

# Assessment of Cyanobacterial diversity in paddy fields and their capability to degrade pesticides

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Submitted By

*Arun Kumar*

Enrolment No. 907/13

Under the Supervision of

*Dr. Jay Shankar Singh*

Assistant Professor

DEPARTMENT OF ENVIRONMENTAL MICROBIOLOGY  
SCHOOL FOR ENVIRONMENTAL SCIENCES  
BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY  
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VIDYA VIHAR, RAEBARELI ROAD, LUCKNOW-226 025  
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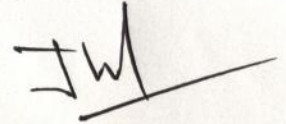
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God and  
My Beloved  
Family*

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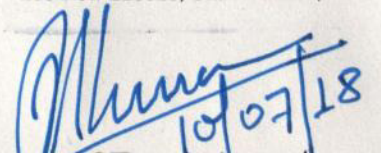
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**(Jay Shankar Singh)**

Dr. Jay Shankar Singh  
Assistant Professor  
Deptt. of Environ. Microbiology  
B.B. Ambedkar (Central) University  
Lucknow-226025, Uttar Pradesh, India

Supervisor



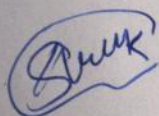
**Head of Department**

*Dr. Rajesh Kumar*  
Professor & Head

Department of Environmental Microbiology  
Babasaheb Bhimrao Ambedkar University  
(A Central University)  
Raibareli Road, Lucknow-226025

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**Arun Kumar**

S/o Mr. Chandrabhan Singh

HNo. 13, Surya Nagar, Near SBI Colony, Delhi Road,

District- Saharanpur-247001,

Uttar Pradesh, India

Mobile: 7830259839

E-mail: potter.kumar7@gmail.com

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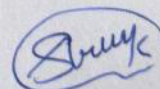
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Place: Lucknow

Date: 10/07/2018



**Arun Kumar**

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## ABBREVIATIONS & SYMBOLS

<b>CAT</b>	Catalase
<b>CPF</b>	Chlorpyrifos
<b>CO<sub>2</sub></b>	Carbon dioxide
<b>Cm</b>	Centimeter
<b>°C</b>	Degree Celsius
<b>DNA</b>	Deoxyribonucleic acid
<b>DETP</b>	Diethylthiophosphoric acid
<b>EC</b>	Electrical Conductivity
<b>EDTA</b>	Ethylenediaminetetraacetic acid
<b>GC-MS</b>	Gas Chromatography Mass Spectrophotometry
<b>GSH</b>	Glutathione
<b>g</b>	Gram
<b>g/L</b>	Gram per litre
<b>H<sub>2</sub>O<sub>2</sub></b>	Hydrogen peroxide
<b>μ</b>	Miu
<b>M</b>	Molarity
<b>mL</b>	Mililitre
<b>Min</b>	Minute
<b>nm</b>	Nanometer
<b>-</b>	Negative
<b>NIST</b>	National Institute of Standards and Technology
<b>NADPH</b>	Nicotinamide adenine dinucleotide phosphate
<b>OD</b>	Optical density
<b>+</b>	Positive
<b>%</b>	Percentage
<b>ppm</b>	Parts per million
<b>POD</b>	Peroxidase
<b>rpm</b>	Revolution per minute
<b>RT</b>	Retention time
<b>RNA</b>	Ribonucleic acid
<b>SOD</b>	Superoxide dismutase
<b>TCP</b>	Trichloro-2 pyridinol

<b>UV</b>	Ultra violet
<b>U/mL</b>	Unit per milliliter
<b>H<sub>2</sub>O</b>	Water
<b>w/v</b>	Weight over volume

*CHAPTER 1*  
*Introduction*

### **1.1 Paddy crop and their importance**

Paddy is one of the important cereal crops in the world that provides staple food for nearly more than half of the world's population. However 90 % of the world's rice is produced and consumed in the Asian region where only 6 countries (China, India, Indonesia, Bangladesh, Vietnam and Japan) comprising 80% of the world's production and consumption. The FAO estimated the world paddy production in 2017 to 754.6 million tonnes (500.8 million tonnes, milled basis); that is slightly more than in 2016 to 748.0 million tonnes (496.7 million tonnes, milled basis). Although, rice production has been more than tripled since 1961 during the era of green revolution; but during last two decades, rice production was almost stagnant in spite of introduction of new technologies, hybrid rice varieties and improved irrigation facilities. However, in spite of change in food pattern, it is estimated that to over 800 million tonnes production of rice by 2030 and 525 million tons by 2050 (IRRI, 2000). In many Asian countries, rice fulfils more than 40 % of daily calorie intake of total calories from food consumption (Timmer and Dawe, 2007; Timmer et al., 2010). Although the percentage of consumption of rice decreased during several years but still rice is the main component of Asian diet. Some Asian countries also export the rice as primary agricultural products, which contribute to economy, but in terms of overall contribution in GDP; it contribute only 1-4 % in 2007.

India is world's second largest rice producing country with highest harvested area worldwide of 44.5 million hectares (24% of total cropped area of the country) and produced 159.016 million tonnes paddy (106 million tonnes, milled basis); which

is 42% of total food grain production and 45% of total cereal production (USDA, 2017). But having highest harvested area, the yield still much lower than many other rice producing countries; it needs to be increased despite the limited options for expanding the area or irrigation coverage. Around 65% of the total population in India depends on rice and it accounts for 40% of their food production (Bishwajit et al., 2013).

## **1.2 History of paddy cultivation**

The paddy (*Oryza sativa*) belongs to family *Gramineae* (grass family). In general it is a short living crop, completes their life span in average 3-7 months, depending on the variety and the climate where it is grown. Paddy is generally considered a semiaquatic annual grass, although in the tropics it can survive as perennial, producing new tillers from nodes after harvest. Its length varies from 0.4 m to over 5 m in some flooded rice varieties. Paddy crop requires a hot and humid climate; and best suited for regions which have high humidity, prolonged sunshine and an assured supply of water. The average temperature required throughout the crop cycle ranges from 21 to 37° C during different stages. At ripening, the paddy plant has a main stem and several tillers. Each productive tiller bears a terminal flowering head or panicle. Paddy crop cycle duration is divided into vegetative phase (including germination, seedling, and tillering stages) and the reproductive phase (including panicle initiation and heading stages).

In India, paddy is mainly grown in two types of soils i.e., (i) uplands and (ii) low lands. The method of cultivation of rice in a particular region depends largely on factors such as situation of land, type of soils, irrigation facilities, and availability of labourers' intensity and distribution of rainfalls. The paddy is grown with the

following methods: (i) dry or semi-dry upland cultivation: (a) broadcasting the seed or (b) sowing the seed behind the plough or drilling; (ii) wet or lowland cultivation: (a) transplanting in puddle fields, or (b) broadcasting sprouted seeds in puddled fields.

### **1.3 Paddy crop and use of pesticides**

Apart from variations in temperature and soil nutrients, paddy is also affected by a large number of insects and diseases; which is responsible for rice production in terms of yield and quality. Oerke (2006), Savary et al. (2012) and Donatelli et al. (2017) estimated that 40% reduction due to diseases and pests in tropics and subtropics. If the post-harvest losses (15-20%) are also added, the situation becomes even more alarming.

Insects are responsible for plentiful crop damage; they attack almost all portions of the paddy plant and also in all stages of plant growth. In paddy crop, these insects are in common i.e. (1) root feeders, (2) stem borers, (3) leafhoppers and plant hoppers, (4) defoliators, and (5) grain sucking insects. Some insects also attack rice grains in storage. Rice diseases are mainly caused by fungi, bacteria or viruses. Stunting is one of the symptoms; others are; colour changes, wilting or abnormal development of certain plant parts. These symptoms can be found in all the parts of the plant. The most common and most severe diseases in rice are blast, rice yellow mottle virus (RYMV) and bacterial leaf blight.

Unlike in other crops, weeds also common in paddy crop, but paddy fields are favours the growth and reproduction of wide variety of terrestrial, aquatic, and semiaquatic weeds. However, weeds are not responsible for direct crop loss but they compete with rice for light, nutrients, water, and other growth requirements. They reduce yields, lower the market value of the crop by reducing quality, and increase the

cost of harvesting, drying, and cleaning. Common weeds that can be seen in rice fields are: *Echinochloa colonum*, *Echinochloa crusgali*, *Cynodon dactylon*, *Eleusine indica*, *Dactyloctenium aegyptium*, *Setaria glauca*, *Cyperus rotundus*, *Caesulia axillaris*, *Eclipta alba*, *Euphorbia herita*, *Solanum nigrum*, *Leucos aspera*, *Phyllanthus niruri*, *Lippia nodiflora*, etc.

Some study also showed that elevated CO<sub>2</sub> can increase the levels of simple sugars in rice leaves and lower their nitrogen content; result in more damage caused by many insects, which consume more leaves to meet their metabolic requirements of nitrogen. Thus, any attack will be more severe. Apart from increased CO<sub>2</sub> concentration, due to global warming, temperature also increasing, leads to more numbers of pest attacks because it favours the better survival during winter season.

Since, the green revolution, the pesticides were used frequently at alarming level during last 2-3 decades, to control the pests, weed and diseases. In India, pesticide use for crop protection and public health goes to the extent of 85,000 tonnes per year. Of these, 77.8% are insecticides, and the rest are fungicides, herbicides, rodenticides, fumigants and miscellaneous pesticides. Of this, paddy, which occupies about 24% of the crop area, accounts for 17.2% of the pesticides used (Mamthanayagam and Sharinila, 2004). For the management of pests, weeds and diseases in paddy crop, a large number of insecticides, herbicides and fungicides are applied; which can disturb the pattern and distribution of flora & fauna in paddy field ecosystem. Major insecticides used in paddy crop are: carbofuran, dioxathion, cypermethrin diazinon, **chlorpyrifos**, monocrotophos and malathion; while fungicides are: edifenfos carbendazim and propiconazole. The weedicides commonly used in paddy crop are: butachlor, pretilachlor, pyrazosulfuronethyl, oxyflurofen, anilofos, trifluralin, 2,4-d, thiobencarb, propanil and phenoxaprop-p-ethyl.

### **1.4 Impact of pesticides on agro-ecosystem and environment**

The maximum portion of pesticides applied in paddy crop production lies unused in soil and contributes environmental contamination. Paddy growers often spray pesticides up to five to six times in one cropping season while only two applications may be sufficient. The pesticide also be taken up by non-target flora and fauna, leaches in to soil, and could be contaminate groundwater or potable water. These pesticides residues along with paddy flood water drained into irrigation canals; leads to further contamination in to rivers and lakes. The pesticides also creates a bigger problem i.e. biomagnification, when they are accumulated in beneficial organisms like fish.

### **1.5 Cyanobacterial diversity in paddy fields**

The paddy fields considered as an ecosystem and provide diverse habitats for the variety of microorganisms. These habitats are microenvironments which exhibit different physico-chemical and biological properties. Having such heterogeneity of the habitats influences the structure and diversity of microbial communities in the paddy field ecosystem and facilitates various microbiological processes; that are agronomically and biogeochemically important for paddy field ecosystem (Kimura, 2000).

Cyanobacteria (Blue-Green-Algae, BGA) are one of the main microorganisms that naturally inhibit the paddy field ecosystem. They are a highly diverse group of prokaryotic microorganisms which performs oxygenic photosynthesis (Kulasooriya, 2011). Some cyanobacteria also possess specialized cells called heterocyst which are capable to fix atmospheric nitrogen; so they can be used as natural bioinoculants. Kaushik (1994) reported that amount of nitrogen contributed by cyanobacteria to rice

crop varies from 20-30 kg/ha. It is also reported that cyanobacteria can improve soil characteristics by, modifying texture size and subsequent aeration (Ibrahim, 2007), increasing phosphorus content (Fuller and Rogers, 1952) and enhancing carbon content and water holding capacity (Richert et al., 2005).

Cyanobacteria naturally inhabit on the surface of paddy field soil as well as flood water. Anand and Hopper (1995); and Vijayan and Ray (2015), reported *Anabaena*, *Arthrospira*, *Cylindrospermum*, *Chroococcus*, *Nostoc*, *Lyngbya*, *Oscillatoria*, *Gleothoece*, *Gleocapsa*, *Microcystis*, *Merismopedia*, *Synechocystis*, *Leptolyngbya*, *Westilopsis*, *Syctonema*, *Phormidium*, *Trichodesmium*, *Haplosiphon* and *Fischerella* in paddy field of Kerala. In paddy fields of Nagaland, Singh et al. (1997) recorded maximum number of species of *Microcystis* and heterocystous genera as *Anabaena* and *Nostoc*. Some of the common cyanobacteria inhabiting paddy field are *Anabaena*, *Aulosira*, *Calothrix*, *Gleotrichia*, *Cylindrospermum*, *Nostoc*, *Fischeiella*, *Scytonema*, *Tolypothrix* and *Wolleea* (Rai, 2001). Various workers have studied the cyanobacterial flora of paddy fields in our country (Ahmed et al., 1999; Tiwari et al., 2000; Nayak et al., 2001; Kaushik and Prasanna, 2002; Mishra and Pabbi, 2004; Choudhury and Kennedy, 2005; Rai, 2006; Nayak and Prasanna, 2007) and few attempts have also been carried to explore their diversity in the state of Orissa and reported cyanobacteria are: *Chroococcus*, *Fischerella*, *Nostoc*, *Lyngbya*, *Oscillatoria*, *Apanothece*, *Microcystis*, *Microcoelus*, *Aulosira*, *Syctonema*, *Phormidium*, *Aphanocapsa*, *Microchaete*, *Dactylacoccopsis*, *Symploca*, *Pseudoanabaena*, *Schizothrix* and *Hydrocoleum* (Bhakta et al., 2006; Dey and Bastia, 2008; Choudhary, 2009; Choudhary and Bimal, 2010; Dey et al., 2010). In Punjab region, *Anacystis*, *Anabaena*, *Cylindrospermum*, *Chroococcus*, *Fischerella*, *Nostoc*, *Lyngbya*, *Oscillatoria*, *Phormidium*, *Apanothece*, *Synchocystis*, *Westilopsis*,

*Microcystis*, *Plectonema*, *Dactylacoccopsis*, *Synechococcus*, *Spirulina* and *Wolleea* were also reported. In Madhya Pradesh region, the cyanobacterial genus were reported are: *Anabaena*, *Aphanocapsa*, *Arthrospira*, *Cylindrospermum*, *Chroococcus*, *Fischerella*, *Nostoc*, *Lyngbya*, *Oscillatoria*, *Gleotheca*, *Gleocapsa*, *Microcystis*, *Westilopsis*, *Plectonema*, *Syctonema*, *Phormidium*, *Microchaete*, *Symploca*, *Stichosiphon*, *Trichodesmium*, *Haplosiphon*, *Stigonema* and *Fischerella*. Bhardwaj and Baruah (2013) reported *Anabaena*, *Aphanocapsa*, *Cylindrospermum*, *Chroococcus*, *Nostoc*, *Lyngbya*, *Oscillatoria*, *Apanothece* *Microcystis*, *Syctonema*, *Phormidium*, *Microchaete* and *Microcoelus* in Assam region. In Chattishgarh region, Singh et al. (2014) reported *Anabaena*, *Cylindrospermum*, *Chroococcus*, *Gleotheca*, *Gleocapsa*, *Merismopedia*, *Nostoc*, *Lyngbya*, *Oscillatoria*, *Apanothece* *Microcystis*, *Arthrospira*, *Anabaenopsis* *Tolypothrix*, *Rivularia*, *Calothrix* and *Microcoelus*. Denoboyina and Shivakumar (2013) reported *Anabaena*, *Cylindrospermum*, *Chroococcus*, *Gleocapsa*, *Nostoc*, *Lyngbya*, *Oscillatoria*, *Tolypothrix*, *Rivularia* and *Calothrix*.

### **1.6 Cyanoremediation of pesticides**

Pesticides cause damaging effect on growth, photosynthesis, nitrogen fixation, biochemical composition and metabolic activity of paddy field cyanobacteria (Moustafa and Helling, 2002). Pesticide application is responsible for oxidative stress in cyanobacteria and also induced the production of reactive oxygen species (ROS), including superoxide radicals ( $O_2^-$ ), hydroxyl radicals (OH) and hydrogen peroxide ( $H_2O_2$ ). These reactive oxygen species (ROS) affect the cell membranes, proteins and nucleic acids; and could cause enzyme inactivation, protein denaturation, lipid peroxidation and DNA mutation and also damages the cellular components through oxidative (Imlay et al., 1998; Vandana et al., 2001). Maximum work related to effects

of pesticides on cyanobacteria is available with pesticides such as DDT, lindane, parathion, endosulfan, **chlorpyrifos**, glyphosate, molinate, monocrotophos and PCP.

Singh (1973) isolated cyanobacteria i.e. *Cylindrospermurn* sp., *Aulosira fertilissirna*, and *Plectonema boyanurn* from paddy fields; which were able to tolerate lindane (commercial perspiration) up to 80 pg/mL conc. Singh (1973) also found that the growth of these cyanobacteria did not affected by the lindane concentrations up to 20 pg/mL. It is also reported that *Anabaena* sp. PCC7120 and *Nostoc ellipsosporurn*, degraded A-HCH to a mixture of 1,2,3-and 1,2,4-trichlorobenzenes and forms an intermediate pentachlorocyclohexene (Kuritz and Wolk, 1995).

Lee et al. (2003) investigated that *Anabaena* sp. strain PCC 7120 and *Anabaena flos-aquae* degraded the endosulfan into endodiol (main end product) and endosulfan sulphate (trace amount). Although endodiol is a non-toxic metabolite to fish and other organisms, but endosulfan sulfate is much toxic like parent compound endosulfan and also persists much longer tolerance into soil environment in comparison to endosulfan (Kennedey et al., 2001).

Megharaj et al. (1987, 1994), Orus and Marco (1991) and Subramanian et al. (1994) isolated *Nostoc*, *Oscillatoria* and *Phomidium* from methyl parathion enriched soil and grew in media supplemented with methyl parathion or other organophosphorus pesticides as a sole source of organic phosphorus and nitrate. They found that these cyanobacteria were able to utilize phosphorus from the pesticide for their growth and development. Ibrahim et al. (2014) observed that *Anabaena oryzae*, *Nostoc muscorum* and *Spirulina platensis* were able to degrade and utilized malathion pesticide as a source of phosphorous. It is also found that under aerobic conditions *Anabaena* sp. PCC 7120 strain was able to reduce the nitro-group of methyl parathion to an amino group via a nitroso group intermediate (Barton et al., 2004).

Megharaj et al. (1994) said that cyanobacteria were able to oxidize the nitro-group of para-nitrophenol accompanied by the release of nitrite into growth media. But enzymes involving in this process are unknown and further use of this released nitrite is likely to depend on the activity of nitrite reductase. Similar observations that nitrogen metabolism and phosphorus utilization from organophosphorus pesticides are interconnected (Subramanian et al., 1994); however, the researchers did not examine the possible effects of various sources of fixed nitrogen on biodegradation of organophosphorus pesticides (Kuritz, 1999).

Lipok et al. (2007; 2009) investigated that *Spirulina* sp. was able to degrade glyphosate and stated that the rate of glyphosate utilization from the aqueous medium was independent of its initial concentration. Lipok et al. (2007) also suggested that the degradation mechanism of glyphosate by *Spirulina* sp. might be different from the mechanism shown by the other bacteria. It is also reported that *Anabaena* sp., *L. boryana*, *Microcystis aeruginosa* and *Nostoc punctiforme* are able to utilize the glyphosate as the only source of phosphorus (Forlani et al., 2008). Dyhrman et al. (2006) observed that marine cyanobacteria *Trichodesmium erythraeum* showed existence of phosphorous-dependent glyphosate transformation.

Although a number of reports confirmed about phosphorous-dependent glyphosate transformation; but reports on the utilization of glyphosate as a source of nitrogen by cyanobacteria are not yet available in the literature. Ravi and Balakumar (1998) investigated the glyphosate degradation by *Anabaena variabilis* and also claimed that extracellular phosphatases are responsible in the hydrolysis of C-P bond of glyphosate; however, this claim has not been supported by the other researchers. In relation of this, it is stated that extracellular phosphatases have no significant role in glyphosate degradation (Forlani et al., 2008). Arunkumara et al. (2013) stated that

cyanobacterial strains having the ability to use these phosphatases as a source of phosphorus could be applied for decontamination of pesticides.

El-Nahhal et al. (2013) investigated the ability of cyanobacterial mats to degrade the pesticide acetochlor; and found that these cyanobacterial mats very efficient in degradation of acetochlor at high concentration. It is reported that *Phormidium valderianum* BDU 20041 strain is able to tolerate chlorpyrifos with oxido-reductase enzymatic activity to degrade the chlorpyrifos (Palanisami et al., 2009). Thengodkar and Sivakami (2000) investigated that *Spirulina platensis* are able to tolerate chlorpyrifos up to 80 ppm and mineralize chlorpyrifos into TCP (3, 5, 6-trichloro-2-pyridinol) through the enzyme alkaline phosphatase (ALP). It is also reported that cyanobacterium *Synechocystis* sp. PUPCCC 64 strain was able to degrade chlorpyrifos into 3,5,6-trichloro-2-pyridinol (Singh et al., 2011).

### 1.7 Chlorpyrifos: General, Structure and mode of action

Chlorpyrifos (O, O-diethyl O, 3, 5, 6-trichloro-2- pyridol phosphorothioate) is one of the most widely used organophosphorus insecticides (Lee et al., 2012). The properties of chlorpyrifos are given in **Table 1.1**.

**Table 1.1** Chemical characteristics of chlorpyrifos

Chemical name	Molecular weight	CAS registry number	Specific gravity	Vapour pressure at 25 °C (MPa)	Activity soil sorption coefficient (mg/g)	Solubility at 25 °C in water (mg/L)	Half life
O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate	350.62	2921-88-2	1.398 at 43 °C	2.49	849	1.39	6-120 days in soil, 3-9 days in water

Chlorpyrifos is a broad spectrum insecticide, which is applied to kill a wide variety of insects. It was introduced in 1965 (Hayes and Laws, 1990). Primarily chlorpyrifos is used to kill mosquitoes in the immature, larval stage of development; but it is no longer registered for this use. Chlorpyrifos is effective in controlling a variety of insects, including cutworms, corn rootworms, cockroaches, grubs, flea beetles, flies, termites, fire ants, and lice (US EPA, 1986). It is commonly applied as an insecticide on cereals, cotton, field, fruit, nut and vegetable crops, and well as on lawns and ornamental plants (US EPA, 1984; Berg, 1986). It is also registered for direct use on sheep, turkey, for horse site treatment, for treatment of dog kennels, and for domestic dwellings, farm buildings, storage bins, and commercial establishments.

It is available in emulsifiable concentrate, dust, flowable, pellet, spray, granular and wettable powder formulations. Chlorpyrifos kills insects upon ingestion by affecting the nervous system. It affects the nervous system by inhibiting the breakdown of acetylcholine (ACH), a neurotransmitter (Manahan, 1992). Chlorpyrifos binds to the active site of the cholinesterase (ChE) enzyme, which prevents breakdown of ACH in the synaptic cleft; resulted in the accumulation of ACh in the synaptic cleft causes overstimulation of the neuronal cells, which leads to neurotoxicity and eventually death (Ragnarsdottir, 2000; Singh and Walker, 2005). Chlorpyrifos also interferes with other enzymes, such as carboxylesterases and A-esterases; but functional role of these enzymes is not well understood, although they occur in many mammalian systems. The mechanism of toxicity of chlorpyrifos resembles with other organophosphate insecticides such as malathion and parathion, thus, chlorpyrifos would not be effective against organophosphate-resistant insect populations.

Chlorpyrifos also able in cholinesterase inhibition in humans at high enough doses; result in the overstimulation the nervous system causing nausea, dizziness, confusion. But at very high exposures (e.g., accidents or major spills) it could be cause respiratory paralysis and death. So it should be primary concern for manufactures to aware about occupational exposure to chlorpyrifos. To prevent from this, current chlorpyrifos labels require information to wear additional personal protective equipment (chemical resistant gloves, coveralls, respirators) during workers handling and applying chlorpyrifos, and also restricting entry into treated fields for 24 hours up to five days.

Aysal et al. (2004) reported the chlorpyrifos residues in food chain. During supervised trials on rice conforming to Good Agricultural Practise conducted in Columbia, the Philippines, Thailand, Vietnam, and India, maximum chlorpyrifos residue level of 0.5 mg/kg for rice, a supervised trials median residue of 0.12 mg/kg, and a highest residue level of 0.28 mg/kg were estimated. Chandra et al. (2010) reported chlorpyrifos residue in cauliflower in range of 0.024–0.07 mg/kg and brinjal in range of 0.018–0.021 mg/kg. Berg (1986) estimated LD50 for chlorpyrifos in rat body was 82–270 mg/ kg.

These reports are evident that indiscriminate use of chlorpyrifos may cause serious human health problems. There is growing concern about the toxicological and environmental risks associated with chlorpyrifos residues. The persistent nature of the insecticide is a health hazard, and thus, there is a need to detoxify this moiety (Mukherjee et al., 2004).

### **1.8 Cyanoremediation of chlorpyrifos**

A number of researchers studied the effect of chlorpyrifos on cyanobacteria (Palanisami et al., 2009; Singh et al., 2013; Kumar et al., 2014). It is evident that

insecticides altered the enzyme activities associated with antioxidant defence mechanism through inducing the production of singlet oxygen and other active oxygen species at various sites of photosynthetic electron transport chain and responsible for oxidative damage (Halliwell, 1987; Bagchi et al., 1995; Palanisami et al., 2009). In response to this, Cellular systems scavenge these active oxygen species by invoking antioxidative machinery such as superoxide dismutase, catalase, peroxidase, etc. (Patel and Chakrabarti, 1982; Büyüksönmez et al., 1998; Palanisami et al., 2009).

Kumar et al. (2014) investigates the effect of chlorpyrifos on the antioxidant level and changes in the fatty acid profile of *Chroococcus turgidus* NTMS12 and found increase in the activity of antioxidant enzymes proline, SOD and CAT. Under chlorpyrifos exposure conditions, *Chroococcus turgidus* NTMS12 showed in change its fatty acid profile by lowering the level of unsaturated fatty acids. It could be strong and effective defence mechanisms i.e. increased activity of cellular antioxidants and lowering the levels of cellular unsaturated fatty acids by cyanobacteria against the pesticide-induced stress.

It is also reported that phosphatases play an important role in the biodegradation of chlorpyrifos (Madhuri and Rangaswamy, 2002; Thengodkar and Sivakami, 2010). Although few reports on the metabolization of pesticides by cyanobacteria are available (Subramanian et al., 1994; El-Bestawy et al., 2007; Lee et al., 2003), but detailed mechanism of chlorpyrifos degradation in cyanobacteria is not fully known from the paddy fields therefore, we selected cyanobacteria diversity from paddy soils and its efficacy to the tolerance and degradation of chlorpyrifos pesticide in laboratory with the following objectives.

**1.9 Objectives**

1. Analyses of soil physico-chemical parameters and nutrients status of the selected paddy fields.
2. Isolation and identification of existing cyanobacterial diversity from paddy fields.
3. Screening of pesticide tolerant cyanobacterial strain(s).
4. Testing the efficacy of the selected cyanobacteria for pesticide degradation.
5. Analyses of the pesticide degraded end product by cyanobacteria.

# *CHAPTER 2*

## *Review of Literature*

### **2.1 Recent trends of paddy cultivation**

Paddy crop is widely grown in Asian countries mainly in tropics and subtropics, except a notable presence in African and South American countries. In last 10 years, from 2007-17, on average 691.69 million tonnes of paddy (rough rice) produced in the world (USDA, 2017). In 2017, China was the largest producer of paddy with 29% of world production; followed by India (23%) and Indonesia (10%). Currently paddy is approximately grown on 161.27 mha and it is increasing at a rate of 0.4% per year during the period of 2007–17 (USDA, 2017). However Indonesia, Myanmar, Vietnam and Sub-Saharan Africa show notable increase in paddy area; while there is a decrease in rice area in China and South America.

Rice is a staple food for over half of the world's population especially in Asian region. Rice accounts for over 20 per cent of global calorie intake. The world rice production has been increased from 433 million tonnes in 2007 to 486 million tonnes in 2017. Apart from increasing rice production per capita consumption of rice is decreasing in some Asian countries like Japan, Taiwan and the Republic of Korea. It may be due to growing economic prosperity and urbanization or change in food pattern. But, in some Asian countries such as Afghanistan, North Korea, Nepal and Vietnam, where nearly more than one fourth of the population is still poor and totally depend up on rice. Currently the Asian population is increasing at 1.09 % per year; result in the increase in demand for rice (for total consumption but not per capita consumption). So it is crucial to keep the pace of the rice production with annual

growth rate for rice consumption more through yield increase rather than area expansion.

## **2.2 History of paddy: Origin, migration and modern improvement**

Paddy (*Oryza Sativa*) is an annual crop; but it is also grown as a perennial crop in some tropical areas such as Brazil, Japan, Thailand, Taiwan, USA and some parts of India and harvest a ratoon crop for up to many years. Paddy or rice plant considered a tropical plant because of well grow in wet, humid climate, though it is not a tropical plant. In relation to origin of paddy or rice plant, there are many opinions; one says it is a descendent of wild grass that was most likely cultivated in the foothills of the far Eastern Himalayas, another believes that it may have originated in southern India, then spread to the north of the country and then onwards to China.

After China, it arrived in Korea, the Philippines (about 2000 BC) and then Japan and Indonesia (about 1000 BC) (Anonymous, 2012). In 327 BC, Alexander the Great invaded India; it is supposed that rice reached in Greece. Arab travellers took it to Egypt, Morocco and Spain and then it spreads all across Europe. Portuguese took rice to their colonies in West Africa and then it travelled to America through the ‘Columbian Exchange’ of natural resources. Thereafter the journey of rice continues with the Moors taking it to Spain in 700 A. D. and then the Spanish brought rice to South America at the beginning of 17th century.

In Indian subcontinent, rice cultivation accounts for more than a quarter of the cultivated land (2015-16). Rice is a very essential part of the daily meal in the southern and eastern parts of India. In the northern and central parts of the subcontinent, where wheat is mostly used, rice has its own place and is cooked daily as well as on festivals and special occasions. Historians believe that while the *Indica*

variety of rice was first domesticated in the foothills of the Eastern Himalayas (i.e. north-eastern India), and the japonica variety was domesticated in southern China which was introduced to India. Perennial wild rice still grows in some parts of Assam and Nepal.

In 1952, Dr. K. Ramiah initiated a programme for incorporating genes for fertilizer response from temperate japonica rice varieties from Japan into indica strains at the Central Rice Research Institute (CRRI), Cuttack. Major aim was to select from segregating populations of indica x japonica crosses, lines which showed the ability to utilize effectively about 100 kg of N per hectare. Introduction of Dee-Gee-Woo-Gen dwarfing gene from Taichung Native 1 in our tropical rice resulted in crossing the yield barrier as the yield jumped from 3 tonnes/ha to 7 tonnes/ha. Semi-dwarf indica rice's like Taichung Native 1, IR8 and Jaya provided the initial material for the High Yielding Varieties Programme. Now the country has a large number of high yielding rice varieties, suitable for cultivation under irrigated and rainfed conditions. India is the second country in the world to commercialize hybrid rice technology.

Since its origin, the spread of paddy cultivation is extensive and paddy is now being grown wherever water supply is adequate and ambient temperature is suitable. The paddy grain is covered with a woody husk or hull, which is indigestible and is to be removed in the first step during processing for making the rice edible. Paddy cultivation is well suited to countries and regions with low labour costs and high rainfall, as it is labour intensive to cultivate and requires ample water. Paddy can be grown practically anywhere, even on a steep hill or mountain.

### **2.3 Paddy cultivation in India**

The most common practice used for cultivating paddy is simply flooding the fields or after and then transplanting the young seedlings. This simple method needs proper management of damming and channelling of water that flooding is maintained during the crop. However flooding inhibits the robust growth of weeds and pest plants that could not survive in submergence. While flooding is not compulsory for the paddy cultivation, but all other all irrigation practices need higher effort in weed and pest control during crop. In India paddy is cultivated in all terms from hills to mountains, rain-fed to irrigate; so mainly four distinct types (<https://farmer.gov.in/imagedefault/pestanddiseasescrops/rice.pdf>) of paddy cultivation methods is developed:

- 1. Irrigated:** Irrigated paddy is the primary type found in East Asia. Irrigated ecosystems provide 75 % of global rice production. In India, the total area under irrigated rice is about 22.00 million hectares, which accounts about 49.5 % of the total area under paddy crop in the country. Rice is grown under irrigated conditions in the states of Punjab, Haryana, Uttar Pradesh, Jammu & Kashmir, Andhra Pradesh, Tamil Nadu, Sikkim, Karnataka, Himachal Pradesh and Gujarat. Irrigated rice is grown in bounded (embanked) paddy fields.
- 2. Rain-fed upland:** Generally upland zones are found in Asia, Africa, and Latin America. In India, the total area under upland rain fed paddy in the country is about 6.00 million hectares, which accounts 13.5 % of the total area under rice crop in the country. Upland rice areas lies in eastern zone comprising of Assam, Bihar, Eastern M.P., Orissa, Eastern U.P., West Bengal and North-Eastern Hill region. Upland rice fields are generally dried, unbounded and

directly seeded. Land utilized in upland rice production can be low lying, drought-prone, rolling, or steep sloping.

- 3. Rain-fed lowland:** Rain-fed low-land rice is grown in such areas as East India, Bangladesh, Indonesia, Philippines, and Thailand, and is 25 % of total rice area used worldwide. In India, low land rice area is about 14.4 million hectares, which accounts 32.4 % of the total area under rice crop in the country. Production is variable because of the lack of technology used in rice production.
- 4. Flood prone:** Flood-prone ecosystems are prevalent in South and Southeast Asia, and are characterized by periods of extreme flooding and drought. The paddy yields are low and variable. Flooding occurs during the wet season from June to November, and rice varieties are chosen for their level of tolerance to submersion.

#### **2.4 Effect of climate change on paddy cultivation**

Before starting discussion on impact of pests and diseases on paddy yields; there is another challenge i.e. climate change, which is not directly related to reduction in yields but it could be worse in the future. It is expected that in comparison of temperatures of 1980-1999, surface air temperature will be increased around 1.4-5.8 °C in relation to at the end of 21<sup>st</sup> century. Most of the paddy is currently grown in the areas where temperatures are above the optimal for growth (28/22 °C) and further increase in mean temperature or incidents of high temperatures during sensitive stages may reduce paddy yields drastically. In tropical regions, high temperature is already concerning factor or stress that affects the rice productivity through reductions in weight and quality of rice grains. There are several approaches to overcome high temperature stress such as early sowing of paddy cultivars or

selection of early maturing cultivars that temperature is not so high during grain filling. But these approaches not so successful in long term, when high temperature stress events would be more frequent and severe in the future climate. Although high temperature stress tolerant paddy cultivars developed for better alternative approach, it could be done through systematic understanding of genetics, biochemical, and physiological processes for identifying and selecting traits, and enhancing tolerance mechanisms in paddy cultivars.

### 2.5 Pests, diseases and weeds occurred in paddy and control measures

Paddy cultivation affected by a large variety of pests, diseases and weeds during the life span of a paddy plant which could be responsible for the approx. 40% reduction in the paddy yield in in tropics and subtropics (Oerke, 2006; Savary et al., 2012; Donatelli et al., 2017). For controlling these pests, diseases and weeds, a numerous pesticides is applied. Here is list of pests, diseases in various stages of paddy cultivation along with control measures or particular pesticide recommended (Table 2.1 & 2.2).

**Table 2.1** Common pests reported from paddy fields

Stage	Pests	Control measures
Nursery	Stem-borer ( <i>Scirpophaga incertulas</i> ), gall midge ( <i>Orseolia oryzae</i> ), root-knot nematode ( <i>Meloidogyne graminicola</i> ), and white tip nematode ( <i>Aphelenchoides besseyi</i> )	Phorate 10 G, Fipronil 0.3 G, Chlorpyrifos 20 EC, Quninalphos 25 EC
Vegetative Stage	Stem-borer ( <i>Scirpophaga incertulas</i> )	Cartap 4 G, Phorate 10 G, Fipronil 0.3 G, Chlorpyrifos 10 G
	Gall midge ( <i>Orseolia oryzae</i> )	Fipronil 0.3 G, Phorate 10 G
	Green leafhopper ( <i>Nephotettix malayanus</i> )	Carbaryl 50 WP, BPMC 50 EC, Acephate 50 WP, Ethofenprox 10 Ec, Imidacloprid 200 SL, Thiamethoxam 25

		WG, Clothianidin 50 WDG, Phorate 10 G
	Hispa ( <i>Dicladyspa armigera</i> )	Triazophos 40 EC, Phosalone 35 EC, Chlorpyrifos 20 EC, Quinalphos 25 EC, Ethofenprox 10 EC, Fipronil 5 SC
	Leaf folder ( <i>Cnaphalocrocis medinalis</i> )	Chlorpyrifos 20 EC, Cartap 50 WP, Quinalphos 25 EC, Acephate 50 WP, Fipronil 5 SC, Phosalone 35 EC, Carbaryl 50 WP, Triazophos 40 EC, Cartap 4 g
	Whorl maggot	Fipronil 0.3 G, Chlorpyrifos 20 EC
	Case worm	Carbaryl 50 WP, Carbaryl dust
	Mealy bug ( <i>Brevinnia rehi</i> )	Phorate 10 G granules
<b>Reproductive Stage</b>	Stem-borer	Cartap 50 WP, Chlorpyrifos 20 EC
	Brown planthopper ( <i>Nilaparvata lugens</i> ), White backed planthopper ( <i>Sogatella furcifera</i> )	Imidacloprid 200 SL, Thiamethoxam 25 WG, Ethofenprox 10 EC, Acephate 50 WP, BPMC 50 EC. Carbaryl 50 WP
	Green leafhopper ( <i>Nephotettix malayanus</i> )	Imidacloprid 200 SL, Thiamethoxam 25 WG, Ethofenprox 10 EC, Acephate 50 WP, BPMC 50 EC, Carbaryl 50 WP
	Leaf folder	Cartap 50 WP, Chlorpyrifos 20 EC, Phosalone 35 EC, Quinalphos 25 EC
	Ear-cutting caterpillar/ cut worm ( <i>Mythimna separate</i> )	Quinalphos 25 EC, Chlorpyrifos 20 EC, Carbaryl 50 WP, Phosalone 35 EC
	Leaf/Panicle mite ( <i>Steneotarsonemus spinki</i> )	Sulphur wettable powder, Dicofol, Profenophos 50 EC
	Gundhi bug ( <i>Leptocorisa oratorius</i> )	Carbaryl 50 WP, Dust Malathion or Carbaryl

**Table 2.2** Diseases caused by different microbial agents to paddy crop

Disease/ crop stage/ seasons	Casual organisms	Control measures
<b>Leaf blast</b> Nursery and vegetative Kharif and Rabi	<i>Magnaporthe oryzae</i>	Tricyclazole 75 WP, Carbendazim 50 WP, Tricyclazole 75, Carpropamid 30 SC, Isoprothiolane 40 EC, Iprobenphos 48 EC, Propiconazole 25 EC, Kasugamycin-B 3 SL, Carbendazim 50 WP

<b>Neck blast</b> Flowering and after Kharif/Rabi	<i>Pyricularia grisea</i>	Tricyclazole 75 WP Carpropamid 30 SC Isoprothiolane 40 EC Iprobenphos 48 EC Propiconazole 25 EC Carbendazim 50 WP
<b>Sheath blight</b> Panicle initiation to booting Kharif/Rabi	<i>Rhizoctonia solani</i>	Validamycin 3 L Thifluzamide 24 SC Hexaconazole 5 EC Propiconazole 25 EC Carbendazim 50 WP
<b>Brown spot</b> Vegetative stage Kharif/Rabi	<i>Helminthosporium oryzae</i>	Carbendazim (12%) + Mancozeb (63%) combination 75 WP, Carbendazim 50 WP Mancozeb (63%) 75 WP, Mancozeb 75
<b>False smut</b> Post-flowering stage Kharif	<i>Ustilaginoidea virens</i>	Propiconazole 25 EC, Chlorothalonil 75 WP, Copper oxychloride
<b>Sheath rot and grain discoloration</b> Post-flowering stage Kharif	<i>Sarocladium oryzae</i>	Mancozeb 75 WP, Captan 50 WP, Propiconazole 25 EC, Hexaconazole 5 EC, Thiophanate methyl 70 WP
<b>Stem rot</b> Panicle initiation to booting Kharif	<i>Sclerotium oryzae</i>	Iprobenphos 48 EC. Carbendazim 50 WP, Thiophanate methyl 70 WP, Isoprothiolane 40 EC
<b>Foot rot/ Bakanae</b> Vegetative Stage Kharif	<i>Gibberella Fujikuroi</i>	Captafol 80% Mancozeb 75 WP, Carbendazim 50 WP
<b>Bacterial blight</b> Panicle initiation to booting Kharif	<i>Xanthomonas oryzae</i>	Apply judicious level of fertilization (60-80 kg N/ha with required level of potassium) without sacrificing the yield.
<b>Rice tungro disease</b> Nursery, tillering Kharif	<i>Rice tungro bacilliform virus</i> (RTBV)	Phorate 10 G, Fipronil 0.4 G, Carbaryl 50 WP, Fipronil 5 EC

Unlike pests and diseases, weeds do not harm the paddy crop directly but they compete with paddy for nutrients and space; result in the reduction in paddy yield. Here are also list of various weeds such as grasses, sedges and broad leave plants that commonly occurred in paddy crop (Table 2.3).

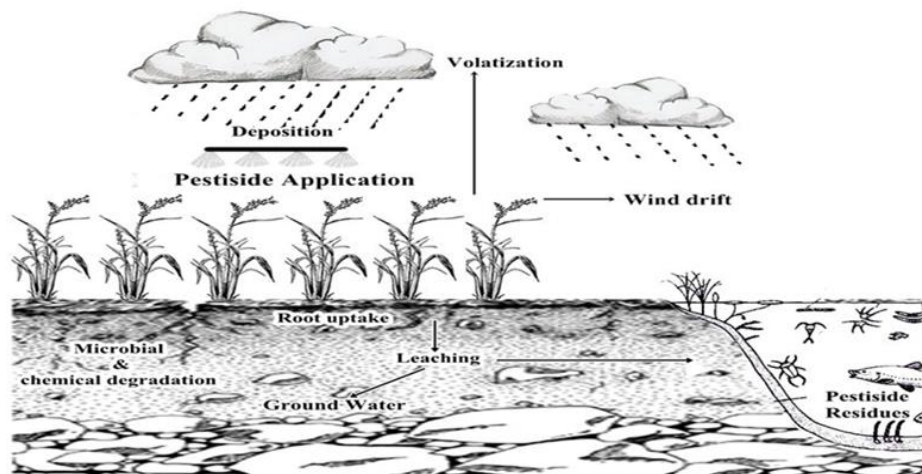
Table 2.3 Weeds reported in paddy fields

SNo.	Common name	Botanical name	Control measures
<b>Grasses</b>			
1.	Bansawan	<i>Echinochloa colonum</i> , <i>Echinochloa crusgali</i>	<b>Pre planting stage</b> Glyphosate, paraquat and glufosinate
2.	Doob grass	<i>Cynodon dactylon</i>	
3.	Bankodo	<i>Eleusine indica</i>	
4.	Makra	<i>Dactyloctenium aegyptium</i>	
5.	Bottle grass	<i>Setaria glauca</i>	
<b>Sedges</b>			
1.	Motha	<i>Cyperus rotundus</i>	<b>Pre emergence stage</b> Butachlor, pretilachlor pretilachlor + fenclorim, oxadiazon, pendimethalin
2.	Thukaha (Gurguja)	<i>Caesulia axillaries</i>	<b>Post emergence</b> Bispyribacbispyribac, fenoxaprop + safener, fenoxaprop + ethoxysulfuron, metsulfuron + chlorimuron, azimsulfurom, penoxsulam, penoxsulam + cyhalofop
3.	Bhangaria	<i>Eclipta alba</i>	
4.	Bari dudhi	<i>Euphorbia herita</i>	
5.	Ban makoy	<i>Solanum nigrum</i>	
6.	Gumma	<i>Leucces aspera</i>	
7.	Hazardana	<i>Phyllanthus niruri</i>	
8.	Mokana	<i>Lippia nodiflora</i>	

## 2.6 Fate of pesticide in paddy agroecosystem

Pesticides are applied in paddy cultivation through spraying, dusting or spreading; only some part of the pesticides are taken up by pests or crop plants; remaining pesticide residue suspended in the soil. These pesticide residues either can be transformed in to less toxic products through chemical reactions and microbial actions in the soil/ can also be mineralized through sorption onto soil organic matter and clay minerals/ also be lost to atmosphere through volatilization. The pesticides that are not degraded, immobilized, detoxified, or removed; they further contaminate

water sources or sometimes in bio-accumulated into plant parts or animal tissues through food chain (Babu et al., 2003; Waliszewski, 2008).



**Figure 2.1** Fate of synthetic pesticides in agro- and aquatic ecosystems

(Kumar and Singh, 2017)

The major loss pathways of pesticides to the environment are volatilization in to the atmosphere and aerial drift, runoff to surface water bodies in dissolved and particulate forms, and leaching into groundwater basins (Figure 2.1).

## 2.7 Impact of pesticides on ecosystem and environment

Paddy growers applied much hazardous insecticides such as organophosphates and organochlorine in more amounts and also in many times than recommended; sometimes up to five to six times in one crop cycle while only two applications may be sufficient. McLaughlin and Mineau (1996) pointed out the problem of over-use of pesticide and its consequences such as contamination of ground water, surface water, soils and food, and the consequent impacts on wildlife and human health. There are some impacts that could be aroused from indiscriminate and over-use pesticide; and

their incidence and magnitude depend on the types of pesticides, frequency and quantities applied, and their persistence. Here are the following:

- 1) Deleterious impacts on health due to direct or indirect exposure to pesticides;
- 2) Contamination of ground and surface waters through runoff and seepage;
- 3) Bioaccumulation of pesticide residues through the food chain;
- 4) Development of pesticide resistant pest populations, leads to more efficient and consistent pest outbreaks;
- 5) Negative impact on populations and diversity of beneficial insects like parasites and predators, that could be helpful in effective pest control strategies and further helps in reduction in pesticide use;
- 6) Also leads to decrease in microbial diversity in the paddy soil agro-ecosystem that play great role in soil fertility and lowers chemical fertilizer use.

Although synthetic pesticide helps in controlling agricultural pests and diseases; result in the increase paddy crop production. They are also used in control of vector borne diseases to insure better human health. But these pesticides cause serious health problems to human and also responsible for disruption of agro- and aquatic ecosystems (Forget, 1993; Igbedioh, 1991).

### **2.7.1 Soil contamination**

The unused remain of these pesticides is suspended in the soil in several days depending upon the solubility and half-life of pesticide; cause soil contamination. Pesticide residue can be suspended as persistent or degraded or transformed forms in the soil. It is evident that pesticides can be degraded and transformed into variety of transformation products (TPs) (Barcelo' and Hennion, 1997; Roberts and Hutson, 1999). Further pesticide residues and their transformation products can be absorbed

by the soils to different extent; it is primarily depend upon the pesticide characteristics, soil moisture, soil pH, and soil texture. Akhtar et al. (2009) suggested that pesticide residues are bitterly adsorbed by the soils that are rich in clay or organic matter. Organic matter content is the most important factor that influences the rate or absorption by the soil; it is found that greater the organic matter content, the greater the adsorption of pesticides and their transformation.

### **2.7.2 Water contamination**

Groundwater or surface water contamination due to pesticide residues is widespread problem. Pesticide residue could be transported to ground water or surface water through leaching or runoff from treated plants and soil (Kumar and Singh, 2017). Leaching of pesticide residues can be downward, upward, or sideways; and this is influenced by various factors such as characteristics of the soil and pesticide, and their interaction with water either it is from irrigation or rainfall. Leaching can be enhanced by these factors: (a) the water solubility of pesticide; (b) sandy nature of soil; (c) event of rainfall or irrigation shortly after spraying; (d) the pesticide residues is not better adsorbed by the soil. Kole and Bagchi (1995) reported that 58% of drinking water samples taken from various hand pumps and wells around Bhopal, India were found to be traces of organochlorine pesticides above the EPA standards. It is costly and complex process to remove or clean up the groundwater polluted with toxic pesticide residues (Waskom, 1994; O'Neil, 1998; US EPA, 2001).

### **2.7.3 Effects on beneficial soil microorganisms**

Soil microorganisms bacteria and fungi, play a vital role for the plants in terms of transformation of atmospheric nitrogen into nitrates, which plants can use; enhancing bioavailability on nutrients. Pesticides also have deleterious impact on

beneficial soil microorganisms which play a great role in retaining soil fertility and plant growth promotion through secretion of various compounds (Singh et al., 2016). In absence of these beneficial soil microorganisms soil started to lose its fertility and capability to support plant growth. Savonen (1997) suggested that it may takes days, months or years to be absorb, degrade or escape after application of pesticides; but after a while, there aren't enough populations of beneficial soil organisms to retain the soil nutrients that are essential for soil health.

#### **2.7.4 Effects on non-target organisms**

The widespread introduction of pesticides to control pests and diseases of paddy crop (bacterial and fungal pathogens, weeds, nematodes, snails, insects, and rodents) has undoubtedly increased paddy yields. But these pesticides also have potential to change the composition of paddy agroecosystem. Paddy agro-ecosystems also support populations of many organisms such as insects, plants, fish, birds, and other wildlife, which have their different role in agro-ecosystem (Singh et al., 2014). These non-target organisms are badly affected by the pesticide contamination in inside or outside the agroecosystem. When pesticides are continuously applied into the crop; it can be responsible for the extinction of useful organisms present in the agro-and aquatic ecosystems.

#### **2.7.5 Effects on Human health**

Indiscriminate and unsafe use of pesticides can cause serious health problems like poisoning of farmers and farm workers, cardiopulmonary, neurological and skin disorders, fatal irregularities, miscarriages, lowering the sperm count of applicators, etc. This pesticide poisoning can be acute and chronic poisoning:

1. According to Bodekar and Dummler (1993) and Alavanja et al. (2004), these are some symptoms that considered as acute pesticide poisoning i.e. fatigue, headaches and body aches, skin discomfort, skin rashes, poor concentration, feelings of weakness, circulatory problems, dizziness, nausea, vomiting, excessive sweating, impaired vision, tremors, panic attacks, cramps, etc., and in severe cases coma and death.
2. According to WWF (2002), UNEP (2004) and Terre Des Hommes (2011), these are symptoms that are considered as chronic poisoning or long-term effects i.e. include weakening of the immune system and effects on the reproductive system, which can lead to miscarriage, still birth, and premature birth or to low birth weight.

#### **2.7.6 Effects on cyanobacteria**

Cyanobacteria are the major community that naturally existed in the paddy fields which have distinctive role in the paddy agroecosystem. It is well studied that due to pesticides physiological and biochemical processes of cyanobacteria such as growth, photosynthetic pigments and enzyme activities has been affected significantly. Palanisami et al. (2009) found that during degradation, pesticides releases singlet oxygen and other active oxygen species (ROS-) at various sites of photosynthetic electron transport chain upon. To cope up these active oxygen species, cyanobacteria releases antioxidant enzymes such as superoxide dismutase, catalase, peroxidase, etc.; that helps the cyanobacterial cells in tolerate the pesticide stress.

#### **2.8 Cyanobacterial diversity in paddy fields**

Cyanobacteria are cosmopolitan photoautotrophic microorganisms which play great role in nitrogen-fixing in various ecosystems. Paddy fields are unique

agroecosystem (Prasanna et al., 2009) that provide favourable conditions for cyanobacteria to flourish (Roger and Reynaud, 1982). Nayak and Prasanna (2007) suggested that in the paddy fields, cyanobacteria contributes very well to soil fertility in terms of the improvement in physico-chemical, biological and soil-water relations. Cyanobacteria could be a treated as soil bio-indicator (Zancan, 2006) and they also help in soil conditioning (Choudhary and Bimal, 2010). There are many studies related to cyanobacterial diversity in paddy fields of India (Ahmed et al., 1999; Tiwari et al., 2000; Nayak et al., 2001; Kaushik and Prasanna, 2002; Mishra and Pabbi, 2004; Choudhury and Kennedy, 2005; Rai, 2006; Nayak and Prasanna, 2007; Dey et al., 2010; Selvi and Sivakumar, 2011; Nongbri and Syiem, 2012; Danaboyina and Sivakumar, 2013; Singh et al., 2014; Vijayan and Ray, 2015).

Nayak and Prasanna (2007) reported 12 cyanobacteria genera in which 8 were heterocystous i.e. *Anabaena*, *Nostoc*, *Westiellopsis*, *Calothrix*, *Scytonema*, *Aulosira*, *Hapalosiphon*, *Cylindrospermum* and 4 were non-heterocystous i.e. *Phormidium*, *Oscillatoria*, *Lyngbya* and *Aphanocapsa*. Dey et al. (2010) observed genera of *Aphanocapsa*, *Chroococcus*, *Microcystis*, *Anabaena*, *Nostoc*, *Cylindrospermum*, *Spirulina*, *Oscillatoria*, *Phormidium*, *Lyngbya*, and *Microcoleus*, but *Oscillatoria*, *Phormidium*, *Cylindrospermum* are well dominant in the paddy fields of Mayurbhanj district of Orissa. Nayak and Prasanna (2007) and Dey et al. (2010) also suggested that pH is an influential factor that could be affects the growth, establishment and diversity of cyanobacterial populations; it is generally observed that neutral to slightly alkaline pH favours the growth of cyanobacteria. A positive correlation between the soil pH and cyanobacterial population mostly non-heterocystous also observed by Nayak and Prasanna (2007), which opposes with this approach of heterocystous forms generally more abundant at alkaline. Sevi and Sivakumar (2011) studied the

diversity of non heterocystous cyanobacteria in paddy fields of Cuddalore District of Tamilnadu and reported 25 species of 10 genera. It is also observed that three genus *Oscillatoria*, *Spirulina* and *Lyngbya* are dominant, while genus *Gloeocapsa* shows rare presence. The predominance of non-heterocystous cyanobacteria also indicated the lower nitrogen content in the paddy fields.

Choudhary (2011) reported these cyanobacterial genera *Calothrix*, *Fortiea*, *Gloeotrichia*, *Microchaete*, *Plectonema*, *Rivularia*, *Scytonema* and *Tolypothrix* in the paddy fields of Muzaffarpur district, Bihar. It is also observed that populations of heterocystous cyanobacteria increased with the paddy crop progression. It may be due to development of dense canopy of paddy which prevents the sunlight reaching to the surface of the soil; resulted in the depletion of nutrients particularly nitrogen. Choudhary and Bimal (2010) also confirmed similar phenomenon of cyanobacterial diversity while working with the fertilized and unfertilized paddy fields.

In different part of Meghalaya, cyanobacteria genera *Nostoc*, *Anabaena*, *Fischerella*, *Calothrix*, *Cylindrospermum*, *Gloeocapsa*, *Plectonema*, *Scytonema*, *Stigonema*, *Tolypothrix*, and *Westiellopsis* were reported (Nongbri and Syiem, 2012). In the paddy fields of Guntur district of Andhra Pradesh, following cyanobacterial genera *Chroococcus*, *Gloeotheca*, *Gloeocapsa*, *Oscillatoria*, *Phormidium*, *Microcoleus*, *Lyngbya*, *Anabaena*, *Cylindrospermum*, *Calothrix*, *Scytonema* and *Stigonema* were reported (Danaboyina and Sivakumar, 2013).

Following cyanobacterial genera including heterocystous i.e. *Calothrix*, *Anabaena*, *Anabaenopsis*, *Scytonema*, *Cylindrospermum*, *Tolypothrix*, *Rivularia*, *Chroococcus*, *Nostoc* and non-heterocystous i.e. *Oscillatoria*, *Apanothece*, *Microcystis*, *Arthrospira*, *Gleotheca*, *Gleocapsa*, *Merismopedia*, and *Microcoelus*

were reported in Chattishgarh (Singh et al., 2014). In the paddy wetlands of Kerala, Vijayan and Ray (2015) reported following cyanobacterial genera of *Leptolyngbya*, *Chroococcus*, *Gloeotheca*, *Gloeocapsa*, *Synechocystis*, *Microcoleus*, *Oscillatoria*, *Nostoc*, *Phormidium*, *Lyngbya*, *Anabaena*, *Scytonema*, *Westiellopsis*, *Merismopedia*, *Spirulina* and *Microcystis*.

## **2.9 Cyanoremediation of pesticides**

Pesticide degradation is a complex approach, involves mainly three steps (Hatzios, 1991): (a) Step I consists of oxidation, reduction, or hydrolysis: In this step pesticide becomes more water soluble and may convert into less toxic pesticide metabolites; (b) Step II consists of conjugation of a pesticide or pesticide metabolites to a sugar, amino acid or glutathione: In this step pesticide and pesticide metabolites become further more water soluble and help in conversion of less toxic compounds to parent pesticide compound. (Armstrong, 1994; Marrs, 1996); (c) Step III consists of conversion of phase II metabolites into secondary conjugates- These secondary conjugates are also nontoxic.

Lal et al. (2010) suggested that organochlorine pesticides (chlorinated hydrocarbon chemicals) widely used agrochemicals in control of various agricultural, horticultural pests and also used for public health. El-Bestawy et al. (2007) and Gonzalez et al. (2012) observed that these pesticides also cause deleterious effects in applied agroecosystems, and pesticide residues are retained by the crop plants and non-target organisms.

Lindane (A-hexachlorocyclohexane (HCH) formulation) is the commonly used organochlorine pesticide in paddy crop for the control of pests (Abdullah et al. 1997). Alexander (1994); and Majewski and Capel (1995) investigated that lindane is

highly persistent in soils and even their residues can be traced in the air, rain, and surface water long after its application. It is reported that paddy field cyanobacteria *Cylindrospermurn* sp., *Aulosira fertilissirna* and *Plectonema boyanurn* tolerate the commercial lindane up to 80 pg/ml concentration (Singh, 1973). It is also investigated that *Anabaena* sp. PCC7120 and *Nostoc ellipsosporurn* are able to degrade lindane (A-HCH) via formation pentachlorocyclohexene as an intermediate (Kuritz and Wolk, 1995). However the growth rate cyanobacteria not largely affected by Lindane up to 20 pg/ml concentrations (Singh 1973).

Lee et al. (2003) investigated the potential of *Anabaena* sp strain PCC 7120 and *Anabaena flos-aquae* to degrade endosulfan and found that these two cyanobacteria not only successfully tolerate the endosulfan. But these two cyanobacteria are able to transform endosulfan into less toxic endodiol (primary product) and endosulfan sulfate (trace amount). Kennedy et al. (2001) reported that although endodiol is a non-toxic to fish and other organisms. But endosulfan sulfate showed same toxicity as parent compound endosulfan and it has longer persistence than endosulfan in the soil and environment.

Kanekar et al. (2004) suggested that organophosphorous insecticides (esters of phosphoric acids) also known as organophosphates; are used for the control of sucking, chewing and boring insects, spider and mites, aphids and other pests. Majewski and Capel (1995) observed that although organophosphate shows less persistence than organochlorine pesticides. But their residues also detected in air and water due to indiscriminate use by the farmers. Megharaj et al. (1994) investigated that organophosphates transformed in to para-nitrophenol (more toxic and persistent) through non-enzymatic hydrolysis in the aquatic environments.

Doggett and Rhodes (1991), Megharaj et al. (1994) and Subramanian et al. (1994) suggested that organophosphorus pesticides do not cause much impact on the growth of cyanobacteria up to working concentrations and concentrations present in wastewaters. Megharaj et al. (1987), Orus and Marco (1991), Megharaj et al. (1994) and Subramanian et al. (1994) exposed three cyanobacteria *Nostoc*, *Oscillatoria* and *Phormidium* with methyl parathion or other organophosphorus pesticides to investigate that whether they use organophosphates as a sole source of organic phosphorus and nitrate. It is observed that that *Nostoc*, *Oscillatoria* and *Phormidium* are able to use phosphorus from the organophosphorus pesticides for their growth and development.

The cyanobacteria are able to convert the nitrogroup of para-nitrophenol in to nitrite; but it is not fully known about the enzyme responsible for this process (Megharaj et al., 1994). The enzyme nitrite reductase is responsible for the assimilation of that released nitrite. A link also observed between the nitrogen metabolism and phosphorus utilization from organophosphorus pesticides. But possible effects of nitrogen metabolism on biodegradation of organophosphorus pesticides not analysed by the researchers (Subramanian et al., 1994; Kuritz, 1999).

The *Phormidium valderianum* BDU 20041 successfully tolerate the chlorpyrifos stress through increased activity of oxido-reductase enzymes (Palanasami et al., 2009). Thengodkar and Sivakami (2000) observed that *Spirulina platensis* are also successfully tolerate the chlorpyrifos up to 80 ppm and also able to transform chlorpyrifos in to TCP (3, 5, 6-trichloro-2-pyridinol) as primary metabolite through the action of alkaline phosphatase (ALP) enzyme. It is also supported by Singh et al. (2011) that *Synechocystis* sp. Strain PUPCCC 64 degraded the pesticide chlorpyrifos into TCP (3, 5, 6-trichloro-2-pyridinol). Another important organophosphates

pesticide malathion which is also widely used in control of various pests. Ibrahim et al. (2014) grew *Anabaena oryzae*, *Nostoc muscorum* and *Spirulina platensis* under high concentration of malathion and observed that they are able to use malathion as a source of phosphorous. Barton et al. (2004) also suggested that under aerobic conditions, *Anabaena* sp. Strain PCC 7120 able to convert nitro group of methyl parathion to an amino group via a nitroso group intermediate.

The *Spirulina* sp. able to degrade glyphosate in great extent and the degradation potential of *Spirulina* sp. did not affected by its initial concentration in the aqueous medium (Lipok et al., 2007). It is also suggested that the pathway used by *Spirulina* sp. for the degradation for glyphosate may be different the pathway which already exhibited in the other bacteria. It is further re-confirmed by Lipok et al. (2009) that *Spirulina platensis* able to degrade and metabolize glyphosate. Forlani et al. (2008) further investigate the ability of *Anabaena* sp., *Lyngbya boryana*, *Microcystis aeruginosa* and *Nostoc punctiforme* to use glyphosate as the only source of phosphorus. It is also observed that cyanobacteria *Trichodesmium erythraeum* also showed presence of phosphorous-dependent glyphosate transformation (Dyhrman et al., 2006).

However, there are no such reports are available on cyanobacteria use glyphosate as a source of nitrogen. During working with *Anabaena variabilis*, Ravi and Balakumar (1998) observed that extracellular phosphatases hydrolyzed the C-P bond of glyphosate, but this claim has not been supported so far by the other authors. Extracellular phosphatases may play considerable role in the glyphosate degradation (Forlani et al., 2008). Arunkumara et al. (2013) stated that the ability of cyanobacteria to use this organophosphate as a source of phosphorus could effectively implied for the cleanup of the pesticides.

Table 2.4 Cyanobacteria species responsible for degradation of various pesticides

Synthetic pesticide	Cyanobacteria	References
2,4- D (Dichlorophenoxyacetic acid)	<i>Anabaena fertilissima</i> , <i>Aulosira fertilissima</i> , <i>Westiellopsis prolifica</i>	Kumar et al., 2013
2,4-DNP (Dinitrophenol)	<i>A. variabilis</i> <i>A. cylindrica</i>	Hirooka et al., 2006
Anilofos	<i>Synechocystis</i> sp. Strain PUPCCC 64	Singh et al., 2013
Acetachlor	Cyanobacteria mat consisting of <i>Phormidium</i> sp. and <i>Oscillatoria</i> sp.	El-Nahhal et al., 2013
Carbaryl	<i>Calothrix berevissima</i>	Habib et al., 2011
Carbendizm	<i>Oscillatoria</i> sp.	Ravindran et al., 2000
Carbofuran	<i>A. sphaerica</i> , <i>Nostoc hatei</i> , <i>W. prolifica</i>	Jha & Mishra, 2005
Chlorpyrifos	<i>P. valderianum</i> , <i>Spirulina platensis</i> , <i>Synechocystis</i> sp. strain PUPCCC64	Palanishami et al., 2009 Thengodhar & Sivakami, 2010 Singh et al., 2011
Cypermethrin	<i>Oscillatoria</i> sp.	Ravindran et al., 2000
Endosulfan	<i>Anabaena</i> sp. PCC 7120 <i>A. flos-aquae</i> <i>A. fertilissima</i>	Lee et al., 2003 Kumar et al., 2012
Fenamiphos	<i>Nostoc muscorum</i> , <i>Anabaena</i> sp.	Caceres et al., 2008
Glyphosate	<i>S. platensis</i> , <i>N. punctiforme</i> , <i>M. aeruginosa</i> , <i>L. boryana</i>	Forlani et al., 2008; Lipok et al., 2009; Arunkumara et al., 2013
Isoproturon	<i>A. inaequalis</i>	Mostafa & Helling, 2001
Lindane	<i>M. aeruginosa</i> , <i>Pseudoanabaena limnetica</i> <i>Anabaena</i> sp. strain PCC 7120 <i>N. elliposporum</i>	Gonzalez et al., 2012 Kuritz & Wolk, 1995 El-Bestawy et al., 2007
Malathion	<i>A. oryzae</i> , <i>N. muscorum</i> , <i>S. platensis</i> <i>Anabaena</i> sp. atrain PCC 7120	Ibrahim et al., 2014
Methyl parathion	<i>A. fertilissima</i> , <i>W. prolifica</i>	Barton et al., 2004
Penycuron		Kumar et al., 2013

### 2.10 General structure and mode of action of chlorpyrifos

Chlorpyrifos is the chemically known as 0, 0-diethyl 0-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate with CAS (Chemical Abstracts Service) registry number is 2921-88-2.1. It has molecular weight, specific gravity and vapour pressure of 350.62, 1.398 (at 43 °C) and at 2.49 MPa (25 °C) respectively. The solubility of

Chlorpyrifos in water is 1.39 mg/L (25 °C). Chlorpyrifos is moderately persistent in nature and have half-life of 60-120 days in soil and 3-9 days in water.

Chlorpyrifos is a widely used organophosphate insecticide. It is used for variety of purposes from agricultural food and feed crops to nonagricultural uses such as pest control in golf course turf, industrial plants and vehicles, wood furniture, fence posts and utility poles; and also for public health to control mosquitoes and fire ants. It is also registered for indoor residential use but only in case of containerized baits control (US EPA, 2006).

Chlorpyrifos is primarily known as a non-systemic insecticide and it kills the insects through direct contact, ingestion, and inhalation (Tomlin, 2006). Smegal (2000) suggested that it affects the nervous system of insects by inhibiting the breakdown of acetylcholine (ACh), a neurotransmitter. It is observed that chlorpyrifos occupied the active site of the cholinesterase (ChE) enzyme (stops breakdown of ACh in the synaptic cleft). It causes overstimulation of the neuronal cells due to accumulation of ACh in the synaptic cleft, leads to neurotoxicity or may be death (Karanth and Pope, 2000).

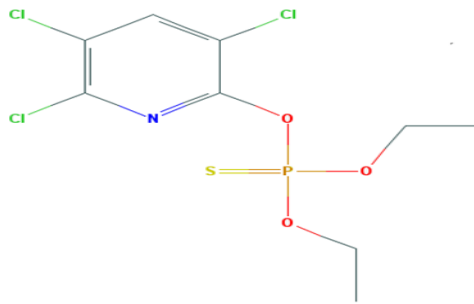
Chlorpyrifos is moderately persistent in soils with half-life of 6-120 days, but it is also found in the soils over one year after it is applied in the fields. The persistence of chlorpyrifos in the soil may depend up on several factors such as the formulation, rate of applied rate, soil type and climate (Kamrin, 1997). It is also observed that chlorpyrifos strongly binds to soils or simply it is moderately immobile in the soil. Although chlorpyrifos has low water solubility, but Roberts and Hutson (1999) suggested that soil bound chlorpyrifos degrade into TCP and other metabolites through UV light, chemical hydrolysis, dechlorination, and by the action soil

microbes. Unlike Chlorpyrifos, TCP also found to bind to the soil particles and showed moderate persistence in the soils (Kamrin, 1997).

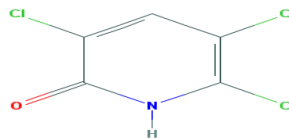
### **2.11 Chlorpyrifos degradation**

Racke et al. (1990; 1996) suggested that chlorpyrifos showed more persistence than other organophosphorus compounds. A number of studies evident that microorganisms especially bacteria were investigated for the degradation of chlorpyrifos such as *Pseudomonas diminuta* (Munnecke et al., 1975), *Micrococcus* sp. (Guha et al., 1997), *Flavobacterium* sp. (Mallick et al., 1999), *Alcaligenes faecalis* DSP3 (Yang et al., 2005), *Stenotrophomonas* YC-1 (Yang et al., 2006), *Klebsiella* sp. (Ghanem and Sharma, 2007), *Sphingomonas* sp. Dsp-2 (Li et al., 2007); and *Pseudomonas fluorescence*, *Brucellamelitensis*, *Bacillus subtilis* and *Bacillus cereus* (Lakshmi et al., 2008). Mallick et al. (1999) isolated *Arthrobacter* sp. from methyl parathion-contaminated soil and further investigate the degradation capability against Chlorpyrifos. Hussain et al. (2007) observed that soil fungi, *Trichoderma viride* and *Aspergillus niger* able to degrade chlorpyrifos.

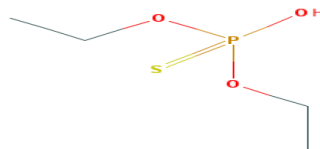
There are other chlorpyrifos-degrading fungi, such as *Phanerochaete chrysosporium*, *Aspergillus terreus*, and *Verticillium* sp. DSP which showed the promising results (Jayashree and Vasudevan, 2007). Chen et al. (2012) concluded that *Cladosporium cladosporioides*



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



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



**DETP (Diethylthiophosphoric acid)**

**Figure 2.2** Degradation pathways of Chlorpyrifos

strain Hu-01 able to metabolize completely chlorpyrifos and their degradation product TCP. In most studies up to recent various bacteria transform chlorpyrifos by hydrolysis to produce TCP and diethylthiophosphoric acid (DETP). Due to more persistence nature, TCP accumulated in the aqueous media, while in some studies it is also observed some bacteria able to utilize DETP as a source of carbon and phosphorus (Singh et al., 2003; 2004). It is also observed that *Flavobacterium* sp. ATCC 27551 and *Arthrobacter* sp. very efficiently degrade the chlorpyrifos in the aqueous media in which chlorpyrifos is provided as a sole carbon source. Some studies showed that *Enterobacter* strain B-14, *Alcaligenes faecalis*, and *Klebsiella* sp. degrade chlorpyrifos and utilize chlorpyrifos as sole carbon source, while other studies also emphasised on that *Flavobacterium* sp. ATCC 27551 and *Arthrobacter* sp. completely metabolize the Chlorpyrifos (Jilani and Khan, 2004). Getzin (1981) and Gomez et al. (2007) also suggested that *Bacillus* sp. and *Micrococcus* sp. has the capability to degrade chlorpyrifos.

### **2.12 Degradation of chlorpyrifos by cyanobacteria**

There are few reports are available on the degradation of chlorpyrifos by cyanobacteria (Palanishami et al., 2009; Thengodhar and Sivakami, 2010; Singh et al., 2013; Kumar et al., 2014); but still mechanism of chlorpyrifos degradation in cyanobacteria is not fully known. Some reports reported the cyanobacteria able to transform chlorpyrifos in to TCP (Singh and Walker, 2006; Singh et al., 2013); while some reports also suggested that cyanobacteria might be able to total degradation of chlorpyrifos without production of earlier reported degradation products (Kumar, 2011). Thengodkar and Sivakami (2010) and Singh et al. (2011) also reported the same phenomenon of total degradation of pesticides without releasing degradation products.

## *CHAPTER 3*

# *Analyses of Soil Physicochemical Parameters and Nutrients Status of the Selected Paddy Fields*

**3.1 Introduction**

Paddy crop grown mostly in tropical conditions; but it is also successfully cultivated in temperate and sub-tropical conditions. In soils, it is cultivated in almost all soil types such as alluvium, red-yellow, red loamy, hill and sub-montane, terai, laterite, red sandy, mixed red and black and medium and shallow black soils. But due to good porosity and sandy and clay in nature, alluvial soils provide good drainage and other favourable conditions for paddy cultivation. They are found in flood plains and deltas of big rivers, coastal plains, fans and terraces. Every group of soils are different in nature and composition. And even within one group of soils may be different in nature or composition such as alluvial soils; which depends on the nature and the degree of weathering of the parent sediments. These differences resulted in the differences in the fertility status of these different soils, which further responsible for the differences in productivity of paddy.

The soil fertility is a significant parameter, which affect the plant growth. The fertility of soil can be determined by availability of N, P, K, organic and inorganic materials, micronutrients and water. In view of the importance of soil physico-chemical parameters, pH affects the availability of micro-nutrients present in soil (Williams, 1990).

Brady and Weil (2002) suggested that low pH favours the high solubility of micronutrients, while high pH declines the solubility and availability of micronutrient to the plant. The pH of the soil generally increased with the depth. Therefore, high and low pH values could be responsible for crop failure due to imbalance in

concentration of ions in the soil (Kumar et al., 2011). Electrical conductivity (EC) is the evaluation of the concentration of soluble salts in the soil; generally known as salinity (Wagh et al., 2013). De-Neve et al. (2000) reported that EC changes with the moisture content soil particles. It could be very helpful to monitor the mineralization of organic matter present in the soil.

Nitrogen is an essential macronutrient for the plant growth. There are several factors that affects the nitrogen content in the soil i.e. crop uptake, immobilization by microorganism and its loss through volatilization (Defoer et al., 2000). It can be supplemented by the use of inorganic nitrogen fertilizers or manures. Parham et al. (2002) compared manures treated soil with inorganic fertilizer treated soil and observed that inorganic fertilizer treated soil showed higher nitrogen content. But due to use of excessive inorganic nitrogen fertilizers in the agriculture, farmers are facing major problem i.e. soil acidification (Jain and Singh, 2014).

Phosphorus is another essential macronutrient that is necessary for the plant growth and metabolism (Solanki and Chavda, 2012; Wagh et al., 2013). Singh and Rathore (2013) suggested that the availability of phosphorus content of the soil is depend up on the topography; soils in higher topographic position shows higher available phosphorus content as compared to soils occurring on lower topographic position. It is also reported that the soil enriched with high organic matter content facilitates the better supplies of organic phosphate for plant uptake in comparison of soils with low organic content (Miller and Donahuer, 2001).

Organic matter content is another characteristic that indicates the fertility of soil and it is also affects the availability of the other nutrients. It is helpful in binding of soil particles, prevents the soil from erosion. Hodes (1996) suggested that high organic matter content in the soil could be helpful in lowering of the pH. It is also

reported that organic matter content of the soil content decreased from surface to subsoil due to the process of levelling (Helburg et al., 1978). It also acts as food for soil microbes and thereby helps in enhancing their populations (Brady, 1996).

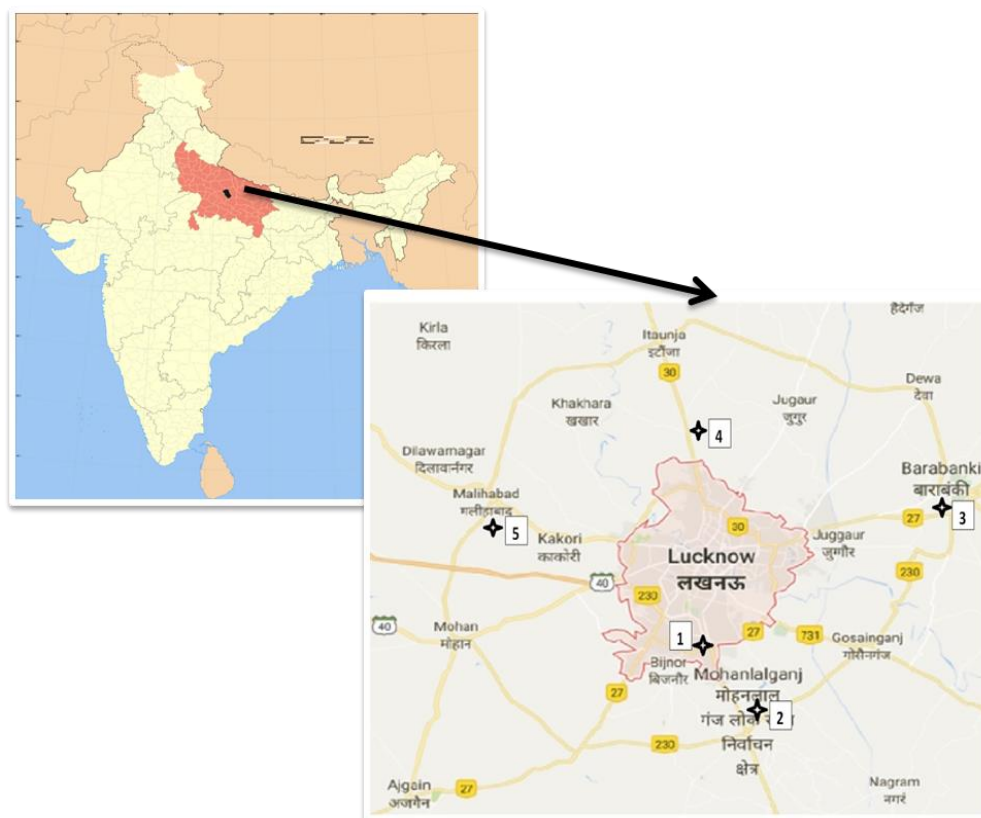
### 3.2 Materials and methods

#### 3.2.1 Study sites

Soil samples were collected from paddy fields from the adjoining areas of Lucknow city, Uttar Pradesh (India). The selected sites for present study were:

**Table 3.1** Location of study sites

SNo	Study sites	Co-ordinates
1.	BBAU Campus	26°46'03.0"N, 80°55'36.8"E
2.	Mohanlalganj	26°42'00.9"N, 80°59'18.4"E
3.	Malihabad	26°54'46.3"N, 80°43'27.6"E
4.	Bakshi ka Talab	26°58'59.3"N, 80°56'41.3"E
5.	Barabanki	26°54'12.8"N, 81°10'21.3"E



**Figure 3.1** Location of study sites

### **3.2.2 Collection of soil samples**

Soil samples from each site were collected from the subsurface horizon (2–15 cm) at three different points and mixed thoroughly. The collected soil samples were brought to the laboratory in polythene bags, air dried and sieved before analyses. The soil texture of all the sites was sandy-clay in nature.

### **3.2.3 Physico-chemical analysis of soil samples**

The physicochemical properties of soil such as Temperature, pH and Electrical conductivity; and Nutrient status as Total-N, -P and Organic-C contents were measured as per standard procedures.

#### **3.2.3.1 Temperature**

The temperature of soil is directly linked to the temperature of the atmosphere because soil is an insulator for heat flowing between the solid earth and the atmosphere.

##### **3.2.3.1.1 Procedure**

1. Cleaned the surface of probe rod of thermometer.
2. Inserted the probe rod of digital thermometer in to soil up to 5-6 inches. Note the temperature immediately.
3. Washed the probe rod with distilled water.
4. Again inserted the probe rod in to the soil, and note the temperature.
5. At least 2-3 reading were taken per location.

#### **3.2.3.2 Soil pH**

##### **3.2.3.2.1 Apparatus**

pH meter with a range of 0-14 pH, Pipette/dispenser, Beaker, Glass rod.

**3.2.3.2.2 Reagents**

Buffer solutions (pH 4, 7 and 9)

Deionized water

**3.2.3.2.3 Procedure**

1. Air dried soil for 1-4 days depending on the relative humidity and soil properties. Then grind the air-dried soil to pass 2 mm sieve and mix well.
2. Calibrated the pH meter, using buffers of pH 7.0, pH 4.0 and pH 9.2.
3. Weighed 10.0g of air dried soil sample into 50 or 100 ml beaker, add 20ml deionized water.
4. Allowed the soil to deionized water without stirring and then thoroughly stir for 10 second using a glass rod.
5. Stirred the suspension for 30 minutes and record the pH on the calibrated pH meter.

**3.2.3.3 Electrical conductivity**

Electrical conductivity (EC) is expressed in units of milli-Siemens per meter (mS/m). Soil EC measurements may also be reported in units of deci-Siemens per meter (dS/m), which is equal to the reading in mS/m divided by 100.

**3.2.3.3.1 Apparatus**

Electrical conductivity meter, Beakers (25 ml), Erlenmeyer flasks (250 ml) and pipettes, Filter paper

**3.2.3.3.2 Reagents**

0.01M Potassium chloride solution: Dried potassium chloride at 60<sup>0</sup> C for two hours. Weighed 0.7456 g of it and dissolve in distilled water and make the

volume to one litre. This solution gives an electrical conductivity of 1.412 mS/cm at 25<sup>0</sup>C.

### 3.2.3.3.3 Procedure

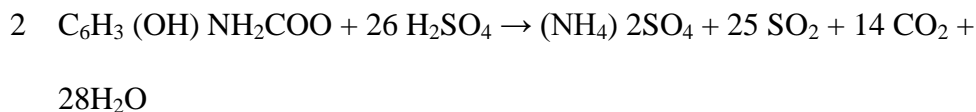
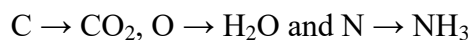
1. Took 20 g soil into 50 ml Erlenmeyer flask, add 40 ml of distilled water, stirred well flask the flask and filtered through Whatman No.1 filter paper. The filtrate kept for the measurement of conductivity.
2. Washed the conductivity electrode with distilled water and rinsed with standard KCl solution.
3. Calibrated the EC meter with KCl solution into a 25 ml beaker and dip the electrode in the solution and adjusted the conductivity meter to read 1.412 mS/cm.
4. Washed the electrode and dip it in the soil extract.
5. The reading in mS/cm of electrical conductivity is a measure of the soluble salt content in the extract, and an indication of salinity status of this soil. The conductivity can also be expressed as mmhos/cm.

### 3.2.3.4 Total-N

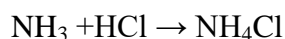
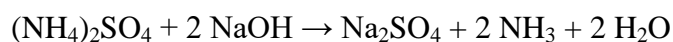
#### 3.2.3.4.1 Principle

The Kjeldahl method (Kjeldahl, 1883) permits the available nitrogen to be precisely determined in the plant and in the soil. The method of determination involves three successive phases which are:

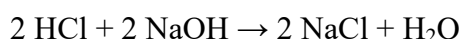
**Digestion:** Digestion of the organic material is carried out by digesting the sample with Con. H<sub>2</sub>SO<sub>4</sub> in the presence of CuSO<sub>4</sub>.H<sub>2</sub>O as a catalyst and K<sub>2</sub>SO<sub>4</sub> which raise the digestion temperature. The organic material decomposes into several components i.e.,



**Distillation:** The Ammonia content of the digest is determined by distillation with excess NaOH and absorption of the evolved NH<sub>3</sub> is in standard HCl.



**Volumetric Analysis:** The excess of standard HCl is titrated against standard NaOH using Methyl Red as an indicator. Through acid-base titration, decrease in the multi equivalence of acid determined which gives a measure of the N content of the sample. The end point is determined by a change of colour from pink to yellow.



#### 3.2.3.4.2 Apparatus

Nitrogen distillation unit, conical flask, pipettes and burette etc

#### 3.2.3.4.3 Reagents

0.32% KMnO<sub>4</sub>: Dissolved 3.2 g of KMnO<sub>4</sub> in water and make the volume to one litre.

2.5% NaOH: Dissolved 25 g of sodium hydroxide pellets in water and make the volume to one litre.

2% Boric acid: Dissolved 20 g of boric acid powder in warm water by stirring and dilute to one litre.

Mixed Indicator: Dissolved 0.066 g of methyl red and 0.099 g of bromocresol green in 100 ml of ethyl alcohol. Add 20 ml of this mixed indicator to each litre of 2% boric acid solution.

0.1M Potassium Hydrogen Phthalate: Dissolved 20.422 g of the salt in water and dilute to one litre. This is a primary standard and does not require standardization.

0.02M H<sub>2</sub>SO<sub>4</sub>: Prepared approximately 0.1M H<sub>2</sub>SO<sub>4</sub> by adding 5.6 ml of conc. H<sub>2</sub>SO<sub>4</sub> to about 1 litre of distilled water. From this, prepare 0.02M H<sub>2</sub>SO<sub>4</sub> by diluting a suitable volume (20 ml made to 100 ml) with distilled water. Standardize it against 0.1M NaOH solution.

0.1M NaOH. Dissolved 4gm of NaOH pellets in 100 ml distilled water.

#### **3.2.3.4.4 Procedure**

1. Weighed 20 g of soil sample in 800 mL Kjeldahl flask.
2. Moisten the soil with about 10 mL of distilled water, washed down the soil, if any, adhering to the neck of the flask.
3. 100 mL of 0.32% of KMnO<sub>4</sub> solution added in the flask.
4. A few glass beads or broken pieces of glass rod added.
5. Added 2-3 mL of paraffin liquid, avoiding contact with upper part of the neck of the flask.
6. Measured 20 mL of 2% boric acid containing mixed indicator in a 250 mL conical flask and placed it under the receiver tube. Dipped the receiver tube in the boric acid.
7. Ran tap water through the condenser.

8. 100 mL of 2.5% NaOH solution added and attached immediately to the rubber stopper fitted in the alkali trap.
9. Switched on the heaters and continue distillation until about 100 mL of distillate is collected.
10. First removed the conical flask containing distillate and then switch of the heater to avoid back suction.
11. The distillate titrated against 0.02M H<sub>2</sub>SO<sub>4</sub> taken in burette until pink colour starts appearing.
12. Ran a blank without soil.
13. Carefully removed the Kjeldahl flask after cooling and drained the contents in the sink.

#### **3.2.3.4.4 Calculations**

##### **(a) Blank:**

Volume of HCl taken for blank = a mL

Volume of NaOH used = b mL

Volume of HCl consumed by liberated NH<sub>3</sub> present in blank = a – b = z mL

##### **(b) Sample:**

Volume of HCl taken for sample = v mL

Volume of NaOH used = u mL

Volume of HCl consumed by liberated NH<sub>3</sub> present in sample = v – u = w mL

Volume of HCl consumed for NH<sub>3</sub> liberated by sample only = w – z = y mL

1000 mL 1N HCl = 1000 mL 1 N NH<sub>3</sub> = 17 g NH<sub>3</sub> = 14 g N

$$1 \text{ mL } 1\text{N HCl} = 1 \text{ mL } 1 \text{ N NH}_3 = 0.014 \text{ g N}$$

$$1 \text{ mL } 0.1 \text{ N HCl} = 1 \text{ mL } 0.1 \text{ N NH}_3 = 0.0014 \text{ g N}$$

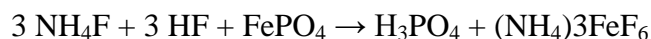
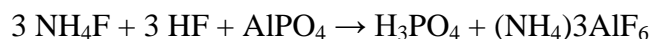
$$\text{Weight of Nitrogen in 20 g of Sample} = y \times 0.0014 \text{ g N} = q \text{ g N}$$

$$\begin{aligned} \text{\% of N in sample} &= \frac{q \times 100}{20} \\ &= p \text{ \%} \end{aligned}$$

### **3.2.3.5 Total-P**

#### **3.2.3.5.1 Principle**

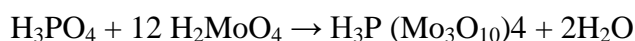
The total-P was measured according to the method of Dickman and Bray (1940). The underlying principle of this method is that the soil is shaken with an extracting solution of 0.03 N NH<sub>4</sub>F in 0.025 N HCl, which dissolves the fraction of phosphorus roots. Ammonium Fluoride complexes with Al and Fe ions in the acid solution with the consequent release of Phosphorus held by the soil, by these trivalent ions. Thus the combination of HCl and NH<sub>4</sub>F helps in removing easily acid soluble forms of phosphorus, largely Calcium Phosphates and a portion of aluminium and iron phosphates. The reactions in acid solution may be represented as follows,



In the above reactions AlPO<sub>4</sub> and FePO<sub>4</sub> represents various hydrated and hydroxyl phosphates of Al + Fe including any absorbed or precipitated surface layer on oxides of Al, Alumino-silicates and an oxide of Fe. HCl results in the dissociation of more active calcium, phosphate and in addition to this, it prevents the precipitation

of phosphorus (as calcium phosphate) which has been dissolved by ammonium fluoride.

In the filtered extract phosphorus is estimated calorimetrically by adding ammonium molybdate and thereafter, reducing the molybdenum phosphate complexes with stannous chloride in the acidic medium. The hetero-complex (phosphorus molybdate) is formed by coordination of molybdate ions with Phosphorus as the central coordinating atom, the oxygen of the molybdate being substituted for that of PO<sub>4</sub>. The reaction may be represented as follows.



The heteropoly complexes, before reduction gives a yellow colour to their water solution. In the solution of low Phosphorus concentration, the molybdate is partially reduced to a characteristic blue colour. The intensity, a measure of concentration of Phosphorus in the test sample is read on colorimeter.

#### **3.2.3.5.2 Apparatus**

Conical flask (500 ml), Pipettes (2, 10 and 20 ml), Burette (50 ml), Whatman No. 42 filter paper.

#### **3.2.3.5.3 Reagents**

1 N Ammonium fluoride: Dissolved 37 g NH<sub>4</sub>F in distilled water and dilute the solution to 1 L. Store this solution in a polyethylene bottle.

0.5 N HCl: Dissolved 20.2 mL Con. HCl to a volume of 500 mL with distilled water.

Extracting solution: Added 15 mL 1N  $\text{NH}_4\text{F}$  and 25 mL 0.5 N HCl to 450 mL distilled water. This gives a solution of 0.03 N  $\text{NH}_4\text{F}$  and 0.025 N HCl.

Dickman and Bray's reagent: Dissolved 15 g of ammonium molybdate  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$  in 300 mL distilled water. Warmed to about  $60^\circ\text{C}$ , filter and cool. To that add 34.2 mL of conc. HCl and make up the volume to 1 L. This is 1.5% solution of ammonium molybdate in the HCl.

Stannous chloride ( $\text{SnCl}_2 \cdot 2 \text{H}_2\text{O}$ ) solution: Dissolved 2 g  $\text{SnCl}_2 \cdot 2 \text{H}_2\text{O}$  crystals in 8.3mL conc. HCl, dilute to 100 mL and store in amber bottle. This is 40 %  $\text{SnCl}_2$  stock solution. A piece of tin metal if added will keep the stock solution for long.

Standard phosphorous solution: 0.439 g of potassium dihydrogen phosphate is weighed into a 500 ml standard flask. Added approximate 25 mL Con.  $\text{H}_2\text{SO}_4$  to it and make up the solution. 2 mL from this solution pipetted out into a 10 mL standard flask and make up the solution. This gives 2 ppm stock solution of phosphorus.

#### **3.2.3.5.4 Procedure**

##### **(a) Extraction:**

1. Weighed 5 g soil and transfer it to a 100 mL conical flask.
2. 50 mL extract solution added to the soil.
3. Shook the content for exact 5 minutes, and filter through Whatman No. 42 filter paper.
4. Prepared a blank in which all the reagents are added similarly, except the soil.

**(b) Estimation (Dickman and Bray, 1940):**

1. Took 7.0 and 25.0 mL standard flasks.
2. Labelled one as “sample” another one as “blank” and all others as 0.2, 0.4, 0.6, 0.8 and 1.0.
3. About 5.0 mL of soil extract pipetted out into one 25mL standard flask which is labelled as sample.
4. 5 mL Dickman and Bray’s reagent taken using 5 ml pipette and transferred that into 25.0 mL standard flask which contain the soil extract. To that 7.5 mL boric acid added.
5. Took the standard phosphorus solution in a clean burette.
6. From this burette 1, 2, 3 etc upto 5 mL standard phosphorus solution add in previously labelled 25.0 mL standard flasks (except in blank).
7. Pipetted out 5.0 mL “Dickman and Bray’s reagent” and transferred that into each 25.0 mL standard flask containing “standard phosphorus solution”. To that 7.5 mL boric acid added.
8. Took a test tube full of distilled water and added through the neck of the flask down to remove the adhering ammonium molybdate. Mixed the content thoroughly and kept.
9. Finally added 1mL  $\text{SnCl}_2$  working solution with immediate mixing and make up to the mark with distilled water once again, mix the solution thoroughly.
10. Similarly a blank was also prepared. Measured the intensity of blue colour just after 10 minutes at 690 nm.
11. A graph plotted between absorbance against the concentration in ppm and determined the concentration of P in soil samples from the standard curve.

This is very important that colour starts fading after about 15-20 minutes at development of colour.

#### **3.2.4.5.5 Calculation**

$$\text{ppm P in soil} = \text{ppm P in solution (gram graph)} \\ \times \frac{\text{Total extracting solution used(ml)}}{\text{Weight of soil}}$$

#### **3.2.3.6 Organic-C**

##### **3.2.3.6.1 Principle**

The determination of soil organic carbon is based on the Walkley-Black chromic acid wet oxidation method (Walkley and Black, 1934). The soil organic matter is oxidised by 1 N  $\text{K}_2\text{Cr}_2\text{O}_7$  solution. The reaction is assisted by the heat generated when two volumes of  $\text{H}_2\text{SO}_4$  are mixed with one volume of the dichromate. The remaining dichromate is titrated with ferrous sulphate. The titre is inversely related to the amount of C present in the soil sample.

##### **3.2.3.6.2 Apparatus**

Conical flask (500 mL), pipette (50mL), burette, reflux condenser

##### **3.2.3.6.3 Reagents**

0.5 N ferrous ammonium sulphate: Dissolved 196 g ferrous ammonium sulphate in 800 mL distilled water containing 20 mL  $\text{H}_2\text{SO}_4$  and diluted the solution to 1 L.

1N Potassium dichromate solution: Dissolved 49.04 g potassium dichromate in distilled water and dilute the solution to 1 L.

Diphenylamine indicator: 0.5 g diphenyl amine dissolved in a mixture of 20 ml of water and 100 ml of concentrated sulphuric acid.

Phosphoric acid

#### **3.2.3.6.4 Procedure**

1. Took 1.0 g of soil in a 500 mL conical flask.
2. 10 mL of 1N  $K_2Cr_2O_7$  solution added in to flask and shake to mix it.
3. Then added 20 mL conc.  $H_2SO_4$  and swirl the flask 2 or 3 times.
4. Allowed the flask to stand for 30 minutes on an asbestos sheet for the reaction to complete.
5. 200 mL of water poured to the flask to dilute the suspension. Filter if it is expected that the end point of the titration is not to be clear.
6. Added 10 mL of 85%  $H_3PO_4$  and 1 mL of diphenylamine indicator and back titrate the solution with 0.5 N ferrous ammonium sulphate, till the colour flashes from violet through blue to bright green.  $H_3PO_4$  gives sharper endpoint, by making the colour change, distinct through a flocculating effect.
7. Note the volume of ferrous ammonium sulphate.
8. Run the blank titration (without soil) in similar manner.

#### **3.2.3.6.5 Calculation**

$$\% \text{ of Organic carbon in soil, } R = \frac{W \times (V_1 - V_2) \times N \times 0.003 \times 100}{W} \times C$$

Where,

W - Weight of Sample

$V_1$  - Blank Titre value

$V_2$  - Titre value of the Sample

N - Normality of  $K_2Cr_2O_7$  (Here it is 1N)

C - Correction Factor (1.334, 1.724)

### **3.2.4 Statistical analysis**

All parameters were investigated in triplicates and the values are given as means  $\pm$ SE. Further the data were analysed by one-way ANOVA according to Duncan's multiple range test ( $P=0.05$ ) to find the significance level.

## **3.3 Results**

### **3.3.1 Soil physico-chemical properties**

ANOVA revealed significant differences ( $P<0.001$ ) in soil physico-chemical characteristics due to sites (Table 3.1). In the collected soil samples, temperature varied from 28.4°C (BBAU campus) to 30.4°C (Malhiabad). The lowest pH (7.5) was recorded at Barabanki site, while highest pH (8.7) was noted for the Mohanlalganj site. The electrical conductivity (EC) of soil samples ranged from 0.11 ms/cm (Malhiabad) to 0.29 ms/cm (Mohanlalganj).

Among the nutritional status of collected soil samples total-N ranged from 0.11 % (Mohanlalganj) to 0.22 % (Bakshi ka Talab). Total-P was recorded from 0.5 % (Mohanlalganj) to 0.7 % (Bakshi ka Talab). The Organic-C was noted from 0.43 % (Mohanlalganj) to 0.81 % (Bakshi ka Talab).

**Table 3.2** Physico-chemical properties of soil samples collected from different selected sites of Lucknow district and adjoining area. The values given are means of 3 independent experiments  $\pm$ SE. N=15 (5 sites  $\times$  3 replicates).

Parameters	BBAU campus	Mohanlalganj	Barabanki	Bakshi ka Talab	Malhiabad	Significance level
Temperature (°C)	28.4 $\pm$ 0.08 <sup>a</sup>	28.5 $\pm$ 0.06 <sup>a</sup>	29.4 $\pm$ 0.04 <sup>b</sup>	30.1 $\pm$ 0.02 <sup>c</sup>	30.4 $\pm$ 0.04 <sup>c</sup>	P < 0.001
EC (ms cm <sup>-1</sup> )	0.25 $\pm$ 0.05 <sup>a</sup>	0.29 $\pm$ 0.03 <sup>b</sup>	0.25 $\pm$ 0.02 <sup>c</sup>	0.12 <sup>b</sup> $\pm$ 0.03	0.11 <sup>b</sup> $\pm$ 0.05	P < 0.001
Soil pH	8.4 $\pm$ 0.07 <sup>a</sup>	8.7 $\pm$ 0.01 <sup>a</sup>	7.5 $\pm$ 0.06 <sup>b</sup>	8.1 $\pm$ 0.01 <sup>c</sup>	7.9 $\pm$ 0.07 <sup>b</sup>	P < 0.001
Total-N (%)	0.17 $\pm$ 0.06 <sup>a</sup>	0.11 $\pm$ 0.08 <sup>b</sup>	0.12 $\pm$ 0.03 <sup>b</sup>	0.22 $\pm$ 0.02 <sup>c</sup>	0.14 $\pm$ 0.06 <sup>d</sup>	P < 0.001
Total-P (%)	0.7 $\pm$ 0.03 <sup>a</sup>	0.5 $\pm$ 0.04 <sup>b</sup>	0.6 $\pm$ 0.01 <sup>c</sup>	0.7 $\pm$ 0.07 <sup>a</sup>	0.6 $\pm$ 0.02 <sup>c</sup>	P < 0.001
Organic-C (%)	0.69 $\pm$ 0.07 <sup>a</sup>	0.43 $\pm$ 0.04 <sup>b</sup>	0.60 $\pm$ 0.09 <sup>c</sup>	0.81 $\pm$ 0.05 <sup>d</sup>	0.64 $\pm$ 0.07 <sup>a</sup>	P < 0.001

EC=electrical conductivity. Values of each row followed by the same letter(s) are not significantly different according to Duncan's multiple range test (P=0.05).

### 3.4 Discussion

Morales et al. (2011) suggested that soil pH also influenced by the soil moisture content; leads change in the chemical equilibrium which further affects the form and effectiveness of soil nutrient elements present in the soil. In tropics, flooding commonly practiced for the paddy cultivation; it is observed that flooding facilities the availability of phosphorus and potassium; but also reduces the availability of nitrogen, sulphur and zinc (Fan et al., 2008; Das et al., 2016). It is also observed that phosphorous availability initially enhanced by flooding in lowland rice soils and it is used by paddy to fulfil phosphorous requirement (Gupta et al., 2007). Reeves (1997) suggested that soil organic content could be a key soil physicochemical parameter to assess quality of soil and its maintenance is crucial for the productivity and long-term stability of paddy agroecosystem (Carter, 2002).

The variation in soil physico-chemical properties across different selected sites (Table 3.1) may effects the soil quality and fertility of the paddy agro-ecosystem (Schoenholtz, 2000; Tale and Ingole, 2015; Kekane et al., 2015). Several physico-chemical parameters like soil temperature, pH, electrical conductivity, total -N, -P and organic-C leads to quality and nutrient status of the paddy field soil of adjoining areas of Lucknow district (Uttar Pradesh). Results showed that all the five sampling sites i.e. BBAU campus, Mohanlalganj, Malihabad, Bakshi ka Talab and Barabanki have suitable pH, EC and also good amount of nutrients (N, P and C) which well supported the cyanobacterial growth in paddy agro-ecosystem (Kumar et al., 2018). There are various factors that affect the distribution and abundance of cyanobacteria in the paddy field but pH is an important factor and it has been observed that an increase in pH along with decrease in light intensity and less nitrogen favoured cyanobacterial growth (Kanniayan and Kumar, 2004). This information about physico-chemical parameters of paddy field soil and its correlation with the cyanobacterial diversity could be very useful for further study. A wide variation in soil physico-chemical characteristics across different selected sites could be due to variation in anthropogenic activities and consequently variation in occurrence of different cyanobacterial species.

## *CHAPTER 4*

# *Isolation and Identification of Existing Cyanobacterial Diversity from Paddy Fields*

**Isolation and identification of existing cyanobacterial diversity from paddy fields**

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**4.1 Introduction**

Cyanobacteria are the tiny prokaryotes that successfully survived through 1.5 billion years (Kulasooriya, 2011; Prasanna et al., 2009, Singh et al., 2016). This was the reason behind the remarkable flexibility and adaptability to the extremes of temperature, salinity, pH, drought, radiance, pollution and nutrient suitability. These aspects also remained through the evolution deliberated cosmopolitan nature cyanobacteria and their far reaching role in maintaining ecological balance in natural ecosystems and agro-ecosystems.

In the agro-ecosystems, cyanobacteria occurred even at 20 cm depth with pronounced effect on the surface soil layer (Goyal, 1996). Nowadays they have gaining much attention in agriculture as an input, which improves and enhance the fertility of paddy field soil (Singh, 1973; Goyal and Goyal, 1998; Singh et al., 2016). Kaushik (1994) reported that cyanobacteria as bioinoculants able to reduce the nitrogenous fertilizer consumption up to 30%. Cyanobacterial could provide fixed nitrogen to paddy crop in the range of 20-30 kg/ha.

Cyanobacteria naturally inhibit the paddy agro-ecosystem and it existed not only on the surface of paddy soil as well as flood water. These are the common paddy field cyanobacteria such as *Anabaena*, *Aulosira*, *Calothrix*, *Gleotrichia*, *Cylindrospermum*, *Nostoc*, *Fischeiella*, *Scytonema*, *Tolypothrix* and *Wolleea* (Ahmed et al., 1999; Tiwari et al., 2000; Nayak et al., 2001; Kaushik and Prasanna, 2002; Mishra and Pabbi, 2004; Choudhury and Kennedy, 2005; Rai, 2006, Nayak and Prasanna, 2007; Dey et al., 2010; Selvi and Sivakumar, 2011; Nongbri and Syiem,

2012; Danaboyina and Sivakumar, 2013; Singh et al., 2014; Vijayan and Ray, 2015). Kannaiyan (1985) reported incident of cyanobacterial blooms just after transplanting the paddy saplings in fields due to use of fertilizers or ploughing, or both; and also better availability of sunlight. It is investigated that flooding affects the distribution of cyanobacteria in paddy fields and also observed that cyanobacterial population increased by about 4-5 times during flooded period as compared to pre-flood and post-flood conditions (Begum et al., 1988).

There are various factors that affect the distribution and abundance of cyanobacteria in the paddy fields; pH is an important factor. Kannaiyan (1990) and Kannaiyan and Kumar (2004) observed that during the vegetative growth of paddy crop, an increase in pH along with decrease in light intensity and less nitrogen favoured cyanobacterial growth.

## **4.2 Materials and Methods**

### **4.2.1. Media composition**

The medium used throughout the study was BG-11media which has following constituents:

NaNO <sub>3</sub>	1.5 g
K <sub>2</sub> HPO <sub>4</sub>	0.04 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.075 g
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.036 g
Citric acid	0.006 g
Ferric ammonium citrate	0.006 g
EDTA (disodium salt)	0.001 g
NaCO <sub>3</sub>	0.02 g
Trace metal solution A <sub>5</sub> *	1.0 mL

Agar (if needed)	10.0 g
Distilled water	1.0 L

\*Trace metal solution A<sub>5</sub>

H <sub>3</sub> BO <sub>3</sub>	2.86 g
MnCl <sub>2</sub> ·4H <sub>2</sub> O	1.81 g
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.222 g
NaMoO <sub>4</sub> ·2H <sub>2</sub> O	0.39 g
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.079 g
Co(NO <sub>3</sub> ) <sub>2</sub> ·6H <sub>2</sub> O	49.4 mg
Distilled water	1.0 L

#### 4.2.2 Assessment of cyanobacterial population (MPN Method)

The cyanobacterial population was assessed through MPN method (Pepper et al., 1995). In brief 10 g of soil sample was suspended in 95 mL of BG-11 medium and a serial dilution of 10<sup>-2</sup> to 10<sup>-6</sup> was prepared. From dilution 10<sup>-3</sup>, 10<sup>-4</sup> and 10<sup>-5</sup>, 1.0 mL was transferred to test tubes containing 9 mL BG-11 medium for each sample in triplicates. These tubes were incubated for 4 weeks in culture racks with 14/10 photoperiod and the number of cyanobacteria was enumerated.

#### 4.2.3 Isolation and purification of cyanobacteria strains

Cyanobacteria were isolated by serial dilution and plating method (Stanier et al., 1971). The organism was grown photoautotrophically in batch cultures in BG-11 medium in 250 mL Erlenmeyer flasks. The cultures were maintained in a culture room at 28°C±2°C. The surface of culture vessels was illuminated with fluorescent tubes giving photon flux of 40 W CFL with light/dark cycle of 14/10 h. The culture vessels were hand-shaken four to five times daily to keep the cultures in homogenous state.

#### 4.2.4 Identification of cyanobacterial species

Identification of purified cyanobacterial species were done based on their morphology as per the methods of Desikachary (1959) and Komarek and Anagnostidis (1998; 2005).

### 4.3 Results

#### 4.3.1 Cyanobacterial population

In the paddy field soil, the cyanobacterial counts varied from 430 g<sup>-1</sup> (Mohanlalganj) to infinite (Bakshi ka Talab) (Table 4.1). On the basis of nutritional status of soil samples, better Nitrogen, phosphorous and carbon content favours the cyanobacterial growth. The paddy field soil of Mohanlalganj was poor in Nitrogen, phosphorous and carbon content; had the lowest cyanobacterial growth (430 g<sup>-1</sup>), while paddy field soil of Bakshi ka Talab was good in Nitrogen, phosphorous and carbon content; had infinite cyanobacterial growth.

**Table 4.1** Cyanobacterial population at different study sites

S No.	Study sites	MPN (g <sup>-1</sup> dry soil)
1.	BBAU Campus	2100
2.	Mohanlalganj	430
3.	Barabanki	930
4.	Bakshi ka Talab	Indefinite
5.	Malhiabad	1500

#### 4.3.2 Identification and distribution of cyanobacterial strains

Isolated cyanobacterial strains were identified as *Synechococcus* sp., *Gloeocapsa* sp., *Anabaena* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoleus* sp., *Gloeotheca* sp., *Oscillatoria* sp., *Gleotrichia* sp., *Aphanothece* sp. (Figure 4 (a-j)). The distribution of cyanobacterial strains were in Table 4.2.

**Table 4.2** Distribution of cyanobacteria in paddy field soil of selected sites of Lucknow city and adjoining area, Uttar Pradesh.

SNo.	Cyanobacteria	BBAU campus	Mohanlalganj	Barabanki	Bakshi ka Talab	Malhiabad
1.	<i>Synechococcus</i> sp.	-	-	+	+	+
2.	<i>Gloeocapsa</i> sp.	+	-	-	+	-
3.	<i>Anabaena</i> sp.	+	+	+	-	+
4.	<i>Aphanocapsa</i> sp.	-	+	-	+	-
5.	<i>Chroococcus</i> sp.	-	-	+	+	-
6.	<i>Microcoleus</i> sp.	-	-	-	+	-
7.	<i>Gloeothece</i> sp.	+	-	-	-	+
8.	<i>Oscillatoria</i> sp.	-	-	+	+	-
9.	<i>Gloeotrichia</i> sp.	-	-	-	+	-
10.	<i>Aphanothece</i> sp.	+	+	+	-	+

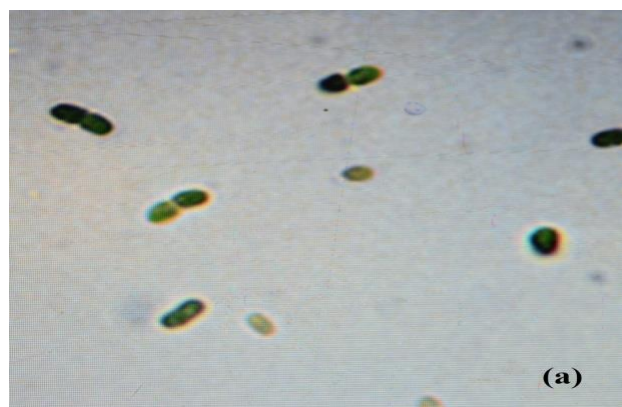
### 4.3.3 Characteristics of isolated cyanobacterial strains

#### *Synechococcus* sp. (Nageli, 1848)

Order: Chroococcales

Family: Chroococcaceae

Cells oblong, cylindrical or ellipsoidal, erect, seldom slightly bent with rounded apices, single or in colonies of 2, rarely in fours, mucilage envelope absent or a very thin and narrow one present; division transverse.



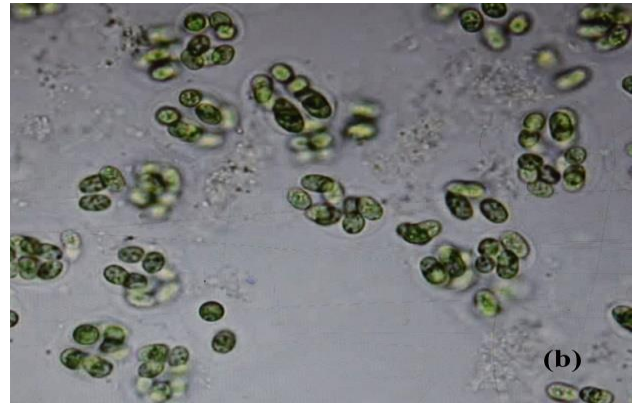
**Figure 4.1(a)** *Synechococcus* sp

***Gleocapsa* sp.** (Kutzing, 1843)

Order: Chroococcales

Family: Chroococcaceae

Cells spherical, 2-8 in colonies, seldom many, with a number of concentric special envelopes; colonies single or many together forming an expanded mass, individual sheaths lamellated, cell division very regularly in three directions, cells in large



colonies often with secondary colonies, **Figure 4.1 (b) *Gleocapsa* sp.**

arranged irregularly; occasionally with nanno-cytes, resembling *Aphanocapsa*-stage; spores with firm thick walls often formed in a number of species.

***Anabaena* sp.** (Bornet and Flahault, 1887-88)

Order: Nostocales

Family: Nostocaceae

Sub-family- Anabaenae

Trichomes uniformly broad throughout or apices alone somewhat attenuated, sheath absent or more or less diffluent, forming a free, torn or floccose or soft mucilaginous thallus; heterocysts generally intercalary; spores single or in long series, formed from near the heterocysts or in between the heterocysts.

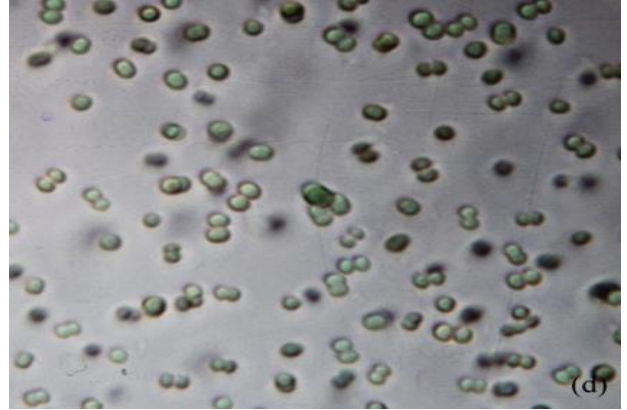
**Figure 4.1 (c) *Anabaena* sp.**

***Aphanocapsa* sp.** (Nageli, 1848)

Order: Chroococcales

Family: Chroococcaceae

Cells spherical or nearly so, many loosely arranged without an order, forming a formless gelatinous mass, often a few cm in diameter, mucilage homogenous, colourless, cells often with thin more or less gelatinized individual sheaths; division

**Figure 4.1 (d)** *Aphanocapsa* sp.

in two (or various?) directions, often two, four and sometimes many with in a common mucilageous envelop of the parent cell; nannocytes present in some species, formed by repeated division.

***Chroococcus* sp.** (Nageli, 1848)

Order: Chroococcales

Family: Chroococcaceae

Cells spherical or sub-spherical, Hemispherical, after division in small groups of 2-4 individuals, sometimes 8-16, rarely single, in a gelatinous or mucous matrix; sheath of individual cells distinct, firm, generally lamellated, in some homogenous, persistently or irregularly broken; reproduction by cell division and fragmentation of colonies; division of cells in three directions. Nannocytes occasionally seen.

**Figure 4.1(e)** *Chroococcus* sp.

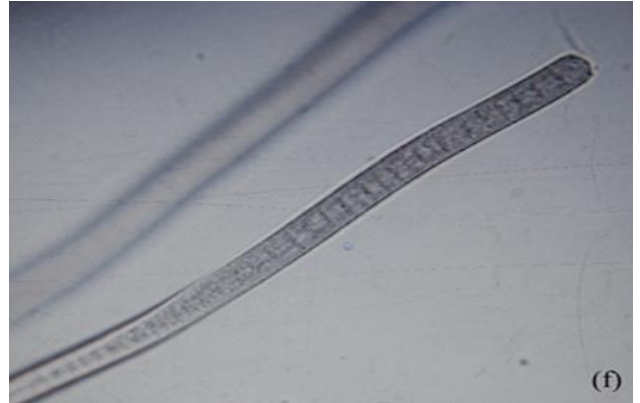
arly broken; reproduction by cell division and fragmentation of colonies; division of cells in three directions. Nannocytes occasionally seen.

***Microcoleus* sp.** (Gormont, 1892)

Order: Nostocales

Family: Oscillatoriaceae

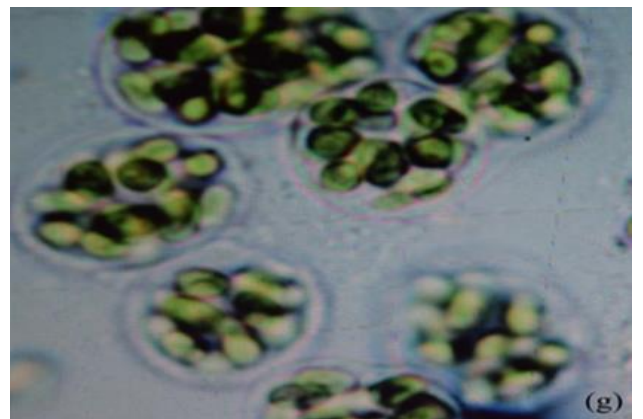
Filamentous unbranched or sparsely branched; sheath mostly colourless, more or less regularly cylindrical, not regularly, not lamellated, sometimes when old, gelatinizing; trichomes very many in each sheath, densely aggregated, often coiled or contorted like a rope; ends straight, mostly attenuated; end more or less conical seldom capitate.

**Figure 4.1 (f)** *Microcoleus* sp.***Gloeothece* sp.** (Nageli, 1848)

Order: Chroococcales

Family: Chroococcaceae

Cells cylindrical to ellipsoidal, straight or bent, not attenuated at the ends, but broadly rounded, in small colonies or forming large thallus, division of cells at right angles to the longitudinal axis, sometimes the daughter cells divide in all the three directions; sheath structure and colony structure as in *Gloeocapsa*; nannocytes present.

**Figure 4.1 (g)** *Gloeothece* sp.

***Oscillatoria* sp.** (Gomount, 1892)

Order: Nostocales

Family: Oscillatoriaceae

Trichome single or forming a flat or spongy free-swimming thallus, sheath absent, rarely with more or less very delicate sheath, motile, mostly by a creeping movement causing rotation on the longitudinal axis; end of trichome distinctly marked, pointed, bent like a sickle or coiled more or less like a screw.



**Figure 4.1(h)** *Oscillatoria* sp.

Hormogones formed by the division of the trichome.

***Gloeotrichia* sp.** (Bornet and Flahault, 1886-87)

Order: Nostocales

Family: Rivulariaceae

Thallus spherical or hemispherical, solid, sometimes when old inflated and follow; filamentous radial more or less parallel, often with false branches; sheath at the base firm, only gelatinizing on the outside, soft to mostly diffluent; trichomes with a distinct trichothallic growth; heterocysts



**Figure 4.1(i)** *Gloeotrichia* sp.

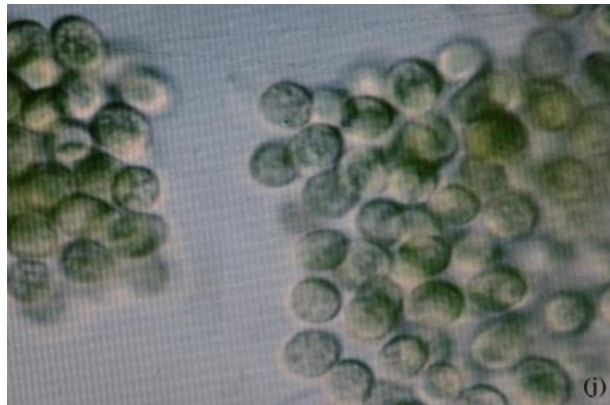
basal; spores at the base of the trichome, single or a few next to the heterocyst; hormogones present.

*Aphanothece* sp. (Nageli, 1848)

Order: Chroococcales

Family: Chroococcaceae

Cell ellipsoidal to cylindrical, straight, or slightly bent, many in a more or less shapeless expanded thallus, mucilage homogenous, occasionally with lamellated individual envelopes, often gelatinizing; division transverse. Nannocytes presents.



**Figure 4.1(j)** *Aphanothece* sp.

#### **4.4 Discussion**

This chapter deals with identification and quantification of cyanobacteria present in paddy fields of adjoining areas of Lucknow city, Uttar Pradesh. This study provides not only to the information on the diversity and occurrence of cyanobacteria in the region but also gives the opportunity to know their distribution, abundance and dominancy. However conventional methods widely used for the identification of cyanobacteria have depends up on morphological characteristics observed under the microscope. Lu et al. (1997) and Murayama-kayano et al. (1998) suggested that the morphology of cyanobacteria might be change due to different environmental conditions and the phase of growth of the organism. This morphological variation may lead to difficulty and errors in cyanobacterial identification (Murayama-kayano et al., 1998).

The cyanobacterial population in the paddy fields of adjoining areas of Lucknow city, Uttar Pradesh has shown a moderate diversity. About 10

cyanobacterial genera i.e. *Synechococcus* sp., *Gloeocapsa* sp., *Anabaena* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoleus* sp., *Gloeotheca* sp., *Oscillatoria* sp., *Gleotrichia* sp., and *Aphanothece* sp. were observed (Kumar et al., 2108). Similar observations were reported by Nayak et al. (2001), Kaushik and Prasanna (2002), Mishra and Pabbi (2004), Rai (2006), Nayak and Prasanna (2007), Dey et al. (2010), Selvi and Sivakumar (2011), Nongbri and Syiem (2012), Singh et al. (2014) and; Vijayan and Ray (2015) in different regions of India.

Among these cyanobacterial genera, only *Anabaena* sp. and *Gleotrichia* sp. were heterocystous while rest *Synechococcus* sp., *Gloeocapsa* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoleus* sp., *Gloeotheca* sp., *Oscillatoria* sp., and *Aphanothece* sp. were found to be non-heterocystous. It is not necessary that only cyanobacteria having heterocyst can perform nitrogen fixation; it is well established that non-heterocystous are able to fix nitrogen. This attribute of non-heterocystous cyanobacteria could be exploited in the selection of cyanobacteria as efficient biofertilizer in paddy fields.

The present study indicates that the soil conditions in the paddy fields of the adjoining areas of Lucknow city, Uttar Pradesh are favourable to cyanobacterial growth; there are no exceptional variations in these soil features from those reported to other parts of India. On the basis of observations, non-heterocystous cyanobacteria were more abundant in the paddy fields of selected paddy fields.

## *CHAPTER 5*

# *Screening of Pesticide Tolerant Cyanobacterial Strain(s)*

**5.1 Introduction**

Chlorpyrifos is a widely used organophosphate insecticide. It is white or colourless in colour, crystals in nature and has rotten eggs or garlic odour. Chlorpyrifos is not only used in used in agriculture for various pests to protect feed and food crops, but also used for public health for disease vectors like mosquitoes, and roundworms (US EPA, 2006). It kills the pests through by blocking the enzyme acetylcholinesterase that controls the acetylcholine. So when the enzyme activity is inhibited, the nervous system malfunctioned and due to this it causes death of the pest (Smegal, 2000; Tomlin, 2006). When chlorpyrifos applied in the paddy agroecosystem; it suspended into the soil and it can take 6 to 120 days for the disappearance of chlorpyrifos or chlorpyrifos residues from the agro-ecosystems where they are used.

The microbes including bacteria, actinomycetes and cyanobacteria are found to be very successful for the clean-up of the pesticides (Kumar and Singh, 2017). Kumar and Singh (2018) observed that cyanobacteria *Synechococcus* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoelus* sp. and *Oscillatoria* sp. are able to successfully tolerate the chlorpyrifos pesticide.

**5.2 Material and methods****5.2.1 Chlorpyrifos**

Commercial grade chlorpyrifos Eldrin TC (Chlorvip 20%, w/v), manufactured by Crystal Crop Protection Pvt. Ltd. and purchased from the local market. It is used for the screening of chlorpyrifos-tolerant cyanobacterial strains.

### **5.2.2 Screening for the chlorpyrifos-tolerant cyanobacterial strains**

Chlorpyrifos uptake experiments were conducted in 250 mL Erlenmeyer flasks containing 100 mL cultures supplemented with 5 ppm chlorpyrifos (double the recommended dose of field application). At this concentration, there is 20% inhibition in the growth of the organism. So the selected concentration, at one hand, is more than the recommended field application dose, and on the other hand, it is not causing profound effect on growth of the organism. Exponentially growing cultures (8 days old culture) were inoculated to get initial absorbance 0.010 at 680 nm (12.5 µg chl/mL culture). At regular intervals of 2 days, extending up to 14 days, 5 mL samples were withdrawn and growth was measured spectrophotometrically as the increase in absorbance of the cultures with a Thermo Scientific Evolution 201 UV-Visible spectrophotometer (Thermo Fisher Scientific, USA).

### **5.2.3 Growth Inhibition (%)**

According to Ref. (El-Nahhal et al., 1988; El-Nahhal et al., 2016), % growth inhibition (GI) which represents toxicity was calculated as follows:

$$\% \text{ GI} = 100 * [(OD_c - OD_t) / OD_c]$$

Where OD<sub>c</sub> and OD<sub>t</sub> are the optical densities of the control and the treated samples respectively

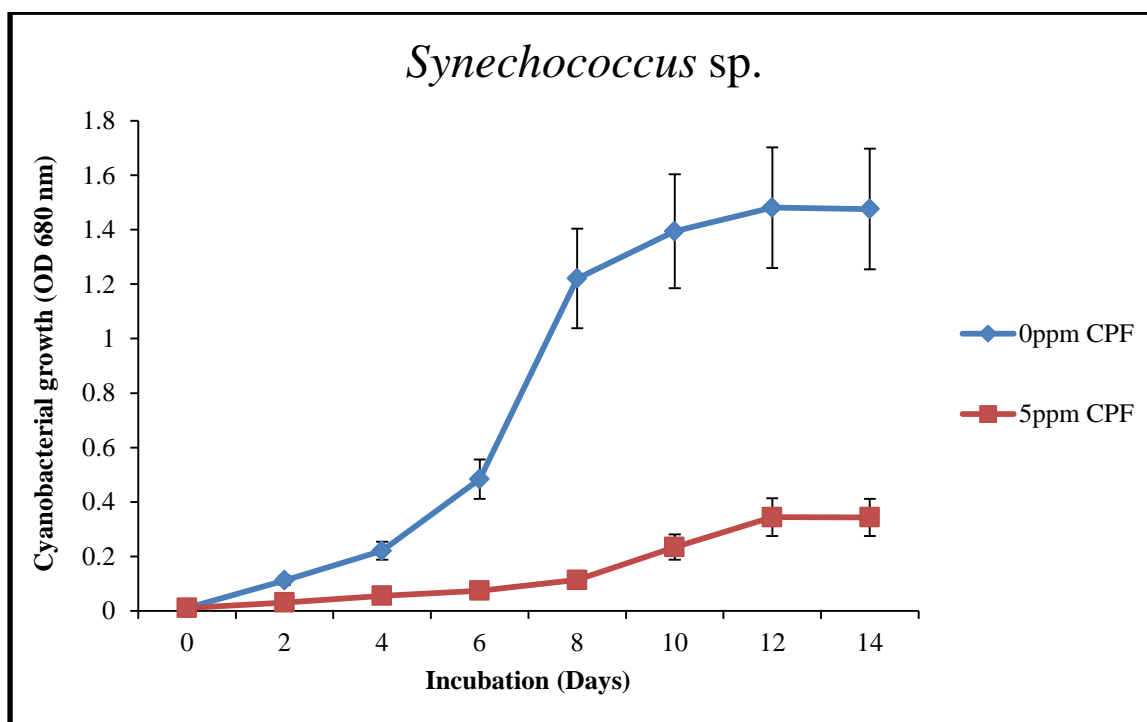
### **5.2.4 Statistical analysis**

All parameters were investigated in triplicates and the values are given as means ±SE. Further the data were analysed by one-way ANOVA according to Duncan's multiple range test (P=0.05) to find the significance level.

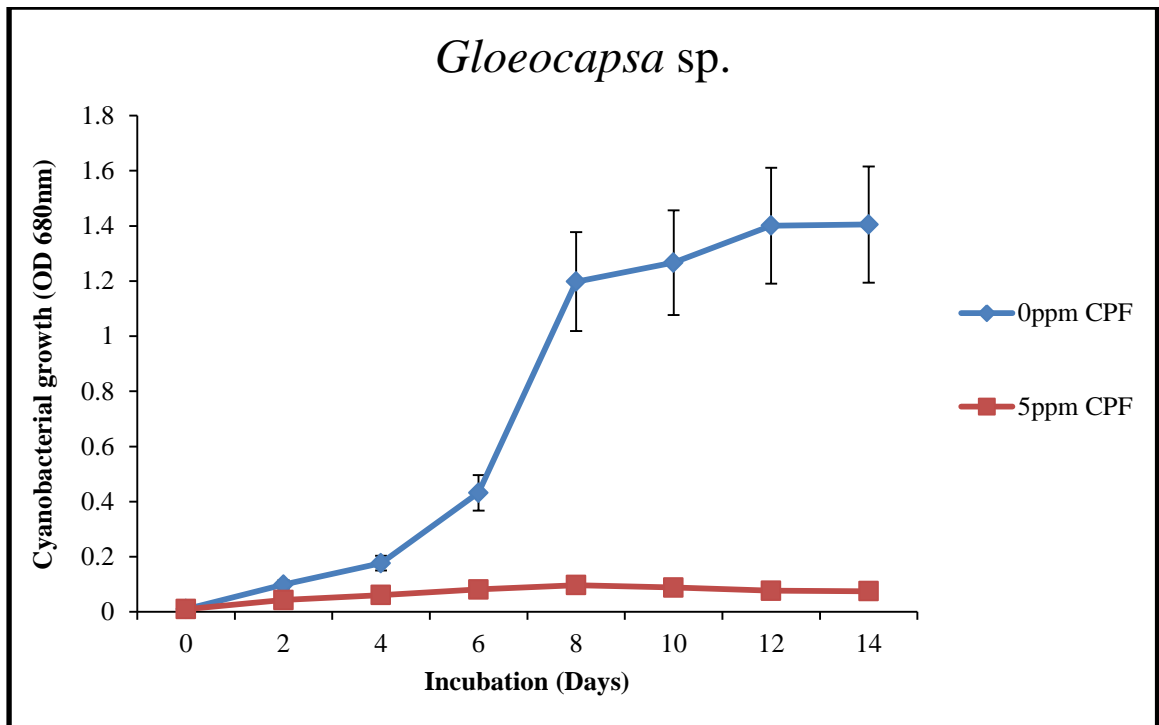
### 5.3 Results

#### 5.3.1 Growth of cyanobacterial strains in chlorpyrifos

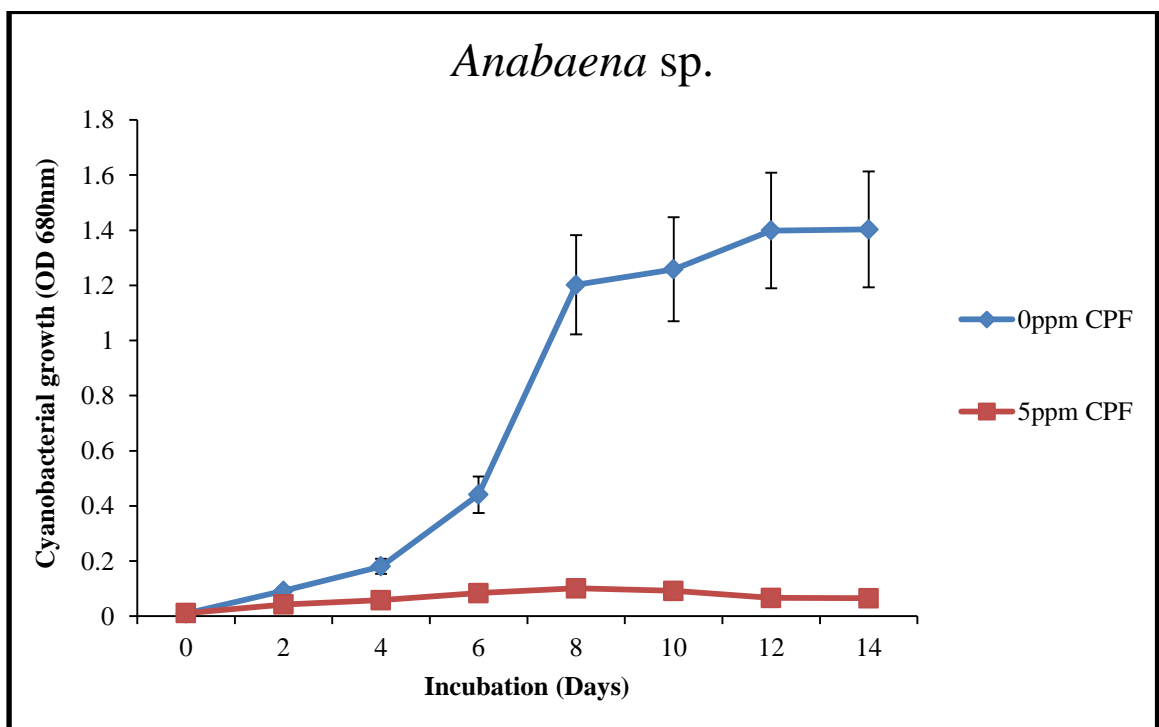
The growth of cyanobacterial strains in concentration  $5 \text{ mg L}^{-1}$  of chlorpyrifos is shown in Figure 5.1 (a-j). The growth of cyanobacterial strains *Synechococcus* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoleus* sp. and *Oscillatoria* sp. increased regularly from OD 0.010 on day 0 to OD 0.340, 0.408, 0.433 0.511 and 0.421 on day 14 and after day 14 was showing stationary stage, while some cyanobacterial strains *Gloeocapsa* sp., *Anabaena* sp., *Gloeotheca* sp., *Gloeotrichia* sp. and *Aphanothece* sp. increased from OD 0.010 on day 0 to OD 0.097, 0.101, 0.103, 0.121 and 0.104 on day 8 and after day 8 was showing declining in growth. *Synechococcus* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoleus* sp. and *Oscillatoria* sp. were observed chlorpyrifos tolerant effectively (Kumar et al., 2018) which was further confirmed by calculating growth inhibition of isolated cyanobacteria.



**Figure 5.1(a)** Growth of *Synechococcus* sp. at 5 ppm chlorpyrifos concentration



**Figure 5.1(b)** Growth of *Gloeocapsa* sp. at 5 ppm chlorpyrifos concentration



**Figure 5.1(c)** Growth of *Anabaena* sp. at 5 ppm chlorpyrifos concentration

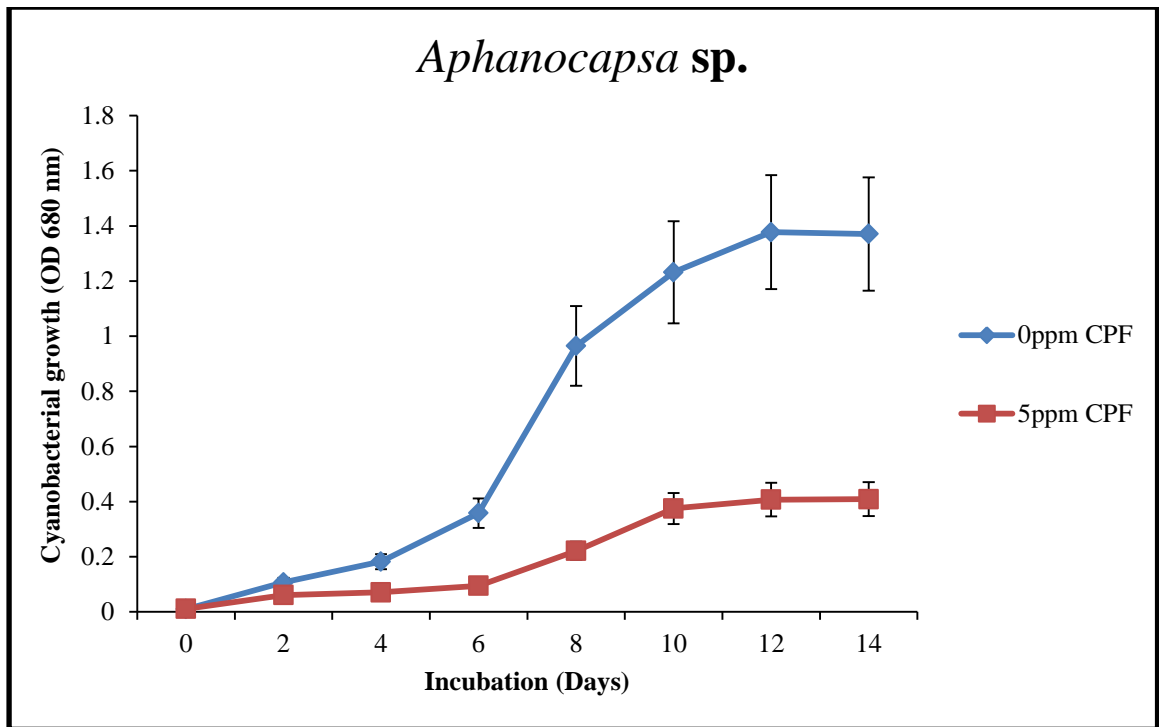


Figure 5.1(d) Growth of *Aphanocapsa* sp. at 5 ppm chlorpyrifos concentration

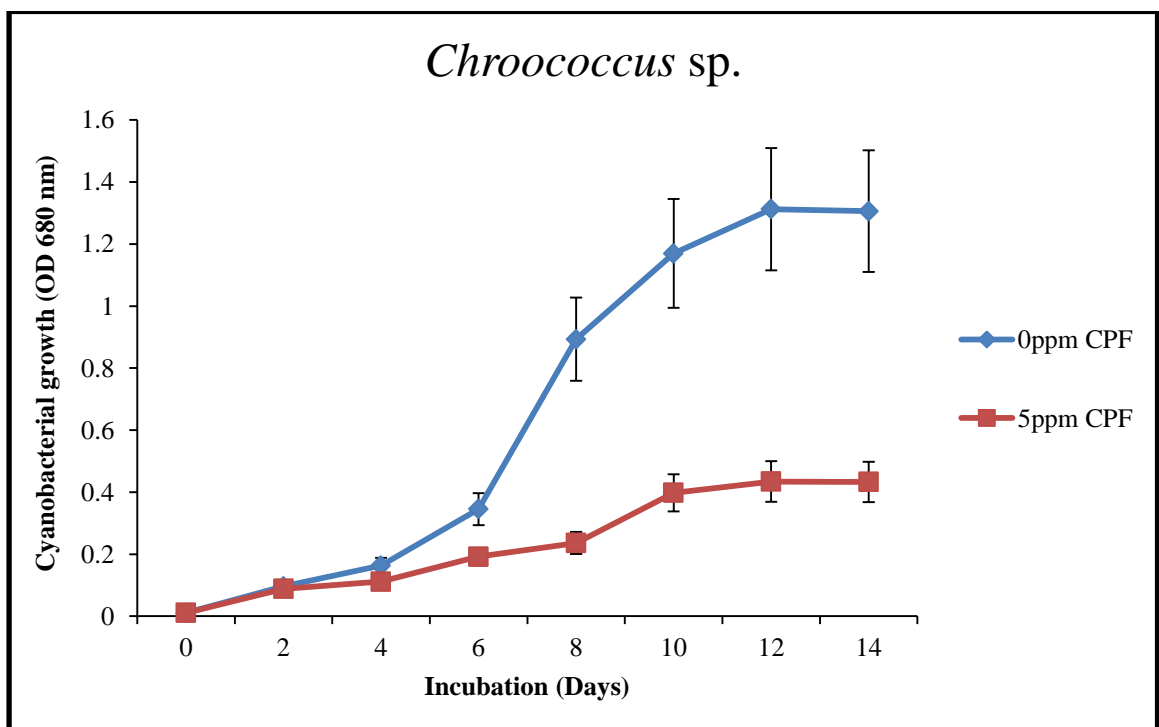


Figure 5.1(e) Growth of *Chroococcus* sp. at 5 ppm chlorpyrifos concentration

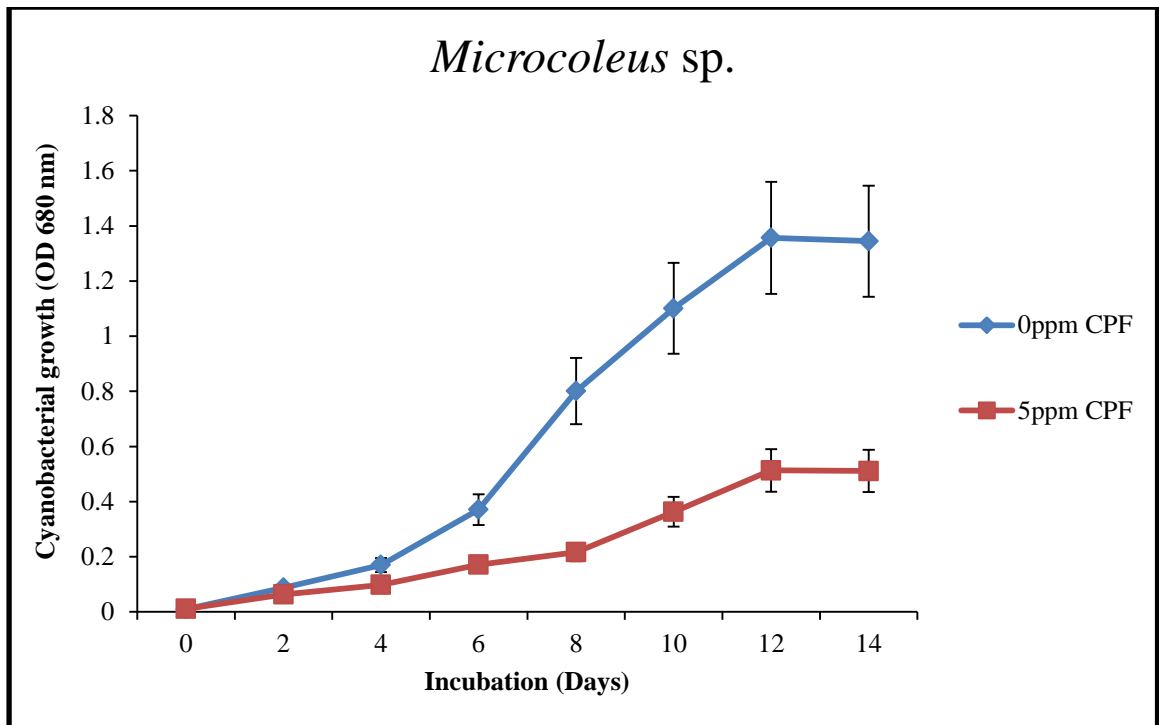


Figure 5.1(f) Growth of *Microcoleus sp.* at 5 ppm chlorpyrifos concentration

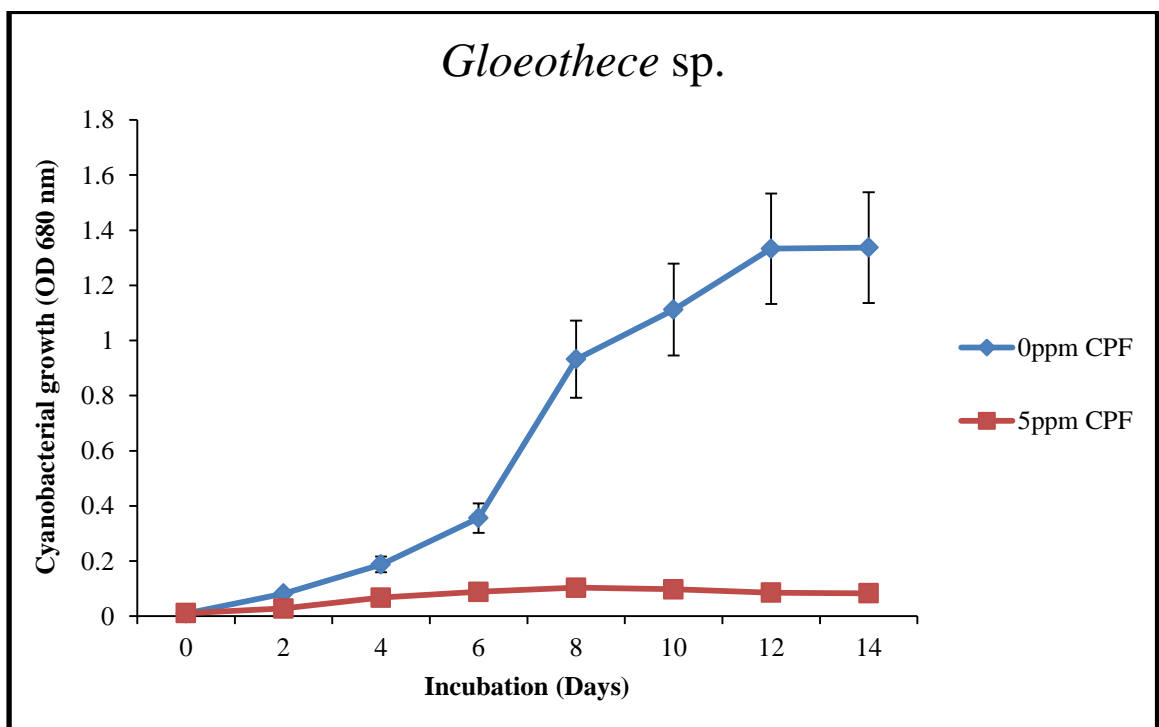


Figure 5.1(g) Growth of *Gloeotheca sp.* at 5 ppm chlorpyrifos concentration

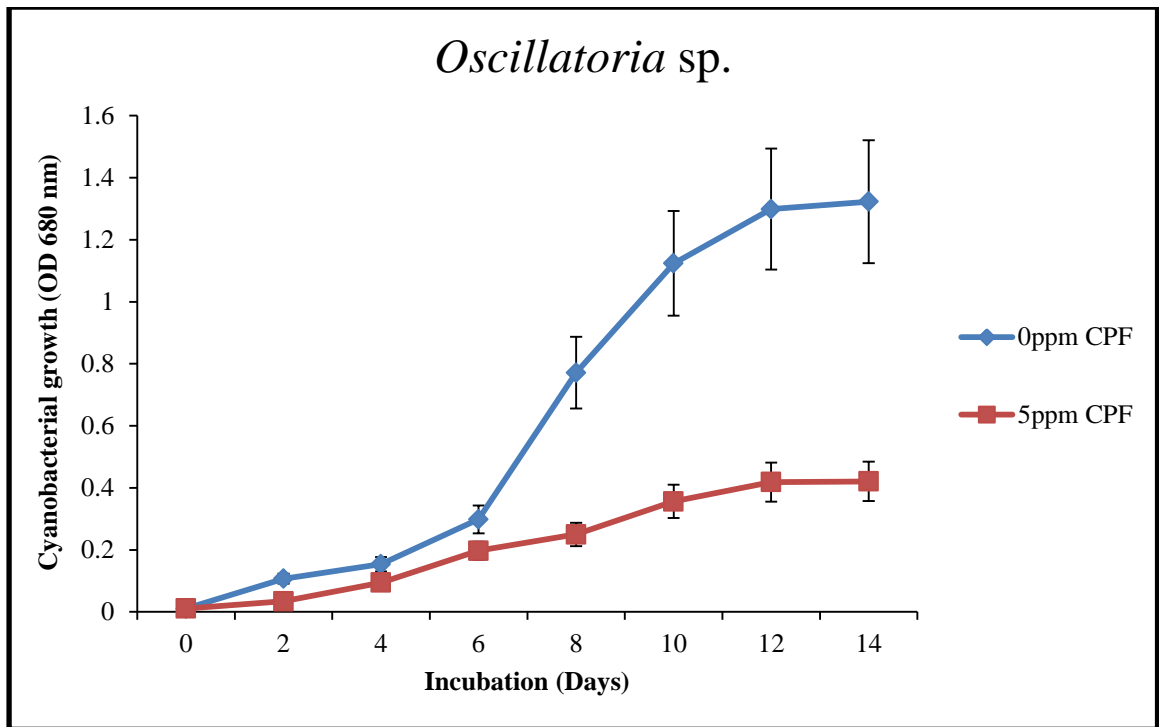


Figure 5.1(h) Growth of *Oscillatoria* sp. at 5 ppm chlorpyrifos concentration

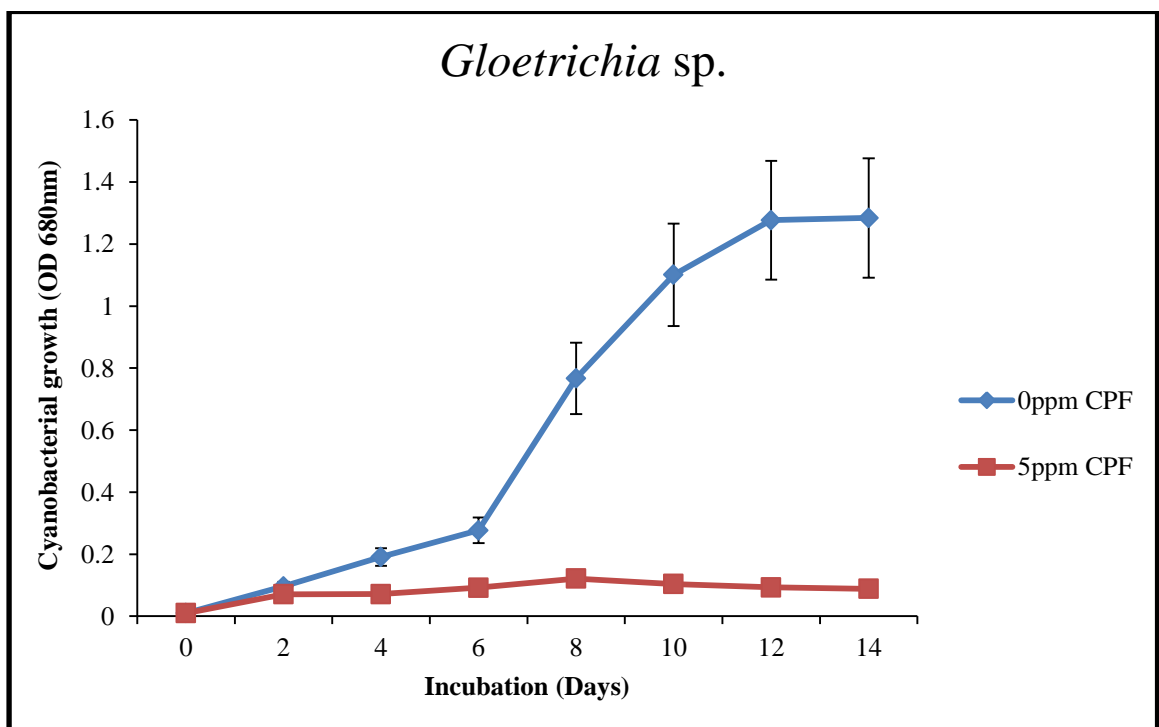
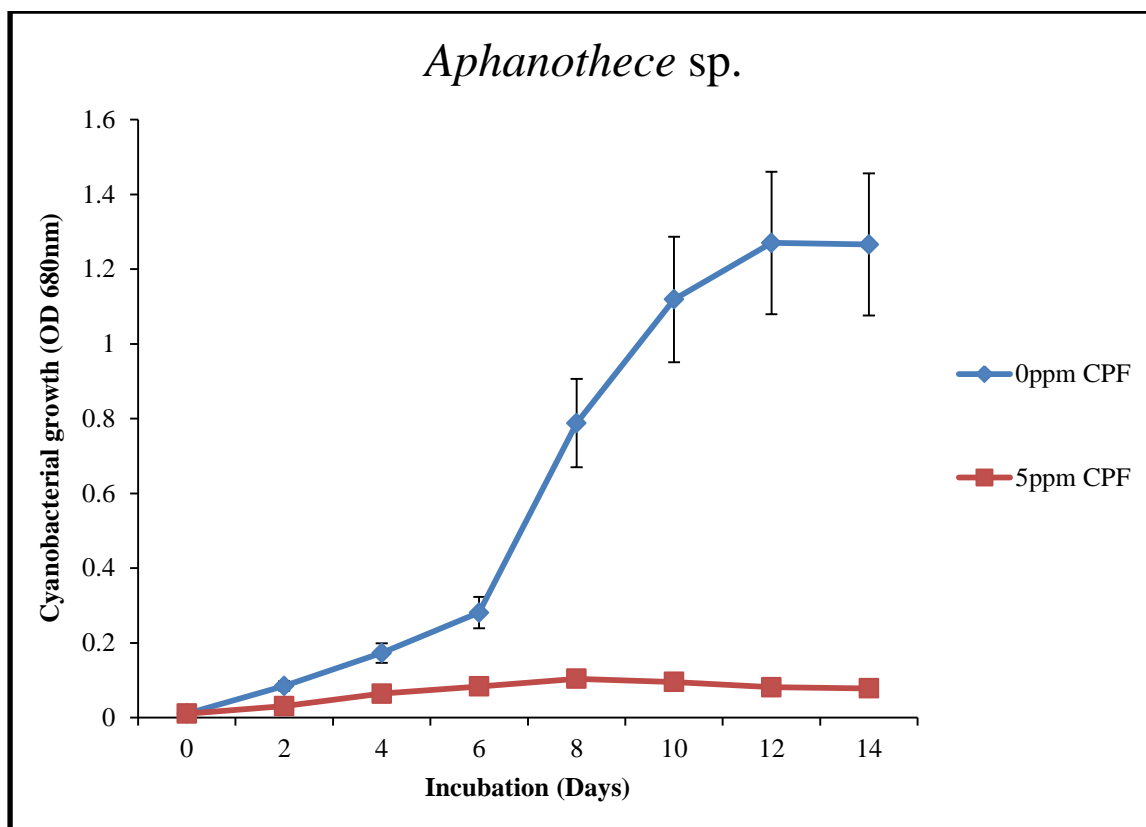


Figure 5.1(i) Growth of *Gloetrichia* sp. at 5 ppm chlorpyrifos concentration



**Figure 5.1(j)** Growth of *Aphanothece* sp. at 5 ppm chlorpyrifos concentration

### 5.3.2 Growth inhibition (%)

The cyanobacterial growth inhibition calculated against 5 ppm chlorpyrifos (Table 5.1). The cyanobacterial strains *Synechococcus* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoleus* sp. and *Oscillatoria* sp. showing growth inhibition of 77 %, 70 %, 67 %, 62 % and 68 % while *Gloeocapsa* sp., *Anabaena* sp., *Gloeotheca* sp., *Gloeotrichia* sp. and *Aphanothece* sp. showing growth inhibition 95 %, 95 %, 97 %, 93 % and 94 %. So it was confirmed that *Synechococcus* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoleus* sp. and *Oscillatoria* sp. could be chlorpyrifos tolerant which were further used for the study of their chlorpyrifos tolerant efficacy.

**Table 5.1** Growth Inhibition of isolated cyanobacterial strains

SNo.	Cyanobacteria	Growth inhibition (%)
1.	<i>Synechococcus</i> sp.	77
2.	<i>Gloeocapsa</i> sp.	95
3.	<i>Anabaena</i> sp.	95
4.	<i>Aphanocapsa</i> sp.	70
5.	<i>Chroococcus</i> sp.	67
6.	<i>Microcoleus</i> sp.	62
7.	<i>Gloeothece</i> sp.	97
8.	<i>Oscillatoria</i> sp.	68
9.	<i>Gloeotrichia</i> sp.	93
10.	<i>Aphanothece</i> sp.	94

#### 5.4 Discussion

In this study the growth of isolated cyanobacterial strains was observed at minimum conc. 5 ppm chlorpyrifos which is double the recommended dose of field application (2.5 ppm) for the screening of chlorpyrifos tolerant cyanobacteria (Singh et al., 2013; Kumar et al., 2018). *Synechococcus* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoleus* sp. and *Oscillatoria* sp. were showed regularly growth from day 1 to day 14; while *Gloeocapsa* sp., *Anabaena* sp., *Gloeothece* sp., *Gloeotrichia* sp. and *Aphanothece* sp. were able to sustain the growth after day 8 due to inhibitory effect of the chlorpyrifos. Further to support this results inhibition % calculated for all the isolated cyanobacterial strains, in which *Synechococcus* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoleus* sp. and *Oscillatoria* sp. also showed less inhibition to chlorpyrifos in compared to *Gloeocapsa* sp., *Anabaena* sp., *Gloeothece* sp., *Gloeotrichia* sp. and *Aphanothece* sp.

Pawar (2015) isolated five pesticide tolerant cyanobacterial genera *Anabaena* sp., *Synechocystis* sp., *Gloeothece* sp., *Synechococcus* sp., *Oscillatoria* sp., and *Chroococcus* sp. from pesticide contaminated agricultural soil; and further screened for monocrotophos and endosulfan pesticide tolerance. It is also observed that non-heterocystous genera were more dominated over heterocystous cyanobacterial genera in the pesticide contaminated soils. Similar observations were also found in this study that all chlorpyrifos tolerant cyanobacteria *Synechococcus* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoleus* sp. and *Oscillatoria* sp. were non-heterocystous. So it was established that *Synechococcus* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoleus* sp. and *Oscillatoria* sp. were chlorpyrifos tolerant cyanobacteria, which could be used for further studies related to investigate the efficacy of these chlorpyrifos tolerant cyanobacteria strains.

## *CHAPTER 6*

# *Testing the Efficacy of the Selected Cyanobacteria for Pesticide Degradation*

**6.1 Introduction**

The chlorpyrifos is a moderate persistent organophosphorous pesticide, which is known to be easily accumulated in the soils and tend to bind with clayey particles, shows further resistance to transformation or degradation (US EPA, 2006; Smegal, 2000; Tomlin, 2006). Cyanobacteria are able to tolerate the chlorpyrifos pesticide at a certain level, but for the efficient degradation of chlorpyrifos; it is very useful to investigate the maximum tolerable concentration of cyanobacteria against the increasing pesticide concentration. Similar studies of inhibitory effects of insecticides investigated by using cyanobacteria *Synechococcus leopoliensis* (Van Donk et al. 1992), *Anabaena sphaerica*, *Nostoc hatei*, and *Westiellopsis prolifica* (Jha and Mishra 2005), *Phormidium valderianum* (Palanisami et al. 2009) and *Spirulina platensis* (Thengodkar and Sivakami 2010). It also can be helpful for the better planning and implementation of bioremediation strategies that cyanobacteria would be cost- and time- effective for removal of pesticide contamination (Kumar and Singh, 2017).

**6.2 Material and methods****6.2.1 Efficacy of chlorpyrifos-tolerant cyanobacterial strains**

Chlorpyrifos uptake experiments were conducted in 250 mL Erlenmeyer flasks containing 100 mL cultures supplemented with 5, 7.5, 10, 15 and 20 ppm chlorpyrifos. Exponentially growing cultures were inoculated to get initial absorbance 0.010 at 680 nm (12.5 µg chl/mL culture). At regular intervals of 2 days, extending up to 14 days, 5 mL samples were withdrawn and growth was measured spectrophotometrically as the increase in absorbance of the cultures with a Thermo Scientific Evolution 201 UV-Visible spectrophotometer (Thermo Fisher Scientific, USA).

### 6.2.2 Growth Inhibition (%)

According to Ref. (El-Nahhal et al., 1988; El-Nahhal et al., 2016), % growth inhibition (GI) which represents toxicity was calculated as follows:

$$\% \text{ GI} = 100 * [(OD_c - OD_t) / OD_c]$$

Where OD<sub>c</sub> and OD<sub>t</sub> are the optical densities of the control and the treated samples respectively

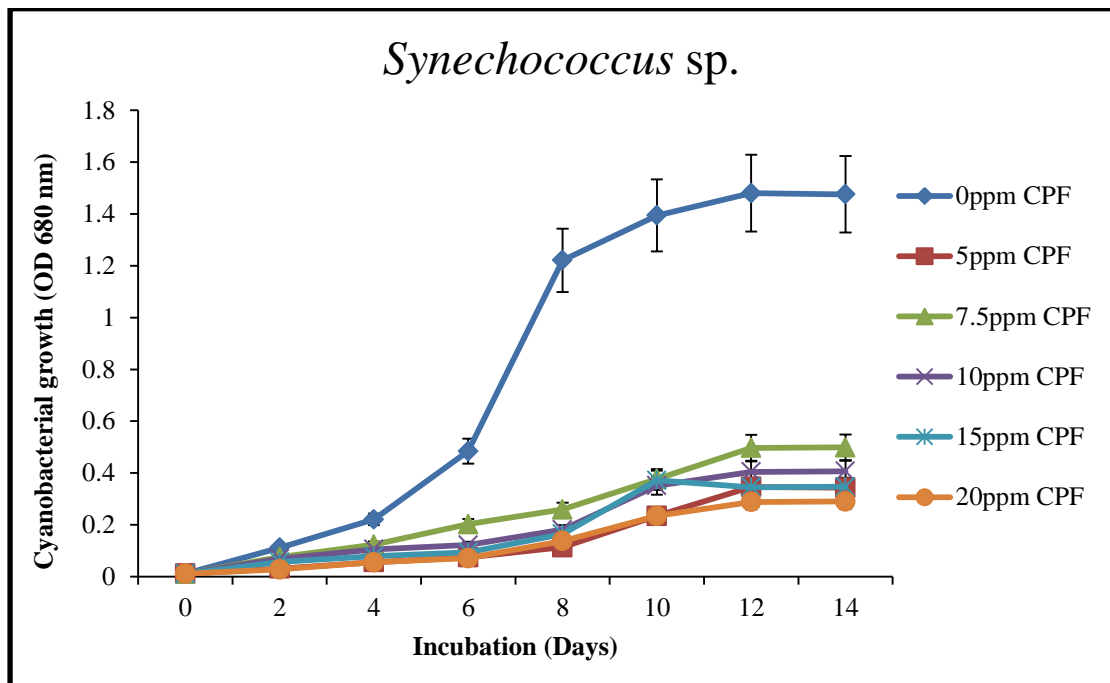
### 6.2.3 Statistical analysis

All parameters were investigated in triplicates and the values are given as means  $\pm$ SE. Further the data were analysed by one-way ANOVA according to Duncan's multiple range test (P=0.05) to find the significance level.

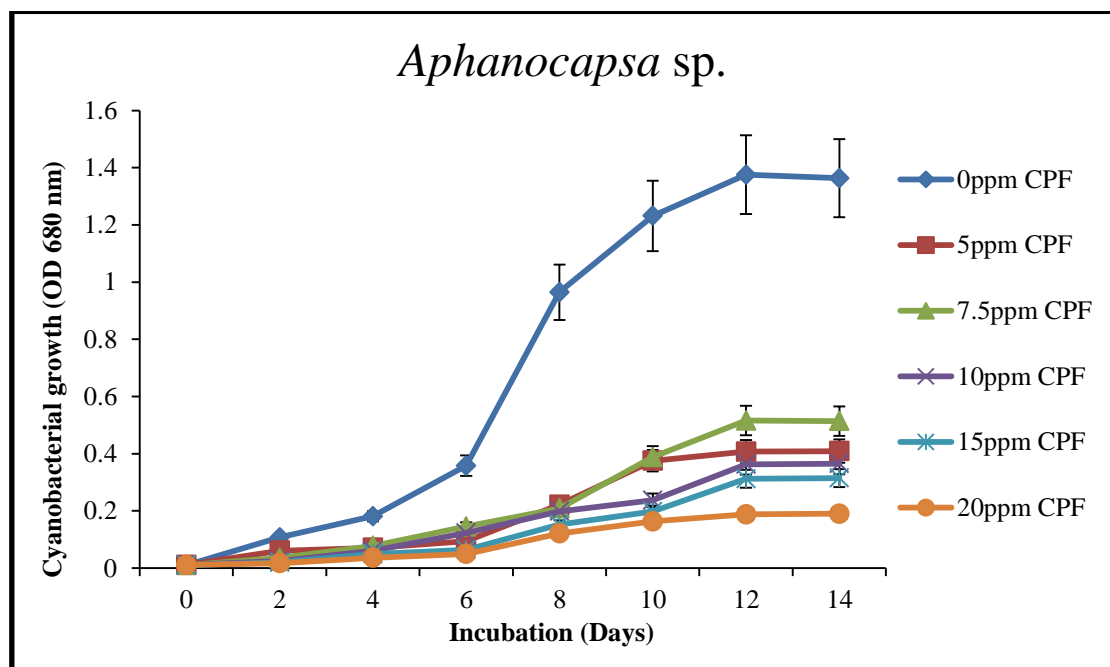
## 6.3 Results

### 6.3.1 Efficacy of chlorpyrifos-tolerant cyanobacterial strains

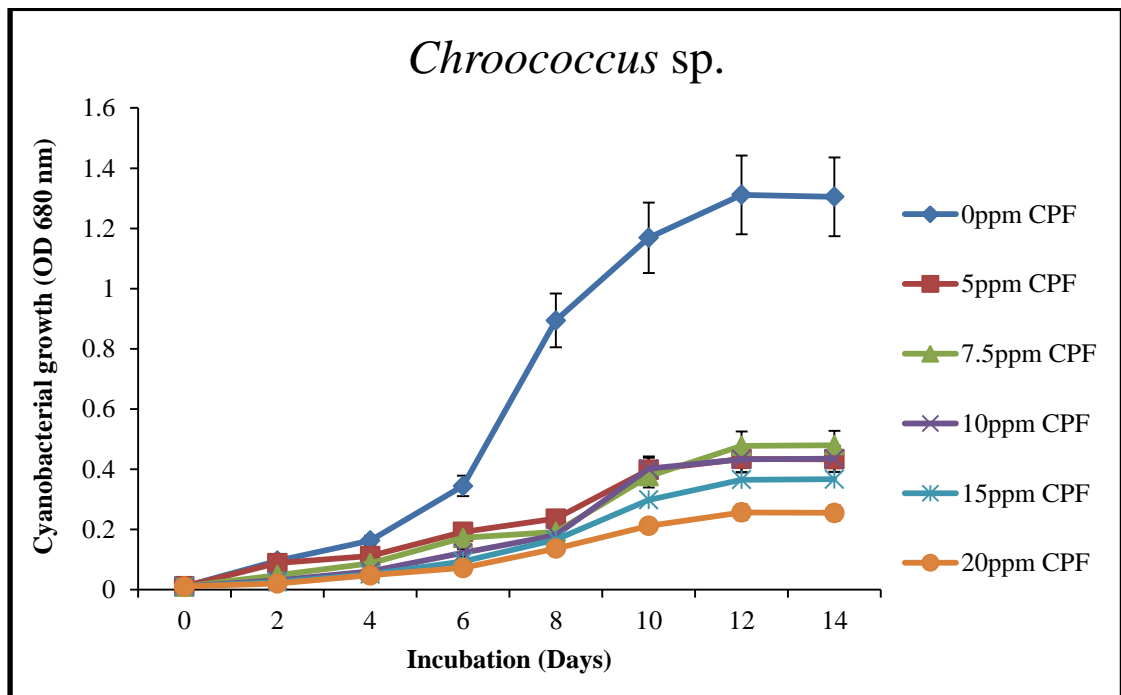
*Synechococcus* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoleus* sp. and *Oscillatoria* sp. were treated with 5, 7.5, 10, 15 and 20 ppm chlorpyrifos. At 5, 7.5, 10 ppm chlorpyrifos concentration, *Synechococcus* sp. (OD 0.343, 0.499, 0.407), *Aphanocapsa* sp. (OD 0.409, 0.514, 0.365), *Chroococcus* sp. (OD 0.433, 0.480, 0.435), *Microcoleus* sp. (OD 0.511, 0.465, 0.415) and *Oscillatoria* sp. (OD 0.421, 0.567, 0.436) showed similar growth pattern respectively; but at 15 and 20 ppm, *Aphanocapsa* sp. (OD 0.315, 0.190) and *Microcoleus* sp. (OD 0.290, 0.209) showed some slow growth in compare to *Synechococcus* sp. (OD 0.347, 0.290), *Chroococcus* sp. (OD 0.367, 0.255) and *Oscillatoria* sp. (OD 0.403, 0.359). In *Synechococcus* sp., *Chroococcus* sp. and *Oscillatoria* sp. which showed better growth at 15 and 20 ppm; *Oscillatoria* sp. showed maximum growth compared to *Synechococcus* sp. and *Chroococcus* sp.



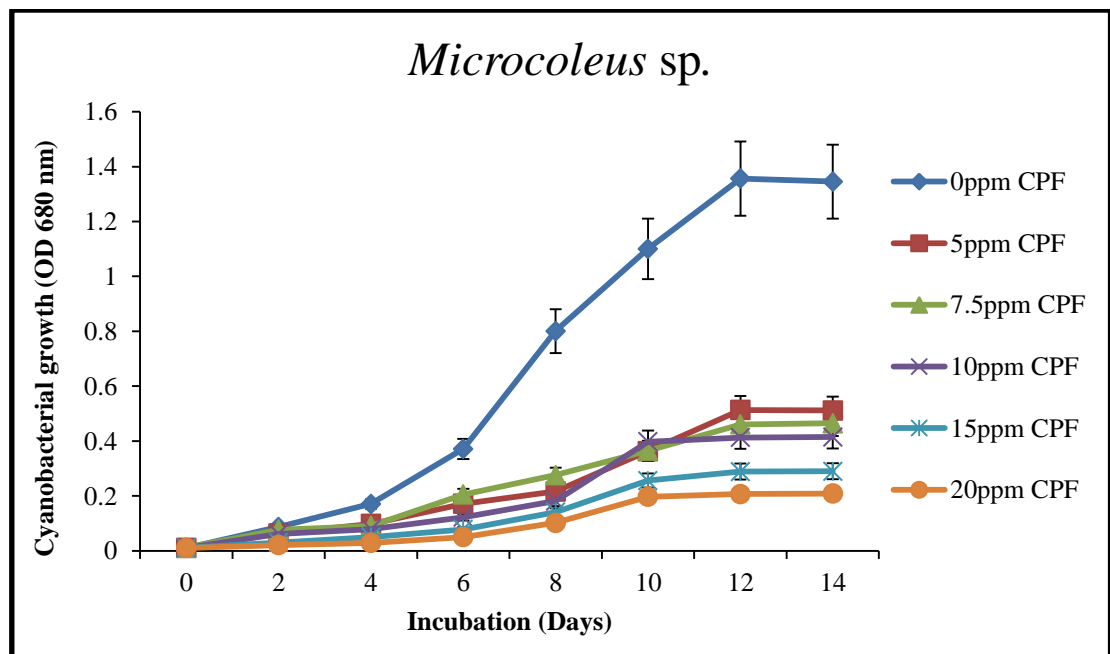
**Figure 6.1(a)** Growth curve of *Synechococcus* sp. against different concentration of chlorpyrifos



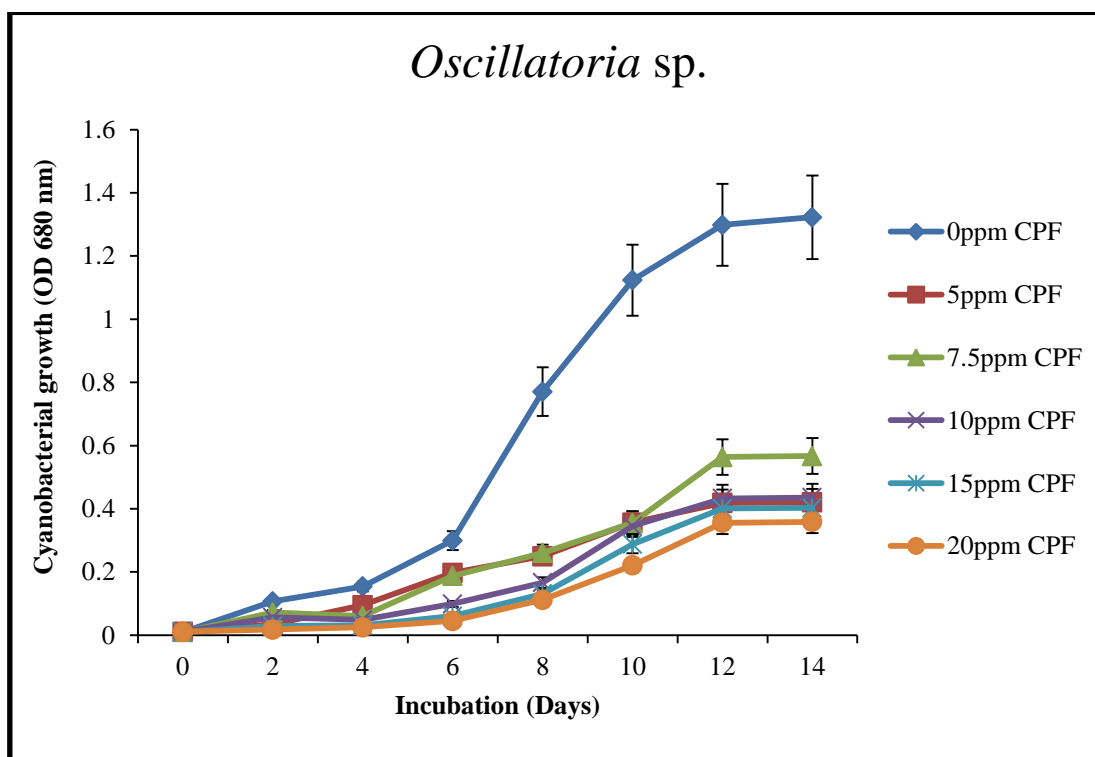
**Figure 6.2(b)** Growth curve of *Aphanocapsa* sp. against different concentration of chlorpyrifos



**Figure 6.1(c)** Growth curve of *Chroococcus sp.* against different concentration of chlorpyrifos



**Figure 6.1(d)** Growth curve of *Microcoleus sp.* against different concentration of chlorpyrifos



**Figure 6.1(e)** Growth curve of *Oscillatoria* sp. against different concentration of chlorpyrifos

### 6.3.2 Growth inhibition (%)

Growth Inhibition calculated against 5, 7.5, 10 and 20 ppm chlorpyrifos at day 14 (Table 6.1). At 5, 7.5, 10 and 20 ppm, *Synechococcus* sp. showed the growth inhibition of 77, 66, 72, 76 and 80 %; *Aphanocapsa* sp. showed the growth inhibition of 70, 70, 73, 77 and 85 %; *Chroococcus* sp. showed the growth inhibition of 67, 63, 67, 72 and 80 %; *Microcoleus* sp. showed the growth inhibition of 62, 65, 69, 78 and 84 %; and *Oscillatoria* sp. showing growth inhibition of 68, 67, 67, 70 and 73 %. After compared the growth inhibition % in *Synechococcus* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoleus* sp. and *Oscillatoria* sp.; it was supported the growth curve of the chlorpyrifos cyanobacteria and *Oscillatoria* sp. less

growth inhibition compared to *Synechococcus* sp. and *Chroococcus* sp. So *Oscillatoria* sp. proved potential cyanobacteria that could be used for the further study of analysis of degradation of chlorpyrifos.

**Table 6.1** Growth inhibition of chlorpyrifos tolerant isolated cyanobacterial strains.

SNo.	Growth inhibition (%)	5 ppm CPF	7.5 ppm CPF	10 ppm CPF	15 ppm CPF	20 ppm CPF
1.	<i>Synechococcus</i> sp.	77%	66%	72%	76%	80%
2.	<i>Aphanocapsa</i> sp.	70%	70%	73%	77%	85%
3.	<i>Chroococcus</i> sp.	67%	63%	67%	72%	80%
4.	<i>Microcoleus</i> sp.	62%	65%	69%	78%	84%
5.	<i>Oscillatoria</i> sp.	68%	67%	67%	70%	73%

#### 6.4 Discussion

Salman and Abdul-Adel (2015) investigated the ability of *Oscillatoria* sp. to grow and tolerate in the high concentrations level of the herbicide glyphosate. The ability of *Nostoc punctiforme*, *Nostoc calcicola*, *Anabaena variabilis*, *Gloeocapsa* sp. and *Aphanocapsa* sp. along with *Nostoc muscorum* ISU investigated that against increasing concentrations (0–100 mgL<sup>-1</sup>) against common paddy field herbicides Arozin, Butachlor, Alachlor and 2,4-D (Singh and Datta, 2006). Das (2008) observed that *Nostoc muscorum* and *Calothrix parietina* able to tolerate organophosphate pesticide dimethoate 30 EC (Rogor) up to 10 ppm; *Anabaena variabilis* up to 2 ppm and *Westiellopsis prolifica* up to 5ppm.

From the results, it was indicated that there is reduction in growth of chlorpyrifos tolerant cyanobacterial strains *Synechococcus* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoleus* sp. and *Oscillatoria* sp. with increasing chlorpyrifos concentration; this phenomenon well supported by Ibrahim and Essa (2010) and

Ghadai et al. (2010) who investigated the growth of seven cyanobacterial strains with different concentrations of organophosphorus pesticides. They observed that low concentrations of organophosphorus pesticides induced the growth of cyanobacterial strains, but further higher concentrations organophosphorus pesticides could be reduced the growth of cyanobacterial strains. Further analysing the growth of *Synechococcus* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoleus* sp. and *Oscillatoria* sp.; it was found that at lower concentration 5, 7.5, 10 ppm of chlorpyrifos, there is no clear distinction in the growth curve which indicated the potential cyanobacteria that tolerate the maximum concentration of chlorpyrifos. But at higher concentration 15 and 20 ppm, *Aphanocapsa* sp., and *Microcoleus* showed lesser growth in compared to *Synechococcus* sp., *Chroococcus* sp. and *Oscillatoria* sp.; which suggested that *Aphanocapsa* sp., and *Microcoleus* would not further to sustain the chlorpyrifos. Further analysing the growth curve of rest of the cyanobacterial strain *Synechococcus* sp., *Chroococcus* sp. and *Oscillatoria* sp.; *Oscillatoria* sp showed the maximum growth at higher concentration of chlorpyrifos. After investigation of efficacy of chlorpyrifos tolerant cyanobacterial strains, *Oscillatoria* sp. found that potential cyanobacteria which would use further to study the impact of chlorpyrifos on the physiology of *Oscillatoria* sp. and also analysed for the degradation of chlorpyrifos by *Oscillatoria* sp.

## *CHAPTER 7*

# *Analyses of The Pesticide Degraded End Product by Cyanobacteria*

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## 7.1 Introduction

Chlorpyrifos significantly affected the growth and physiology of cyanobacteria (Palanisami et al., 2009; Singh et al., 2013; Kumar et al., 2014). It is well observed that chlorpyrifos interferes with the enzyme activities associated with antioxidant defense mechanism. Chlorpyrifos induced the production of singlet oxygen and other active oxygen species, responsible for oxidative damage (Halliwell, 1987; Bagchi et al., 1995; Palanisami et al., 2009). In response to this, cyanobacteria use the antioxidative machinery such as superoxide dismutase, catalase, peroxidase, etc. to scavenge these active oxygen species (Patel and Chakrabarti, 1982; Buyuksonmez et al., 1998; Palanisami et al., 2009).

Chlorpyrifos considered more persistent than other organophosphorus insecticides (Racke et al. 1990; 1996). A number of bacteria investigated for the degradation of chlorpyrifos such as *Pseudomonas diminuta*, *Micrococcus* sp., *Flavobacterium* sp., *Alcaligenes faecalis* DSP3, *Stenotrophomonas* YC-1, *Klebsiella* sp., *Sphingomonas* sp. Dsp-2, *Pseudomonas fluorescence*, *Brucellamelitensis*, *Bacillus subtilis*, *Bacillus cereus* (Munnecke et al., 1975; Guha, et al., 1997; Mallick et al., 1999; Yang et al., 2005, 2006; Ghanem and Sharma, 2007; Li et al., 2007; Lakshmi et al., 2008). In most studies up to recent various bacteria transform chlorpyrifos by hydrolysis to produce TCP and diethylthiophosphoric acid (DETP).

There are few reports are available on the degradation of chlorpyrifos by cyanobacteria (Palanishami et al., 2009; Thengodhar and Sivakami, 2010; Singh et al., 2013, Kumar et al., 2014); but still mechanism of chlorpyrifos degradation in

cyanobacteria is not fully known. Some reports reported the cyanobacteria able to transform chlorpyrifos in to TCP (Singh and Walker, 2006; Singh et al., 2013); while some reports also suggested that cyanobacteria might be able to total degradation of chlorpyrifos without production of earlier reported degradation products (Kumar, 2011). Thengodkar and Sivakami (2010) and Singh et al. (2011) also reported the same phenomenon of total degradation of pesticides without releasing degradation products.

## **7.2 Material and methods**

### **7.2.1 Genomic DNA preparation, PCR amplification and 16s rRNA gene sequencing analysis**

#### **7.2.1.1 DNA extraction**

##### **7.2.1.1.1 Reagents**

50mM Tris-HCL, pH 8.0 & 7.5

50 mM EDTA

3 M sodium acetate (pH 5.2)

95% Ethanol

Chloroform

0.5 % SDS

##### **7.2.1.1.2 Procedure**

1. The exponentially grown (8 day's old) cyanobacteria centrifuged and further transferred in the mixture of 5 mL of 50 mM Tris Buffer (pH 8.0) and 50 mM EDTA each.

2. Then cyanobacterial cells freeze at the temperature of -20 °C.
3. Add 0.5 mL of 250 mM Tris Buffer (pH 8.0) and 10 mg/mL lysozyme in the freeze cells suspension, was added, and thawed at room temperature. When thawed, were placed on ice for 45 min.
4. 1 mL of 0.5 % SDS, 50 mM Tris (pH 7.5), and 0.4 M EDTA and 1 mg/mL proteinase K was added to the cell suspension and placed in 50 °C water bath for 60 min.
5. After this, the bacterial cells were extracted with 6 mL of Tris-equilibrated phenol and were centrifuged at 10,000 rpm for 15 min. Obtained top layer was transferred to new tube.
6. Add 0.1 volume of 3 M Na-acetate (mix gently), then add 2 volume of 95 % ethanol (mix by inverting).
7. Rolled out DNA was transferred to 5 mL of 50 mM Tris (pH 7.5), 1 mM EDTA, 200 g/mL RNase and dissolved overnight by rocking at 4 °C.
8. DNA was extracted by mixing with equal volume of chloroform by inverting and centrifuged at 10,000 rpm for 5 min. The top layer was transferred to a new tube.
9. Add 0.1 volumes of 3 M Na-acetate to tube, mix gently, then add 2 volume of 95 % ethanol and mixed by inverting.
10. DNA was spool out and dissolved in 2 mL of 50 mM Tris (pH 7.5) and 1 mM EDTA and finally, the purity of DNA was checked by electrophoresis and spectrophotometric analysis.

**7.2.1.2 PCR amplification and 16S rRNA gene sequencing analysis**

Cyanobacterial biomass was scrapped from BG-11 agar plates and the total genomic DNA was prepared from exponentially grown cyanobacterial cultures by alkaline lysis method as described by Kapley et al. (2001). The 16S rRNA gene from isolated bacterium was amplified using about 5 µl DNA as template and universal primers (27F) 5'-AGAGTTTGATCMTGGCTCAG-3' and (1492R) 5'-CGGTTACCTTGTTACGACTT-3' and 1180 bp product was amplified for the cyanobacterial strain. The reaction mixture contained 1 µL template DNA, 0.25 µL forward primer, 0.25 µL reverse primer, 25 µL master mix and rest nuclease free water in a final volume makeup of 50 µL. The thermocycling reactions were carried out by using Veriti® 96-Well Thermal Cycler (Applied Biosystems, USA) as initial denaturation at 95 °C for 2 minutes followed by 35 cycles of denaturation for 30 seconds at 95 °C, annealing for 30 seconds at 52 °C, extension for 2 minutes at 72 °C and final extension for 15 minutes at 72 °C. The PCR product was gel purified using 1 % agarose gel at 100V for 60 min and gel was visualized under UV light.

**7.2.1.3 Phylogenetic analysis**

The evolutionary history was contingent using the Neighbor-Joining method (Saitou and Nei, 1987). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (Tamura et al., 2004). The analysis involved 11 nucleotide sequences and all positions with more than 95 % site coverage were included, fewer than 5 % alignment gaps, missing data,

and ambiguous bases were allowed at positions. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

#### **7.2.1.4 Nucleotide sequence accession numbers**

The partial sequences obtained were subjected to BLAST analysis using the online option available at [www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1997), suggesting the identity of the isolates. The final sequences obtained were submitted to NCBI for obtaining accession no.

### **7.2.2 Photosynthetic pigments**

#### **7.2.2.1 Chlorophyll-a and carotenoids**

##### **7.2.2.1.1 Principle**

Cyanobacteria rely on chlorophyll-a and phycobilin protein to capture light and turn light energy into sugar it needs. Chlorophyll-a is a greenish pigment that makes photosynthesis possible by passing on charged electrons to other molecules to manufacture sugar. Carotenoids include pigments like carotenes and xanthophylls.

##### **7.2.2.1.2 Reagents**

80% acetone or methanol

##### **7.2.2.1.3 Procedure**

10 ml of cyanobacterial culture was taken and centrifuged at 5000 rpm for 10min. Pellet was washed twice in distilled water. Then pellet was suspended in 10 ml of methanol and vortexed thoroughly. The tubes were incubated in a water bath at 60°C for 1hr in dark with occasional shaking. Then the reaction mixture was centrifuged at 5000 rpm for 10min. The process was repeated to ensure complete

extraction. Absorbance of the supernatant was taken at 660, 645 and 450 nm on UV-Vis spectrophotometer and 80% acetone was used as blank.

#### **7.2.2.1.4 Calculations**

The amount of chlorophyll a (Chl a) was calculated according to Holm (1954) whereas total carotenoids (Car) were quantified as per Myers and Kratz (1955):

$$Chl\ a = 12.25 A_{660} - 2.35 A_{645}$$

$$Chl\ b = 20.31 A_{645} - 4.91 A_{660}$$

$$Chl\ a + b = 17.76 A_{645} + 7.34 A_{660}$$

$$Car = 4.69 A_{450} - 0.267 Chl\ a + b$$

#### **7.2.2.2 Phycobiliproteins (PBS)**

##### **7.2.2.2.1 Principle**

Phycobiliproteins (PBS) were extracted in phosphate buffer (50 mM, pH 7.0) by freeze-thawing as described elsewhere (Bennett and Bogorad, 1973).

##### **7.2.2.2.2 Reagents**

0.1M Phosphate buffer

##### **7.2.2.2.3 Procedure**

8 days old cyanobacterial culture taken and centrifuged it at 3000-4000 rpm for 10-15 min. Pellet is suspended into 3-5 ml newly prepared 0.1M Phosphate buffer and kept in -20°C for 24-48 h in plastic screw-capped tubes. (Avoid use of glassware during freezing at -20°C). After this step it is kept at below 5°C for 24-36 h for thawing. This process may be repeated once (in case of thick sheathed cyanobacteria). Cyanobacterial suspension then minced in the homogenizer for 10min approx.,

centrifuged the homogenate at full speed for 10 min, after this the absorbance of the supernatant taken at 652, 615 and 562 nm. 0.1M Phosphate buffer used as blank.

#### **7.2.2.2.4 Calculations**

By putting the values of absorbance at different wavelength in the given formula, will provide concentration of phycobilin protein in mg/ml,

$$C - PC (C - Phycocyanin) = \frac{A_{615} - 0.474(A_{652})}{5.34}$$

$$APC (Allophycocyanin) = \frac{A_{652} - 0.208(A_{615})}{5.09}$$

$$C - PE (C - Phycoerythrin) = \frac{A_{562} - 2.41(PC) - 0.849(APC)}{9.62}$$

Where,

5.34= Extinction co-efficient

5.09 = Extinction co-efficient

9.62= Extinction co-efficient

### **7.2.3 Antioxidant enzymes**

#### **7.2.3.1 Super oxide dismutase**

##### **7.2.3.1.1 Principle**

Superoxide dismutase (SOD) activity measured as inhibition of reduction of nitroblue tetrazolium chloride (NBT) into GSH-phenazinemethosulphate-nitroblue tetrazolium formazon (blue colour crystals) photochemically (Beauchamp and Fridovich, 1971).

**7.2.3.1.2 Reagents**

Nitroblue tetrazolium (0.014%)

100 mM sodium phosphate buffer (pH 7.8)

Methionine (3%)

Na<sub>2</sub>CO<sub>3</sub> (23.4%)

Riboflavin (0.015%)

EDTA (2 mM)

**7.2.3.1.3 Procedure**

8 days old cyanobacterial culture taken and centrifuged at 5000 rpm, that thick cell suspension was obtained. The pellet was washed twice with double distilled water, resuspended in a small volume of 100 mM phosphate buffer (pH 7.8). Cyanobacterial cells were disintegrated with a sonicator by giving 5 pulses (5 micron amplitude) each of 1 min, with 30 sec intervals. Then supernatant centrifuged (15,000 rpm for 20 min, at 4°C) which was used as an enzyme extract. The assay mixture prepared by mixing together 27 mL of 100 mM sodium phosphate buffer (pH 7.8), 1.5 mL of methionine (3%), 1 mL of NBT (0.014%), 1 mL of Na<sub>2</sub>CO<sub>3</sub> (23.4%) and 1.5 mL of EDTA (2 mM). To 2.7 mL of reaction mixture, 0.1 mL enzyme extract and 0.2 mL of riboflavin (0.015%) were added.

After mixing, test tubes containing reaction mixture were illuminated for 5 min under fluorescent lights (44.5 mE). Then reaction mixture was kept in the dark. Tubes containing reaction mixture with enzyme extract served as blank and were kept in the dark. Control tubes without enzyme extract served as controls and were kept in

the light. The absorbance of solution was measured at 560 nm. One unit of SOD activity is the amount of enzyme required to inhibit the reduction of NBT in light by 50%.

### **7.2.3.2 Peroxidase**

#### **7.2.3.2.1 Principle**

Peroxidase activity was determined spectrophotometrically at 25 °C with a UV-visible spectrometer at 470nm using guaiacol as the substrate and H<sub>2</sub>O<sub>2</sub> as the hydrogen donor (Gahagen et al., 1968; Hemeda and Klein, 1990).

#### **7.2.3.2.2 Reagent**

Phosphate Buffer 0.1 M (pH 7.0),

Guaiacol solution 20 mM,

H<sub>2</sub>O<sub>2</sub> (0.042%; 12.3 mM)

#### **7.2.3.2.3 Procedure**

8 days old cyanobacterial culture taken and centrifuged at 5000 rpm, that crude extract was obtained. The substrate mixture prepared by mixing 10 ml of 1% guaiacol, 10 ml of 0.3% hydrogen peroxide and 100 ml of 0.05M sodium phosphate buffer (pH 6.5). Then a total volume of 3 ml assay mixture prepared by mixing 2.87 ml substrate mixture, 0.1 ml crude extract, and 0.03 ml antioxidant solution. Since peroxidase activity assay using guaiacol as a substrate is very sensitive and rapid, it is important to use the right levels of enzymatic activity in the extract. The enzyme source a reagent blank was prepared with 0.03 ml deionized water instead of antioxidant (control sample). One unit of activity is defined as a change in absorbance

of 0.001 min<sup>-1</sup>. The resulting oxidized (dehydrogenated) guaiacol is probably more than one compound and depends on the reaction conditions. The rate of formation of guaiacol dehydrogenation product is a measure of the POD activity and can be assayed spectrophotometrically at 420 nm.

#### **7.2.3.2.4 Calculations**

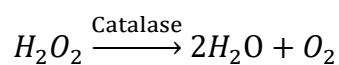
Since the extinction coefficient of guaiacol dehydrogenation product at 436 nm under the conditions specified is 6.39/mmol, the enzyme activity per litre of extract is calculated as follows:

$$\text{Enzyme activity units/litre} = (500/\Delta t)$$

#### **7.2.3.3 Catalase**

##### **7.2.3.3.1 Principle**

Catalase activity determined by analysing the formation of yellow colour complex, due to H<sub>2</sub>O<sub>2</sub> & ammonium molybdate OD at 405nm, via a UV Spectrophotometer. The decrease in OD represents the action of catalase on H<sub>2</sub>O<sub>2</sub>, due to which ammonium molybdate didn't able to form complex with H<sub>2</sub>O<sub>2</sub> (Goth, 1991; Korolyuk et al., 1988).



##### **7.2.3.3.2 Reagent**

Hydrogen Peroxide (0.065 M H<sub>2</sub>O<sub>2</sub>),

Sodium-Potassium Phosphate Buffer, pH 7.4 (60 mmol/L),

Ammonium Molybdate 32.4 mmol/L (NH<sub>4</sub>)<sub>6</sub> Mo<sub>7</sub>O<sub>24</sub> .4 H<sub>2</sub>O)

### 7.2.3.3.3 Procedure

All reagents and serum were brought to 37°C. Sample, blank 1, blank 2, and blank 3 test tubes were prepared then pipetted into test tubes.

Reagents	Sample (µL )	Blank 1 (µL )	Blank 2 (µL )	Blank 3 (µL )
Serum	50	50	-	-
Substrate( H <sub>2</sub> O <sub>2</sub> )	1000	1000	1000	-
Phosphate buffer	-	-	50	1050
Ammonium molybedate	1000	1000	1000	1000

Tubes were incubated for 60 second at 37°C. Absorption was read at 405 nm against blank 3.

### 7.2.3.3.4 Calculations

Serum CAT activity (kU/l) =  $[A (\text{Blank 1}) - A (\text{Sample}) / A (\text{Blank 2}) - A (\text{Blank 3})] * 271$

271

Where:

Blank 1 = Control;

Blank 2 = Standard;

Blank 3 = Reagent Blank;

271 = Constant

## 7.2.4 Glutathione

### 7.2.4.1 Principle

(GSH) was determined according to the modified Ellman's method (1959).

**7.2.4.2 Reagents**

100 mM  $\text{KH}_2\text{PO}_4$

1 mM EDTA (pH 7)

1.5 mg/ml DTNB Stock Solution

40 mg/ml NADPH Stock Solution

0.16 mg/mL NADPH working solution

5% 5-Sulfosalicylic Acid (SSA) Solution

**7.2.4.3 Procedure**

The cyanobacteria culture was initially deproteinized with the 5% Sulfosalicylic Acid Solution, centrifuged to remove the precipitated protein, and then analysed for glutathione. The plate reader is set at 412 nm with kinetic read at 1 minute intervals for 5 minutes. The reaction scheme for the sample is done as shown in the Table below. Every test performed in duplicate. The first 2 wells should contain only 10  $\mu\text{l}$  of the 5% SSA Solution as a reagent blank. Add 2  $\mu\text{l}$  volumes of the sample in duplicate into separate wells and make it up to 10  $\mu\text{l}$  by adding 8  $\mu\text{l}$  5% SSA. Add 150  $\mu\text{l}$  of the Working Mixture to each well with a multichannel pipette. Mix by pipetting up and down. Incubate 5 minutes at room temperature and then add 50  $\mu\text{l}$  of the diluted NADPH Solution with a multichannel pipette. Mix by pipetting up and down. Use the Micro-plate reader which is already set at 412 nm to measure the absorbance in each well.

Sample measured	Mix and incubate 5 minutes Start			NADPH 0.16 mg/mL
	Sample volume	5% SSA	Working mixture	
Reagent Blank	-	10 $\mu$ L	150 $\mu$ L	50 $\mu$ L
Sample	2 $\mu$ L	8 $\mu$ L	150 $\mu$ L	50 $\mu$ L

## 7.2.5 Proline

### 7.2.5.1 Principle

Proline content was measured according to the method of Bates et al. (1973).

### 7.2.5.2 Reagents

Sulphosalicylic acid (3%)

Std.Proline

Ninhydrin reagent

Toluene

### 7.2.5.3 Procedure

The experiment is done at room temperature except of specific indication. To generate a standard curve, a serial concentration of Proline is made in 3% sulphosalicylic acid: 1  $\mu$ M, 10  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M, 150  $\mu$ M, 200  $\mu$ M, 300  $\mu$ M, 1 ml for each dilution. Each 500  $\mu$ l standard solution is added with 500  $\mu$ l acetic acid and 500  $\mu$ l ninhydrin reagent in 5 ml tube and boil for 45 min, and then cooled in ice for 30 min. Add equal volume toluene to each sample and vibrate for 1 min, and then centrifuge at 1,000 x g for 5 min. Measure the optical density of toluene solution at

520 nm by spectrophotometer and make the standard curve. Twenty mL of culture cyanobacteria sample centrifuged washed with double distilled water and then homogenized with 2 ml of 3% sulphosalicylic acid in tube. Centrifuge homogenized samples at 5,000 x g for 5 min, and then collect the supernatant. The supernatant is treated as steps 2 and 3, and measure the optical density of sample, and then calculate the content of proline using the standard curve (Bates et al., 1973).

### **7.2.6 GC-MS Analysis**

#### **7.2.6.1 Conditions for GC-MS**

The GC operating parameters were: capillary column BP (100% dimethyl polysiloxane), length 30 m, internal diameter 0.25 mm; oven temperature 250°C; injection temperature 270°C; detector temperature 290°C; detector, ECD; carrier gas and flow, Helium, 1 mL/min.

#### **7.2.6.2 Extraction**

Exponentially growing washed cultures of the cyanobacteria were inoculated in 500 mL Erlenmeyer flasks containing 250 mL medium supplemented with chlorpyrifos (10 mg/L). After 5 days incubation, biomass was harvested by centrifugation (5,000×g) and supernatant kept. Cell pellet was washed with distilled water to remove any insecticide residues adhering to the cell surface. The biomass and cell wash were saved for detection of chlorpyrifos products. Cells were disintegrated with the help of a sonicator (Soniprep 150, Sanyo, UK) by giving 30 pulses (5 µm amplitude) each of 1 min, with interval of 30 s. Chlorpyrifos residue was extracted from the supernatant, biomass wash, and cell-free extracted three times from the supernatant with dichloromethane, followed by three times with hexane. The collected

solvents were allowed to dry and the final volume of extracted chlorpyrifos was made in hexane. Chlorpyrifos and its degradation products were analysed by capillary gas chromatography–mass spectrometry in selected ion monitoring mode (GC-MS/SIM) (Wong et al. 2010). In this technique, mass spectrometer was set to scan over range of 1 unit. A plot of the ions current resulting from this very small range of mass was detected and plotted.

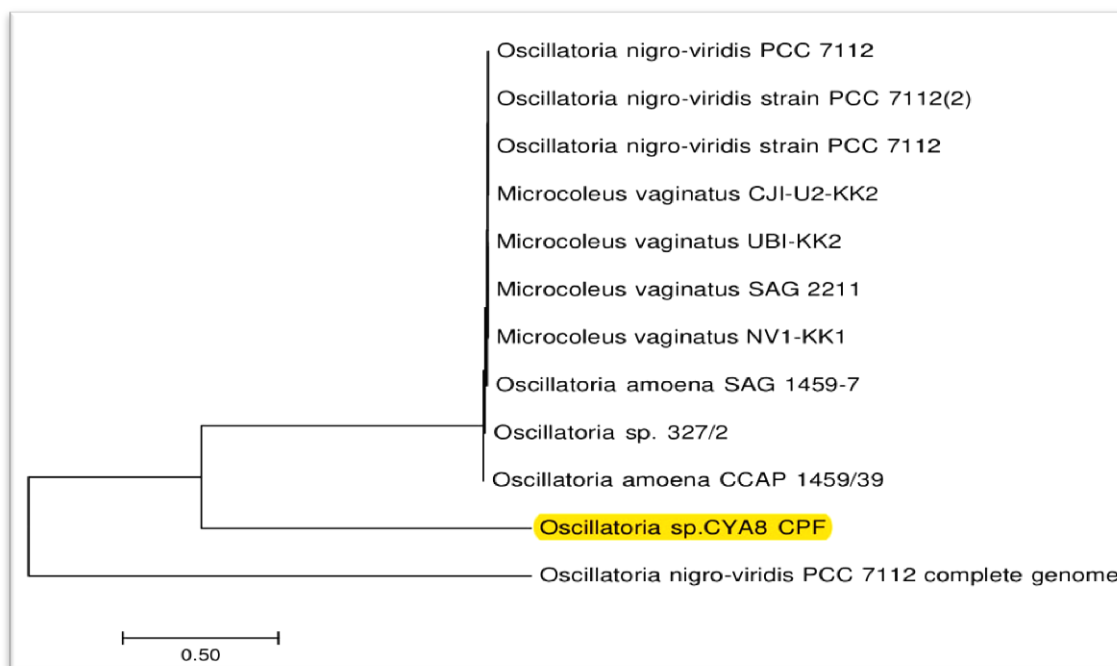
### **7.2.7 Statistical analyses**

All parameters were investigated in triplicates and the values are given as means  $\pm$ SE. Further the data were analysed by one-way ANOVA according to Duncan's multiple range test ( $P=0.05$ ) to find the significance level.

## **7.3 Results**

### **7.3.1 Phylogenetic tree of *Oscillatoria* sp.**

It is generally accepted that a 16S rRNA sequence similarity of  $\geq 97\%$  is a reasonable level for grouping the bacteria into species. The BLAST analysis of sequences obtained cyanobacterial strains *Oscillatoria* sp. showed 98-99% similarity with *Oscillatoria* genera. This similarity values clearly indicates that the cyanobacterial strain *Oscillatoria* sp. was of genus *Oscillatoria* sp. and identified as *Oscillatoria* sp., respectively as shown in the Figure 7.1. The partial 16S rRNA gene sequences of was submitted to GenBank and an accession number **MH 392711** was assigned with strain *Oscillatoria* sp. CY8 CPF.



**Figure 7.1** Phylogenetic tree showing the relationship of *Oscillatoria* sp. with its neighbouring species

### 7.3.2 Photosynthetic pigments

It was observed that chlorpyrifos caused inhibition of synthesis/ degradation of photosynthetic pigments in *Oscillatoria* sp. CYA8 CPF. Table 7.1 showed that after 5<sup>th</sup> day Chlorophyll a decreased from 2.44 mg L<sup>-1</sup> to 0.145 mg L<sup>-1</sup> against increasing conc. of chlorpyrifos, similarly Carotenoids also decreased from 0.088 mg L<sup>-1</sup> to 0.051 mg L<sup>-1</sup>. Among Phycobiliproteins: Phycocyanin, Allophycocyanin and Phycoerythrin pigments decreased against increasing conc. of chlorpyrifos. Phycocyanin decreased from 2.07 mg L<sup>-1</sup> to 0.81 mg L<sup>-1</sup>, Allophycocyanin decreased from 3.62 mg L<sup>-1</sup> to 1.95 mg L<sup>-1</sup> and Phycoerythrin decreased from 1.86 mg L<sup>-1</sup> to 0.66 mg L<sup>-1</sup>.

**Table 7.1** Photosynthetic pigments ( $\text{mg L}^{-1}$ ) of *Oscillatoria* sp. CYA 8 CPF treated with different concentrations of chlorpyrifos at 5<sup>th</sup> day.

Pigments (on 5 <sup>th</sup> day)	0 ppm	5 ppm	10 ppm	ANOVA
Chlorophyll a	0.24±0.05 <sup>a</sup>	0.19±0.05 <sup>b</sup>	0.14±0.01 <sup>c</sup>	P < 0.001
Carotenoids	0.08±0.01 <sup>a</sup>	0.06±0.01 <sup>b</sup>	0.05±0.0 <sup>c</sup>	P < 0.001
Phycocyanin	2.07±0.05 <sup>a</sup>	0.92±0.02 <sup>b</sup>	0.81±0.06 <sup>c</sup>	P < 0.001
Allophycocyanin	3.62±0.01 <sup>a</sup>	2.11±0.05 <sup>b</sup>	1.95±0.01 <sup>c</sup>	P < 0.001
Phycoerythrin	1.86±0.05 <sup>a</sup>	1.15±0.01 <sup>b</sup>	0.66±0.04 <sup>c</sup>	P < 0.001

Values of each row followed by the same letter(s) are not significantly different according to Duncan's multiple range test (P=0.05).

### 7.3.3 Antioxidant enzymes

To protect cyanobacteria from these harmful ROS-, cyanobacteria released more antioxidant enzymes like SOD, POD and CAT, which helps the cyanobacteria to tolerate chlorpyrifos stress. Increased level of antioxidant enzymes SOD, POD and CAT indicated that chlorpyrifos stress stimulated the generation of reactive oxygen species (ROS<sup>-</sup>). These ROS<sup>-</sup> are extremely harmful to cyanobacteria at high concentrations (Mittler, 2002; Meriga et al., 2004; Srivastava and Dubey, 2011).

SOD, POD and CAT activities were induced significantly with higher concentration of (Figure 7.2 a-c). The *Oscillatoria* sp. CYA 8 CPF showed increase of 69 and 100% activities of SOD in 5 and 10 ppm of chlorpyrifos, respectively, at 12 hour compared to control without chlorpyrifos (Figure 7.2 A). POD activity was increased by 54 and 81% (Figure 7.2 B) whereas activity of CAT increased by 31 and 65% (Figure 7.2 C) in 5 and 10 ppm chlorpyrifos compared to controls, respectively, at 12 hour. The results indicated that these antioxidant enzymes played a significant role to overcome the oxidative stress caused by chlorpyrifos.

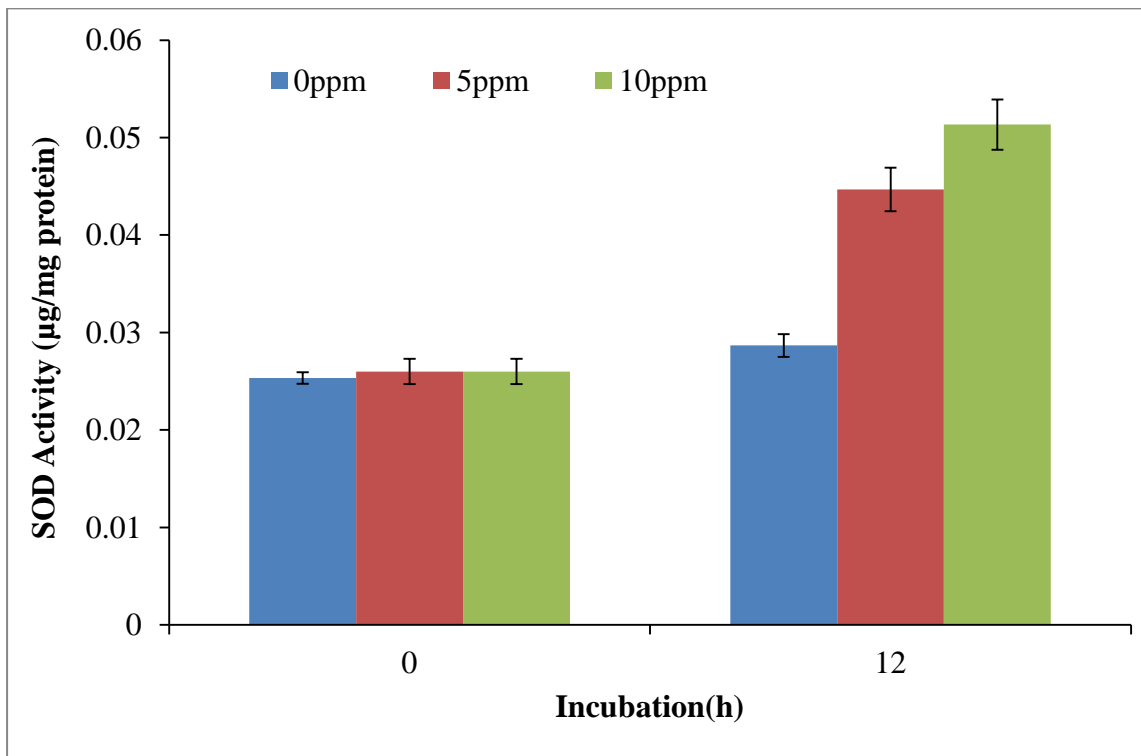


Figure 7.2 (a) SOD (superoxide dismutase) activity

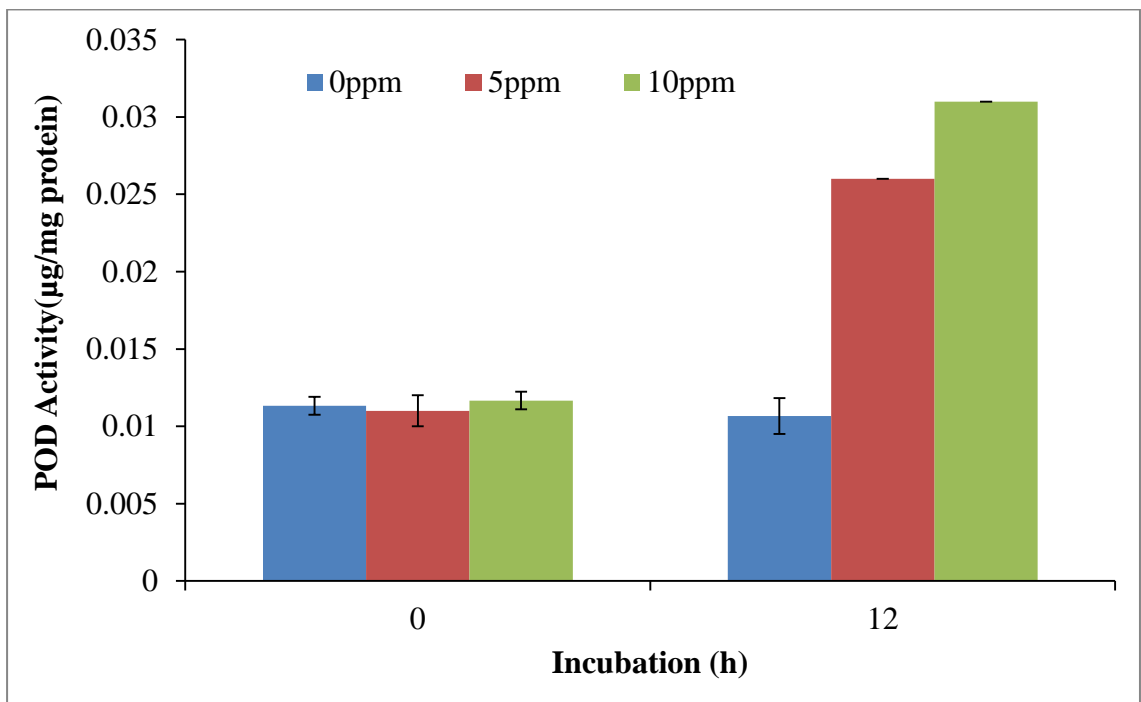
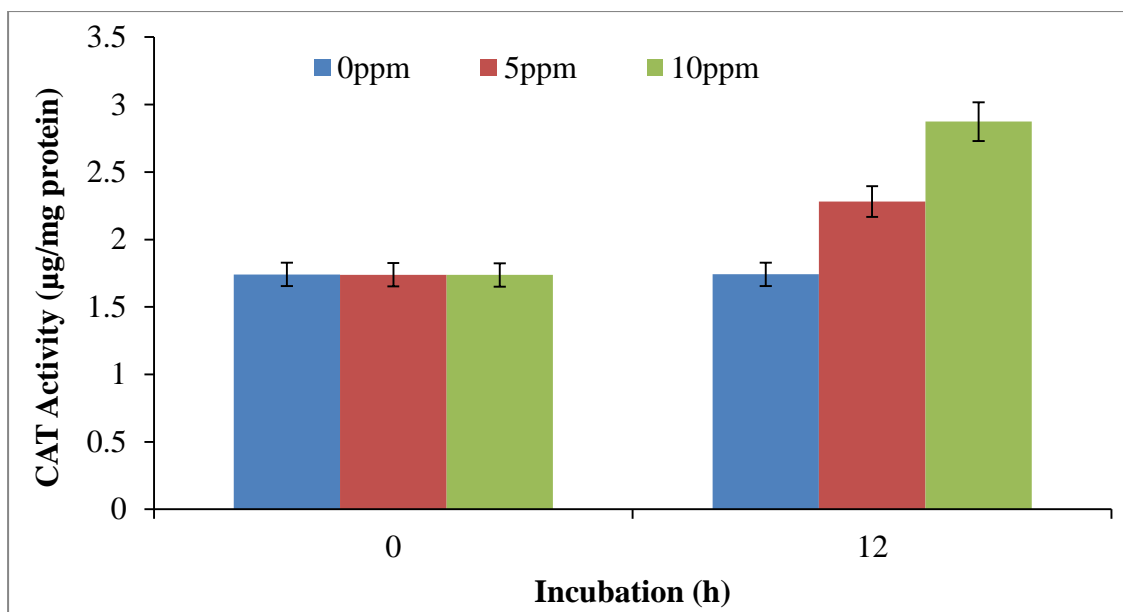


Figure 7.2 (b) POD (peroxidase) activity



**Figure 7.2 (c)** CAT (catalase) activity

#### 7.3.4 GSH

GSH content in *Oscillatoria* sp. CYA8 CPF decreased by 14 and 35 % with 5 and 10 ppm of chlorpyrifos (Figure 7.3), at 12 hour compared to control without chlorpyrifos respectively. Asada (1999) suggested that GSH (reduced form of glutathione) helps lowering the level of  $H_2O_2$  through the conversion in to glutathione peroxidase. So the decreased level of GSH indicated that it might be helping in reduction of  $H_2O_2$ .

#### 7.3.5 Proline content

Proline content *Oscillatoria* sp. CYA8 CPF increased by 42 and 73 % with 5 and 10 ppm of chlorpyrifos (Figure 7.4), at 12 hour compared to control without chlorpyrifos respectively. Fatma et al. (2007) suggested that due to environmental pollution, cyanobacteria accumulated intracellular proline to alleviate from oxidative stress. So increased level of Proline content indicted the cyanobacteria used proline accumulation to protect from the chlorpyrifos stress.

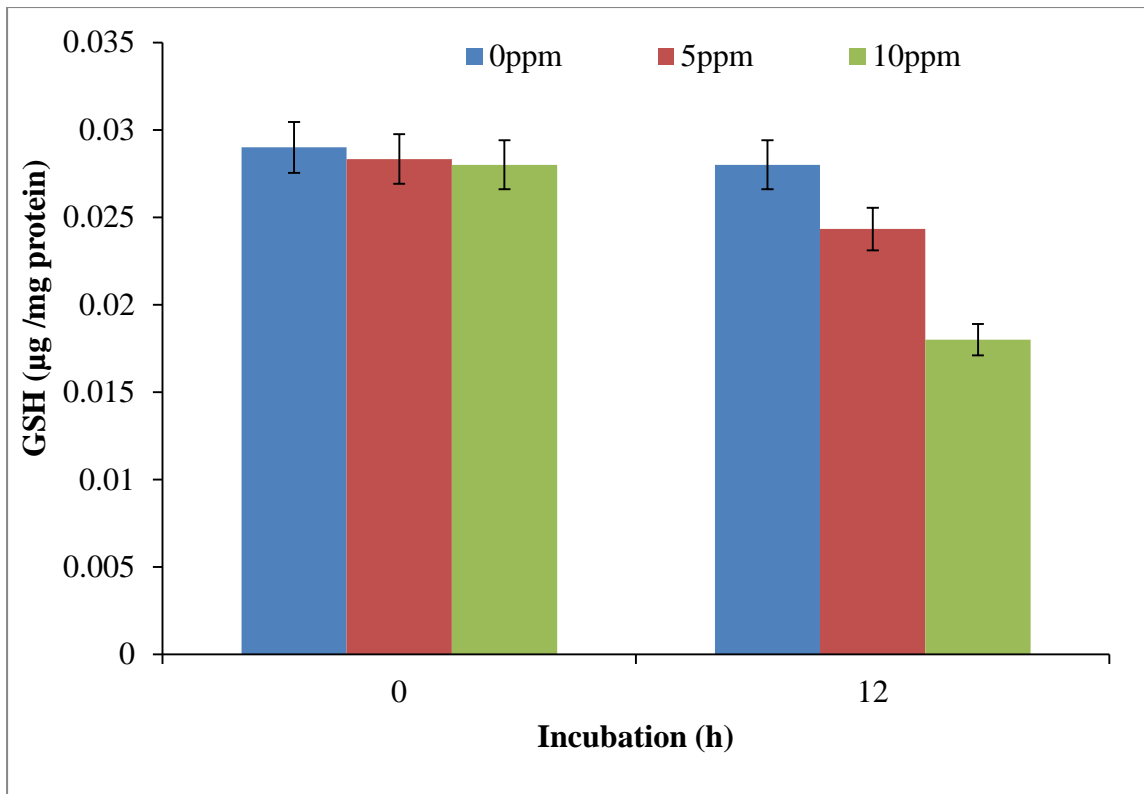


Figure 7.3 GSH activity

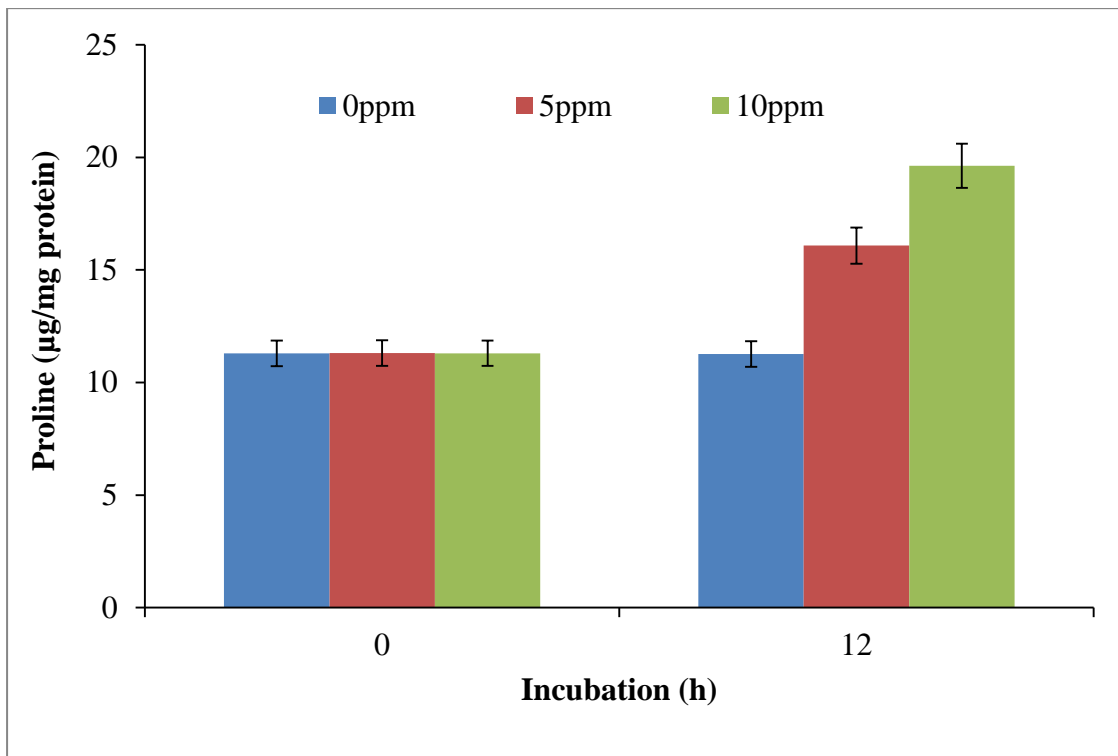


Figure 7.4 Proline activity

**7.3.6 GC-MS Analysis**

After analysing GC-MS chromatogram of Standard Chlorpyrifos, Supernatant and cell extract; chlorpyrifos peak is detected at RT 23.572, RT 22.880 and RT 22.9886 minutes the GC-MS chromatogram of Standard chlorpyrifos, supernatant and cell extract respectively. But there is no clear detection of end product of chlorpyrifos were detected such as TCP and DETP, which were reported in to the previous studies related to degradation of chlorpyrifos. So *Oscillatoria* sp. CYA8 CPF did not form any degradation product or nor it is released into the medium, but *Oscillatoria* sp. CYA8 CPF intake good amount of chlorpyrifos which observed by a clear and good peak in GS-MS chromatogram of cell extract. The GC-MS spectra obtained showed that the chlorpyrifos might be degraded to some small metabolites which could not be identified using the available library database. The presence of chlorpyrifos was observed in between RT 22.880 to RT 23.572 minutes but no any intermediate was identified. This indicated that chlorpyrifos is probably completely metabolized by the *Oscillatoria* sp. CYA8 CPF into smaller intermediates.

Thengodkar and Sivakami (2010), (Kumar, 2011) and Singh et al. (2011) reported the same phenomenon in case of organophosphorous pesticides (chlorpyrifos and anilofos). So *Oscillatoria* sp. CYA8 CPF was able to degrade chlorpyrifos completely without forming any clear end products which were reported in earlier studies.

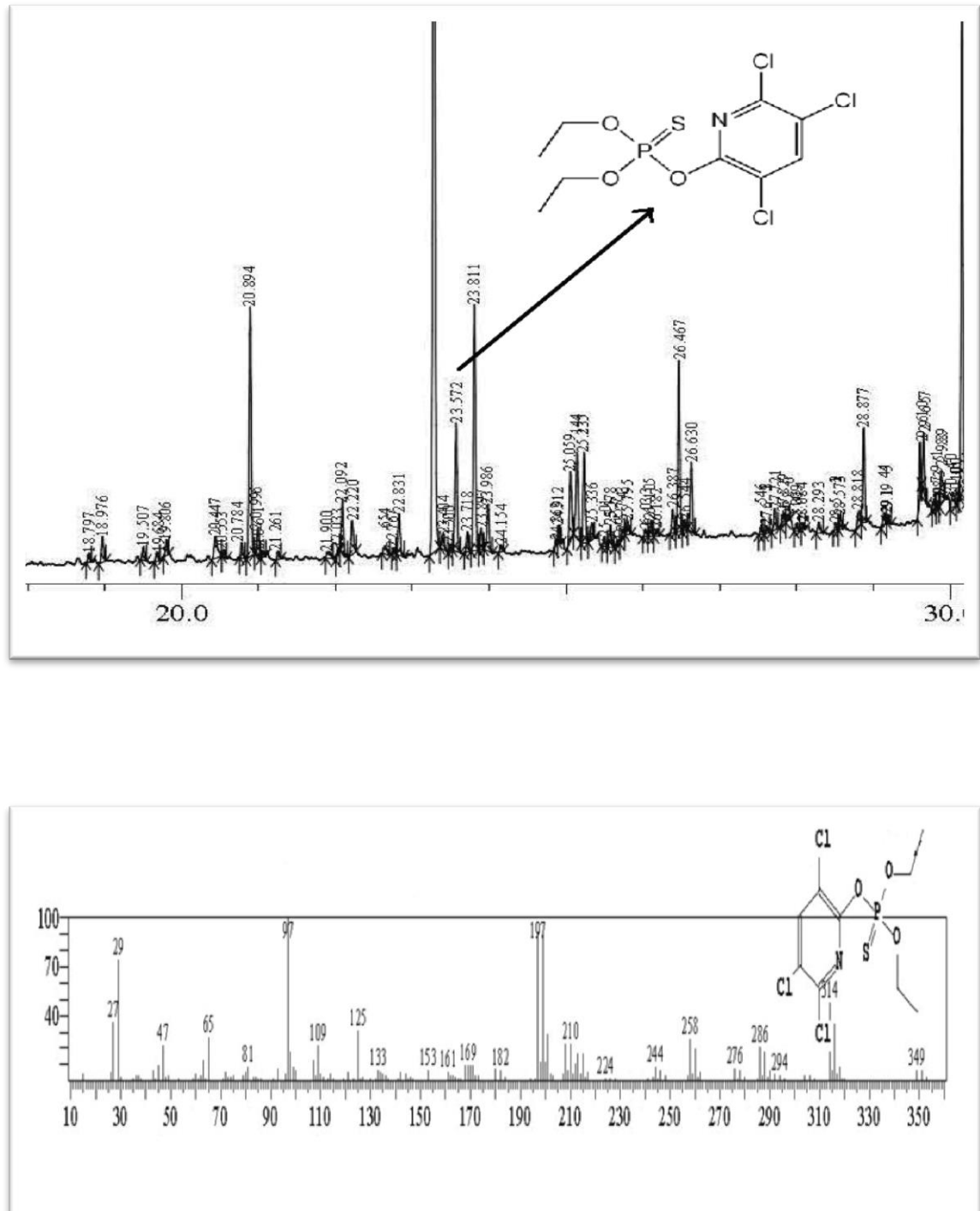


Figure 7.5 (a) GC-MS Chromatogram of standard chlorpyrifos

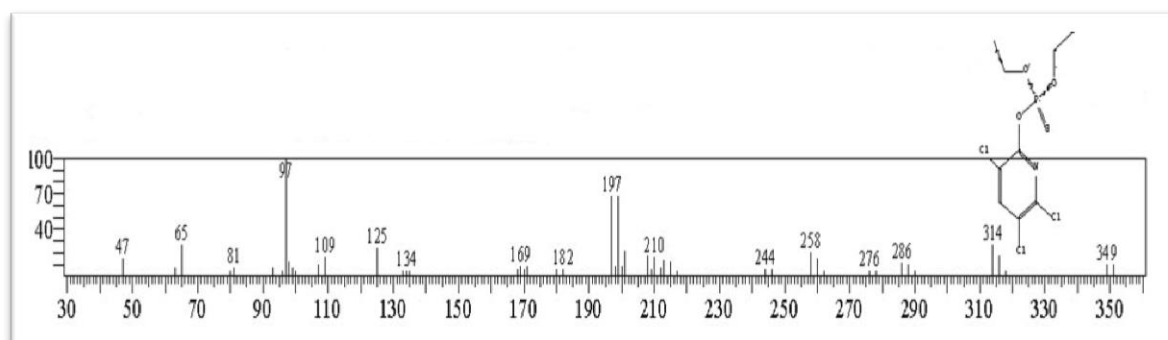
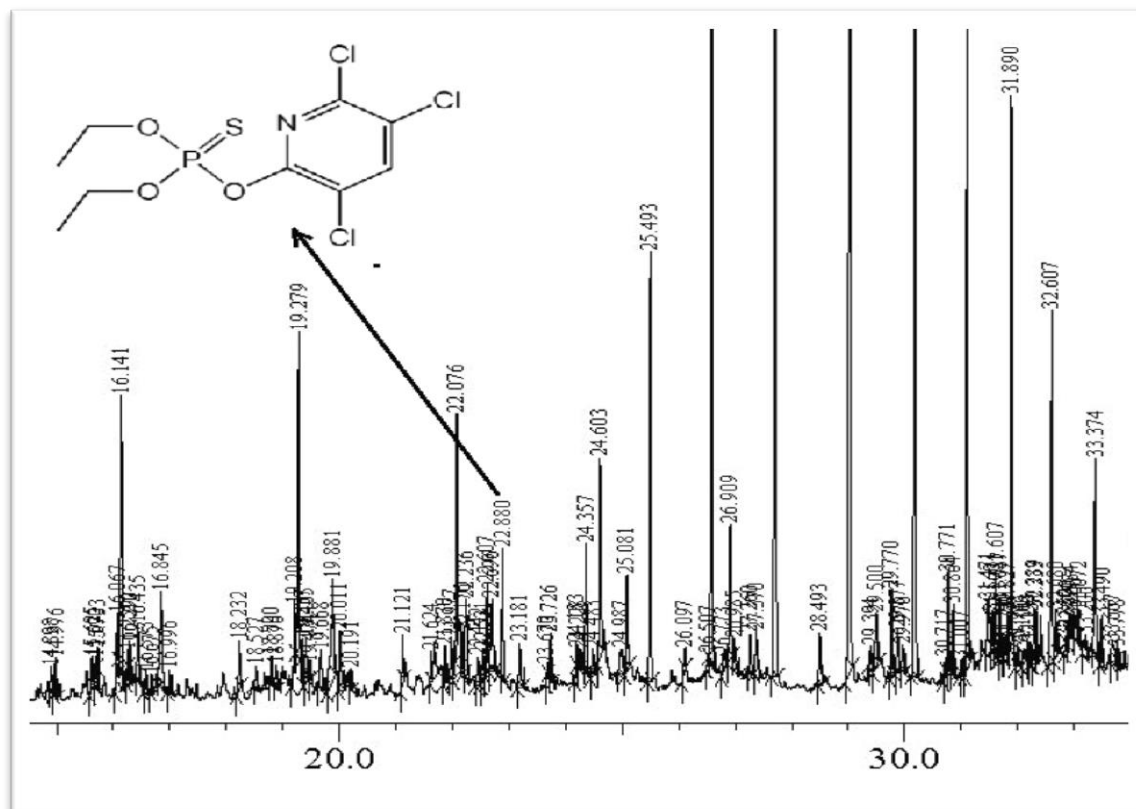


Figure 7.5 (b) GC-MS Chromatogram of supernatant

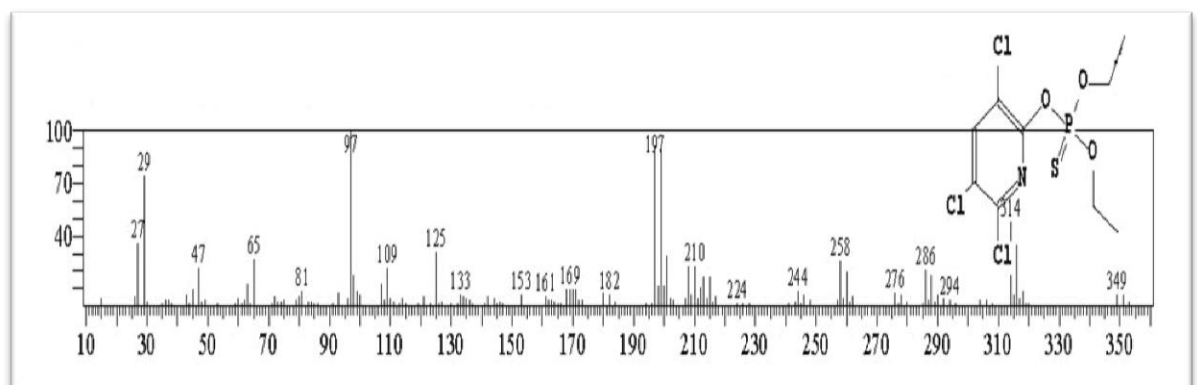
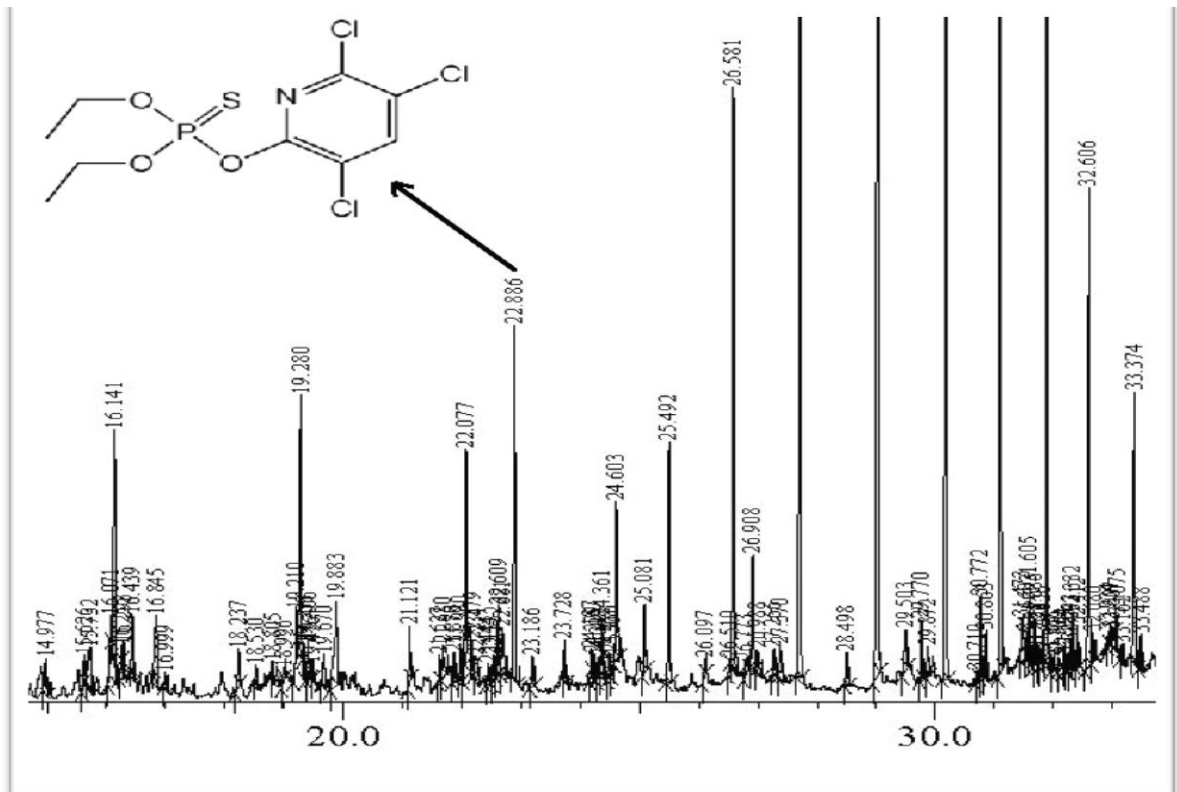


Figure 7.5 (c) GC-MS Chromatogram of cell extract

## **7.4 Discussion**

### **7.4.1 Phylogenetic tree of *Oscillatoria* sp. (CY8)**

Although all cyanobacterial strains identified according to the methods of Desikachary, 1959; Komarek and Anagnostidis (1998, 2005); which is well used for the identification of cyanobacteria. But the morphology of cyanobacteria may change depending on environmental conditions and the phase of growth of the organism (Lu et al., 1997; Murayama-kayano et al., 1998). This morphological variation may lead to difficulty and errors in cyanobacterial identification (Murayama-kayano et al., 1998). So molecular techniques 16S rRNA sequencing used for the identification of potential cyanobacteria *Oscillatoria* sp. CY8 CPF which was best in among of other isolated cyanobacterial strains from paddy fields of adjoining areas of Lucknow city, Uttar Pradesh.

### **7.4.2 Photosynthetic pigments**

Due to chlorpyrifos stress, photosynthetic pigments in *Oscillatoria* sp. CYA8 CPF were declined in significant manner. Regarding this, a number of reports are available regarding the effect of pesticides on the photosynthetic pigments in cyanobacteria (Abou-Waly et al., 1991; Babu et al., 2001; Mohapatra and Schiewer, 2000; Mostafa and Helling, 2002; Porsbring et al., 2009; Singh et al., 2013; Kumar et al., 2014) which also observed that pesticide have the deleterious effect on the photosynthetic pigment i.e. Chl a, carotenoids and phycobilioproteins (phycocyanin, allophycocyanin and phycoerythrin); which is crucial for the growth of cyanobacteria.

The degradation of photosynthetic pigments could be also due to the pesticide thylakoid membrane interaction. Mohapatra and Schiewer (2000) observed

that organophosphorous insecticides induced the changes in the thylakoid membrane in the *Synechocystis* PCC 6803 resulted in the decrease in the pigment content. Mostafa and Helling (2002) also agreed to this finding and also suggested that decrease in photosynthetic pigments could be due to the inhibition of pigment synthesis directly by the insecticide or accelerated degradation of pigments due to increased.

### **7.4.3 Antioxidant enzymes**

*Oscillatoria* sp. CYA8 CPF showed increased activity of antioxidant enzymes SOD, POD and CAT which indicated that to overcome the oxidative stress caused by the chlorpyrifos. Srivastava et al. (2005), Wiktelius and Stenberg (2007) suggested that that cyanobacteria have an antioxidant defence system, comprises of several enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidases (POD) and ascorbate peroxidase (APX); which protect the cells from the deleterious effect of reactive oxygen species (ROS), including superoxide radicals ( $O_2^-$ ), hydroxyl radicals (OH) and hydrogen peroxide ( $H_2O_2$ ). Imlay et al. (1998), Vandana et al. (2001) suggested that these reactive oxygen species (ROS) could be very harmful for cell membranes, proteins and nucleic acids; cause enzyme inactivation, protein denaturation, lipid peroxidation and also DNA mutation.

### **7.4.4 Glutathione**

In this study, low content of GSH in *Oscillatoria* sp. CYA8 CPF indicated that glutathione used for the protection from the oxidative stress caused by chlorpyrifos. Glutathione stored in the cyanobacterial cells in the reduced form i.e. GSH and it is necessary that glutathione must be available in reduced form (GSH) to prevent the cells from the harmful effects of ROS induced oxidative stress (Larson,

1988; Meyer, 2008; Briviba et al., 1997). Higher utilization of GSH under oxidative stress caused by a pesticide was also reported earlier (Tozum and Gallon, 1979; Karni et al., 1984).

#### **7.4.5 Proline content**

Under chlorpyrifos stress, increase in proline content in *Oscillatoria* sp. CYA8 CPF indicated that it might be used as strategy to overcome the pesticide stress. Bates et al. (1973), Alia and Saradhi (1993), Choudhary et al. (2007) and Kumar et al. (2008) proposed that under variety of stress conditions such as water, salt, drought, heavy metal and other toxicants in plants; increased accumulation of proline in is one of the adaptive roles to reduce free radical generation. Unlike in plants, cyanobacteria also used this strategy of proline accumulation under various stress. Working with *Nostoc muscorum*, Galhano et al. (2011) suggested that intracellular proline could be an indicator for stress tolerance capacity due to being a good hydroxyl and singlet oxygen scavenger. It is also was observed that endosulfan induced the proline accumulation in nitrogen fixing cyanobacteria *Nostoc muscorum*, *Anabaena variabilis* and *Aulosira fertilissima* in compared to control (Kumar et al., 2008).

#### **7.4.6 GC-MS Analysis**

From the GC-MS studies, it is well indicated that *Oscillatoria* sp. CYA 8 CPF able to completely degrade the chlorpyrifos. For the analysis of degradation studies, the samples were analysed by GC-MS technique. The GC-MS analysis showed the presence of chlorpyrifos in between RT of 22.880 to 23.572 minutes. The comparison with standard library of mass spectral database confirmed the matching of mass/charge ratio v/s relative intensity at in between of RT 22.880 to 23.572 minutes

for the samples to standard spectra of chlorpyrifos. The mass spectra obtained showed that the chlorpyrifos was degraded to some small metabolites which could not be identified using the available library database (Singh et al., 2013, Kumar et al., 2014). The presence of chlorpyrifos was observed at between RT of 22.880 to 23.572 minutes but no any intermediate was identified. This indicated that chlorpyrifos is probably completely metabolized by the isolates into smaller intermediates.

# *CHAPTER 8*

## *Summary and Conclusions*

Paddy crop is one of the important cereal crops of the world in terms of providing staple food for more than the 50 % of the world's populations. In Asia where 90 % of paddy is cultivated and consumed especially in countries like China, India, Indonesia, Bangladesh, Vietnam and Japan. Due to having major crop of Asian regions, paddy (rough rice) the basic staple for the majority of the population, including the region's million's of poor peoples. India is the second largest producer of the paddy where in north-east and south regions paddy is cultivated more than two or three times and imparts major portion in the daily diet in these regions.

Paddy is the crop which needs enough water to grow or mainly depends upon the rain for the good yields but due to global warming, there would be significant danger to the paddy production in terms of non-availability of good rainfall and arable land. Paddy crop is also affected by number of pests such as insects, diseases and weeds; which affects the paddy production in many folds in terms of loss in yields and quality. Although paddy growers uses variety of pesticides for the control of these but pesticides not only control the pests but also disrupt the paddy agro-ecosystem. Pesticides are responsible for the loss of beneficial organisms which play significant in the healthy ecosystem and pesticide residue which further creates problems in paddy ecosystem; somehow pollutes the nearby aquatic ecosystems.

Paddy agro-ecosystem has the many micro-habitats which supports the many types of communities of organisms such as microorganisms. Microbes play a beneficial role in maintaining healthy paddy agroecosystem by recycling of nutrients and soil fertility enhancement. The other beneficial microbes together with the

photoautotrophic cyanobacteria or blue green algae (BGA) account about 60% of total biomass and have been considered as most successful and abundant photosynthesizers on this Planet. The cyanobacteria have immense potential to contribute a very crucial role in agriculture and ecosystem services. A number of investigations have been conducted with objectives to improve our knowledge on the diversity, dynamics and roles of efficient soil microorganisms and their beneficial activities in the development of agricultural production. The advanced green-technologies such as bio-fertilizers consisting of cyanobacteria could be a powerful tool to remove soil contaminants, soil fertility restoration and enhanced agricultural production, rendering the paddy agro-systems more stress-tolerant. Furthermore, cyanobacteria can help to reduce the amount of energy input demands in the form of chemical fertilizers and mitigation of stress agro-ecosystems restoration. Now it well proved that the cyanobacteria are one of the important beneficial microorganisms of the paddy field agro-ecosystem. The cyanobacteria act as a bio-inoculants for the paddy crop, make available more nutrients and increase the quality of soil to the crops. Now days due to heavy dependence on pesticides to control pests in paddy crop, cyanobacteria also affected by the deleterious impacts of these pesticides. However, pesticides badly affected the cyanobacterial populations existed in the paddy fields; but some cyanobacterial genera were known to tolerate these pesticides in some extent and also able to degrade in lesser toxic intermediates.

So paddy field cyanobacteria could be promising entities to remediate pesticide contamination in paddy field agro-ecosystem can be implied in other areas related to remediation of various pollutants. The present research work describes the multidimensional role of cyanobacteria for reclamation and removal of pesticides of soil and sustainable paddy agriculture development. To investigate the ability of

cyanobacteria to degrade the pesticides; there is firstly needs of the systematic study of cyanobacterial diversity in paddy fields and then to check paddy field cyanobacteria against pesticides. The sites selected for the study of the adjoining areas of Lucknow city, Uttar Pradesh were:

<b>SNo</b>	<b>Study sites</b>	<b>Co-ordinates</b>
1.	BBAU Campus	26°46'03.0"N, 80°55'36.8"E
2.	Mohanlalganj	26°42'00.9"N, 80°59'18.4"E
3.	Malihabad	26°54'46.3"N, 80°43'27.6"E
4.	Bakshi ka Talab	26°58'59.3"N, 80°56'41.3"E
5.	Barabanki	26°54'12.8"N, 81°10'21.3"E

Soil samples were collected from the selected sites and the physico-chemical properties of soil such as temperature, pH, electrical conductivity (EC) and nutrient status such as total N, -P and organic-C contents were measured as per standard procedures. All the five sampling sites BBAU Campus, Mohanlalganj, Malihabad, Bakshi ka Talab and Barabanki showed suitable pH, EC and sufficient nutrients (N, P and C) which could be helpful for the good cyanobacterial growth in paddy agro-ecosystem (Kumar et al., 2018). This information about physico-chemical parameters of paddy field soil could be very useful to correlate with the cyanobacterial diversity.

The cyanobacterial diversity in the paddy fields of BBAU Campus, Mohanlalganj, Malihabad, Bakshi ka Talab and Barabanki was found to follow no regular pattern. There were different diversity of cyanobacteria in the paddy fields of the adjoining areas of Lucknow city, Uttar Pradesh; it was 430 g<sup>-1</sup> to infinite. The survey of paddy fields of adjoining areas of Lucknow city, Uttar Pradesh showed

moderate cyanobacterial diversity. Further 10 cyanobacterial genera *Synechococcus* sp., *Gloeocapsa* sp., *Anabaena* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoleus* sp., *Gloeotheca* sp., *Oscillatoria* sp., *Gleotrichia* sp., and *Aphanothece* sp. were isolated. The study has endorsed the ideal nature of paddy field for cyanobacterial growth.

These isolated cyanobacteria were further treated with chlorpyrifos, a widely organophosphate insecticide which applied in agriculture for the control of various insects and as well as in public health for control of disease vectors like mosquitoes. It is also used for controlling termites in wood fences and furniture. The mode of action of chlorpyrifos is like inhibiting the acetylcholinesterase enzyme which controls the acetylcholine (a neurotransmitter). Due to this communication between the nerve cells disrupted, leads to the nervous system malfunction and finally causes death of the pest. All the 10 isolated cyanobacteria grown in BG-11 medium containing 5 ppm chlorpyrifos (a minimum dose that is double the recommended dose in paddy fields) for the 12-14 days for the screening of chlorpyrifos tolerant cyanobacteria. The growth of *Synechococcus* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoleus* sp. and *Oscillatoria* sp. showed the regular increase up to day 14 while the growth of *Gloeocapsa* sp., *Anabaena* sp., *Gloeotheca* sp., *Gleotrichia* sp. and *Aphanothece* sp. showed increase up to day 8 and further declined. After analysing the growth curve and inhibition % of all cyanobacteria, *Synechococcus* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoleus* sp. and *Oscillatoria* sp. proved to be chlorpyrifos tolerant cyanobacteria.

The chlorpyrifos tolerant cyanobacteria *Synechococcus* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoleus* sp. and *Oscillatoria* sp. were investigated to study the efficacy of these cyanobacteria to tolerate chlorpyrifos in greater amount. All

cyanobacteria were treated further against 5, 7.5, 10, 15 and 20 ppm chlorpyrifos with control without no chlorpyrifos. At 5, 7.5, 10 ppm chlorpyrifos concentration, *Synechococcus* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoelus* sp. and *Oscillatoria* sp. showed similar growth pattern respectively; but at 15 and 20 ppm, *Aphanocapsa* sp. and *Microcoelus* sp. showed some slow growth in compare to *Synechococcus* sp., *Chroococcus* sp. and *Oscillatoria* sp.. In *Synechococcus* sp., *Chroococcus* sp. and *Oscillatoria* sp. which showed better growth at 15 and 20 ppm; *Oscillatoria* sp. showed maximum growth compared to *Synechococcus* sp. and *Chroococcus* sp. So *Oscillatoria* sp. proved potential cyanobacteria that could be used for the further study of analysis of degradation of chlorpyrifos.

The 16S rRNA sequencing of *Oscillatoria* sp. further confirmed the cyanobacteria was *Oscillatoria* sp. which the showed 98-99 % similarity with *Oscillatoria* genera. The partial 16S rRNA gene sequences of was submitted to GenBank and an accession number MH 392711 was assigned with strain *Oscillatoria* sp. CYA8 CPF.

Chlorpyrifos stress caused degradation of photosynthetic pigments chlorophyll a, carotenoids phycobiliproteins (phycocyanin, allophycocyanin and phycoerythrin) in *Oscillatoria* sp. CYA8 CPF. To protect from the chlorpyrifos stress, *Oscillatoria* sp. increased the production of antioxidant enzymes SOD, POD and CAT, which played a significant role to overcome the oxidative stress caused by chlorpyrifos.

There was in reduction in GSH content in *Oscillatoria* sp. CYA8 CPF which indicated that GSH (reduced form of glutathione) helps lowering the level of H<sub>2</sub>O<sub>2</sub> through the conversion in to glutathione peroxidase. So the decreased level of GSH indicated that it might be helping in reduction of H<sub>2</sub>O<sub>2</sub>. While Proline content in

*Oscillatoria* sp. increased which indicated the *Oscillatoria* sp. CYA8 CPF used proline accumulation to protect from the chlorpyrifos stress.

*Oscillatoria* sp. CYA8 CPF did not form any degradation product or nor it is released into the medium, but *Oscillatoria* sp. CYA8 CPF, intake good amount of chlorpyrifos which observed by a clear and good peak in GS-MS chromatogram of cell extract. The GC-MS spectra obtained showed that the chlorpyrifos might be degraded to some small metabolites which could not be identified using the available library database.

Finally it may be concluded that cyanobacteria have proven track record of restoring soil fertility they can be applied for refining and enhancing soil fertility of disturbed soil. However, there is still scope for further improvement in the cyanobacterial bio-technology and better exploitation of these microbes for better soil improvement process and paddy agricultural productivity. It is proposed that co-inoculations of paddy agricultural lands or degraded lands with selected pesticide tolerant cyanobacteria can be a suitable bio-formulation for improving the soil characteristics and soil detoxification. Nevertheless, there is dearth of information regarding the possible role of cyanobacteria in combination with other microbes in relation to their impact on removal of various pesticides from the paddy fields. Application of pesticide tolerant strains of cyanobacteria offer an ample scope in the field of sustainable paddy agriculture production and enriching soil fertility of degraded lands.

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*SCIENTIFIC  
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## List of Publications

### Review/Research articles

Singh JS, **Kumar A**, Rai AN, Singh DP (2016) Cyanobacteria: A precious bio-resource in Agriculture, Ecosystem, and Environmental sustainability. *Front Microbiol* 7: 529.

Singh JS, Koushal S, **Kumar A**, Vimal SR, Gupta VK (2016) Book Review: Microbial inoculants in sustainable agricultural productivity- Vol. II: Functional Application. *Front Microbiol* 7: 2105.

**Kumar A**, Kaushal S, Saraf SA, Singh JS (2017) Cyanobacterial Biotechnology: An opportunity for sustainable industrial production. *Climate Change Environ Sustain* 5(1): 97-110.

**Kumar A**, Kaushal S, Saraf SA, Singh JS (2018) Screening of Chlorpyrifos (CPF) tolerant cyanobacteria from paddy field soil of Lucknow, India. *Int J Appl Adv Sci Res* 3(1): 100-105.

**Kumar A**, Kaushal S, Saraf SA, Singh JS (2018) Microbial bio-fuels: a solution to carbon emissions and energy crisis. *Front Biosci Landmark* 23: 1789-1802.

### Book Chapters

**Kumar A**, Singh JS (2016) Microalgae and cyanobacteria biofuels: A sustainable alternate to crop-based fuels. In: *Microbes and Environmental Management*. Singh JS, Singh DP (eds.), Studium Press Pvt. Ltd., New Delhi. pp. 1–20.

**Kumar A**, Singh JS (2017) Cyanoremediation: A green-clean tool for decontamination of synthetic pesticides from agro- and aquatic ecosystems. In: *Agro-Environmental Sustainability- Vol. II: Managing Environment Pollution*. Singh JS, Seneviratne G (eds.), Springer, Heidelberg. pp. 59-83.

## Scientific achievements

### Workshop & Conferences attended

International Conference on Environmental Technology and Sustainable Development: Challenges & Remedies (IET&SD) on Feb 21-23, 2014.

International Workshop on Bridging Development Divide for Inclusive Growth through Science, Technology and Innovation (BRIDGES) on Jan 16-17, 2015.

Hands-on-Training on SEM, FTIR, FPLC and Ion Chromatography at University Science Instrumentation Center, Babasaheb Bhimrao Ambedkar University, Lucknow on Feb 18-20, 2015.

National conference on Recent Trends in Applied Microbiology, Human Health & Environment on March 27-28, 2015.

National conference on Climate Change and Sustainable Development: Emerging Issues and Mitigation Strategies (CCSD-2015) on November 23-24, 2015.

National Symposium on Impact of Climate Change on Plant-Microbe Interactions and its Implications (ICCPMI-2015) on December 18-19, 2015.

57<sup>th</sup> Annual Conference of AMI & International Symposium on Microbes and Biosphere: What's New What's Next on November 24-27, 2016.

Seven days workshop on "Gene Cloning & Its Expression, To Produce Genetically Modified Organisms" on November 20-26, 2017.

### Professional Memberships

Life member of Association of Microbiologists of India (AMI)

Life member of Indian Science Congress Association (ISCA)

**Poster Presentation**

**Arun Kumar**, Jay Shankar Singh (2016) “Isolation of Cyanobacterial strains from paddy field soil and their screening for degradation of pesticide Chlorpyrifos”. 57th Annual conference of AMI, Gauhati University, Guwahati, Assam, India. **Poster presentation**

**Arun Kumar**, Jay Shankar Singh (2015) “Cyanobacteria: A potential biological control agent towards plant pathogenic bacteria and fungi”. ICCPMI-2015, BHU, Varanasi. **Poster presentation**

**Arun Kumar**, Jay Shankar Singh (2015) “Cyanobacteria (BGA): A clean-green tool for safe agriculture and environmental stability”. Recent Trends in Applied Microbiology, Human Health & Environment, Bundelkhand University, Jhansi. **Poster presentation**

# Microalgae and Cyanobacteria Biofuels: A Sustainable Alternate to Crop-based Fuels

ARUN KUMAR AND JAY SHANKAR SINGH\*

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## ABSTRACT

*Fossil fuels reserves are finite and also responsible for the Green House Gas emissions and ultimately lead to global warming. So there is need to develop sustainable alternative energy sources which not only able to fulfill the fuel demand of present and future generations but also be environmentally safe and economically viable. Although crop based bio-fuels are currently in used but they have limitation such as requirement of arable land, high inputs and replacement of food crops that may leads to further pressure on sustainable agriculture. Microalgae and cyanobacteria are currently considered as potential candidate for bio-fuel production because of their rapid growth rate, CO<sub>2</sub> fixation ability and high production capacity of lipids and at the same time they also do not compete with food or feed crops, and can be produced on non-arable land. Microalgae and cyanobacteria have broad bio-fuel potential as they can be used to produce hydrogen, liquid fuels (bio-diesel, bio-ethanol) and bio-methane. This chapter reveals the potential of microalgae and cyanobacteria for efficient production of bio-fuels; cultivation and harvesting techniques, large scale production of bio-fuels and role of genetic engineering in betterment of their quantity and quality production of bio-fuels.*

**Key words:** Biodiesel, Bio-ethanol, Bio-methane, Cyanobacteria, Microalgae

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<sup>1</sup> Department of Environmental Microbiology, B.B. Ambedkar (Central) University, Lucknow - 226025, Uttar Pradesh, India

\* Corresponding author: E-mail: jayshankar\_1@yahoo.co.in



# Cyanobacteria: A Precious Bio-resource in Agriculture, Ecosystem, and Environmental Sustainability

Jay Shankar Singh<sup>1\*</sup>, Arun Kumar<sup>1</sup>, Amar N. Rai<sup>2</sup> and Devendra P. Singh<sup>3</sup>

<sup>1</sup> Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar University, Lucknow, India, <sup>2</sup> Department of Biochemistry, North-Eastern Hill University, Shillong, India, <sup>3</sup> Department of Environmental Science, Babasaheb Bhimrao Ambedkar University, Lucknow, India

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### \*Correspondence:

Jay Shankar Singh  
jayshankar\_1@yahoo.co.in

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Keeping in view, the challenges concerning agro-ecosystem and environment, the recent developments in biotechnology offers a more reliable approach to address the food security for future generations and also resolve the complex environmental problems. Several unique features of cyanobacteria such as oxygenic photosynthesis, high biomass yield, growth on non-arable lands and a wide variety of water sources (contaminated and polluted waters), generation of useful by-products and bio-fuels, enhancing the soil fertility and reducing green house gas emissions, have collectively offered these bio-agents as the precious bio-resource for sustainable development. Cyanobacterial biomass is the effective bio-fertilizer source to improve soil physico-chemical characteristics such as water-holding capacity and mineral nutrient status of the degraded lands. The unique characteristics of cyanobacteria include their ubiquity presence, short generation time and capability to fix the atmospheric N<sub>2</sub>. Similar to other prokaryotic bacteria, the cyanobacteria are increasingly applied as bio-inoculants for improving soil fertility and environmental quality. Genetically engineered cyanobacteria have been devised with the novel genes for the production of a number of bio-fuels such as bio-diesel, bio-hydrogen, bio-methane, synga, and therefore, open new avenues for the generation of bio-fuels in the economically sustainable manner. This review is an effort to enlist the valuable information about the qualities of cyanobacteria and their potential role in solving the agricultural and environmental problems for the future welfare of the planet.

**Keywords:** agriculture, bioremediation, beneficial microbes, bio-fertilizers, cyanobacteria

## INTRODUCTION

The present world population of about 7.2 billion is expected to cross 9.6 billion by the end of year 2050. In order to provide food to all by that times, the annual production of cereals needs a jump of about 50%, i.e., from 2.1 billion tons per year to ~3 billion tons per year. This onerous target puts enormous pressure on agriculture sector to achieve the food security. But such a quantum

**Abbreviations:** BGA, blue green algae; BOD, biological oxygen demand; CCS, carbon capturing and storage; COD, chemical oxygen demand; EPS, extracellular polymeric substances; FYM, farm yard manure; GHGs, green house gases; WHC, water holding capacity.



# Book Review: Microbial Inoculants in Sustainable Agricultural Productivity- Vol. II: Functional Application

Jay Shankar Singh<sup>1\*</sup>, Sumit Koushal<sup>1</sup>, Arun Kumar<sup>1</sup>, Shobhit R. Vimal<sup>1</sup> and Vijai K. Gupta<sup>2</sup>

<sup>1</sup> Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar (Central) University, Lucknow, India, <sup>2</sup> Molecular Glyco-biotechnology Group, Discipline of Biochemistry, School of Natural Sciences, National University of Ireland Galway (NUI), Galway, Ireland

**Keywords:** agriculture, cyanobacteria, inoculants, microbes, mycorrhiza

## A Book Review on Microbial Inoculants in Sustainable Agricultural Productivity- Vol. II: Functional Application

by D. P. Singh, H. B. Singh, and R. Prabha, (New Delhi; Heidelberg; New York, NY; Dordrecht; London: Springer), 2016, 308 pages, ISBN: 978-8132226420.

Ecologically sustainable agricultural practices are essential to ensure food security, and efficient agriculturally beneficial microbes (microbial inoculants) are playing potential role in sustainable crop production due to their immense plant growth promoting attributes, better adaptability to survival under stresses, and other uses that result in attenuating the pesticides/fertilizers use in agriculture. However, the unpredictable biogenic and abiogenic soil factors that determine the nature and magnitude of the microbial inoculants responses and survival after their delivery in the field conditions remain unresolved.

Soil microbes via several processes play indispensable roles in the supply of valuable nutrients to crop plants (Bashan et al., 2014). The presence of beneficial microbial communities in the rhizosphere minimizes the susceptibility to crop diseases (Singh, 2015). Because of ability to produce plant growth promoting and other molecules from secondary metabolism, beneficial microbes are widely used as commercial bio-inoculants (Singh et al., 2016a). However, a great diversity of valuable microbial inoculants continues to be revealed, and little is known about the potential applications of new efficient microbial formulations that have been described.

The book *Microbial Inoculants in Sustainable Agricultural Productivity Vol. II Functional Applications* essentially addresses the field usage of microbial agents (biofertilizers, biostimulants, biopesticides) for boosting agriculture sustainability. In this volume, a total of 19 chapters have been distributed over 308 pages. It contains the relevant topics contributed by the well-known researchers from different universities and institutes. Entire chapters in different subject areas contributed by the leading authors can be grouped into four parts. The first part (chapter 1–11) highlights the use of bio-inoculants in management of crop plant stresses. This section provide satisfactory information about diverse group of microbes (rhizobia, cyanobacteria, actinomycetes, mycorrhiza, endophytes, etc.) that have been developed as microbial inoculants with beneficial functions at different levels and many chapters have touched on commercial production for applications in field conditions for farmers' benefits. Recently, plant growth promoting rhizobacteria (PGPRs) and cyanobacteria have gained attention for their indispensable role in restoration ecology and sustainable agriculture (Singh and Strong, 2016; Singh et al., 2016b). Therefore, selection of such efficient microbial strains with well-defined PGP mechanisms can be exploited in development of bio-fertilizer/bio-pesticide inoculants for achieving consistent and economical results under field conditions. Furthermore, these microbial inoculants with tested results can be considered as integrated nutrient management system to sustain agricultural productivity with no adverse environmental impact. Though, these bio-agents play a significant role in soil nutrient management, the knowledge about their roles, functioning and the mechanism

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Banaras Hindu University (BHU), India

### \*Correspondence:

Jay Shankar Singh  
jayshankar\_1@yahoo.co.in

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# Cyanobacterial Biotechnology: An Opportunity for Sustainable Industrial Production

Arun Kumar<sup>1</sup> • Sumit Kaushal<sup>1</sup> • Shubhini A. Saraf<sup>2</sup> • Jay Shankar Singh<sup>3\*</sup>

**Abstract** In this communication, a comprehensive commentary on contribution of cyanobacteria and micro-algae in well being of humans and environment has been postulated. In the recent years cyanobacteria and micro-algae have gained much more attention because of their valuable biotechnological applications. These valuable bio-agents are potential source of various compounds such as exopolysaccharides, lipids, proteins, vitamins, sterols, enzymes, pharmaceuticals and other valuable life supporting chemicals. Secondary metabolites derived from cyanobacteria and micro-algae have been reported to be beneficial as antibacterial, antifungal, antiviral, anticancer, anti-malarial and immunosuppressive properties and can open new doors to develop more effective drugs in agriculture, industrial and human welfare. Some cyanobacteria and micro-algae can accumulate polyhydroxyalkanoates intracellularly; having similar properties to polyethylene and polypropylene. These biodegradable plastics can be alternative to oil-derived thermoplastics incoming future. An inclusive knowledge on the diversity, physiology and inherent genome organisation of cyanobacteria and their beneficial genetic manipulations may be helpful to provide better opportunity for sustainable industrial products and environmental development. This article remarks meaningful information about the possible uses of cyanobacteria and micro-algae in industry sector and also expresses an outlook on the challenges and future prospects of cyanobacteria and micro-algal biotechnology.

**Keywords** Biotechnology, Cyanobacteria, Colorants, Micro-algae, Nutrition, Pharmaceuticals, PHA,

## 1. Introduction

Cyanobacteria (Blue-Green Algae, BGA) comprise a highly diverse group of prokaryotic microorganisms

throughout the globe performing oxygenic photosynthesis (Garcia-Pichel and Pringault, 2001; Kulasooriya, 2011). Oxygen generated by cyanobacteria helped the ancient reducing atmosphere of the earth to an oxidising one (Olson, 2006) and facilitates the evolution of biodiversity on the planet Earth. It is also suggested that origin of chloroplasts of eukaryotic algae and higher plants have developed from endosymbiotic relationships by cyanobacteria. This event in the early evolution of life has stimulated the advent of oxygen tolerant flora and fauna capable of aerobic respiration, resulted in the predominance of oxygenic and aerobic species diversity globally (Kulasooriya, 2011). Cyanobacteria have been reported to flourish in different morphological forms ranging from unicellular to filamentous (Castenholz and Phylum, 2001). While unicellular cyanobacteria exist as single cells, suspended or benthic, or aggregate while filamentous structures either thin or thick, single trichome or bundles with or without a sheath (Abed *et al.*, 2009).

Micro-algae are evolutionarily diverse, unicellular, eukaryotic organisms, use sunlight to produce biomass and oxygen from carbon dioxide (CO<sub>2</sub>), water and simple mineral nutrients (Kulasooriya, 2011). In most of the terrestrial (paddy soils), aquatic ecosystems (marine waters, hypersaline, brackish waters, soda lakes, freshwater etc.) and extreme environments (hot springs and Polar Regions), cyanobacteria and micro-algae are the primary producers at the base of the food web in an ecosystem. Cyanobacteria have been reported to exist as symbiont in association with a variety of organisms, that is the marine diatom *Rhizosolenia*, leaves of *Azolla* and the roots of *Cycas* (Thajuddin and Subramanian, 2005; Abed *et al.*, 2009). Cyanobacteria and micro-algae, an interesting natural source of diverse bioactive compounds, can be used as functional ingredients of several medicines, cosmetics and new

<sup>1</sup>Research Scholar, <sup>2</sup>Assistant Professor, Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar (Central) University, Lucknow-226025, Uttar Pradesh, India

<sup>3</sup>Professor, Department of Pharmaceutical Sciences, Babasaheb Bhimrao Ambedkar (Central) University, Lucknow-226025, Uttar Pradesh, India

\*Corresponding author E-mail id: jayshankar\_1@yahoo.co.in

# Chapter 4

## Cyanoremediation: A Green-Clean Tool for Decontamination of Synthetic Pesticides from Agro- and Aquatic Ecosystems

Arun Kumar and Jay Shankar Singh

**Abstract** Immense use of synthetic chemicals in agriculture has deleterious effects on the environment even outside agro-ecosystem, microbial biodiversity, water bodies, and on life especially at the end of food chain, including humans. Therefore, there is a need to develop some viable and eco-friendly tools to remove these lethal chemicals from the environment. Bioremediation has been considered as a less-expensive alternative to physical and chemical means to decontaminate and degrade the pesticides from the contaminated sites. A number of microorganisms such as bacteria, fungi, actinomycetes, and cyanobacteria have been reported to degrade the pesticides. However, cyanobacteria (formally known as blue-green algae—BGA), the only known group of prokaryotes, capable of oxygenic photosynthesis and ubiquitous in distribution, have the remarkable ability to survive in harsh environments. Therefore, cyanobacteria could be a potential bioagent in degradation of noxious chemicals including pesticides. As a bioremediating agent, cyanobacteria have some advantages over other microbes in bioremediation, i.e., phototrophic nature makes them self-sufficient in growth, ability to fix nitrogen, and ease in biomass recovery. Some efficient and potential cyanobacterial genera such as *Anabaena*, *Leptolyngbya*, *Microcystis*, *Nostoc*, *Spirulina*, and *Synechocystis* have been found to tolerate and degrade various pesticides and herbicides. Biodegradation capabilities of cyanobacteria can be improved through genetic engineering, which can be exploited as cost-effective and eco-friendly remediation technology. This review focuses on the potential of cyanobacteria in the biodegradation of synthetic chemical residues from agro- and aquatic ecosystems.

**Keywords** Bioremediation • Cyanobacteria • Ecosystems • Insecticides • Synthetic Pesticides • Cell Immobilization

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A. Kumar

Department of Environmental Microbiology, School for Environmental Sciences,  
B.B. Ambedkar (Central) University, Raibareilly Road, Lucknow, Uttar Pradesh 226025, India

J.S. Singh (✉)

Department of Environmental Microbiology, Babashaeb Bhimrao Ambedkar (Central)  
University, Lucknow, Uttar Pradesh, India

e-mail: [jayshankar\\_1@bbau.ac.in](mailto:jayshankar_1@bbau.ac.in)



## SCREENING OF CHLORPYRIFOS (CPF) TOLERANT CYANOBACTERIA FROM PADDY FIELD SOIL OF LUCKNOW, INDIA

Arun Kumar\*, Sumit Kaushal\*, Shubhini A. Saraf\*\* &  
Jay Shankar Singh\*

\* Department of Environmental Microbiology, BB Ambedkar (Central) University,  
Lucknow, Uttar Pradesh

\*\* Department of Pharmaceutical Sciences, Babasaheb Bhimrao Ambedkar (Central) University,  
Lucknow, Uttar Pradesh

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### Abstract:

In this study, paddy field soil, collected from different locations of Lucknow, India i.e. BBAU campus, Mohanlalganj, Barabanki, Bakshika Talab and Malihabad. The cyanobacterial populations in soil samples of selected sites were maximum at Bakshika Talab (Indefinite number  $g^{-1}$  soil) and minimum at Mohanlalganj (430  $g^{-1}$  soil). Total 10 cyanobacteria *Synechococcus* sp., *Gloeocapsa* sp., *Anabaena* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoelus* sp., *Gloeotheca* sp., *Oscillatoria* sp., *Gleotrichia* sp. and *Aphanothece* sp. were isolated from paddy field soil. For the screening of Chlorpyrifos (CPF) pesticide tolerant cyanobacteria, all the isolated cyanobacterial strains were grown on BG-11 media containing 5 ppm of CPF concentration. Among 10 cyanobacteria, only *Synechococcus* sp., *Aphanocapsa* sp., *Chroococcus* sp., *Microcoelus* sp. and *Oscillatoria* sp. were able to tolerate CPF. These CPF tolerant cyanobacteria will be used further for the study related to efficacy of the strains to tolerate CPF.

**Key Words:** Paddy Field Soil, Chlorpyrifos, Cyanobacteria, Pesticide Stress & Pesticide Tolerance.

**Abbreviations:** CPF-Chlorpyrifos; MPN- Most Probable Number; WHO-World Health Organization; FAO-Food and Agriculture Organization

### Introduction:

Paddy is a major cereal crop in the world and rice is a staple food for nearly 3.5 billion people worldwide (IRRI, 2013). Currently worldwide rice production has been estimated about 481.04 million tonnes which is 2.06 million tonnes (0.43%) less than previous year's rice production 483.1 million tonnes (USDA, 2017). However, the present rate of rice production would not be enough to satisfy the food demand coming generation population. Apart from pressure of increasing rice production, paddy cultivation is affected by several pests mediated diseases that are responsible for almost 37 % crop yield losses (Sparks et al., 2012). To deal with this situation of pest mediated paddy yield loss, the farmers are using extensively the chemicals like pesticides. These pesticides not only pollute soils and food grains but also harmful for the loss of beneficial micro-flora including cyanobacteria (Kumar and Singh, 2017).

In paddy fields, cyanobacteria contributes in enhancing the soil fertility by fixing atmospheric nitrogen and releasing some plant growth promoting substances, extra cellular polysaccharides and also solubilizing phosphates (Singh et al., 2016). A number of reports are available related to pesticide-induced inhibitory effects on cyanobacteria and stated that it influence their growth, photosynthesis, nitrogen fixation, biochemical composition and metabolic activities (Singh et al., 2011, Singh et al., 2013, Kumar et al., 2013; Kumar et al., 2014; Kumar and Singh, 2017). However, only few study demonstrated the capability of cyanobacteria for the degradation of pesticides (Lee et al., 2003; Barton et al., 2004; El-Bestawy et al. 2007; Cáceres et al., 2008; Singh et al., 2011; Singh et al., 2013). Therefore, it is need of the hour to investigate the role of cyanobacteria regarding its tolerance level and degradation of insecticides/pesticides used in the paddy agriculture.

Chlorpyrifos (CPF), a broad spectrum insecticide, is extensively used for the control of foliar insects in paddy fields (Lee et al., 2012). It is moderately persistent in nature and could be remains active in soil for 20-90 days along with 10-60 days of half-life (Lakshmi et al., 2008; Singh et al., 2011). It has been reported that CPF degradation in the soil is affected by some factors such as soil pH, moisture, temperature and initial pesticide concentration. It is well known that volatilization, hydrolysis and microbial degradation are the common routes by which CPF is assimilated in soil or environment. Aysal et al. (2004) during an investigation found that the CPF residues entered in significant quantity in food chain. In 2004, WHO and FAO conducted good agricultural practices in Columbia, Philippines, Thailand, Vietnam and India to estimate the maximum residue level of CPF in rice grains through supervised trials using CPF median residue ranged from 0.12 to 0.28 mg/kg. The data showed the maximum CPF residue level of 0.5 mg/kg in the rice. It is also reported that CPF residue in some vegetables with the range of 0.024–0.07 mg/kg and 0.018–0.021 mg/kg in Cauliflower and Brinjal (Chandra et al., 2010). The LD<sub>50</sub> for CPF in rat has been reported to be 82–270 mg/kg body weight (Berg 1986; US Environment Protection Society, 1984). Sun and Chen (2008) found CPF residue in the farmed fish because of existence in fish feed and they also confirmed dietary accumulation of CPF by indoor carp study.

## Microbial bio-fuels: a solution to carbon emissions and energy crisis

Arun Kumar<sup>1</sup>, Sumit Kaushal<sup>1</sup>, Shubhini A. Saraf<sup>2</sup>, Jay Shankar Singh<sup>1</sup>

<sup>1</sup>Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar (Central) University, Lucknow-226025, Uttar Pradesh, India, <sup>2</sup>Department of Pharmaceutical Sciences, Babasaheb Bhimrao Ambedkar (Central) University, Lucknow-226025, Uttar Pradesh, India

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### 1. ABSTRACT

Increasing energy demand, limited fossil fuel resources and climate change have prompted development of alternative sustainable and economical fuel resources such as crop-based bio-ethanol and bio-diesel. However, there is concern over use of arable land that is used for food agriculture for creation of biofuel. Thus, there is a renewed interest in the use of microbes particularly microalgae for bio-fuel production. Microbes such as micro-algae and cyanobacteria that are used for biofuel production also produce other bioactive compounds under stressed conditions. Microbial agents used for biofuel production also produce bioactive compounds with antimicrobial, antiviral, anticoagulant, antioxidant, antifungal, anti-inflammatory and anticancer activity. Because of importance of such high-value compounds in aquaculture and bioremediation, and the potential to reduce carbon emissions and energy security, the biofuels produced by microbial biotechnology might substitute the crop-based bio-ethanol and bio-diesel production.

### 2. INTRODUCTION

During 1990-2013, the primary energy demand of the world was increased by 55% to

13,560 million tons and is projected to grow about 45% by 2040 (1). According to US EIA (2), worldwide consumption of energy derived from fossil fuels will grow about 177 quadrillions British thermal units (BTUs) in the year 2040. If the fossil fuels had kept the same share the CO<sub>2</sub> emissions will increase from 32.3 billion metric tons in 2012 to 35.6 billion metric tons in 2020. To cope up this energy-related CO<sub>2</sub> emissions, the dependence on fossil fuel should be reduce before arriving vulnerable consequences of climate change. Recently in 2016, all the nations gathered and agreed to keep a global temperature this century well below 2.0 °C. Some largest carbon emission nations such as China, US and India have also started to promote renewable energy in great amount. This can be considered as a better approach to reduce the fossil fuel consumption to minimize the carbon emissions.

The crop-based biofuels could also be another option which can help to provide us a clean-green environment as well as energy security. Currently, crop-based biofuels such as ethanol and biodiesel, successfully used in many countries, helps in the reduction of carbon emissions. However, crop-based biofuels have their limitations as they may influence