EVALUATION OF MONOCROTOPHOS RESIDUES IN SOILS OF BATHINDA, PUNJAB AND ITS BIODEGRADATION THROUGH MICROBIAL ISOLATES OF CONTAMINATED SOIL

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BY

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CERTIFICATE

I declare that the dissertation entitled "**EVALUATION OF MONOCROTOPHOS RESIDUES IN SOILS OF BATHINDA, PUNJAB AND ITS BIODEGRADATION THROUGH MICROBIAL ISOLATES OF CONTAMINATED SOIL**" has been prepared by me under the guidance of Dr. Dhanya M. S., Assistant Professor, Centre for Environmental Science and Technology, School of Environment and Earth Sciences, Central University of Punjab. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

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ABSTRACT

Evaluation of Monocrotophos residues in Soils of Bathinda, Punjab and its Biodegradation through microbial isolates of Contaminated Soil

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The soil samples for the study were randomly collected from ten different villages (Bhagibander, Dhadde, Gurusar Sahnewala, Jajjal, Jassi, Malkana, Mahinangal, Sangat Kalan, Talwandi sabo, Teona Pujarian) of Bathinda. The questionnaire survey provided the information on pesticide history and consumption. All the villages had the history of monocrotophos (MCP) usage for crops like cotton, rice and legumes and two villages Dhadde and Teona Pujarian had current monocrotophos usage. The MCP residue of 0.08 μg/kg was detected only in Teona Pujarian soil sample. The screening of all soil samples for the microbes with tolerance for different monocrotophos concentrations of 50 mg/l to 1000 mg/l in pikovskaya media was done by enrichment and adaptation method. The two bacterial isolates named as D_1, T_1 and four fungal isolates D_2, D_3, T_2, and T_3 were isolated from Dhadde (D) and Teona Pujarian (T) soil at MCP concentration of 1000mg/L. The morphological and biochemical test results for bacterial isolates from Teona Pujarian, T_1 and Dhadde, D_1 were found similar to Pseudomonas sp. and Serratia sp. respectively. The fungal isolates were found to have similar morphological characters to Aspergillus sp. The isolate T_1 showed the degradation potential of 88.9 % in media and 87.4% in the MCP spiked soil after 7 days for MCP concentration of 1000 mg/L. The increase in P solubilisation and detection of traces of MCP residues and volatile fatty acids like palmitic acid, stearic acid, etc. indicated MCP mineralization by the isolate. This isolate could be useful in on-site bioremediation of the monocrotophos contaminated soil.

(Disha Mishra) **(Dr. Dhanya M. S.)**

DEDICATION

This dissertation is dedicated to all the people who never stop believe me and who along with God, have been my 'footprints in the sand'

My Uncle: Lt. Mr. D. K. Mishra

&

My Parents

My Loving Sisters and Brothers

To my Teacher, who taught me to get up after a fall and start again

Finally, this dissertation is dedicated to all those who believe in the richness of learning

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Disha Mishra

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LIST OF ABBREVIATIONS

Sr. No.	Full form	Abbreviation
1	Active ingredient	a.i.
2	Chemical Oxygen Demand	COD
3	Colony forming Unit	cfu
4	Monocrotophos	MCP
5	Optical density	OD
6	Organophosphorus	OP
7	Phosphorous solubilizing bacteria	PSBs
8	Phosphorous solubilizing microbes	PSMs
9	Pikovskaya media	PKV
10	Volatile fatty acids	VFAs

CHAPTER 1

INTRODUCTION

In India pesticides are detected as marked input for the agricultural process for attaining self sufficiency in the food grain production. The progressive enhancement in the agricultural activities throughout the country is linked with intensive pesticides application as every year almost 30% of crop loss is being recorded due to pest infestation. For year 2010 the consumption of organophosphorus pesticide in India accounts for 16133.62 tonnes (FAOSTAT, 2012). The most commonly used pesticides are dimethoate, endosulfan, quinalphos, monocrotophos, phorate, chlorpyrifos, diazinon, methyl parathion etc (Patil and Katti, 2012). Few of them are considered as highly hazardous category of pesticides and causes serious health impacts. The pesticide consumption in India is 0.57 kg/ha while Punjab is on 5810 MT for the year 2009-10 (SoIAR, 2011-12). The organophosphate pesticides became one of the major pesticide consuming groups in agriculture after the ban on many of the chlorinated and carbamate pesticides. Organophosphorus insecticides are esters of phosphoric acid which include aliphatic, phenyl and heterocyclic derivatives and have one of the basic building blocks as a part of their complex chemical structure. Among all OP compounds, chloropyrifos, monocrotophos and quinalphos attain top position in Indian market. The organophosphoric (OP) compounds share maximum of 38% of total usage of pesticides in the world, but the problem of pesticides residue is very common in India. In India the usage pattern of pesticides is having a different scenario than other countries as major share is skewed towards the insecticides alone. Among the insecticides the organophosphorus compounds like parathion, methyl parathion, phorate, monocrotophos, chlorpyrifos, dimethoate are used frequently for various cash crops. But indiscriminate usage of pesticides leads to depletion of essential macro and micronutrients from soil, residue persistence, leeching to ground water, and bioaccumulation in the food, milk and blood and make environment more vulnerable towards qualitative and quantitative degradation.

Monocrotophos (MCP) which is classified under highly hazardous category, and is reported still under usage rather than banned on its application due to its broad spectrum nature. It is widely used for vegetables, cotton, sunflower, tobacco plants to control common pest (aphids, caterpillars, Helicoverpa sp., mites, jassids, budworm, scale and stem borer) and even locusts. Monocrotophos is a systemic OP insecticide and acaricide belonging to the vinyl phosphate group. The maximum consumer of pesticides is cotton crop followed by paddy and wheat. The major cotton growing states of India are Maharashtra, Punjab, Haryana, Rajasthan (Choudhary and Gaur, 2010). The MCP is widely used in the cotton crops of this zone and residues of MCP are found in soil and food. According to WHO (2009), the ingestion of 120 mg monocrotophos can be fatal to humans and acute oral lethal dose (LD_{50}) for rats is 14 mg/kg. The mode of entry of monocrotophos is absorption following ingestion, inhalation and skin contact. When inhaled, it affects the respiratory system and may trigger bloody or runny nose, coughing, chest discomfort, difficulty in breathing or shortness of breath and wheezing due to constriction or excess fluid in the bronchial tubes. Skin contact with organophosphates may cause localized sweating and involuntary muscle contractions. Eye contact will cause pain, tears, pupil constriction and blurred vision. Severe poisoning will affect the central nervous system, producing lack of coordination, slurred speech, loss of reflexes, weakness, fatigue, involuntary muscle contractions, twitching, tremors of the tongue or eyelids, eventually paralysis of the body extremities and the respiratory muscles (WHO, 2009).

Thus indiscriminate and injudicious usage pattern is directly degrading the environmental components like soil, water and air. Also their persistence in soil further leads to bioaccumulation in the tissue, blood and milk along with disturbing the harmony of soil environment. Thus it is essential to remove these contaminants from soil so that environmental hazard can be prevented. Microbial metabolism posses immense potential to degrade that contaminants to less toxic compounds. The degrading microbes utilize these OP as a source of carbon, nitrogen, phosphorus and energy thereby converting xenobiotics into carbon dioxide, ammonia or water but for environmental point there should be complete mineralization. The biodegradation strategy applies very less impact on natural ecosystem. Thus it becomes necessary

to explore the process of biodegradation, types of microbial community, development of efficient and cost effective methods of decontamination of hazardous contaminated soil including pesticides. Further this would help to remove persistent residue from soil, which would not further enter into food chain and many of the lethal disease like cancer can be prevented up to some extent.

In the soil environment microbial degradation as the result of microbial metabolism was found major reason for the degradation of these xenobiotics. The soil microorganisms posses a great potential for the removal of these toxic chemicals from the environment. The complete biodegradation in the soil is mediated by the action of many microbes and converting them into less toxic end products like mainly CO_2, H_2O, NH_3 etc. The first microorganism that could degrade organophosphorus compounds was isolated in 1973 and identified as Flavobacterium sp. (Sethunathan et al., 1973). Various bacterial and fungal strains were isolated from a range of habitats that are actively involved in the biodegradation of wide range of OP compounds (Singh and Walker, 2006). The extra cellular enzymatic activity is responsible for complete mineralization of MCP and number of isolates are reported for its biodegradation (Kavikarunya, 2012; Das and Anitha, 2011; Jia et al., 2006; Singh and Singh, 2003; Bhadbhade et al., 2002a).

The Malwa region which is the heartland for cotton production is suffering with the high pesticides exposure along with the residue in different matrices of environment. Pesticide consumption in Punjab is 923 g/ha (PSoER, 2007) as compared to the average consumption of 381 g/ha at India level (SoIAR, 2012). The MCP consumption particularly have been decreased from the 1622 MT to 1242 MT from year 2001 to 2006 yet various lethal after effects have been noticed due to long term application on the each and every segment of population (WHO, 2009). The residue contaminated soil also affecting adversely to plants and microorganisms activity. Therefore the adaptation of biodegradation approach for its decontamination from soil is ecofriendly and cost effective technology.

The present study is formulated for analysis of MCP residues in different soils of Bathinda and also exploring the degradation of MCP in the soil by isolates from the

contaminated soil. This would further help in developing the bioremediation technique for the pesticides contaminated sites of the region.

Objectives:

The objectives of the study were formulated as follows:

1. To analyze Monocrotophos residues in different soil samples of Bathinda region
2. To isolate microflora with Monocrotophos degrading potential from soils of Bathinda
3. To study the degradation rates of Monocrotophos by isolated bacterial microflora

CHAPTER 2

REVIEW OF LITERATURE

Pesticides are those substances which are used to control, destroy, repel or attract pests in order to minimize their detrimental effects (EPA, 2012). The Indian agriculture utilizes 0.381 kg a.i./ha of pesticides and produces about 1.6 % of total world production (Arora et. al., 2011). India ranks 12th in world for pesticides usage along the annual production accounts for 90000 tones and in turn largest producer of pesticides in Asia (Patil and Khatti, 2012). The major pesticides consuming states are Andhra Pradesh, Gujarat, Maharashtra followed by Punjab and Karnataka. Among all the crops grown in India, pesticide consumption in the cotton ranks first followed by paddy (22.8%), sorghum (8.9%), vegetables (7%), wheat (6.4%), pulses (2.8%) and others (7.6%) (Singhal, 2003). The insecticides shared for 52 per cent, herbicides 33 per cent and fungicides 15 per cent of the total consumption pattern of pesticides in India. Based on chemical nature of products, the market comprises 16 per cent organochlorines, 50 per cent organophosphates, 4 per cent carbamates, 19 per cent synthetic pyrethroids, 1 per cent biopesticides and 10 per cent others.

2.1 Pesticides consumption and its residues in Punjab

The injudicious and indiscriminate usage of highly toxic and persistence pesticides brought high production along with negative effects due to the introduction of green revolution in Punjab. The per hectare pesticides consumption in Punjab is 923 g/ha followed by Haryana 843 g/ha, Andhra Pradesh 548 g/ha, Tamil Nadu 410 g/ha (Kaur and Sinha, 2011). Punjab accounts only 1.5% of the total land mass of the country but it had total 15 % pesticide usage of the country. Alone the Malwa region (The cotton belt) utilizes 75% of the total pesticides consumption of Punjab (PSoER, 2005). About 60 % of total pesticides usage is insecticides and 90% of that was reported for crops like cotton, rice and vegetables (Singh, 2002).

The residue of DDT and HCH were reported in wheat flour from Jalandhar, Patiala, Sangrur, Faridkot, Chandigarh, Amritsar district with its amount for more than permissible limit (Joia et al., 1978). The blood samples and vegetables from Patiala

region were detected with residues of phosphamidon, dichlorovos, quinalphos above minimum residue limits (Chattophadya, 1998). Most of the vegetables and food commodities were found with the residues of chlorpyrifos, endosulfan, monocrotophos, quinalphos with the 18% of above tolerance limit. Singh (2004) have reported the residue of chlorpyrifos in cotton seed sample with the amount of 0.019 mg/kg in Bathinda district while ethion residue was in the amount of 0.066 mg/kg in Mansa district of Malwa region. The water and vegetable samples collected from the Talwandi sabo block have been detected the residues of endosulfan, heptachlor, chlorpyrifos, aldrin, ethion etc. (Singh, 2002). Most of the blood samples from the villages Jajjal, Balloh, Mahinangal from Bathinda district and Dher village from Ropar district had the residue of organochlorine pesticides and MCP in most of the blood samples was reported 0.095 µg/gm which is four times higher than short term exposure limit for human (Mathur et al., 2005).

The study conducted in the Jalandhar and Moga district also revealed that 28% of the farmers were not aware with the instruction for the pesticides usage and 64.5% are unaware about the recommended dose of the pesticides. He also reported that 75.5% of the farmers were using the empty containers of pesticides for household activities and 54% were not aware about the ill effect of pesticides misuse and overdosing (Sharma, 2005). A survey conducted by Singh (2008) in 30 villages of eight districts of Malwa region i.e. Faridkot, Muktsar, Bathinda, Mansa, Sangrur, Barnala, Firozpur and Moga which are considered as high risk zone due to cancer mortality and the villages were associated with cotton cropping pattern and was found to have strong linkage with cancer deaths. Thakur (2008) reported the cancer prevalence in Malwa region with the excessive usage of pesticides by comparing cancer deaths of 52 per lakh and 30 per lakh of population studied in 129 villages from Talwandi sabbo and Chamkursahib. As cotton crop require more application of pesticides thus there were more chances to exposure of pesticides (Singh, 2008). According to Kaur and Sinha, (2011) the rising instances of abortion, early menarche, foetal abnormalities, nervous system disorder and early ageing was due to the higher pesticides levels in Punjab.

2.2 Pesticides entry to the environment

The prolonged and indiscriminate application of pesticides for increasing the yield in the agriculture sector lead to the contamination in different environmental matrices. Pesticides and these degradative products enter the atmosphere either by application drift, post-application vapour losses or wind erosion of pesticide treated soil. Water transport of pesticides can occur through wet deposition, run-off from surfaces, and infiltration of water through the ground, ditches, storm sewers, drains, rivers, and open water current The long repeated and consistent manner application of pesticides to the soil affects soil biological parameters, soil enzymatic properties and microbial population etc. (Das and Mukherjee, 2000; Singh et al., 1999; Shetty and Magu, 1998). The bioaccumulation of pesticides and its transformation to byproducts also affect non target organisms. Due to pesticides application the structure and chemistry of degraded soil was altered with little organic matter for microbial growth and also become vulnerable to soil erosion (Chowdhury et al., 2008).

The impact of pesticides on the soil environment depends on the mode of application, bioavailability of chemical, soil interaction with compound, persistency of compound etc. The effect of different pesticides i.e. phorate, carbofuran, carbosulfan, thiomethoxam, imidacloprid, chlorpyriphos and monocrotophos applied on soyabean was studied on the soil microflora by Sarnaik et al. (2006). The rhizobial count was increased by the seed and foliar application of carbofuran, thiomethoxam, phorate and chlorpyrifos while imidacloprid application decreased its counts. The phosphorus solublizing bacteria were also found to decrease significantly with pesticides application. The application of MCP after 30 days had no adverse effect on the total bacterial count of the soil but the repeated application had resulted decrease in the rhizobial count of the soil (Sarnaik et al., 2006). The population of cellulolytic bacteria, Pseudomonas bacteria and fungi was affected by the application of glyphosate and paraquat over a period of 2.5 year of application (Weckert et al., 2007). The herbicide application had directly affected the microbial community, while the usage of insecticides and nematicides was also responsible for short term fluctuation in the microbial populations (Pandey and Singh, 2004). The treatment of 2, 4-D to the soil

had been resulted to the decreased microbial biomass by 15-20% due to the prolonged applications of trifluralin (Dumontet and Perucci, 1992) and atrazine were also reported to cause a decline in microbial biomass. The decline in enzymatic activities of dehydrogenase, urease, arylsulfate, phosphatase etc. were reported by application 2,4-D, atrazine, glyphosate (Sannino and Gianfreda, 2001 and Rai, 1992). The nitrogen mineralization and nitrification rates were reduced by the application of herbicides i.e. diuron, fluometuron, prometryn and metolachlor on the cotton crop (Gupta et al., 2000).

The transformation products of DDT, endosulfan, endrin, heptachlor, lindane had persistence nature. Transformation byproducts of banned pesticides are still present in the bound manner with soil particle (Aktar et al., 2009). Vig et al. (2008) reported the insecticides like dimethoate, monocrotophos, deltamethrin, endosulfan, cypermethrin, triazophos application on the cotton crop soil from the village of Punjab area was responsible for temporary decline in the bacterial population. The short time iron reduction capacity was recorded with the application of endosulfan and cypermethrin. The substrate level respiration decline with the MCP application whereas basal respiration also decrease with the dimethoate application. The history and half life of some organophosphate insecticides in soil were described in Table1.

2.3 Structure and properties of Monocrotophos

Monocrotophos (MCP) is non-specific systemic organophosphorus (OP) insecticide and acaricide, used to control common pest like helicoverpa sp., budworm, stem borer, aphids, caterpillars, mites, jassids, scales and locusts. It is manufactured from mono-chloro-monomethylacetoacetamide and trimethyl phosphate and quickly enters in the plant tissue due to water solubility (Tomlin, 1994). It is widely used for crops like cotton, sugarcane, groundnut, maize, rice, soybean, vegetables, ornamentals and tobacco etc.

Table 1: History, Toxicity and Half-life period of some common organophosphorus pesticides

Name	Type	Year of introduction	Average Half-life period in soil (days)	Mammalian LD_{50} (mg kg^{-1})
Chlorpyrifos	Insecticide	1965	10–120	135–163
Parathion	Insecticide	1947	30–180	2–10
Methyl parathion	Insecticide	1949	25–130	3–30
Monocrotophos	Insecticide	1965	14-21	18–20
Dicrotophos	Insecticide	1965	45–60	15–22
Diazinon	Insecticide	1953	11–21	80–300
Dimethoate	Insecticide	1955	2–41	160–387
Fenitrothion	Insecticide	1959	12–28	1700
Glyphosate	Herbicide	1971	30–174	3530–5600
Coumaphos	Acaricide	1952	24–1400	16–41
Fenamiphos	Nematicide	1967	28–90	6–10
Ethoprophos	Nematicide	1966	3–30	146–170

Source: (Singh and Walker, 2006)

This organophosphate insecticide had high oral and moderate dermal toxicity and mode of action is the inhibition of choline esterase activities (Skripsky and Loosli, 1994). It has a half-life of 7 days in exposed sun light (Smith, 1993).

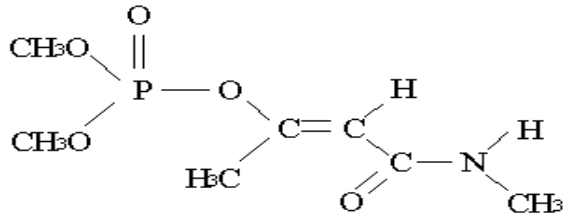
Biodegradability of MCP is dependent on the pH of the soil. The half-lives of 26, 134, and 131 days were calculated at pH 9, 6, and 3, respectively at 25 ^{0}C, (Lee et al., 1990). The half-life of MCP solution of 2 mg/L at pH 7.0 and 38°C was reported to be 23 days while 80 minutes at pH 4.6 and 100°C.

The EPA classifies monocrotophos as a Class I toxic chemical and highly toxic to birds with the LD_{50} in birds being 0.9–6.7 mg/ kg (IPCS, 1993). Table 2 gives chemical and physical properties of MCP.

Table 2: Physico-chemical characteristics of MCP

Common name	Monocrotophos
IUPAC name	Dimethyl (E)-1-methyl-2-(methylcarbamoyl) vinyl phosphate
Molecular formula	$C_7H_{14}NO_5P$
Molar mass	223.2 g/mol
Density	1.33 g/cm^3
Melting point	Technical grade: 25-30°C, pure:54-55°C
Boiling point	120 °C at 0.0005 mmHg
Vapour pressure	0.29 mPa (20°C)
Physical state	Colorless, hygroscopic crystals
Solubility	Soluble in water, acetone and alcohol; very slightly soluble in mineral oils.

Chemical structure:



2.4 Environmental fate of MCP

MCP gets degraded in the environment very easily due to its water solubility. The hydrolysis of MCP is pH dependent as at higher pH the rate of hydrolysis was found faster with the half lives of 17-20 days at pH 9. The degradation MCP in the aerobic soil conditions was fast having the half life between 1-7 days. N-methyl acetoacetamide, O-desmethyl-monocrotophos, N-(hydroxymethy1)-monocrotophos, 3-hydroxy-N-methylbutyramide were also reported as degradation products of MCP in soil (Lee et. al., 1990). The degradative pathway of MCP suggested by Kanekar et al., 2004 (Fig.1) had CO_2 and inorganic phosphates as final end products.

When pesticides are introduced to any segments of environment they become mobile in the environment. Pesticide characteristics like water solubility, adsorption on the soil, mode of application, pesticide persistence, physical, chemical and biological characteristics of soil were important factor for deciding the fate of pesticides in the environment (Pal et al., 2006). The pesticide formulation, method, and rate of application, topography of application site, amount and type of vegetation and groundcover, weather conditions also affect distribution and degradation of pesticides. The fate of pesticides was decided by several mechanism in the environment like transformation in which biological and chemical processes help in changing the structure of original compound, transfer process helps in the distribution of pesticides between solids and liquids/ gases (soil and soil water/air) where as through transport movement occur from one environmental compartment to another,

such as the leaching of pesticides through soil to ground water, volatilization into the air, or runoff to surface water. The movement of pesticides occurs from the application sites to non target sites through volatilization spray drift, runoff, leaching, absorption and crop removal, created problems like economic loss to farmers, pests resurgence, and environmental contamination (Waite et al., 2002).

Figure 1: Pathway for degradation of MCP (Source: Kanekar *et al*., 2004)

2.5. Mechanism for pesticides fates in the soil environment

The fate of pesticides in soil is governed by several mechanisms like retention, volatilization and degradation. The degradation comprises mainly of chemical degradation, photo degradation and biodegradation.

The adsorption of pesticides to the soil particles was due to hydrogen bondings, ion exchanges, interactions with metallic cations, polar interactions, charge transfers, london-vander waals dispersion forces and hydrophobic effects (Calvet et al., 1989). The amount of pesticide adsorbed to the soil varies with the type of pesticide, soil, moisture, soil pH, and soil texture. The presence of bio pores and high organic matter promotes the retention of pesticides (Larsbo et al., 2009). The distribution coefficient (K_d) and sorption coefficient (K_{oc}) mainly determines sorption and desorption of pesticides on the soil particles and repeated application of pesticides could increase their sorption and formation of bound, non-extractable residues, which further leads to persistence.

The transformed gas product from solids/liquids can move from the initial application site to atmosphere by volatilization. The moisture content of the soil, the pesticide's vapor pressure, sorption, water solubility and windy weather affects the rate of volatilization from soil. The post application volatilization results further in significant pesticide input into the troposphere up to several days/weeks after application.

The pesticides are broken down by microbes, chemical reactions, and light or photo degradation. The chemical degradation occurs when a pesticide reacts with water, oxygen, or other chemicals in the soil by the process of hydrolysis, oxidation-reduction etc. and the rate and type of chemical reactions that occur are influenced by the binding of pesticides to the soil, soil temperatures, pH level etc. The pH plays crucial role by affecting ionizable pollutants and reactive surface sites. In soils, oxygen causes auto oxidation or weathering through the production of radical $O^{2-.}$ (Larson and Weber, 1994). The hydrolytic reactions are mainly catalyzed by acid or

alkali therefore soil pH and water availability are key factor. The surface bound metal ions and clay minerals also catalyzed the hydrolysis reaction by forming chelates, or bidentate complex while oxidation mechanisms in soils may be facilitated by action of oxidative enzymes (Dec and Bollag, 2000). The high reactivity and frequency of manganese oxides and hydroxides in soils were also responsible for carrying the oxidative reactions (Li et al., 2003). The pesticide reduction was possible in hypoxic and anoxic conditions which occurred mainly in poorly drained soils, riparian zones, wetlands or flooded areas and sediments. The reductive species might be metals, ions or organic matter (Borch et al., 2010), transition metal coenzymes (Kappler and Haderlein, 2003) and extracellular bio chemicals.

The nature of light, intensity, exposure time and structure of pesticides affects the photo degradation. The direct photolysis excite the pesticides itself to transform it while indirect photolysis involves the reaction with other excited chemicals to transform the pesticide. The uniform availability of sunlight on the upper soil layers catalyzes maximum photoreactions there. Many of OP compounds undergo reductive cleavage, photohydrolysis, transesterification etc. in presence of sunlight.

Biodegradation of toxic chemicals or xenobiotics performs mainly by the action of microbes which utilizes them as source of nutrient or energy and in turn converts them into less harmful byproducts like carbon dioxide and water. Biodegradation of pesticides is a complex process and completes in several steps catalyzed by specific enzymes that found internally or externally in microbial cells. This process provides both carbon and energy for the growth and reproduction of microbes. Soil organic matter, texture, and site characteristics such as moisture, temperature, aeration, and pH-all affect microbial degradation. Many bacterial and fungal species were isolated from different habitats which were found efficient in biodegradation of pesticides in soil environment. Bacterial sp. like Pseudomonas sp., Burkholderia gladioli, Bacillus sp., Serratia liquefaciens, Serratia marcescens, Sphingomonas sp., Enterobacter sp., Ochrobactrum sp., Sphingobium sp. and fungal sp. like Aspergillus niger, Trichosporon sp., Verticillium sp. DSP, Penicillium miczynskii, Trichoderma sp.etc. were documented for degradation of pesticides in various environment (Ortega et al.,

2010; Cycon et al., 2009; Malghani et al., 2009; Li et al., 2007; Xu et al., 2007; Bhalerao and Puranik, 2007; Qiu et al., 2006; Singh et al., 2004).

The pesticides metabolisms inside the living beings are performed under several steps catalyzed by a specific enzyme at each step. In the primary step the functionalization is done via the hydrolysis, oxidation and reduction reaction which adds some special functional groups such as OH, NH_2, SH and COOH, to the parent compound. This produces metabolites with some modified physicochemical and biological properties. In the secondary process the conjugation of produced activated metabolites with the cell constituents takes place through a synthetic process. This metabolite is distributed and sequestered by the organisms, or excreted. In the tertiary steps through oligomerization and secondary conjugation, a heavy molecular weight or bound compound is produced which further incorporated and stabilized within the cells. By the action of all these reactions the mobility and toxicity of pesticides get reduced up to some extent.

Pesticide metabolism in the environment is also governed by co metabolism in which organisms grow at the expense of a co substrate to transform the pesticide without deriving any nutrient or energy for growth from the process. The cometabolism produces modification in the structure and nature of pesticides which can create drastic change in the bioavailability and mobility in soil. Some organisms can use cometabolized products completely for their growth but sometimes they accumulate and become more toxic (Tixier et al., 2002). The synthesis includes conjugation and oligomerization. The conjugation produces endogenous substrate via methylation, acetylation, or alkylation. These compounds can be excreted from the living cells, or stored. During oligomerization, a pesticide combines with itself, or with other xenobiotic residues (proteins, soil organic residues). Consequently, they give high-molecular weight compounds, which are stable and often incorporated into cellular components (cell wall) or soil constituents (soil organic matter).

2.6. Factors affecting Microbial Degradation

2.6.1. Soil characteristics: The biodegradation depends on of soil physicochemical properties such as moisture, redox conditions, temperature, pH, organic matter, nutrients amount of clay and various management practices that affect microbial activity and chemical diffusion in soils (Pal et al., 2006). Soil organic matter serves as major place for the sorption /desorption of pesticides and it also provides suitable environment for growth of microbes which in turns helps in the degradation (Briceno et al., 2007; Spark and Swift, 2002). The minerals in the soil as silicates, oxides, hydroxides also produce various adsorption sites for pesticides in soil (Calvet, 1989). Degradation rate was slower in acidic soils than in neutral and alkaline soils (Singh et al., 2006). The charge on the pesticides also affects the adsorption of pesticides in the soil as the rate of pesticide adsorption found decreases when the increase in density of soil aggregates (Chaplain et al., 2008). The retention of pesticides in the running systems depends upon the transport characteristics and residence time of water (Pot et al., 2011; Pot et al., 2005). The organic matter also becomes more hydrophilic with greater sorption potential for hydrophilic pesticides with increase in water content (Roy et al., 2000). The degradation of pesticides like isoproturon, benzolin-ethyl, and glyphosphate was found to be increased with the increase in soil moisture (Schroll et al., 2006).

2.6.2. Environmental factors: Temperature plays an important role in the mineralization of various metabolites through microbes as all the reactions are enzymatically governed and activity of enzymes always temperature dependent. The rates of various biochemical reactions in soil is regulated by temperature as rise in 10 ^{0}c temperature may doubles the rate of reaction. However the sorption of pesticides is also dependent on the temperature (Alletto et al., 2006). The degradation of HCH was found to be effective at temperature of 15-30^{0}C (Siddique et al., 2002). The presence of suitable nutrient conditions, absence of any toxic metals promotes the degradation capability of microorganisms. The optimum condition for biodegradation was summarized in Table 3.

Table 3: Suitable condition for biodegradation

Parameters	Optimum value for degradation
Soil moisture	25-28% of water holding capacity
Soil pH	5.5.-6.5
Oxygen content	Aerobic, minimum soil air pores space of 10%
Nutrient content	N and P for microbial growth
Temperature (°C)	15-45 ^{0}c
Contaminants, heavy metals	Not too toxic
Type of soil	Low clay or silt content

2.7. Microbial degradation of pesticides

The fate of pesticides released in the soil is often decided by the soil microbial community as they utilize it as the source of energy. Schmimmel et al. (1983) based on degradation studies with aqueous solution and sediments, concluded that microorganisms play an important role in degradation. The first microorganism that could degrade organophosphorus compounds was isolated in 1973 and identified as Flavobacterium. sp. (Sethunathan,1973). A wide range of bacterial and fungal species had been documented for their capability for organophosphorus compound cleavage by the action of similar enzymes known as organophosphate hydrolase or phosphotriesterase. Engineered microbes are also been tested for the degradation of OP pollutants in the soil. The use of microbial culture for the bioremediation of pollutants is considered as environment friendly and viable approach and requires having knowledge of all physiological, microbiological, ecological, biochemical and molecular aspects involved in pollutant transformation (Iranzo et al., 2001). Several pure cultures and mixed cultures of bacterium were isolated which have ability to use the pesticides as the sole sources of carbon, nitrogen or phosphorus (Singh and Kuhad, 2000). Among the organophosphorus compounds, glyphosate, chlorpyrifos,

parathion, methyl parathion, diazinon, coumaphos, monocrotophos, fenamiphos and phorate are majorly used as they are found to have capability of biodegraded in the soil. In the OP compound the phosphorus is either present in the form of phosphate ester or phosphonate which are vulnerable towards the hydrolysis. Major steps involved in the biodegradation of OP compounds are the hydrolysis, oxidation, alkylation and dealkylation (Singh et al., 1999). Even there is not more study have been reported for further degradation as the hydrolysis decreases the level of mammalian toxicity further. It is observed that the cleavage of phosphoester yields mono esters and finally inorganic phosphates.

2.8. Degradation of organophosphorus (OP) compounds

Several bacteria, a few fungi and cyanobacteria, had been isolated that can use OP compounds like chlorpyriphos, monocrotophos, glyphosate, chlorpyrifos, parathion, methyl parathion, diazinon, coumaphos, fenamiphos etc. as a source of carbon, nitrogen or phosphorus. The enhanced degradation of chlorpyrifos in to diethyl thiophosphoric acid (DETP) and 3, 5, 6-trichloro-2-pyridinol (TCP) by the Enterobacter sp. was reported by Singh et al., 2004. Arthobacter and Flavobacterium ATCC 27551 sp. was found to mineralize chlorpyrifos completely with in 24 h and 48 h of incubation (Mallick et al., 1999). The capability of monoculture and mixed culture of four different bacterial sp. like Pseudomonas sp., Staphylococcus sp., Flavobacterium sp. and Streptococcus sp. was tested and it was found that monoculture of Pseudomonas sp., and mixed culture was able to degrade chlorpyriphos up to 77% and 85% respectively after 30 days (Kumar, 2011).

Bacillus subtilis, Pseudomonas aeruginosa, Bacillus cereus, Micrococcus luteus and Galactomyces geotrichum were incubated with liquid nutrient medium containing chlorpyrifos for 3 days and Bacillus subtilis found capable of 87.5% degradation while other microorganisms viz. Bacillus cereus, Micrococcus luteus and Galactomyces geotrichum showed about 75%, 72.50% and 58.75% degradation respectively (Bhuimbar et al., 2011). Five bacterial species of Pseudomonas aeruginosa AF137358, P. aeruginosa AF531099, P. aeruginosa AY264292, Pseudomonas nitroreducens EF107515 and Pseudomonas putida AF291048 were isolated from the

effluent storage tank of a pesticides manufacturing industry by Latifi et al. (2012) and it was found that Pseudomonas aeruginosa AF137358 was able to grow upto 2000 mg/L of chlorpyrifos concentration and the growth rate in presence of chlorpyriphos had showed fast in the initial period of incubation duration. Several chlorpyrifos degrading fungi, such as Phanerochaete chrysosporium, Aspergillus terreus, Verticillium sp., Trichoderma harzianum and Penicillium brevicompactum were reported to utilize chlorpyrifos as sources of phosphorus (Nair and Pradeep, 2007).

A soil bacterium Serratia sp. was isolated which is having the capacity to utilize methyl parathion up to 2.25 mM as a sole source of carbon and phosphorus in the minimal media. The bacterial isolates of Bacillus pumilus T1 isolated from contaminated sites showed 70% degradation of methyl parathion within 24 h at the concentration of 500 mg/L (Ali et al., 2012). Acinetobacter radioresistens USTB-04 was found able to degrade 130 mg/L and 1200 mg/L of methyl parathion within 2 h and less than 4 d respectively (Liu et al., 2007).

The isolates Pseudomonas putida W and Burkholderia gladioli Y in mineral salt media supplemented with profenofos was able to mineralize profenofos upto 92% by Pseudomonas putida W and upto 87% by Burkholderia gladioli Y after the incubation period of 96 h. The degradation study in soil by the same isolates showed70% profenofos degradation within 5 days while degradation reaches 96.06% and 99.37% within 25 days by Pseudomonas putida and Burkholderia gladioli respectively (Malghani et al., 2009).

The fenamiphos and chlorpyrifos degrading microbial consortia were used to check their degrading potential in various insecticides. The fenamiphos was found completely degraded but the chlorpyrifos was found to be accumulated as a degradation product of trichlorophenol. The chlorpyrifos degrading Enterobacter sp. was found to able to mineralize other OP compound like parathion, diazinon coumaphos and isazofos in water samples but it could not degrade fenamiphos. The soil inoculated with microbes showed 50% degradation within 2 days (Singh and Walker, 2006). Chanika et al. (2011) reported two bacteria identified as

Pseudomonas putida and Acinetobacter rhizosphaerae were able to degrade fenamiphos.

In the study of the tetrachlorvinphos (TCV) degradation was found by the isolates from contaminated soil of Mexico identified as Stenotrophomonas malthophilia, Proteus vulgaris, Vibrio metschinkouii, Serratia ficaria, and Yersinia enterocolitica by Ortizhernandez, (2010). The consortia was able to degrade the TCV by 57% in the minimal media in 36 h of incubation. But the TCV removal upto 98% was observed by each strain. The metabolite of TCV hydrolysis was also identified as 1-(2,3,4) trichlorophenylethanone from the culture media of all strains after 10 days. (Ortiz-Hernández et al., 2010)

Najavand et al. (2012) isolated Pseudomonas aeruginosa NL01 sp. from diazinon contaminated soil of north of Iran. The species was found capable of degrading diazinon upto 200 mg/L. The OP degrading enzyme organophosphorus hydrolyze as OPH NL01 was also extracted from bacterial culture which shows potential to hydrolyze OP compound and convert them into less toxic products.

Thabit and Naggar (2012) isolated Pseudomonas aeruginosa, Bacillus amyloliquefaciens, Staphylococcus sciuri, Bacillus pseudomycoides, Bacillus licheniformis from vegetable agricultural soil and were tested for their ability to degrade malathion in liquid media. The half-life values (RL_{50}) were 16.68 days for Pseudomonas aeruginosa, 20.27 days for Bacillus amyloliquefaciens, 21.33 days for Staphylococcus sciuri, 12.72 days for Bacillus pseudomycoides and 12.49 days for Bacillus licheniformis respectively while control value was 27.50 days. Monocarboxylic and dicarboxlyic acids were detected as end product of malathion degradation.

Wang et al. (2012) isolated Agrobacterium sp. strain Yw12 from activated sludge was found capable to degrade the methyl parathion (50 mg/L) within 2 h and its degradation product p-nitrophenol with in 6 h completely. It was further found able to utilize phoxim, methamidophos, chlorpyrifos, carbofuran, deltamethrin and atrazine as carbon and energy source.

2.9. Degradation study of Monocrotophos

The isolation of microbes tolerant to different concentration of MCP ranged from 50 to 300 mg/L was studied by Jain et al. (2012). The most tolerant strain of fungal isolate isolated from 300 mg/L and was identified as Aspergillus niger (MCP1). The optimized condition for growth was MCP concentration of 150 mg/L, pH of 8, and temperature of 30^0C. MCP was served as the source of phosphorus and energy as the 37% increase in the growth was recorded by the isolates in the presence of MCP. The degradation kinetics revealed that 90 % of MCP was degraded by the isolates after 10 days of incubation. The half life of compound was found to be 75.87 h under optimized parameters for growth of isolate in the media supplemented with MCP.

Kavikarunya (2012) isolated Pseudomonas fluorescens, Bacillus subtilis and Klebsiella sp., from the paddy field of Annamalai Nagar having the history of repeated pesticide application. The factors such as temperature, pH, carbon and nitrogen sources were also had effect on the degradation of MCP. Klebsiella sp. had shown growth by utilizing MCP at temperature of 35^0C and pH 6. The maximum growth was observed in dextrose and malt extract respectively as carbon and nitrogen sources.

Das and Anitha (2011) also reported degradation capability of Aspergillus sp. in the media supplemented with 0.5 % (w/w) MCP for 8 days at 28^0C. The GC-MS analysis revealed presence of several metabolites such as behenic acid, stearic acid, palmitic acid, carycnic acid, n-nonoic acid, enantic acid were formed by enzymatic synthesis in the fungus.

Bhalerao and Puranik (2009) carried out the degradation study for monocrotophos (MCP) contaminated soil from different geographical sites and the study was done in media enriched with monocrotophos concentration ranged from 100 to 500 mg/L. The seven isolates from the contaminated soil which are having the capability of MCP tolerance only one isolate Aspergillus oryzae ARIFCC showed maximum tolerance against 900 mg/L of MCP with degradation potential of 70% of MCP in the first 50 h of incubation and reached up to undetectable after the incubation time of 168 h. The mineralization of MCP resulted in end product such as carbon dioxide, ammonia and

phosphates. The mineralization of MCP was found closely related with MCP concentration and microbial growth. The formation of end product through the degradation of MCP was performed by the action of phosphatase enzyme present in the microbial cells. The increased concentration of phosphatase was found correlated with the disappearance of MCP from media.

Jia et al. (2006) isolated a bacterial strain of Paracoccus sp. from the sludge of waste water containing MCP. It was observed that under aerobic conditions 79.92% of MCP was disappeared after first 6 h of incubation along with the nitrate reduction in the sample along. The phosphatase and amide enzymes, responsible for the degradation of MCP was found constitutive expressed. The degradation intermediates formed during the MCP degradation was also served as the substrate for the denitrification mechanism for Paracoccus sp. The addition of these strains in fluvo-aquic soil and high-sand soil resulted in MCP degradation with the half life of less than 20 days and 10 days respectively.

Singh and Singh (2003) isolated Pseudomonas aeruginosa F10B and Clavibacter michiganense subsp. insidiosum SBL 11, from soil and that were capable of utilizing MCP as a sole source of phosphorus. These two strains were able to degrade technical MCP in culture up to 98.9 and 86.9%, respectively, and pure MCP up to 79% and 80%, respectively, within 24 h at 37^0C. The optimal concentration of MCP for the normal growth was found to be 500 mg/L.

Gundi and Reddy (2006) studied the degradation rate of MCP applied as commercial formulation in two different type red alfisol and black vertisol soil of the groundnut and cotton field respectively. About 96.5–98.4% disappearance of MCP was recorded in both soils after 10 days of application of 10 µg MCP /g soil. The rate constants and half-lives for the degradation of monocrotophos in black vertisol and red alfisol soils were found to be 0.0753 and 0.0606 /day and 9.20 and 11.40 days, respectively. The hydrolyzed product of MCP N-methyl acetoacetamide was reported to be 3.8–4.82 µg/g soil. But the higher amount of MCP was recovered at the MCP concentration of 100 µg/g at 10 and 20 day intervals. They also observed that the sterilization

increased the persistence of monocrotophos in terms of half-lives from 9–11 days to 29–45 days in both soils.

The bioremediation of effluent of MCP manufacturing unit containing MCP was done from the microbial culture of Arthrobacter atrocyaneus, Bacillus megaterium and Pseudomonas mendocina that were previously isolated from MCP exposed soil. B. megaterium and P. mendocina showed maximum removal of MCP (68–76%) and reduction in COD (62–68%) at 35°C, but for A. atrocyaneus, MCP removal and COD reduction were founded maximum at 30°C. In the aerated culture maximum removal of MCP (75–80%) and reduction in COD (53–60%) by all three cultures was reported. This indicated the effect of temperature and aeration on MCP degradation (Bhadbhade et al., 2002a).

The seventeen isolates were obtained from enrichment and adaptation method from MCP contaminated soil by Bhadbhade et al., 2002b. The isolates A. atrocyaneus MCM B-425 and B. megaterium MCM B-423 were found capable of MCP degradation 93% and 83%at the concentration of 1000 mg/L of MCP with in 8 day of incubation at 30^0C. Two isolates viz. A. atrocyaneus MCM B-425 and B. megaterium MCM B-423 showed maximum removal 78.94% and 82.53%, respectively within 48 h at 30^0C. Methylamine, volatile fatty acids, ammonia, phosphates, carbon dioxide were detected as the end product of MCP mineralization.

CHAPTER 3

MATERIALS AND METHODS

3.1 Selection of study sites and survey on Insecticides usage pattern

Bathinda district is considered as heartland of Malwa region which is primarily linked with the cultivation of cotton, rice and wheat. The criteria for selecting sampling sites was mainly the usage pattern of insecticides for the various crops and reports about the MCP residues in the soil of different villages of Bathinda district (Mathur et al., 2005). The usage of different organophosphorus insecticides like monocrotophos to different crops also resulted in the residue accumulation in vegetables, food, soil, water, milk etc.

The survey was based on the information obtained from the randomly selected farmers from each of the randomly selected sampling sites. The qualitative and quantitative survey with the help of questionnaire (Appendix A) was done for obtaining knowledge about the usage pattern and bioaccumulation potential of various insecticides. The data about the history, usage pattern, dosage and mode of application of pesticides in fields were collected. The repeated application of monocrotophos was noticed in various crops like cotton, rice and leguminous plants. The sites of sampling were given in Table 4 and its location map (Appendix B) to represent the location of sampling sites.

3.2 Collection, storage and preparation of soil sample

The samples of soils were collected from various villages of Bathinda district which were situated within 10 km distance from the Central University of Punjab-Bathinda. The sampling was done in the month of August-September, 2012. The soil was collected from fields using soil auger. The samples were air dried and stored in plastic containers and part of that was stored under -20^0C for further use.

Table 4: Details of sampling sites

Sl. No.	Study site	Block	Geographical location		Abbreviation used in the study
			Latitude	Longitude	
1	Bhagibander	Talwandi sabo	29.918566°	75.111802°	BW
2	Dhadde	Rampura	30.179441°	75.249323°	DHA
3	Gurusar Sahnewala	Sangat	30.085573°	74.841940°	GS
4	Jajjal	Talwandi sabo	29.967887°	75.030448°	JAJ
5	Jassi	Bathinda	30.154768°	74.966929°	JAS
6	Malkana	Talwandi sabo	29.937501°	75.034013°	MAL
7	Mahinangal	Talwandi sabo	30.023614°	75.018034°	MN
8	Sangat Kalan	Bathinda	30.210337°	74.874598°	SANT
9	Talwandi sabo	Talwandi sabo	29.985408°	75.083659°	TS
10	Teona Pujarian	Talwandi sabo	29.928094°	75.077176°	TEO

3.3. Analysis of physico-chemical properties of soil

The parameters for the soil samples were analyzed for basic characteristics of soil. All the soil samples were characterized for the following parameters using standard methods.

3.3.1 pH: The soil suspension in distilled water in ratio of 1:5 was prepared, stirred thoroughly and kept for 10 min. The pH of the soil solution was measured using pH meter (Mettler Toledo) (Jackson, 1973). In the soil – water system the hydrogen ion dissociates from the soil surface to the solution. The conc. of H^+ will further give rise to acidity or alkalinity to the soil solution.

3.3.2 Electrical Conductivity: The soil suspension was made as described earlier and EC was measured using EC meter of Mettler Toledo (Jackson, 1973). The conductivity meter measures the rise in the flow of current which are present in the soil water system. It is measurement of the electric current due to the migration of ions of the solution.

3.3.3 Moisture content:

The gravimetric loss in the sample before and after drying is considered as measurement of total moisture in soil (Jackson, 1973).

$$Moisturecontent(\%) = \frac{(\text{Initial weight of soil} - \text{Final weight of soil dried after 8h at 103℃})}{\text{Initial Weight of soil (g)}} \times 100$$

3.3.4 Organic Carbon: The organic matter present in the soil is oxidized by using potassium dichromate and concentrated sulfuric acid. The excess dichromate is determined by back titration with standard ferrous ammonium sulphate and the quantity of substances oxidized is calculated from the amount of dichromate reduced (Wakley and Black, 1934).

$K_2Cr_2O_7 + 4H_2SO_4 \longrightarrow K_2SO_4 + Cr_2(SO_4)_3 + 4H_2O + 3O$

$C^0 + 2O \longrightarrow CO_2$

$2FeSO_4 + H_2SO_4 + O \longrightarrow Fe_2(SO_4)_3 + H_2O$

The organic carbon content was calculated by the formula:

$$\text{Organic Carbon}(\%) = \frac{S \times 0.003 \times 1.3 \times 100}{\text{Weight of soil (g)}}$$

Where, S =

Volume of $K_2Cr_2O_7$ reduced (ml)

$$= \frac{(\text{Blank Titre value} - \text{Sample Titre value})}{\text{Blank Titre value}} \times \text{volume of } K_2Cr_2O_7 \text{ used} \times \text{N of } K_2Cr_2O_7$$

Total Nitrogen: The estimation of total nitrogen was done by wet digestion method using Khejldhal apparatus followed by distillation (Jackson, 1973). The strong acid digestion by addition of sulfuric acid helps in the conversion of organic nitrogen into $NH4^{+}$-N. The addition of several salts or metal catalyst speeds up the digestion process. The addition of excess base further convert NH_4^{+} to NH_3 which condensed and collected in boric acid and is titrated against standard H_2SO_4 using mixed indicator to determine the amount of ammonia liberated. The total nitrogen is determined by the equation:

$$\text{Total Nitrogen}(\%) = \frac{\text{V} \times 0.014}{\text{weight of soil (g)}} \times 100$$

Where, V= volume of 0.1 N H_2SO_4 used for neutralization of NH_3 in boric acid in ml

3.3.5 Total Phosphorus: The estimation of P involved preparation of soil extract followed by the colorimetric determination. A known amount of soil was weighed and digested with di-acid mixture (HNO_3-$HClO_4$ in 10:4) on the hot plate according to process of Jackson (1973). Digested material was cooled, diluted with distilled water, filtered through Whatman filter No.1 to get the clear extract. The aliquot was made up to a known volume and the P content was determined by Vandomolybdophosphoric acid method using UV-VIS spectrophotometer SHIMADZU, UV-2450).

$$\text{Total Phosphorus (\%)} = \frac{Conc.\,of\ P\ from\ standard\ curve\ \times ml\ of\ extract \times final\ volume}{10^6\ \times\ \text{aliqout taken from digested sample}\ \times \text{weight of sample (g)}} \times 100$$

3.3.6 Potassium content: The sample extract was done as per procedure described for P estimation and K was determined directly by SYSTRONICS flame photometer following the method described by Piper (1950).

3.4 Biological properties of soil: The biological properties of soil are very important for maintaining the harmony of the growth of plants as well as microbial community. The soil microbial biomass and soil enzymes are indicators of changes in soil quality resulting from the inappropriate farming practice. The presence of healthy microbial community is responsible for the detoxification of various xenobiotics from soil environment. The microbial biomass carbon and soil enzymes were determined in all the soil samples to check their biological properties.

3.4.1. Microbial biomass carbon (MBC): The MBC was determined using fumigation and extraction method (Anderson and Ingram, 1993). The fumigation kills the microorganisms without affecting nonliving part of soil organic matter. The MBC was calculated against non fumigated controls. The residual dichromate is measured by back titration with ferrous ammonium sulphate solution using phenanthroline as an indicator.

3.4.2. Dehydrogenase: The dehydrogenase enzyme helps in the oxidation of organic matter in soil. Dehydrogense activilty helps in the CO_2 release, proteolytic and nitrification activity, in soil. Soil microbes reduces 2,3,5 Triphenyl tetrazolium chloride (TTC) to 2,3,5 triphenyl formazan (TPF) , which concentration was measured spectrophotometrically (UV-VIS SHIMADZU- 2450). The Dehydrogenase activity was measured by the method described by Casida et al. (1964).

$$\mu g \text{ TPF } g^{-1} \text{ soil } 24\ h^{-1} = \frac{\text{Amount of TPF from standard curve } (\mu g)}{\text{dry weight of soil } (g)}$$

3.4.3. **Phosphatase:** Phosphatase enzyme catalyzes the hydrolysis of both esters and anhydrides of phosphoric acid. When soil is incubated with buffer sodium p-nitrophenyl phosphate solution and toluene released p- nitrophenol (yellow colored) which was estimated colorimetrically (SHIMADZU-UV 2450) (Tabatabai and Bernmer, 1969). The enzyme activity was expressed as µg PNP g^{-1} soil h^{-1}.

3.4.4 Urease: Urease enzyme released from all living and disintegrated microbial cells and act as extracellular enzyme in soil. Ammonium nitrogen released on incubation of soil with buffered urea solution was measured colorimetrically (Mc Garity and Mayers, 1967). Ammonia reacts with an alkaline solution of phenol and sodium hypochlorite to form a blue colored complex known as indophenols in the presence of sodium nitropruside as catalyst. The enzyme activity was expressed as µg NH_3-N g^{-1} soil h^{-1}.

3.5 Insecticide selected:

The Monocrotophos (MCP) an OP insecticide was selected for the study. The MCP (99.5% purity) was procured from Sigma-Aldrich. The different concentration of 50, 100, 250, 500, 750, 1000 mg/L was prepared in distilled water and used for degradation potential. The commercial grade MCP (MONOCRID) 36 % SL formulation (53% w/w) was purchased from local supplier of Bathinda was also used for the study.

3.6 Microbial population in soil

3.6.1 Serial dilution of the soil samples: Isolation of microbial population from soil was done using serial dilution method. A one gram of soil sample was mixed with the 10 ml of sterile distilled water. The dilution was made upto 10^{-10} using diluent (sterile water).

3.6.2 Preparation of media and plates: The bacterial population count of soil was done in culturing in nutrient agar media. The media was prepared using the ingredients and autoclaved at 121^0C temperature, 15 psi pressure for 20 minutes, cooled and agar plates were prepared aseptically.

Composition of Nutrient Agar:

Peptone – 5 g

Beef extracts – 3 g

Sodium chloride – 5 g

Agar - 15 g

Distilled water - 1 L

pH – 6.8

3.6.3 Inoculation and Spreading: A 100 µl of aliquot from each dilution was transferred to the nutrient agar and pikovskaya agar plates in triplicates by using spread plate method. The plates were incubated for 72 h and microbial count was done at regular time interval.

3.6.4 Enumeration of microbial population: The bacterial population in the soil samples was determined by counting colony forming unit (cfu) in nutrient agar plates in triplicates by colony counter by viable plate count technique at regular time interval upto 72 h (Thompson, 1989). The plate count method assumed each colony as each viable bacterial cell. Technique was based on that the each colony represents the individual cells and all the cell grows in the suitable incubation conditions. At different time interval of 24, 48 and 72 h the colonies were counted using colonymeter. The dilution showing the count enough to count accurately was further selected for enumeration of microbial count.

$$cfu/g = \frac{\text{Number of colonies}}{volume\ plated \times \text{Dilution factor}}$$

3.7 Enumeration of Phosphorus Solublizing Microorganisms (PSMs): The growth of PSMs was checked using in pikovskaya Media in triplicates agar plates (Sundara Rao and Sinha, 1963). The microbial count in terms of cfu was enumerated same as described for nutrient agar media. The turbidimetric or spectrophotometric analysis was also carried out in broth culture to study the trend in growth pattern. It is an indirect measurement of cell density and was done at 600 nm by using SHIMADZU-UV-2450.

Composition of Pikovskaya media (PKV):

Dextrose - 10 g

Yeast extract - 0.5 g

Ammonium sulphate - 0.5 g

Potassium chloride - 0.2 g
Magnesium sulphate - 0.1 g
Manganese sulphate - 0.0001g
Ferrous sulphate - 0.0001g
Tri calcium phosphate – 5 g
Agar – 15 g
Distilled water -1 L
pH - 7±0.5

3.8 MCP residue analysis in the soil: The residue of MCP in the soil samples was estimated using High performance liquid chromatography (HPLC -DIONEX – ULTIMATE 5000). The metabolites of MCP were checked by using the Gas Chromatography – Mass Spectroscopy (SHIMADZU-QP2010 PLUS). The description about the preparation of samples and operating parameters are described below:

3.8.1 Sample preparation for MCP residue analysis: The soil samples were extracted for analysis of pesticides residue detection as per Vig et al. (2001). A 50 g soil sample was weighed in duplicates and then mixed with 50 ml of acetone containing 1 ml of 1N ammonium acetate. Then the solution was shaken for 30 min in a mechanical shaker. The extract was filtered using Buchner funnel followed by washing with acetone. The filtered solution was pooled up and then mixed with 500 ml of 2% anhydrous Sodium sulfate. The solution was then transferred into the separatory funnel and shaken well. This solution was further partitioned three times using dichloromethane (total 100 ml). The volume of the pooled extracts were reduced to less than 5 ml with the help of rotary evaporator. This was passed through a fine layer of sodium sulfate and used for further HPLC analysis.

3.8.2 Quantification of Residue: The quantification of MCP in the soil extracts were done by using HPLC (DIONEX- ULTIMATE 5000). The HPLC system was equipped with UV-VIS detector and analytical column of specification ACCLAIM120 –C18

(5µm, 120 A^0- 4.62×250 mm). The calibration was done with different dilution of MCP of 0.01 to 100 mg/L from stock solution of 1000 mg/L prepared in HPLC grade water. The operating parameters for residue detection with the mobile phase of acetonitrile and water (20: 80 w/v), isocratic flow rate of 1 ml/min, column temperature 20^0C, detector temperature 30^0C, UV absorption 230 nm and injection volume of 20 µl/m (Lee and Kwon, 2004).

3.8.3. GC-MS analysis of soil samples

The soil samples with MCP residue detection and those with present MCP usage history were further subjected for the determination of metabolites using GC-MS analysis (SHIMADZU-QP2010 PLUS with TD 20) with column specification of Rtx-5 MS (30m X 0.25 mm id X 0.25 µm). The chromatographic conditions were as follows; the injection was in split less mode with the injector and column oven temperature of 270^0C and 100^0C, respectively. The oven temperature was increased 100^0C at 6 ml/min upto 250^0C hold for 6 min, followed by increase upto 300^0C at the rate of 25 ml/min. Helium was used as carrier gas at the column head pressure 93.6 kPa with the linear velocity of 40.9 cm/sec and column flow of 1.21 ml/min. The compound detected was compared with standard library of NIST (mass spectral database) and the external standard of MCP was used for the identification of residue of desired compound by comparing with its retention time.

3.9 Tolerance of microbial population in soil samples at the different concentration of MCP

The soil samples were having the history for monocrotophos application, so the microbial population present was developed tolerance against the MCP. Thus the level of tolerance in each sample was checked on the basis of microbial count in MCP supplemented media**.**

3.9.1 Screening of samples for MCP degradation: The growth was checked in PKV media supplemented with the different concentrations of MCP (99.5% pure). The phosphorus solublizing microbes which had the capability to utilize P in PKV

media for its growth, had potential for the degradation of MCP being an OP compound. Since PKV is specific for the growth of PSMs so the MCP usage and degradation can easily recorded. A 100 µl of supernatant obtained from all soil samples of 10^{-1} dilution were directly added as inoculum to the PKV agar and broth supplemented with the 50 mg/L MCP.

3.9.2 The adapted culture of 72 h (Anusha et al., 2009) from broth was used as inoculum for each successive concentration of MCP of 100, 250, 500, 750 and 1000 mg/L. The turbidimertic growth of microbial cell was measured by spectrophotometer at 600 nm at different concentration of MCP. For enumeration of cfu/g by viable plate count method a 100 µl of 72 h old culture from 50 mg/L was spread to PKV agar plates with different concentration of MCP.

3.9.3 Selection of sample: The screening for higher MCP tolerance was done by discarding those with poor growth in agar plates and broth. The samples showing fast colony growth on the maximum concentration of MCP were selected for the isolation of pure isolates. The plates were further incubated for 7 day to check the growth of fungal isolates.

3.10 Isolation of MCP resistant strain: The samples showing the growth at the maximum concentration of MCP (1000 mg/L) supplemented media were selected for further isolation process. Each bacterial and fungal colonies were picked carefully and streaked in the nutrient agar media and potato dextrose agar media separately. It was also streaked on PKV media supplemented with 1000 mg/L MCP for confirming its growth at higher concentration.

3.11 Culturing of potential Isolates: The colonies from the plates and fungal spores were further inoculated on PKV broth media. The 10 µl of 24 hr old inoculum was inoculated on PKV agar for culturing. It was also sub cultured on nutrient agar media for studying its morphological and biochemical characterization.

After subculturing was done for three to four times on PKV agar and slants, the inoculum was prepared in PKV broth and 24 h old culture was used as inoculums.

Also 10 µl was again spreaded on the PKV agar plates supplemented with 1000 mg/L MCP for reconfirming the tolerance of isolates.

3.12 Identification of the Isolates: The bacterial isolates were identified on the basis of morphological and biochemical tests. The fungal isolates were identified on the basis of color and morphological characters.

3.12.1 Morphological characterization of Isolates: The colony morphology, color, shape, texture was visually identified on nutrient agar and PKV agar plates. It was correlated with typical characters of bacteria in PKV media and fungal isolates in PDA media.

3.12.1.1. Gram staining: The gram staining was performed for the differentiation between gram positive and gram negative bacteria. The smear was made by spreading the culture and air dried and heat fixed. Then it was flooded with crystal violet for 20 sec only. It was then washed with distilled water and again stained with grams iodine solution and stand for one minute. Then it was washed with 95% of ethanol as decolorizing agent followed by washing with distilled water. Then the smear was covered with safranin for 1 minute. Then the slide was washed gently with distilled water, air dried and was microscopically examined under oil immersion.

3.12.1.2. Endospore forming capability: The endospores forming capability was checked after giving the heat shock to the single colony at 80^0c for 15 min. Then the colony was further streaked on the nutrient agar plates. Appearance of the growth after 24 hour will indicate its endospore forming capability.

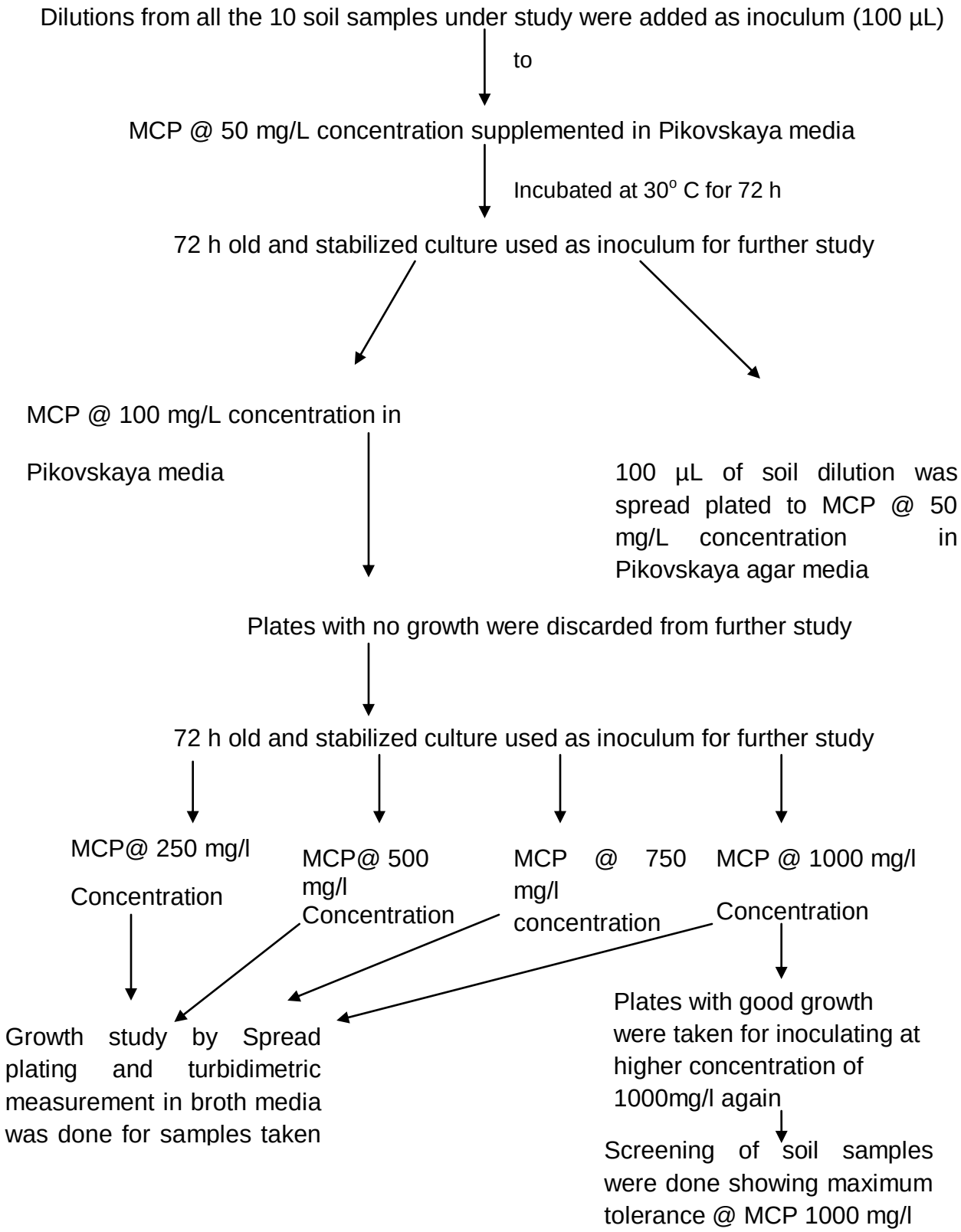


Fig 2: Flow diagram for the screening microbial population from soil samples to MCP tolerance

3.12.2. Biochemical characterization: Various biochemical tests were performed based on the fermentation abilities, presence of certain enzymes, and certain biochemical reactions. Qualitative observations were obtained from tests results.

3.12.2.1. Catalase test: A single colony of the isolates was immersed in the 3% H_2O_2 solution and the immediate active bubbling was ensured the catalase production capability. Organisms capable of producing catalase enzyme broke down the hydrogen peroxide, and the resulting O_2 production produces bubbles in the reagent drop, indicating a positive test.

3.12.2.2 Utilization of different Carbon sources: Ability of bacteria to utilize different carbon sources through fermentation reactions produces acidic products which was responsible for change in color of media. The isolates were inoculated in the media containing beef extract; 3g/L, peptone; 5g/L, with carbon sources in form sucrose, glucose, lactose, mannitol; 5g/L separately. The pH was adjusted up to 7.00. After this the tubes were inoculated and incubated at 37^0C for overnight. The production of yellow color will give the positive test. The appearance of bubble in the durhams tubes will indicate the production of gas.

3.12.2.3 Motility test: The motility was checked in the Sulphide Indole Motility medium (peptone 20 g/L, beef extracts 8g/L, ferrous ammonium sulfate 0.2 g/L, sodium thiosulfate 0.3 g/L, agar 3.5 g/L) and pH was adjusted 7.6. The media was transferred in the test tubes and autoclaved. Then a single colony was inoculated by stabbing it with the help of needle over a half distance of tube. The tubes were incubated at 37^0C for overnight. The motility was examined by the spread growth in the media. A positive result is indicated by diffuse or cloudy growth mainly at the top and bottom of the stabbed region.

3.12.2.3 Urea hydrolysis test: The test was performed after inoculation in the Stuart's urea broth (Yeast extract; 0.1 g, KH_2PO_4; 9.1 g, K_2HPO_4; 9.5 g, Urea;20 g, phenol red; 0.1 g in 1L) and observed for change in the color from yellow to pink. Production of hydrolytic enzyme urease by the isolates in the media will split the urea into ammonia and then in alkaline environment the color of media will turn pink.

3.12.2.4 Citrate utilization test: The single colony was streaked in Simmon citrate agar slants (Sodium chloride, 2g/L; Sodium citrate, 2g/L; Ammonium phosphate, 1g/L; K_2HPO_4, 1g/L; $MgSO_4$, 0.2g/L; bromothymol blue, 0.08g/L, agar, 15 g/L) and incubated for 24 h and, then observed for change in color from green to blue. Utilization of citrate as carbon source and changed it in to alkaline sodium carbonate will turn the color of media.

3.12.2.5 MR-VP test: The inoculation was done in MR-VP broth (peptone, 5g/L; K_2HPO_4, 5g/L; dextrose, 5g/L) and incubated 37^0C for 24 h. After this the VP tubes were taken out and 1 ml of 40% KOH and 4 ml of 5% of alpha-naphthol was added and tubes were aerated for 30 min and observed for appearance of red color. After 48 h the Methyl red reagent was added in tubes and observed again for appearance of red color. The accumulation of acidic end products due to fermentation of glucose will change the color of media after addition of methyl red. The VP test was also due to cherry red color appearance of complex formed between Diacetyl and guanidine components of peptone.

3.12.2.6 Starch hydrolysis test: The test was performed in the nutrient agar media supplemented with starch. The isolates were previously grown in trypticase soy broth and inoculate in starch agar plates. The plates were incubated for 48 h and after that the plates were flooded with iodine solution and allow the iodine to remain in the contact for minimum for 1 h. The indication of clear zone ensured the positive test for starch hydrolysis.

The temporary identification of isolates was done using Bergey's Manual of Bacteriology based on the result obtained from morphological studies and biochemical characteristics. The fungal identification was done according to previous studies and was based on its morphological characteristics.

3.13 Phosphorus solublization by bacterial isolates: Then isolates were inoculated in PKV broth inoculated in triplicates and incubated on the rotary shaker at 120 rpm for 10 days at 30^0C. The sample was centrifuged at 10000 rpm, for 15 min to remove bacterial cells and other insoluble materials. Then the soluble

phosphorus content was determined spectrophotometrically by using chlorostannous reduced molybdophosphoric acid blue method at 660 nm (Jackson, 1973). Molybdophosphoric acid was formed and reduced by stannous chloride to blue color. This indicated the potential to solublize insoluble tri calcium phosphates in the media. The pH drop was also measured in the media using pH meter after 10 days.

3.14 Pesticides degradation by bacterial isolates in the media: The pikovskaya media containing 1000 mg/L of MCP concentration was inoculated with isolates. The samples were drawn at regular interval to check the growth ability spectrophotometrically at 600 nm. The residual MCP was determined by HPLC system in the culture media.

3.14.1 Extraction and estimation of MCP: For MCP residue, the sample was drawn and extracted twice with equal volume of ethyl acetate. The extractants were centrifuged at 3000 rpm for 15 minute. Then the supernatant was again washed using equal volume of ethyl acetate. Then it was filtered and extract was dried over anhydrous sodium sulfate followed by filtration through syringe filter. The filtrate was evaporated up to dryness and redissolved into 20 µl HPLC grade dichloromethane (Ortiz-Hernández et al., 2010). The samples was inject in HPLC for quantification of residual MCP at the same operating parameters as described.

3.15 Quantification of available phosphorus in soil

The soil sample of TEO and DHA from which the isolates had been isolated were taken in polythene bags and autoclaved at 121^0C for 20 minutes to kill the microbes present in it. The inoculums was prepared with isolates of D_1 and T_1 separately and added to DHA and TEO respectively at inoculums volume of 2% and 10%. Then bags were incubated at 30^0C upto 7 days. At different time interval the samples were drawn and the available phosphorus content was estimated using sodium bicarbonate method (Olson et al., 1954). The intensity of blue color was estimated spectrophotometrically at 660 nm. The available phosphorus content in the soil was

an indirect determination the ability of isolates to solublize the phosphorus present in the MCP.

The extent of solublization of phosphorus in soil was selected and the efficient isolate was screened out to test its ability to degrade the MCP in soil.

3.16. Estimation of phosphatase enzyme in the MCP supplemented soil

The degradation of MCP was related with the activity of phosphatase enzyme in the soil. The PSBs were capable of producing of Phosphatase which is responsible for mineralization of organophosphates. Each sample was supplemented with commercial grade MCP and pure PSB isolates at inoculums volume of 2% and 10%. Then samples were incubated at 30^0C up to 7 days. The phosphatase activity was measured at different time interval using standard method of Tabatabai and Bernmer (1969).

3.17. MCP degradation in soil by the efficient Isolate

The sample screened on the basis of its solublization capability of phosphorus from MCP was further selected for the determination of percent degradation capability of MCP. The extraction procedure and HPLC/GC process were same as discussed earlier. The samples supplemented of pure isolates and spiked with the 1000 µg/g of MCP were taken for residue analysis. The presence of MCP after 7 day was confirmed by GC-MS analysis while the quantification of percent degradation was done through HPLC.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Summary of data obtained from Questionnaire survey: The information about pesticides consumption, dosage, mode of application, cropping pattern of farm was obtained from the questionnaire survey (Appendix A) conducted in ten different villages of Bathinda district.

4.1.1. Pesticides consumption pattern: In the randomly selected ten villages of Bathinda district the pesticides consumption information comprised of history, usage pattern, dosage and mode of application of pesticides in the fields (Table 5). The main crops grown in the field were cotton, rice followed by vegetables. The major pesticides used in the villages were traizophos, acetamiprid, acephate, monocrotophos etc. for prevention from the pest infestation in the field. The farmers mainly followed a single or multiple pesticides application. Majority of the pesticides were under the slightly and moderately hazardous class as per WHO classification. The trend was showing about 50% consumption of slightly and moderately hazardous pesticides while the traizophos and monocrotophos are under highly hazardous group with consumption of 57.14% and 28.57% respectively (Fig.3).

4.2. Usage pattern of Monocrotophos in the sampling sites

All the villages under study had a history of the monocrotophos application to the crop (Table 6). Two villages namely Dhadde (DHA) and Teona Pujarian (TEO) were reported to have current usage of monocrotophos either in alone or mixed formulation. The application was mainly for cotton and guar crop. The application was three applications per crop period. In the DHA the MCP application was reported 15 days back while in TEO it was only 1 day prior to the sampling (Fig. 4). The mode of application was mainly found in foliar spray to the crop. It was inferred that most of the farmers were unaware about the impacts of pesticides on the human health, soil and water environment and the application have been noticed without any precautionary measure.

Table 5. Crops and Pesticides used in sampling sites

Sl. No.	Name of the village	Area of sampling site (acre)	Crop grown At sampling site	Growth Stage of Crop at sampling time	Pesticides used	WHO Category	
						Class	Hazardous nature
1	Bhagi Bander	1	Cotton	Flowering	Acetamiprid	Class lll	Slightly
2	Dhadde	2	Cotton	Flowering	Monocrotophos	Class lb	Highly
3	Gursar Sahnewala	2.5	Cotton	Flowering and Fruiting	Traizophos	Class lb	Highly
					Dichlorvos	Class lll	Slightly
					Imidacloprid	Class ll	Moderately
4	Jajjal	5	Cotton	Flowering	Traizophos	Class lb	Highly
					Acetamiprid	Class lll	Slightly
5	Jassi	3	Cotton	Flowering	Imidacloprid	Class ll	Moderately
6	Malkana	7.5	Cotton	Fruiting	Acephate	Class ll	Moderately
					Triazophos	Class lb	Highly
7	Mahinangal	7	Rice	Boot leaf stage	Biferthin,	Class ll	Moderately
					Traizophos	Class lb	Highly
8	Sangat Kalan	2	Cotton	Flowering	Acetamiprid	Class lll	Slightly
					Dichlorvos	Class lll	Slightly
9	Teona Pujarian	3	Guar	Flowering	Monocrotophos	Class lb	Highly
					Acephate	Class ll	Moderately
10	Talwandi sabo	9.5	Cotton	Flowering	Acetamiprid	Class lll	Slightly

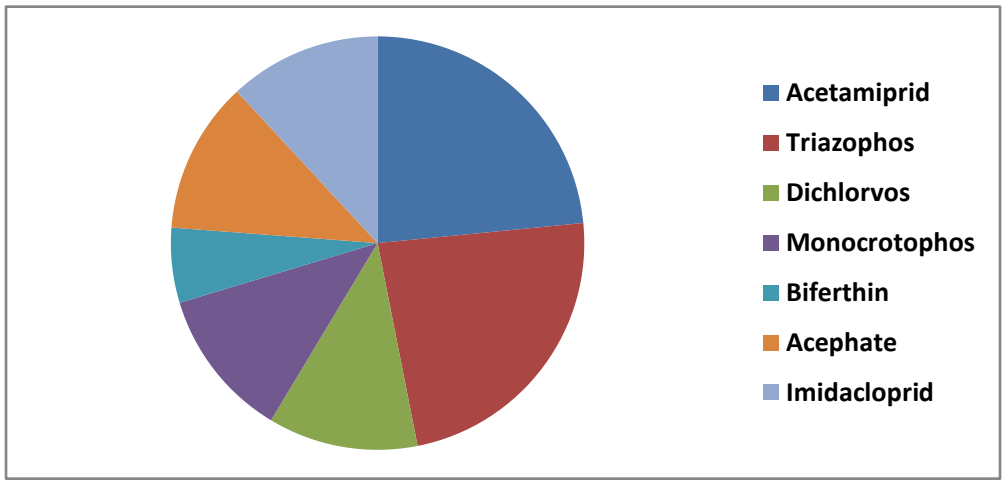


Fig. 3: Pattern of Pesticides application in sampling sites

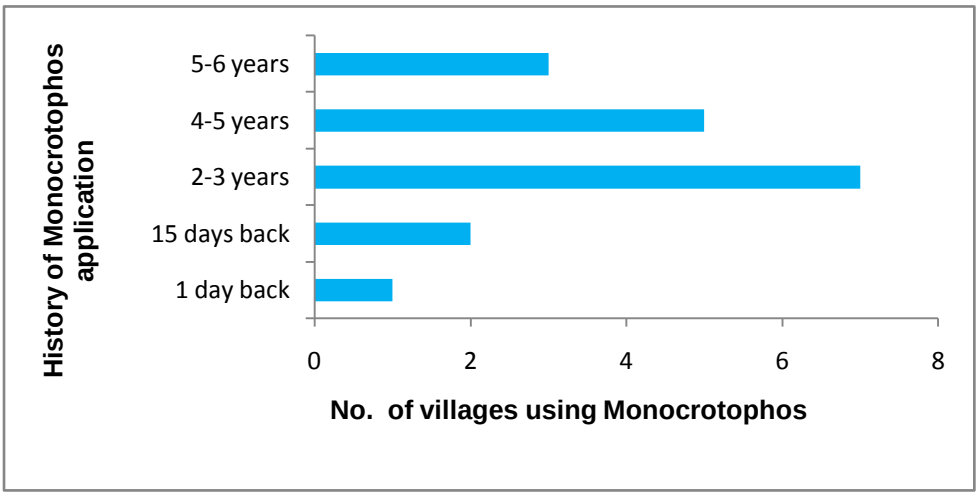


Fig. 4: History of MCP usage in sampling sites

Table 6. History of MCP application in the sampling site

Sl. No.	Village Name	Crops in the sampling site			Current MCP application			History of MCP usage
					MCP usage	No. of application	Sampling time	
		Cotton	Rice	Beans				
1	Bhagi bander	+	–	–	N	–	–	2-3 years back
2	Dhadde	+	+	–	Y	3	After 2nd application	15 day before sampling
3	Gursar Sahnewala	+	+	–	N	–	–	2-3 years back
4	Jajjal	+	+		N	–	–	5-6 years back
5	Jassi	+	–	–	N	–	–	5-6 years back
6	Malkana	+	–	–	N	–	–	4-5 years back
7	Mahinangal	+	+	–	N	–	–	2-3 years back
8	Sangat Kalan	+	+		N	–	–	2-3 years back
9	Teona Pujarian	+	+	–	Y	3	After 2nd application	1 day before sampling
10	Talwandi sabo	–	–	+	N	–	–	2—3 years back

Y= Yes, N= No

4.3 Analysis of soil properties: The physico-chemical and biological properties were characterized for the soil of villages under study. The soils of Bathinda district mainly developed under semi arid to arid environment which were commonly sandy loamy to silty in texture.

4.3.1 Physico-chemical properties: The physico-chemical properties were showing not very much variation in the all parameters as all the soil was developed under same climatic conditions of the region. The pH range of all soil samples were in alkaline range from 8.5 to 9.6 while of EC was 2.1 to 3.3 (Table 7). The content of organic carbon was maximum in JAS (0.77%) followed by TS and MN and range was 0.04% to 0.77%. The macronutrient content like nitrogen, phosphorus and potassium was not in very wide range. The content of nitrogen was showing similar trends as of organic carbon while the phosphorus content was found maximum in the DHA followed by TEO and MN. The potassium content was higher than other nutrients in the soil as the soil potassium was found in the range of 46.4 to 28.2 mg/kg. In present survey, analysis of all the soil samples were showing alkaline in nature along with low nitrogen and phosphorus content, medium organic carbon, high in potassium content (Khokhar et al., 2012, PSoER, 2005).

The alkaline and sandy nature of soil of Bathinda district was mainly developed under arid and high temperature conditions. The low organic content present in all the samples was due to high temperature condition prevailing in the area and similar results were also observed by Verma et al. (2005). All the samples were under the category of low nitrogen content. Majority of samples were also had low available phosphorus content due to high pH where phosphorus was not easily available. The results also indicated that the high potassium content in all the soil samples are due to more occurrence of K rich illite mineral present in soil of the area as per study of Kanwar (1961). A part from irrigation of fields with the high dissolved potassium content in the groundwater of the region might also be a reason for the high K in these soils (Patel et al., 2000).

Table 7. Physico-chemical properties of soils in sampling sites

S.N.	Sample Name	Moisture(%)	pH	EC (mS/cm)	TOC (%)	Total N (%)	Total P (mg/kg)	Total K (mg/kg)
1	BW	15.3±0.13	8.9±0.06	2.60±0.15	0.27±0.01	0.022±0.001	43.5±1.39	46.4±0.25
2	DHA	10.0±0.12	9.6±0.06	2.10±0.20	0.27±0.10	0.018±0.009	66.1±0.48	28.2±0.25
3	GS	9.40±0.13	8.5±0.09	2.50±0.40	0.32±0.01	0.027±0.002	42.4±0.47	40.6±0.36
4	JAJ	6.37±0.14	9.2±0.05	3.30±0.10	0.04±0.01	0.005±0.002	37.4±0.71	46.4±0.32
5	JAS	8.65±0.05	9.2±0.05	3.3±0.10	0.77±0.01	0.073±0.001	31.6±0.74	43.3±0.33
6	MAL	16.20±0.17	9.0±0.19	2.27±0.32	0.36±0.03	0.032±0.001	45.8±0.95	43.3±0.25
7	MN	13.30±0.20	8.7±0.09	3.15±0.15	0.37±0.15	0.033±0.002	55.6±0.91	38.5±0.15
8	SANT	9.0±0.23	9.3±0.11	2.32±0.56	0.28±0.04	0.020±0.001	42.1±0.28	38.3±0.30
9	TEO	13.30±0.20	9.3±0.04	2.60±0.15	0.32±0.06	0.029±0.001	60.1±0.47	32.4±0.20
10	TS	15.80±0.06	9.0±0.08	3.20±0.32	0.38±0.02	0.033±0.002	41.6±0.66	29.6±0.10

Results are in the form of Mean ± S.E.

BW= Bhagi Bander, DHA= Dhadde, GS= Gursar Sahnewala, JAJ= Jajjal, JAS= Jassi, MAL= Malkana, MN= Mahi Nangal, SANT= Sangatkalan, TEO= Teona Pujarian, TS= Talwandi Sabo

4.3.2 Biological properties: The analysis of biological properties of the soil reveals that The soil enzyme activity were showing the maximum dehydrogenase activity in the DHA while the phosphatase activity was highest in GS and the urease activity was found maximum in JAJ. The trend of enzymatic activity was showing that the all soils were having high phosphatase activity than dehydrogenase. The range of phosphatase enzyme was in the range of 7.68 $\times 10^2$ to 1.08 $\times 10^2$ mg pNp g^{-1} h^{-1} (Table 8). The soil enzymes are produced by the microbes present in soil hence considered as important indicator of soil microbial oxidative capacity. The application of repeated and continuous manner of pesticides creates pressure on the microbial count thus attributed towards low dehydrogenase and urease activity in soil. All the soil samples were having the history of pesticides application that is ultimately affecting the enzymatic activity of soils. In contrast the high phosphatase activity present in all the samples might be due to high pH and low phosphorus content in the soil. The pesticides application and high pH was found responsible for the increase in the alkaline phosphatase activity in all the samples. Apart from that low P content in the soils might be reason for favoring high phosphatase activity in soil for making P in available form was suggested by Nath and Samanta (2012). The inhibition in enzyme activity has also documented due to pesticides application by Sebiomo et al. (2011) and Andrea et al. (2000).

The microbial biomass content (MBC) was found in the range of 1 to 9 µg/kg (Table 9). The value of microbial quotient was found maximum in TEO while lowest in the GS and decline in microbial biomass content due to high rate of herbicides application was studied by Subhani et al. (2000). The MBC content and Microbial Quotient value are the important index of the activity of soil microorganisms. The low MBC in soil due to poor nutrient status and low organic matter present in the soil and pesticides induced environmental stress in the soil. Ayansina and Oso (2006) accounted the low microbial quotient of the soil due to low soil organic matter content mainly as the pesticides application have reported to have significant changes in the percent organic matter content in soil.

Table 8. Enzymatic activity in soils of sampling sites

S.N.	Sample Name	Dehydrogenase (µg TPF g^{-1} h^{-1})	Phosphatase (mg pNp g^{-1} h^{-1})	Urease (µg NH_3-N) g^{-1} h^{-1}
1	BW	3.33×10^2	1.52×10^2	1.85×10^2
2	DHA	4.44×10^2	4.45×10^2	1.70×10^2
3	GS	2.56×10^2	7.68×10^2	1.5×10^2
4	JAJ	4.02×10^2	5.40×10^2	2.27×10^2
5	JAS	3.04×10^2	1.1×10^2	2.77×10^2
6	MN	3.49×10^2	1.08×10^2	2.24×10^2
7	MAL	2.0×10^2	6.0×10^2	1.21×10^2
8	SANT	1.15×10^2	6.18×10^2	1.42×10^2
9	TEO	1.58×10^2	5.68×10^2	2.40×10^2
10	TS	2.61×10^2	5.50×10^2	2.18×10^2

Table 9. Microbial Biomass Carbon (MBC) content in soils of sampling sites

S.N.	Sample Name	TOC (µg/kg)	MBC (µg/kg)	Microbial Quotient
1	BW	2.9	3	0.12
2	DHA	2.8	6	0.23
3	GS	3.3	3	0.11
4	JAJ	4.1	1	0.25
5	JAS	7.8	1.5	0.2
6	MN	3.9	9	0.25
7	MAL	3.7	3	0.09
8	SANT	2.7	7	0.26
9	TEO	3.1	1.1	0.35
10	TS	3.9	7	0.19

TOC= Total Organic Carbon, MBC= Microbial Biomass Carbon

BW= Bhagi Bander, DHA= Dhadde, GS= Gursar Sahnewala, JAJ= Jajjal, JAS= Jassi, MAL= Malkana, MN= Mahinangal, SANT= Sangatkalan, TEO= Teona Pujarian, TS= Talwandi Sabo

4.4 Enumeration of Microbial population in the soil: The nutrient agar media facilitates the growth of all types of bacteria. While the pikovskaya media (PKV) is defined for the growth of phosphorus solublizing microorganisms.

4.4.1 Growth count of microorganism in nutrient agar

The highest population was observed in MAL followed by the MN, TS, JAJ, DHA while least observed in the TEO (Table 10). The microbial count was in the range of 16×10^8 to 6.2×10^8 cfu/g in all the samples after 72 h. The similar count was also observed by Janssen et al. (2002). There were slight difference in total population of bacteria in the samples and the increase in the cfu count was found as the incubation period increases (Fig. 5). The low bacterial count in the TEO might be due to application of pesticides immediate before sampling as the application of pesticides also creates pressure on the population of bacteria naturally. The applications of mixed formulation of pesticides in the repetitive manner greatly influence the population of microorganisms in the soil (Chu et al., 2008).

Table 10. Growth count of microbes in nutrient agar

Sl. No.	Sample Name	Viable count × 10^8 at regular interval (cfu/g)		
		24 h	48 h	72 h
1	BW	4.2	5.5	7
2	DHA	7	10	12
3	GS	8.9	9.5	11
4	JAJ	7.5	9	12
5	JAS	11.9	12.8	14
6	MAL	10	15.1	16
7	MN	10	13.5	15
8	SANT	3.5	4.9	6.6
9	TEO	5	6	6.2
10	TS	10	13.2	15

BW= Bhagi Bander, DHA= Dhadde, GS= Gursar Sahnewala, JAJ= Jajjal, JAS= Jassi, MAL= Malkana, MN= Mahi Nangal, SANT= Sangatkalan, TEO= Teona Pujarian, TS= Talwandi Sabo

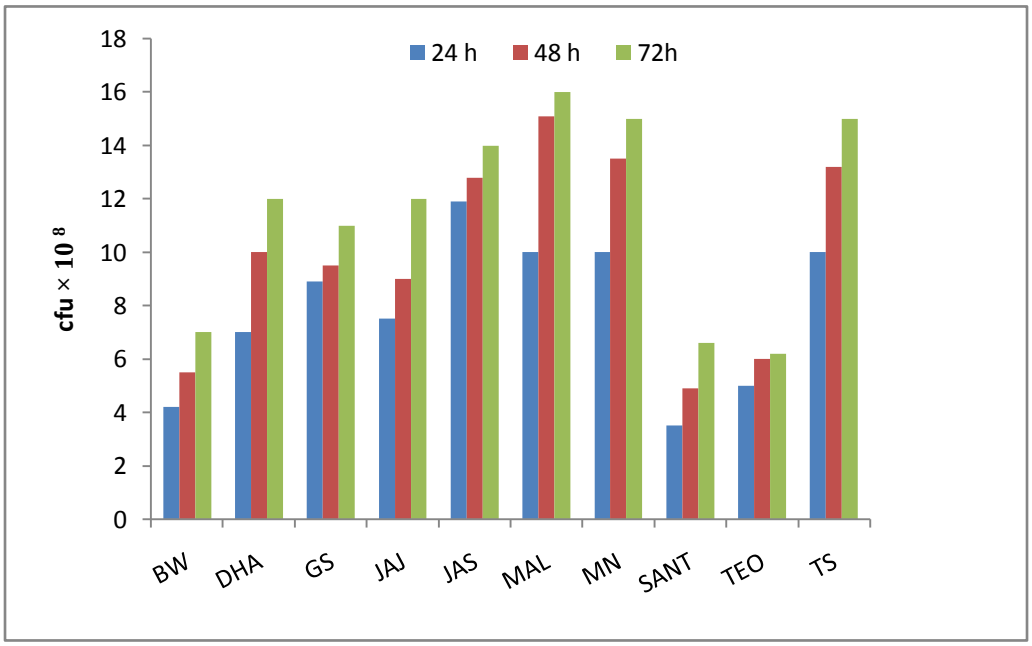


Fig. 5: Natural bacterial population in nutrient agar

4.4.2 Growth of microorganism in Pikovskaya media

The microbial count in pikovskaya media was less than that of growth in nutrient agar because it is specific media for the phosphorus solublizing microbes (PSMs). In the initially at 24 h the range of viable count was found 1.7×10^3 to 6.9×10^3 cfu/g which was increased upto 8×10^3 after the incubation of 72 h. The growth of PSBs were observed highest in JAS followed by BW, SANT, MN and the least was in JAJ, MAL in terms of cfu/g and biomass OD 600 after 72 h of incubation (Table 11). The suitable environmental conditions, nutrient availability and physicochemical properties are main factors which influence the growth of phosphorus solublizing microbes. The cfu was found increasing from the 24 to 72 hour of incubation due to solublization of phosphorus from tricalcium phosphates in media and make it available for growth (Fig. 6). The growth of phosphorus solublizing bacteria (PSBs) were found in the range of 4.0×10^3 to 8.0×10^3 after 72 h which was also accounted by Seshachala and Tallapragada (2012), Kannapiran and Ramkumar (2011), Vikram et al. (2007). After the incubation up to 3-4 days fungal growth was also observed. The comparison in growth trend of total bacterial count to the PSMs count is showing harmony with the work of Suliasih and Widawati (2005).

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Table 11. Growth count of Phosphorus Solublizing Bacteria (PSBs) in pikovskaya media

S.N.	Sample Name	Growth in PKV media (OD 600)		Viable count × 10^3 at regular interval (cfu/g)		
		24 h	48 h	24 h	48 h	72 h
1	BW	0.713	0.965	5.5 × 10^3	6.9 × 10^3	7.5 × 10^3
2	DHA	0.355	0.524	3.5 × 10^3	4.0 × 10^3	5.0 × 10^3
3	GS	0.436	0.665	4.0 × 10^3	5.8 × 10^3	6.5 × 10^3
4	JAJ	0.263	0.312	2.5 × 10^3	3.6 × 10^3	4.0 × 10^3
5	JAS	0.885	0.989	6.9 × 10^3	7.0 × 10^3	8.0 × 10^3
6	MAL	0.247	0.342	1.7 × 10^3	3.0 × 10^3	4.0 × 10^3
7	MN	0.75	0.96	5.0 × 10^3	6.2 × 10^3	6.8 × 10^3
8	SANT	0.706	0.906	5.4 × 10^3	6.0 × 10^3	6.9 × 10^3
9	TEO	0.44	0.563	4.0 × 10^3	4.2 × 10^3	4.8 × 10^3
10	TS	0.53	0.56	3.8 × 10^3	4.2 × 10^3	4.8 × 10^3

BW= Bhagi Bander, DHA= Dhadde, GS= Gursar Sahnewala, JAJ= Jajjal, JAS= Jassi, MAL= Malkana, MN= Mahi Nangal, SANT= Sangatkalan, TEO= Teona Pujarian, TS= Talwandi Sabo

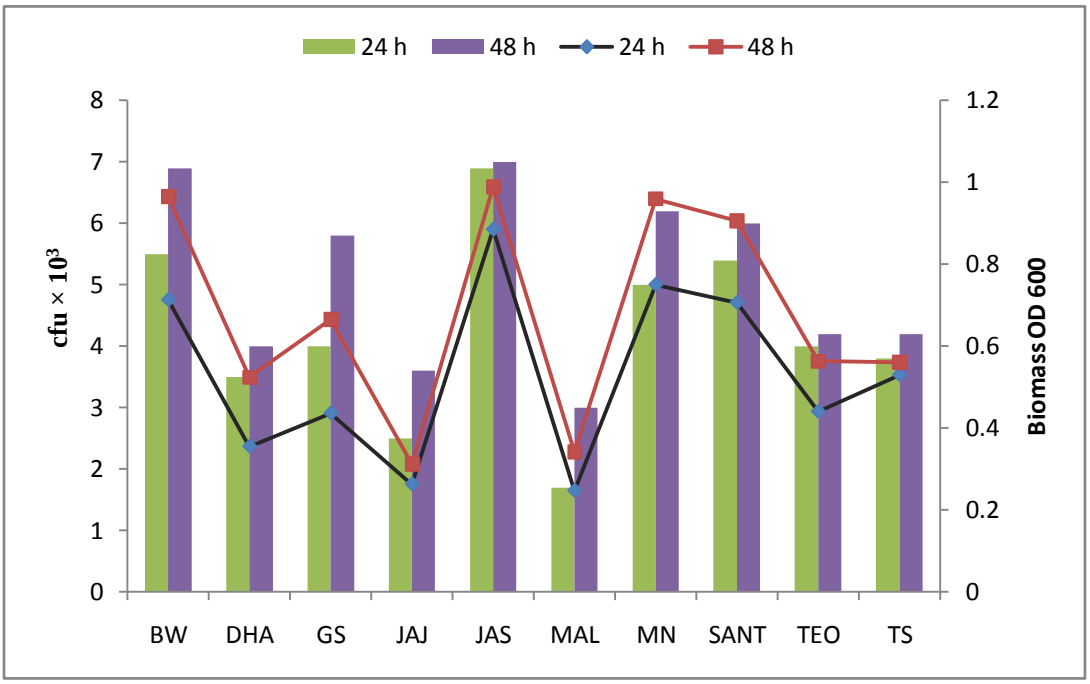

Fig.6: Biomass OD and cfu count of Phosphorus Solublizing Bacteria

4.5 MCP residue in soil of different sampling sites

The results of HPLC analysis revealed the presence of MCP only in TEO soil out of the samples from ten villages. The MCP was detected in small amount of 0.08 µg/g in TEO at the R.T. of 7.9 minute (Fig. 7). The presence of MCP in the sample might be because of the sampling done one day after the MCP application and hence the chances of MCP degradation were very less. The low value of MCP concentration in the soil might be due to foliar application of MCP which is unable to enter in the soil in due course of time. Another reason may be due to faster dissipation and water soluble nature of MCP.

In the soil sample of DHA had no residue of MCP was observed due to the biodegradable nature of MCP. The sampling was done 15 days after the MCP application. The hydrolysis of monocrotophos was found quicker at higher pH with the half life of 17 to 26 days in soil (Lee et al., 1990). So that the alkaline nature of soil might favored the degradation of MCP in soil. So there might be very much chances of complete degradation of MCP in soil samples. Vig et al. (2001) also observed the same trend for MCP residue detection in Punjab soils applied with MCP and other OP pesticides.

Table 12. MCP residue in soil samples by HPLC analysis

Sl. No.	Sample Name	MCP concentration (µg/g)
1	BW	ND
2	DHA	ND
3	GS	ND
4	JAJ	ND
5	JAS	ND
6	MAL	ND
7	MN	ND
8	SANT	ND
9	TEO	0.08
10	TS	ND

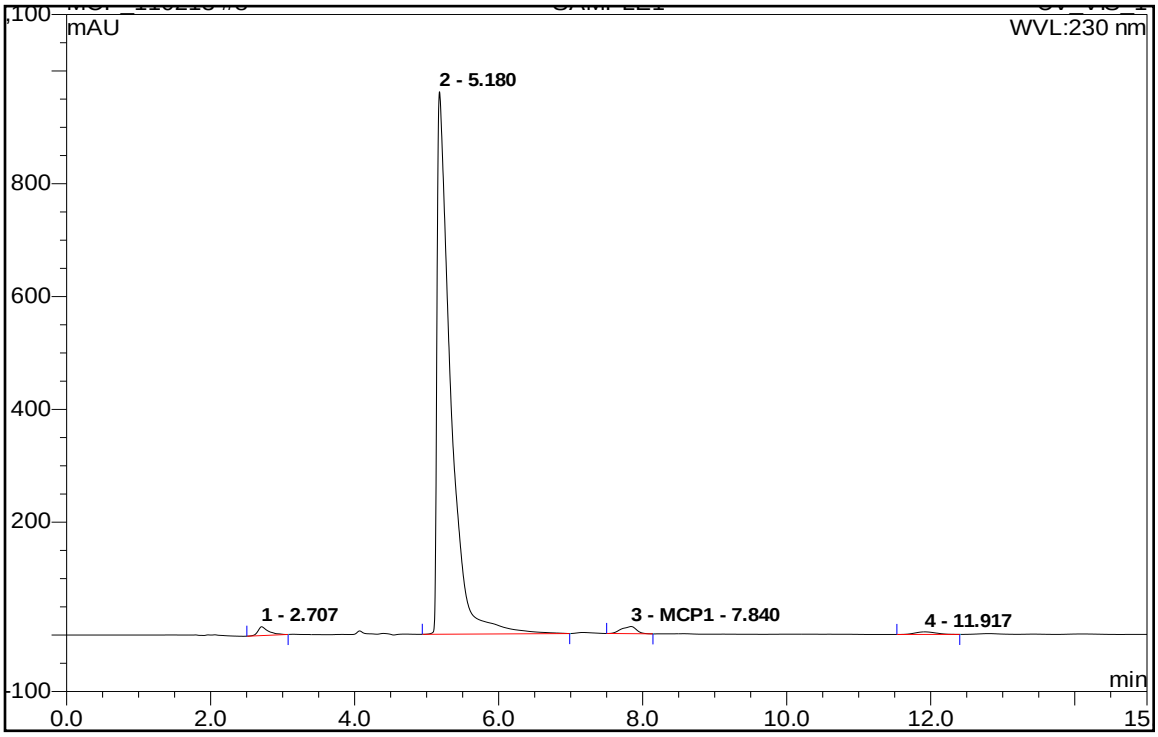

Fig 7: HPLC Chromatogram showing residue detected in TEO

4.6 GC- MS analysis of soil samples

The GC-MS analysis was done to determine the monocrotophos residues, metabolites and other compounds present in the soil samples. The retention time for monocrotophos was detected at 15.9 minutes with the standard MCP after comparing the mass spectra with the standard mass/charge spectrum. The MCP residue was not detected in DHA soil samples might be due to its complete degradation. The accelerated and pH dependent degradation of MCP was also reported in alkaline environment by Lee et al. (1990). The DHA soil showed presence of palmitic acid in maximum peak area (12%) at RT of 19.27 minute. While other components detected were stearic acid, erucic acid, caproic acid, di-n-octyl phthalate, squalene etc. (Appendix C).

The TEO soil showed the presence of erucic acid with maximum peak area (44.99%) at RT of 27.18 minutes. Several other compounds like stearic acid, palmitic acid, behenic acid, sqalene, arachidic acid, methyl dihydromalvalate, other esters, alkanes

and terpenes were also detected in the sample (Appendix D). Due to the heterogeneous soil environment and microbial activities volatile fatty acids, esters and terpenes were found as main compounds in soil.

4.7 Screening of microbial population in soil samples with MCP as sole source of Phosphorus

The growths of PSMs were observed in the PKV media supplemented with MCP at the concentration of 50 mg/L as source of phosphorus. The growth was determined in terms of biomass and cfu/g. The sequential screenings of samples were done with different concentration of MCP ranging from 100 to 1000 mg/L to check the tolerance power of PSMs against MCP so that the best tolerant isolate could be isolated.

The population of PSBs in the media supplemented with the MCP was found less than the population growth in pikovskaya media as the introduction of MCP in the media as sole source of phosphorus affects the growth of microbes. The cfu/g was also found more in uncontaminated soil than contaminated soil with refined petroleum products in the study done by Olalemi and Arotupin (2012). Another studies also founded that the application of various pesticides severely inhibit the bacterial counts (Sultan et al., 2013; Ayana et al., 2011; Bindhya et al., 2009).

Initially at the MCP concentration of 50 mg/L all 10 soil samples have shown growth in 24 hour and the growth was increased after 48 hour. At 24 h the biomass OD was also found maximum in TEO (0.325) followed by JAJ, BW and GS with the lowest in TS (0.025). The increase in biomass OD was noticed after incubation of 48 h in the range of 0.787 to 0.120. At 24 h the sample of TEO have showed rise in cfu upto 5×10^2 while the sample of BW, JAJ, MN, SANT have shown similar growth trend after 24 h (Table 13).The sharp increase in the growth was observed in the JAJ, SANT and TS after incubation of 48 h. The maximum count was observed in JAJ (9×10^2) followed by SANT, TEO, MN and TS at the time period of 48 hr. The cfu count and biomass growth (OD 600) was showing correlation in growth trends after increase in incubation hour (Fig.8). At the MCP conc. of 50 mg/L the percent microbial population that of pikovskaya media was ranging 1.45 to 12.94 % which gets increase up to 25%

of population in pikovskaya media after incubation of 48 h (Table 14). The increase in the population of PSBs was might be due to adaptation towards MCP.

Table 13: Biomass growth and cfu at 50 mg/L of MCP in soil

Sl. No.	Sample Name	Biomass OD (600 nm) at MCP 50 mg/L		cfu after adaptation for 72 h in PKV agar + MCP (50 mg/L)	
		24 h	48 h	24 h	48 h
1	BW	0.256	0.336	3.0×10^2	5.0×10^2
2	DHA	0.126	0.274	2.0×10^2	3.0×10^2
3	GS	0.247	0.355	4.0×10^2	6.0×10^2
4	JAJ	0.319	0.787	3.0×10^2	9.0×10^2
5	JAS	0.022	0.185	1.0×10^2	2.5×10^2
6	MAL	0.121	0.243	2.2×10^2	2.7×10^2
7	MN	0.240	0.603	3.0×10^2	7.0×10^2
8	SANT	0.222	0.646	3.0×10^2	7.2×10^2
9	TEO	0.325	0.633	5.0×10^2	7.0×10^2
10	TS	0.025	0.120	2.0×10^2	6.0×10^2

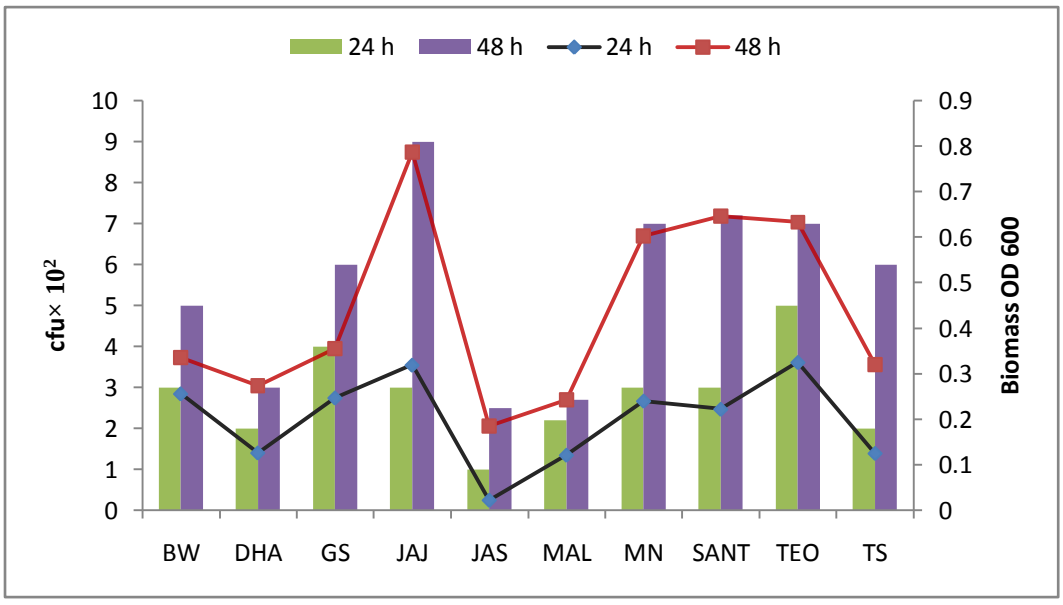

Fig. 8: Biomass growth and cfu at 50 mg/L of MCP

Table 14: Percentage of population showing tolerance at MCP concentration of 50 mg/L

Sl. No.	Sample Name	% of PSM population tolerance to MCP at 50 mg/L	
		24 h	48 h
1	BW	5.51	7.25
2	DHA	5.71	7.50
3	GS	10.0	10.34
4	JAJ	12.0	25.0
5	JAS	1.45	3.57
6	MAL	12.94	9.0
7	MN	6.0	11.29
8	SANT	5.56	12.0
9	TEO	12.5	16.66
10	TS	5.26	14.28

The microbes which get adapted in 72 h to 50 mg/L MCP were further screened for its tolerance at 100 mg/L of MCP supplemented in the media. Out of 10 adapted soil samples only 8 were found able to show growth. At this concentration of MCP the cfu count of DHA was maximum 12×10^2 followed by the GS, JAJ and MAL after 24 and 48 h (Table 14). The biomasses OD of samples were in the same manner with maximum for DHA and minimum for MN and SANT and JAS. The soil samples of MN and SANT have showed no colonies for both the time period with less colony count in JAS (Fig. 9). The maximum cfu growth in the DHA might be due to regular application of MCP in DHA soil which helped the microbes to develop tolerance (Table 6). The TEO has not showed any significant changes in microbial count at higher concentration due to adaptation developed after 72 h in 50 mg/L. The samples showing no colony growth were further screened for higher concentration.

After comparing the adaptation percent at 100 mg/L of MCP it was found that BW, TEO and TS had showed decreased in growth count while GS, DHA, JAJ and MAL showed increase in the growth. The increase was ranged from 6 to 1.4 folds than 50 mg/L.

Table 15. The growth of microorganisms in 100 mg/L of MCP

Sl. No.	Sample Name	PKV+MCP (100 mg/L)		PKV+MCP (100 mg/L)	
		24 h	48 h	24 h	48 h
1	BW	0.124	0.145	1.8×10^2	3.0×10^2
2	DHA	0.136	0.244	5.0×10^2	12.0×10^2
3	GS	0.285	0.294	10.0×10^2	11.0×10^2
4	JAJ	0.309	0.349	5.5×10^2	6.0×10^2
5	JAS	0.077	0.177	0.8×10^2	1.0×10^2
6	MAL	0.204	0.256	3.6×10^2	4.5×10^2
7	MN	0.018	0.021	No visible growth	No visible growth
8	SANT	0.045	0.056	No visible growth	No visible growth
9	TEO	0.205	0.326	5.0×10^2	7.0×10^2
10	TS	0.138	0.045	1.9×10^2	1.1×10^2

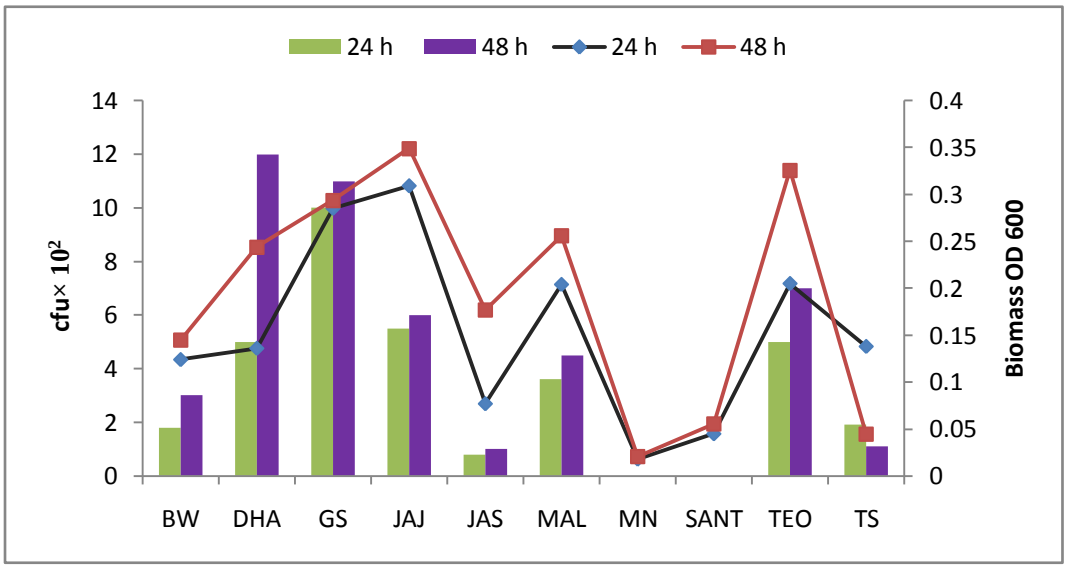

Fig. 9: Biomass growth and cfu at 100 mg/L of MCP

With the increase in concentration of 250, 500, 750 mg/L of MCP there was no biomass growth in any sample after incubation of 24 h (Table 16). Out of 7 adapted samples at lower concentration of MCP, only 6 soil samples were able to show growth at concentration of 250 and 500 mg/L of MCP after 48 h. After get adapted at 250 mg/L maximum cfu count was in DHA and JAJ while TS showed very less cfu as

no tolerance was developed (Fig.10). The DHA had showed maximum tolerance at 500 mg/L of MCP followed by JAJ, TEO and BW. The samples BW, JAJ, MAL and TEO showed less cfu count at increased concentration from 250 to 500 mg/L of MCP. The GS showed no colonies at 250 and 500 mg/L. At the 500 mg/L only DHA showed increase in cfu with the value of 17×10^2 cfu/g (Fig.11). The biomass OD at 600 found decreased after 48 h for increase in concentration from 250, 500 and 750 mg/L of MCP for most of the soil samples (Table 17).

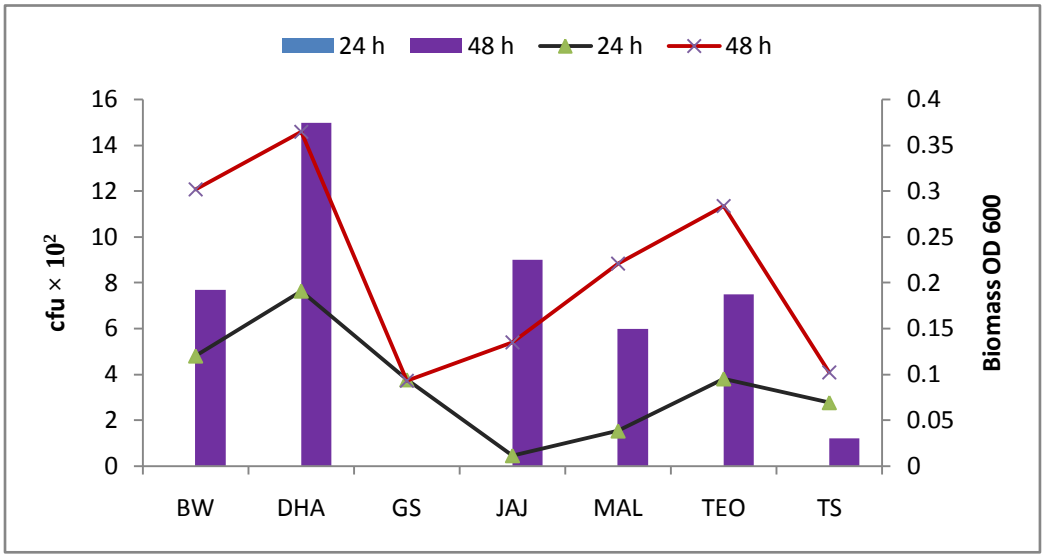

Fig 10: Biomass growth and cfu at 250 mg/L of MCP

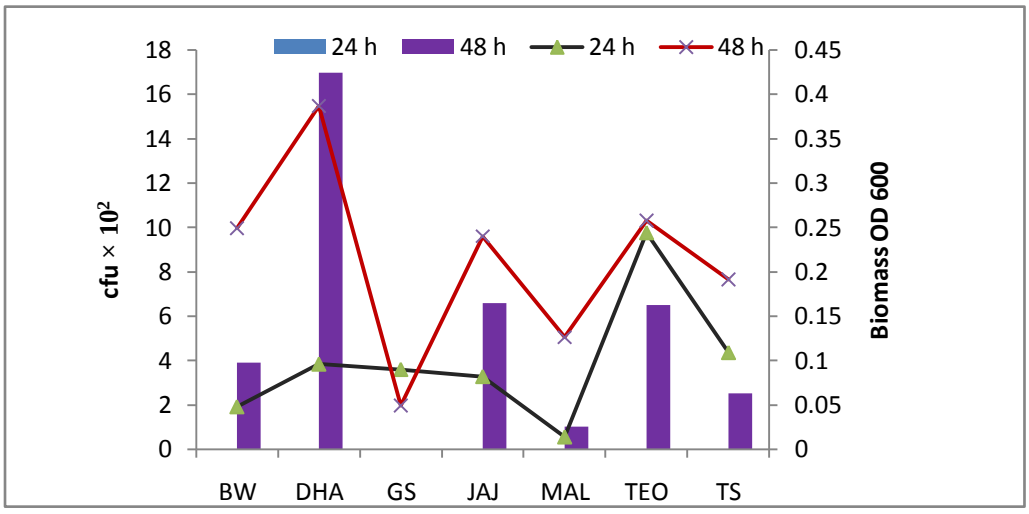

Fig 11: Biomass growth and cfu at 500 mg/L of MCP

Table 16. Microbial count at different concentration of MCP

Sl. No.	SAMPLE	MCP(250 mg/L)		MCP(500 mg/L)		MCP(750 mg/L)		MCP(1000 mg/L)	
		24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
1	BW	No visible growth	7.7×10^2	No visible growth	3.9×10^2	No visible growth	2.2×10^2	No visible growth	1×10^2
2	DHA	No visible growth	15×10^2	No visible growth	17×10^2	No visible growth	2.9×10^2	No visible growth	2.5×10^2
3	GS	No visible growth	No growth	No visible growth	No visible growth	No visible growth	No growth	No visible growth	No visible growth
4	JAJ	No visible growth	9×10^2	No visible growth	6.6×10^3	No visible growth	2×10^2	No visible growth	1.1×10^2
5	MAL	No visible growth	6×10^2	No growth	1×10^3	No growth	No growth	No growth	No growth
6	TEO	No visible growth	7.5×10^2	No visible growth	6.5×10^3	No visible growth	6×10^2	No visible growth	5×10^2
7	TS	No visible growth	1.2×10^2	No visible growth	2.5×10^2	No visible growth	No visible growth	No visible growth	No visible growth

Table 17. Biomass growth at different concentrations of MCP

Sl. No.	Sample Name	Biomass OD MCP 250 mg/L			Biomass OD MCP 500 mg/L			Biomass OD MCP 750 mg/L		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
1	BW	0.12	0.302	0.124	0.048	0.249	0.206	0.006	0.187	0.039
2	DHA	0.191	0.365	0.142	0.096	0.387	0.257	0.119	0.264	0.241
3	GS	0.094	0.093	0.021	0.09	0.049	0.004	0.044	0.036	0.004
4	JAJ	0.011	0.135	0.09	0.082	0.24	0.247	0.069	0.198	0.178
5	MAL	0.038	0.221	0.201	0.014	0.126	0.114	0.089	0.102	0.045
6	TEO	0.095	0.284	0.213	0.244	0.258	0.227	0.219	0.262	0.245
7	TS	0.069	0.102	0.012	0.109	0.191	0.179	0.082	0.163	0.046

At the 750 mg/L of MCP in the media as sole source of P only 4 adapted soil samples were able to utilize MCP as source of phosphorus. The increase in cfu count was drastically high in the TEO than DHA which showed relatively less tolerance at higher concentration of MCP (Fig.12). The samples of GS, MAL and TS were failed to show any colonies after 48 h. The growth of more tolerant PSBs was more in the TEO followed by DHA, BW and JAJ after incubation of 48 h (Table 15). But for DHA and TEO after 72 h the biomass OD attained a constant phase at the concentration of 500 and 750 mg/L (Table 17). The lower concentration of pesticides had less effect on the population than the higher concentration that affect the microbial population severely was also observed by Sultan et al. (2013) and Bindhya et al. (2009).

The adapted samples at lower concentration failed to show the growth at higher concentration of 1000 mg/L. Out of 4 adapted soil samples none had showed cfu at 24 h. The cfu count was less than that of at 500 mg/L but the TEO had showed maximum cfu followed by DHA, JAJ and BW after 48 h (Fig. 12-13). The samples of DHA and TEO showed the increased biomass concentration after the incubation of 7 day (Table 18) and were found able to grow much fast growth at 1000 mg/L of MCP (Fig. 13).

The growth pattern for adapted bacterial population to MCP in DHA and TEO followed similar trend. Table 17 showed fast increase in growth at higher concentration of 1000 mg/L of MCP as sole source of P in media. The soil of DHA and TEO had more tolerance power towards MCP as the microbes were under the stress of continuous application of MCP (Table 6). It enabled them to use MCP as carbon and phosphorus source and hence facilitates its biodegradation. Therefore the samples of DHA and TEO were further screened for their ability to degrade MCP in media and soil.

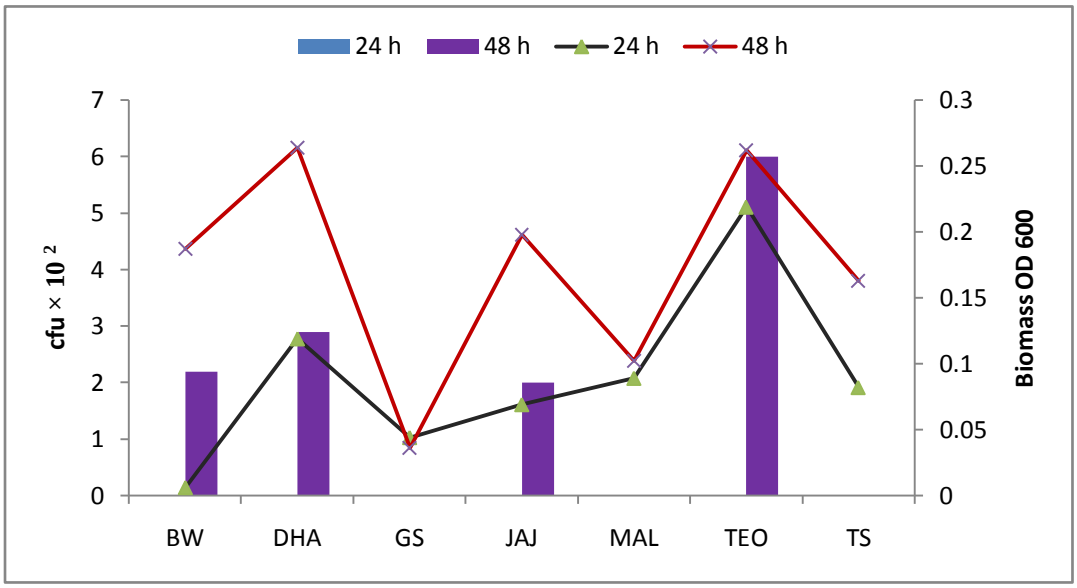

Fig 12: Biomass growth and cfu at 750 mg/L of MCP

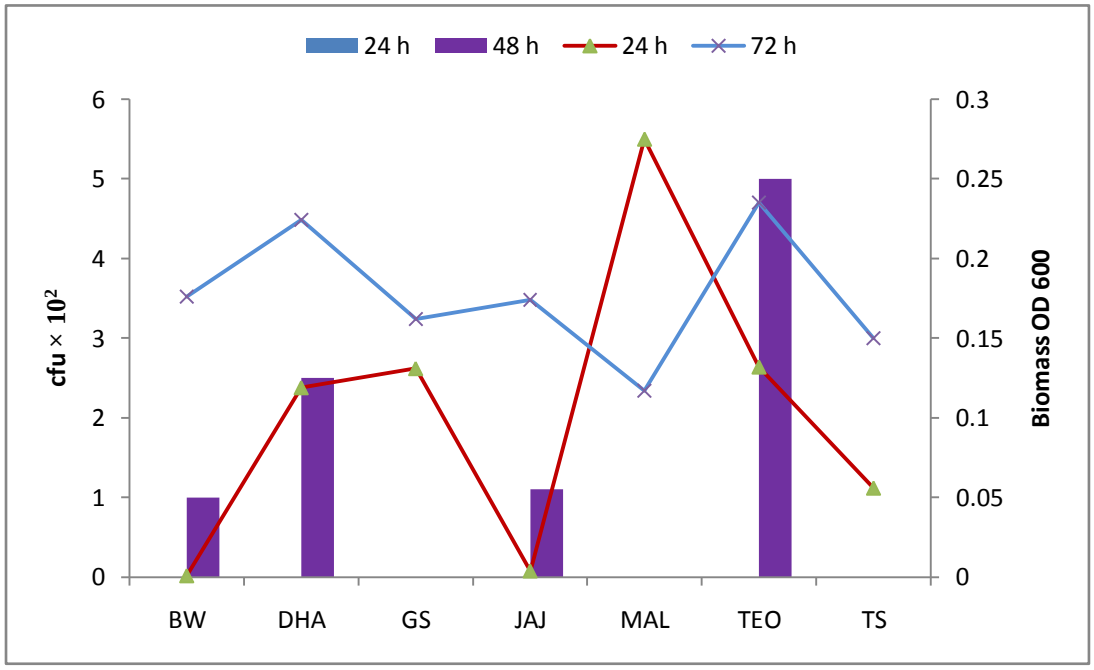

Fig 13: Biomass growth and cfu at 1000 mg/L of MCP

Table 18. Biomass growth at MCP conc. of 1000 mg/L in different samples

Sl. No.	SAMPLE NAME	Biomass OD at MCP 1000 mg/L			
		24 h	72 h	120 h	168 h
1	BW	0.001	0.176	0.194	0.226
2	DHA	0.119	0.224	0.228	0.316
3	GS	0.131	0.162	0.108	0.121
4	JAJ	0.004	0.174	0.265	0.285
5	MAL	0.004	0.174	0.265	0.285
6	TEO	0.132	0.235	0.249	0.332
7	TS	0.056	0.15	0.226	0.194

The growth at 250 mg/L was noticed increase in both DHA and TEO. Maximum increase was 3.16 fold in TEO followed by BW and DHA after 48 h than 100 mg/L. At higher concentration ranged from 500 to 1000 mg/L all the samples showed decrease in growth. After the concentration of 500 mg/L the decline of 0.07 fold for TEO while 0.8 fold for DHA was found. The sample of GS, MAL and TS was showed almost decrease of 1 fold than the previous concentration of 500 mg/L. After comparing the growth count at 1000 mg/L to the previous concentration of 750 mg/L most of sample failed to grow while for DHA and TEO less decrease in growth count and range was 0.4 fold to 0.16 fold was noticed.

Omar and Sater (2001) had also documented that low rate pesticides application less effect on the total population of soil microbes while the high concentration had significant inhibitory impact on the total bacterial population in soil. The pesticides like methyl parathion, dimethoate and endosulfan showed inhibitory effect on the population of Azotobacter chroococcum, Azospirillum brasilense and Beijerinckia indica was observed by Sudhakar et al. (2000). It indicated that the growth of bacterial population was affected by the pesticides application and the growth response varied with soil characteristics, concentration and characteristics of

pesticides. Initial application of pesticides may result in the decrease of activity of microorganisms but later the activity increased due to the utilization of degradation products for cell proliferation. Also the behavior of microbes varied with the nature, dosage and amount of organophosphates compounds.

4.8: Biomass Growth (OD 600) of screened samples of DHA and TEO

The bacterial isolates from two samples DHA and TEO were taken for further study based on its trend of tolerance and adaptation at higher concentration of MCP. The biomass concentration and cfu count clearly showed that both the samples had the growth at lower concentration of 50 and 100 mg/L (Table 13-15). The soils of DHA and TEO had the adaptation and tolerance potential for higher concentration of MCP also.

The bacterial cfu count of population from the sample of DHA was maximum at 500 mg/L of MCP at 48 h. At the higher concentration of 250 to 1000 mg/L no visible cfu count at 24 h but after get adapted for 48 h the cfu count increased tremendously upto 500 mg/L. Thereafter a drastic decrease was noticed for the concentration 750 mg/L to 1000 mg/L (Fig. 14). The changes in biomass concentration had showed correlation with the microbial growth count after 24 h and 48 h.

The trend of microbial growth was similar for TEO but the increase in cfu was much higher than DHA. The maximum cfu was found in 250 mg/L and that decreased for the concentration of 500 to 1000 mg/L but at the highest concentration of 750 and 1000 mg/L more cfu was found in TEO than DHA (Fig. 15). The microbes from TEO had more or very less constant decrease in biomass concentration at higher concentration. It showed similar adaptation pattern for both isolates at the concentration of 250 to 1000 mg/L but the growth trend was more stabilized for TEO at higher concentration of MCP.

The rise in TEO was more at higher concentration which might be because of developed tolerance at higher concentration of MCP. The DHA was also showed adaptation but the capability to grow at higher concentration was less than the TEO.

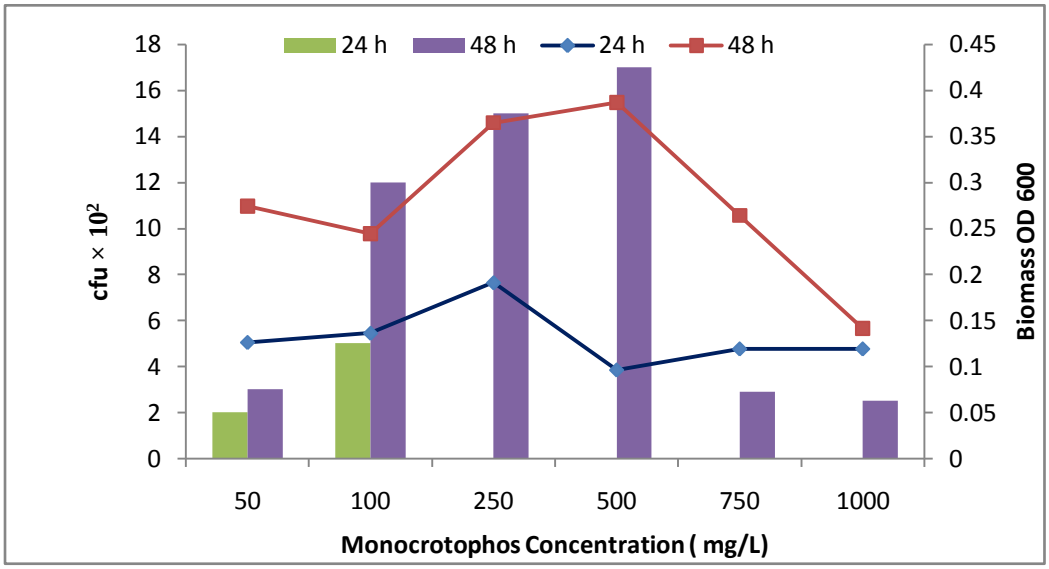

Fig. 14: Biomass OD 600 of DHA at different MCP conc.

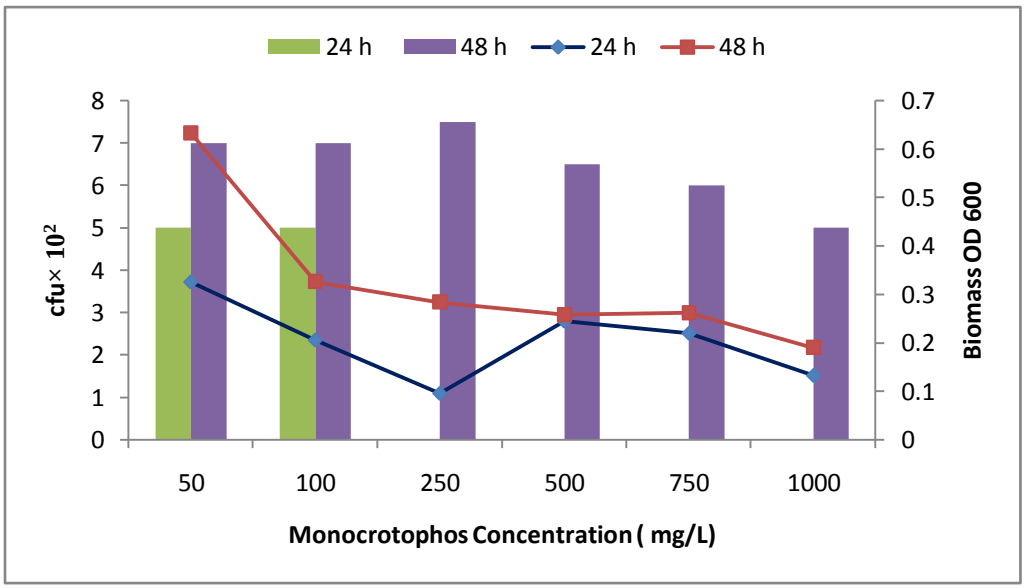

Fig. 15: Biomass OD 600 of TEO at different MCP conc.

4.8: Isolation of MCP degrading PSMs

The two soil samples DHA and TEO were further selected for isolation study at higher concentration of 1000 mg/L of MCP. The growth was confirmed at 1000 mg/L for both the samples and the isolates adapted at 1000 mg/L of MCP were carefully picked and inoculated to nutrient agar and PDA agar plates separately. The bacterial isolates were further sub cultured three to four times on nutrient agar for isolation of pure colonies. For fungal isolates subcultured on the PDA plates were further incubated for 5-7 days.

One bacterial and two fungal isolates were carefully isolated from the soil of TEO and were designated as T_1, T_2 and T_3 respectively (Fig. 16. a, b, c).

One bacterial and two fungal isolates were carefully isolated from the soil of DHA and were designated as D_1, D_2 and D_3 respectively (Fig. 17. a, b, c).

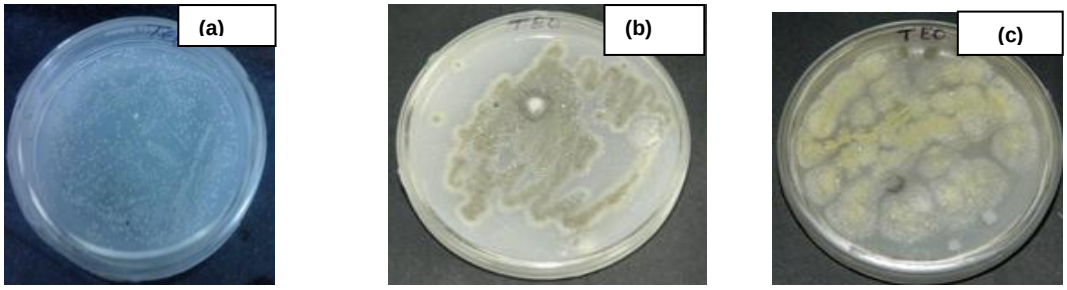

Fig: 16 (a) Isolate T_1; (b) Isolate T_2; (c) Isolate T_3

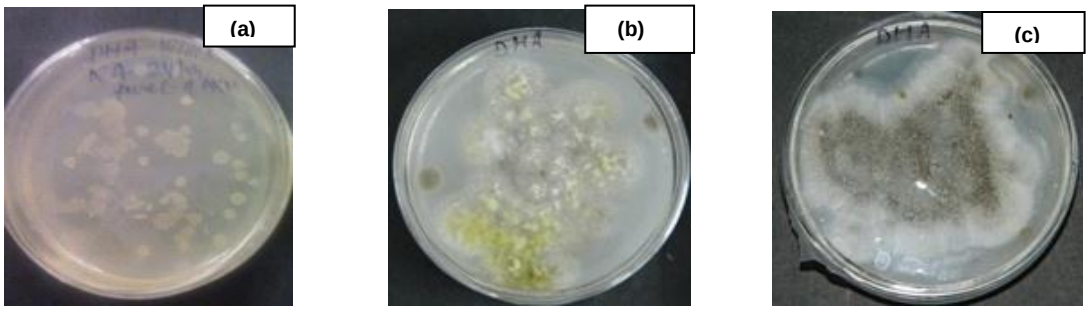

Fig: 17 (a) Isolate D_1; (b) Isolate D_2; (c) Isolate D_3

4.9 Identification of Isolates: Table 19 and 20 represented the morphological and biochemical characters of bacterial isolates of D_1 and T_1. The colony color, shape and colony morphology was similar for D_1 and T_1.

The bacterial isolate T_1 was creamy white colored, round and rod shaped, gram negative. The isolate was found nonmotile, endospore forming (Fig. 18). The isolate was able to produce oxygen with catalyze enzyme because of oxidative behavior (Fig. 19 a). The isolates were found able to ferment the glucose and sucrose while unable to utilize lactose, mannitol and glycerol (Fig. 19 b). The Isolate was capable of utilizing citrate as sole carbon source with the production of alkaline bicarbonates and carbonates as metabolic product of citrate (Fig. 19 c).

The isolate D_1 was also creamy white colored, round and rod shaped, gram negative, nonmotile, non endospore forming (Fig. 17 a) and unable to produce catalase enzyme. The isolate was also able to ferment glucose (Fig. 19 b) and sucrose while unable to utilize lactose, mannitol, and glycerol.

Both the isolates were unable to produce urease enzyme for splitting urea and unable to perform mixed acid fermentation in MR-VP broth. The starch hydrolysis with the production of alpha amylase enzyme was also showed negative results in both the isolates. The fungal isolates namely D_2 and D_3 were yellowish and black white respectively while the morphological pattern was showing powdery growth. The isolate T_2 and T_3 were of green and white color respectively.

The summary of morphological characters, biochemical tests results of the bacterial and fungal isolate are given in Table 19 and 20.

Table 19. Morphological characteristics of bacterial isolates

Characteristics	**Isolate D_1**	**Isolate T_1**
Colony color	Creamy white, round	Creamy white, round
Gram reaction	Gram –ve	Gram –ve
Cell shape	Rod	Rod
Colony morphology	Opaque and flat	Opaque and flat
Endospores	–	+

Fig 18: Endospore forming ability of Isolates

Table 20. Biochemical characteristics of MCP resistant bacterial Isolates

Characteristics	Isolate D_1	Isolate T_1
Motility	-ve	-ve
Catalase	–	+
Glucose	+	+
Sucrose	–	+
Lactose	–	–
Urea hydrolysis	–	–
Methyl red	–	–
Vogas Proskauer	–	–
Starch hydrolysis	–	–
Citrate	+	+
Mannitol	–	–
Glycerol	–	–

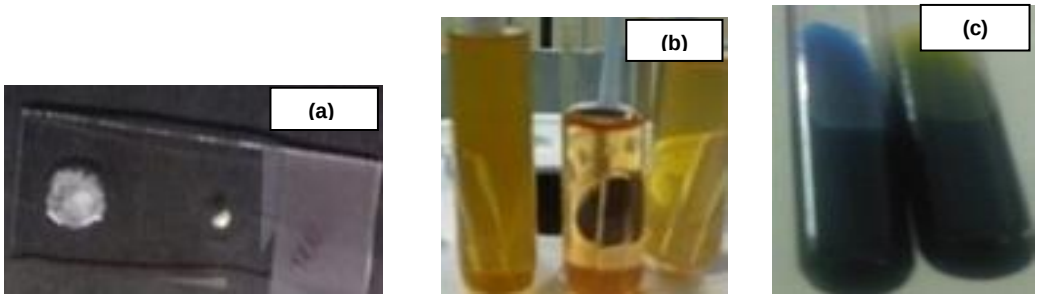

Fig 19 (a) Catalase test for Isolate $T_1$and D_1; (b) Glucose utilization test for D_1 and T_1

(c) Citrate utilization test

Table 21: Characteristics of Fungal isolates

Isolate name	Color	Morphology
D_2	Yellowish White	Powdery spores
D_3	Black	Powdery spores growth
T_2	Green	Powdery mat like
T_3	Yellowish White	Powdery spores

The isolates found similar characters of:

- T_1 may be Pseudomonas sp., D_1 may be Serratia sp. according to the Bergey's Manual of bacteriology. The isolate T_1 was also found similar characteristics to phosphorus solublizing bacteria studied by Tripti et al. (2012), Widiastuti (2008).
- The D_2 and T_3 were the same isolates based on the similarity of color and spores formation. The fungal isolates might be belonging to Aspergillus sp. as this sp. had been well documented for the degradation of MCP as well as phosphorus solublizing. While the color and morphological characters of isolates D_3 and T_2 showed similarity to Aspergillus niger and Aspergillus flavus respectively (Das and Anitha, 2011, Bhalerao and Puranik, 2009).

Due to fast growth pattern and ease of handling the bacterial isolates were further selected for further degradation studies of MCP.

4.10 P solublization potential of Pure isolates in PKV media: The phosphorus solublizing capability of the isolates were determined by solublization of 0.5% of insoluble tricalcium phosphate (TCP) supplemented in the media. The results indicated that in the media inoculated with pure isolates the concentration of available P was more than uninoculated after 10 days of incubation along with pH drop. The extent of TCP solublization was in 456 mg/L DHA and 490 mg/L in TEO (Table 21). Both the isolates were found to capable of solublizing the bound phosphorus into the available form. The sharp decrease in the pH upto 4±0.5 in media was in concurrence with the solublization of TCP. The solublization of TCP was due the acidification reaction in most of soils resulting in the phosphorus solublizing capability of Isolates. The extent of P solublization after 7 days was 58 µg/ml and 250 µg/ml by Aspergillus and Penicilium respectively in media supplemented with 0.5% of TCP with negatively correlated with pH of media was noticed by Pradhan and Sukla (2005). The study of

Yadav et al. (2011) and Chakkaravarthy (2010) also reveals the ability of PSBs to solublized TCP in different ranges of 9.6-136.6 mg/L and increase in the solublization potential from 438 µg/ml to 512 µg/ml with increase in Incubation days from 7 to 21 days, respectively. The acidic condition promotes the solublization of TCP (Maheshwar et al., 2012; Yadav et al., 2011; Seshadri et al., 2002) because the PSBs were well documented for the production of organic acid in the medium (Fankem et al., 2006; Rodrı´guez and Fraga 1999). The isolates clearly showed their ability to solublized TCP so there are chances of their ability towards degradation of other P containing xenobiotics and make them bioavilable toward degradation in soil environment.

Table 22. Phosphorus solublization efficiency of isolates

Sl.No.	Sample	pH change		Tri-Calcium Phosphates Solublized (mg/L)
		Initial	Final	
1	TEO	7.00±0.5	4.10±0.5	490±0.64
2	DHA	7.00±0.5	4.00±0.5	456±0.75

Results are expressed in the form Mean±S.E.

4.11 Growth pattern of Isolate T_1 and Isolate D_1 in MCP supplemented media

The growth pattern of pure isolates were determined from biomass growth at different MCP concentration of 100, 500, 1000 mg/L. The more growth was at lower concentration of 100 mg/L which reduced after 500 mg/L and 1000 mg/L (Fig. 20-21). The growth trend indicates maximum growth after 24 h due to more availability of MCP in media and thereafter the growth was found decreased as the nutrient concentration decreased by the utilization of isolates population. Both the isolates exhibit the similar growth due to developed tolerance against MCP and capable of utilizing the MCP as P source for their growth. The pure isolates were able to utilize MCP more efficiently even at higher concentration and showed more biomass OD than mixed culture of DHA and TEO after 24 h (Fig.14-15). The similar results were obtained using high doses than conventional doses of pesticides which failed to support the growth at higher concentrations (Jain et al., 2012, Rani et al., 2008).

Figures (20 and 21) indicate the decrease in growth due to reduced rate of substrate utilization at higher concentration of pesticides. At lower concentration the isolates were more capable in solubilizing the MCP than at the higher concentration.

Table 23. Biomass growth of Isolate D_1 and T_1

MCP conc. (mg/L)	Biomass growth of Isolate D_1 (g/L)			Biomass growth of Isolate T_1 (g/L)		
	24 h	48 h	72 h	24 h	48 h	72 h
100	0.008	0.005	0.004	0.003	0.001	0.001
500	0.008	0.004	0.003	0.003	0.001	0.001
1000	0.007	0.002	0.002	0.002	0.001	0.009

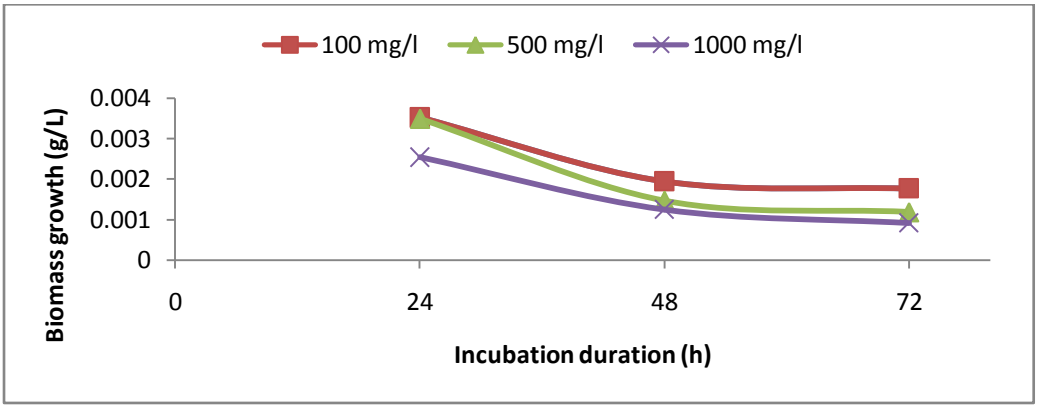

Fig 20: Growth Pattern of Isolate T_1

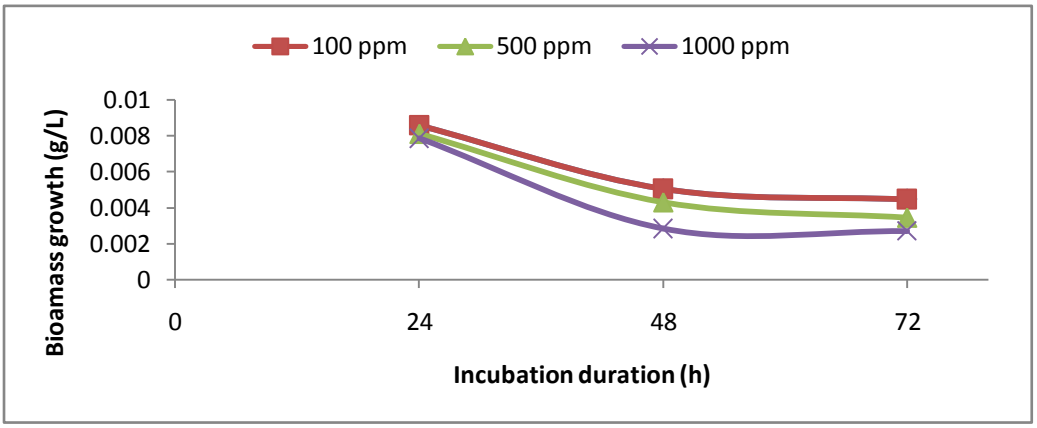

Fig 21: Growth Pattern of Isolate D_1

4.12 MCP Degradation potential of Isolates in PKV media

The growth pattern of isolates indicated the capability of degrading MCP in media as they were found able to utilize it as source of carbon and phosphorus. The TEO samples had showed constant rise in biomass and microbial count at higher concentration. So the degradation study was carried out at higher concentration of 1000 mg/L in pikovskaya media with isolate T_1.

The ability of the isolates was checked to degrade the MCP in the media as sole source of P by utilizing it for growth. The HPLC results showed 88.9% degradation with the residue concentration of 111.1 mg/L of MCP in the PKV media by isolate T_1 after incubation up to 7 days (Fig. 22). It indicated the efficiency of utilization of MCP by isolates as phosphorus source for growth. Several studies had also been shown the potential of bacterial isolates for the degradation of several other OP pesticides (Kumar, 2011, Bhagobaty and Malick, 2008). The complete or degradation upto 75-90 % after 7 days of incubation was also reported by Jain et al. (2011), Das and Anitha (2011), Jia et al. (2006), and Bhadbhade et al. (2002b).

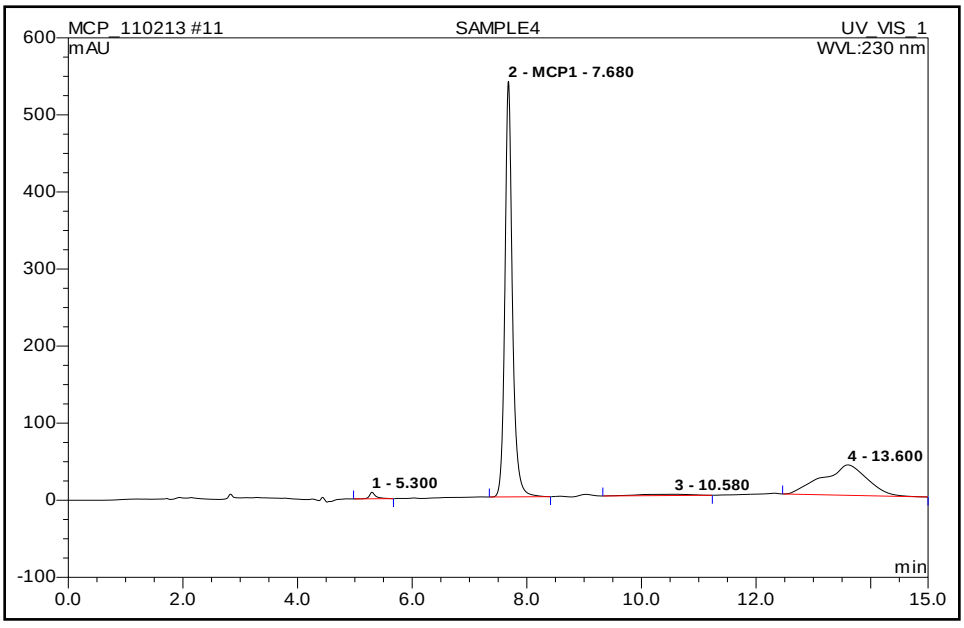

Fig. 22: HPLC chromatogram of extract from Pikovskaya media supplemented with 1000 mg/L MCP inoculated with isolate T_1 after 7 day

4.13 GC-MS analysis of the culture media inoculated with T_1

The mass spectra obtained from GC-MS of media with 1000 mg/L of MCP after 7 day inoculated with isolate T_1 had the similar compounds reported by other workers (Das and Anitha 2011; Bhalerao et al., 2009; Bhadbhade et al., 2002b). The GC-MS analysis detected 14 compounds (Appendix E) in which traces of MCP was detected. The mass spectrum of MCP is given Fig. 23 and other VFAs and hydrocarbons in Table 24. The phthalic acid was found in maximum peak area (89.62%) followed by palmitic acid, nonadecane, hexadecane, 3- octadecane, These compounds indicated presence of bacterial cells in the sample.

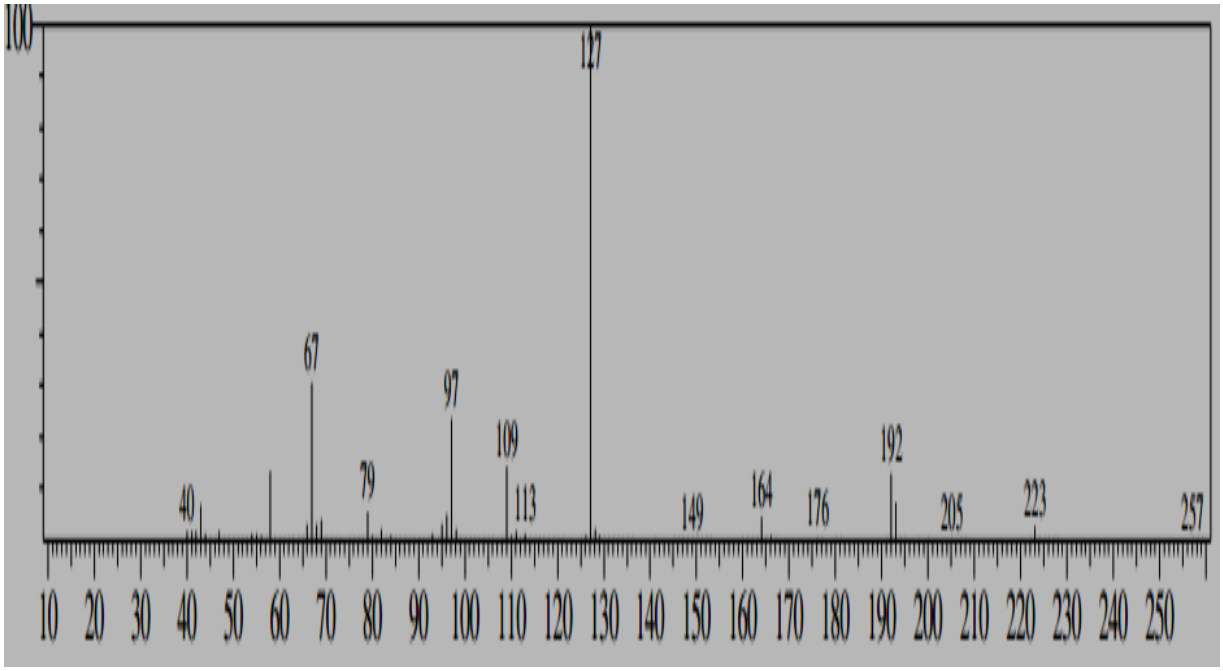

Fig 23. Mass spectrum of MCP detected in media

Table 24: Compound detected in media

Sl. No.	RT(min)	Name of compound	Molecular formula	Molecular weight	Peak area (%)
1	8.89	3- Hexadecane	$C_{16}H_{34}$	226.44	1.24
2	12.75	3-Octadecane	$C_{18}H_{38}$	254.49	1.39
3	14.7	Monocrotophos	$C_7H_{14}NO_5P$	223.16	0.36
4	19.2	Palmitic acid	$C_{16}H_{32}O_2$	256.42	0.33
5	19.69	Nonadecane	$C_{19}H_{40}$	268.52	1.36
6	27.04	Di-n-octyl phthalate	$C_{24}H_{38}O_4$	390.56	0.31
7	27.84	Phthalic acid	$C_6H_4(CO_2H)_2$	166.14	89.62

4.14 Quantification of Available Phosphorus in soil supplemented with MCP

The P solublization potential by the isolates was determined by the estimation of available P in the soil supplemented with commercial grade MCP. The content of P solublization was simultaneously estimated in inoculated and sterilized soil as control at regular time interval. The effect of inoculums volume of 2% and 10% was studied.

The trend of P solublization was found increasing with the increase in the time. After 72 h the available phosphorus content in DHA 2% inoculated with isolate D_1 was 21.71 mg/kg which increased up to 35.43 mg/kg at the end of 168 h. DHA 10% have shown more available P content initially but the content of P solublized was found less than DHA 2% with the 51.12%. The difference in the activity of solublization was not varied much with the inoculums volume. The rise was more upto 144 h after that the isolates showed less rise in solublization behavior.

The P solublization capability of isolate T_1 was also in the same trend with the rise in available P content. Inoculum volume 2% showed rise in available P with the initial P content of 23.57 mg/kg which showed constant rise upto 44.43 mg/kg at the end of 168 h. TEO with inoculums volume 10% was observed with 18.43 mg/kg at 72 h and the rise was noticed up to 40.43 mg/kg. It was found that T_1 showed constant rise in the P solublization up to 168 h but the rise was more in inoculums volume of 2% than 10% by the end of 168 h (Table 25).

The phosphorus solublization potential of isolates was varying with the inoculums volume. DHA 2% and 10 % showed similarity in the trend of phosphorus solublization. While TEO 10% was found to have less solublization potential than TEO 2%. TEO 2% showed maximum rise in the percent phosphorus solublization of 67.97% at the end of 168 h (Fig. 24). Therefore TEO 2% was found more efficient among all the treatments. So that TEO 2% was further selected for the determination of MCP degradation capability in soil.

The soil environment shows very heterogeneous and complex behavior towards microbial activity. Hence the degradation of MCP was indirectly measured as ability to hydrolyze the organophosphorus pesticide in soil. Being the PSMs, these isolates were able to degrade the MCP in soil, by utilizing it as the source of phosphorus and thereby releasing phosphorus from the MCP through the action of various enzymes like phosphatase present in the cells (Hasan, 1999).

In a study neutral to slightly acidic pH was found to have significant effect on the microbial population and alkaline phosphatase activity in soil (Nath and Samanta, 2012). The isolates were found able to degrade the MCP. The potential of isolates in solublization of MCP was summarized in the Table 25 given below.

Table 25. Phosphorus solublization potential of Isolate D_1 and T_1

Sl. No.	Sample name / Incubation duration(h)	Available P (mg/kg)				% P solublization			
		DHA 2%	DHA 10%	TEO 2%	TEO 10%	DHA 2%	DHA 10%	TEO 2%	TEO 10%
1	72 h	21.71	35.71	23.57	18.43	31.58	32.83	36.06	28.19
2	96 h	27.71	39.71	25.29	21.00	40.31	38.65	38.69	32.12
3	120 h	32.28	44.00	29.57	24.43	46.96	44.90	45.24	37.37
4	144 h	32.00	47.42	33.57	32.71	46.55	49.87	51.36	50.04
5	168 h	35.43	48.28	44.43	40.43	51.54	51.12	67.97	61.85

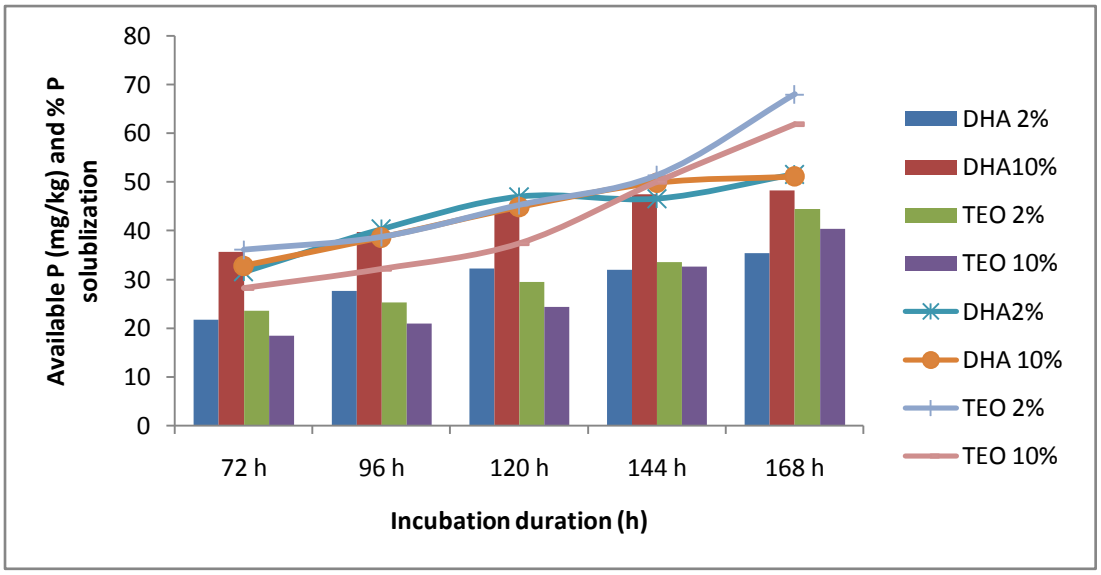

Fig. 24: Phosphorus solublization potential of Isolate D_1 and T_1

4.15 Activity of phosphatase enzyme in soil supplemented with MCP

The activity of phosphatase enzyme was estimated as the production of mg pNp/g/h and it showed concurrence with the solublization of phosphorus in soil. The activity was increased upto 144 h after that the activity was decreased. Maximum activity was found in TEO 2% with 24.09 mg pNp/g/h while the minimum was at 72 h with the 2.32 mg pNp/g/h (Fig. 25). With increase in time the increase in phosphatase was showed with the increase in soluble phosphorus content. The role of phosphatase was also noticed for the degradation of MCP by Bhadbhade et al. (2002a) and Rosenberg and Alexander (1979). The increase in the concentration of available phosphorus might be

due to degradation of MCP and phosphatase was found responsible for the hydrolysis of P–O alkyl and P–O aryl bonds present in MCP.

Table 26. Phosphatase activity at different time interval

S.N.	Sample Name	Phosphatase activity (mg pNp/g/h × 10^2)				
		Time (h)				
		72	96	120	144	168
1	DHA 2%	3.22	6.23	9.77	14.09	8.18
2	TEO 2%	2.32	7.36	15.45	24.09	19.54
3	DHA 10%	7.25	18.18	18.86	20.22	16.28
4	TEO 10 %	10.45	14.65	20.9	20.45	13.63

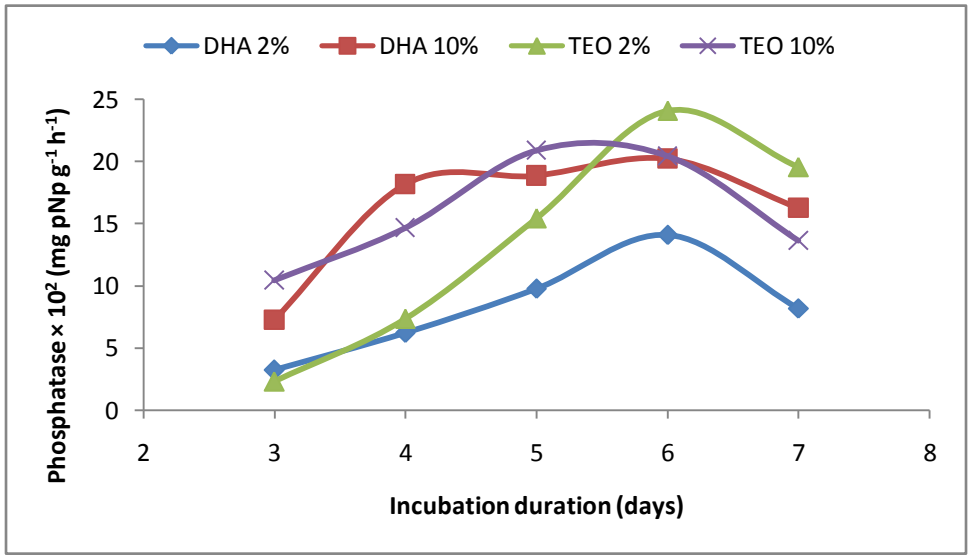

Fig. 25: Phosphatase activity in soil supplemented with MCP

4.16 Estimation of MCP residues in the soil

The residual MCP in soil spiked with 1000 mg/L of MCP and inoculated with 2% of isolate T_1 was estimated using HPLC system. The percent degradation was approx 87.4% with the residue of 126 mg/L. GC chromatogram also showed the MCP residues in the soil after 7 days of incubation. The nature of compounds in soil was found in harmony with the results media.

4.17 GC-MS analysis of soil samples supplemented with 1000 mg/L and inoculated with isolate T_1

The major compounds detected by GC-MS analysis of TEO soil sample spiked with 1000 mg/L MCP inoculated with isolate T_1 incubated for seven days was given in Appendix F. The chromatogram showed the presence of MCP residues similar to media inoculated with isolate T_1 which had the mass spectrum of MCP (Fig 23). Several other compounds of VFAs like stearic acid, palmitic acid, oleic acid, phthalic acid, esters, terpenes and nonadecane were also detected in the spiked TEO soil. The presence of these compounds in culture media inoculated with microbes was in accordance with the reports of Das and Anitha (2011).

The higher dosages of pesticides were affected the growth count of microbes. Only two samples have showed tolerance at maximum 1000 mg/L of MCP concentration. While the phosphorus solublizing capability was found more in TEO than DHA. The isolates were also found capable of degrading MCP in media as well as in soil by utilizing MCP as source of phosphorus**.** The higher amount of volatile fatty acids, alkanes, esters and terpenes were detected in soil and media inoculated with isolate. Therefore the results obtained showed the tolerance in the microorganism isolated from the soil with continuous application of monocrotophos.

SUMMARY

Present study was formulated to study the biodegradation of monocrotophos by microbial community of contaminated sites. The sampling was done from randomly selected villages of Bathinda district. The salient findings of the study were as follows:

- The questionnaire survey revealed that the most of farmers had applied insecticides like traizophos, acetamiprid, acephate, monocrotophos etc. for different cash crops. These insecticides were mainly belonging to slightly hazardous class except monocrotophos and triazophos which were under highly hazardous group.
- All the villages studied had the history of monocrotophos usage. The current usage of monocrotophos was seen in two villages namely Dhadde and Teona Pujarian were observed as present usage of monocrotophos. The farmers followed three applications per crop period.
- The physico-chemical properties of soil samples shown that the soil were loamy and silty with alkaline pH, low carbon content ranging from 0.04 to 0.77%, low nitrogen, moderate phosphorus and high potassium content ranging from 0.018 to 0.073%, 37.4 to 66.1 mg/kg, 29.6 to 46.4 mg/kg respectively .
- The biological characterization of soil samples revealed low microbial biomass (9 to 1 μg/g of soil) and low soil enzymatic properties for dehydrogenase. But the phosphatase activity was found more in all soil samples in comparison to other enzymes.
- The total bacterial population in soil was found to be 6.2 to 16×10^8 cfu/g in all the samples after 72 h and the populations of phosphorus solublizing microbes were found to be 1.7 to 6.9×10^3 cfu/g which increased to 8×10^3 after the incubation of 72 h.
- The residue of monocrotophos was detected in soil samples from Teona Pujarian (TEO) with a concentration of 0.08 μg/g of soil while no residues were

detected in other soil samples which might be due to its faster degradation in alkaline soil pH.

- The GC-MS analysis of soil samples detected the volatile fatty acids such as stearic acid, erucic acid, palmitic acid, oleic acid, phthalic acid and nonadecane in soil samples and media supplemented with MCP.
- The screening of all the soil samples was carried out for tolerance against monocrotophos at different concentration from 50 to 1000 mg/L for the microbial growth.
- At lower concentration the microbial population from all the soil samples showed growth. But at 100 mg/L only 8 soil samples (BW, DHA, GS, JAJ, JAS, MAL, TEO and TS) showed growth. At increasing concentrations of 250 mg/L and 500 mg/L only 6 soil samples (BW, DHA, JAJ, MAL, TEO and TS) had shown growth.
- The higher concentration of 750 mg/L and 1000 mg/L favored growth of microbes in only 4 soil samples (BW, DHA, JAJ and TEO).
- The two soil samples of DHA and TEO that showed maximum microbial count at highest concentration were further selected for isolation study.
- Two bacterial isolates named as D_1 and T_1 and four fungal isolates D_2, D_3, T_2, T_3 were isolated from DHA and TEO samples.
- The morphological and biochemical characteristics showed that the isolate T_1 similar to Pseudomonas sp., and D_1 similar to Serratia sp.
- The fungal strains of D_2 and T_3 based on their morphological characteristics was found resemblance with Aspergillus sp. while the isolates D_3 and T_2 showed similarity to Aspergillus niger and Aspergillus flavus respectively. Due to ease of handling and fast growth time period only bacterial isolates were further selected for study.
- The phosphorus solublizing capability of the isolates revealed that the isolate T_1 was more efficient than D_1 and found capable of solubilizing TCP was 490 mg/L supplemented with 0.5 per cent of tricalcium phosphate.
- The phosphorus solublization from monocrotophos was also studied in the soil by both the isolates D_1 and T_1 at different inoculums volume of 2 % and 10%

(v/w). The isolate T_1 with 2% of inoculum volume showed constant rise in available phosphorus of 44.43 mg/kg of soil after 168 h incubation at 30^0C.

- The isolate T_1 was further taken for the study and showed 88.9% of degradation potential in media supplemented with 1000 mg/l of monocrotophos.
- The degradation potential of TEO 2% in soil supplemented with 1000 mg/L resulted in 87.4% of degradation after 7 days.
- The phosphatase activity related with the hydrolysis of monocrotophos helped in the degradation of MCP that found increased till 144 h and afterwards the activity was decreased.

The continuous application of pesticide developed tolerance in microbes and also the development of microbes adapted to higher concentration to the pesticides. The phosphorus solublizing microbes were found capable of degradation of monocrotophos in culture media and in soil due to their inherent capability of utilizing phosphorus, energy and nutrient source. These isolates can be used to degrade the pesticide at contaminated sites.

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Appendix A: Questionnaire Format for Sampling

QUESTIONNAIRE FORMAT

Name of the farmer:

Address:

Date of sample collection:

1. Area of the farm
   - ◯ 1 ha
   - ◯ 3 hectare
   - ◯ ….. Acres

2. Location of the farm
   - ◯ District,
   - ◯ Taluka,
   - ◯ Village

3. Crops you are cultivating
   - ◯ Cotton
   - ◯ Sugarcane
   - ◯ Vegetables
   - ◯ Rice
   - ◯ Wheat

4. Season for various Crops in a year

5. Present crop and its present status

6. ◯ Sowing ◯ Already sown ◯ Date of sowing ◯ Flowering ◯ Fruting ◯ Mature stage

7. Whether multi-crops are growing

   ◯ in same field ◯ in different fields ◯ crop rotation (one after another)/after a gap period)

8. No. of Crops in rotation in same field for a year

9. Problems faced in cultivation
   - ◯ Pest
   - ◯ Diseases
   - ◯ Irrigation
   - ◯ Weed growth

10. Chemicals used
    - ◯ Fertilizer
    - ◯ Pesticides
    - ◯ Herbicide

11. Plant protection measures applied
    - ◯ Herbicides
    - ◯ Insecticides
    - ◯ Fungicides

12. Name of pesticides ◯ Nuvacron ◯ Power ◯ Any other

13. Source of pesticides (where you buy):

14. Purpose of application Pest name:

15. Frequency of application in a year

16. Quantity of pesticide purchased per year

17. Have you heard of Pesticide name:
    ◯ Monocrotophos ◯ Nuvacron ◯ Power ◯ Monokill
    ◯Monocare ◯ Monoplus ◯ Rasayanphos ◯Monocill

18. Dosage of application

19. Mode of application ……◯ Broadcasting ◯ Spraying

20. Time of application ◯ Flowering ◯ Fruiting

21. Success of application ◯ Yes ◯ No

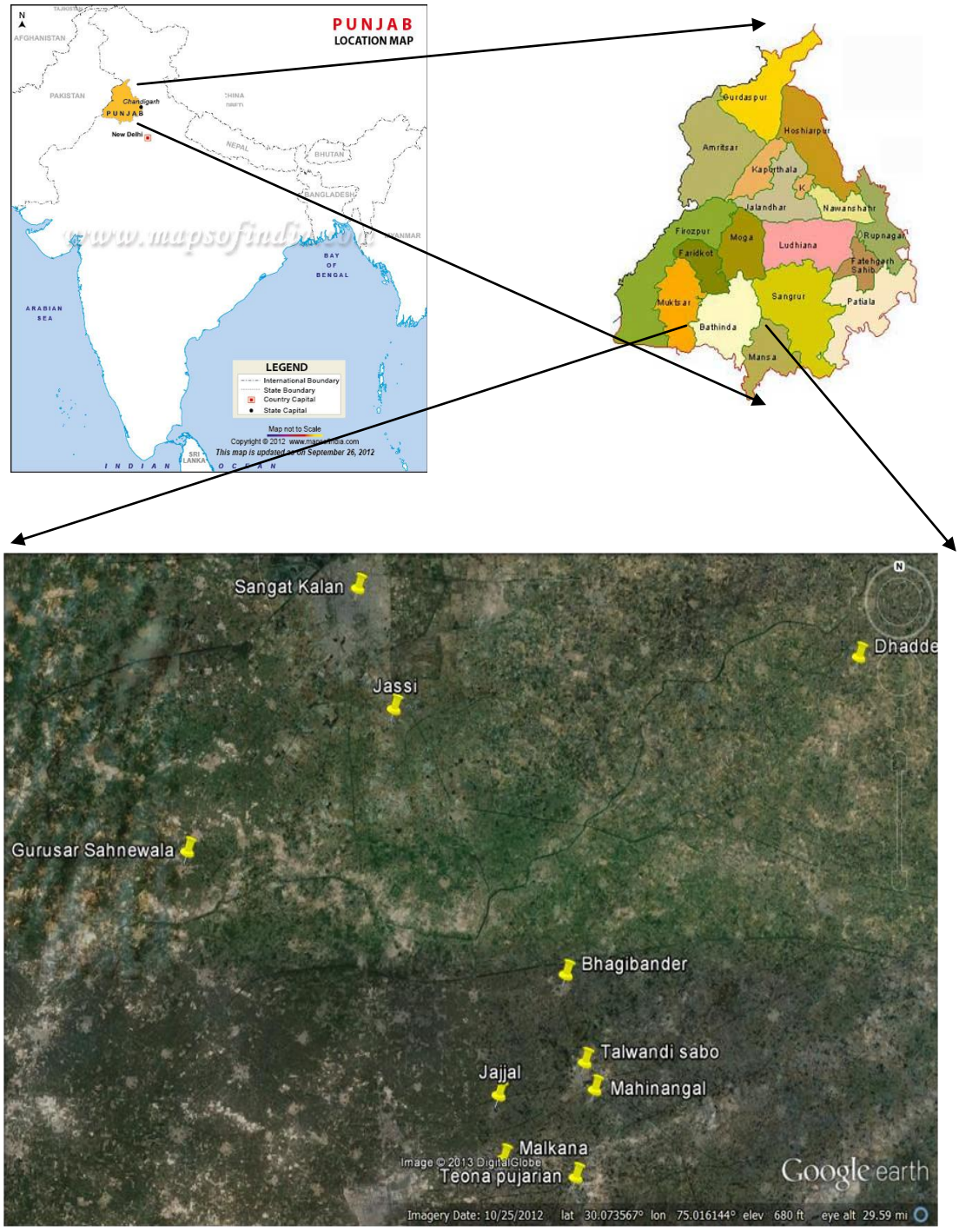
22. Any side effects noticed in soil/crop ……..

23. Precautions taken for application

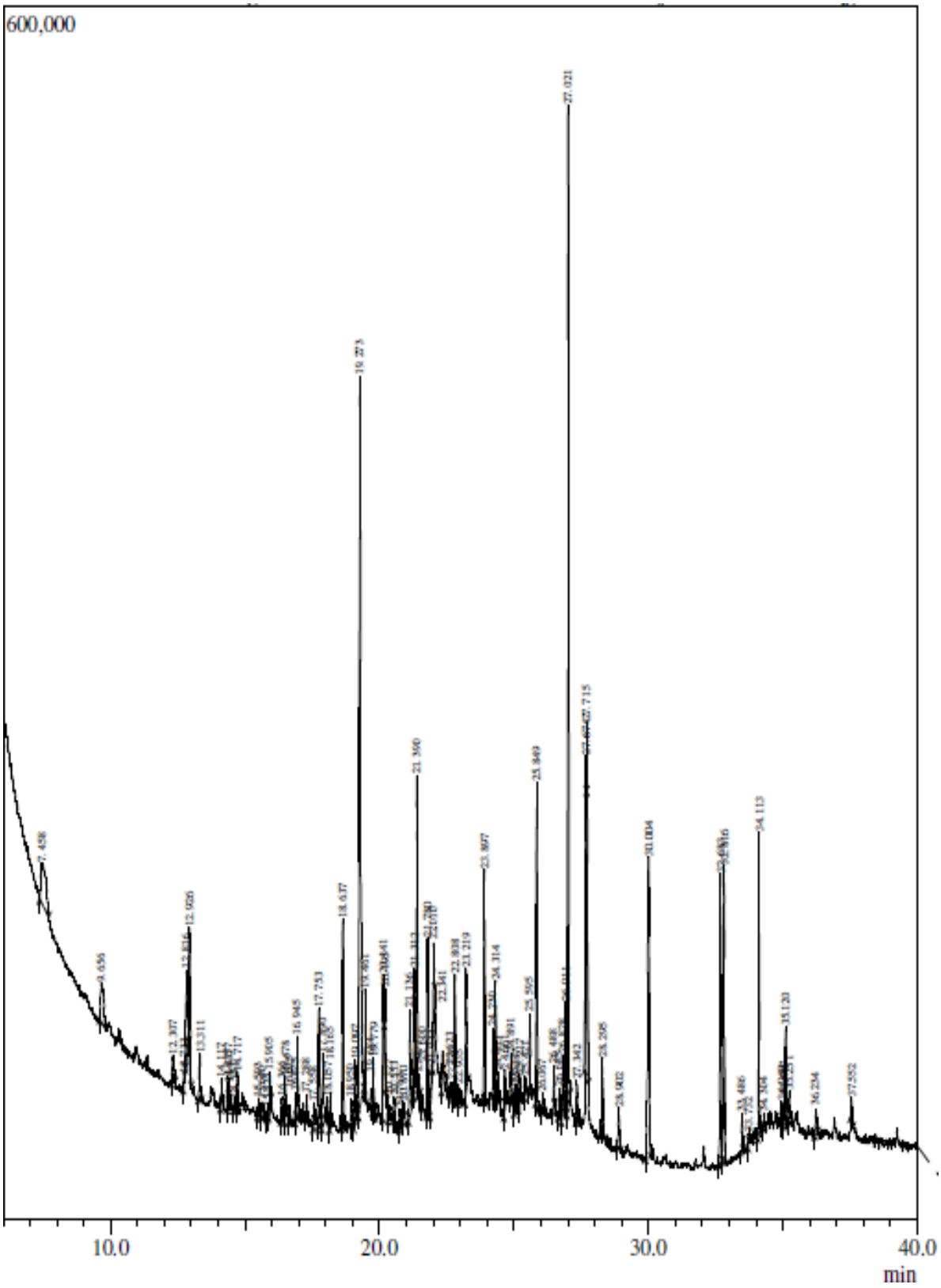
24. Any health problems after application (if farmer himself/labourer)

25. Any subsidies for chemicals (pesticides)

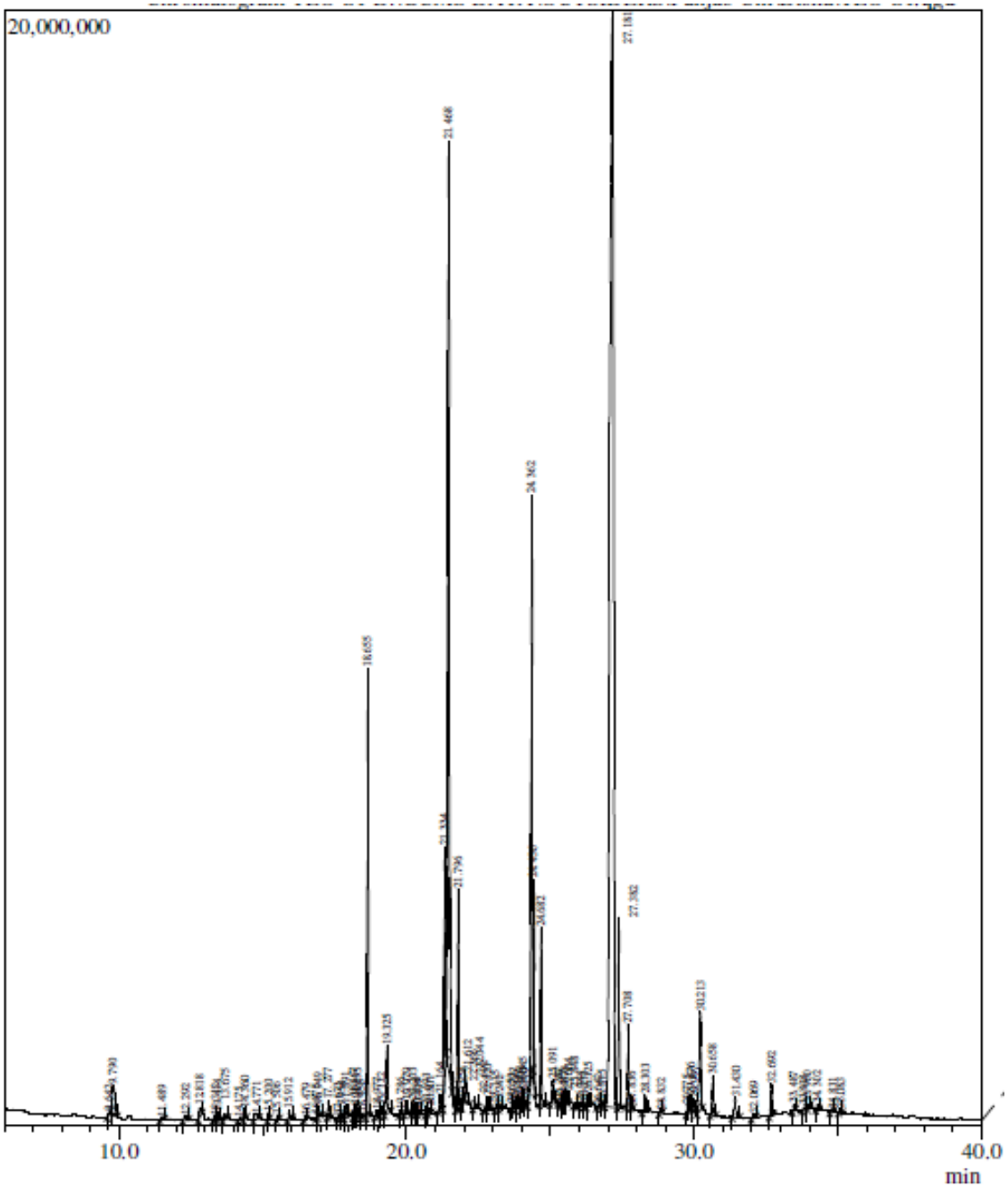
Appendix B: Location Map of sampling sites in Bathinda district



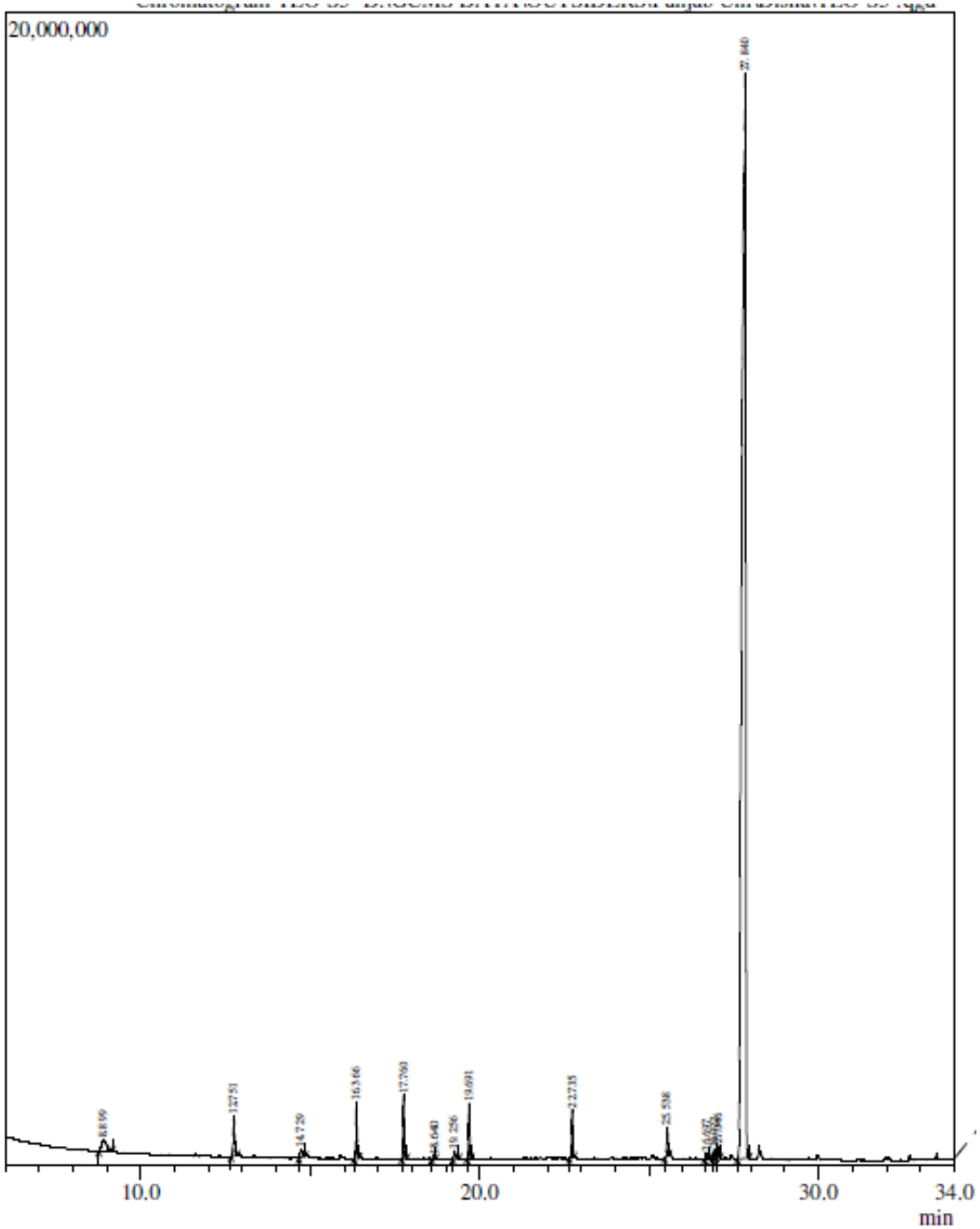
APPENDIX C: GC-MS Chromatogram of DHA soil sample



APPENDIX D: GC-MS Chromatogram of TEO soil sample



Appendix E: GC-MS chromatogram of PKV media inoculated with pure Isolate T_1



Appendix F: GC-MS chromatogram of soil samples supplemented with 1000 mg/L MCP and inoculated with isolate T_1