MOLECULAR APPROACHES FOR CHLORPYRIFOS DEGRADATION

PhD Thesis

In fulfillment of the requirements for the

Doctorate in Philosophy (PhD)

Submitted by

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CERTIFICATE

This is to certify that the present research work entitled " ***Molecular Approaches for Chlorpyrifos Degradation****" being submitted to Thapar University, Patiala, Punjab, in the fulfillment of the requirements for the award of the degree of Doctorate in Philosophy at Thapar University, Patiala, embodies authentic and faithful record of original research carried out by Ms C. Vidyalakshmi, student of PhD (Biotechnology) at Department of Biotechnology and Environmental Sciences, Thapar University, Patiala. She has worked under our supervision and the matter embodied in this thesis work has not been submitted, in part or full, for the award of any other degree or diploma of Thapar University or any other university.*

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I hereby declare that the thesis entitled **"Molecular Approaches for Chlorpyrifos Degradation"** which is being submitted to the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, in partial fulfillment of the requirement for the degree of **Doctor of Philosophy**, has previously not formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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ACKNOWLEDGEMENT

God helps those who help themselves by being there with us and blessing us at all times, provided we dare to dream and dare to achieve those dreams. If we recognize and utilize the gifts God gives us, stay focused on our goals, keep positive thoughts, start the day with prayers praising his glory, he who moves the world, will definitely move the world for us. First of all I thank the God almighty for giving me a breakthrough whenever I broke down and blessing me with what ever I have ever achieved.

Next I thank someone I revere as much, my supervisor, guide, a godfather sent for me by the angels above, Prof Sunil Khanna, who molded me from an amateur learner to an independent researcher. It was his constant encouragement, support and advices that made me achieve the objectives of this thesis work. It is with his blessings which made me succeed through it all. No words can ever suffice his role in my accomplishments.

When you feel the whole world slipping beneath your feet, a hand that holds you through and lifts you up cannot be counted anything less than god's blessing. I will always be indebted to the advices and support rendered by my supervisor, Dr N Tejo Prakash, under various tough circumstances during the tenure of my PhD.

I wish to express my most sincere gratitude to The governing body of Thapar University, Director Dr Abhijit Mukherjee, former Directors Prof M P Kapoor & Dr S C Saxena, Deputy Director Dr K K Raina and Dean RSP Dr Susheel Mittal, DOSA Dr N.K Verma and DOAA Dr R.K.Sharma for allowing me to proceed with the PhD thesis work in the premises of the university.

I am also thankful to The Registrar Col. Jagdev Singh, Deputy Registrar (F&A) Mr D.K. Aggarwal & the finance section, Assistant registrar (Academics) Mr Gurbinder Singh & the academic section, Mr Manmeet, Mr Malhotra, Mr Ashwani, Mr Hariish, Mr Handa and Mr Yadav for helping with the smooth proceeding of the administrative needs associated with the work.

I am also thankful to Ms Rama Verma, Ms Shalini Batra, Ms Gurvinder Kaur and Ms Shweta Goel for those memorable days of stay in the hostel. I am also thankful to Sh B.P Chauhan, Head Central Library and all the other members of the library and Dr Rajesh Kumar and the computer center for allowing me to use the facilities.

I am really grateful to The Head, all the faculty members and all the staff members of the Department of Biotechnology and Environmental Sciences, Thapar University for enlightening me with their knowledge and inputs. I am also grateful to Dr Ranjana Prakash, School of Chemistry and Biochemistry, for her amicable endeavors, advices and support. A special thanks to Anita ma'm for her well-wishes at all points of time. I also wish to thank all the members of the tissue culture unit at TIFAC-CORE for the help rendered. My heartfelt thanks to Mr Anil Kumar for helping me learn the use of randomized block design. I am also thankful to Mr Ashok Kumar, Mr Jai Prakash and Mr Ram Neval for all the assessorial help.

I am grateful to the Department of Biotechnology (DBT) for the financial grant and to the scientists at PAU, Ludhiana for giving me the protocol for analysis of pesticide residues in plants.

The support of friends in every walk of life also acts as an essential component for success. A trustable friend all through the way, I'm thankful to Mohit, who has always helped me with his valuable advices, patient listening and guidance through all obstacles. I am also thankful to my friends Dr Smitha, Mr Anoop, Mr Machiavelli, Mr Saurabh, Ms Henna, Mr Sunny and Mr Atul for standing by me through the testing times. I am also thankful to my colleagues Ms Bella and Mr Anshu for support during the experimental work.

For any successful accomplishment, the support, faith and blessings of near and dear ones is a must. I am totally indebted to my parents, husband, brother and all other family members for their love, patience, understanding and endurance during the whole tenure of the work.

Dedicated to

My "Parents" for their continuous love, support and faith

My Brother "Vishak" for his constant encouragement and belief

My Husband "Prakash" for his unconditional love and trust

My Son "Pranav" for making my life more beautiful than ever

ABBREVIATIONS

%	percentage
≥	equal to or greater than
° C	degree Celsius
° F	degree Fahrenheit
μg	microgram
μl	microliter
μm	micrometer
2, 4-D	2, 4-dichlorophenoxyacetate
Amp	Amplifier/ Amplification
Amu	Atomic Mass Unit
BHA	Bushnell Hass Agar
BHB	Bushnell Hass Broth
BHC	Benzene hexachloride
Bp	Base Pair
$CaCl_2$	Calcium Chloride
cm	Centimetre
CO_2	Carbon-di-oxide
Cpp	Chlorpyrifos
DCM	Dichloromethane
DDT	Dichloro-diphenyl trichloroethane
DNA	Deoxyribonucleic acid
DNase	DNA nuclease
DNSA	Dinitrosalicilic acid
dNTP	Deoxyribonucleotide triphosphate
ECD	Electron Capture Detector
et al	*et alia*
Etc	Etcetera
g	Gravitational constant
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
gel-doc	Gel documentation
gm	Gram
Ha	Hectare
Hr/ hrs	Hour/ Hours
IPTG	Isopropyl-beta-D-thiogalactopyranoside
Kan	Kanamycin
Kb	Kilobase
Kg/kg	Kilogram
L	Litre
LA	Luria Agar
LB	Luria Broth
Log	Logarithm
m	Meter
mg	Milligram
$MgCl_2$	Magnesium Chloride
min	Minutes
ml	Millilitre
mm	Millimeter

mMol	Millimolar
MPD	Methyl-parathion Degrading
Nal acid	Nalidixic acid
ng	Nanogram
nm	Nanometer
Nmol	Nanomolar
O.D	Optimum Density
OPD	Organophosphate Degrading
P_2O_5	Phosphorus Pentoxide
PCB	Polychlorinated biphenyls
PCR	Polymerase Chain Reaction
pH	"potenz"/ potential + Hydrogen
rDNA	Ribosomal DNA
REP	Repetitive Extragenic Palindrome
RNase	RNA nuclease
ROSE	Rapid One Step Extraction
rpm	Revolutions per minute
TAE	Tris Acetate EDTA
Taq	*Thermus aquaticus*
TCE	Trichloroethane
TCP	3, 5, 6-trichloro-2-pyridinol
TE	Tris EDTA
TMP	3, 5, 6-trichloro-2-methoxypyridine
UV	Ultraviolet
UV-Vis	Ultraviolet and Visible light
V	Volt
v/v	volume by volume
Vs	Versus
w/v	weight by volume
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

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1. INTRODUCTION

Ubiquitous in nature, the world of microorganisms fascinates everyone. Man has made many inventions in every century to harvest the potential of these tiny inhabitants of nature to his best advantage. Starting from manure, followed by pesticides of yesterday to bio-pesticides of today, man has come a long way in developing nutritive and protective strategies in agricultural practices in every way possible. In this process, he has developed many scientific methods to increase the crop yield, manifold. Synthetic fertilizers are added to the soil to improve soil fertility and increase crop yield. Similarly the yield is being increased by protecting agricultural crops and their products from a large number of plant and animal organisms, which damage crops both in the fields and during storage. Chemical means of control are faster and more effective than organic means. However, excessive use of synthetic chemicals especially pesticides has severely contaminated the environment.

Among various inventions facilitating modern agriculture, pesticide remains the most widely used resource for yesterday, today and for the future. Pesticides are chemical compounds, which kill or repel harmful plant and animal organisms, the pests. Originally pesticides were used for crop protection but now they are also used for controlling vectors carrying disease-causing organisms and to protect the domestic animals and humans from diseases. The importance of pesticides was realized at the end of Second World War, when two synthetic chemicals, DDT and 2, 4-D, were used on a large scale.

1.1 Types of Pesticides:

Pesticides may be contact or systemic, specific or non-specific. They can be classified based on the nature of organisms they control or based on their chemical structure.

Based on their *chemical structure*, the pesticides are classified as

1.1.1 Organochlorines

These are basically the organic compounds that have been chlorinated with several atoms of chlorine per molecule. The common examples are DDT (Dichloro-diphenyl trichloroethane), BHC (Benzene hexachloride), aldrin and endosulfan. All these compounds are lipophilic in nature and show much affinity for the fatty tissues of animals. They are also not biodegradable and thus remain as such in the fields. They enter into the body of the animals and man through the food chain, and are quite harmful to them. Their accumulation in the environment and also their affinity towards the animals pose serious problems to the living beings. Due to their excessive use and toxic effects, some organochlorines have been banned and the attention has shifted to the increased use of organophosphates.

1.1.2 Organophosphates

The term “organophosphates” is used generically to include organic compounds that contain a phosphorus atom. These are the organic esters of phosphoric, thiophosphoric and other phosphoric acids. The insecticidal properties of these chemicals were discovered at about the same time as that of DDT. These compounds are widely used as insecticides and to control agricultural pests and disease vectors. Overall, organophosphates account for ~ 38% of total pesticides used globally. Organophosphates show a strong effect on the nervous system. Studies have revealed that organophosphorus pesticides are more toxic to living systems, than organochlorines (Khanna, 1995) as these are more water-soluble, so the amount reaching the aquatic environment is high. Some of the major ones used in India are chlorpyrifos, malathion, parathion, fenitrothion etc.

1.1.3 Carbamates

They are the organic esters of the hypothetical carbanic acid. In their structure, they are quite similar to acetylcholine and, therefore, they have a strong affinity for the enzyme acetylcholine esterase. Some of the carbamates are: carbaryl, carbofuran, aldicarb, propoxur.

1.1.4 Pyrethroids

Pyrethroids insecticides have their origins in the naturally occurring insecticides in pyrethrum extracts obtained from the flowers of chrysanthemum species (*Chrysanthemum cinerariaefolium* and the name pyrethrin is applied to the crude naturally occurring pyrethroid mixture. Pyrethrum extract is a mixture of three closely related insecticidal esters of chrysanthemic acid and the three corresponding esters of pyrethric acid formed by the alcohols cinerolone or pyrethrolone. The compounds currently in use owe their origin to the ingenuity of synthesis chemists whose structural variations on the theme of naturally occurring pyrethrins led to extremely effective insecticides, which are widely used in crop protection.

1.1.5 Triazines

The triazine herbicides are derivatives of *s*-triazine manufactured by the reaction of cyanuric chloride with appropriate amines. They are a group of herbicides derived from urea. The triazines are inhibitors of photosynthesis. They are mostly used for controlling weeds in tea, tobacco, and cotton fields. Some of these are simazine, atrazine etc.

Based on the *nature of organisms* they control, the pesticides are classified as

1. **Fungicides**-that kill the fungi,
2. **Herbicides or weedicides**-that kill the weeds,
3. **Insecticides**-that kill the insects,

4. **Nematicides**- that kill the nematodes,
5. **Rodenticides**- that kill the rodents.

1.1.6 Fungicides

Sulfur maintains its position as the leading fungicide in terms of tonnage. Considerable amounts of copper fungicides are still in use. Ethylenebisdithiocarbamates are used in large amounts as organometallic complexes. The triazole fungicides have come into prominence and demonstrate systemic activity. They act by inhibiting cell membrane ergosterol biosynthesis, thus stopping development of the fungus.

1.1.7 Natural Products

Natural products have provided novel structural leads for many classes of pesticide chemicals. Plant extracts and products of microbial metabolism offer promising sources of activity. Several new materials with complex structures have been derived from the products of fermentation like strobilurins, spinosads, and the avermectins. Abamectin, an insecticide and acaricide, is a mixture of avermectins, a group of related compounds produced by fermentation of *Streptomyces avermitilis.*

An Indian tree, Azadirachta *indica* A. Juss. (syn.*Melia azadirachta* L.), is being utilized as a valuable insecticidal source. Extracts derived from the neem tree and the extracts of the seeds possess a wide spectrum of biological activity, including pesticidal activities. Generally the biological activity of neem oils is highly correlated with their content of azadirachtin, the major insecticidal and active component of neem extracts.

1.2 Mode of Action

Every pesticide has a characteristic mode of action, usually disruption of a vital body process, by which it kills the pest. Most pesticides attack the nervous system of the body. For example, organophosphates and carbamates are known as "cholinesterase-inhibiting pesticides", because they kill by interfering with the enzyme “cholinesterase” vital for nerve transmission and so interfering with the conduction of nerve impulses in the nervous system. Some pesticides inhibit energy production. A few examples are hydramethylnon, sulfluramid, and sulfluryl fluoride. Some herbicides affect Photosystem II in the process of photosynthesis.

1.3 Distribution of Pesticide Use in India

India is now among the largest pesticide consumers in the world. The total pesticide consumption estimated in 2000-2001, in India alone was 63,348 metric tones. Out of this, the consumption of organochlorines was 40% and the consumption of organophosphorus pesticides was 30%. Annual consumption of pesticides in India increased in the early ‘90s with an average of 0.5kg/ha. Now with all the precautious measures and use of bio-organics,

consumption of pesticides has decreased to 0.44 kg/ha, which still is very significant (http://agri.mah.nic.in/agri/extension/html/Chap8.html). Benzene Hexa Chloride (BHC), γ-isomer (Lindane), is only in use today and represents 50% of the total volume of pesticides consumed every year. It is used mainly in public health programs. Use of DDT, Heptachlor, Aldrin and many other organochlorines is banned now, in many countries.

Among the organophosphates, Chlorpyrifos is the maximum consumed pesticide in India, with maximum use (54 percent) being on cotton (http://agri.mah.nic.in/ agri/extension/html/Chap8.html).

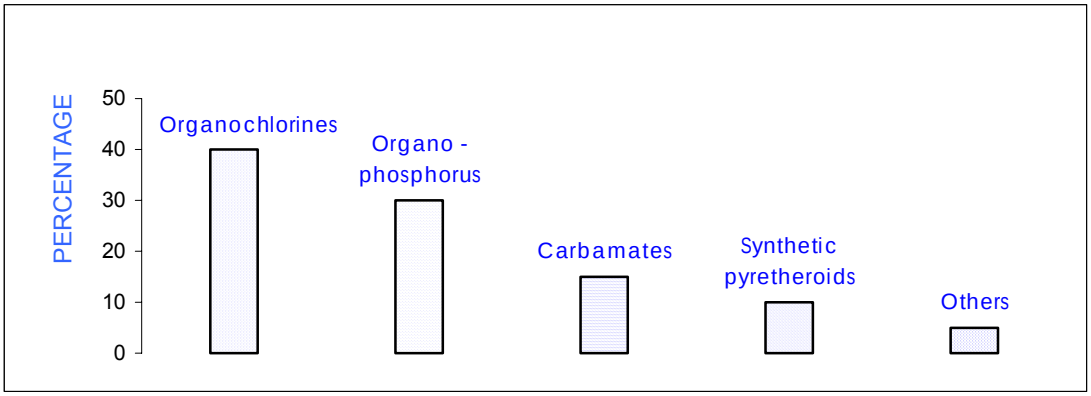


Fig1: Distribution of pesticide use in India

1.4 Benefits of Pesticides

The greatest benefit of pesticides even today is to kill crop pests and thus, increase the production of food and other crops to feed the increasing world population. The green revolution in India was dependent on the production of high yielding varieties of crops devoid of pest attack, which was only possible with the use of pesticides. Pesticides are also increasingly used to kill the insects that act as carriers or vectors of many human diseases. Some of the common diseases that are thus controlled by the killing of their vectors are malaria, filaria, sleeping sickness and dengue fever.

1.5 Problems Posed by Pesticides

Pesticides are potent poisons which pose a threat to wildlife, may affect non-target organisms which come in contact and may cause mutations in target species that are exposed but not killed leading to development of pesticide resistance. Exterminating the natural predator population may lead to emergence of new pests previously kept under control and thus, an imbalance in the ecosystem may occur. Pesticides are generally less biodegradable and do not stay where they are used, leaving residues and breakdown products in the soil, air, surface and ground water, bottom sediment, food and non-target organisms. Cumulative effects of pesticide exposure are much varied and may be

disastrous. Every year 4,000 to 20,000 people die from pesticide poisoning, the bulk of them being farmers. Around one million poisoning cases are reported annually.

1.6 Pesticide Degradation

Pesticide degradation occurs via physical, chemical as well as biological pathways. Some pesticides are solely degraded by one of these methods while a combination of these methods applies for others. There are many of the possible fate processes for a pesticide. These processes can be grouped into those that affect persistence, including photo degradation, chemical degradation and microbial degradation, and those that affect mobility, including sorption, plant uptake, volatilization, wind erosion, runoff, and leaching. Pesticide persistence and mobility are influenced by the properties of the pesticide. The soil environment, site conditions, weather, and application method influence the properties of a pesticide. Some of the most important properties of a pesticide that can be used to predict environmental fate include half-life, soil sorption coefficient, water solubility, and vapor pressure.

1.6.1 Pesticide Persistence

Pesticide persistence is usually expressed in terms of "half-life". This is the typical length of time needed for one-half of the total amount applied to degrade. Soil half-lives can be grouped into three major categories: (a) "non persistent" pesticides have a typical soil half-life of less than 30 days; Examples include Aldicarb, Malathion, 2, 4-D, Captan, Methyl Parathion, Dalapon, Oxamyl. (b)"moderately persistent" pesticides have a soil half-life of 30 to 100 days; examples include Aldrin, Atrazine, Carbaryl, Carbofuran, Diazinon, Glyphosate, Fonofos,Parathion, Heptachlor, and (c) "persistent" pesticides have a half-life of more than 100 days; examples include DDT, Lindane, Endosulfan, Bromacil, Chlordane, Paraquat. The rate of degradation in natural environments is highly dependent on pesticide chemistry as well as environmental factors. Solar insulations, temperature, soil and water pH, microbial activity, and other soil characteristics can significantly affect the breakdown of pesticides.

1.6.2 Solubility and Mobility of pesticides in soil

Pesticide movement in soils depends on many factors, including some, which may be influenced by the particular chemical properties of the pesticide. Some pesticides are much more soluble in water than others; and highly soluble pesticides tend to be removed from the soil in runoff water or by leaching. Common pesticides like DDT and BHC have a long life due to their low solubility in water and their relatively high solubility in fats. Because of this low water solubility, these pesticides do not contaminate ground water and hence are usually not present in drinking water. On the other hand pesticides like carbofuran enters surface water as a result of runoff from treated fields and enters ground water by leaching of

treated crops. Leaching studies have shown pesticides like Aldicarb, Chloramben, carbofuran, Fenuron, 2,4-D, Terbacil are highly mobile/ leachable in various soils, whereas, Carbaryl, Monolinuron, Prometone, Ametryn, Diuron, Prometryn, Trietazine, Chlorpropham, Monuron, Simazine, Dichlobenil, Atrazine, Fluometuron, Cynazine, Propazine leach moderately. Pesticides like DDT and Chlorpyrifos with high partition coefficients of 243,000 and 13,490 respectively are tightly bound to soil and do not leach significantly to lower layers.

1.6.3 Physico- Chemical Degradation

Physical or chemical degradation of pesticides can occur when the pesticide is directly exposed to water, oxygen, or other chemicals. When soil pH becomes extremely acidic or alkaline, it may result in increased chemical degradation. When a pesticide's functional groups are attached with weak or labile bonds, it can degrade more rapidly. Sunlight can also break down pesticides (photo degradation). Factors affecting pesticide photo degradation include the intensity of the sunlight, length of exposure, the application site and/or method, and the chemical properties of the pesticide. Some well-known examples of pesticides easily degradable by physico-chemical methods include bromacil which in particulate phase is removed physically from air by wet and dry deposition. Photodegradation of this pesticide may occur in natural waters containing sufficient concentrations of sensitizing agents. Similarly, chemical degradation of pesticide promecarb in moist soil is expected to proceed via hydrolysis. Diazinon, dimethoate, chlortoluron, isoproturon, metoxuron, vinclozolin and diuron are also easily degradable by ozonation. Carbaryl is weakly sorbed and generally considered to be easily degradable in soil. When exposed to UV light, amitrole breaks down to form CO_2, urea and cyanamide.

Volatilization is another route of dissipation of pesticides from soil. This process is highly temperature dependent; thermophilic temperatures typically increase pesticide losses. Examples of pesticides randomly lost by volatilization include 1, 3-dichloropropene, 2, 4-D esters, and thiocarbamates like Eptam and Nortron. Dinitroanilines (e.g. Prowl, Treflan) vapourize from moderate – high levels depending on temperature conditions. Dichlobenil volatilizes (vaporizes) readily so it can contaminate air in areas where it is used. Dicamba salt, benomyl, diuron, and bensulide all are relatively nonvolatile. Adding water may also break many labile bonds (hydrolysis), and this process may or may not be enzyme catalyzed.

Enzymatic Hydrolysis

There are combinatorial methods whereby physico-chemical processes are combined with biological catalysts, the enzymes. Malathion is an example of an insecticide containing many

labile bonds that may be broken using hydrolytic enzymes (for example, esterase and phosphatase). Other pesticides capable of hydrolytic degradation are: carbamate pesticides, urea derivatives, pyrethroids, diazinon, dicamba, dichloropicolinic acid, dimethoate, phenylalkanoic ester, dimethoate, phenylalkanoic pyrazon, atrazine, linuron, propanil, and 2, 4-D. Two other classes of enzymes, mono- and dioxygenases, are also commonly associated with pesticide degradation. These enzymes introduce one or two oxygen atoms, respectively, into the structure of a pesticide. This oxidation process often makes the pesticide more amenable to further degradation by increasing its water solubility, thereby increasing its bioavailability. However, it is well established that for some pesticides microbial degradation is the major and sometimes, the only means of total removal from the environment.

1.6.4 Microbial Degradation/ Bioremediation

Microbial degradation is the breakdown of chemicals by microbial organisms. The role of microorganisms in maintaining steady-state concentrations of environmental chemicals is well established. Microorganisms play an important role in the degradation of xenobiotics particularly the pesticides. Microbial degradation occurs when fungi, bacteria, and other microorganisms in the soil use pesticides or other organic materials as food (carbon) or other energy source, or consume them along with other sources (co-metabolites) of food or energy. Organic matter content, moisture, temperature, aeration, and pH of soil all affect microbial degradation. Microbial activity is high in warm, moist soils with neutral pH. Protocols that use microorganisms to degrade or remove organic materials and sequester inorganic ions are called “Bioremediation”. Bioremediation of soil contaminated with pesticides is one approach wherein the xenobiotic residues in the soil are mitigated to a certain extent. Effective bioremediation, utilizing microbial inoculation, has the following basic and absolutely essential requirements:

1. Oxygen at a residual level of 1 ppm or more
2. Essential inorganic nutrients
3. Microbes and substrate must be in contact
4. Water - either salt or fresh

Under carefully controlled conditions, bioremediation is a practical and cost effective method to remove hydrocarbons and other xenobiotics compounds from contaminated surfaces and sub-surfaces. The primary responsibility of microbes is to recycle organic material, but they must be present in sufficient quantities and diversity in order to accomplish this task. Microorganisms have been seen to degrade certain aromatic hydrocarbons and oxidize

certain halogenated benzenes. Toluene-grown cells when incubated with fluorobenzene, chlorobenzene, bromobenzene, or iodobenzene, have shown to degrade the compounds and accumulate corresponding 3-substituted catechols in the culture medium. Mixed microbial populations have shown chlorination of diphenols to 1, 2, 3- and 1, 2, 4-trichlorobenzenes. A *Pseudomonas* species and an *Achromobacter* species have shown ability to grow with toluene as the sole source of carbon and energy. Xenobiotics such as DDT, PCB and TCE co-metabolise, while, for some compounds like Dichlorvos, chlorpyrifos, metalochlor microbial degradation is the major route of dissipation from soil.

2. LITERATURE REVIEW

2.1 Microbial mechanism of degradation of organophosphate pesticides

The mechanism of degradation of organophosphates by microorganisms may be hydrolytic, reductive and sometimes, oxidative (Lal, R., 1982). P=O and P=S containing organophosphorus insecticides are degraded by hydrolytic processes in the microbes. For e.g. phorate is metabolized in *Pseudomonas sp. (isolate P)* and *Thiobacillus thioxidans* by hydrolytic pathways (Ahmed & Casida, 1958). Microorganisms also hydrolyze parathion to p-nitrophenol (Sethunathan & Yoshida, 1972, 1973). The reduction of the nitro group to amine has been reported during metabolism of organophosphates as in the conversion of parathion into aminoparathion in microorganisms (Cook, 1957). *Bacillus sp.1* inactivates both parathion and parathion-methyl through reduction of the nitro group to an amino group (Yasuno *et al*, 1965).

The oxidative reactions are less common in microbes maybe because of lack of a defined mixed-function oxidase system in them (Matsumura, 1975). Oxidation reactions involving the conversion of parathion to paraoxon and the opening of the aromatic ring of p-nitrophenol have been reported in microorganisms (Munnecke & Hsieh, 1974; Sudhakar *et al*, 1976; Sethunathan *et al*, 1977).

It is assumed that the decomposition of organophosphorus pesticides occurs by the secretion of microbial enzymes, specifically phosphatases, into the soil (Skujins, 1967; Kaufman, 1970). These pesticides have many structural similarities with naturally occurring compounds and so, the microbe's inherent enzymes may act upon these pesticides; these enzymes may be constitutive (Kaufman and Kearney, 1965) or may require induction (Alexander, 1965). It has been seen by Heuer *et al* (1976) that the enzymes applied on sterile soil does not act on the absorbed insecticides on soil surfaces even after multiple applications. A major organophosphorus insecticide and acaricide degraded by microbial action and in use on a wide variety of farmlands and households around the world is chlorpyrifos.

2.2 CHLORPYRIFOS

Chlorpyrifos is a versatile broad-spectrum organophosphate insecticide, extensively used all over the world to control a wide range of insects and other arthropod pests on crops, soil, livestock and for vector control in pastures, operations in construction of buildings and in other domestic uses in agricultural (e.g. corn and cotton), and urban (e.g. turf grass, indoor) scenarios. It can be used on a wide range of crops, including pome fruit, stone figs, citrus fruits, nut crops, strawberries, figs, bananas, vines; vegetables like potatoes, beet, tobacco,

soybeans and on sunflowers, sweet potatoes, groundnuts, rice, cotton, cereal maize, sorghum, asparagus, glasshouse and outdoor ornamentals, mushrooms and in forestry. From studied consumption patterns, it has been seen that chlorpyrifos is used on corn, 39%; alfalfa, 6%; cotton, 3%; sorghum, 1%; citrus and deciduous fruits/nuts, 21% and non-agricultural uses, 31% (Spectrum Laboratories, 2001)

Chlorpyrifos is used for soil treatments to control soil pests such as wireworms, white grubs, mole crickets, Maulwurf's Grill, garden symphylans, seed corn maggots, cereal maggots, beet root weevils, the Atomaria, Blaniulus, Tipula and cutworms in Europe, in Near East and South Africa for maize, spring cereals, beans, onions, tobacco, turnips, bulb flowers and carrots. It is also used for foliar treatments to control insect pests of rape, cereals, potatoes, fodder crops, beans, peas and deciduous fruits such as apples and pears in these places. In Canada, this pesticide is used to control soil insect pests of lettuce, onions and carrots and Tobacco plants. In Japan, Thailand, Philippines, India and Malaysia, chlorpyrifos usage is mainly for controlling insect pests of apples, pears, rice, tobacco, cotton, Chinese cabbage and kale as foliar treatments. High concentrations of chlorpyrifos with short exposures are more effective against insect larvae. Wettable-powder formulations are more effective than emulsifiable formulations in general as disappearance of chlorpyrifos was faster when applied as an emulsifiable concentrate (Davis and Kuhr, 1976) formulated as 20% and 48%. In high organic matter the efficacy of chlorpyrifos is less.

Chlorpyrifos has low water solubility in water and is highly soluble in organic solvents like acetone, benzene, chloroform, methanol and iso-octane. It has been found to be highly flammable. Chlorpyrifos burns and decomposes into hydrogen chloride, ethyl sulfide, diethyl sulfide, and nitrogen oxides.

2.3 Toxicological effects of chlorpyrifos

Chlorpyrifos is a triple action insecticide i.e. chlorpyrifos is effective by contact, ingestion and vapor action and it is not systemic (Spectrum Laboratories, 2001). Exposure to chlorpyrifos has reportedly caused death by respiratory failure or cardiac arrest or disability in humans (Environmental Research Laboratory, 1984). Lesser exposures may cause headache, dizziness, extreme weakness, unsteady movements, tiny pupils, twitching, tremor, nausea, slow heartbeat, anemia, bronchitis, fluid in the lungs, and sweating (EXTOXNET, 1993). All chlorpyrifos formulations may be fatal if swallowed (Davis & Kuhr, 1976). Excessive exposure to the solvent-based product may cause eye and upper respiratory irritation, central nervous system depression, increased sensitivity to epinephrine and irregular heartbeats. Chlorpyrifos is a cholinesterase inhibitor affecting this enzyme in brain or blood, although differing in its response within different animal species (Smith, 1966) but the

process may be injurious to brain (Ecobichon, 1994). Chlorpyrifos is one of the leading causes of acute insecticide poisoning in the US (US EPA, 1995).

TABLE1: Physico-chemical properties of chlorpyrifos

Generic Name	Chlorpyrifos
Chemical Name	O, O-diethyl O (3, 5, 6-trichloro-2-pyridyl) phosphorothioate
Trade Names	DURSBAN, PHOSPHORODITHIOIC ACID, BRODAN, COROBAN, DANUSBAN, PIRIDANE
Molecular Formula	$C_9H_{11}Cl_3NO_3PS$
Molecular weight	350.59
Density at 43.5°C	1.398 gm/cc
Melting Point	41-42°C
Boiling Point	160°C
Soil sorption coefficient	8498 ml/gm
Physical existence	Colorless/ white granular crystal
Odor	Mild mercaptan
Intermediate vapor pressure at 25°C	2.7 X 10^{-3} Pa
Water solubility	1.4 mg/ L

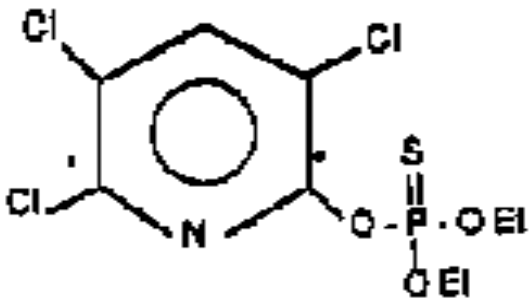


Fig2: Chemical Structure of Chlorpyrifos

2.3.1 Acute Toxicity

Chlorpyrifos is moderately toxic to humans (US EPA 1989; WHO, 1996-1997). Poisoning from chlorpyrifos may affect the central nervous system, the cardiovascular system, and the respiratory system (OHS, 1986). It is also a skin and eye irritant (OHS, 1991). Inhalation of chlorpyrifos may cause absorption of the insecticide through the mucous membranes, resulting in systemic intoxication (OHS, 1986). Acute oral dose of 300mg/kg in man has been seen to affect the peripheral nerve and cause pareshtesia, resulting in muscle weakness and later coma (Davis & Kuhr, 1976). The oral LD50 for chlorpyrifos in rats is 82 to 270 mg/kg, in mice is 60 mg/kg, 1000 mg/kg in rabbits, 32 mg/kg in chickens, 500 to 504 mg/kg in guinea pigs, and 800 mg/kg in sheep (Hartley and Kidd, 1983; Gosselin *et al* 1984; Berg, 1986 and OHS, 1991). The dermal LD50 in rats is greater than 2000 mg/kg (The Dow Chemical Company. 1986), and 1000 to 2000 mg/kg in rabbits (Hartley and Kidd, 1983; Berg, G. L., ed. 1986). Toxicological studies with chlorpyrifos indicated acute toxicity of the metabolite 3, 5, 6-trichloro-pyridinol in rats and mice (Gerbig and Emerson, 1970 a, b).

2.3.2 Chronic Toxicity

Effects reported in workers repeatedly exposed to chlorpyrifos include impaired memory and concentration, disorientation, severe depressions, irritability, confusion, headache, speech difficulties, delayed reaction times, nightmares, sleepwalking and drowsiness or insomnia. Influenza-like condition with headache, nausea, weakness, loss of appetite, and malaise has also been reported (OHS, 1991). In human volunteers, pest control operators and spray workers, chlorpyrifos showed inhibition of plasma/ RBC cholinesterase (TOXNET, 1975-1986; ACGIH, 1986). Chronic, low-level exposure to organophosphates adversely affects children's developing nervous systems, resulting in lower cognitive function, behavior disorders, and other subtle neurological deficits and is known to disrupt the autonomic nervous system leading to the hypothesis that organophosphates are related to respiratory disease in children and therefore may be among the preventable causes of childhood asthma (Eskenazi *et al*, 1999). When technical chlorpyrifos was fed to dogs at doses 2 years, increased liver weight occurred with signs of cholinesterase inhibition. Rats and mice showed no adverse effects other than cholinesterase inhibition (USEPA, 1989).

2.3.3 Reproductive Effects

EPA has determined that chlorpyrifos does not adversely affect reproduction (USEPA 1984, 1989). No effects on reproduction occurred in a 3-generation study with rats or study on other animals fed dietary doses as high as 1 mg/kg/day (ACGIH, 1986; USEPA 1984, 1989) though there was a slight increase in the number of deaths of newborn offsprings (Hayes, 1982) as once in the bloodstream, chlorpyrifos may cross the placenta (OHS, 1991). There

may also be deformed heads, faces, eyes and genitals in such exposed children (Sherman, 1995).

2.3.4 Teratogenic Effects

No major teratogenic or other adverse effects to offspring were found when pregnant rats were fed with chlorpyrifos though minor skeletal variations and a decrease in fetal length occurred at the highest dose tested (EXTONET, 1993).

2.3.5 Mutagenic Effects

EPA has determined that chlorpyrifos is not mutagenic (USEPA, 1989), but mutagenic effects were observed in fruit flies given oral concentrations of 50 parts per billion (ppb) of chlorpyrifos for 3 days (NIOSH, 1981-1986). Also, it has been reported that at a dose of 2mg/kg, it has been found to be mutagenic (affects lymphocytes in mouse, and human sister chromatid exchange). A dose of 500μg/kg affects normal cells (Davis & Kuhr, 1976).

2.3.6 Carcinogenic Effects

EPA has determined that chlorpyrifos is not carcinogenic. There was no increase in the incidence of tumors when rats were fed or when mice were fed with chlorpyrifos (USEPA, 1989). However, another research has identified immune system abnormalities in individuals following chlorpyrifos exposure. Higher than usual frequencies of allergies and sensitivities to antibiotics together with atypical abundances of certain types of lymphocytes (decreases in T cells and increases in CD26 cells) were found in patients one to five years after chlorpyrifos exposure leading to autoimmunity (Thrasher and Madison, 1994).

2.4 Fate in Humans and Animals

As with other phosphorothioate esters chlorpyrifos is rapidly absorbed, metabolized and excreted by mammals following oral administration and so is the case with its major metabolite 3, 5, 6-trichloro-2-pyridinol. In humans, chlorpyrifos and its principal metabolites are eliminated relatively rapidly following a single dose (Nolan *et al*, 1984) and it does not have a significant bioaccumulation potential (NYSDEC, 1986). Chlorpyrifos may bio-concentrate (BCF = 2.50 to 3.54) at very low levels in other ecological systems (Howard, 1989). It is readily absorbed into the bloodstream through the gastrointestinal tract if it is ingested, through the lungs if it is inhaled, or through the skin if there is dermal exposure (USEPA, 1984). Chlorpyrifos was found in its original form in the blood, brain and liver of a 61-year old man who lived only one day after accidentally eating this material (Hayes, 1982). Analyses have showed that chlorpyrifos retention occurs only in the fatty tissues of the mammals (Smith *et al*, 1967b, McKellar *et al* 1972, Cheng *et al*, 1989). Chlorpyrifos is eliminated primarily through the kidneys in urine, unless stored in fat tissue (Hayes, 1982; Hayes and Laws, 1990) and detoxified quickly in rats, dogs and other animals (Worthing,

1983; USEPA, 1986) with TCP as the major metabolite but TCP does not inhibit cholinesterase and it is not mutagenic (Worthing, 1983; USEPA, 1989). Unlike chlorpyrifos, residues of the pyridinol in fat were trivial, which was consistent with its much greater polarity. Small tissue residues (<0.3 mg/ L) were present and occurred mainly in the biological systems involved with urinary excretion, i.e. liver, kidney and blood (Branson and Litchfield, 1971a, b, Smith *et al,* 1970). In rats, products excreted have been identified as 3, 5, 6-trichloro-2-pyridyl phosphate, 3, 5, 6-trichloro-2-pyridinol and traces of chlorpyrifos and desethylation is believed to be the metabolic pathway, confirmed by the study of Branson and Litchfield, 1971a, b.

Chlorpyrifos has been detected in the milk of cows for 4 days following spray dipping and in salt marsh snails after exposure (NAS, 1982; Hayes and Laws, 1990). When an emulsion formulation was used, residues were found immediately in treated animals following treatment and for up to 3 weeks afterwards (McEwen and Stephenson, 1979).

2.5 Ecological Effects

Chlorpyrifos may affect other organisms also. It may be toxic to algae in the soil. Chlorpyrifos is moderately to highly toxic to birds, highly toxic to freshwater fish, aquatic invertebrates and estuarine and marine organisms (USEPA, 1989) and to sea bottom dwellers (Schimmel, 1983). Chlorpyrifos at 1.5 μg/gm reduced the population of ammonium oxidizers, denitrifiers, and nitrate oxidizers in soil (Sivasithamparam, 1969). Inhibition of nitrogenase by chlorpyrifos has been observed in a number of asymbiotic nitrogen – fixers (Hegazi *et al,* 1979). Some wildlife and non-target species like insects and honeybees are also affected (Hartley and Kidd, 1983; USEPA, 1984). A study reported that practically all non-target insects died after chlorpyrifos application in rice fields (Guenzi, 1974). Chlorpyrifos application also significantly reduced root production, tardigrade and nematode populations and altered microbial community structure and function (Susan *et al*, 2002). Soon after application most of the relatively water-insoluble chlorpyrifos is absorbed on the marine organisms, especially more quickly on those with large respiratory surfaces (Hurlbert *et al*, 1970). As with many organophosphorus insecticides, biocidal activity of chlorpyrifos (Dursban) could be due to its oxygen analogue, diethyl 3, 5, 6-trichloro-2-pyridyl phosphate, a highly reactive compound and a strong inhibitor of cholinesterase activity (Smith *et al* 1966).

2.6 Residues of chlorpyrifos

A US national survey of pesticides and their metabolites in the US found that the primary chlorpyrifos breakdown prodFiguct, 3, 5, 6-trichloro-2-pyridinol was the second most commonly detected chemical in food (Cox, 1994). Chlorpyrifos is regularly detected in UK

fruit and vegetables. The report for 1997 shows that one sample out of 15 of Spanish celery was detected at 0.06 mg/kg which exceeded the maximum residue limit of 0.05 mg/kg (WPPR report, 1998).

Another US research showed that if a child played in his/her home a week after chlorpyrifos application, there was a danger that he/she would be overexposed to chlorpyrifos. The researchers were still finding chlorpyrifos residues on toys two weeks after application (Gurunathan *et al*, 1998). Based on the findings of this and other researchers, the estimated chlorpyrifos exposure levels from indoor spraying in US for children are about 21-119 times above than the US recommended reference dose of 3 mg/kg/day from all sources (Davis and Ahmed).

A number of scientists studied the persistence of chlorpyrifos and its oxygen analogue on field plots (Leuck *et al*, 1968, Winterlin *et al*, 1968, Myers *et al*, 1968, Dishburger *et al*, 1969, Johnson *et al*, 1969 and Hurlbert *et al*, 1970,) and reported that there was no evidence of accumulated residues of chlorpyrifos or its oxygen analogue after ~10 days of multiple foliar applications of chlorpyrifos. Analysis of residues done by some Canadian scientists using the analytical method of Braun (1971) for chlorpyrifos and McKellar (1971) for the pyridinol also indicated less residue accumulation. Similar were the results from Europe, the Near East, Japan, Thailand, Malaysia, and the Philippines. Chlorpyrifos may be toxic to some plants, such as head lettuce (McEwen and Stephenson, 1979). Even if residues are present, they remain on plant surfaces for approximately 10-14 days (Harding, 1979; Thomson, 1982).

2.7 Metabolic fate of Chlorpyrifos

2.7.1 Abiotic Degradation

Extensive studies have been carried out on the abiotic degradation of chlorpyrifos in soil, grasslands, plants and animals. Smith *et al* (1967 a, b) studying the fate of chlorpyrifos in animals and plants found that the parent compound disappears via excretion, volatility, catabolism and possibly photodecomposition. Dealkylation, oxidation, hydrolysis, dechlorination and ring fragmentation are involved (Davis & Kuhr, 1976).

Transformation in plants

The metabolic fate of chlorpyrifos has been investigated in plants (Smith *et al*, 1967a, 1967b) and data indicate an oxidative and/or hydrolytic breakdown yielding des-mono-ethyl chlorpyrifos, desethyl chlorpyrifos, 3,5,6-trichloro-2-pyridinol, ethyl-3, 5, 6-trichloro-2-pyridyl phosphate and 3, 5, 6-trichloro-2-pyridyl phosphate and further degradation products (Smith *et al*, 1967a). Studies on the absorption, translocation and metabolism in plants show that for all practical purposes uptake of the chemical or its degradation products into plants does

not occur, either through the foliage or through the roots (Smith *et al*, 1967a, 1967c) though some later data indicate that this insecticide and/or its soil metabolites, can accumulate in certain crops (USEPA, 1984b). Losses occur mostly by vaporization from the leaf surface and some chlorpyrifos may be sorbed on the outside of the plant roots. TCP may or may not be taken up by the plant depending on pH of the soil medium. Information is limited on chlorpyrifos toxicity to freshwater plants, although algal blooms frequently follow its field application (USEPA, 1986).

Degradation in Air and Water

Chlorpyrifos is known to react in the vapor phase/air with the photo chemically produced hydroxyl radical ($t_{1/2}$ = 13.74 hrs) and get transformed, but it does not react with ozone (Spectrum Laboratories, 2001). When released to water, chlorpyrifos gets significantly partitioned from water column to sediments and this hydrolysis rate is markedly enhanced by the presence of sufficient concentrations of Cu^{+2} ions (Spectrum Laboratories, 2001). The measured hydrolysis half-life at 25°C at/near neutral conditions is 35-78 days (Howard, 1989). Immediately after entering open waters, emulsifiable concentrates and wettable powders tend to produce a large increase in chlorpyrifos concentrates in water, with residual concentrations of chlorpyrifos maintained in open waters for long periods of time (EXTONET, 1993). Chlorpyrifos enters freshwater and saltwater ecosystems primarily as spray drift. It is also carried on eroded soil particles from treated areas (USEPA, 1986). If soil with adsorbed chlorpyrifos is carried by runoff, surface water may be contaminated (Rao, 1983). In water, chlorpyrifos readily adsorbs to suspended sediment and bottom materials. Volatilization is probably the primary route of loss of chlorpyrifos from water. Photo degradation of chlorpyrifos is not expected to be significant in deep waters, during winter, or in waters which sunlight can not penetrate(Howard, 1989).Research suggests that this insecticide is unstable in water, and the rate at which it is hydrolyzed increases with temperature, decreasing by 2.5 to 3-fold with each 10 degrees C drop in temperature. The rate of hydrolysis is constant in acidic to neutral waters, but increases in alkaline waters.

Degradation in Soil

Chlorpyrifos is moderately persistent (USEPA, 1989), adsorbs strongly to soil particles and it is not readily soluble in water (USDA, 1990; Racke, 1992). Being relatively immobile in soils, it is unlikely to leach significantly or to contaminate groundwater (Rao, 1983; USEPA, 1984, 1989; Racke, 1992).

The half-life of chlorpyrifos in soil is usually between 60 and 120 days, but can range from 2 weeks to over 1 year, depending on the soil type, climate and other conditions (Hartley and Kidd, 1983; USEPA, 1984a; Howard, 1989). Its half-life depends maximally on application

rate (Racke *et al*, 1994). Chlorpyrifos may remain biologically active in soil up to six months. Dosage rates, pH, soil type, soil moisture content, temperature and insecticide formulation are factors which affect biological persistence (Whitney 1967, Read 1976, Tashiro & Kuhr 1978).

In aerobic soils with soil pH from 5.4 to 7.4, the soil half-life of chlorpyrifos varies from 11 to 141 days, as chlorpyrifos undergoes a pH-independent hydrolysis reaction over the environmentally relevant pH range of 4-7.5. Chlorpyrifos is less persistent in the soils with a higher pH as it degrades by alkaline hydrolysis above pH 8. But, the higher rate of degradation of chlorpyrifos at alkaline pH attributed to chemical hydrolysis was falsified by Racke *et al* in 1996, when they concluded that the relationship between high soil pH and chemical hydrolysis was weak and that other factors like soil silt content might be important in determining environmental fate (Singh and Walker, 2006).

In anaerobic soils, the half-life is 15 days in loam and 58 days in clay soil (USEPA, 1989). In another study, 2.6 and 9.3% of the chlorpyrifos applied to sand or silt loam soil remained after 30 days (Racke, 1992). Chlorpyrifos is the most stable on peat moss, Ca-montmorillinite, and in soils high in organic matter or low in clay content (Getzin, 1981) though the faster degradation in air-dry soils by chemical hydrolysis has been attributed to clay-catalyzed hydrolysis (Awasthi 1997).

Volatilization from soil surfaces especially at higher temperatures contribute significantly to its loss from soil, though base-catalyzed and surface-catalyzed hydrolysis are the major degradative routes (L.W.Getzin, 1981). Chlorpyrifos may be extremely ephemeral or be quite persistent depending on the soil and its degradation in soil has been found to be biphasic (Baskaran, Kookana & Naidu, 1999). Knuth & Heinis, 1992 found that the majority of the chlorpyrifos applied as an emulsifiable concentrate to littoral enclosures was initially in the aqueous phase but after quickly dissipating from the water, trace amounts remaining were primarily associated with the sediment phase.

Chlorpyrifos is the most widely used soil termiticide and it exhibits long persistence in soil when applied at termiticidal rates with significantly retarded degradation at the 1000 μg/g compared to the 10μg/g rate of application (Racke *et al*, 1994). Hance & McKone (1971) have speculated that a limitation in the number of abiotic reaction sites for pesticide degradation may be responsible for the phenomenon.

Chlorpyrifos undergoes hydrolysis even when exposed to UV light or to sunlight, in the presence of moisture. When treated on moist soils, the volatility half-life of chlorpyrifos was 45-163 hours, with 62-89% of the applied chlorpyrifos remaining on the soil after 36 hours. Chlorpyrifos is more effective in moistened soil than in dry soil but retains its activity in both

soils. It has been seen that chlorpyrifos is more easily degradable in dry soils than under wet conditions (Getzin, 1981).

2.7.2 Biotic Degradation

While chlorpyrifos is susceptible to chemical hydrolysis and photodecomposition, biochemical mechanisms rather than physical factors probably purge most of the chlorpyrifos from the ecosystem. Chlorpyrifos degrades faster with increasing temperature presumably because higher temperature promotes both biological and non-biological/ chemical degradation of the insecticide (Whitney, 1967). Comparisons of degradation and decay rates of chlorpyrifos in autoclaved soils and non-autoclaved soils showed that microorganisms played an important role in breakdown of this pesticide and its major metabolite (Getzin, 1981). Chlorpyrifos is stable under normal storage conditions (Singh and Walker, 2006). Microbial degradation may contribute to chlorpyrifos removal in some natural waters also. Extension of chlorpyrifos persistence in soil could be due to lack of ease of initiation of microbial degradation (Racke *et al*, 1990) or inhibition of microbial degradation of chlorpyrifos at its high concentrations, which may be due to direct pesticide effect on soil microbial community or due to alteration of the soil environment like drop in soil pH (Racke *et al*, 1994). Fumigation of soil samples completely inhibited hydrolysis of chlorpyrifos, suggesting an involvement of soil micro-organisms (Singh *et al*, 2003).

Degradation of chlorpyrifos by Algae, Fungi and Yeast

Zidan and Ramdan in 1976 were able to grow *Aspergillus* and *Penicillium* well on malt medium containing different concentrations of chlorpyrifos (Lal, 1982). Jones and Hastings (1981) reported the metabolism of 50 mg/ L chlorpyrifos by cultures of *Trichoderma harzianum, Penicillium vermiculatum, and Mucor sp*. Lal and Lal in 1987 observed some degree of degradation by the yeast *Saccharomyces cervisiae*. Only half the initial chlorpyrifos was recovered 12 h after the cultures were inoculated with 1-10 mg/ L chlorpyrifos. Bumpus *et al*, 1993 reported mineralization of chlorpyrifos by *Phanerochaete chrysosporium*. Several species of *Aspergillus sp., Trichoderma harzianum and Penicillium brevicompactum* have been reported to utilize chlorpyrifos as sources of phosphorus and sulfur (Omar, 1998). White-rot fungi *Hypholoma fasciculare*e and *Coriolus versicolor* degraded chlorpyrifos in 'biobed' composting (Bending *et al*, 2002). Swati & Singh in 2002 isolated strains of *Aspergillus flavus and Aspergillus niger* isolated from agricultural soil with previous history of chlorpyrifos use and reported biomineralization of chlorpyrifos in liquid medium. Disappearance of chlorpyrifos from cultures of *Chlorella vulgaris* was also reported by Mukherjee *et al*, in 2004. Yu *et al* in 2006 isolated and characterized a *Verticillium sp.* capable of degrading chlorpyrifos. They found that the degradation of chlorpyrifos by the

fungal strain in mineral salt medium increased almost linearly with increasing concentrations of chlorpyrifos (r^2 =0.9999), suggesting that the degradation is subjected to pseudo-first order kinetics, while in the controls the hydrolysis percentages of chlorpyrifos were found to be less than 5%. They further used the cell free extracts of the strain to detoxify chlorpyrifos in vegetables.

Bacterial degradation of chlorpyrifos

Till 1995, bacterial degradation of chlorpyrifos was just suggested, but no attempts had been made to isolate chlorpyrifos degrading bacteria. It was always reported that chlorpyrifos is not affected by accelerated biodegradation in retreated soils (Racke *et al*, 1990; Mallick *et al*, 1999) unlike other pesticides like diazinon (Sethunathan 1971; Sethunathan and Pathak 1972), parathion (Sethunathan 1973), methyl parathion and fenitrothion (Misra *et al*, 1992). Even Racke and Coats (1988) found that chlorpyrifos was neither metabolized nor co-metabolized by *Arthrobacter sp*. and even 30 mg kg^{-1} of chlorpyrifos was inhibitory to organisms introduced in soil and hence, efforts to isolate chlorpyrifos-degrading microorganisms from chlorpyrifos-acclimatized soils were not successful (Racke *et al*, 1990). Other reports also indicated the resistance of chlorpyrifos to degradation in pure (Hirakoso, 1969) and mixed (Sethunathan and Pathak, 1972) cultures of microorganisms. Thus, there have been no reports of enhanced degradation of chlorpyrifos since its first use in 1965 until recently (Singh and Walker, 2006).

Some later reports indicated disappearance of chlorpyrifos from medium containing bacteria and postulated possible degradation of the pesticide. The possible metabolism by two lactic acid bacteria (*Lactobacillus bulgaricus and Streptococcus thermophilus*) was reported by Shaker *et al* in 1988. The synthetic culture medium, containing 7.4 ppm chlorpyrifos in which the bacteria were grown displayed a 72-83% loss of chlorpyrifos after 96 h. Ivashina in 1986 studied chlorpyrifos disappearance in the presence of several microbial cultures maintained in liquid media containing 10 ppm chlorpyrifos and found that the dissipation was more rapid in a glucose supplemented media containing *Bacillus sp.* than in control media containing no microorganisms and that the chlorpyrifos disappeared from the microbial cultures in a linear fashion over two week period.

Some recent reports indicate mineralization of chlorpyrifos by bacteria. A *Micrococcus sp.* was isolated from a Malathion enriched soil was later seen to degrade chlorpyrifos in liquid media (Guha *et al*, 1997). The major report came in 1999, when Mallick *et al* in 1999 reported that *Flavobacterium sp*. ATCC 27551, isolated from diazinon-retreated rice fields by Sethunathan and Yoshida, 1973 and *Arthrobacter* sp., isolated from a flooded soil retreated with methyl parathion by Misra *et al* in 1992, could degrade chlorpyrifos also rapidly. The

Flavobacterium sp. was known to be versatile and capable of degrading common P-O-C linkage containing organophosphorus compounds like diazinon, parathion, methyl parathion, fenitrothion, coumaphos and diisopropyl fluorophosphate (Sethunathan and Yoshida 1973, Adhya *et al*, 1981, Karns *et al*, 1986 and Attaway *et al*, 1987). Brown, 1980 had said that the phosphotriesterases of the *Flavobacterium sp.*, used in this study, could hydrolyze several phosphorothioate insecticides including chlorpyrifos. The *Arthrobacter* sp. hydrolyzed not only chlorpyrifos and methyl parathion, but also parathion and fenitrothion. However, there are only two reports till today on organisms from enrichments, capable of degrading chlorpyrifos.

Singh *et al*, 2003 came up with the first report of an *Enterobacter* strain B-14 capable of enhanced degradation of chlorpyrifos from an Australian soil and noted that this strain showed greatest similarity to *Enterobacter asburiae* based on 16S rDNA studies of the bacterium. The degradation in United Kingdom soils was mediated by the co-metabolic activities of the soil microorganisms and addition of *Enterobacter* strain B-14 (10^6 cells/ gm soil) from Australian soil to the United Kingdom soils with a low indigenous population of chlorpyrifos degrading bacteria and 35mg/kg chlorpyrifos enhanced the degradation rate than that in noninoculated soils. This strain was able to utilize chlorpyrifos as sole sources of carbon and phosphorus and was also able to utilize parathion, diazinon, coumaphos and isazofos.

Yang *et al*, 2005 isolated a new strain, an *Alcaligenes faecalis*, from contaminated soils, capable of utilizing chlorpyrifos as well as parathion and diazinon as sole sources of carbon and phosphorus. Addition of *Alcaligenes faecalis* (10^8 cells/ gm soil) to a soil with 100 mg/kg chlorpyrifos resulted in higher degradation rate than the one obtained from noninoculated soils. Soil used for cabbage growing in combination with inoculation of this strain showed enhanced microbial degradation of chlorpyrifos.

2.7.3 Products and Pathways of Chlorpyrifos Degradation

Degradation of chlorpyrifos occurs through both abiotic & biotic processes and microorganisms play an important role not only in the degradation of the parent compound but also in the subsequent metabolism of breakdown products. Hydrolyzed by any process, chlorpyrifos is degraded to a primary metabolite, 3, 5, 6-trichloro-2-pyridinol (TCP), which is a major product (Macalady & Wolfe, 1983) which is then further degraded to the secondary metabolite, 3, 5, 6-trichloro-2-methoxypyridine (TMP). The other product formed along with TCP is O, O-diethyl phosphorothioic acid (DETP), which undergoes further decomposition to diols and triols and ultimately cleavage of the ring to fragmentary products (Smith, 1968).

In most cases described to date, the hydrolysis by aerobic bacteria transforms chlorpyrifos to DETP and TCP, which in turn accumulate in the culture medium without further metabolism. This transformation reaction removes chlorpyrifos and its mammalian toxicity but yields compounds that are not metabolized by the microorganisms that produce them (Richins *et al*, 1997, Mallick *et al*, 1999, Horne *et al*, 2002, Wang *et al*, 2002 and Singh and Walker, 2006). Cook *et al* (1978a) isolated several dialkylthiophosphonic acid degrading bacteria from sewage sludge and one among them, *Pseudomonas acidovorans*, was able to use DETP as a sole source of sulfur (Cook *et al*, 1980). But a variety of isolates that could use phosphorothionate or phosphorodithionate compounds as a sole source of phosphorus were unable to degrade these compounds as a source of carbon (Rosenberg & Alexander, 1979).

Repeated treatment with chlorpyrifos over many years in an Australian soil resulted in development of some opportunist microorganisms with the capability to use TCP, a metabolic by-product of some organochlorine compounds (Robertson *et al*, 1998, Singh *et al*, 2000, Singh and Walker, 2006). *Enterobacter sp*. isolated by Singh *et al*, 2003 from this Australian soil showed enhanced degradation of chlorpyrifos to TCP and DETP and it utilized DETP as a source of carbon and phosphorus and other organophosphorus insecticides as a source of phosphorous (Singh *et al*, 2003, 2004). However this strain was also not able to degrade TCP (Singh *et al*, 2004), as has been in other instances mentioned previously in many other reports. TCP adsorbs weakly to soil particles and has high water solubility and therefore is expected to be bioavailable for degradation. However, TCP is only moderately mobile and highly persistent in soils (USEPA, 1989) and hence prevent the enhanced degradation of chlorpyrifos in soil. From their experiments, Singh and Walker, 2006 concluded that in high pH soils, the microbial community transforms chlorpyrifos co-metabolically into its metabolite TCP and as TCP contains three chlorine atoms on the pyridinol ring, to break this ring, chlorine atoms have to be removed (Feng *et al*, 1997), and free chlorine has toxic effects on the microorganisms. Thus TCP metabolism may be toxic to microorganisms; so normally enhanced degradation does not occur with repeated enrichment of soil with chlorpyrifos.

The only known organisms to be able to degrade TCP include the *Alcaligenes faecalis* isolated by Yang *et al*, 2005 and the TCP degrading bacterium isolated on TCP by Feng *et al*, 1997. Feng *et al* (1997) isolated a *Pseudomonas sp.* which can mineralize TCP in liquid medium and later suggested that TCP was metabolized by a reductive dechlorination pathway (Feng *et al*, 1998). In this pathway, TCP is first reductively dechlorinated into chlorodihydro-2-pyridone, which is further dechlorinated to tetra-hydro-2-pyridone. Ring

cleavage of this compound resulted in formation of maleamide semialdehyde, which is metabolized to water, carbon dioxide, and ammonium ions. This is the only instance where a pure culture of bacteria capable of mineralizing TCP has been shown but this organism is not a chlorpyrifos degrader. Microbial degradation of analogous compounds such as pyridine and hydroxypyridine has been researched and reviewed extensively (Shukla, 1984, Sims & O'Loughlin, 1989, Kaiser *et al*, 1996). It has been reported that white-rot fungi can degrade certain chlorinated compounds by reductive de-chlorination (Aiken & Logan, 1996, Mougin *et al*, 1996, Singh & Kuhad, 1999, 2000) and this could be a method of mineralization of TCP by these TCP degrading bacteria (Singh and walker, 2006).

The earlier proposed mechanism by Smith *et al* 1966 indicated chlorpyrifos degradation till formation of the primary metabolite. As was seen, the primary mechanism of chlorpyrifos conversion was hydrolytic. Conversion of TCP to TMP may be a direct methylation process or may involve a number of intermediates. Singh and Walker in 2006 have proposed a pathway of chlorpyrifos degradation based on degradation of analogous compounds and literature available (Fig 3).

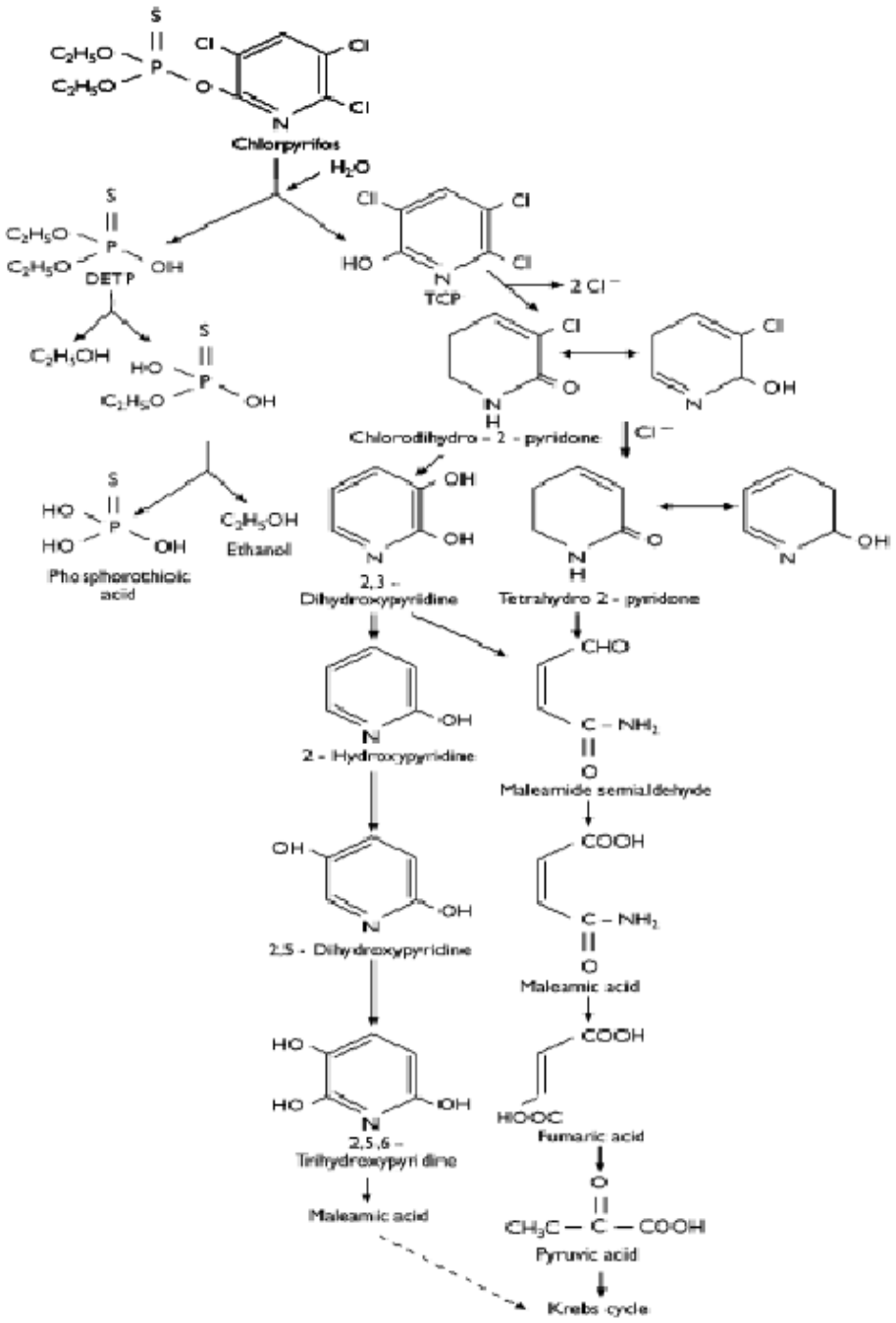


Fig3. Proposed pathways for chlorpyrifos degradation by microorganisms (Singh and Walker, 2006).

When the conversion of one compound to another is believed to occur through a series of intermediates, the steps are indicated by dotted arrows.

DETP → diethylthiophosphate; TCP → trichloropyridinol.

2.7.4 Genes and Enzymes involved in Chlorpyrifos Degradation

Researchers since 1970s started reporting the enzymatic detoxification of organophosphates with bacterial phosphodiesterase from *Haemophilus influenzae* (Macfadyen *et al*, 1988), *Escherichia coli* (Imamura *et al*, 1996), and *Burkholderia caryophylli* PG2982 (Dotson *et al*, 1996). The phosphodiesterase from *B. caryophilli* differed in sequence to that in the first two bacteria (Dotson *et al*, 1996). With the identification of organophosphate degrading organisms like *Pseudomonas diminuta* capable of hydrolyzing parathion & *Flavobacterium sp*. (Sethunathan & Yoshida, 1973, Serdar *et al,* 1982) capable of degrading diazinon, with similar genetic mechanism of degradation of organophosphates and broad specificity of the enzyme organophosphate hydrolases (OPHs) which are responsible, the process of detoxification became an interesting aspect to study. Havens and Rase (1991) circulated a 0.25 % aqueous (EC) solution of chlorpyrifos through a packed column containing immobilized parathion hydrolase enzyme obtained from *Pseudomonas diminuta.* Approximately 25 % of the initial dose was degraded after 3 h of constant recirculation through the column. Many other such experiments were reported in the 1980s and 1990s.

However, none of these microbes were reported to contain enzymes that degraded chlorpyrifos. Many reports were then published for degradation of related organophosphates like Diazinon, Parathion, and Methyl Parathion by isolated bacterial strains (Sethunathan, 1972, Sethunathan and Yoshida, 1973, Misra *et al*, 1992 and Zhongli *et al*, 2001) or by using common OPHs. Plasmid involvement was indicated in these hydrolysis reactions (Serdar *et al*, 1982, Mulbry *et al*, 1986 and Mulbry *et al*, 1987), and homology was found in the sequences present in the *opd* gene of different involved organisms. Various researchers had carried out cloning of this opd gene and its related opdA/ mpd genes (Zhongli *et al*, 2001; Horne *et al*, 2002; Yang *et al*, 2006). Siddavattam *et al*, 2003 reported that the gene cluster found in the *Flavobacterium sp.* involved a transposon-like organization. Horne *et al* in 2002 reported the identification of a new *opd* gene, which they named as *opdA*, from an *Agrobacterium* isolate degrading coumaphos, capable of hydrolyzing dimethyl substrates at a higher rate than OPH, the rate of hydrolyzation of diethyl OPs remaining unaffected. Studies found higher copy numbers of opd (organophosphate degrading) gene in higher pH soils (Singh *et al*, 2003). Chlorpyrifos degrading *Enterobacter* strain had a different genetic mechanism of chlorpyrifos degradation than what have been reported previously using opd genes (Singh *et al*, 2004). During their studies on protein profiles of the isolate *faecalis* grown under different conditions, Yang *et al*, 2005 found that there was a new band in the profile for the strain grown in chlorpyrifos containing medium, which they concluded to be an

enzyme involved in degrading chlorpyrifos and that this enzyme was induced by chlorpyrifos while TCP degrading enzymes were constitutive in the organism.

2.8 Rationale behind the work

Some recent reviews on chlorpyrifos make us ponder on the usage and degradation steps necessary to eliminate this pesticide from the environment. A report by Centre for Science and Environment's Down to Earth in 2003 indicated that the residues of chlorpyrifos were present in bottled drinking water and leading cold drink brands like Coke and Pepsi. The startling 2005 CSE Report by Mathur and Agarwal on “Analysis of Pesticide Residues in blood samples from villages of Punjab” indicated high levels of chlorpyrifos in 85% of the samples. The presence of chlorpyrifos on regularly consumed vegetables has been seen. Pesticide news, 2001a mentions hundreds of fish death in River Ouse in Southern England and its 2001b report indicates this pesticide even in street vended staple food items in Accra, Ghana’s capital city. Numerous abnormalities in newborns have been associated with exposure to this pesticide. An US environmental Protection Agency report also indicates reduction in Amphibian populations and malformations in their embryo, thus disturbing the food chain.

Also, research is scarce on

a) Biodiversity studies on chlorpyrifos degraders
b) Microcosm and *in-situ* remediation of chlorpyrifos contaminated sites, collaborating it with population dynamics of introduced microorganisms
c) Chlorpyrifos degrader degrading its major metabolite TCP
d) Eco-friendly, cost effective and convinient method of bioremediation of chlorpyrifos contamination
e) Completely elucidated metabolic pathway of chlorpyrifos degradation

Keeping all these aspects in mind, the objectives of this study were framed.

3. OBJECTIVES

- Development and screening of efficient aerobic microbial system(s) capable of degrading organophosphorus pesticide chlorpyrifos
- Elucidation of metabolic pathway of chlorpyrifos degradation
- To optimize conditions for enhanced degradation of the selected pesticide in soil microcosm
- Development of molecular markers for tracking the fate of the introduced efficient degraders of chlorpyrifos in soil and in American Cotton Plants

4. MATERIALS AND METHODOLOGY

MATERIALS

4.1a Chemicals and reagents:

Standards of chlorpyrifos (chlorpyrifos) and 3, 5, 6-trichloro-2-pyridinol (TCP), and antibiotics were purchased from Sigma-Aldrich, USA. Commercial grade chlorpyrifos containing 20% active ingredient was purchased from Rallis India, Mumbai. General chemicals were obtained from Hi-Media, India. Solvents were purchased from Merck, Germany. Other chemicals were of highest analytical grade.

4.1b Restriction enzymes, markers and Taq polymerase

All restriction enzymes, primers, DNA markers (1kb and 100bp) and *Taq polymerase* were of Qiagen and used as per the manufacturers' instructions.

4.1c Seeds

Sterile American cottonseeds were procured from Mahyco seeds, Maharashtra.

4.1e Primers sequences

Rep1R - 5'IIIICGICGICATCIGGC3'

Rep 2R - 5'ICGTCTTATCIGGCCTA3'

E8 F Forward - 5'AGAGTTTGATCCTGGCTCAG3'

E1429R Reverse - 5'GGTTACCTTGTTACGACTT3'

OPD forward - 5'GATCGTGGATCCTCGATCGGCACAGGCGATCGG3'

OPD reverse - 5'GATCGTAAGCTTTCATGACGCCCGCAAGGTCGG3'

OPDA forward - 5'GATCGTCTGCAG CCAATCGGTACAGGCGATCTG3'

OPDA reverse - 5'GATCGTAAGCTTTCATCGTTCGGTATCTTGACGGGGAAT3'

MPD forward - 5'GAATTCATATGCCCCTGAAGAAC3'

MPD reverse - 5'CGGAATTCTCACTTGGGGTTGAC3'

4.1f Bacterial culture

E.coli DH5∝ was obtained from IMTECH, Chandigarh.

Strains P, M19, M119, D113, Q1a, Q2a, Q2b and Q2c were isolated from chlorpyrifos contaminated soils.

METHODOLOGY

4.2 Preparation of standard stock solutions

Standard stock solutions and buffers like 1M potassium dihydrogen orthophosphate (pH 6.5), 1M dipotassium hydrogen phosphate (pH 6.5), were prepared in double distilled water at room temperature. The pH was adjusted using standard electrode which had been standardized using pH 4.0 and pH 7.0 buffer solutions. These stock solutions were stored at

4°C. Stock solutions of X-gal, IPTG and antibiotics (Kanamycin, Ampicillin, Chloramphenicol, Nalidixic acid, carbenicillin) 100mg/ml were prepared, filter sterilized and stored in -20°C ultra freeze, along with stock solutions of technical 3, 5, 6-trichloro-2-pyridinol (100mg/ml in acetone).

4.3 Preparation of media

Chlorpyrifos was prepared as 100mg/ml stock in acetone just before use. Potassium phosphate buffer was prepared according to need from 1M stock of potassium dihydrogen orthophosphate (pH 6.5) and 1M stock of dipotassium hydrogen phosphate (pH 6.5). The pH was rechecked and adjusted to 6.5, if needed and autoclaved. Glycerol (50% v/v) and 1% (w/v) glucose solution were autoclaved separately. For preparation of solid media, addition of 1.5 %(w/v) agar to basal medium was done. Before pouring, chlorpyrifos was added to the media and poured plates were left to solidify overnight.

4.4 Maintenance of the culture

Bacteria were grown in Luria broth, harvested and washed with 10mM potassium phosphate buffer (pH 6.5). Pellet was re-suspended in 50 % (v/v) glycerol and kept frozen at -20°C and -80°C, till further use. The purity of the culture was examined periodically by dilution streaking on Luria agar plates and by Gram staining.

4.5 Collection of Soil samples

Soil samples (eleven in number) were collected from ten paddy fields and a formulation site in Punjab, India. The formulation site in Dera Bassi Village of Punjab was the site where technical chlorpyrifos was procured and mixed with emulsifiers and surfactants to make various commercial formulations. The source of soil samples is indicated in Table 2. Among the ten field samples, some were from fields repeatedly sprayed with the pesticide chlorpyrifos for at least two years. These sandy loam soils had ≥ 30-40% water holding capacity and most were from waterlogged paddy fields. Topsoil was collected from the first 15 cm in sterile polythene bags, air dried, and used for developing aerobic consortia capable of utilizing chlorpyrifos as sole carbon and energy source, and also co-metabolically with glucose as carbon source, by enrichment procedures.

4.6 Development of Consortia by enrichment procedure

Soils (10 gram) were added to nutrient broth (100ml) with 25mg/L chlorpyrifos and incubated at 37°C, 130 rpm, in a rotary shaker for a week. After a week, the flask was withdrawn; soil was allowed to settle. The turbidity in the supernatant was transferred to a sterile flask with basal medium (Bushnell Haas Broth), with 50 mg/L chlorpyrifos, with and without 0.01%, 0.05%, 0.1% & 0.5% (w/v) glucose. The inoculated flasks were incubated for seven days in a rotary shaker at 37°C, 130 rpm. Repeated regular transfer with 2% (v/v) inoculum, into

fresh basal broth, were made and through five transfers enabled the development of the various aerobic consortia.

4.6.1 Glucose utilization studies

After selection of the medium, growth studies were carried out, by taking the absorbance at 550nm in an UV-Vis spectrophotometer. Growth profile was also studied along with glucose utilization using dinitrosalicilic acid (DNSA) method for the determination of Reducing Sugars (Murao *et al*, 1979).

4.6.2 Extraction and analysis of chlorpyrifos

Flasks containing basal medium (50ml), chlorpyrifos (50 mg/L) and 0.1% (w/v) glucose were inoculated (2% v/v) with developed consortia. After repeated time intervals, the residual pesticide was extracted using equal volume of ethylacetate: phosphoric acid (97:3) into a glass crucible using pasteur pipettes (2ml). The solvent system was referred from Racke *et al*, 1990 and the process was standardized. The solvent was evaporated, using rotary flash evaporation under an exhaust fume chamber and sample was re-dissolved in acetone (1ml) and quantified using Gas chromatography (Perkin-Elmer) equipped with electron capture detector and a capillary column, PE-5, (30m * 0.2mm I.D * 0.25 μm film thickness). The program was set as 150°C (2min) to 280°C @ 8°C/min & held for 5 min at 280°C. The injector temperature was 300°C and the detector temperature was 350°C. Volumes of 0.5 µl were injected and peak area measurements were used to quantify the insecticide residues.

4.7 Pure culture studies

4.7.1 Isolation of chlorpyrifos degrading Bacteria

The five developed consortia were tested for their capacity to degrade chlorpyrifos. The flasks with culture in basal medium with 50 mg/L chlorpyrifos and 0.1% (w/v) glucose were incubated at 37°C, 130 rpm in a rotary shaker along with negative control flasks with only basal medium containing 50 mg/L chlorpyrifos and 0.1% (w/v) glucose and no culture. Extraction of residual chlorpyrifos was carried out from one flask of each set after 7, 14 and 21 days of incubation as described (Section 4.6.2).

An aliquot of 1ml culture, withdrawn from each consortium after seven days of growth at 37 ºC and 130 rpm, was serially diluted and plated on Luria agar (LA) plates containing 50 mg/L chlorpyrifos. The plates were incubated at 37 ºC for 24-48 hours. Morphologically distinct isolated colonies were picked up and patched on fresh basal agar plates with 50 mg/L chlorpyrifos as carbon source. The well growing patches were repeatedly transferred on basal agar-chlorpyrifos plates five times over a period of 15days, purified by dilution streaking method to get isolates capable of growing and degrading chlorpyrifos. The pure

isolates so obtained were cryopreserved in 50% (v/v) glycerol at -20°C and -80°C ultra freezers.

The degradative ability of these isolates was also estimated as done for the five consortia. Growth studies were carried out for the isolates along with the degradation studies. Growth was studied for the isolates simultaneously with the time-course experiments by taking 2ml from a sample flask before extraction and measuring the absorbance of that sample at 550nm. The rest of the sample was extracted to study degradation.

4.7.2 Tolerance studies

All the cultures and strains were subjected to various concentrations of chlorpyrifos (25, 50, 75, 100, 200, 300 and 500 mg/L respectively) in tubes with basal medium. Growth was monitored by absorbance at 550nm in an UV-Vis spectrophotometer. Tolerance was visually monitored by monitoring turbidity.

4.7.3 Morphological studies and Gram staining

The morphological features (shape, color, shine) of isolates were studied under microscope. Gram staining of the isolates was carried out by standard procedures.

4.8 Chemical determination

4.8.1 Protein estimation

Biuret method was used for estimation of total cell protein with reference to the method of Itzhaki and Gill (1964). Reaction was carried out in the total volume of 3 ml. Sample was added in a tube and volume was made to 2 ml using 10mM Potassium phosphate buffer (pH 6.5). To each sample 1ml of Biuret reagent (Appendix) was added. The reaction mixture was incubated for 10 minutes. The concentration of the protein was calculated from the absorbance values obtained at 310 nm against the standard solution of bovine serum albumin (fraction V).

4.8.2 DNA estimation

Quantity of the DNA in the sample was calculated by reading the sample at 260 nm against a TE (10/1) pH 8.0 blank. An O.D of 1.0 corresponded to 50 μg/ml of DNA. The isolated DNA was also checked for purity by taking ratio of absorbance at 260nm to absorbance at 280nm. Only samples showing a ratio of greater than 1.8 (high purity) were selected for further experiments.

4.9 Whole cell studies

4.9.1 Preparation of whole cells

The various bacterial isolates were grown in Luria broth (1L). The cells were harvested after 24 hours, washed with 10 mM potassium phosphate buffer (pH 6.5), re-dissolved in 5ml of the same buffer and were left overnight for starvation by incubation at 37°C, 120

rpm in a rotary shaker. Protein was estimated for these whole cells by Biuret method (Section 4.8.1) followed by initial uptake studies of chlorpyrifos by the isolates.

4.9.2 Uptake studies

For the uptake studies, to test tubes containing 2 ml of 10 mM potassium phosphate buffer (pH 6.5), chlorpyrifos (25 mg/L) and cellular protein (starved cells- 1mg/ml) were added. Cell (-) controls containing only 2ml of 10 mM potassium phosphate buffer (pH 6.5) and chlorpyrifos (25 mg/L) and cell (+) controls containing 2ml of 10 mM potassium phosphate buffer (pH 6.5), chlorpyrifos (25 mg/L) and dead cells of respective isolates were also set to know the effect of substrate adsorption by the bacterial cells. The reaction was carried out at 37°C and samples were withdrawn after 6 hours and analyzed for residual chlorpyrifos by extraction and GC analysis (Section 4.6.2).

4.9.3 Uptake studies by induction

Time-course experiments were carried out to study the effect of induction with chlorpyrifos on chlorpyrifos uptake, when they were induced after growth, as whole cells and when the cells were induced at growth. Cell (-) controls containing only 2ml of 10 mM potassium phosphate buffer (pH 6.5) and chlorpyrifos (50 mg/L) and cell (+) controls containing 2ml of 10 mM potassium phosphate buffer (pH 6.5), chlorpyrifos (50 mg/L) and dead cells of respective isolates were also set for each set of experimental sample.

For the first experiment, bacterial cells were grown in Luria broth, harvested by centrifugation for 10min at 8000 rpm, 4°C. The pellets were washed with 10 mM phosphate buffer (pH 6.5) and re-suspended in 5ml of the same buffer. These whole cells were starved overnight at 37°C and divided into two sets for uptake studies. One set of starved whole cells (2mg/ml protein) was inoculated into 2ml phosphate buffer containing 50mg/L chlorpyrifos and 2mg/ml protein and time course experiments were carried out up to 12 hours and samples were withdrawn after every 1 hour for residual chlorpyrifos analysis (Section 4.6.2). The other set of whole cells was induced with 5 mg/L chlorpyrifos for 2 1/2 hours and uptake was carried out in similar way. A third set of cells was induced during growth, with 5 mg/L chlorpyrifos, and then uptake studies were carried out as done before.

4.9.4 Substrate Replacement Studies

Whole cell studies were carried out by replacing the substrate chlorpyrifos by its primary metabolite 3, 5, 6-trichloro-2-pyridinol (TCP). Starved whole cells were subjected to a time-course experiment as done before using TCP as substrate. Uptake studies were carried out in 2ml phosphate buffer containing 50mg/L TCP and 2mg/ml protein up to 12 hours. Samples were withdrawn after every 1 hour and residual TCP was analyzed as done for chlorpyrifos ((Section 4.6.2).

4.9.5 Studies of chlorpyrifos and TCP mass spectra by GC-MS

The standard solutions of chlorpyrifos and TCP were subjected to GC-MS to obtain their respective mass spectra. GC-MS analysis was performed using following parameters; Initial oven temperature kept at 150 °C for 2 minute. Oven temperature was programmed to apply the temperature gradient from 150 °C to 280 °C with an increment of 8 °C/min. Finally the oven temperature was held at 280 °C for 5 min. Injector temperature was always maintained at 300 °C. Solvent delay for MS was 2 minutes; lower mass cut off value was 50 amu, higher mass cut off value was 500 amu. Compounds were separated on a PE-5 Elite capillary column (Perkin-Elmer) having the following column dimensions; 30 m length, 0.2 mm. internal diameter and 0.25 μm film thickness. Helium was used as carrier gas at constant flow of 1 ml/min. Product analysis by GC-MS could not be done.

4.10 Soil studies

4.10.1 Antibiotic profile studies

Antibiotic profile was studied on Luria agar plates containing one of the antibiotics (Ampicillin/ Kanamycin/ Carbenicillin/ Chloramphenicol/ Nalidixic acid) in varying concentrations (25 μg/ml, 50 μg/ml, 75μg/ml and 100 μg/ml). Cultures were individually dilution plated on these antibiotic plates and the growth of the isolates was monitored on these plates after 24hrs of incubation at 37°C.

4.10.2 Isolation of bacteria from the microcosms

Soil samples (1 gm) were collected in triplicates after repeated time intervals coinciding with sampling for degradation studies from the experimental microcosm set up. These soil samples were mixed together & soil (1gm/ sample) was re-suspended in 9 ml of sterilized saline (0.84% sodium chloride, w/v) tubes by vigorous vortexing. Serial dilution was carried out in saline tubes and an aliquot of 100μl was plated on Luria agar plates containing kanamycin (50 μg/ml) under sterile conditions of laminar air-flow chamber. The plates were incubated for 48 hours at 37°C. Individual colonies showing the ability to grow on this plate were counted to study the population dynamics, were further patched on LA + Kan (50 μg/ml) plates. Colonies showing the ability to grow on these plates were scored and their DNA was isolated for molecular fingerprinting.

4.10.3 Extraction of chlorpyrifos residues from soil

Chlorpyrifos was extracted from sample soil using Hexane-Acetone (9:1). This solvent system was referred from Awasthi *et al*, 1997 and the process was standardized. For extraction of residual chlorpyrifos, the soil sample was distributed into conical flasks (5gm in each) and 10ml solvent was added. The flasks were incubated at 37°C, 130 rpm in a rotary

shaker for two hours; the organic phase was decanted into a glass crucible using pasteur pipettes (2ml). The sample was re-extracted with additional solvent (total volume 25ml), the solvent was evaporated, using rotary flash evaporation, under an exhaust fume chamber. Samples were re-dissolved in acetone (5ml) and quantified by GC as done earlier (Section 4.6.2).

4.10.4 Optimization of microcosm conditions

For all soil studies, sandy loam garden soil with organic carbon as 0.273 ± 0.0008 %, available phosphorus as 42.40 ± 2.43mg/kg, total nitrogen as 1568.0 ± 550.7 mg/kg was taken. Estimation methods for organic carbon were referred from Walkley and Black, 1934, for available phosphorus from Bray and Kurtz, 1945 and for total nitrogen from Jackson, 1967. The garden soil was autoclaved for three consecutive days for 1 hour, at the same time as the previous day, incubated at 37°C for 24hrs after each autoclaving, re-autoclaved and then used under sterile conditions for sterile studies.

Optimization of required conditions was carried out in 50 mg/kg chlorpyrifos contaminated soil (100gm). Consortia and isolates were grown in Luria Broth (1000 ml) overnight, centrifuged at 10000 X *g* for 10min, washed with 10mM phosphate buffer (pH 7.0) and re-suspended in basal medium (pH 7.0), according to required dilutions. The chlorpyrifos contaminated soil was inoculated with these cultures. Incubation was carried out by varying moisture content of soil (15%, 30%, 40% and 50%) or cell mass of inoculum (with 10^6 cells/ml, 10^7 cells/ml, 10^8 cells/ml & 10^9 cells/ml) and incubation time (5- 60 days). Residual chlorpyrifos was extracted and analyzed (Section 4.10.3) at regular time intervals.

4.10.5 Fingerprinting using molecular markers i.e. REP-PCR (PCR based on Repetitive Extragenic Palindromic sequences)

To track the population survival of inoculated degraders with the concomitant mitigation of chlorpyrifos, the fingerprinting of the bacteria from the experimental soil has been done using molecular markers based on Repetitive Extragenic Palindromic sequences (REP-PCR). The different fingerprinting profiles were matched with the introduced bacterial isolate/s to assess the effective survival of our strains with the success of degradation.

4.10.5.1 Extraction of Genomic DNA for molecular fingerprinting

Genomic DNA of the isolates from soil were extracted by suspending bacterial colonies in sterile distilled water (100 µl) followed by PCR (Gene Amp PCR system 9700) amplification at 95ºC for 5min and then cooling immediately to 4ºC. Centrifugation at 10,000 rpm for 10 min, the supernatant was quantified, diluted appropriately and used directly for PCR amplifications. Genomic DNA was isolated by standard ROSE method (Steiner *et al*, 1995) for the original isolates.

4.10.5.2 Development of molecular markers using REP-PCR

PCR amplification was then carried out with 10ng of the respective DNA under standardized conditions. For this, standardization of the conditions for REP-PCR was carried out, by altering various parameters like concentration of Primers, Taq Polymerase, $MgCl_2$, dNTPs and the temperature conditions. PCR amplification of the DNA was performed in a Perkin-Elmer Thermocycler (Gene Amp PCR system 2700). The reaction consisted of 35 cycles at 94°C for 1min, 40°C for 1min, and 72°C for 2min with 10 nmol of each of the primers REP-1R and REP-2R, 2.5mM $MgCl_2$, 0.2mM dNTPs, and 1Unit Taq Polymerase (Genetix). PCR products (10 µl) were loaded onto a 1% (w/v) agarose gel and run in TAE buffer, pH 7 (Sambrook *et al*, 1987). The gel was run and products were visualized on gel as described in section 4.11.5. The fingerprints were photographed in the gel-doc system.

Simultaneously, from the dilution plated LA+K plates of 10^{-4} dilution of various days of experimental sampling, colonies were patched into LA +K plates. After 24hours, these isolates from patches were inoculated into LB tubes, their DNA was isolated, quantified, appropriately diluted and the isolated DNA was further amplified by REP-PCR using the standardized concentration of the various components in the reaction mixture. A plate with at least 50 colonies was taken for these REP-PCR studies, in each case. The samples were run on 1% (w/v) agarose gel along with a 100bp ladder and the banding pattern was compared with that of the original isolates. The final tabulation of the population by colony counting and the actual population survival by REP-PCR was done and the percentage survival of the two was compared, to know the actual survival during microcosm studies. For the assessment of population survival of inoculated isolates in plots and to know the effect of plant-microbe interaction, REP-PCR of the isolates from plots with and without cotton plants were tracked similarly and compared.

4.10.6 Microcosm Studies

Microcosm studies were conducted with 100gm sterile and non-sterile soil/ 11kg non-sterile soil respectively contaminated with 50 mg/kg chlorpyrifos. Cells were grown, as done during optimization studies and inoculated to make 2x 10^{8} cells/gm soil. Soil was contaminated with 50mg/kg chlorpyrifos and equal volume of basal medium as that in experimental samples was added. This served as 'cell minus' control to monitor the degradation by indigenous microflora, in each experiment. Moisture was maintained throughout the experiments at 30% approximately. The soil samples (5gm each) were withdrawn in triplicates at different time intervals; residual chlorpyrifos was extracted and analyzed (Section 4.10.3). After initial soil studies for chlorpyrifos degradation with 100gm soil,

degradation studies along with population dynamics by antibiotic plating and REP-PCR was carried out for soil samples from 11 kg soil experimental microcosms.

4.10.7 Persistence studies in plots

Two 9m * 6 m plots were sprayed with chlorpyrifos to make a concentration of 50mg/kg (commercial grade from Rallis India). Racking was done thoroughly for aeration and 30% moisture was maintained throughout. The plots were tilled, racked and maintained properly after every few days and any weed/ grass growth was plucked out. Soil samples were taken out in triplicates at three different levels (15cm, 30cm and 45cm) from each plot at intervals of a month over a period of 6 months and studied for the presence of chlorpyrifos by solvent extraction and GC (Section 4.10.3).

4.10.8 In-situ degradation of chlorpyrifos in plots

Nearly 1.3-acre land was divided into small plots of 9m * 6m. These plots were sprayed with chlorpyrifos to a concentration of 50mg/kg (commercial grade from Rallis India, 20% active component). Soil moisture was maintained at 30% approximately, throughout the experiments. The plots were tilled, racked and maintained properly for a few days before sowing. Randomized block design was used for this study. Cultures of consortia and isolates were grown on a mass scale, pelleted, re-suspended in sterile distilled water & inoculum was made ready. Sterile American cottonseeds were procured from Mahyco seeds, Maharashtra. When the cultures were ready for inoculation, some plots were inoculated with consortia and isolates as such while in others cottonseeds (seeds which had been soaked overnight in culture and had swollen and then mixed with jaggery to make a paste) were sown at a depth of 2.5cm and with a distance of 0.5m by length and 1m by width between each other, along with addition of culture. Some plots were left without any further additions as control.

Culture were checked before inoculation to see purity in case of isolates and cfu enumerated as 2-3 x 10^8 per/ml in each case on LA + Kan (50 μg/ml) plates, so that approx. 2-3 x 10^8 cells/gm soil was inoculated into these plots. Samples of soil and plants were taken out in triplicates after regular time intervals from each plot. The soil samples were analyzed for residual chlorpyrifos and population survival was estimated by antibiotic plating and REP-PCR as done before (Sections 4.10.2, 4.10.3, 4.10.4 and 4.10.5).

The sampled plants were air-dried for a few minutes and crushed with pestle –mortal. The powdered plant samples (10gm) were left overnight in acetone (100ml). They were re-suspended in 5% (w/v) brine solution (500ml) so as to dilute aqueous phase, and partitioning was done using 75ml dichloromethane (twice) and 75ml hexane (twice) in a separating funnel (1000ml). After drop-wise elution of the organic phase in a beaker, and its

evaporation to 5ml, the eluent was mixed with silica gel (20gms), activated charcoal (5gms) and anhydrous sodium sulfate (20gms) in a beaker and wet packing of this mix was done in a column with dichloromethane (DCM). 150ml DCM was added on top and drop-wise elution was again carried out, followed by elution with DCM/Hexane (1:1). The samples were dried by rotary flash evaporation, dissolved in 5ml acetone and their GC analysis (Section 4.6.2) was done. A column check with standard chlorpyrifos (50ug) was also done to know the efficiency of extraction by this method using columns and to know whether chlorpyrifos adheres to the charcoal or is eluted out.

4.11 Molecular Techniques

4.11.1 Chromosomal DNA isolation

DNA isolation was carried out by ROSE (rapid one step extraction) method. A loopful of cells of isolates was inoculated into 5ml LB media tubes respectively and incubated at 37°C, 60rpm overnight in a roller drum. After 24hours, mid-log phase cells (2ml) in eppendorf tubes were harvested in a micro centrifuge at 8000rpm, 5min. The supernatant was discarded and the pellet was washed with TE (10/1) buffer (pH8.0). To the pellet, 200µl of ROSE solution was added. These eppendorf tubes were incubated at 90°C for 20min with intermittent shaking and immediately chilled on ice for 5min. This was followed by the addition of 1ml of chloroform/ isoamylalcohol (24:1). The tubes were then kept at room temperature for 30min without disturbing, followed by centrifugation at 10000g for 10min. The top layer containing DNA was siphoned into fresh eppendorfs and treated with DNase free RNase (2µl of 10mg/ml) for half an hour at 37°C incubator without shaking. The DNA was then precipitated with 2.5 volumes of chilled ethanol, at –20°C for an hour. This was centrifuged at top speed for 10min, the supernatant was discarded and the pellet was thoroughly dried in an inverted position in a tissue paper till the adhering drops of fluid are removed. The pure chromosomal DNA was then dissolved in TE (10/1) buffer (50µl, 10mM, pH 8.0). Agarose gel electrophoresis was carried out to visualize this DNA by standard methods (Sambrook *et al*, 1987; Sections 4.11.5.1 and 4.11.5.2). The isolated DNA was quantified and its purity checked by taking ratio of absorbance at 260nm to absorbance at 280nm.

4.11.2 Plasmid DNA extraction

Plasmid isolation of the transformed *E.coli* cells was carried out by the method of Birnboim and Doly (1979). A single transformed colony of each isolate or overnight grown culture of bacterial isolates was inoculated into 2ml LB medium containing Ampicillin (50 µg/ml) in loosely capped 15ml tubes respectively, and these tubes were incubated at 37°C, 60rpm in

a roller drum overnight. The culture was incubated at 37 °C with vigorous shaking until the culture reached the $O.D_{600}$ of 0.5. The culture was centrifuged at 10,000 x *g* for 10 minutes 1.5ml of each culture was taken in separate eppendorf tubes, and centrifugation was carried out in a microfuge at 8000rpm for 5minutes. The supernatant(s) was discarded, and the pellet was thoroughly dried in an inverted position in a tissue paper till the adhering drops of fluid are removed. The pellets were re-suspended in 200 μl of ice-cold solution I (Appendix) by vigorous vortexing to ensure uniform dispersion of the bacterial pellet into this solution. Freshly prepared solution II (200 μl (Appendix)) was then added followed by mixing of the contents by gentle inversion of the tubes for 5mins. The tubes were stored on ice for 5mins, followed by addition of ice –cold solution III (300 μl (Appendix)). The contents of the tubes were mixed again by gentle inversion for 5mins followed by storage on ice for 10mins. The tubes were centrifuged at 12000rpm for 10minutes in a microfuge. The supernatant(s) were then carefully transferred into fresh eppendorf tubes and the double-stranded plasmid DNA was precipitated with equal volume of isopropanol. The contents were mixed well and allowed to stand at room temperature for 15min, followed by centrifugation at 10000 rpm for 10min in a microfuge. The supernatant was then decanted and the pellet was thoroughly dried in an inverted position in a tissue paper till the adhering drops of fluid are removed. The pellets were further air-dried to remove any traces, and finally dissolved in 50μl TE buffer (10mM, 10/1, pH8.0 (Appendix)). Agarose gel electrophoresis was carried out to visualize this DNA by standard methods (Sambrook *et al*, 1987; Sections 4.11.5.1 and 4.11.5.2).

4.11.3 Ligation

The 16S rDNA PCR amplified fragment was cloned into pDrive-cloning vector using a QIAGEN PCR Cloning Kit (Qiagen, India). The components of the kits, 1μl of pDrive Cloning Vector (50 ng/μl), and 5μl of Ligation Master Mix (2x solution) were mixed well for each reaction in 0.5μl eppendorf tubes and 4μl of amplified product was added into each of the tubes, for each of the isolates, total volume was made to 10μl and these tubes were incubated at 8°C in a rotary water bath overnight. The ligated sample was checked by standard methods (Sambrook *et al*, 1987; Sections 4.11.5.1 and 4.11.5.2). Isolation of plasmid DNA from original isolates was done using plasmid isolation kit (QIAprep Spin Miniprep kit from Qiagen) and crosschecking of the inserted ligation product into transformed cells was also done using this kit.

4.11.4 Transformation

4.11.4.1 Preparation of competent cells

Transformation was carried out by the $CaCl_2$ method (Cohen *et al*, 1972). A loop of *E.coli DH5∝* was inoculated into a LB flask (20ml) and left incubated at 37°C, 130 rpm in a rotary shaker. After 24 hours, 200µl was transferred into another fresh LB flask (20ml) and left incubated at 37°C, 130 rpm in a rotary shaker for 2 ½ hrs, till O.D reached nearly 0.5. The 20ml culture was transferred to sterile, disposable, ice-cold 50ml polypropylene tubes and the culture was cooled to 0°C in ice for 10 minutes. The cells were recovered by ultracentrifugation at 8000rpm 10min 4°C, media was decanted, traces of media were removed and the pellet was thoroughly re-suspended in 10ml ice-cold $CaCl_2$ (100mM) and left on ice for 10minutes. Centrifugation (8000rpm, 10min, 4°C) was again carried out followed by pellet dissolution in 1ml $CaCl_2$ (100mM). This was kept on ice for 2 ½ hours.

4.11.4.2 Transformation of competent cells

Aliquots of 100µl of competent cells were distributed into sterile, pre-chilled eppendorf tubes, followed by addition of 4µl ligated product. They were mixed gently and left on ice for 30minutes. After half an hour, the mixture was heat pulsed without agitation at 42°C for 90 seconds in a pre-heated circulating water bath followed by rapid cooling on ice for 2minutes. 1ml LB was added to these eppendorf tubes, and they were incubated at 37°C, without shaking for 1½ hour. 100µl of these transformed cells was then plated on Luria agar plates containing Ampicillin (50µg/ml), X-Gal (2ml/1L from stock) and IPTG (500µl/1L). These plates were incubated at 37°C in incubator overnight. The isolated white colonies were picked up the next day and patched onto fresh Luria agar plates containing Ampicillin (50µg/ml), X-Gal (2ml/1L from stock) and IPTG (500µl/1L). These plates were incubated at 37°C in incubator overnight. Transformed colonies were picked up.

4.11.5 Electrophoresis

4.11.5.1 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out with 0.8% or 1% agarose in TAE buffer (Appendix). Agarose was dissolved in TAE (pH 8.0) buffer by heating for a minute in a microwave. The solution was cooled and poured into the casting tray with the comb, the teeth of which formed the wells. After the gel was set, comb was removed and the gel was placed in the electrophoresis tank with TAE (pH 8.0) buffer. DNA sample was mixed with the loading dye (Appendix) and were loaded into the slot of submerged gel. The gel was run by applying constant current of 60 V for the desired time.

4.11.5.2 Agarose gel staining

Agarose gels were stained with ethidium bromide at a concentration of 100μg/ml in distilled water and visualized. The fingerprints were photographed in the gel-doc system.

4.12 Studies on the genetic aspects

4.12.1 Molecular characterization and Diversity studies using 16S rDNA analysis

PCR amplification using universal primers of 16S rDNA was carried out. The extracted and quantified genomic DNA was appropriately diluted such that 1μl equaled 10ng. PCR was then carried out with 10ng of the respective DNA under conditions standardized in our laboratory. PCR amplification of the DNA was performed in a Perkin-Elmer Thermocycler (Gene Amp PCR system 9700). The reaction mixture containing 25nmol of each of the universal primers for 16S rDNA - 8F and 1429R, 1.5mM $MgCl_2$, 0.2mM dNTPs, and 1.5 Units *Taq Polymerase* (Genetix) and 10ng DNA was amplified at 94°C for 1min, 55°C for 30sec, and 72°C for 30sec, in 35 cycles. The primers amplified the 16S rDNA sequences, universally present in each organism. The amplified products were analyzed by standard procedures (Sambrook *et al*, 1987; Sections 4.11.5.1 and 4.11.5.2).

4.12.2 Construction of Phylogenetic trees

The sequences of the isolates obtained from Bangalore genei were used to construct the phylogenetic trees to study the diversity of chlorpyrifos degraders and to know the relatedness of these species with others in the database. For this Basic Local Alignment Search Tool (BLAST) was used followed by analysis with Molecular Evolutionary Genetics Analysis (MEGA 3.1) and ClustalW software tools and the phylogenetic tree was constructed.

4.12.3 Amplification of opd gene

The amplification of the genomic DNA was carried out with the 10nmol of *opd, opdA and mpd* primers respectively, separately for each reaction mix, 2.5mM $MgCl_2$, 0.2mM dNTPs, and 1Unit Taq Polymerase (Genetix). The temperature conditions of PCR were 94°C for 1min, 60°C for 1min and 72°C for 2min, 35 cycles in Thermocycler (GeneAmp PCR system 2700). The amplified products were analyzed by standard procedures (Sambrook *et al*, 1987; Sections 4.11.5.1 and 4.11.5.2).

5. RESULTS

5.1. Collection of Soil samples

Eleven soil samples were collected from the various paddy fields of Rajpura (3 samples), Dera Bassi (3 samples), Samana (2 samples), Barnala (2 samples) and a chlorpyrifos formulation site in Dera Bassi (1 sample) in Punjab, India (Table 2). The residual chlorpyrifos in the ten paddy field soil samples was in the range of 0.75-1.00 mg/ kg and the residual chlorpyrifos in the soil from mixing site of Dera Bassi was 4.8 mg/ kg. These soils were used for the development of aerobic consortia by enrichment and isolation of chlorpyrifos degrading bacteria. After repeated transfer through five transfers, it was observed that among these eleven soils only microbes from five soils showed the ability to grow on chlorpyrifos (50 mg/ L) in basal medium. This included soil from Barnala (Sample 10 and 11 in Table 2) and Dera Bassi (Sample 4 and 5 in Table 2) including one from the chlorpyrifos formulation site (Sample 7 in Table 2) in Dera Bassi, Punjab. Five developed consortia showing chlorpyrifos degradation were designated as AC, BC, DC, Q1 and Q2 and grown repeatedly on basal media containing chlorpyrifos (50 mg/L).

5.2 Screening of consortia for chlorpyrifos degradation

The five developed consortia were grown in basal medium with varying concentration of chlorpyrifos (25, 50, 75, 100, 200, 300 and 500 mg/L). Tolerance studies indicated that consortia BC, DC, and Q2 (Table 3) were tolerant to even 200 mg/L chlorpyrifos while consortia AC and Q1 were tolerant to only 50 mg/ L chlorpyrifos. It was seen that growth decreased with increase in chlorpyrifos concentration, being negligible at 300 mg/ L and 500 mg/ L. Best growth was seen for all the five consortia at 50 mg/L chlorpyrifos.

The effect of various concentration of glucose (0.01, 0.05 and 0.1% w/v) on growth was studied and at 0.1% (w/v) glucose, enough cell mass (O.D550 = 0. 78-1.01) was observed. The cell mass at 0.01% and 0.05% (O.D550 = 0.12-0.18 and 0.42-0.46) was less and so 0.1% (w/v) glucose was selected for further studies (data not shown).

Growth studies were conducted for seven days with consortia Q2 along with glucose utilization and chlorpyrifos degradation studies. It was observed that glucose was utilized completely within the first 24 hours with growth of 1.12 (OD_{550}) units and degradation of 1.9% for consortia Q2. After three days the chlorpyrifos degradation increased to 8% which further increased to 32% after seven days as compared to 2% and 8% in uninoculated

control (Fig 4) while the growth stabilized at 0.81 (OD_{550}) units after the third day. The experiments were also done for consortia AC, BC, DC and Q1 (data not shown) and it was seen that the trend followed by all the five consortia was similar.

The rate of chlorpyrifos degradation was estimated by growing the consortia AC, BC, DC, Q1 and Q2, in basal medium containing 50 mg/ L chlorpyrifos and chlorpyrifos was extracted at regular time intervals and quantified by gas chromatography to estimate the degradation efficiency of each consortium. Among the five consortia, consortia Q1 and Q2 showed the maximum degradation of 51% and 55% after 14 days of incubation, which further increased to 72 and 70% respectively after 21 days as compared to 15% in uninoculated control after 21 days. Consortia DC showed degradation ability of only 34% on day 14, which increased to 60% after 21 days. Consortia AC and BC showed degradation of 54% and 46% respectively after 21 days, as compared to uninoculated control, which showed abiotic degradation of 15% after day 21 (Fig 5). Consortia Q1, Q2 and DC were the most efficient among the five consortia and were selected for isolation of bacterial isolates.

5.3 Isolation and screening of chlorpyrifos degrading Bacteria

Bacterial isolates capable of degrading chlorpyrifos were obtained by serial dilution of the three best consortia DC, Q1 and Q2 on basal agar plates with chlorpyrifos (50 mg/ L). A total of 55 isolates were obtained on basal agar plates, of which only 25 were positive for chlorpyrifos degradation in aqueous medium with degradation ranging between 2% and 86% (Fig 6). Among these 25 isolates the best 15 isolates showing ≥40% degradation of chlorpyrifos revealed degradation of 71, 68, 78 and 50% for isolates P, M19, M119 and Q2c on day 10, as compared to 8% in control. After 20 days the degradation increased to 86, 83, 84 and 84% respectively (Fig 7a) as compared to 23% in control. Isolates D113, Q1a, Q2a, and Q2b were also found to be efficient with degradation of 48, 65, 64 and 37% respectively after 10 days which further increased to 77, 81, 82 and 80% respectively (Fig 7b) after 20days of incubation. Isolates D15, D234, D320, D244, M117, Q1g and Q1m were found to be less efficient showing <75% degradation of chlorpyrifos after 20days (Fig 7 c & d). The growth of isolates P, M19, M119, D113, Q1a, Q2a, Q2b and Q2c was ~23% higher (data not shown) than that of the rest, concomitant with their chlorpyrifos degrading ability.

When the eight isolates showing efficient degradation were grown in basal medium with varying concentration of chlorpyrifos (25, 50, 75, 100, 200, 300 and 500 mg/L), tolerance studies indicated that isolates P, M119, D113, Q1a, Q2a and Q2c were tolerant to even 200 mg/L chlorpyrifos (Table 4) while isolates M19 and Q2b were tolerant to only 50 mg/ L chlorpyrifos. It was seen that growth decreased with increase in chlorpyrifos concentration,

being negligible at 300 mg/ L and 500 mg/ L. Best growth was seen for all the eight isolates at 50 mg/L chlorpyrifos (Table 4).

5.4 Morphological and 16S rDNA analysis

Morphological studies with the best eight chlorpyrifos degrading isolates, isolates P, Q1a, Q2a, Q2b and Q2c were gram negative rods, Isolate M19 was a gram negative coccus while isolates M119 and D113 were gram positive rods (Table 5).

Based on an analysis of the relatedness of their 16S rDNA sequence with others in the database, P was identified as *Pseudomonas sp.*, M19 as *Brucella sp.*, M119 as *Bacillus sp.*, D113 as *Bacillus sp.*, Q1a/Q2a as *Klebsiella sp.*, Q2b as *Serratia sp.* and Q2c as *Pseudomonas sp.* (Fig 8).To distinguish the species during the experiments, P has been named as *Pseudomonas sp.1*, Q2c as *Pseudomonas sp.2,* M119 as *Bacillus sp.1 and* D113 as *Bacillus sp.2.*

Among the eight isolates showing good chlorpyrifos degradation, *Pseudomonas sp.1, Brucella sp.*, *Bacillus sp.1 and Pseudomonas sp.2* were the most efficient. These isolates were chosen for uptake and microcosm studies.

5.5 Antibiotic profile studies

Antibiotic profile studies were carried out to select the first marker for assessing and tracking introduced population in soil (Table 6). The various isolates were screened for their resistance to ampicillin, kanamycin, carbenicillin, chloramphenicol and nalidixic acid and it was found that all the isolates except the *Pseudomonads* were resistant to nalidixic acid; *Brucella sp.* and *Bacillus sp.1* were resistant to chloramphenicol also. *Bacillus sp.1* and *Pseudomonas sp.2* were resistant to carbeniccilin and all the isolates were found to be resistant to kanamycin. Hence, kanamycin (50µg/ml) was chosen as the first marker for studying the population in soil microcosm and *in situ* soil studies.

5.6 Optimization of Microcosm conditions

Soil moisture content and cell mass of the inoculum were optimized for microcosm studies for all the three consortia and all the four isolates.

Soil moisture content was varied as 15%, 30%, 40% and 50% for consortia Q2 *and Pseudomonas sp.2* respectively and after 5, 10 and 20 days residual chlorpyrifos and cell number were estimated (Fig 9 a & b).

Data showed that as the soil moisture was increased from 15 to 30 there was an increase in chlorpyrifos degradation from 38 & 43% respectively to 68 & 82% respectively for consortia Q2 *and Pseudomonas sp.2* after 20 days. At 40% and 50% moisture, the rates of

degradation were 36 & 57% and 69 & 70% respectively after 20 days for consortia Q2 *and Pseudomonas sp.2*.

Population survival indicated that population at 15% moisture was 30 x 10^9 & 18 x 10^9 (log value=10.48 & 10.26) cfu/ gm soil respectively for consortia Q2 *and Pseudomonas sp.2* while at 30, 40 and 50% moisture, the population was 43 x 10^{11}, 6.7x 10^8 & 1.34 x 10^5 and 66 x10^{11}, 13.18x 10^8 & 1.41 x 10^5(log value=12.64, 7.83 & 5.13 and 14.82, 9.12 & 5.15) cfu/ gm soil respectively for consortia Q2 *and Pseudomonas sp.2* after 20 days. Similar trends were observed with consortia DC and Q1, *Pseudomonas sp.1, Brucella sp.* and *Bacillus sp.1* (data not shown).

Cell mass (10^6 cells/ gm soil, 10^7 cells/ gm soil, 10^8 cells/ gm soil and 10^9 cells/ gm soil) of consortia Q2 *and Pseudomonas sp.2* were respectively inoculated into chlorpyrifos (50mg/ L) contaminated soils with 30% moisture content and chlorpyrifos degradation was followed over a period of 20days (Fig 10 a & b). When rate of degradation was studied with increase in population from 10^6 cells/gm soil to 10^8 cells/ gm soil, rate of degradation increased from 28 to 74% for consortia Q2 and 28 to 80% for *Pseudomonas sp.2* respectively while it was similar for 10^8 and 10^9 cells/gm soil (74% and 76%) respectively. Similar trends were observed with consortia DC and Q1, *Pseudomonas sp.1, Brucella sp.* and *Bacillus sp.1* (data not shown).

Moisture content of soil as 30% and cell mass of inoculum 10^8 cells/ml were chosen for degradation of chlorpyrifos in soil.

Experiments with the population of kanamycin resistant microorganisms indicated the presence of indigenous kanamycin resistant microorganisms in inoculated soil and so molecular markers using REP-PCR was standardized to distinguish the inoculated population from the indigenous soil microorganisms.

5.7 Fingerprinting using molecular markers i.e. Rep-PCR (PCR based on Repetitive Extragenic Palindromic sequences)

Native DNA of the isolates *Pseudomonas sp.1*, *Brucella sp.*, *Bacillus sp.1* and *Pseudomonas sp.2* was amplified by REP-PCR to develop a strain specific fingerprinting profile (photo-1) and this profile was compared with that of the profile of enumerated isolates from inoculated soil.

5.8 Soil studies under laboratory conditions

Soil studies were conducted under sterile and non-sterile conditions with consortia and isolates to ascertain their degradation ability under natural soil conditions. Soil (100gm), contaminated with chlorpyrifos (50 mg/ L) was inoculated with 2 x 10^8 cells/ gm soil of consortia DC, Q1 & Q2 and *Pseudomonas sp.1, Brucella sp.*, *Bacillus sp.1 & Pseudomonas*

sp.2 respectively under sterile, controlled laboratory conditions. Soil samples were withdrawn after regular time intervals, residual chlorpyrifos was extracted with hexane: acetone (10:1) and analyzed for residual chlorpyrifos by GC-ECD.

Consortia Q1 and Q2 showed degradation of 51% each after 10 days under sterile conditions which further increased to 69 & 77% respectively after 30 days (Fig 11a). Consortia DC showed degradation of 32% after 10 days which increased to 43%, as compared to 29% in uninoculated control after 30 days of incubation. Among the four isolates, *Pseudomonas sp.1& Pseudomonas sp.2* showed 38 & 62% degradation after 10 days which further increased to 91 and 89% respectively after 30 days, while *Brucella sp. & Bacillus sp.1* showed 59 & 46% degradation after 10 days which further increased to 74 & 79% respectively after 30 days under sterile conditions as compared to 33% degradation in uninoculated control after 30 days (Fig 11c).

Under non-sterile conditions, consortia DC, Q1 and Q2 degraded 28, 49% and 61% chlorpyrifos on day 12 which further increased to 70, 87 & 86% respectively as compared to 33% in uninoculated control, after 30 days of incubation (Fig 11b) while *Pseudomonas sp.1, Brucella sp.*, *Bacillus sp.1 and Pseudomonas sp.2* showed degradation of 42, 44, 56 & 62 % degradation respectively after 10 days which further increased to 89, 87, 85 and 92% respectively as compared to 34% in uninoculated control, after 30 days of incubation (Fig 11d).

5.9 Uptake studies

Based on aqueous and soil degradation studies *Pseudomonas sp.1*, *Brucella sp.*, *Bacillus sp.1* and *Pseudomonas sp.2* showing the best degradation ability under both aqueous conditions and efficient degradation in soil were selected for uptake studies. *Pseudomonas sp.1*, *Brucella sp.*, *Bacillus sp.1* and *Pseudomonas sp.2* were grown in luria broth, harvested, washed and re-suspended in phosphate buffer and starved overnight for 14 hours and these starved whole cells (1mg/ml protein) were used for chlorpyrifos utilization studies. Initial experiments showed that after 6 hours of incubation, utilization of chlorpyrifos was 13, 50, 45 and 63% with *Pseudomonas sp.1*, *Brucella sp.*, *Bacillus sp.1* and *Pseudomonas sp.2* respectively (Fig 12) as compared to 2% in control without bacterial cells.

5.9.1 *Uptake Studies of chlorpyrifos with whole cells*

Experiments were carried out to study the effect of induction with chlorpyrifos on chlorpyrifos uptake, when the cells were induced at growth and when they were induced after growth, as whole cells. For the first experiment, bacterial cells of *Pseudomonas sp.1, Brucella sp.*, *Bacillus sp.1 and Pseudomonas sp.2* were grown in Luria broth, harvested, washed and re-

suspended in phosphate buffer, starved overnight and divided into two sets for uptake studies. One set of starved whole cells was inoculated into phosphate buffer containing 50 mg/L chlorpyrifos while the other set was induced with 5 mg/L chlorpyrifos for 2 1/2 hours and uptake was carried out in similar way. A third set of cells was induced during growth with chlorpyrifos and then uptake studies were carried out. Un-induced starved whole cells (cell protein 2mg/ml) of *Pseudomonas sp.1*, *Brucella sp.*, *Bacillus sp.1* and *Pseudomonas sp.2* showed 52, 43, 36 and 62% utilization of chlorpyrifos respectively after six hours of incubation which further increased to 84, 68, 60 and 87% at twelve hours as compared to uninoculated control which showed 4 and 13% degradation respectively at sixth and twelfth hour (Fig 13a). After six hours, induced cells of *Pseudomonas sp.1*, *Brucella sp., Bacillus sp.1* and *Pseudomonas sp.2* utilized 68, 50, 45 and 69% of chlorpyrifos respectively which further increased to 93, 77, 69 and 100%, respectively at 12^{th} hour (Fig 13b). Utilization of chlorpyrifos with whole cells of *Pseudomonas sp.1*, *Brucella sp. and Bacillus sp.1 and Pseudomonas sp.2*, induced during growth, showed 65, 50, 45 and 67% uptake which further increased to 90, 75, 67 and 100% respectively at 12^{th} hour (Fig 13c).

5.9.2 *Replacement studies*

Product analysis during utilization of chlorpyrifos did not show the formation of any peak of known compounds for *Pseudomonas sp.1*, *Brucella sp. and Bacillus sp.1*, while one peak was observed with whole cells of *Pseudomonas sp.2* at 4.3 minutes which corresponded to the peak of standard 3, 5, 6-trichloro-2-pyridinol (TCP) identified on the basis of retention time analyzed by GC (Fig 14). Substrate replacement and uptake of TCP with whole cells of *Psudomonas sp.2* showed 40% utilization after six hours which further increased to 67% utilization after 12 hours of incubation (Fig 15). Formation of any metabolite during the utilization of TCP was not detected.

Table 2: Source of environmental samples for enrichment studies

Samples were collected from various fields in villages of Punjab, India, as indicated in column 2 (site) for development of aerobic consortium capable of degrading chlorpyrifos

Sample No.	Soil collection Site
1	Paddy field, Rajpura
2	Paddy field, Rajpura
3	Paddy field, Rajpura
4	Paddy field, DeraBassi
5	Paddy field, DeraBassi
6	Paddy field, DeraBassi
7	Industrial mixing cum dumping site, DeraBassi
8	Paddy field, Samana
9	Paddy field, Samana
10	Paddy field, Barnala
11	Paddy field, Barnala

Table 3: Tolerance level of various consortia to different concentrations of chlorpyrifos

Consortia were grown in basal medium with 0.1% (w/v) glucose and various concentrations of chlorpyrifos (25, 50, 75, 100, 200, 300 and 500 mg/ L respectively) and growth was monitored visually (section 4.7.2).

- \- indicates no growth
- \+ indicates moderate growth
- ++ indicates high growth

Culture	Chlorpyrifos (mg/L)						
	25	50	75	100	200	300	500
Control	-	-	-	-	-	-	-
AC	+	+	-	-	-	-	-
BC	++	++	+	+	+	-	-
DC	++	++	+	+	+	-	-
Q1	++	++	-	-	-	-	-
Q2	++	++	+	+	+	-	-

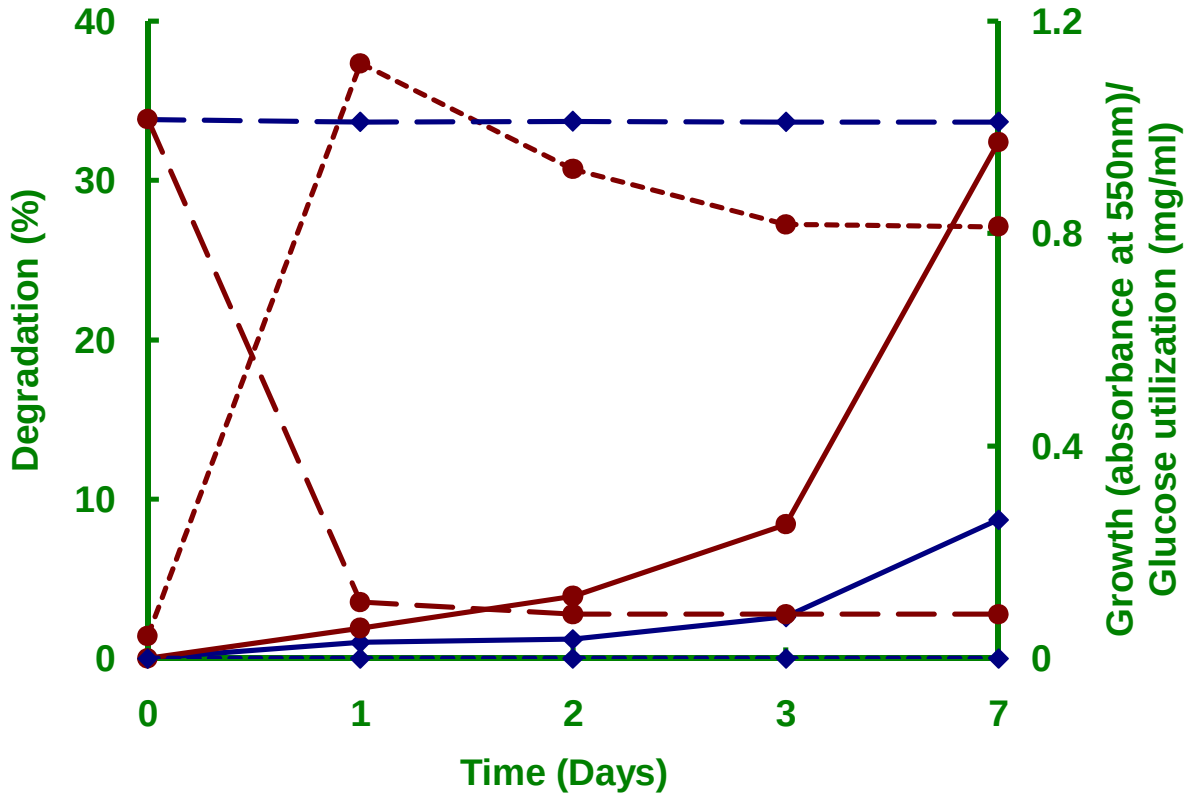


Fig 4: Growth, chlorpyrifos degradation and glucose utilization of consortia.

Developed consortia Q2 was grown in basal medium containing 0.1% (v/v) glucose and 50 mg/L chlorpyrifos. Undegraded chlorpyrifos was extracted with ethyl acetate: phosphoric acid (97:3), dried and analyzed using Gas chromatography with electron capture detector (section 4.6.3).Glucose utilization was studied by DNSA method along with measurement of growth at 550nm (section 4.6.1). Bold lines indicate degradation, dotted lines indicate growth, and serrated lines indicate glucose utilization.

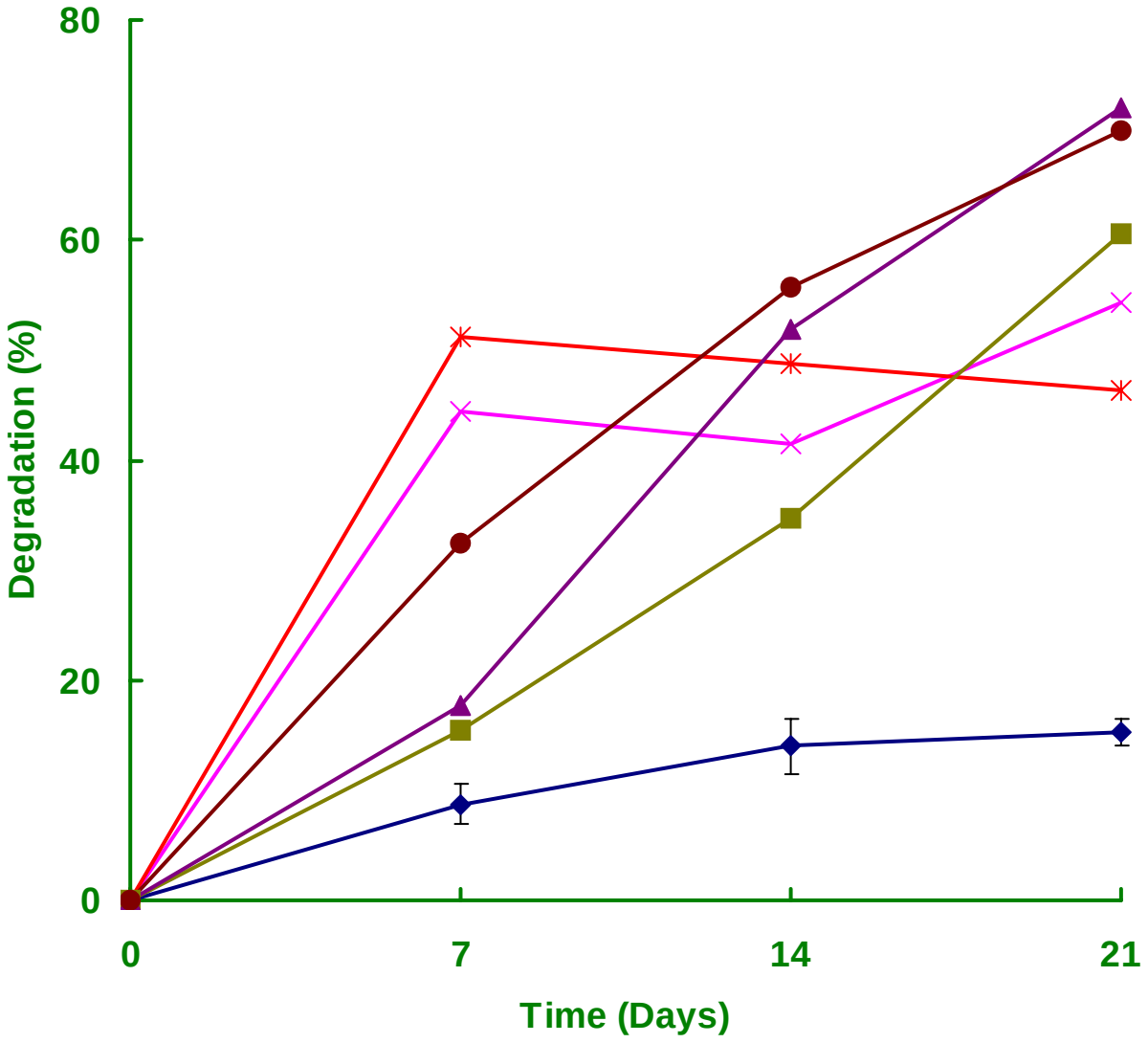


Fig 5: Chlorpyrifos degrading ability of consortia under aqueous conditions

Consortia were developed at 37°C by enrichment procedures from soil samples, in basal medium containing 0.1% (v/v) glucose and 50 mg/L chlorpyrifos. Undegraded chlorpyrifos was extracted with ethyl acetate: phosphoric acid (97:3), dried and analyzed using Gas chromatography with electron capture detector (section 4.6.2).

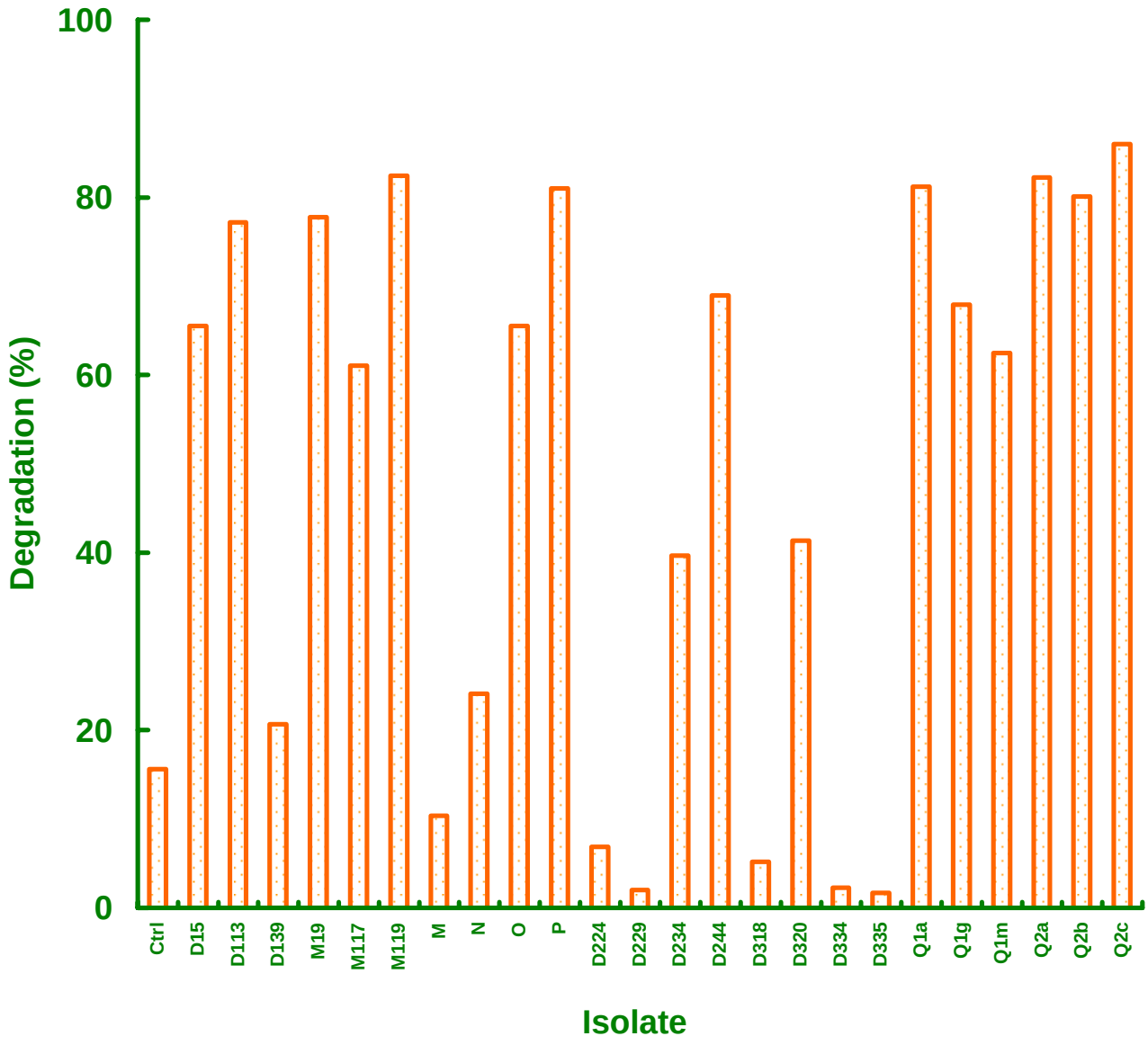


Fig 6: Chlorpyrifos degradation by different isolates under aqueous conditions after 20 days of incubation

Consortia were dilution plated and the efficient degraders of chlorpyrifos were grown on basal medium containing 0.1% (w/v) glucose and 50 mg/L chlorpyrifos at 37 °C, 120rpm on a rotary shaker (section 4.7.1). After 20 days of incubation, the undegraded chlorpyrifos was extracted and analyzed using GC-ECD (section 4.6.2).

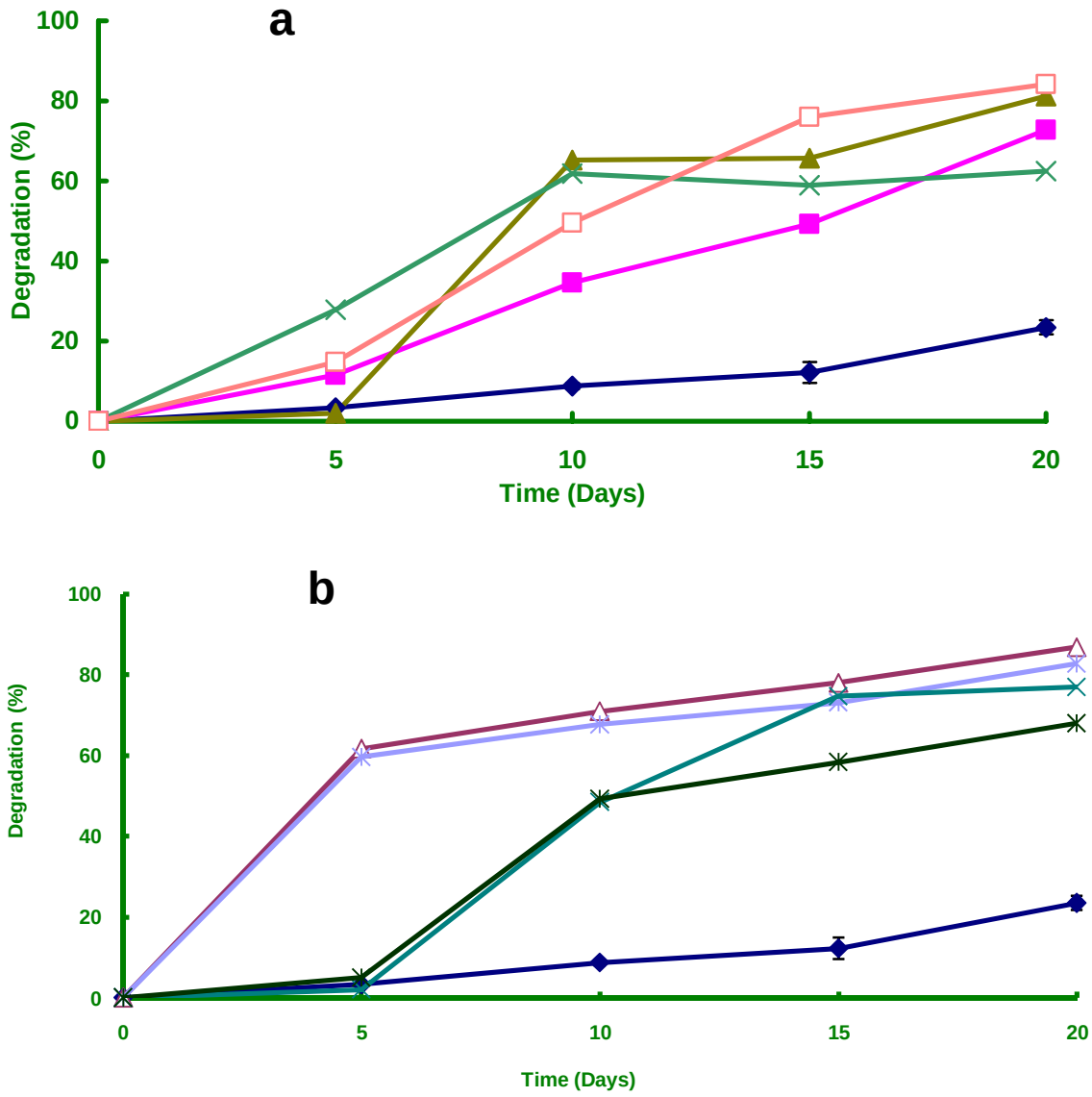


Fig 7 (a & b): Chlorpyrifos degradation by isolates under aqueous conditions

Bacterial isolates a) D320, Q1a, Q1m and Q2c b) P, M19, D113 and Q1g were grown in basal medium containing 0.1% glucose and 50 mg/L chlorpyrifos. After 5, 10, 15 and 20 days of incubation, the undegraded chlorpyrifos was extracted and analyzed using GC-ECD (section 4.6.2).

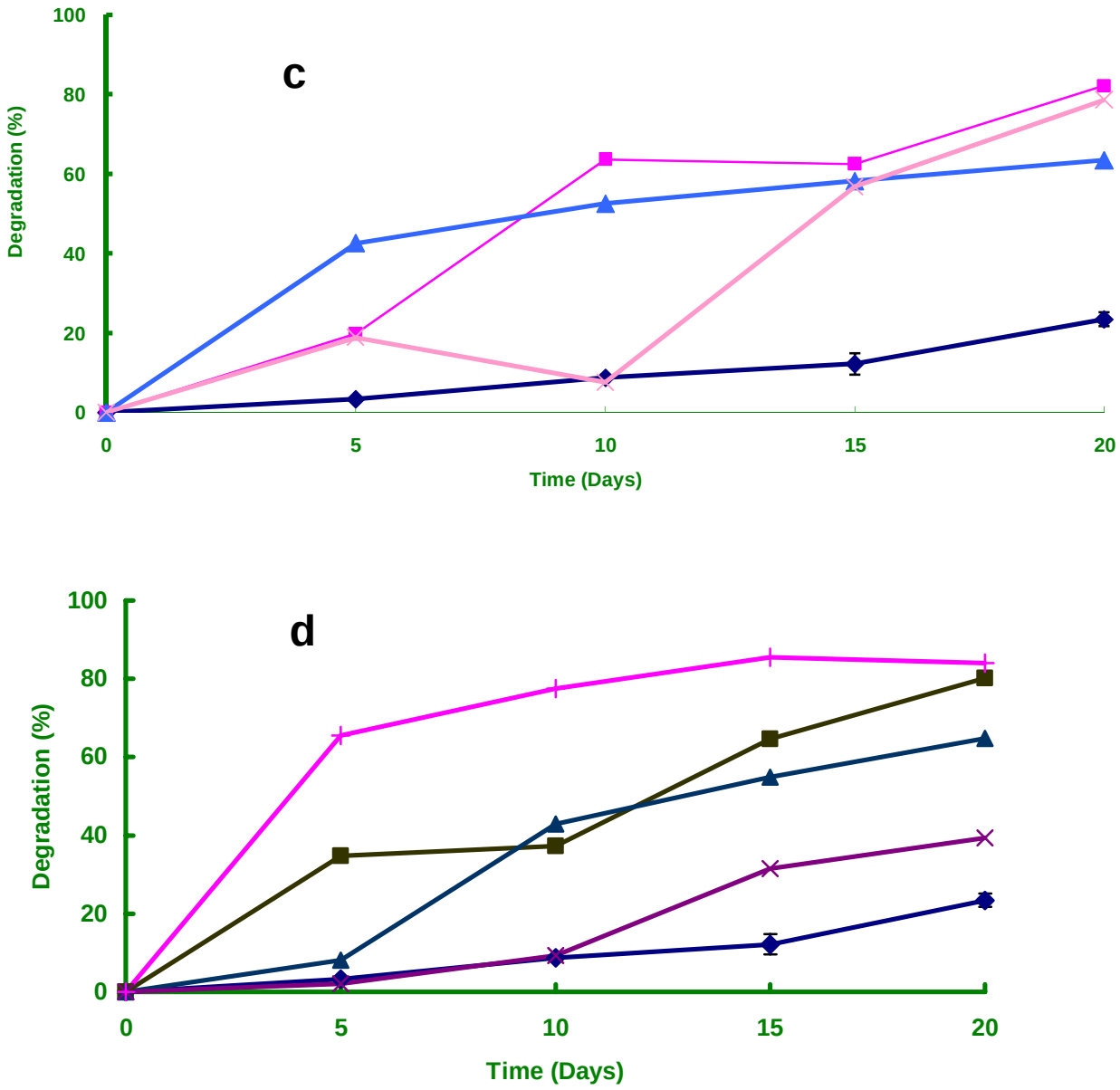


Fig 7 (c & d): Chlorpyrifos degradation by isolates under aqueous conditions

Bacterial isolates c) Q2a, M117 and D234 d) Q2b, D15, D244 and M119 were grown in basal medium containing 0.1% glucose and 50 mg/L chlorpyrifos. After 5, 10, 15 and 20 days of incubation, the undegraded chlorpyrifos was extracted and analyzed using GC-ECD (section 4.6.2).

c) Ctrl Q2a M117 D234

d) Ctrl Q2b D15 D244 M119

Table 4: Tolerance level of various isolates to different concentrations of chlorpyrifos

Isolates were grown in basal medium with 0.1% (w/v) glucose and various concentrations of chlorpyrifos (25, 50, 75, 100, 200, 300 and 500 mg/ L respectively) and growth was monitored visually (section 4.7.2).

- \- indicates no growth
- \+ indicates moderate growth
- ++ indicates high growth

Culture	Chlorpyrifos (mg/L)						
	25	50	75	100	200	300	500
Control	-	-	-	-	-	-	-
P	+	++	+	+	+	-	-
M19	+	++	-	-	-	-	-
M119	++	++	+	+	+	-	-
D113	+	+	+	+	+	-	-
Q1a	+	+	+	+	+	-	-
Q2a	+	+	+	+	+	-	-
Q2b	++	+	-	-	-	-	-
Q2c	++	++	+	+	+	-	-

Table 5: Morphological character of the eight selected isolates

The morphological character of the eight selected isolates (P, D113, M19, M119, Q1a, Q2a, Q2b and Q2c) was studied under microscope, followed by gram staining by standard procedures (section 4.7.3).

Isolate Name	Colony morphology and gram character
P	Gram-negative rods forming slimy mucous colonies with slight yellowish fluorescence
M19	Gram-negative coccus forming extremely small and distinct individual white colonies
M119	gram-positive rods forming white colonies joint together
D113	gram-positive rods forming pale/off-white joint rods
Q1a	gram-negative rods forming yellow joint colonies
Q2a	gram-negative rods forming yellow joint colonies
Q2b	gram-negative rods forming slimy red dots
Q2c	gram-negative rods forming white colonies forming chains

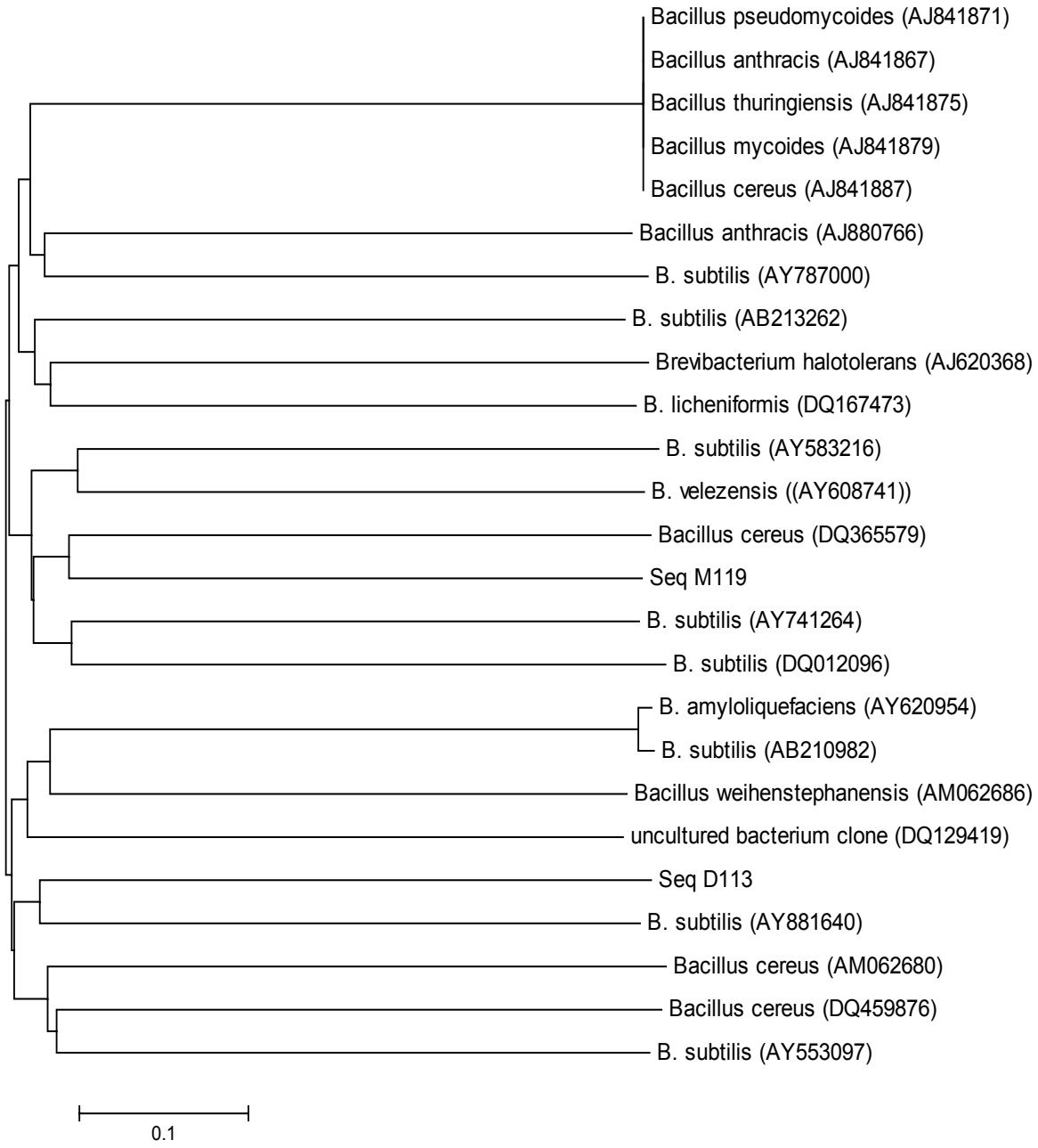


Fig 8 (a): Dendrogram depicting phylogenetic tree of firmicutes

Dendrogram depicting phylogenetic tree based on 16S rDNA gene sequences of the isolates were constructed using BLAST, MEGA 3.1 and ClustalW tools; Gene Bank accession numbers are given in brackets. Our isolates have been submitted to Gene Bank.

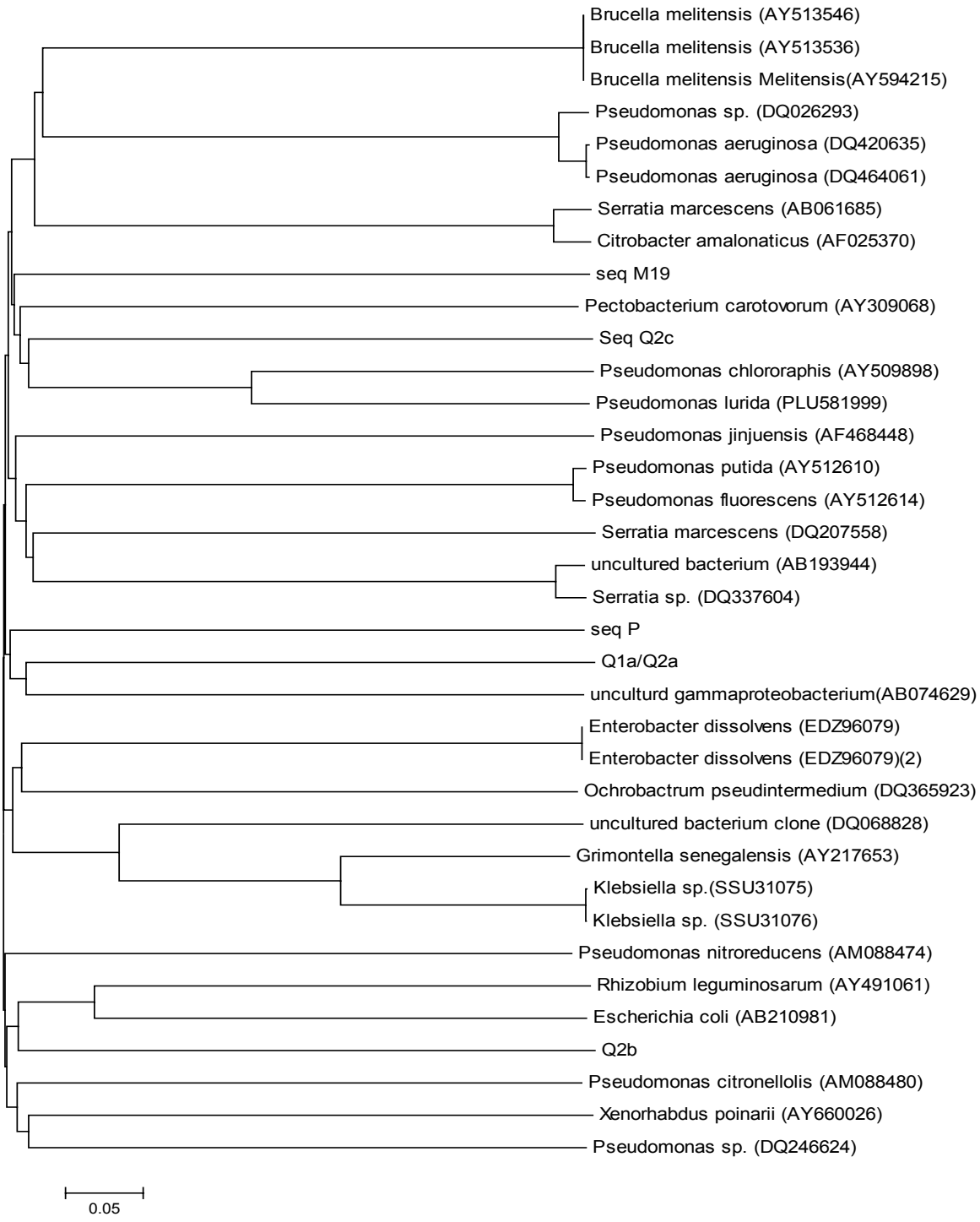


Fig 8 (b): Dendrogram depicting phylogenetic tree of gammaproteobacteria

Dendrogram depicting phylogenetic tree based on 16S rDNA gene sequences of the isolates were constructed using BLAST, MEGA 3.1 and ClustalW tools; Gene Bank accession numbers are given in brackets. Our isolates have been submitted to Gene Bank.

Table 6: Antibiotic profile of consortia and isolates capable of degrading chlorpyrifos

Consortia and isolates were plated on Luria agar plates with various antibiotics (50 μg/ml) and antibiotic resistance indicated by growth on these plates was used as screening to select the first marker for soil microcosm studies (section 4.10.1).

+++ indicates excellent growth ++ indicates good growth

+ indicates moderate growth - indicates that the growth is nil

Culture	Antibiotic (50 µg/ml)				
	Amp	Nal	Kan	Car	Chl
Con DC	-	+	++	-	+
Con Q1	-	+	+++	-	+
Con Q2	-	+	+++	+	-
Brucella sp.	-	+	+++	-	+
Bacillus sp.	-	+	+++	++	+
Pseudomonas Sp.1	-	-	+++	-	-
Pseudomonas Sp.2	-	-	+	++	-

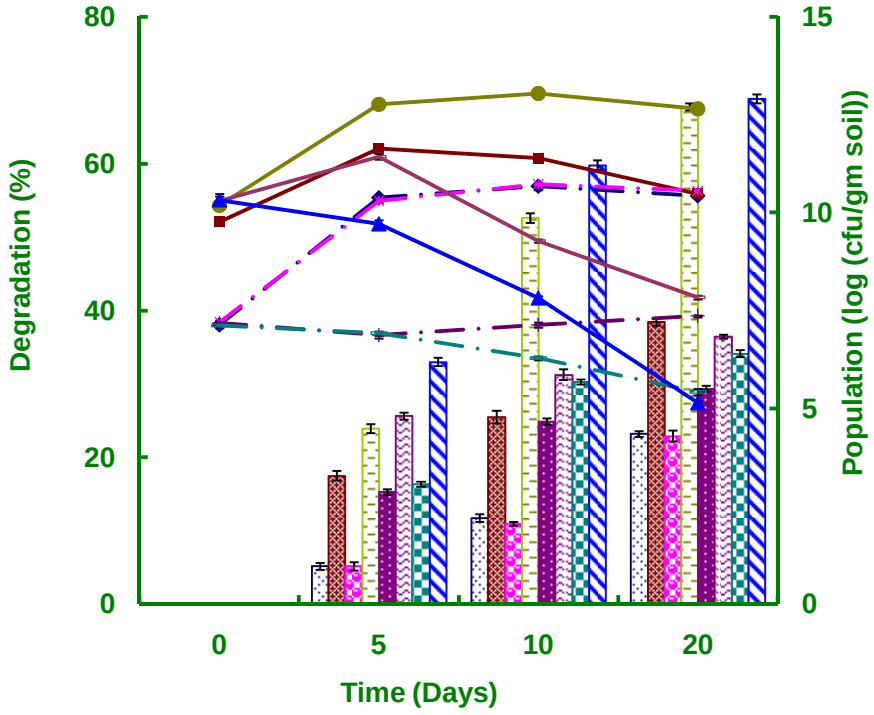


Fig 9 (a): Standardization of moisture content of soil for microcosm studies

Cells of consortia Q2 was inoculated into chlorpyrifos (50 mg/kg) contaminated soil. The moisture content of soil was varied (section 4.10.4). Soil samples harvested at various time intervals were subjected to solvent extraction (section 4.10.3) and GC-ECD analysis (section 4.6.2). Survival of population was simultaneously monitored (section 4.10.2). The bars represent degradation while the lines depict the population of inoculated culture.

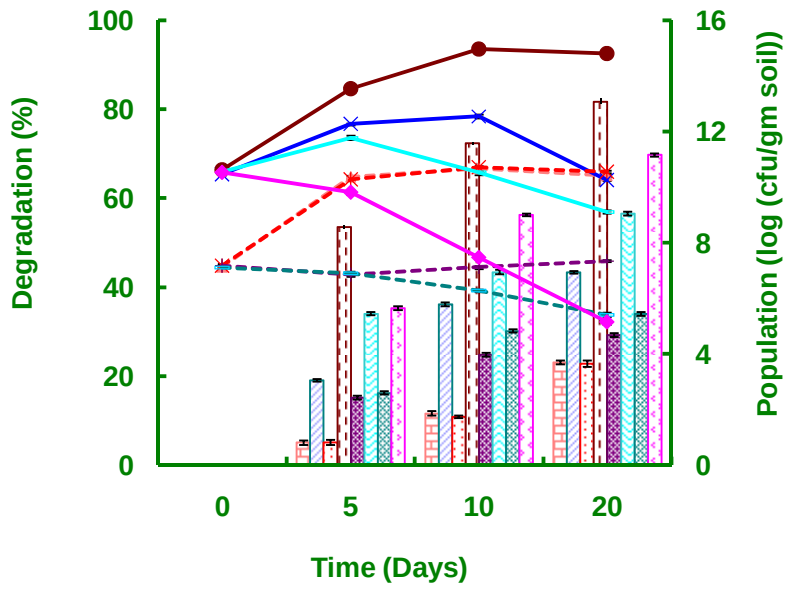


Fig 9 (b): Standardization of moisture content of soil for microcosm studies

Cells of *Pseudomonas sp.2* was inoculated into chlorpyrifos (50 mg/kg) contaminated soil. The moisture content of soil was varied (section 4.10.4). Soil samples harvested at various time intervals were subjected to solvent extraction (section 4.8.3) and GC-ECD analysis (section 4.6.2). Survival of population was simultaneously monitored (section 4.10.2). The bars represent degradation while the lines depict the population of inoculated culture.

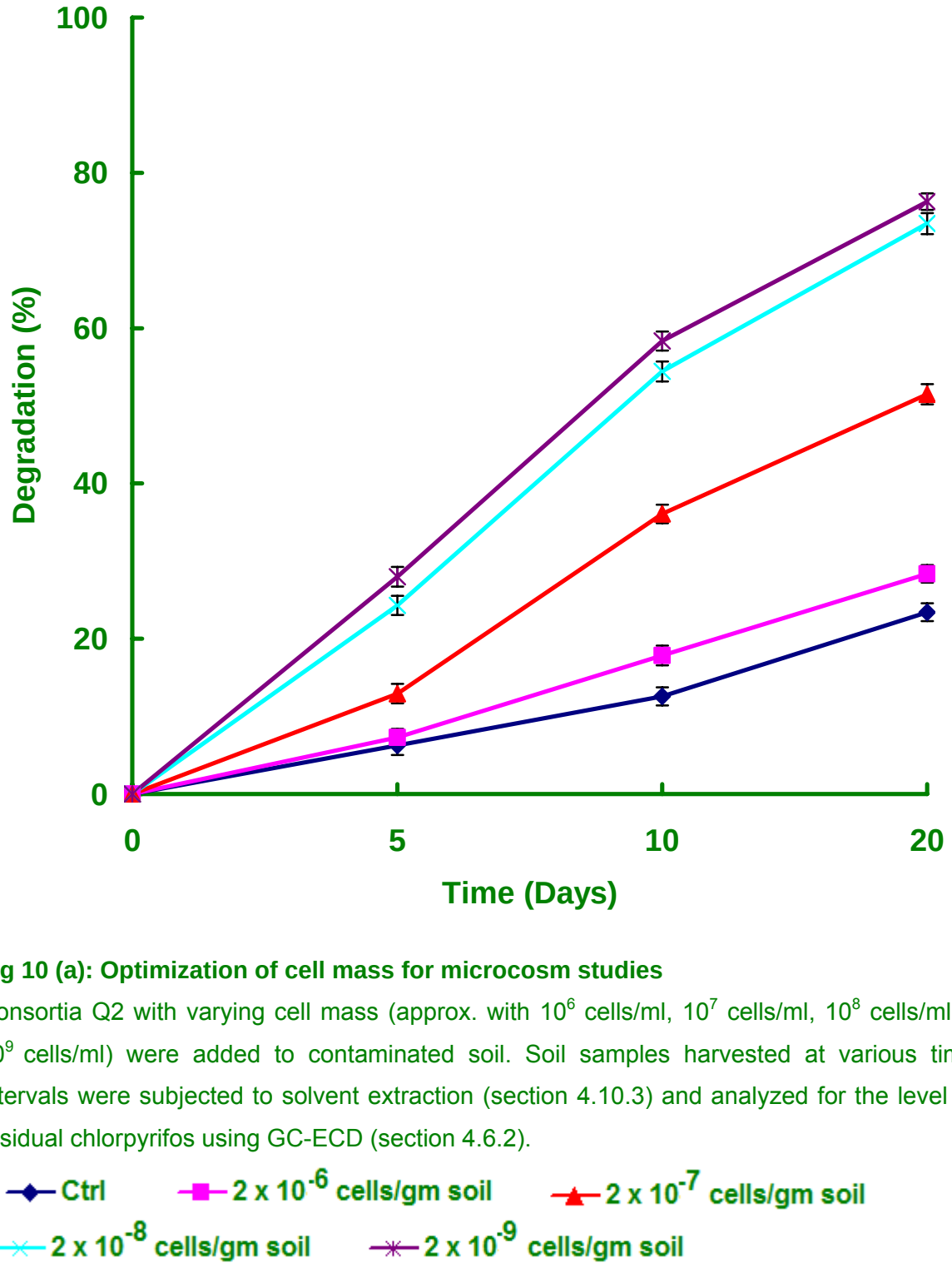


Fig 10 (a): Optimization of cell mass for microcosm studies

Consortia Q2 with varying cell mass (approx. with 10^6 cells/ml, 10^7 cells/ml, 10^8 cells/ml & 10^9 cells/ml) were added to contaminated soil. Soil samples harvested at various time intervals were subjected to solvent extraction (section 4.10.3) and analyzed for the level of residual chlorpyrifos using GC-ECD (section 4.6.2).

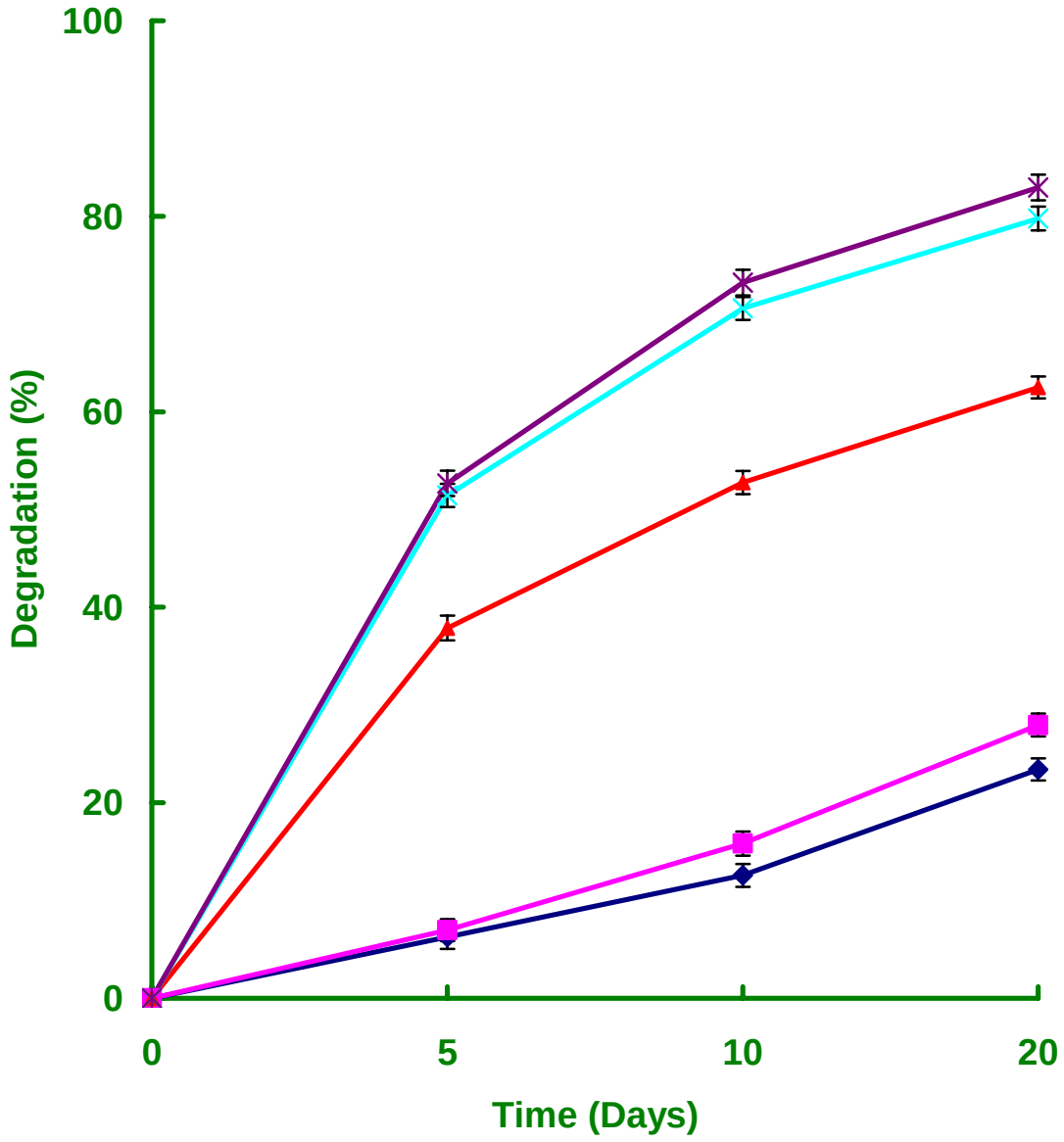


Fig 10 (b): Optimization of cell mass for microcosm studies

Pseudomonas sp.2 with varying cell mass (approx. with 10^6 cells/ml, 10^7 cells/ml, 10^8 cells/ml & 10^9 cells/ml) were added to contaminated soil. Soil samples harvested at various time intervals were subjected to solvent extraction (section 4.10.3) and analyzed for the level of residual chlorpyrifos using GC-ECD (section 4.6.2).

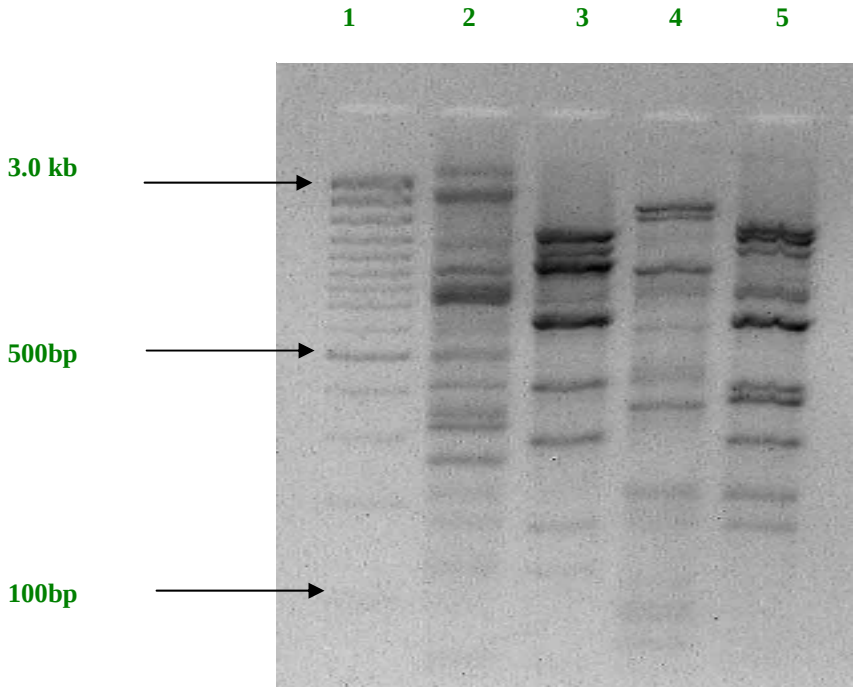


Photo1: REP-PCR of all the four isolates

Pseudomonas sp.1, Brucella sp., Bacillus sp.1 and Pseudomonas sp.2 were grown in Luria broth, their DNA was extracted (section 4.10.5.1) and genomic DNA fingerprints of these bacterial isolates were developed using touchdown PCR with REP primers (section 4.10.5.2). A combination of PCR conditions was used in the present study to generate strain specific DNA fingerprints to select the population released for bioremediation of chlorpyrifos contaminated soils.

Lane 1contains 100bp marker
Lane 2 represents *Pseudomonas sp.1*
Lane 3 represents *Brucella sp.*
Lane 4 represents *Bacillus sp.1*
Lane 5 represents *Pseudomonas sp.2*

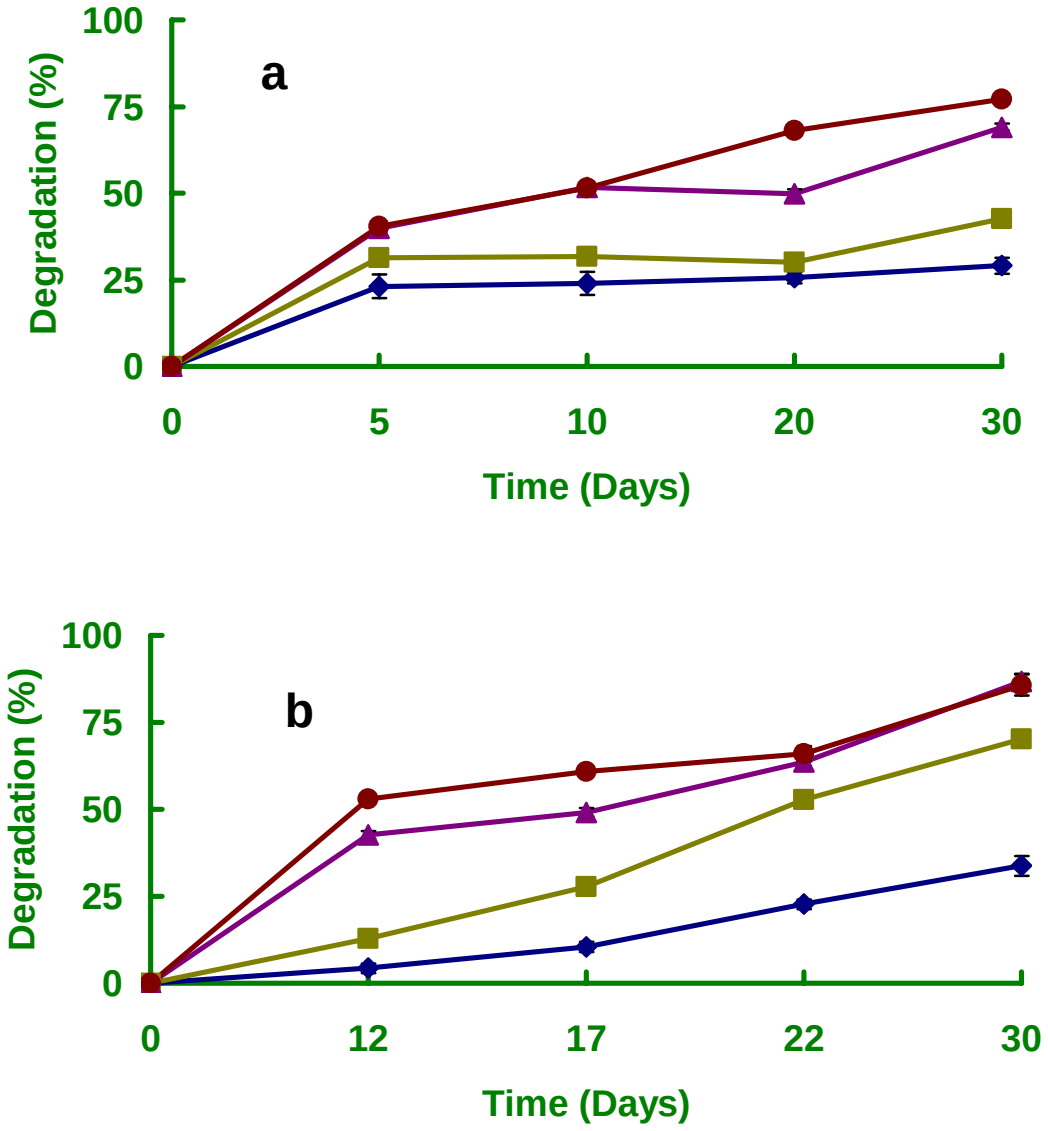


Fig11: Chlorpyrifos degrading ability of consortia under a) Sterile
b) Non-sterile conditions

Fresh garden soil (100gm) was a) sterilized/ b) used as such, contaminated with chlorpyrifos (50mg/kg) and inoculated with (2x 10^{8} cells/ml) consortia DC, Q1 and Q2. Residual chlorpyrifos in soil samples harvested at repeated time intervals were solvent extracted (section 4.10.3) and analyzed by GC-ECD (section 4.6.2).

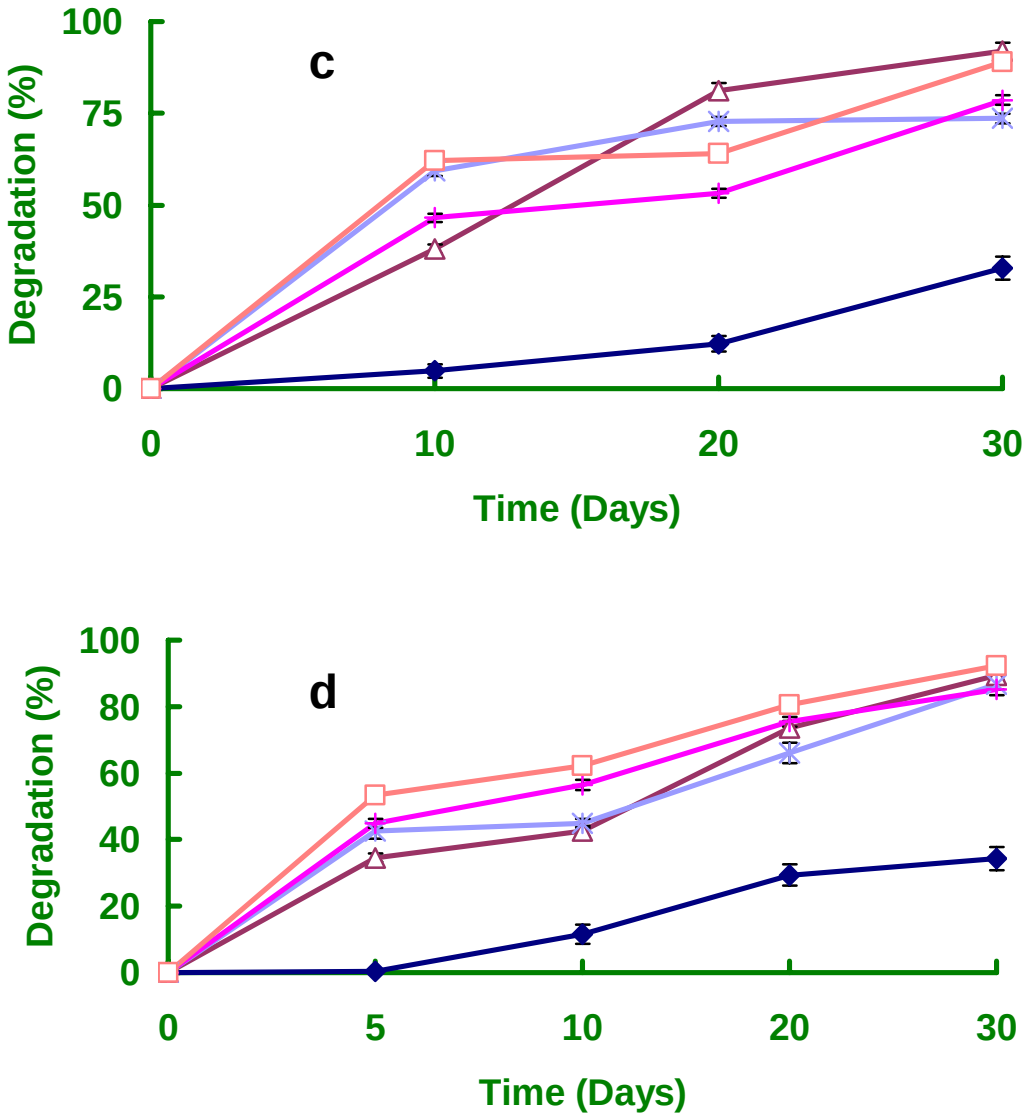


Fig 11: Chlorpyrifos degrading ability of isolates under c) sterile d) non-sterile conditions

Fresh garden soil (100gm) was a) sterilized/ b) used as such, contaminated with chlorpyrifos (50mg/kg) and inoculated with isolate *Pseudomonas sp.1, Brucella sp., Bacillus sp.1 and Pseudomonas sp.2* (2x 10^{8} cells/ml each) respectively. Residual chlorpyrifos in soil samples harvested at repeated time intervals was solvent extracted (section 4.10.3) and analyzed by GC-ECD (section 4.6.2).

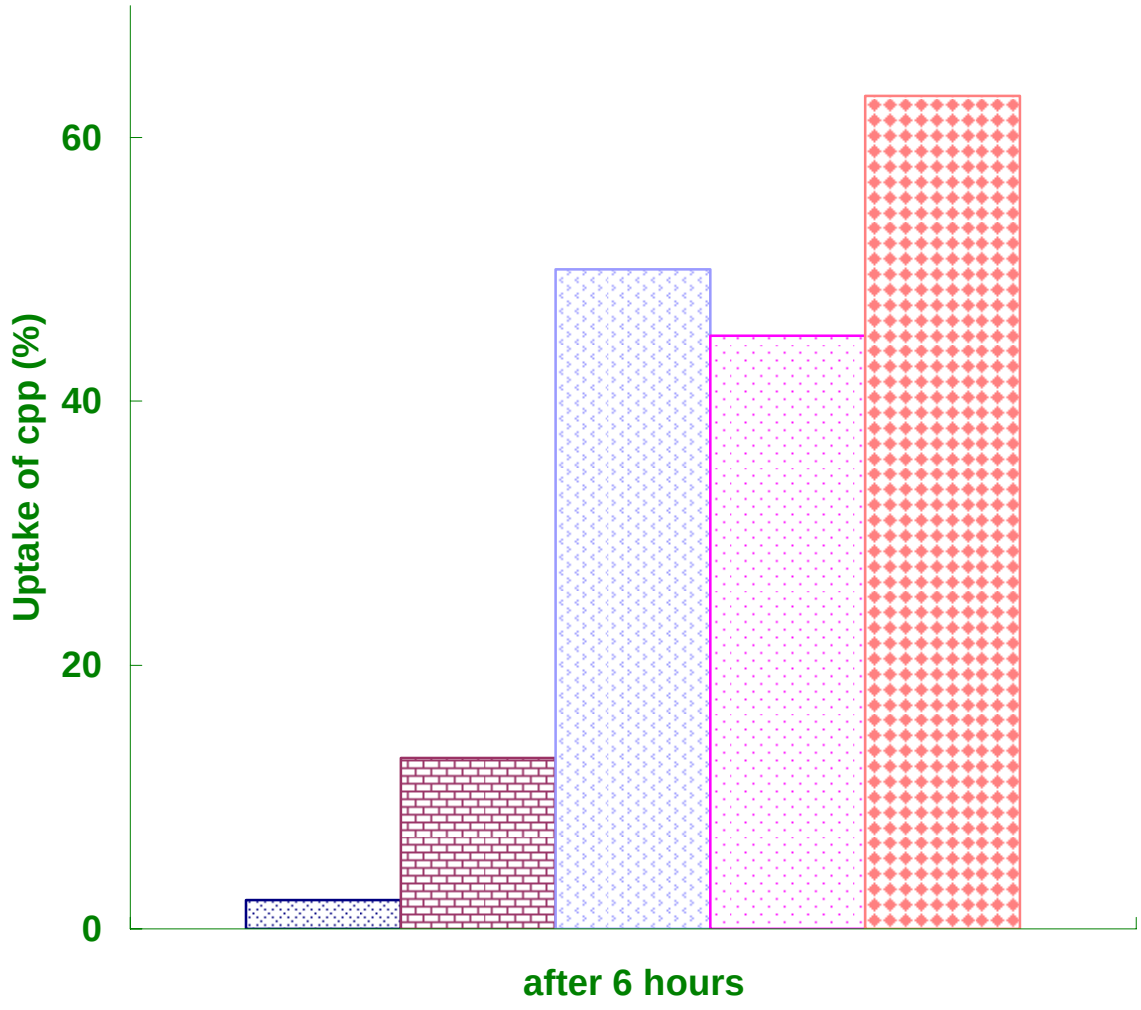


Fig 12: Uptake of chlorpyrifos by whole cells

Starved whole cells (1mg/ml protein) of *Pseudomonas sp.1, Brucella sp., Bacillus sp.1 and Pseudomonas sp.2* were incubated with chlorpyrifos (section 4.9.2). Residual chlorpyrifos was extracted after six hours using ethylacetate /phosphoric acid (97:3) and analyzed using GC-ECD (section 4.6.2).

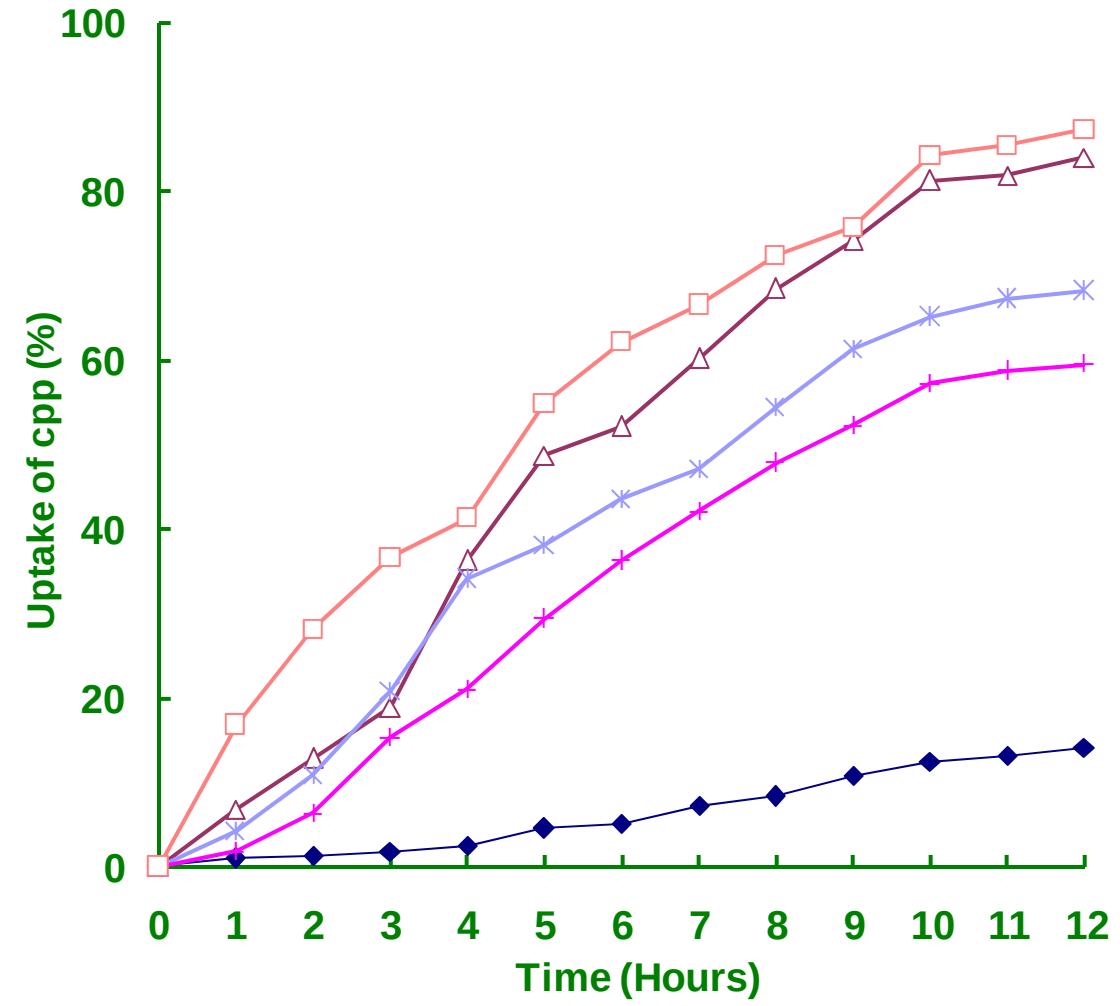


Fig 13 (a): Uptake of chlorpyrifos by un-induced whole cells

Starved whole cells (2mg/ml protein) of *Pseudomonas sp.1, Brucella sp., Bacillus sp.1 and Pseudomonas sp.2* were incubated with chlorpyrifos (section 4.9.3). Residual chlorpyrifos was extracted after every hour upto 12 hours and analyzed using GC-ECD (section 4.6.2).

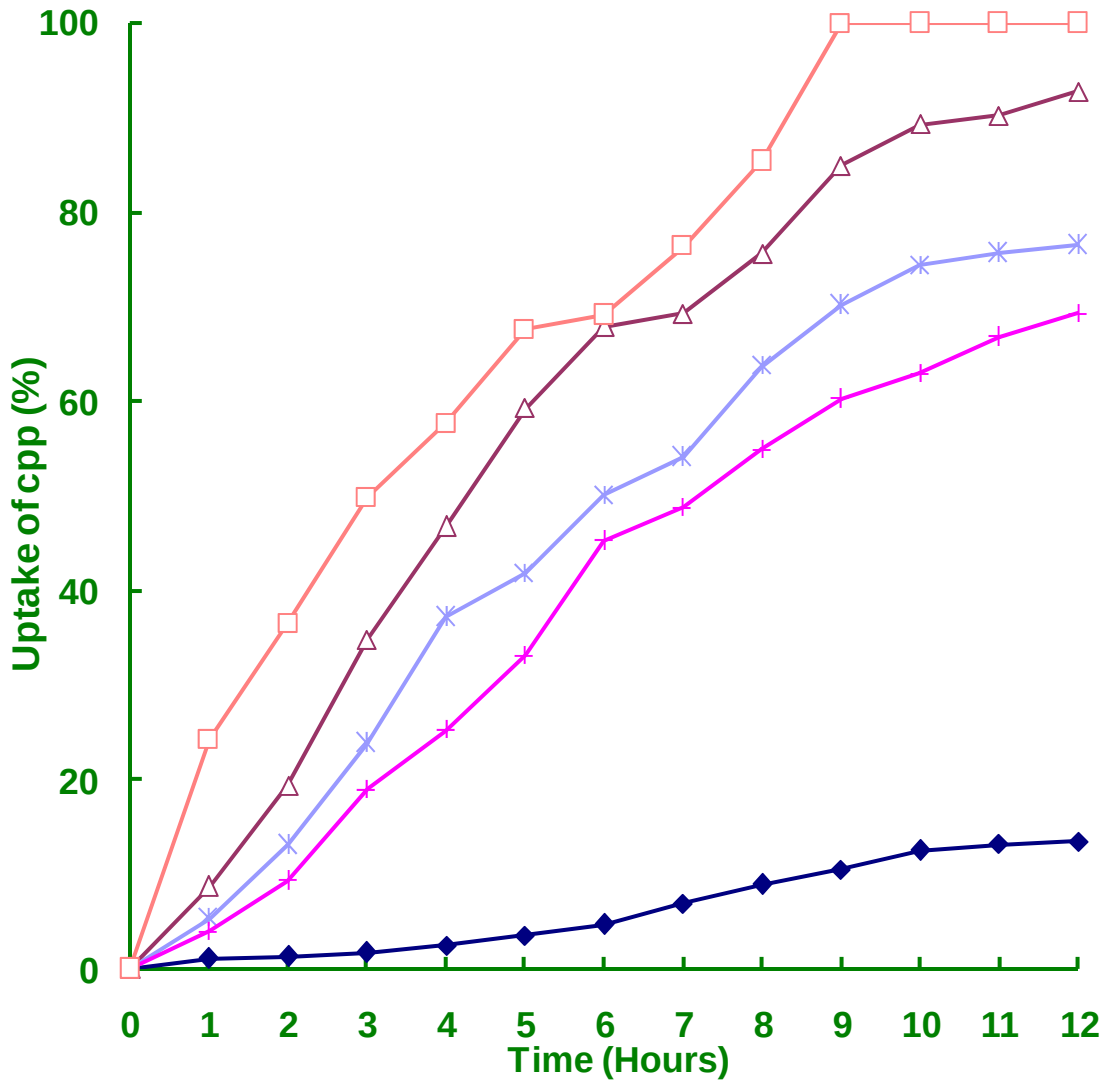


Fig 13 (b): Uptake of chlorpyrifos by induced whole cells

Starved whole cells (2mg/ml protein) of *Pseudomonas sp.1, Brucella sp., Bacillus sp.1 and Pseudomonas sp.2* (section 4.9.3) were induced with chlorpyrifos, then used for uptake studies . Residual chlorpyrifos was extracted after every hour upto 12 hours and analyzed using GC-ECD (section 4.6.2).

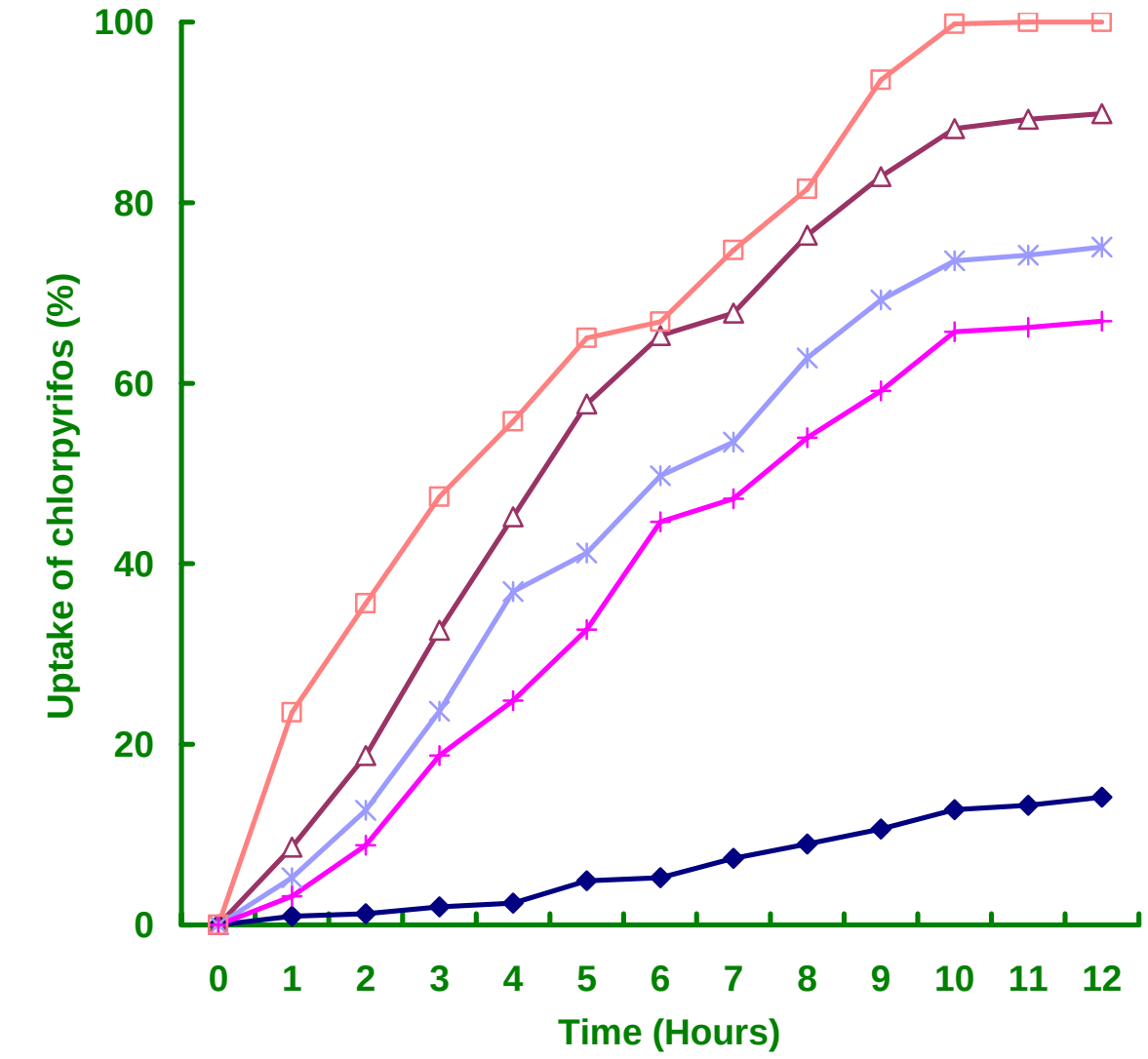


Fig 13 (c): Uptake of chlorpyrifos by cells induced at growth

Cells of *Pseudomonas sp.1, Brucella sp., Bacillus sp.1 and Pseudomonas sp.2* were induced with chlorpyrifos during growth, starved overnight and these whole cells (2mg/ml protein) were incubated with chlorpyrifos (section 4.9.3). Residual chlorpyrifos was extracted after every hour upto 12 hours and analyzed using GC-ECD (section 4.6.2).

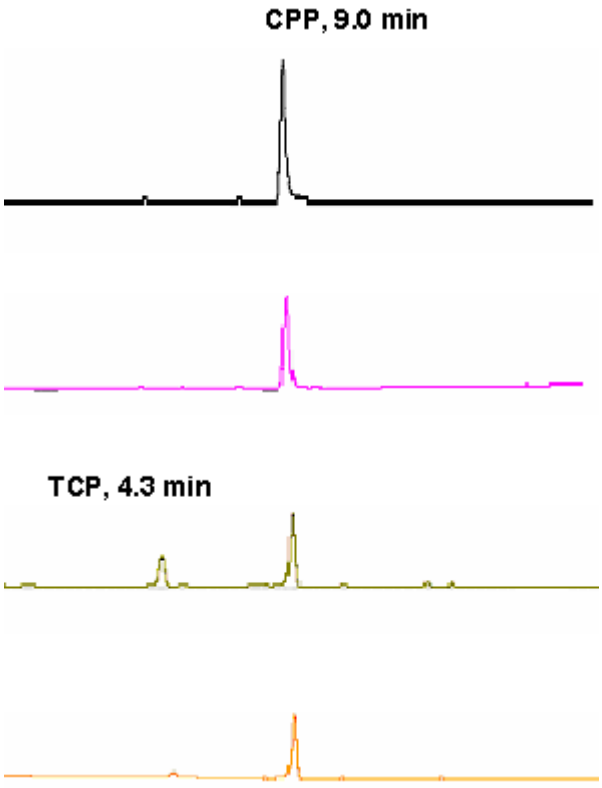


Fig 14: Gas chromatographic profile of metabolites formed during uptake of chlorpyrifos by whole cells of *Pseudomonas sp.2*

Uptake of chlorpyrifos was studied with starved whole cells of isolate *P. sp.2,* after induction with chlorpyrifos (section 4.9.3). The samples were taken after every hour for 12 hours; the residual chlorpyrifos was extracted and analyzed using GC-ECD (section 4.6.2). Metabolite (TCP) formation was thus detected in the chromatogram.

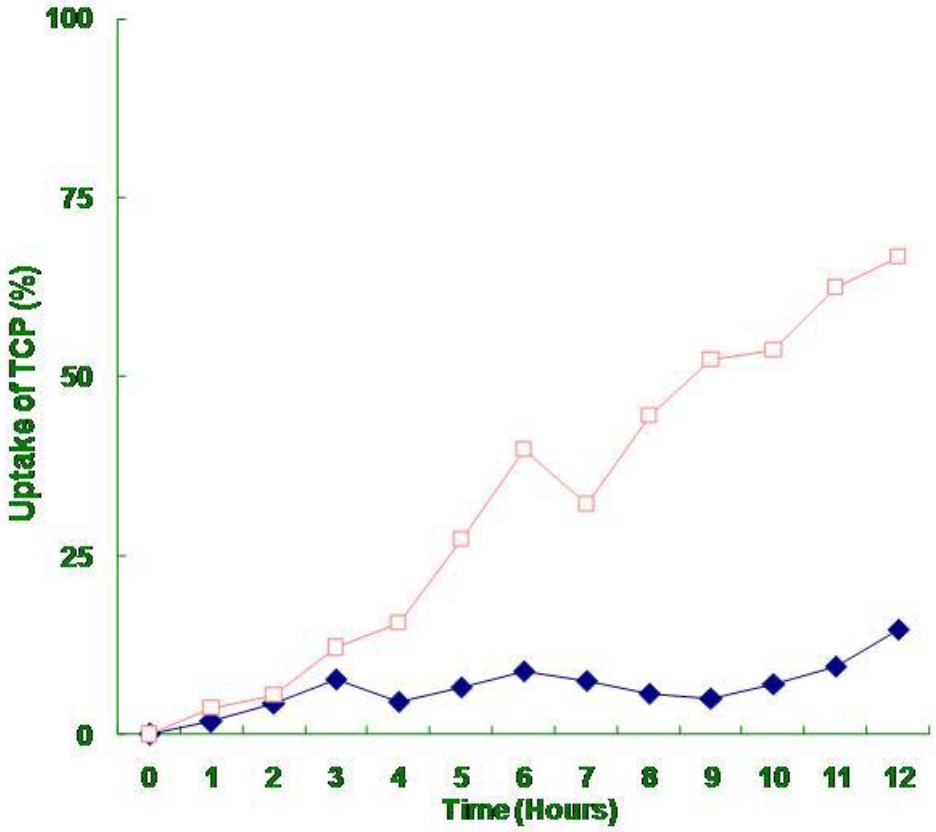


Fig 15: Uptake of 3, 5, 6-trichloro-2-pyridinol (TCP) by whole cells of *Pseudomonas sp.2*

Starved whole cells of *Pseudomonas sp.2* were incubated in basal medium containing 50 mg/ L of 3, 5, 6-trichloro-2-pyridinol (section 4.9.4) and samples were extracted and analyzed using GC-ECD (section 4.6.2).

5.10 Soil Studies

5.10.1 Microcosm Studies

Microcosm studies were conducted with 11kg chlorpyrifos contaminated soil, inoculated with 2 x 10^8 cfu/ gm soil consortia DC, Q1 and Q2, defined consortia MPQ (with equivalent proportions of *Pseudomonas sp.1, Brucella sp., Bacillus sp.1 and Pseudomonas sp.2*) to make an initial population of 3.72 x 10^8, 1.99 x 10^9, 4.89x 10^8 and 5.75 x 10^8 cfu/gm soil respectively and with 2 x 10^8 cfu/ gm soil *Pseudomonas sp.1, Brucella sp., Bacillus sp.1 and Pseudomonas sp.2* to make an initial population of 3.8 x 10^8, 3.63 x 10^8, 3.72 x 10^8 and 3.55 x 10^8 cfu/gm soil respectively.

Consortia Q1 and Q2 showed degradation of 12 & 10% after 10 days and 54 & 66% after 30days of inoculation which further increased to 95 & 94% respectively after 60 days of inoculation. Consortia DC and defined consortia MPQ showed degradation of 14 & 20 % degradation after 10 days and 34 & 49% after 30 days which increased to 89 & 98% after 60 days of inoculation (Fig 16a).

Population of consortia Q1, Q2, DC and MPQ (Fig 16b) decreased from 3.72 x 10^8, 1.99 x 10^9, 4.89x 10^8 and 5.75 x 10^8 (log value 8.57, 9.3, 8.69 and 8.76) cfu/gm soil respectively at inoculation to 6.6 x 10^6, 5.88 x 10^6, 3.23x 10^6 and 5.75 x 10^6 (log value = 6.82, 6.77, 6.51 and 6.76) cfu/gm soil respectively after 30 days, then increased to 1.35 x 10^8, 1.18 x 10^8, 3.63 x 10^7 and 1.51 x 10^8 (log value = 8.13, 8.07, 7.56 and 8.18) cfu/gm soil respectively after 60 days of incubation as compared to control which showed a population of 1.15 x 10^6 (log value= 6.06) cfu/gm soil at 60 days.

A comparative analysis of degradation and population for consortia Q1 and Q2 (Fig 16 c) showed that as the residual chlorpyrifos concentration reduced from 87 and 90% respectively after 10 days to 45 and 34% after 30 days respectively, the microbial cell population also decreased from 2.45 x 10^8 and 1 x 10^7 (log value=8.39 and 7) cfu/gm soil respectively after 10 days to 6.6 x 10^6 and 5.88 x 10^6 (log value=6.82 and 6.77) cfu/gm soil respectively after 30 days.

Among isolates, *Pseudomonas sp.2* and *Brucella sp.* showed 22 & 17% degradation after 10 days and 58 & 45 % degradation after 30 days which further increased to 99% and 98% degradation respectively after 60 days as compared to control, which showed an abiotic degradation of 16, 23 & 26% after 10, 30 & 60 days of inoculation respectively. *Pseudomonas sp.1 and Bacillus sp.1* showed degradations of 22 & 37% after 10 days and 58 & 53 % after 30 days which further increased to 94 & 96% respectively after 60 days respectively (Fig 17 a) as compared to degradation in uninoculated control.

Pseudomonas sp.1, Brucella sp., *Bacillus sp.1 and Pseudomonas sp.2* showed a slight decrease from inoculated population of 3.8 x 10^8, 3.63 x 10^8, 3.72 x 10^8 and 3.55 x 10^8 (log value = 8.58, 8..56, 8.57, 8.55) cfu/gm soil to 2.45 x 10^8, 2.45 x 10^8, 2.39 x 10^8 and 2.45 x 10^8 (log value = 8.39, 8.39, 8.38 and 8.39) cfu/gm soil respectively after 60 days as compared to 1.74 x 10^{87} (log value = 7.24) cfu/gm soil in control (Fig 17 b).

A comparative analysis of degradation with population for *Pseudomonas sp.1 and Pseudomonas sp.2* (Fig 17 c) showed that as the residual chlorpyrifos concentration reduced from 92 and 89% respectively after 5 days to 41% for both after 30 days, the microbial cell population also decreased from 2.69 x 10^8 and 3.23 x 10^8 (log value=8.43 and 8.51) cfu/gm soil respectively after 5 days to 2.29 x 10^8 and 1.82 x 10^8 (log value=8.36 and 8.26) cfu/gm soil respectively after 30 days.

REP-PCR was carried out to ascertain the actual population of the introduced degraders in soil (Photo 2, 3, 4 and 5). For *Pseudomonas sp.1* (Table 7a), population survival was 60, 63 and 67% by antibiotic plating while it was 42, 48 and 46% by molecular fingerprinting after 10, 30 and 60 days respectively. For *Brucella sp.* (Table 7b), population survival was 71, 58 and 64% by antibiotic plating while it was 50, 30 and 38% by molecular fingerprinting after 10, 30 and 60 days respectively. For *Bacillus sp.1* (Table 7c), population survival was 70, 57 and 65% by antibiotic plating while it was 48, 24 and 34% by molecular fingerprinting after 10, 30 and 60 days respectively and for *Pseudomonas sp.2* (Table 7d), population survival was 61, 60 and 68% by antibiotic plating while it was 36, 34 and 40% by molecular fingerprinting after 10, 30 and 60 days respectively.

5.10.2 *In-situ degradation of chlorpyrifos in plots*

Plots were contaminated with 50 mg/kg chlorpyrifos and maintained at 30% moisture. Persistence studies carried out (Fig 19) in plots showed ~ 40% chlorpyrifos degradation after four months.

Plots contaminated with chlorpyrifos were divided into two set, one set was inoculated with cultures of consortia Q1 and Q2 and *Pseudomonas sp.1* and *Pseudomonas sp.2* as such and the other was inoculated with cultures of consortia Q1 and Q2 and *Pseudomonas sp.1* and *Pseudomonas sp.2* along with planting of cotton seeds by randomized block design. Soil and plants were sampled after regular time intervals and analyzed for residual chlorpyrifos along with estimation of population survival by dilution plating on kanamycin plates and REP-PCR.

Comparison of degradation of chlorpyrifos in plots with and without crops on day 25 indicated degradation of 79, 82, 78 and 85% in plots without crops for consortia Q1, Q2 and *Pseudomonas sp.1* and *Pseudomonas sp.2* whereas 99% degradation for all the four

cultures in plots with crops. The population of the four cultures was in the range of 1.77 X 10^7 – 5.62 x 10^7 cfu/ gm soil (log value = 7.25 – 7.75) in plots without crops and 2.39 X 10^7 - 6.76 X 10^7 cfu/ gm soil (log value = 7.38-7.83) in plots with crops on day 25 (Fig 19). No residual chlorpyrifos was found in any of the sampled plants. No chlorpyrifos was detected on the 60th day soil samples from plots with crops and >99% degradation was seen in those without crops.

Metabolite analysis during *in situ* degradation of chlorpyrifos in plots inoculated with *Pseudomonas sp.2* indicated the formation of TCP (2.5mg/L) after 20 days which disappeared to negligible amounts after 30 days (Fig 20 a & b). No other metabolite was detected from any other sample.

During *in situ* studies, REP-PCR of inoculated population indicated that the population of *Pseudomonas sp.1* increased from 15 X 10^6 cfu/ gm soil at the time of inoculation to 20 X 10^6 cfu/ gm soil in plots without crops whereas it increased to 31 X 10^6 cfu/ gm soil in plots with cotton plants on day 25 (Table 1). Similarly, the population of *Pseudomonas sp.2* increased from 16 X 10^6 cfu/ gm soil at the time of inoculation to 26 X 10^6 cfu/ gm soil in plots without crops whereas it increased to 36 X 10^6 cfu/ gm soil in plots with cotton plants on day 25 (Table 8 a, b).

Based on the soil studies and the whole cell uptake studies and literature review, a metabolic pathway of chlorpyrifos degradation by *Pseudomonas sp.2* has been proposed (Fig 21).

5.11 Studies on the genetic aspects

No degradative plasmid was obtained in the chlorpyrifos degrading *Pseudomonas sp., Brucella sp.*, *Bacillus sp.1 and Pseudomonas sp.2*, suggesting that organophosphorus degrading gene might be present on the genomic DNA.

PCR amplification of the genomic DNA of *Pseudomonas sp.1, Brucella sp.*, *Bacillus sp.1 and Pseudomonas sp.2* was carried out with OPD, OPDA and MPD primers. PCR products when analyzed by agarose gel electrophoresis gave no bands on gel. Thus, further quest into the genes involved in the metabolism of chlorpyrifos revealed no definitive results.

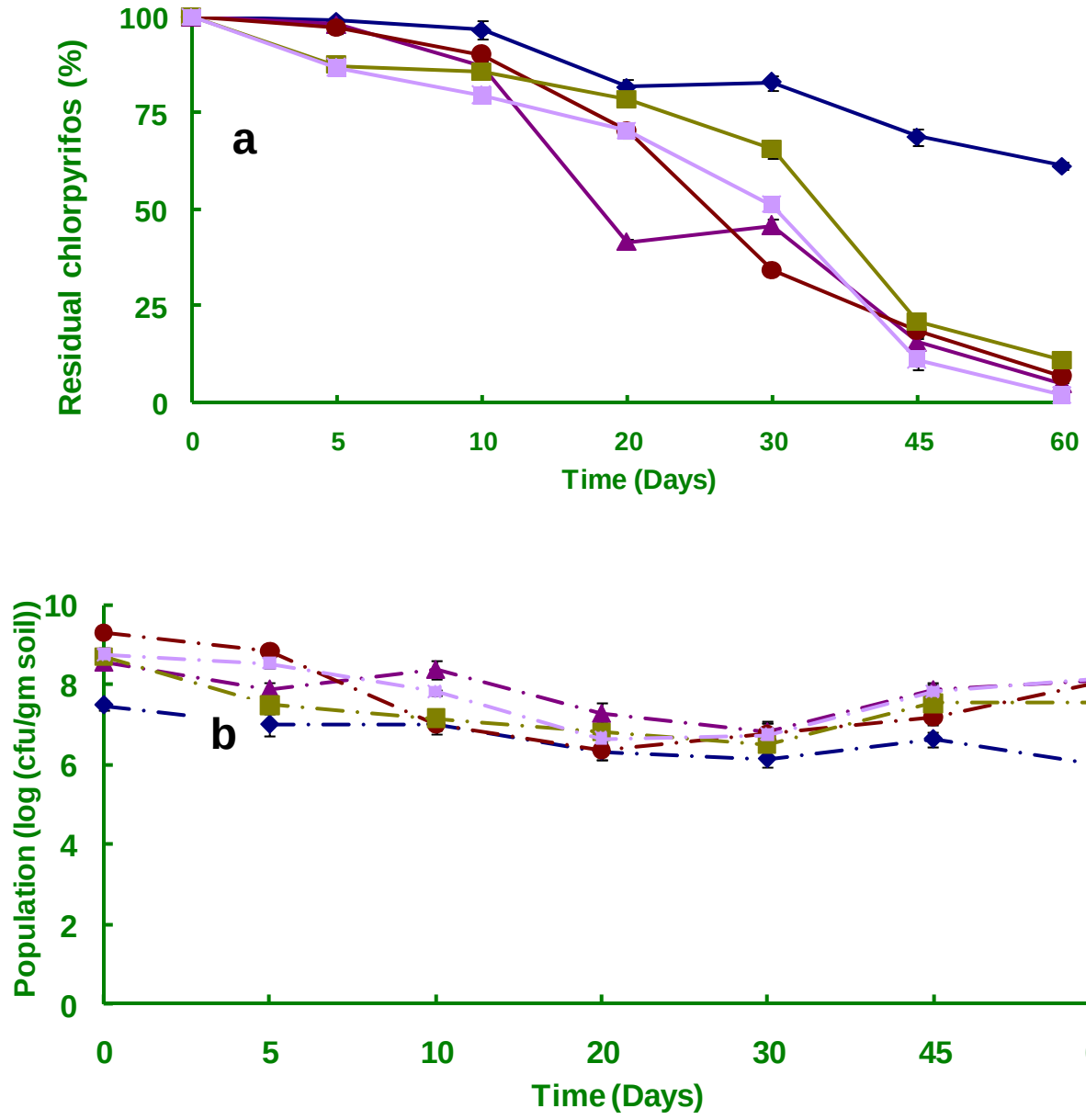


Fig 16 (a): Residual chlorpyrifos in microcosms and (b): Population survival of consortia

Fresh garden soil (11kg) was contaminated with chlorpyrifos (50mg/kg) and inoculated with consortia DC, Q1, Q2 and MPQ (2 x 10^{-8} cfu/gm soil) respectively. Soil samples were harvested at different time intervals and a) residual chlorpyrifos & b) microbial population was estimated. a) Chlorpyrifos was solvent extracted (section 4.10.3) and analyzed by GC-ECD (section 4.6.2) while b) microbial population was monitored as described in section 4.10.2.

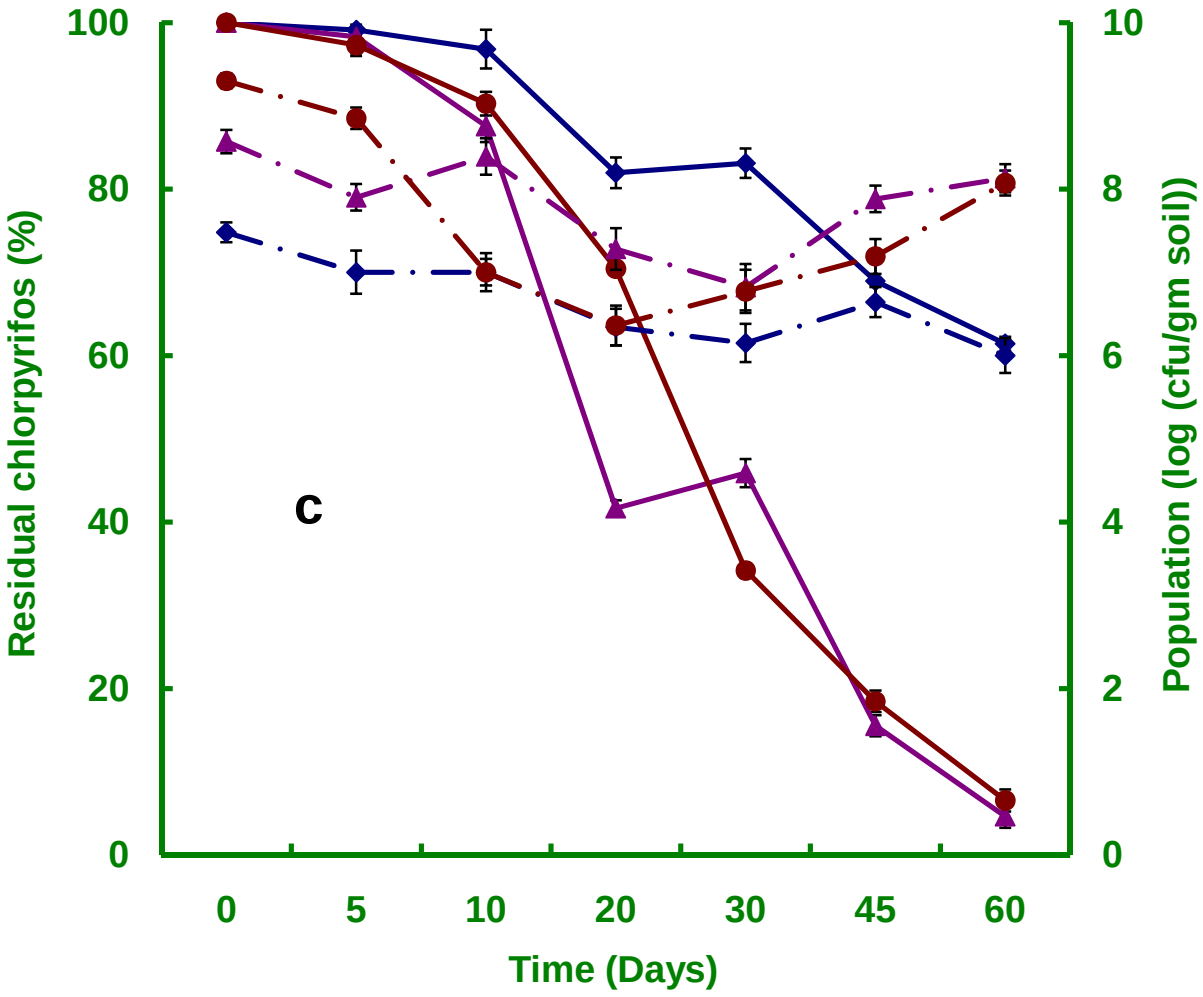


Fig 16 (c): Population survival of consortia and concomitant degradation of chlorpyrifos in microcosms

Fresh garden soil (11kg) was contaminated with chlorpyrifos (50mg/kg) and inoculated with consortia Q1 and Q2 (2 x 10^{-8} cfu/gm soil) respectively. Soil samples were harvested at different time intervals and residual chlorpyrifos & microbial population was estimated. Chlorpyrifos was solvent extracted (section 4.10.3) and analyzed by GC-ECD (section 4.6.2) while microbial population was monitored as described in section 4.10.2. The straight lines indicate residual chlorpyrifos and the serrated lines indicate population survival.

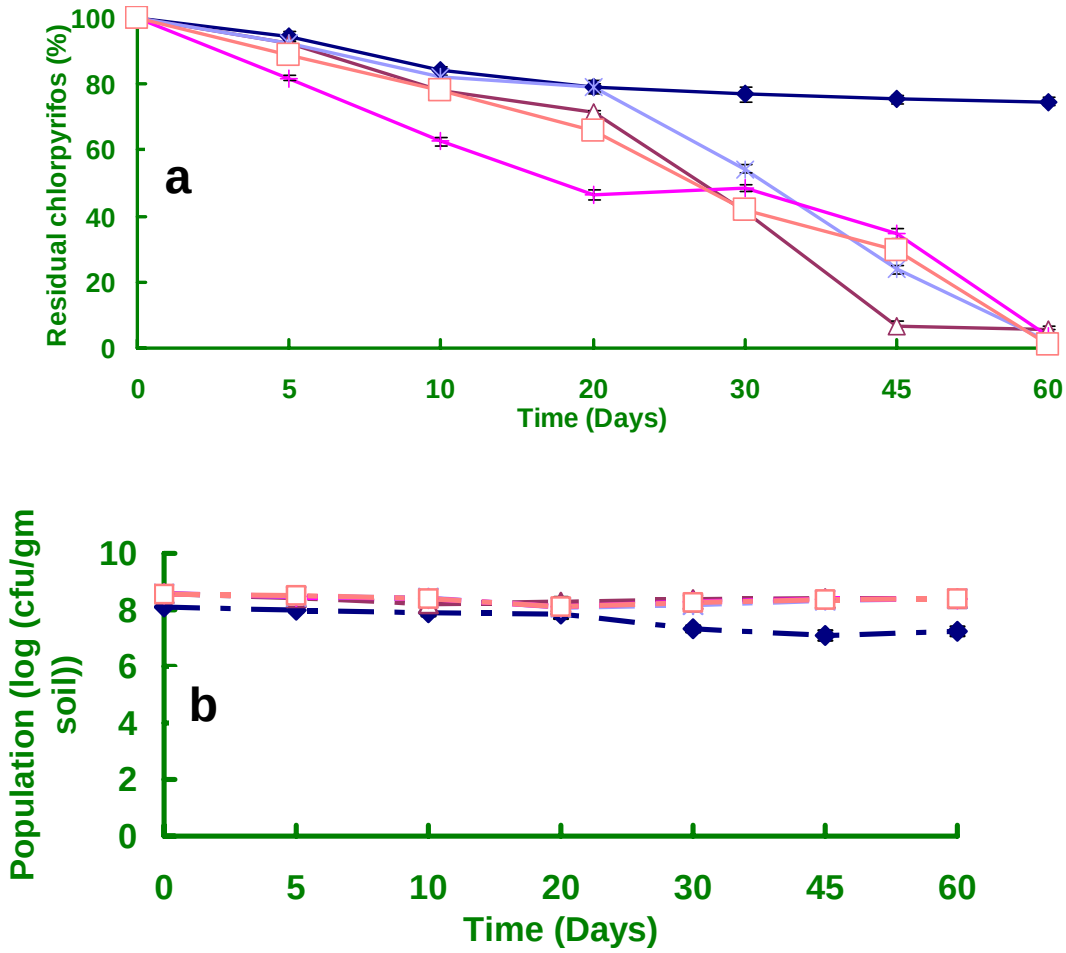


Fig 17 (a): Residual chlorpyrifos in microcosms and (b) Population survival of isolates

Fresh garden soil (11kg) was contaminated with chlorpyrifos (50mg/kg) and inoculated with *Pseudomonas sp.1, Brucella sp., Bacillus sp.1 and Pseudomonas sp.2* (2 x 10^{-8} cfu/gm soil) respectively. Soil samples were harvested at different time intervals and a) residual chlorpyrifos & b) microbial population was estimated. a) Chlorpyrifos was solvent extracted (section 4.10.3) and analyzed by GC-ECD (section 4.6.2) while b) microbial population was monitored as described in section 4.10.2.

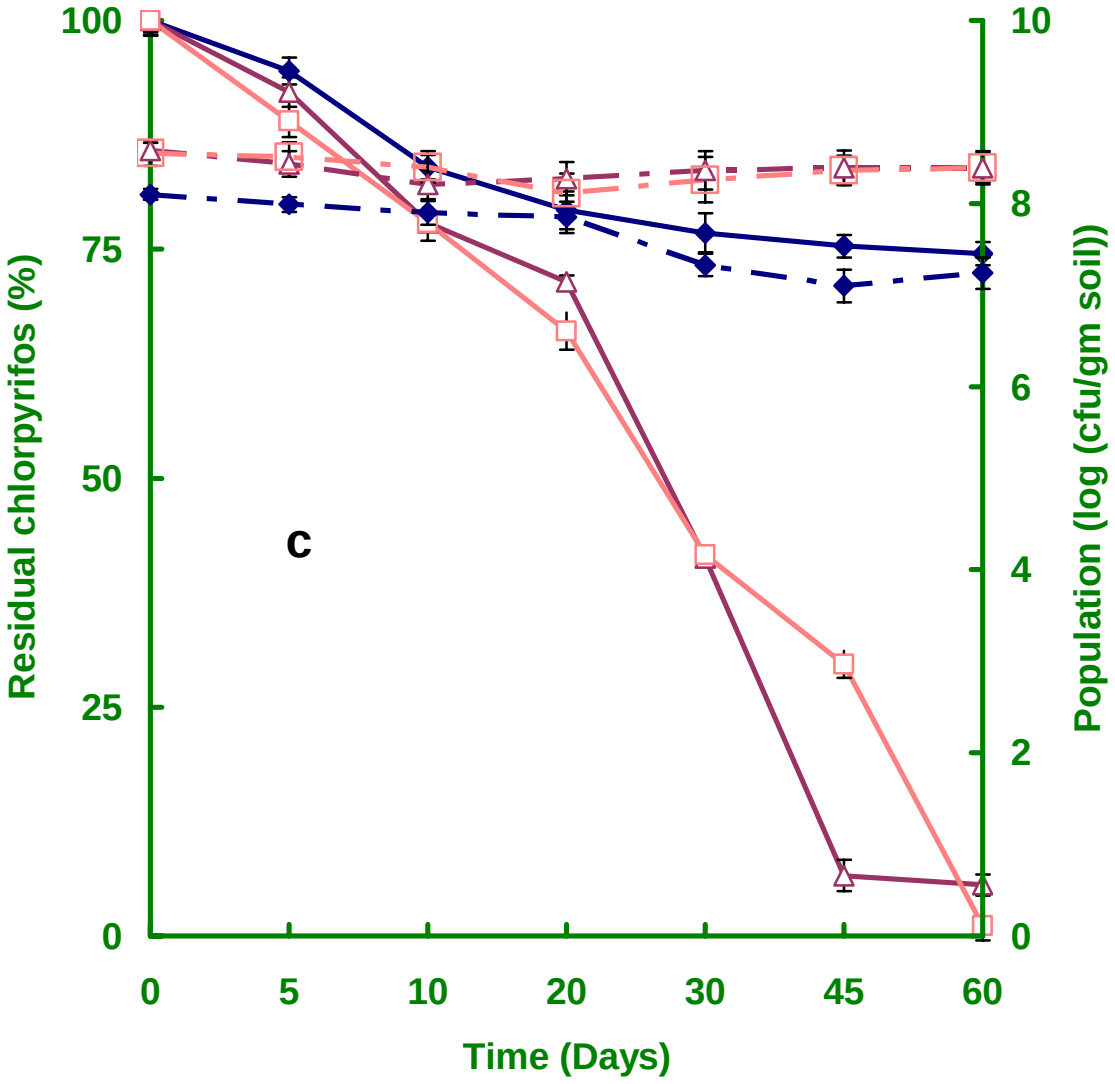


Fig 17 (c): Population survival of isolates and concomitant degradation of chlorpyrifos in microcosms

Fresh garden soil (11kg) was contaminated with chlorpyrifos (50mg/kg) and inoculated with *Pseudomonas sp.1 and Pseudomonas sp.2* (2×10^{-8} cfu/gm soil) respectively. Soil samples were harvested at different time intervals and residual chlorpyrifos was solvent extracted (section 4.10.3) and analyzed by GC-ECD (section 4.6.2) while microbial population was monitored as described in section 4.10.2 straight lines represent residual chlorpyrifos while serrated lines represent population survival.

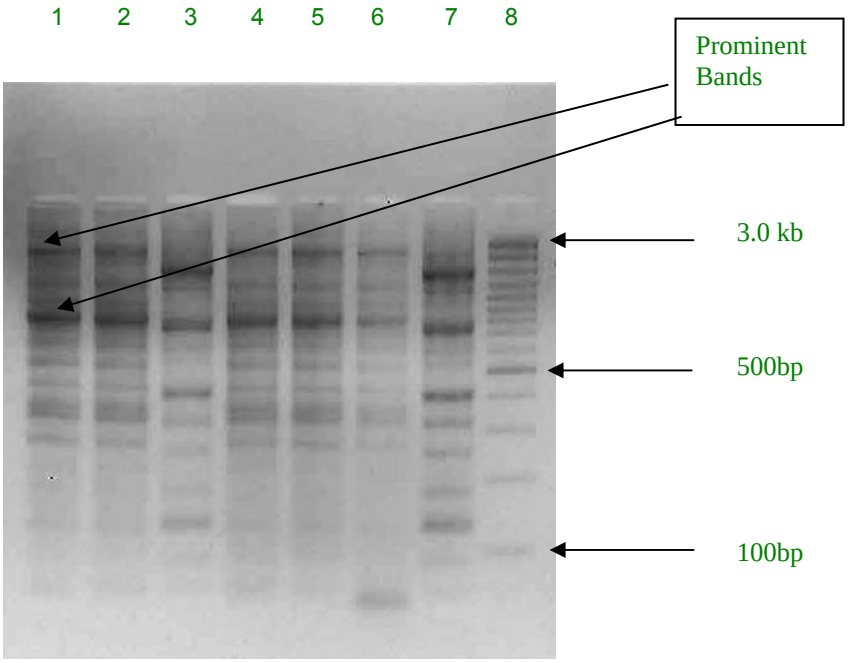


Photo 2: REP-PCR of day 0 of *Pseudomonas sp1.*

Genomic DNA of *Pseudomonas sp.1* was extracted (section 4.10.5.1) along with genomic DNA of isolates from kanamycin plates (section 4.10.2), amplified by REP PCR with REP primers (section 4.10.5.2) and the products (10 µl) were analyzed on agarose gel (section 4.11.5). The population survival of inoculated degraders was tracked and compared (section 4.10.5).

Lane1 – Original *Pseudomonas sp.1* DNA amplified by REP-PCR
Lane 2-7 – Amplified DNA of strains obtained from inoculated soil samples on day 0
Lane 8 – 100bp Marker

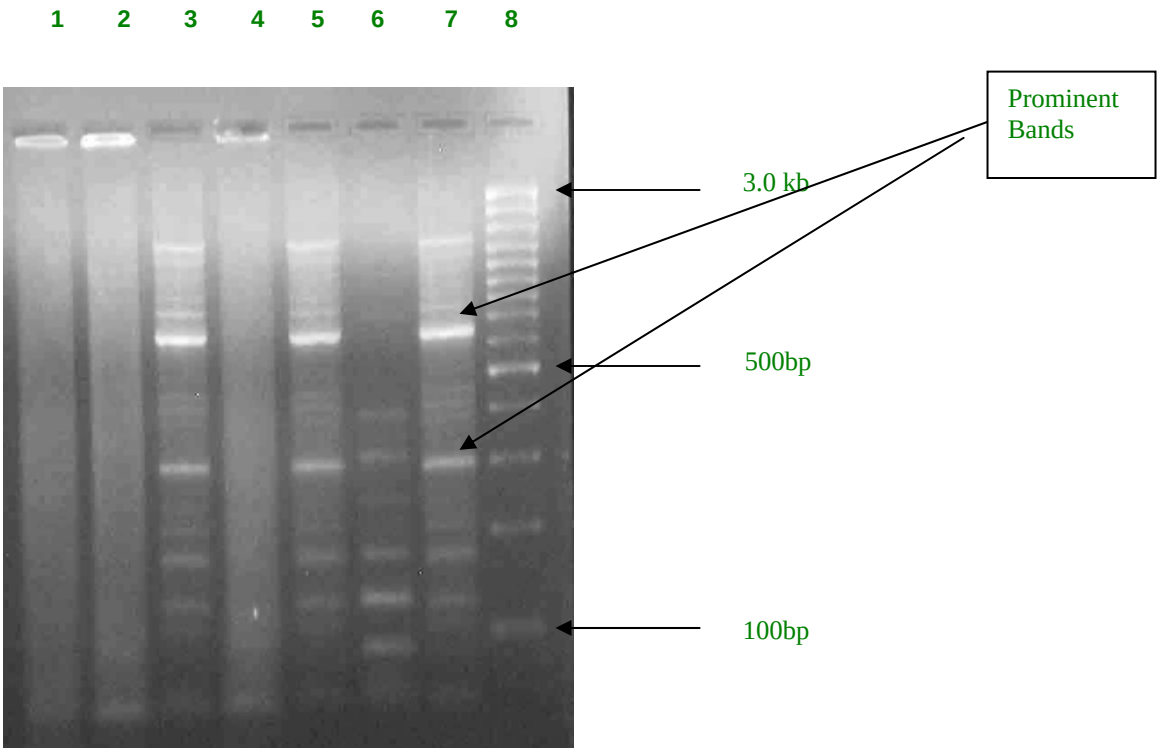


Photo 3: REP-PCR of day 10 of *Brucella sp.*

Genomic DNA of *Brucella sp.* was extracted (section 4.10.5.1) along with genomic DNA of isolates from kanamycin plates (section 4.10.2), amplified by REP PCR with REP primers (section 4.10.5.2) and the products (10 µl) were analyzed on agarose gel (section 4.11.5). The population survival of inoculated degraders was tracked and compared (section 4.10.5).

Lane 1-6 – Amplified DNA of strains obtained from inoculated soil samples on day 10

Lane 7 – Original *Brucella sp.* DNA amplified by REP-PCR

Lane 8 – 100bp Marker

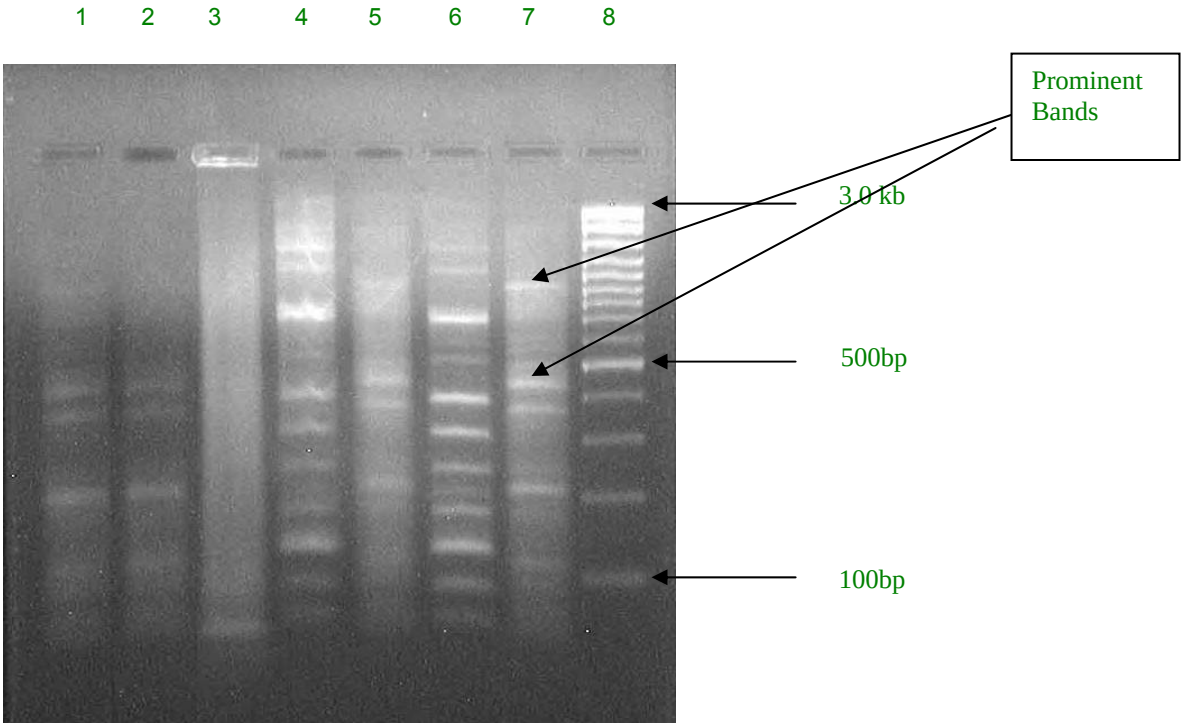


Photo 4: REP-PCR of day 45 of *Bacillus sp.1*

Genomic DNA of *Bacillus sp.1* was extracted (section 4.10.5.1) along with genomic DNA of isolates from kanamycin plates (section 4.10.2), amplified by REP PCR with REP primers (section 4.10.5.2) and the products (10 µl) were analyzed on agarose gel (section 4.11.5). The population survival of inoculated degraders was tracked and compared (section 4.10.5).

Lane 1-6 – Amplified DNA of strains obtained from inoculated soil samples on day 10

Lane7 – Original *Bacillus sp.1* DNA amplified by REP-PCR

Lane 8 – 100bp Marker

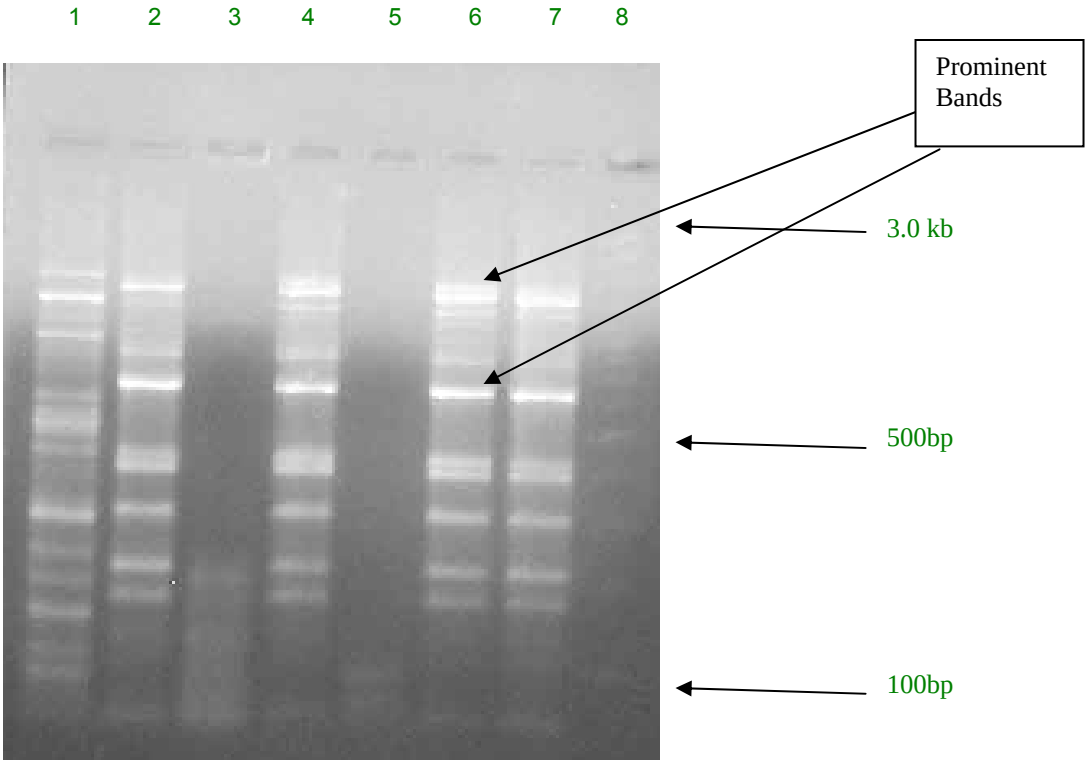


Photo 5: REP-PCR of day 5 of *Pseudomonas sp.2*

Genomic DNA isolate *Pseudomonas sp.2* was extracted (section 4.10.5.1) along with genomic DNA of isolates from kanamycin plates (section 4.10.2), amplified by REP PCR with REP primers (section 4.10.5.2) and the products (10 µl) were analyzed on agarose gel (section 4.11.5). The population survival of inoculated degraders was tracked and compared (section 4.10.5).

Lane 1-6 – Amplified DNA of strains obtained from inoculated soil samples on day 10

Lane7 – Original *Pseudomonas sp.2* DNA amplified by REP-PCR

Lane 8 – 100bp Marker

Table 7(a): Survival table of isolate *Pseudomonas sp.1* in microcosm

Genomic DNA *Pseudomonas sp.1* was extracted (section 4.10.5.1) along with genomic DNA of isolates from kanamycin plates (section 4.10.2), amplified by REP PCR with REP primers (section 4.10.5.2) and the products (10 µl) were analyzed on agarose gel (section 4.11.5). The population survival of inoculated degraders was tracked, tabulated and compared (section 4. 10.5).

Day	Plating (% Survival)	REP (% Survival)
0	100	80
5	80.92	60
10	60.19	42
20	60.53	44
30	63.08	48
45	66.86	46
60	67.93	46

Table 7(b): Survival table of isolate *Brucella sp.* in microcosm

Genomic DNA *Brucella sp.* was extracted (section 4.10.5.1) along with genomic DNA of isolates from kanamycin plates (section 4.10.2), amplified by REP PCR with REP primers (section 4.10.5.2) and the products (10 µl) were analyzed on agarose gel (section 4.11.5). The population survival of inoculated degraders was tracked, tabulated and compared (section 4. 10.5).

Day	Plating (% Survival)	Rep (% Survival)
0	100	70
5	75.67	54
10	71.50	50
20	56.84	28
30	58.64	30
45	62.57	32
60	64.86	38

Table 7(c): Survival table of isolate *Bacillus sp.1* in microcosm

Genomic DNA *Bacillus sp.1* was extracted (section 4.10.5.1) along with genomic DNA of isolates from kanamycin plates (section 4.10.2), amplified by REP PCR with REP primers (section 4.10.5.2) and the products (10 µl) were analyzed on agarose gel (section 4.11.5). The population survival of inoculated degraders was tracked, tabulated and compared (section 4. 10.5).

Day	Plating (% Survival)	Rep (% Survival)
0	100	72
5	79.79	54
10	70.49	48
20	54.24	22
30	57.64	24
45	64.27	28
60	65.56	34

Table 7(d): Survival table of isolate *Pseudomonas sp.2* in microcosm

Genomic DNA of the isolate *Pseudomonas sp.2* was extracted (section 4.10.5.1) along with genomic DNA of isolates from kanamycin plates (section 4.10.2), amplified by REP PCR with REP primers (section 4.10.5.2) and the products (10 µl) were analyzed on agarose gel (section 4.11.5). The population survival of inoculated degraders was tracked, tabulated and compared (section 4.10.5).

Day	Plating (% Survival)	Rep (% Survival)
0	100	72
5	82.53	56
10	61.09	36
20	51.99	28
30	60.01	34
45	66.06	38
60	68.63	40

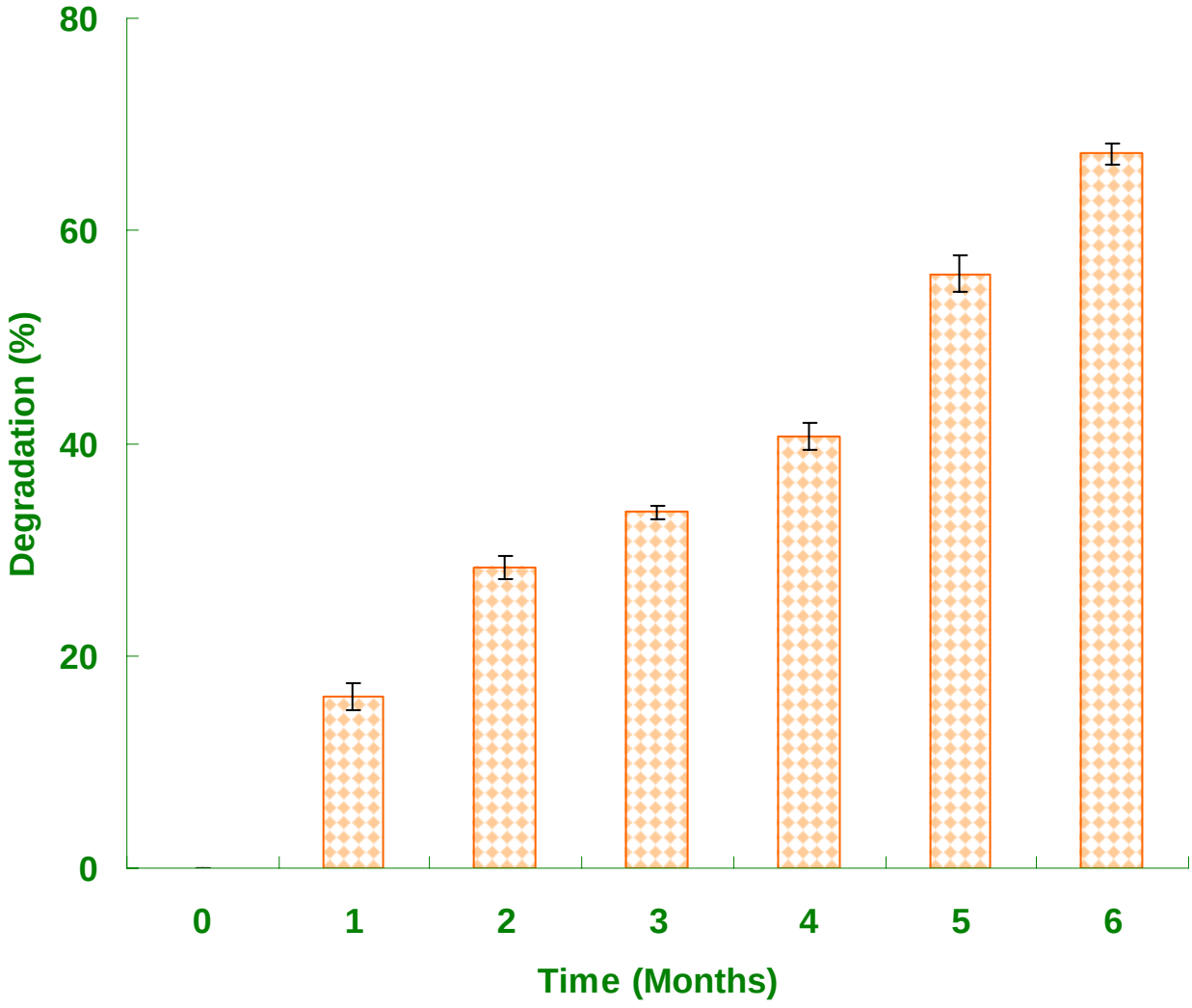


Fig 18: Persistence studies

Plots (54m^2) were sprayed with commercial grade chlorpyrifos (50 mg/kg) and soil was sampled from each plot at intervals of a month (section 4.10.7) and residual chlorpyrifos was extracted by solvent extraction (section 4.10.3) and analyzed by GC-ECD (section 4.6.2).

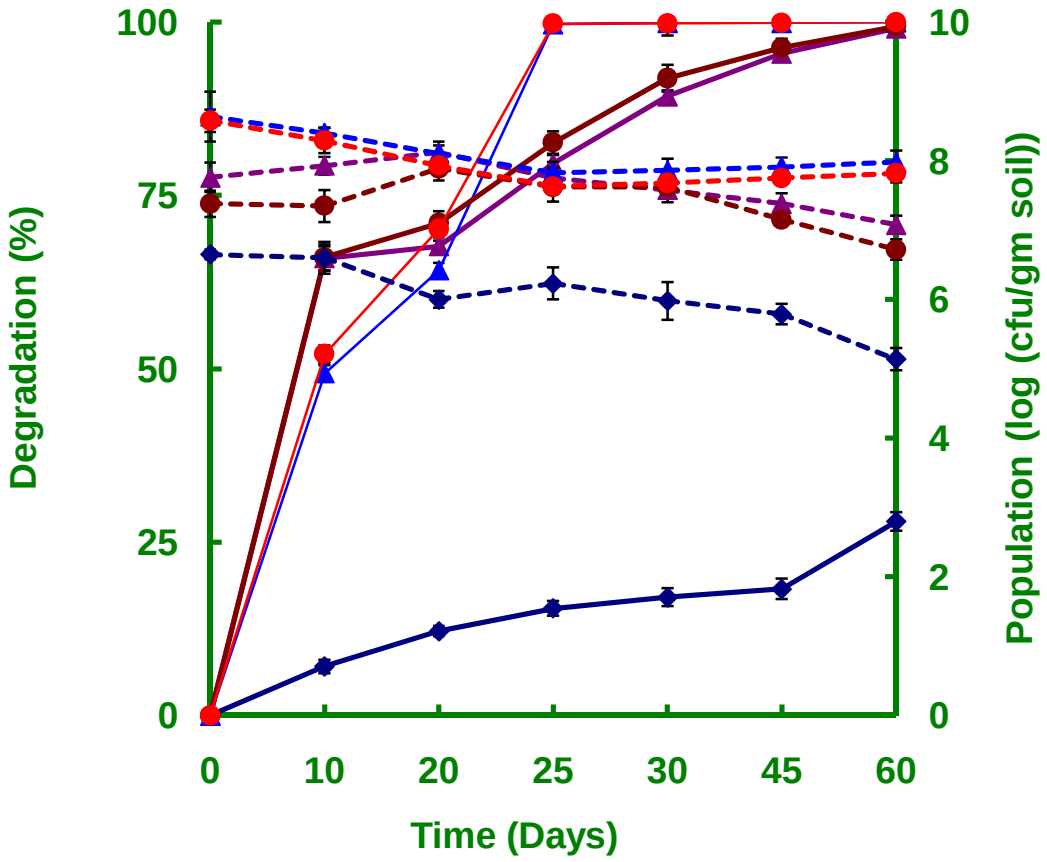


Fig 19 (a): Degradation of chlorpyrifos and population survival of consortia in plots with and without cotton plant

Plots contaminated with chlorpyrifos (50 mg/kg), as such or sown with cotton seeds were inoculated with consortia Q1 and Q2. Chlorpyrifos residues were extracted from sampled soil (section 4.8.3) after repeated time intervals (section 4.10.3) and analyzed using GC-ECD (section 4.6.2). Sampled soil was dilution plated on kanamycin plates (section 4.10.2) and population dynamics was monitored (section 10.54.).

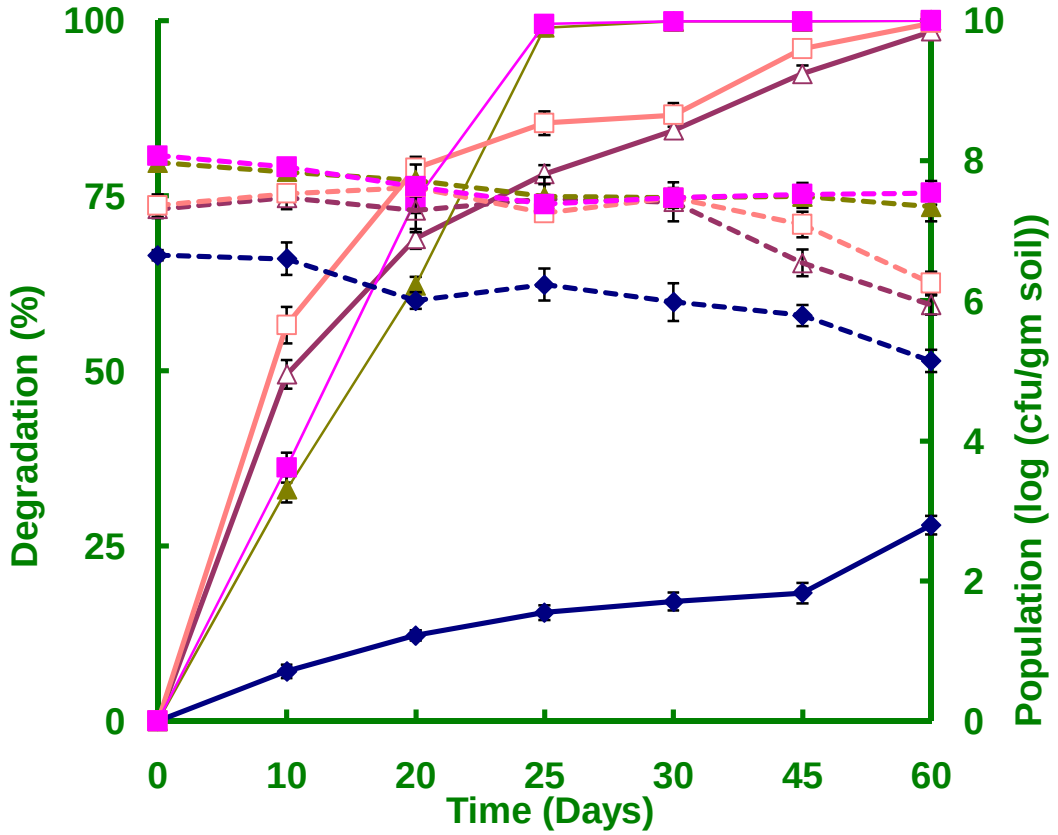


Fig 19 (b): Degradation of chlorpyrifos and population survival of isolates in plots with and without cotton plant

Plots contaminated with chlorpyrifos (50 mg/kg), as such or sown with cotton seeds were inoculated with *Pseudomonas sp.1 and Pseudomonas sp.2*. Chlorpyrifos residues were extracted from sampled soil (section 4.8.3) after repeated time intervals (section 4.10.3) and analyzed using GC-ECD (section 4.6.2). Sampled soil was dilution plated on kanamycin plates (section 4.10.2) and population dynamics was monitored (section 4.10.5).

Table 8 (a): Survival of *Pseudomonas sp.1* in Plots with and without cotton plants, as indicated by REP-PCR

Genomic DNA of plot inoculated *Pseudomonas sp.1* was extracted from kanamycin plates, amplified by REP PCR with REP primers (section 4.10.5) and the products (10 µl) were analyzed on agarose gel (section 4.11.5). The population survival of inoculated degraders in plots, with and without cotton plants, was tracked and compared (section 4.10.5 and 4.10.8).

Day	Without cotton (cfu x 10^6)	With cotton (cfu x 10^6)
0	15.20	15.61
10	28.12	30.26
20	15.06	26.17
25	20.09	31.29
30	20.14	21.17
45	1.75	2.26
60	2.83	4.58

Table 8 (b): Survival of *Pseudomonas sp.2* in Plots with and without cotton plants, as indicated by REP-PCR

Genomic DNA of plot inoculated *Pseudomonas sp.2* was extracted from kanamycin plates, amplified by REP PCR with REP primers (section 4.10.5.2) and the products (10 µl) were analyzed on agarose gel (section 4.11.5). The population survival of inoculated degraders in plots, with and without cotton plants, was tracked and compared (section 4.10.5 and 4.10.8).

Day	Without cotton (cfu x 10^6)	With cotton (cfu x 10^6)
0	15.92	16.03
10	31.31	34.32
20	32.86	46.19
25	25.83	35.83
30	25.81	35.87
45	7.76	8.03
60	0.68	2.02

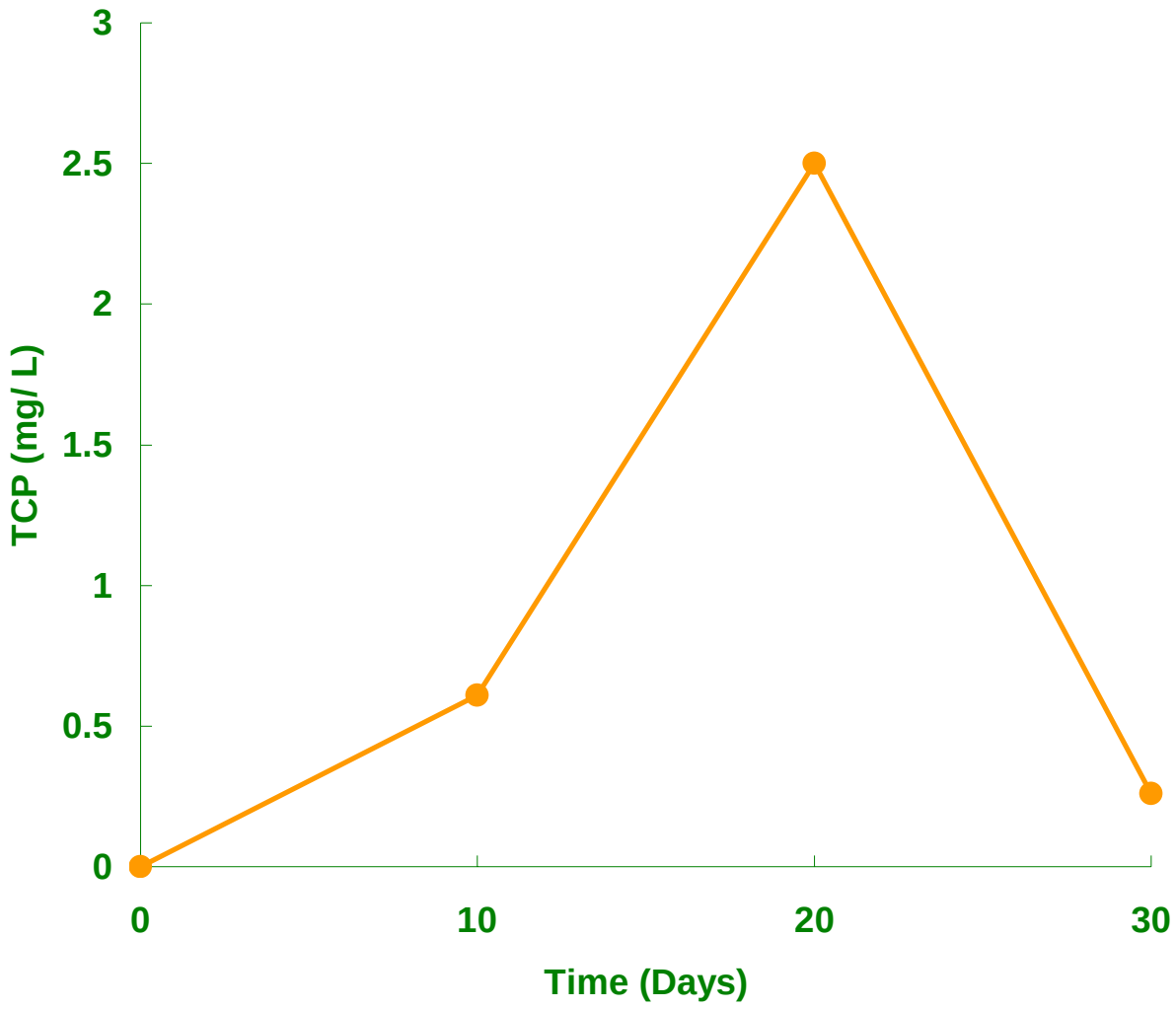


Fig 20 (a): TCP formation and degradation in soil inoculated with *Pseudomonas sp.2*

Cells of *Pseudomonas sp.2* were inoculated into chlorpyrifos contaminated plots (section 4.10.8). Soil samples were withdrawn, residual chlorpyrifos was extracted with solvent system (section 4.8.3) and analyzed by GC-ECD (section 4.6.2). Product formation was seen and residual chlorpyrifos and metabolite TCP were quantified by using the respective standard curves of chlorpyrifos and TCP.

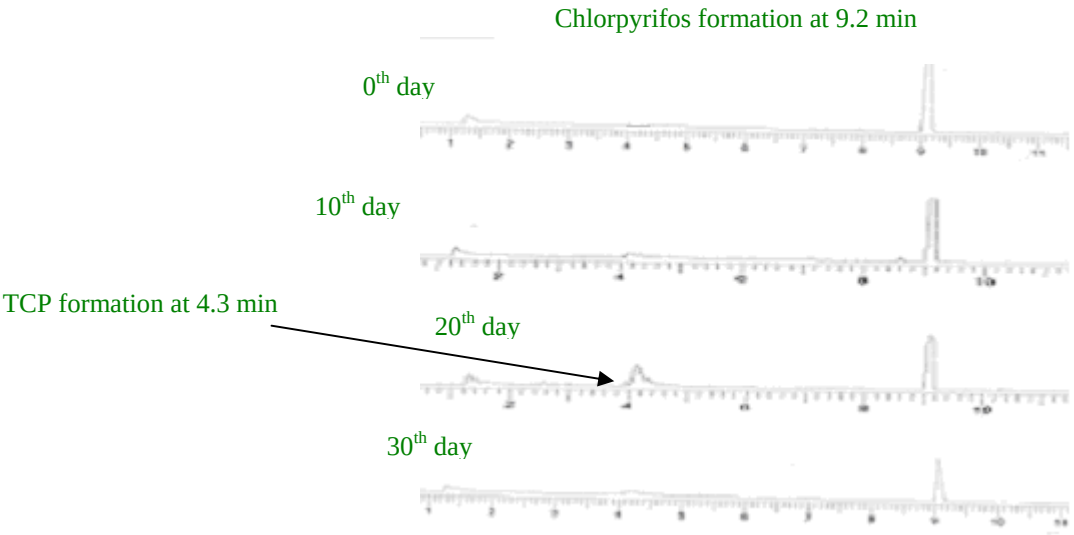


Fig 20 (b): Soil chromatogram indicating formation of 3, 5, 6-trichloro-2-pyridinol (TCP) during in situ degradation of chlorpyrifos

Cells of *Pseudomonas sp. 2* were inoculated into chlorpyrifos contaminated plots (section 4.10.8). Soil samples were withdrawn, residual chlorpyrifos was extracted with solvent system (section 4.10.3) and analyzed by GC-ECD (section 4.6.2). Residual chlorpyrifos and TCP formation was quantified by using the respective standard curves of chlorpyrifos and TCP.

(a)

(b)

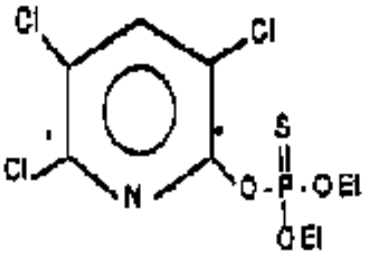
(c)

Photo 6: Different stages of cotton plant growth in fields

(a) A cotton plantlet

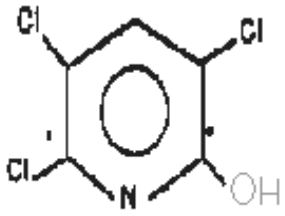
(b) Field with *Pseudomonas sp.2* showing lush growth

(c) Field with consortia Q2 with good growth of cotton



Chlorpyrifos [O, O-diethyl O (3, 5, 6-trichloro-2-pyridyl) phosphorothioate]

O, O-diethyl phosphorothioic acid (DETP)



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

(according to literature)

Maleamic acid/ Pyruvic acid

Krebs cycle

Polar metabolites, CO_2

Fig 21: Proposed metabolic pathway of chlorpyrifos degradation by *Pseudomonas sp.2*

Based on soil studies and uptake studies using whole cells of *Pseudomonas sp.2* this metabolic pathway of chlorpyrifos degradation was proposed with reference from Singh and Walker, 2006 and Feng *et al*, 1998.

6. DISCUSSION

Pesticides have been extensively used for protection of food and cash crops and for vector control in various farmlands and pastures. Due to banning of organochlorines like DDT, Heptachlor etc the trend shifted towards increased usage of various organophosphorus insecticides in the recent years making them the most widely used pesticides in the world at the present time. Organophosphorus compound poisoning is a worldwide health problem with around 3 million poisonings and 200000 deaths annually (Karalliedde and Senanayake, 1999; Sogorb *et al*, 2004). Most of these are neurotoxic upon extensive or prolonged exposure as they inhibit the normal activity of the enzyme acetylcholine esterase needed for proper nervous transmission.

Bulk handling either at manufacturing plants or on application into farmlands occasionally leads to contamination of surface and sub-surface soil and ground water affecting a wide range of water and terrestrial ecosystems including mammals (EPA, 1995; McConnell *et al*, 1999; Cisar & Snyder, 2000; Tse *et al*, 2004). Several organophosphorus compounds are used on animals for the control of body pests and most of them are fat soluble and can thus enter the body readily through the skin and potentially find their way into meat and milk (MAFF/HSE, 1995). Also there are about 200000 metric tons of chemical warfare agents stored or dumped around the world and they need to be destroyed.

Bioremediation is an efficient and cost effective option for decontamination of polluted ecosystems and destruction of these excessive sources of pollution. However, the use of micro-organisms for bioremediation requires an understanding of all physiological, microbiological, ecological, biochemical and molecular aspects involved in pollutant transformation (Iranzo *et al*, 2001). Bioremediation of many air, soil and water bodies contaminated with various xenobiotics have been achieved successfully. Organochlorines and organophosphates are the major class of compounds for which bioremediation strategies are being devised currently. Some of the major organophosphates used all over the world include monocrotophos, phorate, malathion, chlorpyrifos and parathion.

With the recent reports on alarming levels of chlorpyrifos in bottled drinking water, cold drinks, vegetables, farmer's blood, amphibians, fishes etc, chlorpyrifos biodegradation has become an essential area of focus. Only a few recent reports indicate its degradation by microorganisms. Hence the objective of this study was to develop an efficient, cost efficient and reliable chlorpyrifos degrading microbial system and treatment technique for remediation of chlorpyrifos contaminated sites.

Organic pollutants like pesticides and crude oil have been shown to be degraded by consortia (Chapalamadugu and Chaudhry, 1991; Dejonghe *et al* 2003; de Souza *et al*, 1998; Kanaly *et al* 2000; Murtaza *et al,* 2005) and microbial isolates and co-cultures (Awasthi *et al*, 1997; Sutherland *et al*, 2000; Uz *et al*, 2000). Though the environmental fate of chlorpyrifos has also been extensively studied, but unlike other organophosphorus compounds, chlorpyrifos has been reported to be resistant to the phenomenon of enhanced degradation (Racke *et al*, 1990; Mallick *et al*, 1999) in pure (Hirakoso, 1969) and mixed (Sethunathan and Pathak, 1972) cultures of microorganisms and there have been no reports of enhanced degradation of chlorpyrifos since its first use in 1965 until recently (Singh and Walker, 2006). It was suggested that the accumulation of its major metabolite, 3, 5, 6-trichloro-2-pyridinol (TCP), which has anti-microbial properties, acts as a buffer in the soil and prevents the proliferation of chlorpyrifos degrading microorganisms (Racke *et al*, 1990), just like some natural dyes, leaf extracts and medicinal plants prevent proliferation of other microorganisms in soil and various other medium (Khare *et al*, 2005; Rojas *et al*, 2006; Mandal *et al*, 2007). Since only one bacterium has been isolated so far which can degrade TCP in liquid medium, little literature is available on microbial metabolism of TCP also (Singh and Walker, 2006). So it was not only essential to develop a system capable of degrading chlorpyrifos but the need was for a system able to degrade chlorpyrifos as well as its metabolite TCP.

Ivashina (1986) studied chlorpyrifos disappearance by several microbial cultures maintained in liquid media containing 10 mg/ L chlorpyrifos. Munnecke and Hsieh (1975) reported that the enzyme isolated from a mixed microbial culture hydrolyzes chlorpyrifos. The possible metabolism of chlorpyrifos by two lactic acid bacteria (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) was reported by Shaker *et al* (1988). A *Micrococcus sp.* was isolated from a malathion enriched soil which was later reported to degrade chlorpyrifos in liquid media (Guha *et al*, 1997).

Chlorpyrifos has been reported to be degraded co-metabolically in liquid media by *Flavobacterium sp*. and *Pseudomonas diminuta*, which were initially isolated from a diazinon treated field and by parathion enrichment, respectively (Sethunathan & Yoshida, 1973; Serdar *et al*, 1982) and *E. coli* clone (Richnis *et al*, 1997; Wang *et al*, 2005). Also chlorpyrifos has been reported to be utilized as sources of carbon (Singh *et al*, 2003; Yang *et al*, 2005; Ghanem *et al*, 2007), phosphorus (Shelton, 1988; Singh *et al*, 2003, 2004; Yang *et al*, 2005) and sulfur (Cook *et al*, 1980). Thus it appears that chlorpyrifos can be utilized either as a carbon, phosphorus or sulphur source. Singh *et al* (2003) observed the dissipation of chlorpyrifos through co-metabolic activities of the soil microorganisms. Recently, these researchers (Singh *et al*, 2004) reported the enhanced degradation of

chlorpyrifos by an *Enterobacter* strain B-14. Yang *et al* (2005) isolated *Alcaligenes faecalis* DSP3, which is capable of degrading both chlorpyrifos and TCP. A *Klebsiella sp* was isolated by Ghanem *et al*, 2007 from activated sludge which could degrade chlorpyrifos as sole carbon source. These organisms reported from different regions, isolated by different researchers, capable of chlorpyrifos degradation show a predominance of gram-negative bacteria. Attempts to study the diversity at a particular chlorpyrifos contaminated site had not yielded more than one class of bacteria previously (Singh *et al*, 2003; Yang *et al*, 2005).

In the present study, we developed aerobic consortia capable of co-metabolically utilizing chlorpyrifos and studied the diversity of the degraders thus obtained, which utilized chlorpyrifos as carbon source. Among the eleven soil samples collected from the various paddy fields and the formulation site in Punjab, only five showed the presence of chlorpyrifos degrading microorganisms and were from regions with previous history of chlorpyrifos exposure. Chlorpyrifos has been reported to be resistant to the phenomenon of enhanced degradation except for the reports of Singh *et al*, 2003 and Yang *et al*, 2005. But our microorganisms do show the ability of enhanced degradation of chlorpyrifos. Though chlorpyrifos has been reported to be degraded co-metabolically in liquid media by *Flavobacterium sp*. and *Pseudomonas diminuta* (Sethunathan & Yoshida, 1973; Serdar *et al*, 1982) and *E. coli* clone (Richnis *et al*, 1997; Wang *et al*, 2005), these microbes do not utilize chlorpyrifos as a source of carbon. Carbon source is a basic essential requirement for any bacterial cell as carbon is required in the maximum amount for normal cell functioning. Thus the utilization of additional, easily available sources of carbon by bacterial cells during degradation of xenobiotics is very common.

In the present study, bacteria capable of efficient chlorpyrifos degradation from diverse classes including *Pseudomonas sp., Bacillus sp., Klebsiella sp, Serratia sp. and Brucella sp.* have been reported from contaminated soil from the same region (Punjab). Thus, in our studies, though the gram negative bacteria was predominant, but we also found two gram positive degraders belonging to the genus *Bacillus*, of which *Bacillus sp.1* was among the best four isolates degrading chlorpyrifos. While, previous reports indicate only one gram positive bacterium a *Bacillus sp. (*Ivashina, 1986) which was believed to mineralize chlorpyrifos.

Degradation of chlorpyrifos by consortia DC, Q1, Q2 and *Pseudomonas sp.1*, *Brucella sp.*, *Bacillus sp.1* and *Pseudomonas sp.2* in the absence of any competing microorganism was carried out in sterile soil (100gm) whereby the degradation was found to be 43-77% for consortia and 74-91% for the isolates respectively after 30 days, as compared to control. The effect of competing with indigenous soil organisms was then studied in non-sterile soil

(100gm). Here, the degradation was in the range of 70-92% after 30 days, as compared to control.

Chlorpyrifos has been shown to be degraded mainly by hydrolysis and transformed into diethylthiophosphoric acid (DETP) and 3, 5, 6-trichloro-2-pyridinol (TCP), in most of the reports till date. The degradation thus removes chlorpyrifos but yields compounds that are not metabolized by the microorganisms that produce them (Richins *et al*, 1997; Mallick *et al*, 1999; Horne *et al*, 2002b; Wang *et al*, 2002). TCP accumulates in the culture medium causing further toxicity. Other than the *Alcaligenes faecalis* isolated by Yang *et al*, 2005, the only pure bacterial culture known till today is a *Pseudomonas sp.* isolated on TCP by Feng *et al*, 1997. This isolate mineralizes TCP in liquid medium by a reductive dechlorination pathway (Feng *et al*, 1998) ending in water, carbon dioxide and ammonium ions. To break the ring in TCP, chlorine atoms have to be removed (Feng *et al*, 1997) and free chlorine is toxic to microorganisms. That could be the reason that many TCP degraders have not evolved. Yang *et al*, 2005 also proposed that some of the chlorpyrifos degrading enzymes may be inductive as they saw a new band for cells grown in chlorpyrifos when they studied protein profile of cells grown under different conditions. Singh and Walker, 2006 have proposed a possible pathway for microbial degradation of chlorpyrifos, using known degradative pathways of analogous compounds. These researchers have also suggested that metabolic products of chlorpyrifos degradation are TCP and DETP; DETP is further metabolized to ethanol and phosphorothioic acid while TCP is further metabolized through a number of intermediates to pyruvic acid and maleamic acid which then enter krebs cycle. Many such hypothesis of chlorpyrifos degradation exist (e.g.: Smith *et al*, 1967) but no one has as yet elucidated the complete pathway of chlorpyrifos mineralization.

Further exploration in our studies into chlorpyrifos uptake revealed the total utilization of chlorpyrifos by the induced cells of *Pseudomonas sp.2* with formation of only TCP as metabolite, which was believed to be further degraded into unknown metabolites, as no further peak was detected in GC chromatogram during replacement studies with TCP as substrate. The other degraders did not reveal any metabolite formation though they too degraded chlorpyrifos efficiently. The extractions were carried out using non-polar solvents. Hence, we believe that either TCP was degraded by *Pseudomonas sp.2* into unknown polar compounds or the rate of degradation of the other metabolites formed during chlorpyrifos degradation was much faster than that of TCP and so their degradation could not be ascertained by our estimation methods. Also it seems that the rate of formation and utilization of any metabolite formed, which may even be TCP, during chlorpyrifos degradation by *Pseudomonas sp.1*, *Brucella sp.* and *Bacillus sp.1* is also faster and could

not be ascertained. We found that the degradation by induced cells gave enhanced degradation than those by uninduced ones. It could be that some enzymes involved in chlorpyrifos degradation may be induced by chlorpyrifos as also reported by Yang *et al*, 2005.

A modular approach to degradation of a xenobiotic is very essential and effective to estimate the degradation efficiency of inoculated organisms and also to ascertain their population survival in that experimental soil. Pesticide degradation studies using soil microcosms upscaled to plot studies with same microbes would help in deciding the in situ bioremediation strategies to be developed for the clean up of contaminated system. When studied along with crop cultivation, it may also help to assess the effect of plant microbe interaction, if any, under natural conditions.

In soil degradation studies, inoculum size and soil moisture content play a major role along with other affecting factors (Whitney 1967, Read 1976, Tashiro & Kuhr 1978). A community shift occurs whenever a soil is treated with chemicals, hence, it is also essential to know the optimum conditions of pH, and temperature required for any soil degradation studies, the effect of co-metabolism on these communities and the degradation of substrate and survival of the inoculated strains, prior to the release of a microorganism into the natural environment to ascertain the survivability and degradation efficiency of the organism, which can all be ascertained through suitable microcosm studies. To standardize microcosms for future use Angle *et al*, 1994 evaluated survival of a *Pseudomonas aureofaciens* strain in intact soil cores (5.0 x 15 cm PVC core) and disturbed soil microcosms (50 g fresh, sieved soil), which was then compared to data obtained during a field release. They found that an intact soil core microcosm closely simulated field results, from bulk soil and from the rhizosphere of wheat, while a disturbed soil microcosm exhibited a much more rapid decline (within 34 days) in population leading to the conclusion that a small, inexpensive and simple intact soil core microcosm may be appropriate for risk assessment purposes (Angle *et al*, 1994), though a disturbed soil microcosm may also be employed in some cases.

Many researchers have studied the role of inoculated microorganisms into soil in the degradation of chlorpyrifos by fumigating the soil (Robertson *et al*, 1998; Singh *et al*, 2003 & 2004; Yang *et al*, 2005) but it has been reported that with any fumigant treatment a community shift occurs from the naturally present biomass to a community dominated by a particular type of biomass (Ibekwe, 2001). Also, the fumigant may not get completely removed from the experimental soil, causing inhibition of growth of inoculated microorganisms as well. Hence, a sterile microcosm is a better option to ascertain the rate of degradation of chlorpyrifos and other xenobiotics. Getzin (1981) has also studied the

degradation of chlorpyrifos in autoclaved soil and said that autoclaving slowed the rate of decomposition of chlorpyrifos in tested soil, indicating that microbial activities play a role in the degradation of chlorpyrifos.

Soil microcosm studies were conducted for ascertaining the efficacy of our consortia and isolates in natural soil conditions. We observed that a population of 2 x 10^6 cfu/ gm soil and 2 x 10^7 cfu/ gm soil and soil moisture content of 15% failed to effectively degrade chlorpyrifos in contaminated soil, while population of 2 x 10^8 cfu/ gm soil and 30% moisture content were found to be optimum. We also observed that the degradation is highest at 30% moisture content (~82%) & 50% moisture content (~70%) but the population at 50% moisture content is very low. 50% moisture content was waterlogged conditions and hence we believe, it may have become anaerobic and not favorable for our inoculated strains to survive efficiently. It is also known that when released into water, chlorpyrifos has a lower half life (t1/2 = 35-78 days) and it partitions significantly from water column to sediments (Spectrum fact sheet). So, it is possible that either anaerobic degradation is happening or more likely, that chlorpyrifos has partitioned into deeper sediments and was not extracted out efficiently by our methods and hence the error in results showing high degradation in spite of low population at 50% moisture content of soil during standardization. Singh *et al*, (2005) also noted that soil moisture <40% of maximum water-holding capacity slowed down degradation rate and the bacterial isolates were able to rapidly degrade chlorpyrifos between 15 and 35 °C but their degradation ability was sharply reduced at 5 and 50°C. This is similar to the 30% soil moisture and 37°C as the optimum required by our organisms. Microcosm experiments for an ISCB Project (2000-2004) also showed that pesticide degradation was optimal at 35% water holding capacity of the soil and with an incoculum size of 10^8 cells per g soil for *S. paucimobilis* and *A. protophormiae* and the concept of one square meter pilot tests was suitable for disappearance of the target pesticide. Labana *et al*, 2005 studies showed that PNP depletion was quicker when strain RKJ100 cells were induced by pre-exposure to PNP, the efficiency of PNP degradation in soil being dependent on pH, temperature, initial PNP concentration and inoculum size. Here also, a cell density of 2×10^8 colony forming units/g soil was found to be suitable for PNP degradation over a temperature range of 20–40°C and at a slightly alkaline pH (Labana *et al*, 2005). Various factors, including soil pH, temperature, initial fenitrothion concentration, and inoculum size influenced the degradation of fenitrothion by *Burkholderia* sp. FDS-1.The addition of *Burkholderia* sp. FDS-1 at 2×10^6 colony forming units g^{-1} soil was found to be suitable for fenitrothion degradation over a temperature range of 20–40 °C and at a slight alkaline pH (Hong *et al*, 2006).

Soil microcosm studies has been conducted for a number of hydrocarbons like crude oil and naphthalene and pesticides such as isofenphos, fenamiphos and ethoprophos (Chapman *et al*, 1986; Stiriling *et al*, 1992; Karpouzas *et al*, 1999) where the phenomenon of enhanced degradation has been reported. Crude oil degradation by Axenic cultures of *Pseudomonas sp.2* in soil microcosm for 15 days showed degradation in the range of 4.9% to 29.6%, where the major components of the oil were degraded by 6.5% to 70.6% (Okoh, 2003). Inoculation of *Mycobacterium sp.* in microcosms prepared with aquifer samples resulted in rapid degradation of vinyl chloride (VC), whereas native bacteria degraded negligible amounts of VC within the same time period, thus suggesting bio-augmentation potential of the isolate (Fathepure *et al*, 2005). Several microcosm experiments run in parallel indicated that water weathering leads to more effective and rapid elimination of low molecular weight hydrocarbons than microbial mat metabolism, while hydrocarbons of lower volatility were eliminated in higher proportion by microbial mats than by water weathering (Oteyza *et al*, 2006). Soil microcosms designed to study the degradation efficacy for total petroleum hydrocarbon (TPH) of crude oil by *lux*-tagged *A. baumannii* S30 pJES by bioaugmentation showed that TPH levels were reduced from 89.3 to 53.9 g/kg soil in 90 days (Mishra *et al*, 2004). Hence it is important to analyze conditions needed for effective degradation of the xenobiotics in soil before the actual treatment is done.

We conducted microcosm studies for chlorpyrifos degradation with soil (11kg) contaminated with chlorpyrifos (50 mg/ L), moistened to 30% and inoculated with consortia DC, Q1, Q2 & defined consortia MPQ and *Pseudomonas sp.1*, *Brucella sp.*, *Bacillus sp.1* and *Pseudomonas sp.2.* Degradation was found in the range of 34-66% for consortia and 45-58% for the isolates after 30 days. When the studies were further enhanced to i*n situ* in plots, the results indicated that under uncontrolled natural conditions of soil also, effective degradation of chlorpyrifos occurs with our cultures.

Several species of bacteria have been isolated from different environments which degrade organophosphorus compounds in laboratory cultures and in soils (Singh *et al*, 1999, 2006). It has been seen that parathion is rapidly degraded in biologically active soil with a proportional increase in the bacterial population in soils (Nelson, 1982). Degradation of methyl parathion by a *Pseudomonas sp.* in soil and on sodium alginate beads was also reported (Ramanathan & Lalithakumari, 1996). It was observed that mineralization of glyphosate is related to both the activity and biomass of soil microorganisms (Wiren-Lehr *et al*, 1997). More rapid rates of degradation in soil repeatedly treated with the fenamiphos in the laboratory have been reported (Chung & Ou, 1996). Diazinon, monocrotophos, malathion, dimethoate, etc., are being used world-wide and several species of bacteria have

been isolated and characterized that can degrade these compounds in liquid medium and soils.

Enhanced degradation in soil has also been seen for organochlorines like endosulfan where under both flooded and non-flooded conditions in soil, high density inoculum of indigenous phototropic organisms, in the presence of light, transformed α-endosulfan to endosulfan sulphate (Sethunathan *et al,* 2004) which was further enhanced by green algae, *Chlorococcum* sp and *Scenedesm* sp. Other workers have also mentioned the formation and accumulation of endosulfan sulfate by certain fungi in soil by endosulfan metabolism (El Zorgani and Omer, 1974; Martens, 1976; Kullman and matsumara, 1996), which has also been seen in other endosulfan contaminated soils (Kathpal *et al*, 1999; Lee *et al*, 1995; Ghadiri *et al*, 1995). Ochrobacterum sp., Arthrobacter sp., and Burkholderia sp., individually showed *in situ* biodegradation of alpha-endosulfan in contaminated soil microcosm by 61, 73, and 74, respectively, whereas degradation of beta-endosulfan was 63, 75, and 62, respectively, after 6 weeks of incubation over the control which showed 26% and 23 % degradation of alpha-endosulfan and beta-endosulfan, respectively (Kumar *et al*, 2008).

The main objective of *in situ* bioremediation trial in plots with plants was to study the effect of plant-microbe interaction on enhanced degradation of chlorpyrifos. Cotton plants were chosen because of predominant usage of chlorpyrifos on cotton fields in major agricultural areas around Punjab where the studies were carried out. In all soil experiments, the cells were pelleted, washed and re-suspended in sterile water to ensure that no nutrient is added. However, in these plot experiments, where cotton seeds were sown, jaggery was used which may have lead to the general stimulation of soil community. However, the population in plots with and without plants is not very much varied till 20 days and the degradation in plots without crops also shows nearly 99% degradation by day 60, indicating that it is actually the inoculated isolates that are degrading chlorpyrifos. Comparison of degradation of chlorpyrifos in these plots on day 25 indicated degradation in the range of 78-85% after 25 days in plots without crops whereas 99% degradation for all the four cultures was seen in those with crops. Also, the population of all the four cultures was in the range of 7.25 – 7.75 (log value) in plots without crops and 7.38-7.83 (log value) in those with crops on day 25, thus indicating enhanced plant-microbe interaction, i.e. plots showing higher degradation of chlorpyrifos also showed more microbial presence. This is in concurrence with literature, as it has always been seen that rhizospheric soil harbors more microorganisms capable of enhanced xenobiotic degradation abilities. No peak of chlorpyrifos was detected on the 50^{th} day rhizospheric soil samples, indicating mineralization either of the pesticide completely or to non-detectable amounts while 92-95% degradation occurred in plots without plants. No

other peak was found indicating that even if there were any products formed, they were also mineralized within 25days.

Yang *et al*, 2005 treated cabbage growing soils with their strain DSP3 which then showed enhanced degradation of chlorpyrifos, though they did not study whether there was any uptake of chlorpyrifos by the cabbage plants. Flooded soil conditions favored enhanced degradation of parathion in the rhizosphere of rice seedlings (Reddy & Sethunathan, 1983). Enhanced degradation of fenamiphos in the crop fields has been observed in many countries (Stiriling *et al*, 1992; Smelt *et al*, 1996; Meghraj *et al*, 1999). Influence of plants on degradation of diesel in soil was studied by Syal and Ramamurthy in 2003. Soil microcosms treated with carbendazim at concentrations higher than the predicted environmental concentration with a water-only control treatment & studied for plant-microbe interaction showed significant effects on soil dehydrogenase activity, wheat growth and earthworm biomass (Burrows and Edwards, 2004). When vertical soil microcosms flushed with groundwater for *Pseudomonas sp. (isolate P)* was studied, less movement of *isolate P* occurred with downward growth of wheat roots in loamy sand microcosms (Trevors *et al*, 1990). All these reports indicate enhanced plant-microbe interaction in crop fields just like our studies.

In plants there have been reports of delayed seedling emergence, fruit deformities and abnormal cell division upon prolonged exposure to chlorpyrifos (NCAP, 2000). However none of these effects were visually observed in our studies. In fact, all the chlorpyrifos sprayed fields showed better growth than the one without chlorpyrifos. When the crops were analyzed for chlorpyrifos, no chlorpyrifos was found in any of the samples at any stage indicating that no uptake of chlorpyrifos by the plant occurred at any stage.

Studies on the absorption, translocation and metabolism of ^{14}C and ^{36}Cl labeled chlorpyrifos in plants had shown that for all practical purposes uptake of the chemical or its degradation products into plants does not occur, either through the foliage or through the roots and nutrient culture experiments have shown uptake of chlorpyrifos by plant roots to be essentially nil (Smith *et al*, 1967a, 1967c). Many researchers have reported disappearance of chlorpyrifos from Bermuda grass and maize plants (Leuck *et al*, 1968; Johnson *et al*, 1969), from rice and pasture grass (Winterlin *et al*, 1968; Myers *et al*, 1968; Dishburger *et al*, 1969) and from watergrass, sprangletop and smartweed (Hurlbert *et al*, 1970) to nearly negligible levels after only a few days. There has been no evidence of residue build up on plants from repeated application of chlorpyrifos, which is all in total agreement with our studies.

The several factors leading to decline in the microbial population in the field studies over a period of time could be due to nutrition status of the soil, moisture levels, microbial antagonism etc. Apart from competition with the native microflora for nutrition, they must find effective means to survive the nematode and protozoan predators that thrive in such environments. But it has been said that the actively growing roots of the plant release organic compounds, which support growth of the microbial community in the rhizosphere and alter the microbial community creating a distinct community structure in bulk soil with enhanced population density of inoculated strains (Lynch & Whipps, 1990; Bowen *et al*, 1991; Bolton *et al*, 1992 and Kent & Triplett, 2002).

The survival of these inoculated organisms has been studied in a number of different ways but antibiotic plating is the most common method used (Mallick *et al*, 1999; Yang *et al*, 2005). Dilution plating with selective antibiotics was also utilized to monitor the survivability of strains of *A. baumannii* and it was seen that the population of $6.5\pm0.13\times10^8$ cfu/gm soil at day zero (just after bioaugmentation) decreased to $2.09\pm0.08\times10^8$ cfugm soil after 90 days of incubation, confirming the stability of modified organism in soil (Mishra *et al*, 2004). When the survival of *Xanthomonas campestris* pv. was studied under sterile soil microcosms, differences in survival were observed between the EPS producer and its mutant but in non-sterile soil experiments, survival of the organism and the mutant were almost same and bacterial decrease was higher in non-sterile soil microcosms due to the influence of biotic interactions (Lopez *et al*, 1999). The survival of an antibiotic-resistant *Pseudomonas putida* enumerated in field and microcosm soils at 7- to 14-day intervals over 49 d showed survival upto 7 weeks in microcosm soils at a density of 10^4 cfu/g soil, whereas in field soils the population declined to 10^3 cfu/g soil by the fourth week while higher cell densities in the rhizosphere (10^6–10^5 cfu/g fresh root mass) was found when inoculated with seed coating, with no subsequent decrease in numbers (Hirkala and Germida, 2004). Multiple antibiotic resistances were also observed for *Pseudomonas aeruginosa* (Okoh, 2003) and a number of other bacterial isolates from soil.

Antibiotic plating for estimation of population survival has its known advantages and disadvantages. This method acts to screen the inoculated population from the non-resistant indigenous population. However, horizontal gene transfer is a common phenomenon in soil and indigenous soil bacteria may be conferred with multi-drug resistance by the process thus getting selected on the antibiotic plate. So this method may be used as an initial marker for selection but molecular approach may be a better way to have a realistic estimation of the survival of inoculated population under natural conditions. Hence, the combination of soil microcosms with molecular fingerprinting techniques can be very useful as this allows many

replicates to be performed simultaneously with relatively high reproducibility and a useful way of investigating effects of various abiotic and biotic parameters on bacterial community structure. By combining different techniques more detailed information and higher taxonomic resolution can be obtained for the inoculated strains and thus simplifying the estimation of their actual survival in that contaminated soil. An example is the use of Stable Isotope Probing of bacteria based on a markedly different DGGE profile for each species capable of hydrocarbon degradation in contaminated soil (Singleton et al, 2005).

DNA fingerprints based on REP-PCR of isolates were successfully developed to study the population dynamics in conjugation with kanamycin plates. For this DNA of the native isolates and the isolates from soil were subjected to REP-PCR and the original DNA fingerprints of the isolates were compared with the fingerprints of microorganisms isolated from the chlorpyrifos contaminated soil microcosms to score the survival of the population. Molecular fingerprinting profiles indicated that the survival based on antibiotic plates showed higher results (~60-70%) than that by REP-PCR (~30-40%). Population dynamics of inoculated organisms in plots showed that survival of *Pseudomonas sp.1* or *Pseudomonas sp.2* was always better in plots with cotton plants as compared to the plots without cotton plants, leading to enhanced plant-microbe interaction in the rhizospheric soil and so, enhanced degradation of chlorpyrifos in soils with cotton plants. This result is similar to other examples illustrated in literature.

Contrary to the Racke *et al*, 1990 report that there was no evidence for proliferation of microorganisms in soils or in pure cultures of bacteria using chlorpyrifos (Racke *et al*, 1990), our isolates showed an increase in population in the initial 20 days after inoculation concomitant with chlorpyrifos degradation. The extent of proliferation of our population is similar to that of the bacterial isolates in the studies of Mallick *et al*, 1999 but their studies were carried out in liquid media. During studies by Wang *et al* (2005) also the numbers of bacteria and fungi in rhizospheric soil were 1.84-3.18 times larger than those in control. Proliferation also occurred for the chlorpyrifos degrading organisms in the UK soils (Singh *et al*, 2003). Even though only 10^6 cells/ gm soil was required for degradation of chlorpyrifos in the studies by Singh *et al* (2004), these were studied in fumigated soil under laboratory conditions with only 35mg/kg chlorpyrifos, while our experiments were conducted under natural environmental conditions with 50 mg/ kg chlorpyrifos and the inoculated microorganisms had to compete with inherent microorganisms of soil and hence, the difference in inoculum needed for effective degradation of chlorpyrifos. Even in the studies by Yang, 2005, addition of 10^8 cells/ gm soil *Alcaligenes faecalis* was needed for effective bioremediation of 100 mg/kg chlorpyrifos contaminated soil. So far, no one has ever done

such an extensive study of population dynamics of chlorpyrifos degraders especially using molecular markers in conjugation with chlorpyrifos degradation in soil and this is the first report where such comparative analysis has been done.

Bacterial strains like *Flavobacterium and P.dimunita*, that use organophosphate pesticides, synthesize hydrolase enzyme which is encoded by the *opd* gene (Mulbry *et al*, 1986; Harper *et al*, 1988; Mulbry and Karns, 1989 and Siddavattam *et al*, 2003). This gene has been isolated from geographically different locations and taxonomically different species (Singh *et al*, 2006). Recently genes with similar functions and different sequences as that of the *opd* gene, namely *opdA* (Conlin and Miller, 1992 and Horne *et al*, 2002) and mpd (Zhongli *et al*, 2001; Zhang *et al*, 2005 & 2006; Li *et al*, 2006 and Yang *et al*, 2006) have been isolated, characterized, manipulated (Chaudhry & Huang, 1988 and Cho *et al*, 2002) and studied.

In our studies also, an attempt was made to deduce the genetic mechanism responsible for chlorpyrifos degradation in these organisms. Extensive screening of the isolates *Pseudomonas sp.1, Brucella sp.*, *Bacillus sp.1 and Pseudomonas sp.2* did not reveal the presence of any plasmid in any of these chlorpyrifos degraders. Presence of plasmid was not detected in chlorpyrifos degrading strain YC-1, which indicated that the mpd gene responsible was located on the chromosome (Yang *et al*, 2005). Amplification of the native DNA of isolates with OPD, OPDA and MPD primers did not reveal any bands, indicating the absence of known genes of chlorpyrifos degradation.

Singh *et al* in their 2003 studies indicated that among the UK soil and Australian soil tested for presence of known genes of chlorpyrifos degradation, only the UK soil gave a positive signal by DNA probing that indicated that genes similar to *opd* genes were present in this soil but no such signal for DNA hybridization was obtained for Australian soil or isolated degrader indicating presence of unrelated, novel genes. It is very much possible that same is the case with genes involved in the degradation of chlorpyrifos in isolates in our studies.

In summary, five aerobic chlorpyrifos degrading bacterial consortia (AC, BC, DC, Q1 and Q2) were enriched from contaminated soil and isolates obtained from these consortia, *Pseudomonas sp.1, Brucella sp.*, *Bacillus sp.1* and *Pseudomonas sp.2* were capable of showing high efficiency of chlorpyrifos degradation in soil as well as aqueous medium containing chlorpyrifos. Among these, *Pseudomonas sp.2* degrades not only the parent compound but also its major metabolite TCP, thus following a hydrolytic pathway of chlorpyrifos degradation. The degradation of chlorpyrifos by these isolates was concomitant with their survivability in soil, as was demonstrated by ascertaining population dynamics using REP-PCR during microcosm and *in situ* chlorpyrifos degradation studies. Genetic mechanism of chlorpyrifos degradation in these isolates could not be conclusively detected,

hence leaving a speculation of involvement of a novel genetic system in these degraders of chlorpyrifos.

7. SUMMARY

1. Five aerobic consortia (AC, BC, DC, Q1 and Q2) were developed by enrichment from chlorpyrifos contaminated soil. Basal medium containing 50 mg/L chlorpyrifos and 0.1% glucose was found to be the best for the growth of these consortia. Glucose utilization studies indicated that all glucose was utilized within 24 hours of inoculation.
2. Among these five consortia capable of degrading chlorpyrifos, consortia Q1 and Q2 were the best with degradation efficiency of 72 and 70% respectively. Consortia DC had the degradation ability of 60% while consortia AC and BC showed degradation of 54% and 46% respectively after 21 days, as compared to uninoculated control, which had abiotic degradation of 15%.
3. The consortia were serial diluted on basal agar plates and only 25 showed degradation capability between 2-86%. Among the 15 isolates that showed ≥40% degradation of chlorpyrifos, time-course studies showed that isolates P, M19, M119 and Q2c degraded 86, 83, 84 and 84% of chlorpyrifos after 20 days of incubation while isolates D113, Q1a, Q2a, and Q2b degraded 77, 81, 82 and 80% chlorpyrifos respectively as compared to uninoculated control which showed 23% abiotic degradation. The growth of these isolates was ~23% higher than that of the rest concomitant with their ability to degrade chlorpyrifos. The rest of the isolates D15, D234, D244, D320, M117, Q1g and Q1m showed <75% degradation of chlorpyrifos. Thus the best eight Isolates, based on their differential degradative ability of chlorpyrifos degradation, namely P, M19, M119, D113, Q1a, Q2a, Q2b and Q2c were chosen for further studies.
4. Morphological character and gram nature of the isolates showed that P, Q1a, Q2a, Q2b and Q2c are gram negative rods; M19 is a gram negative coccus while M119 and D113 are gram positive rods. The molecular identification based on the 16s rDNA profile lead to the characterization of the isolates. P and Q2c were identified as *Pseudomonas sp.*, M19 as *Brucella sp.*, M119 and D113 as *Bacillus sp.*, Q1a and Q2a as *Klebsiella sp.*, and Q2b as *Serratia sp.*
5. Optimization of microcosm conditions revealed log phase cells with cell mass 2x 10^{8} cells/gm soil as inoculum and 30% moisture content of soil to be the optimum for microcosm studies.
6. Antibiotic profile revealed the various isolates to be resistant to kanamycin (50mg/ L).

7. As high population survival was indicated even by antibiotic plating, estimation of population survival was also carried out using molecular markers based on repetitive sequences in bacteria and touch-down PCR (REP-PCR), to develop strain specific DNA fingerprints of these isolates.
8. Initial soil studies with 100 gm soil contaminated with chlorpyrifos (50 mg/kg), inoculated with (2x 10^{8} cells/ml) consortia DC, Q1 and Q2 and *Pseudomonas sp.1, Brucella sp., Bacillus sp.1 and Pseudomonas sp.2* indicated that consortia Q1 and Q2 degraded 69 & 87% and 77 & 86% chlorpyrifos respectively, under sterile and non-sterile conditions after 30 days of incubation. Among the isolates, *Pseudomonas sp.1, Brucella sp., Bacillus sp.1 and Pseudomonas sp.2* showed 91, 74, 79 & 89% degradation under sterile conditions and 89, 87, 85 & 92% under non-sterile conditions respectively after 30 days of incubation, as compared to control, which had an abiotic degradation of 33% and 34% after 30 days of incubation under controlled laboratory conditions.
9. Based on degradation studies and initial soil studies, *Pseudomonas sp.1*, *Brucella sp.*, *Bacillus sp.1* and *Pseudomonas sp.2* showing the best degradation ability under aqueous conditions and efficient degradation in soil were selected for uptake studies. Experiments were carried out to study the effect of induction on chlorpyrifos uptake, when the cells were induced at growth and when they were induced as whole cells. For the first experiment, bacterial cells of *Pseudomonas sp.1, Brucella sp.*, *Bacillus sp.1 and Pseudomonas sp.2* were grown in Luria broth, harvested, washed and re-suspended in phosphate buffer, starved overnight and divided into two sets for uptake studies. One set of starved whole cells was inoculated into phosphate buffer containing 50 mg/L chlorpyrifos while the other set was induced with 5 mg/L chlorpyrifos for 2 1/2 hours and uptake was carried out in similar way. A third set of cells was induced during growth with chlorpyrifos and then uptake studies were carried out. The uptake by un-induced whole cells of isolates *Pseudomonas sp.1*, *Brucella sp., Bacillus sp.1 and Pseudomonas sp.2* after six hours of incubation was 52, 43, 36 and 62% respectively which further increased to 84, 68, 60 and 87% after 12 hours. In case of induced whole cells of *Pseudomonas sp.1*, *Brucella sp. and Bacillus sp.1 and Pseudomonas sp.2,* at sixth hour, the degradation was 68, 50, 45 and 69%which further increased to 93, 77, 69 and 100% respectively at 12^{th} hour. For cells of *Pseudomonas sp.1*, *Brucella sp. and Bacillus sp.1*, induced at growth with chlorpyrifos, the degradation was 65, 50, 45 and 67% after six hours which further increased to 90, 75, 67 and 100%, respectively after 12 hours of incubation.

10. Product analysis during utilization of chlorpyrifos did not show the formation of any peak of known compounds for *Pseudomonas sp.1*, *Brucella sp. and Bacillus sp.1*, while one peak was observed with whole cells of *Pseudomonas sp.2* at 4.3 minutes which corresponded to the peak of standard 3, 5, 6-trichloro-2-pyridinol (TCP) identified on the basis of retention time analyzed by GC. Substrate replacement and uptake of TCP with whole cells of *P. sp.2* showed 40% utilization after six hours which further increased to 67% utilization after 12 hours of incubation. Formation of any metabolite during the utilization of TCP was not detected.
11. Microcosm studies were conducted with 11kg chlorpyrifos contaminated soil, inoculated with cultures of consortia DC, Q1 and Q2, defined consortia MPQ (with equivalent proportions of *Pseudomonas sp.1, Brucella sp., Bacillus sp.1 and Pseudomonas sp.2*) and *Pseudomonas sp.1, Brucella sp., Bacillus sp.1 and Pseudomonas sp.2*. Consortia Q2 and MPQ degraded 93 & 98% of chlorpyrifos at 60days while consortia Q1 and DC showed 95 and 89% respectively after 60 days as compared to 28% in uninoculated control. Among the isolates, *Pseudomonas sp.2* and *Brucella sp.* showed 99% and 98% degradation while *Pseudomonas sp.1 and Bacillus sp.1* showed 94 & 96% degradation respectively after 60 days as compared to 26% degradation in uninoculated control.

Population was maintained concomitant with chlorpyrifos degradation and there was just a slight decrease from inoculated population of 3.72×10^8, 1.99×10^9, 4.89×10^8 and 5.75×10^8 (log value 8.57, 9.3, 8.69 and 8.76) for consortia Q1, Q2, DC and MPQ to 1.35×10^8, 1.18×10^8, 3.63×10^7 and 1.51×10^8 (log value = 8.13, 8.07, 7.56 and 8.18) cfu/gm soil respectively and 3.8×10^8, 3.63×10^8, 3.72×10^8 and 3.55×10^8 (log value = 8.58, 8..56, 8.57, 8.55) cfu/gm soil to 2.45×10^8, 2.45×10^8, 2.39×10^8 and 2.45×10^8 (log value = 8.39, 8.39, 8.38 and 8.39) cfu/gm soil respectively after 60 days for *Pseudomonas sp.1, Brucella sp.*, *Bacillus sp.1 and Pseudomonas sp.2.* REP-PCR was carried out to ascertain the actual population of the introduced degraders in soil. Antibiotic plating gave a higher estimation of population (64-68%) survival as compared to molecular fingerprinting (34-46%).

12. It is essential for the success of any bioremediation process that the compound as well as its toxic metabolite is removed. When this was ascertained, the studies were continued further in plots (54 m^2) contaminated with chlorpyrifos & some planted with American cotton plants. Plots sprayed with chlorpyrifos (50 mg/ kg) were divided into two sets, one set was inoculated with cultures of consortia Q1 and Q2 and isolates *Pseudomonas sp.1* and *Pseudomonas sp.2* as such and the other was inoculated with

cultures of consortia Q1 and Q2 and isolates *Pseudomonas sp.1* and *Pseudomonas sp.2* along with planting of cotton seeds by randomized block design. Soil samples and plants were withdrawn after regular time intervals and analyzed for residual chlorpyrifos along with estimation of population survival by dilution plating on kanamycin plates and REP-PCR.

13. Comparison of degradation of chlorpyrifos by consortia Q1, Q2 and isolates *Pseudomonas sp.1* and *Pseudomonas sp.2* on day 25 indicated degradation of 79, 82, 78 and 85% in plots without crops, whereas 99% degradation for all the four cultures in plots with crops. The population of all the four cultures was in the range of 7.25 – 7.75 (log value) in plots without crops and 7.38-7.83 (log value) in plots with crops on day 25. The population of *Pseudomonas sp.1* and *Pseudomonas sp.2* indicated by REP-PCR was 7.30 and 7.41 (log value) respectively in plots without cotton and 7.49 and 7.55 (log value) in plots with cotton, on day 25. All these results indicated enhanced chlorpyrifos degradation during effective plant-microbe interaction. No residual chlorpyrifos was found in any of the sampled plants. No peak of chlorpyrifos was detected on the 60^{th} day soil samples from plots with crops and >99% degradation in those without crops.
14. Metabolite analysis during *in situ* degradation of chlorpyrifos in plots inoculated with *Pseudomonas sp.2* indicated the formation of TCP (2.5mg/kg) after 20 days which disappeared to negligible amounts after 30 days. No other metabolite peak was obtained from any of the other samples.
15. Amplification of the native DNA of *Pseudomonas sp.1, Brucella sp.*, *Bacillus sp.1 and Pseudomonas sp.2* was carried out with OPD, OPDA and MPD primers. The sample when analyzed by agarose gel electrophoresis gave no bands on gel, even after repeated trials. Thus, further quest into the genes involved in the metabolism of chlorpyrifos revealed no definitive results.

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APPENDIX

Luria Broth	**g/l**
Casein enzymic hydrolysate	10.00
Yeast extract	5.00
Sodium chloride	5.00
Final pH (at 25°C)	7.0 ± 0.2

Sterlize the media in Autoclave at 121^0C for 15min at 15lb psi pressure

Luria Agar= Luria Broth + Agar (1.5%)

Bushnell Hass Broth

	g/l
Magnesium sulphate	0.2
Calcium chloride	0.02
Monopotassium phosphate	1.00
Dipotassium phosphate	1.00
Ammonium nitrate	1.00
Ferric chloride	0.05
Final pH (at 25°C)	7.0 ± 0.2

Sterlize the media in Autoclave at 121^0C for 15min at 15lb psi pressure.

Bushnell Hass Agar= Bushnell Hass broth + Agar (1.5%)

Potassium – phosphate buffer (1M, pH = 6.5)

	g/l
a) KH_2PO_4 solution	136.09
b) K_2HPO_4 solution	174.18

Added a) 390 ml and b) 610 ml to make the net volume to1000ml.

Adjusted pH with K_2HPO_4 solution.

Biuret reagent:

NaOH	30 g
$CuSO_4$	0.12 g
Total volume	100 ml

Sodium hydroxide was dissolved in ice-bath and cupric sulfate was added slowly with continuous stirring.

Modified ROSE Solution

EDTA (pH=8.0)	3.7 g/L
Tris-HCl (pH=8.0)	1.2 g/L
SDS	1 g/100ml
Polyvinylpolypyrrolidone (PVPP)	1 g/100ml

Plasmid Isolation

Solution I

Glucose	9.0 g/L
Tris-Cl (pH=8.0)	3.0 g/L
EDTA 3.72 g/L	

Solution II

NaOH	8 g/L
SDS	1 g/100ml

Solution III

Potassium acetate (5M)	60 ml
Glacial acetic acid	11.5 ml
Distilled water	28.5 ml

Ethidium bromide stock

Ethidium bromide (1 g) was dissolved in 100ml of distilled water and stored at room temperature.

Agarose gel electrophoresis buffer

Tris-acetate buffer, TAE (50X)

Tris	242 g
Glacial acetic acid	57.1 ml
EDTA (0.5M, pH=8.0)	100 ml

TE Buffer = Tris: EDTA (10:1)

Gel loading dye (6X)

Bromophenol blue	0.25 g/100ml
Xylene cyanol	0.25 g/100ml
Ficoll-type (400,000)	15 g/100ml

The prepared solution was stored at room temperature.

DNSA solution

Solution A:

Sodium potassium tartarate	300 gm
Distilled water	500 ml

Solution B:

3, 5 –dinitrosalicylate	10gm
NaOH solution (2M)	200ml

DNSA solution Solution A + Solution B

Net volume was made to1000ml with distilled water.

Publication record of C. Vidya Lakshmi

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