganisms in pesticide-treated sites since no isolated bacterial strains (Chapter 4) from these soils were capable of utilising CP (OP pesticide) as a source of P. Previous literature has also reported that some bacterial communities have evolved complex systems to survive under phosphorus starvation conditions, accomplished

through hydrolytic cleavage of phosphate ester bonds in OP compounds (Chen *et al.*, 1990, Singh and Walker, 2006, White and Metcalf, 2007, Hirota *et al.*, 2010).

Additional, it should be noted that the abundances in the genes associated with P-metabolism were prevalent enough to mark the difference between pesticide-treated and non-treated sites at all the three metabolic hierarchical levels. This is in agreement with the results of previous chapter (Chapter 3) where the enhanced degradation of CP was maintained in soils despite no exposure to CP after its efficacy loss (about 13 years ago). These results suggested that genes associated with P-metabolism were still enriched in those soils due to the legacy effect of the compound. Likewise, a study by (Singh *et al.*, 2004) reported the isolation of *Enterobacter* strain B-14 from the same sugarcane farm soils (Australian) with the ability to mineralise CP as a source of C and P indicating the presence of P degrading genes in the soils. Thus, the hypothesis that a significantly higher abundance of genes associated with P-metabolism would be expected in pesticide-treated comply with the observed results.

In addition to this, SIMPER analysis revealed that the main distinguishing gene associated with P-metabolism at pesticide-treated sites was that of alkaline phosphatase. This enzyme is involved in hydrolysing the ester bonds between P and carbon (C) (C-O-P ester bonds), releasing inorganic P (Rahmansyah *et al.*, 2009) thus playing a role in P-metabolism. Alkaline phosphatase has been reported for their role in the degradation of the OP pesticides by hydrolysing monoalkyl phosphates (Singh and Walker, 2006, Luo *et al.*, 2009b, Thengodkar and Sivakami, 2010, Falguni and Sharma, 2014) and for their application in biological waste water treatment processes (Xie *et al.*, 2010a). In addition their role as biosensors in the detection of pesticides (Chouteau *et al.*, 2005) and other heavy metals contamination

also has been studied (Awasthi, 2012, Tekaya *et al.*, 2013). The higher abundance of alkaline phosphatase in pesticide-treated sites suggested the enrichment of phosphatase gene and a role of alkaline phosphatase in scavenging inorganic P, under stressed conditions, by degrading OP compounds. However, further analyses at higher resolution is required to tease apart the role of other tri- and bi-ester phosphate metabolising gene(s)/enzyme(s) for they play a direct role in degrading OP compounds. However, above findings should be interpreted with caution because this study suffers from an inherent limitation of field-based studies. It is possible that the differences observed here may be explained by the agricultural practices employed in farm (*e.g*. fertilisation applications and no cultivation in headland soils). These limitations may have an effect in assessing the metabolic functions, making the estimation less accurate. For example, it is worth mentioning here that apart from pesticide effect, the abundance of alkaline phosphate at pesticide-treated sites could also be attributed to the land-use (farming) history and use of phosphorus fertilisers. Thus, metagenomic data needs to be further explored using more soil samples, *e.g*. 3R and 3H (no enhanced CP degradation in fields), to better differentiate the impact of agricultural practices from pesticide treatments. Nonetheless, this study not only provides the information about the relative abundance of the microbial communities in the pesticide-treated sites but also on the distribution of functional genes important for the catabolism of the OP pesticides.

Microbial communities are known to respond to pesticide treatments at the functional level, whereby a shift in the community structure and/or metabolic potential can be observed (Baxter and Cummings, 2008, Wang *et al.*, 2008, Floch *et al.*, 2011, Zabaloy *et al.*, 2012). A major goal in any xenobiotics-microorganisms interaction study is to identify the response of key functional roles being undertaken by the

indigenous soil microbial communities. This study therefore provides additional evidence for developing accurate predictions or ecological theories on relationship between soil microbial diversity and functions into ecosystem models (Allison and Martiny, 2008, Reich *et al.*, 2012). Furthermore, our results based on replicated sampling provide strong confidence in the validity of the observed pattern, which is usually missing in metagenomic studies where differences in characteristics of metagenomes are not necessarily reliable. However, future work is required to investigate temporal-point relationships between soil microorganisms and functional profile in other biomes to assess whether the interaction influences overall ecosystem functioning.

5.5 Conclusion

The present study provides the initial attempt to understand the relationship between bacterial community composition and their potential metabolic functions in pesticide-treated (OP) soils using a metagenomics approach. The study revealed that Proteobacteria and Bacteroidetes were predominantly involved the adaption in the pesticide-treated sites and thus open the possibilities of investigating their role in degradation of OP pesticides. By contrast, Archaea were sensitive to pesticide-treatment and can be further explored for their use as bio-indicators for OP pesticide monitoring *in situ*. These findings further highlight the role of pesticides in influencing distribution of microorganisms in soil systems.

Higher abundances of genes associated with specialised functions including P-metabolism, GTA, iron-acquisition, stress and chemotaxis/motility were found at pesticide-treated sites. In conclusion, the results supported the hypothesis that long-term pesticide application will affect bacterial community composition and functional capabilities. Also, according to my hypothesis of the significantly higher abundance of genes related to P-metabolism at pesticide-treated sites, the results showed marked difference in pesticide-treated and non-treated sites but the magnitude of response was soil dependent. Additionally over-representation of alkaline phosphatase was observed at pesticide-treated (OP) sites which might provide an insight into the OP degradation pathway. Furthermore, detailed analyses of the metagenomic data to finer sub system levels is required to determine the abundance of other phosphatase (bi- and tri-) and any novel enzyme(s) or pathway(s) for their direct role in OP degradation. Given increased anthropogenic activities associated with agricultural practices, and its associated effects impact the microbial system, this study provides a strong framework to develop effective bioremediation

strategies and ecotoxicology impacts of OP contamination, which could be harnessed for future work.

Chapter 6: Final conclusion and future work

6.1 Conclusion

The overarching aim of this study was to investigate various aspects of interactions between model compounds, trichloroethene (TCE), chlorpyrifos (CP) and imidacloprid (IC), and the soil microbial community structure, diversity and functional characteristics. Assessing the impact of xenobiotics on soil microbial communities and their response is of importance because soil microorganisms play a vital role in several biogeochemical cycles and maintain soil health, which are basic requirements for crop production and environmental sustainability. The most important findings of this study were:

- The methanotrophic consortium from Sydney University (SU) soil could co-oxidize TCE in presence of high methane (CH_4) concentration (33%) suggesting a co-metabolic mode of TCE degradation. Of particular interest was the observation that a decrease in CH_4 concentration resulted in the decreased or removal of methanotrophic consortium ability to degrade TCE. The result highlighted the importance of optimum CH_4 feeding strategy to maximise TCE removal and reduce metabolite toxicity effects, which often limits the efficacy of the *in situ* bioremediation process. This finding may provide a framework to engineering models or tools for the effective *in situ* bioremediation of TCE contaminated areas.

- The application of TCE also resulted in the enrichment of T-RF 53, identified as a novel uncultivable indigenous Type I methanotroph (distantly related to

Methylovulum-88%) TCE degrader from Australian soils. Expanded knowledge about the diversity of microorganisms involved in TCE would likely lead to the discovery of novel mechanisms for biodegradation of TCE and new bioremediation technologies based on those mechanisms can be developed for the successful removal of the compound.

- The repeated application of CP and IC at 10 mg/kg in soils with no history of pesticide application (non-treated) and soils with ~20 years pesticide application (pesticide-treated) affected indigenous soil bacterial community composition and broad functions (*e.g.* soil respiration) under lab conditions. The inhibitory effects on microbial community composition persisted for the entire incubation period suggesting that the bacterial community had a low resistance to both CP and IC application.

- Additionally, high rates of CP degradation highlighted the potential risk of reduced pesticide efficacy associated with the repeated application of the pesticides due to the development of enhanced degradation. This could potentially have detrimental impact on sustainability of agro-economy and food security due to loss of pesticide efficacy. Despite no exposure to CP for 13 years (in fields), this study provide evidence that even after 13 years, enhanced degradation capacity of soil microbial community was maintained due to the legacy effect of previous use of the compound. Owing to the widespread use of CP worldwide, this study provide a strong evidence of legacy effects of pesticide on soil microbial communities and the importance of considering long-term management practices in agro-ecosystems.

- The novel CP and TCP degrading bacterial strains, *Xanthomonas* sp. 4R3-M1, *Pseudomonas* sp. 4H1-M3 and *Rhizobium* sp. 4H1-M1 were isolated from Australian soils. These strains retained their degrading ability in a range of different media utilising CP and its primary degradation product (TCP) as a source of carbon (C) and nitrogen (N). However, only *Xanthomonas* sp. 4R3-M1 and *Pseudomonas* sp. 4H1-M3 degraded TCP as a sole C- and N-source when provided externally. The results indicate a promising bioremediation potential of the bacterial strains that remains largely untapped. The efficacy of microorganisms to degrade a certain compound at any particular site is influenced by biotic and abiotic factors and thus is difficult to predict. Therefore, isolation and characterisation of microorganisms with different physiologies and degrading capabilities would likely lead to the discovery of novel mechanisms of degradation and improved knowledge on the degradation process. The expanded knowledge on the biochemical and ecological nature of microbial transformation of the xenobiotics would allow for the development of new and improved bioremediation technologies based on those mechanisms.

- The investigation of impact of pesticides on microbial communities, in soils with no history of pesticide application (non-treated) and soils with ~20 years pesticide application (pesticide-treated) using next-generation sequencing indicated taxonomic and functional adaptations in the soil microbial communities following pesticide treatment. This study represents one of the most comprehensive analyses of soil metagenomes focusing on the impact of pesticide application on the soil microbial communities and the relationship

between the pesticide treatment and the distribution of the microbial taxa and functional genes. The functional diversity was highly correlated with taxonomic diversity across all sites (non-treated and pesticide-treated). The Proteobacteria and Bacteroidetes were predominantly in these adaptions (at pesticide-treated sites), and thus open possibilities to investigate their role in degradation. In addition to this, genes associated with specialised functions (*e.g.* iron-acquisition, stress, motility) were found in higher abundances at pesticide-treated sites. These findings provide novel insights into the responses of soil microbial diversity and functional capabilities to long-term pesticide applications.

- The legacy of CP application on functional capabilities, with particular focus on genes associated with phosphorus (P)-metabolism, was investigated between soil metagenomes of pesticide-treated and non-treated sites. The analyses indicated that there were differences in abundance of P-metabolism genes. Despite generating >520 Mbp shotgun metagenomic data no significant difference on the abundance of organophosphorus (OP) degrading genes was observed. These results indicated the next-generation sequencing did not precisely determine the difference in OP degrading genes and further improvised tools (without need of amplification), *e.g*. metatranscriptomics are required for analysing the profile of expressed genes effectively. In addition to this, alkaline phosphatase associated genes were over-represented in pesticide-treated sites suggesting the capacity of the soil microbial communities to biodegrade simple monoalkyl phosphates which may also

play a role in OP compound degradation. More work is required (at finer hierarchical levels) to tease apart the role of other tri- and bi-ester phosphate.

Overall, this study provides novel insight into the interaction between xenobiotics and the soil microbial communities both at structural and functional levels. The findings from this study should be explicitly considered in developments in xenobiotic regulation and pesticide use in terms of application frequency.

6.2 Future work

As presented in this study, under an optimum CH_4 concentration a novel indigenous uncultivable methanotroph (T-RF 53) was identified from Australian soils with the potential to degrade TCE. Future work should focus on the successful development of TCE bioremedial strategies in field-based research to verify the efficacy of this uncultivable methanotroph (T-RF 53) under natural conditions. If consistent trends are observed, this study can be extended to bioremediate TCE contaminated site *in situ*. Furthermore, future work should include isolating pure methanotrophic cultures from soils under different enrichment conditions because there still remains a lot to be unveiled about their physiology, genetics and genomics.

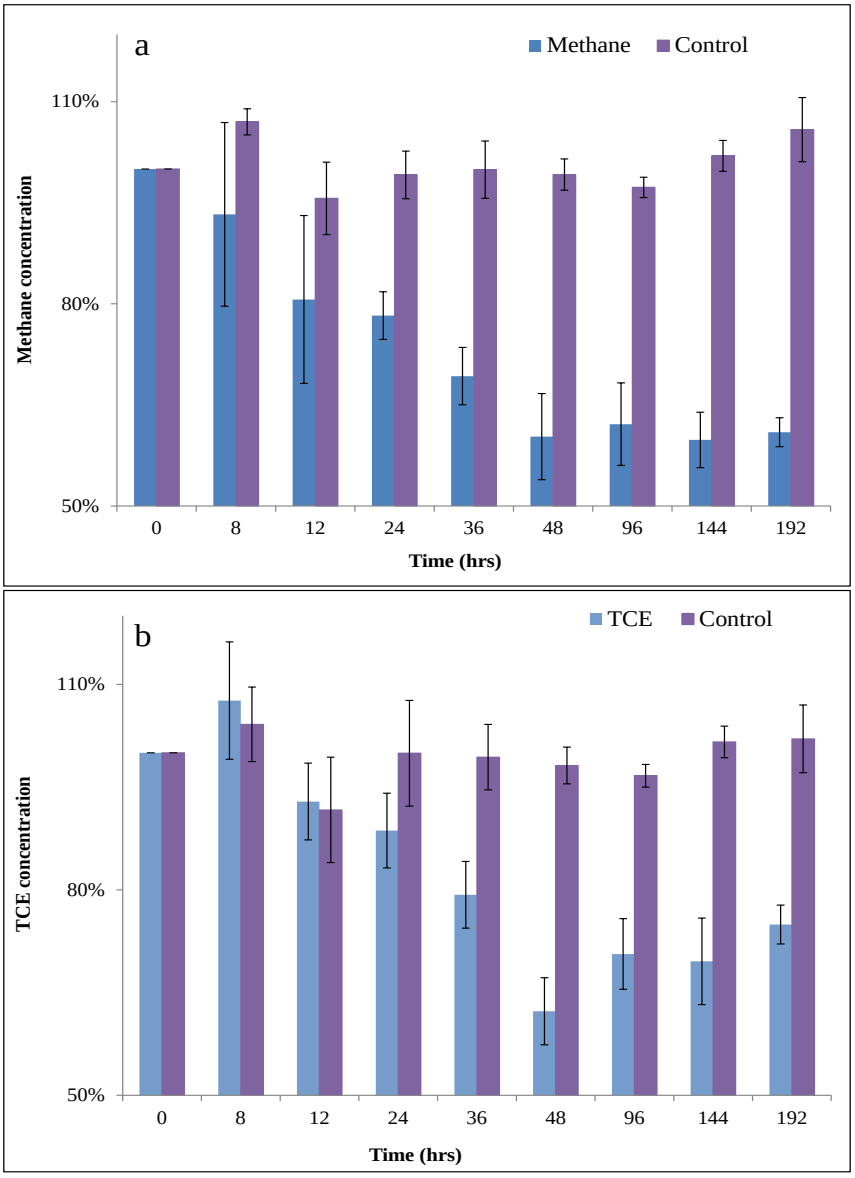
Whilst this study illustrated the significant impact of application of CP and IC on soil bacterial communities and functional capabilities, further research is required to determine impacts on other microbial communities (*e.g.* fungi) and functional groups at different application concentrations. Given that the impact of pesticides is strongly influenced by abiotic conditions, including soil type, temperature and pH, more research can provide required knowledge to establish guidelines for best practices.

For example, soil type can be a major factor in determining the fate of pesticide in the environment, affecting the bioavailability of the compound.

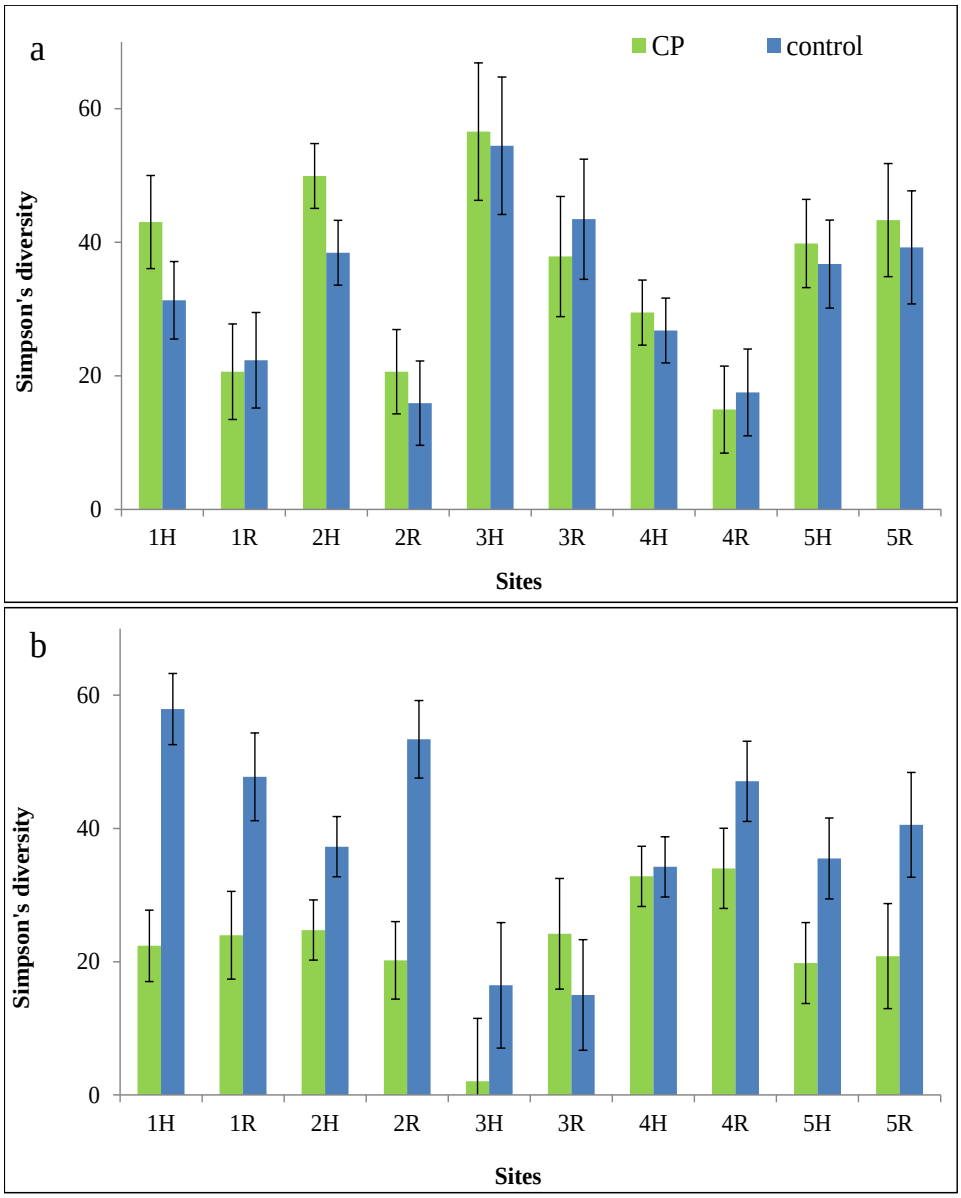
Novel CP and TCP degrading bacterial strains were isolated from Australian sugarcane farm soils through lab screening. Future work could, therefore, focus to determine the optimum pH and temperature of the isolates at different concentrations of CP and TCP for maximum degradation of the compounds. In addition to this, further work is required to study the capacities (activity and survival) of these isolates in contaminated fields by means of bioaugmentation. Scale-up studies (for example using bioreactors) could feasibly be used to assess the potential of the strains to perform as desired at field-conditions treatment facilities. However, it is worth mentioning that most of the microorganisms are uncultivable under laboratory conditions and therefore, the knowledge of their role in degradation remains limited. The use of stable isotope probing (SIP) could be adapted to monitor CP and TCP degrading communities in natural mixed populations. Additionally, characterisation and identification of the degradation pathways, enzymes and genes are other areas for further investigation.

Furthermore, this work reported increased abundance of alkaline phosphatase at pesticide-treated sites and future work should focus investigating the presence of other phosphatases (at finer hierarchical levels) and determining their role in suitable *in situ* bioremediation of organophosphorus contaminated sites.

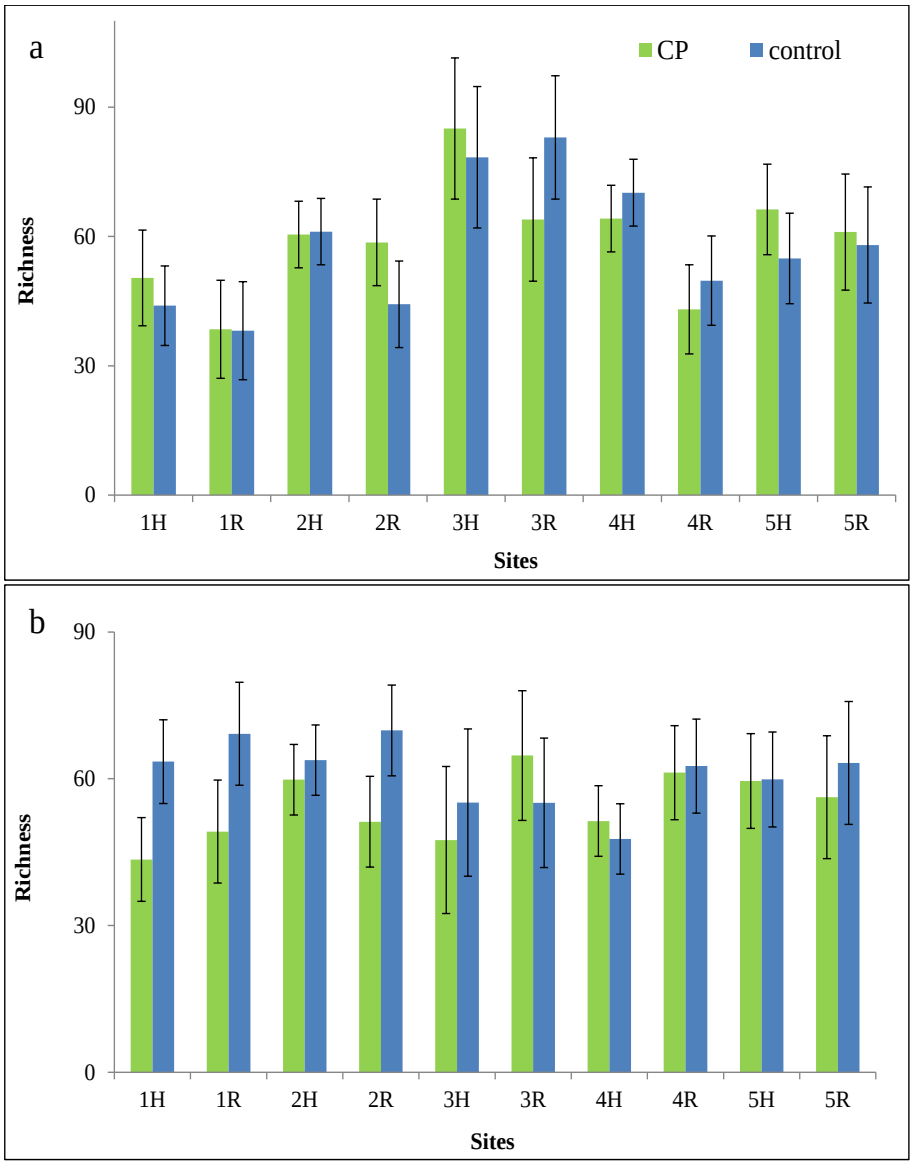
Appendix A.



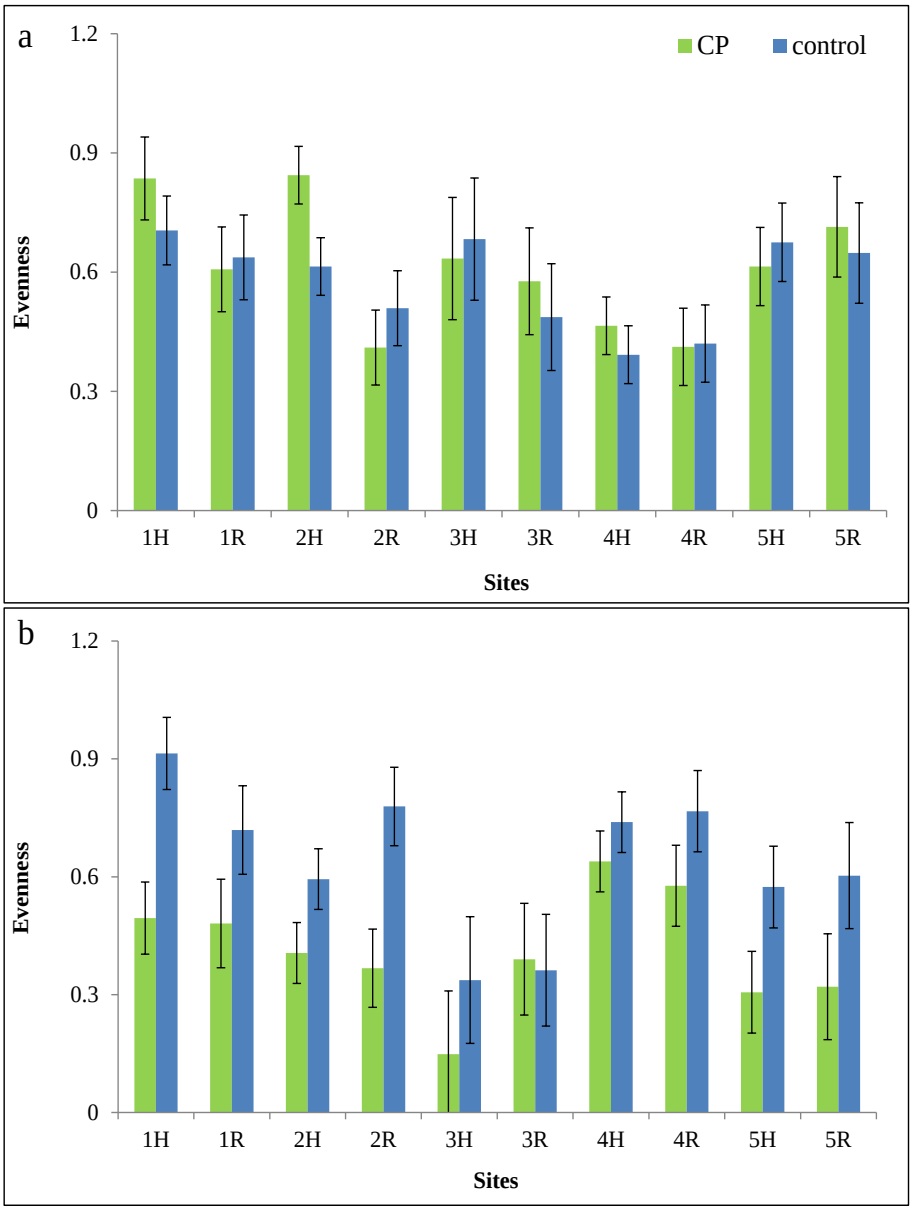
Chapter 2 Figure A-1 Activity of methanotrophic mixed culture in Sydney University (SU) soil under 33% methane concentration. a) Reduction in methane concentration and b) Reduction in TCE concentration. Error bars represent standard error of the mean (n=3). Legends: TCE=trichloroethene



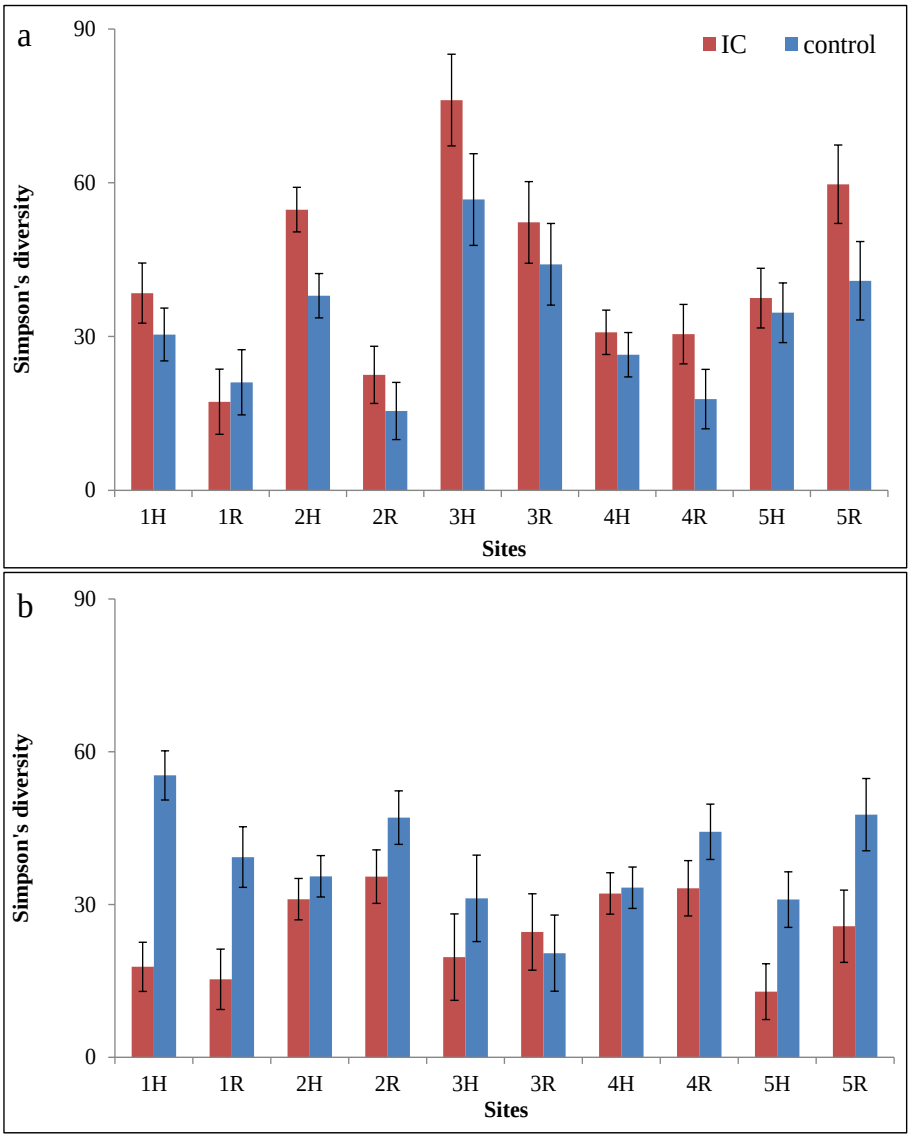
Chapter 3 Figure A-2 Simpson's diversity in soils amended with pesticide CP in the lab a) 0 day and b) 105d with their respective controls. R represents the sites with a history of past pesticides application in the fields and H represents headland not treated with pesticides (in fields). Error bars represent standard error of the mean (n=3). Legends: CP=chlorpyrifos



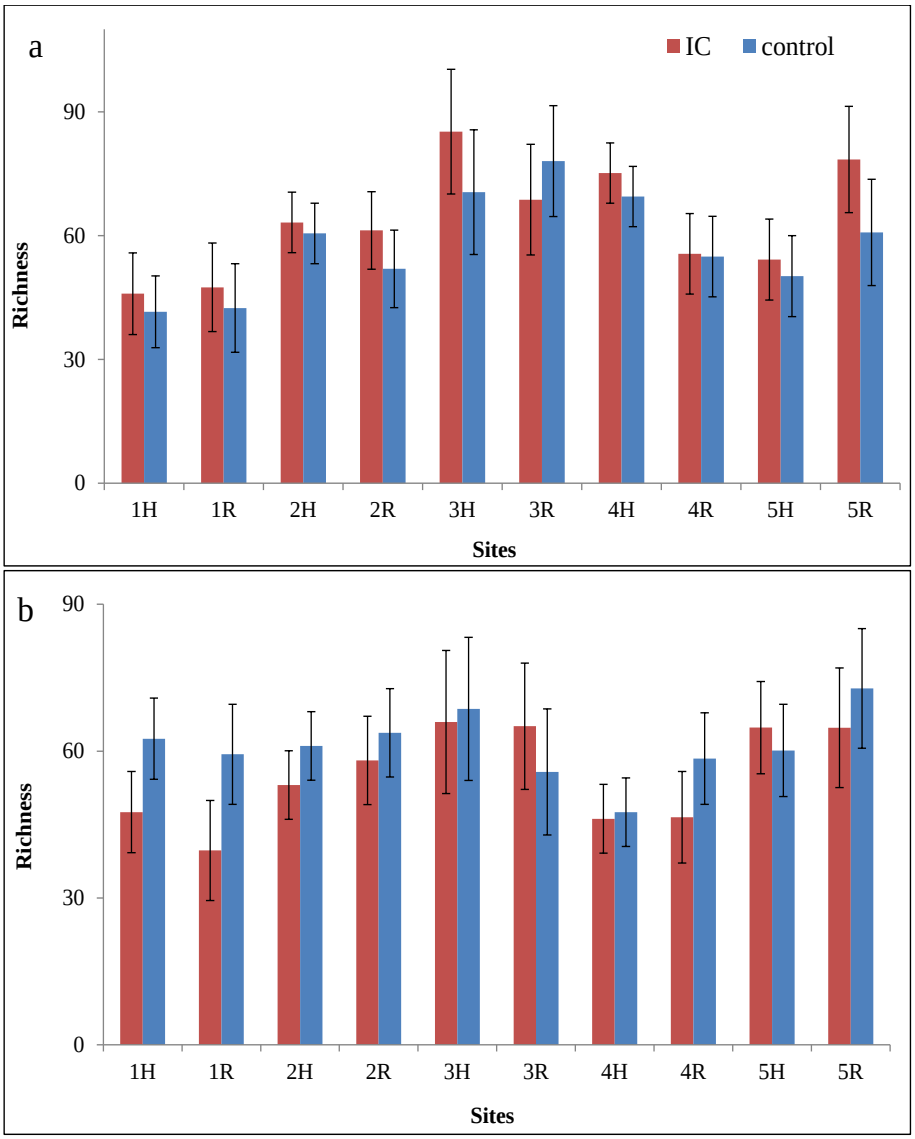
Chapter 3 Figure A-3 Bacterial richness in soils amended with pesticide CP in the lab a) 0 day and b) 105d with their respective controls. R represents the sites with a history of past pesticides application in the fields and H represents headland not treated with pesticides (in fields). Error bars represent standard error of the mean (n=3). Legends: CP=chlorpyrifos



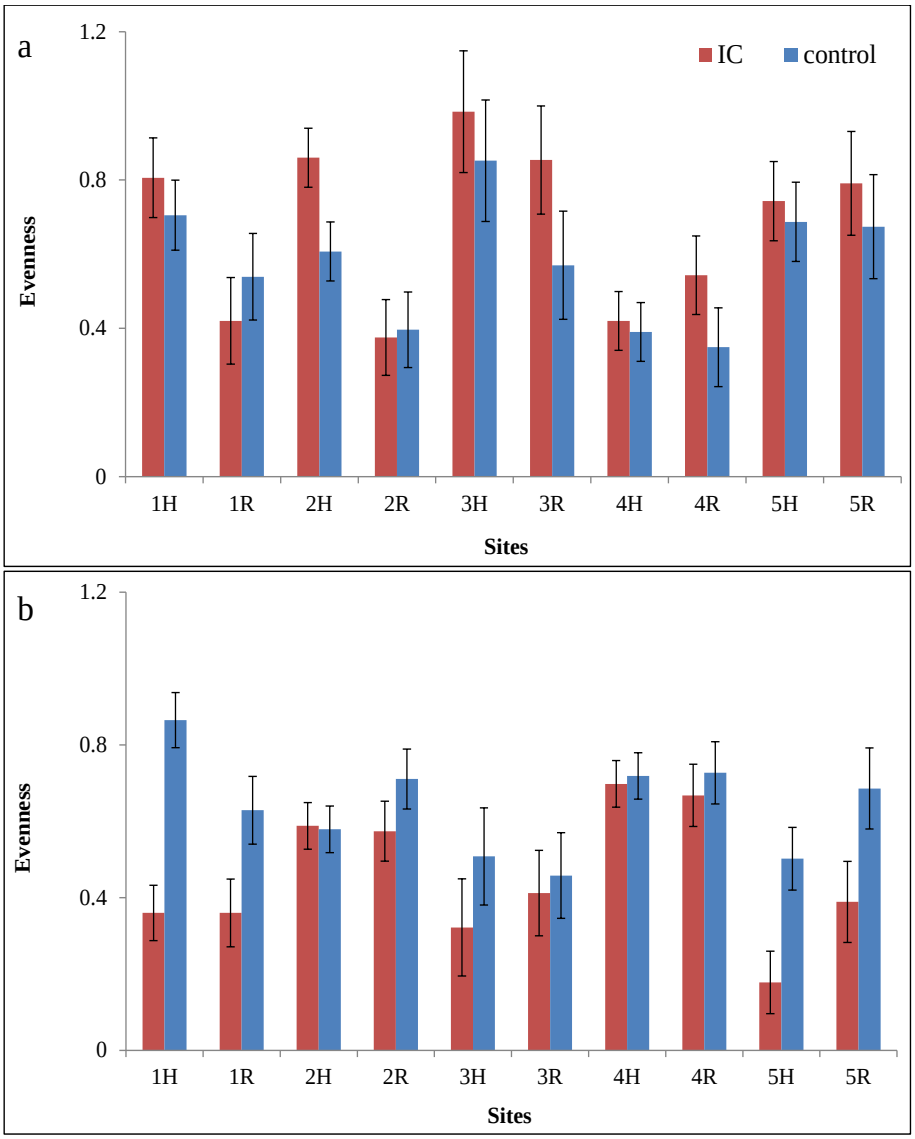
Chapter 3 Figure A-4 Bacterial evenness in soils amended with pesticide CP in the lab a) 0 day and b) 105d with their respective controls. R represents the sites with a history of past pesticides application in the fields and H represents headland not treated with pesticides (in fields). Error bars represent standard error of the mean (n=3). Legends: CP=chlorpyrifos



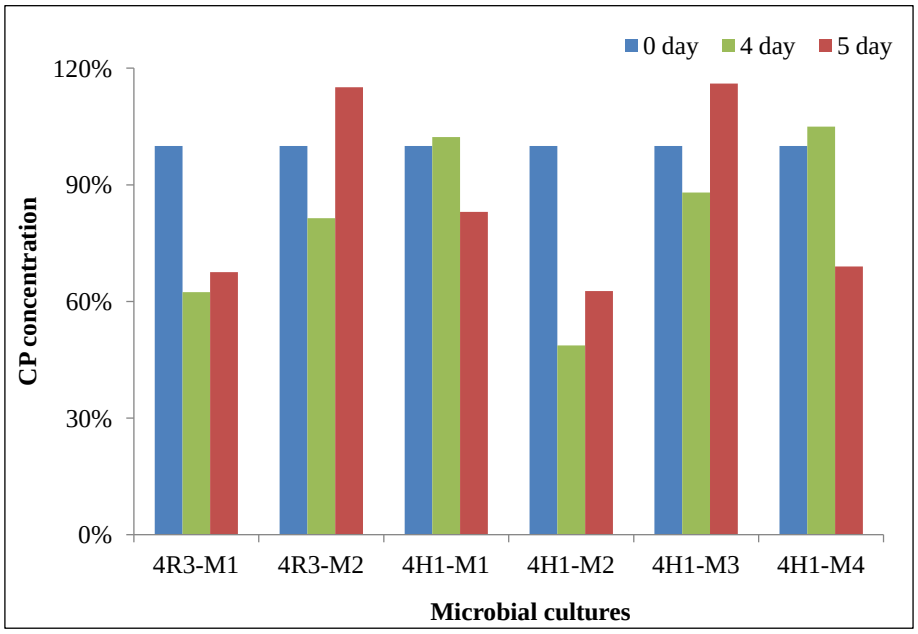
Chapter 3 Figure A-5 Simpson's diversity in soils amended with pesticide IC in the lab a) 0 day and b) 103d with their respective controls. R represents the sites with a history of past pesticides application in the fields and H represents headland not treated with pesticides (in fields). Error bars represent standard error of the mean (n=3). Legends: IC=imidacloprid



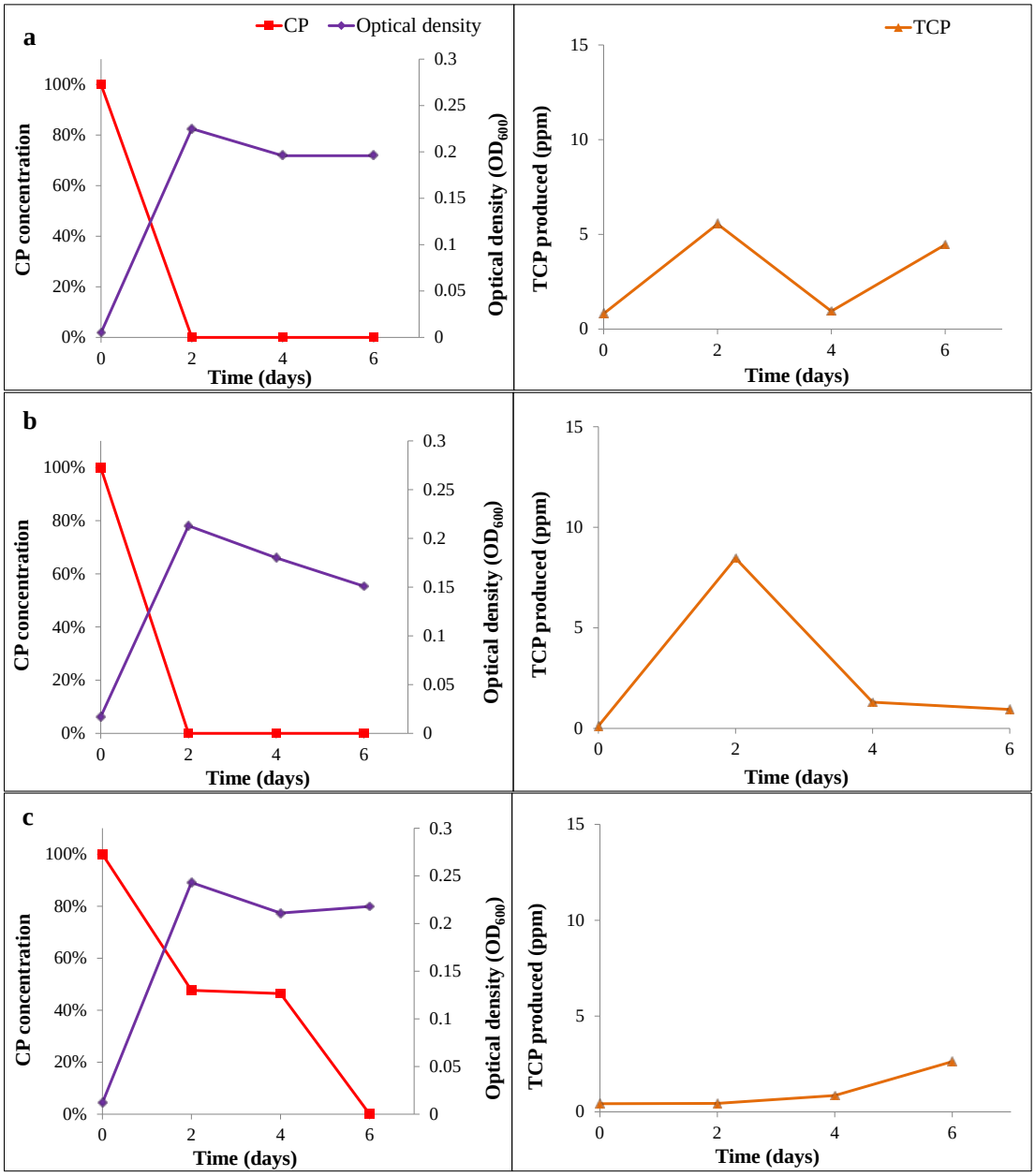
Chapter 3 Figure A-6 Bacterial richness in soils amended with pesticide IC in the lab a) 0 day and b) 103d with their respective controls. R represents the sites with a history of past pesticides application in the fields and H represents headland not treated with pesticides (in fields). Error bars represent standard error of the mean (n=3). Legends: IC=imidacloprid



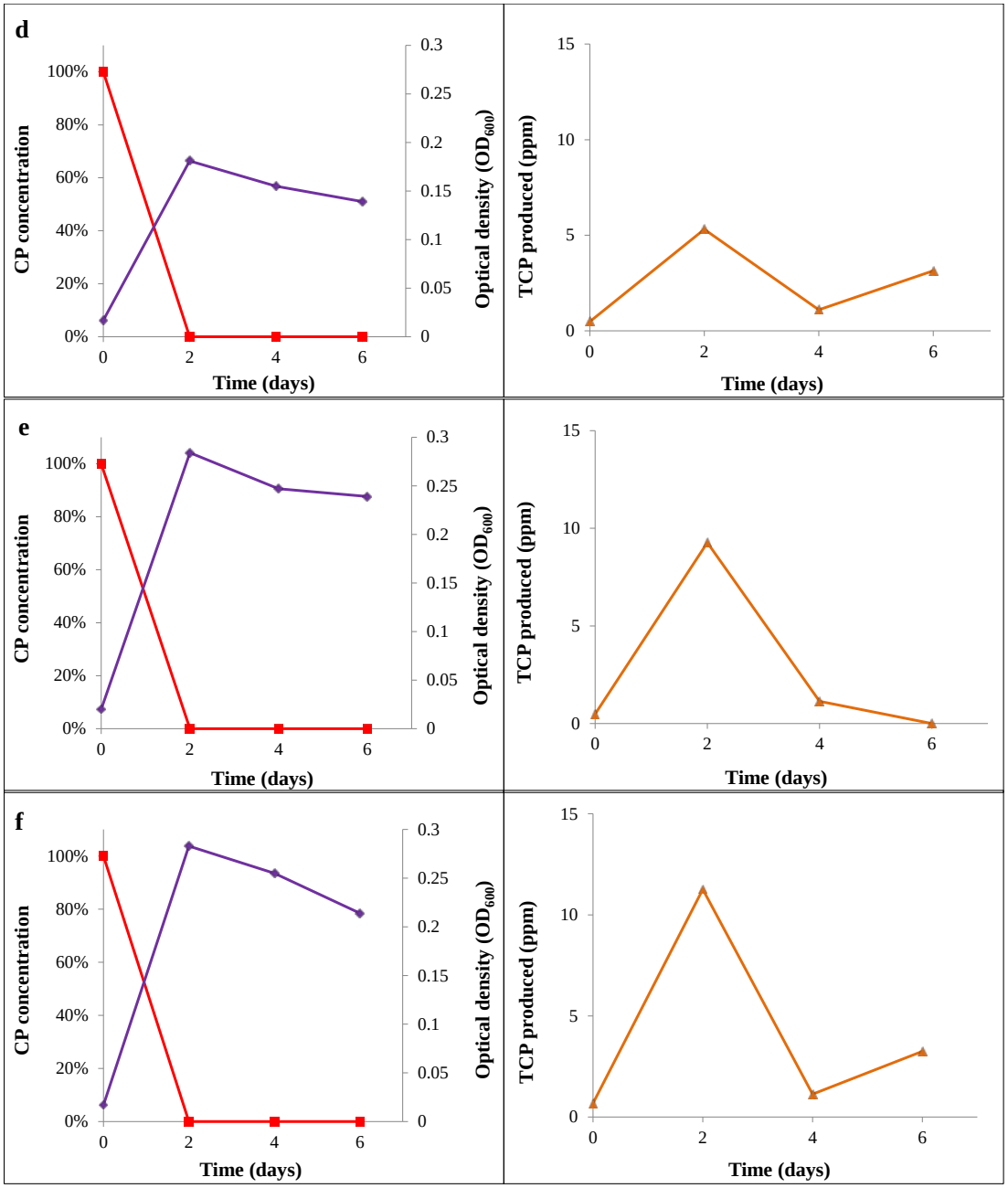
Chapter 3 Figure A-7 Bacterial evenness in soils amended with pesticide IC in the lab a) 0 day and b) 103d with their respective controls. R represents the sites with a history of past pesticides application in the fields and H represents headland not treated with pesticides (in fields). Error bars represent standard error of the mean (n=3). Legends: IC=imidacloprid



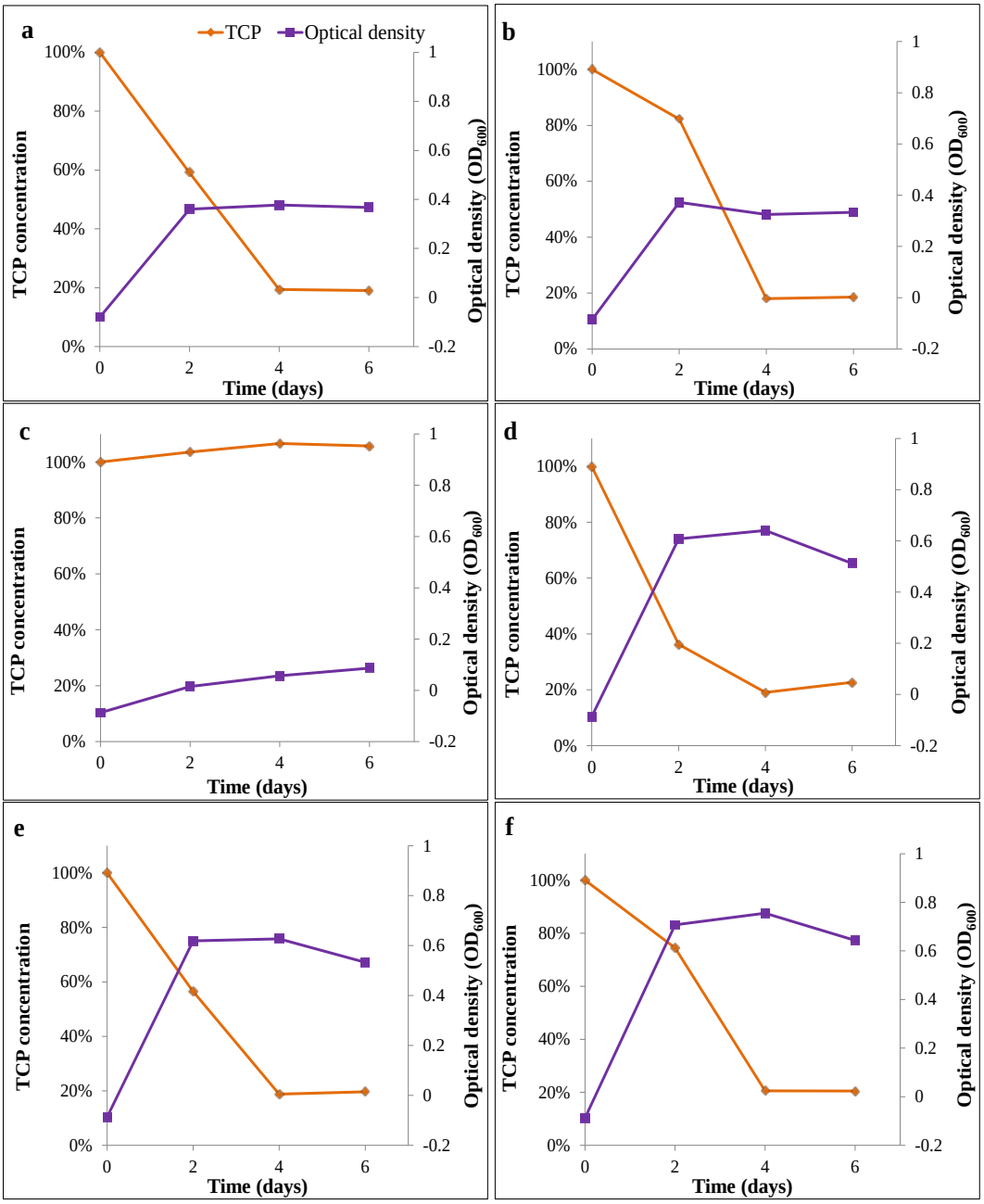
Chapter 4 Figure A-8 Degradation of chlorpyrifos (CP) in MSM+N (without vitamins) when inoculated with six bacterial isolates. After five days of incubation, no degradation was seen in any of the inoculations and in un-inoculated controls (data not shown). Isolates 4R3-M1, 4R3-M2 and 4H1-M4 illustrated a high similarity to members of the genus *Xanthomonas.* 4H1-M3, 4H1-M2 and 4H1-M1 belonged to members of the genus *Pseudomonas*, *Lysobacter* and *Rhizobium*, respectively.


a
CP
Optical density
CP concentration
Optical density (OD600)
Time (days)
TCP
TCP produced (ppm)
Time (days)
b
CP concentration
Optical density (OD600)
Time (days)
TCP produced (ppm)
Time (days)
c
CP concentration
Optical density (OD600)
Time (days)
TCP produced (ppm)
Time (days)

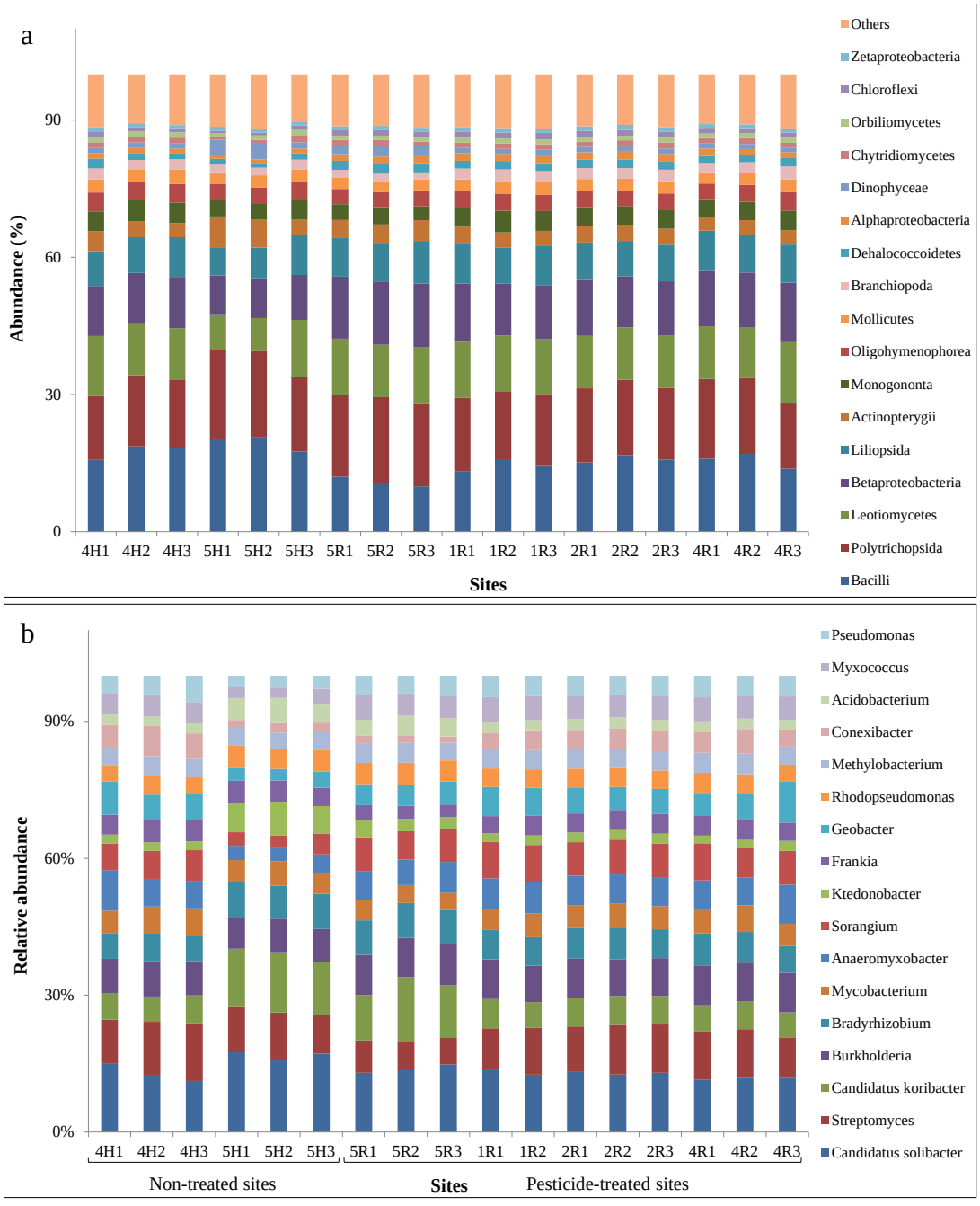
Continued..



Chapter 4 Figure A-9 Bacterial growth, degradation of CP (%) and production of TCP (ppm) in soil extract media (SEM); a) 4R3-M1 (*Xanthomonas* sp.), b) 4R3-M2 (*Xanthomonas* sp.), c) 4H1-M1 (*Rhizobium* sp.), d) 4H1-M2 (*Lysobacter* sp.), e) 4H1-M3 (*Pseudomonas* sp.), and f) 4H1-M4 (*Xanthomonas* sp.). No degradation of CP and TCP production was observed in un-inoculated samples (data not shown). Legends: CP=chlorpyrifos and TCP=3,5,6-trichloro-2-pyridinol



Chapter 4 Figure A-10 Bacterial growth and degradation of TCP in SEM; a) 4R3-M1 (*Xanthomonas* sp.), b) 4R3-M2 (*Xanthomonas* sp.), c) 4H1-M1 (*Rhizobium* sp.), d) 4H1-M2 (*Lysobacter* sp.), e) 4H1-M3 (*Pseudomonas* sp.), and f) 4H1-M4 (*Xanthomonas* sp.). No degradation of TCP was observed in un-inoculated controls (data not shown). Legends: TCP=3,5,6-trichloro-2-pyridinol



Chapter 5 Figure A-11 Relative abundances of sequences across all sites a) Class level, where others represent the classes with <1% abundance and b) top 25% dominant bacterial group at genus level. H represents the non-treated sites and R represents the pesticide-treated sites. Relative abundances are based on the proportional frequencies of the DNA sequences that were classified at the class/genus level. One of the replicate from site 1 (1R3) failed the quality control of MG-RAST at taxonomy level (genus) and was removed.

Chapter 5 Table A-1 Summary of metagenomic data (bp) obtained across sites from each replicate, where H is non-treated sites and R is pesticide-treated sites.

Sample Name	4H1	4H2	4H3	5H1	5H2	5H3
Upload: bp Count	784,365,040	488,929,003	1,182,103,036	552,087,739	2,115,024,565	423,031,308
Upload: Sequences Count	3,405,113	2,128,311	6,524,711	2,233,079	13,466,304	1,820,346
Upload: Mean Sequence Length	230 ± 43 bp	229 ± 42 bp	181 ± 46 bp	247 ± 38 bp	157 ± 24 bp	232 ± 43 bp
Upload: Mean GC percent	63 ± 9 %	63 ± 8 %	63 ± 9 %	61 ± 8 %	61 ± 9 %	61 ± 9 %
Artificial Duplicate Reads: Sequence Count	3,397	994	90,024	2,764	113,225	3,337
Post QC: bp Count	742,496,014	459,900,773	1,085,829,922	518,402,254	1,963,944,299	399,468,284
Post QC: Sequences Count	3,357,636	2,095,947	6,238,415	2,199,615	12,993,485	1,792,924
Post QC: Mean Sequence Length	221 ± 50 bp	219 ± 50 bp	174 ± 52 bp	235 ± 51 bp	151 ± 31 bp	222 ± 50 bp
Post QC: Mean GC percent	63 ± 9 %	63 ± 8 %	63 ± 9 %	61 ± 8 %	61 ± 9 %	61 ± 9 %
Processed: Predicted Protein Features	3,184,241	2,010,166	5,737,824	2,057,643	11,648,094	1,705,129
Processed: Predicted rRNA Features	25,221	16,814	60,955	14,275	111,166	13,483
Alignment: Identified Protein Features	1,235,895	790,282	1,938,380	847,312	3,794,844	673,990
Alignment: Identified rRNA Features	715	488	1,222	466	1,830	432
Annotation: Identified functional Categories	952,405	612,016	1,501,863	656,196	2,902,759	516,076

Continued..

Sample Name	4H1	4H2	4H3	5H1	5H2	5H3
Upload: bp Count	784,365,040	488,929,003	1,182,103,036	552,087,739	2,115,024,565	423,031,308
Upload: Sequences Count	3,405,113	2,128,311	6,524,711	2,233,079	13,466,304	1,820,346
Upload: Mean Sequence Length	230 ± 43 bp	229 ± 42 bp	181 ± 46 bp	247 ± 38 bp	157 ± 24 bp	232 ± 43 bp
Upload: Mean GC percent	63 ± 9 %	63 ± 8 %	63 ± 9 %	61 ± 8 %	61 ± 9 %	61 ± 9 %
Artificial Duplicate Reads: Sequence Count	3,397	994	90,024	2,764	113,225	3,337
Post QC: bp Count	742,496,014	459,900,773	1,085,829,922	518,402,254	1,963,944,299	399,468,284
Post QC: Sequences Count	3,357,636	2,095,947	6,238,415	2,199,615	12,993,485	1,792,924
Post QC: Mean Sequence Length	221 ± 50 bp	219 ± 50 bp	174 ± 52 bp	235 ± 51 bp	151 ± 31 bp	222 ± 50 bp
Post QC: Mean GC percent	63 ± 9 %	63 ± 8 %	63 ± 9 %	61 ± 8 %	61 ± 9 %	61 ± 9 %
Processed: Predicted Protein Features	3,184,241	2,010,166	5,737,824	2,057,643	11,648,094	1,705,129
Processed: Predicted rRNA Features	25,221	16,814	60,955	14,275	111,166	13,483
Alignment: Identified Protein Features	1,235,895	790,282	1,938,380	847,312	3,794,844	673,990
Alignment: Identified rRNA Features	715	488	1,222	466	1,830	432
Annotation: Identified functional Categories	952,405	612,016	1,501,863	656,196	2,902,759	516,076

Continued..

Sample Name	1R1	1R2	1R3	2R1	2R2	2R3
Upload: bp Count	551,722,225	416,295,850	700,158,168	619,531,604	790,158,586	922,747,281
Upload: Sequences Count	2,350,931	1,743,361	2,951,993	2,605,365	3,253,772	3,853,883
Upload: Mean Sequence Length	234 ± 43 bp	238 ± 42 bp	237 ± 42 bp	237 ± 42 bp	242 ± 40 bp	239 ± 42 bp
Upload: Mean GC percent	62 ± 9 %	62 ± 9 %	62 ± 9 %	62 ± 9 %	62 ± 9 %	62 ± 9 %
Artificial Duplicate Reads: Sequence Count	2,908	3,691	2,526	2,506	3,021	3,822
Post QC: bp Count	519,109,689	390,402,344	657,669,970	583,482,725	744,009,960	867,615,159
Post QC: Sequences Count	2,313,565	1,714,557	2,905,315	2,566,028	3,206,595	3,795,241
Post QC: Mean Sequence Length	224 ± 51 bp	227 ± 52 bp	226 ± 51 bp	227 ± 51 bp	232 ± 51 bp	228 ± 51 bp
Post QC: Mean GC percent	62 ± 9 %	62 ± 9 %	62 ± 9 %	62 ± 9 %	62 ± 9 %	62 ± 9 %
Processed: Predicted Protein Features	2,217,986	1,643,489	2,782,451	2,458,394	3,072,861	3,615,555
Processed: Predicted rRNA Features	18,071	13,048	22,654	19,717	24,576	29,140
Alignment: Identified Protein Features	877,144	648,439	1,098,139	978,386	1,250,095	1,433,420
Alignment: Identified rRNA Features	610	432	821	625	885	974
Annotation: Identified functional Categories	681,383	496,864	845,787	757,266	963,516	1,104,912

Chapter 5 Table A-2 Summary of number of features that were annotated by the different databases across sites from each replicate, where H is non-treated sites and R is pesticide-treated sites. These include protein databases, protein databases with functional hierarchy information, and ribosomal RNA databases.

Sample Name	4H1	4H2	4H3	5H1	5H2	5H3	4R1	4R2	4R3
GenBank	1308814	831581	1308814	904997	4127758	714977	1517130	1408229	981952
IMG	1285612	820996	1285612	876331	3989100	692090	1500518	1386520	965773
KEGG	1180285	755905	1180285	810018	3652733	635339	1382521	1277636	890287
PATRIC	1275977	816938	1275977	878269	4028041	687470	1489951	1376903	958813
RefSeq	1327814	848937	1327814	917476	4211500	722065	1548286	1432943	994901
SEED	1130116	721725	1130116	783399	3485704	615298	1325577	1224662	853983
SwissProt	192171	124641	1130116	127645	611020	103315	227504	213808	146464
TrEMBL	1315749	838379	1315749	908020	4138590	716527	1530475	1417603	988609
Eggnog	1056012	671788	1056012	737112	3251446	573542	1228975	1142408	795388
COG	899624	574721	899624	619249	2750473	482732	1051753	976253	681532
KO	460845	298538	460845	308450	1460085	243558	537723	503141	347525
NOG	53240	31930	53240	41355	158119	30792	60517	54496	39357
Subsystems	1572382	1010836	1572382	1060262	4980418	840642	1848709	1717882	1189058
Greengenes	328	192	328	196	1076	167	416	413	246
SILVA LSU	431	309	431	336	1406	304	586	739	528
RDP	355	200	355	216	1182	185	438	431	256
SILVA SSU	447	285	447	305	1611	379	602	747	360

Continued..

Sample Name	5R1	5R2	5R3	1R1	1R2	1R3	2R1	2R2	2R3
GenBank	2118436	2202439	2703729	925788	685220	1148554	1028166	1315531	1508872
IMG	2092458	2181483	2680436	912616	673244	1137534	1014925	1300623	1489901
KEGG	1929073	2014483	2473794	840756	618175	1049231	935977	1195841	1374176
PATRIC	2087407	2169726	2664991	904945	666557	1128728	1006425	1292292	1481821
RefSeq	2167844	2255531	2762670	940718	694600	1172524	1045455	1342173	1537422
SEED	1844070	1921045	2369234	806209	592088	998508	895494	1143826	1310994
SwissProt	321493	336283	427091	139668	100220	169222	151963	191294	221878
TrEMBL	2138988	2223129	2726349	932133	689167	1159340	1035667	1325531	1520540
Eggnog	1737624	1821151	2248403	750949	546038	928102	830934	1059860	1216644
COG	1490497	1562528	1929187	642767	465083	792436	710864	904421	1040006
KO	764617	800181	993413	327966	236778	404364	363794	459262	531932
NOG	84822	90691	109250	36911	27678	46680	41554	53930	60774
Subsystems	2618226	2740144	3429647	1123846	812310	1383000	1246705	1573378	1813186
Greengenes	588	656	753	265	194	378	266	383	435
SILVA LSU	979	932	1103	355	266	557	443	629	663
RDP	616	708	793	291	205	392	293	412	468
SILVA SSU	938	993	1080	369	271	510	418	569	624

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