Potential uptake of Hexachlorocyclohexane (HCHs) by selected plant species from pesticide contaminated soil.

By

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Pesticides - Environmental aspects.

Soil analysis

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FINAL APPROVAL

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DEDICATION

This research is dedicated to my beloved parents whom hard work and prayers have made me able to complete this project and always provide moral support and encouragement.

DECLARATION

I hereby, declare that the work present in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Date

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LIST OF ABBREVIATIONS

Acronym	Abbreviation
APHA	American Public Health Association
OCPs	Organochlorine Pesticides
HCH	Hexachlorocyclohexane
POPs	Persistent Organic Pollutants
DDT	Dichlorodiphenyltrichloroethane
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DCB	Dichlorobenzene
TCB	Trichlorobenzene
CDC	Centre for Disease Control
PCP	Pentachlorophenol
GC-MS	Gas Chromatography Mass Spectrometry
TPH	Total Petroleum Hydrocarbon
SEM	Scanning Electron Microscopy

EDX	Energy-Dispersive X-Ray
HPLC	High-Performance Liquid Chromatography
GC-ECD	Gas-Chromatograph with Electron Capture Detector
ATSDR	Agency for Toxic Substances and Disease Registry
UNEP	United Nations Environment Program
POPRC	Persistent Organic Pollutants Review Committee
NARC	National Agricultural Research Centre
SM	Soil moisture
EC	Electrical Conductivity
TOC	Total organic carbon
SOM	Soil organic matter
MBC	Microbial Biomass Carbon
SPSS	Statistical Package for the Social Sciences.
ANOVA	Analysis of variance

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ABSTRACT

Hexachlorocyclohexane (HCHs) belongs to highly persistent and toxic chemicals. In 2009, HCHs was included in the list of POPs (persistent organic pollutants). According to literature, HCHs were being used as pesticide in Pakistan and the residues of HCHs in soil are still posing a constant source of contamination to the environment, but the real problem is that, there is no strategy for the removal of these outdated and banned stockpiles of HCHs. Phytoremediation is a promising and effective technology that ensures an inexpensive cleanup of contaminated sites. The study is aimed to assess the potential of two different plant species (*Cynodon dactylon* and *Lolium multiflorum*) for the removal of HCHs from soil. Both the species were first germinated and then transferred to soil spiked with three increasing concentrations of HCHs (5mg/kg, 15mg/kg and 25mg/kg) for 60 days. The samples were analyzed by using GC-MS technology. Results showed that these two species can serve as an effective phytoremediation option for HCHs. Furthermore, these species are proved for having potential of dissipating HCHs from soil.

INTRODUCTION

Quality of soil and water is highly degraded by environmental contamination due to rapid increase of urbanization and industrialization (Kamran *et al.,* 2014). Agricultural processes are not the only reason for pesticide contamination but, there are other processes like manufacturing, handling, improper storage, and disposal of pesticides and wastes which contribute to the pesticide contamination of soil. Particularly, environmental contamination with organochlorine pesticides (OCPs) is widespread globally because, after signing Stockholm Convention, many OCP production factories close to cities were abandoned (Gałuszkaa *et al.,* 2011).

At present, organochlorinated pesticides are among the most significant environmental concerns as organochlorine pesticides (OCPs) when come in contact can cause various lethal and adverse health effects, which include carcinogenesis, immunological and reproductive ailments in living beings, humans as well as wildlife (Sanpera *et al.,* 2003).

Presence of hexachlorocyclohexane (HCH) have been reported in all sections of environment, as well as in the remote areas, like the arctic (Daly and Wania 2005; Malik *et al.,* 2010). Hexachlorocyclohexanes (HCHs) are organochlorine pesticides which are being studied most widely. Lindane (γ-HCH) is the most toxic isomer which has been used widely as pesticide to control agricultural pest (Voldner and Li, 1995; Willett *et al.,* 1998). The effects of HCH are not limited to humans and animals, but they are extremely mortal for insects and other soil microorganisms too (Shi *et al.,* 2007). Four major isomers of HCHs (alpha (α), gamma (γ), beta (β) and delta (δ)) are of major environmental significance among eight known isomers of HCHs. (Li *et al.,* 2011). The γ -isomer affects the sprouting and primary growth of some plant species (Verma and Pillai, 1991). The HCHs can also destabilize major processes like photosynthesis, biomass distribution, development and physiology of plants (Audus, 1976; Wieczorek and Wieczorek, 2007). The HCHs in an ecological system are introduced by

LITERATURE REVIEW

The pollution occurred by the usage of POPs is becoming a global issue. Long half-life is a key characteristic of POPs which results into their perseverance in the environment and the high hydrophobicity are the causes of bioaccumulation of POPs in the food chain. Even though POPs like organochlorine pesticides were used years ago, they are still act as a source of soil pollution, which in turn, contaminate the water, crops, and animals (Miglioranza *et al.*, 1999; Nakata *et al.*, 2002; Gong *et al.*, 2004). The use of the OCPs started from 1940s to 1960s in agronomy as well as for control of mosquito. DDT, methoxychlor, dieldrin, chlordane, toxaphene, mirex, kepone, lindane, and benzene hexachloride are the characteristic chemicals of this group (Abhilash and Singh, 2009; Fenner *et al.*, 2013). In past, OCPs have contributed a vital part for improvement of food manufacture for growing global population. But, the massive usage of these pesticides have caused extensive air, water and soil pollution during the last few decades and their deposits are constantly posing a severe threat to human well-being (Abhilash and Singh, 2009).

2.1 Hexachlorocyclohexane (HCHs)

HCHs (Hexachlorocyclohexanes) belongs to the group of OCPs. It is a white crystal-like solid have chemical formula $C_6H_6Cl_6$ and mass 291 g/mol. The HCHs can be found in two forms; one is Technical HCH (comprises of four major isomers) and the other is γ -HCH (Wang *et al.*, 2014). These two forms were used as commercial pesticides formerly, but they are currently banned internationally. Though contaminated regions still exist, mainly the areas which are adjacent to the centers of production (Li, 1999; Van Pul *et al.*, 1999).

2.2 Sources of human exposure

In past, OCPs including DDTs and HCHs were being extensively used in both the under-developing and industrialized world. Nevertheless, these are still being consumed in emerging world for getting desired benefits (Zhou *et al.*, 2008). Currently, lindane (gamma HCH) is a prevalent anthropogenic chemical and frequently used in medication for the cure of human being as well as animals. The residues of HCHs in the environment are evidences of the input of these toxic chemicals in agriculture and consumption by humans. There are many other usages of HCHs such as a pesticide for various crops of fruit and vegetable, for the cure of many diseases.

2.3 Fate of OCPs in the environment

OCPs are the Persistent Organic Pollutants (POPs) as they exhibits high chemical stability and are difficult to transform through biotic and abiotic means. They have the ability to easily accumulate in plants and animal tissues and then can find a way to enter the food chain that pose a severe threat to human health. The extensive usage of HCHs in the past for control of pest in Asia and Africa, leads them to enter the river, estuary and aquatic environment by seepage from agricultural, waste deposits or other means (Wurl and Obbard 2005; Imo *et al.*, 2007). In this new ecological state characterized by huge emissions of anthropogenic pollutants in environment, plants and microorganisms displayed new abilities for the uptake and metabolic breakdown of these pollutants. This provide the basis for development of phytoremediation technologies (Kurashvili *et al.*, 2016).

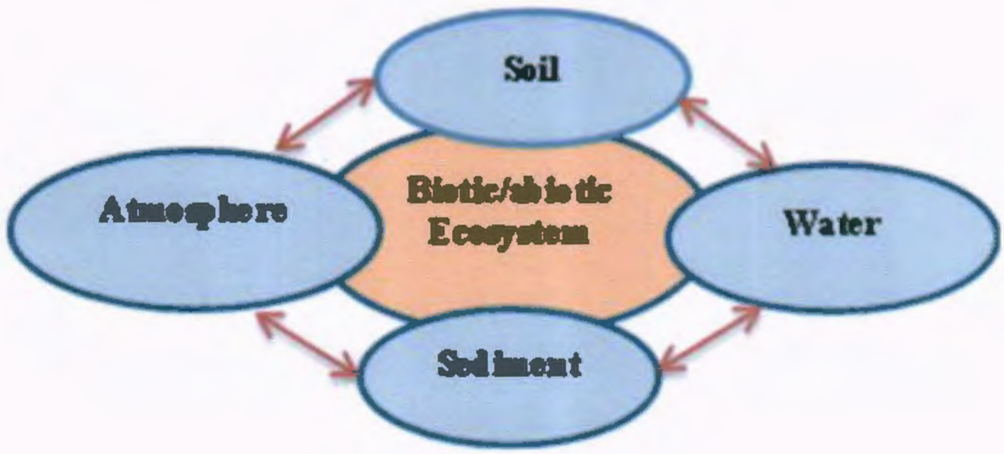


Fig. 1. Fate of HCHs in the environment and its interactions with biotic and abiotic environment (Ahmed *et al.*, 2015).

2.4 Health effects of the Organochlorine pesticides

OCPs are very toxic to human that even a short term exposure to them can cause many illnesses such as tremors, nuisance, faintness, sickness and confusion, weakness in muscle, garbled speech, salivation and panicking. While, contact with OCPs for longer periods cause illnesses to the liver, kidney, central nervous system, thyroid and bladder. Several pesticides are also associated to different types of cancer in wildlife and according to some evidences these can cause cancer in humans too (CDC, 2004).

2.5 Phytoremediation

Phytoremediation refers to a green technology that assures an effective and economical cleanup of contaminated sites (Mitton *et al.*, 2016). Numerous plant species have been proved to be potentially favorable for the phytoremediation of HCH-contaminated soils (Abhilash *et al.*, 2009; Abhilash and Singh, 2010; Kidd *et al.*, 2008).

In many countries HCHs has been banned, but γ-HCH is still being in use in some countries intended for financial benefits and new areas are subsequently being polluted. This widespread usage of persistent and non-degradable chemicals has headed to a world-wide environmental problem. Hence, the uptake and degradation of deadly chemicals by vegetation is a substantial method in knowing the fate of these contaminants in the environment (Bacci *et al.,* 1990). Many plants are identified that can serve as an environmental equalizer by acting as a sink for environmental pollutants. This study was carried out to evaluate the uptake level of HCH isomers in plant samples growing in an industrial area. From the most ample growing plants eight species were collected from the industrial area. These plants were then tested for HCH presence. Results showed the occurrence of HCH remains in almost all of the plant samples. The results indicated that all the plants species were capable to remediate HCHs isomers in their tissues, thus creating an effective sink for HCHs. Among eight species, *Solanum torvum Sw*. showed maximum accumulation of HCH. Hence, these species can serve better for the uptake of HCH in future (Abhilash *et al.,* 2008).

A research study conducted in which two plants species (Cynara scolymus L. and Erica sp.) were evaluated to check their capability for accumulating HCH. Results indicated that the process of accumulation of HCH in plant tissues is isomer-based process, in which accumulation of β-HCH is preferred. The results showed the significance of plants for phytoremediation of OCPs. Further research is still required in this field (Pereira *et al.*, 2008).

Pereira *etal.* (2010) conducted a study to select a plant species for the removal of HCHs from contaminated soil. Soil was spiked with a varied mixture of the main HCH isomers. Nine plants species of economic and agricultural importance were germinated and grown under controlled conditions in the contaminated soil. The results showed plant responded differently to the control sample of soil and the HCHs spiked soil samples. Overall, all the species displayed signs of stress due to the presence of HCHs, but to various extent. The three out of nine species used in the experiment (*Hordeum vulgare L., Brassica sp., Phaseoulus vulgaris L.*) appeared to be best for phytoremediation purposes.

Remains of dichlorodiphenyltrichloroethane (DDT) or its metabolite often identified in agricultural soils and food, the presence of these chemicals poses a high risk to human health. In this research, DDTs contaminated soil was collected from the cotton fields of Zhejiang Province, China, which was contaminated by widespread usage of DDT. 23 genotypes of *Ricinus communis* in remediation and dissipation of DDTs were compared in the co-contaminated soil. The accumulation potential of all the genotypes of the plant varied greatly. The results showed that the plants accumulated DDTs in leaf, stem as well as in root. These results show that *R. communis* can serve as an ideal choice for the removal of DDTs from contaminated soils (Huan *et al.*, 2011).

							showed the highest capacity for pyrene dissipation in soil
8.	Miguel *et al*	2013	A comparative study on the uptake and translocation of organochlorines by Phragmites australis	14C-labeled 1,4-dichlorobenzene (DCB), 1,2,4-trichlorobenzene (TCB) and - hexachlorocyclohexane (HCH)	Phytovolatilization (*Phragmites australis*)	Airflow system	81%, 88%, 88% of the total plant uptake, respectively for DCB, TCB, γHCH

4.	Rani *et al*	2012	Biodegradation of phorate in soil and rhizosphere of Brassica juncea (L.) (Indian Mustard) by a microbial consortium	Phorate	Biodegradation by Microbial consortium and rhizoremediation by *Brassica juncea*	GC-ECD	Biodegradation of phorate in soil in presence of plants alone was 38%
5.	Kurashvili *et al*	2014	Plants and Microorganisms for Phytoremediation o Soils Polluted with Organochlorine Pesticides	Organochlorine pesticides (DDT, Lindane, PCP)	Phytoremediation (*Lathyrussativum, Glycine max, Zea mays, Medicago sativa, Cicer arietinum* and *Lactuca sativa*)	GC-ECD	Obtained results show that alfalfa, maize and soybean have highest tolerance to organochlorine pesticides.
6.	Liu *et al*	2013	Effect of ryegrass (*Lolium multiflorum* L.) growth on degradation of phenanthrene and enzyme activity in soil	Phenanthrene	Phytoremediation by *Lolium multiflorum* L.	HPLC	Phenanthrene degradation rate reached 81.1, 90.4 and 85.0%
7.	D'Orazio *et al*	2013	Phytoremediation of Pyrene Contaminated Soils by Different Plant Species	Pyrene	Phytoremediation by *Medicago sativa, Brassica napus* and *Lolium perenne*	HPLC	Pyrene concentration in soils declined by 32, 30 and 28%, respectively, with *M. sativa, B. napus* and *L. perenne* while, *M. sativa*

sr. no.	Author	Year	Title	Contamination	Remediation strategy	Instrumentation	Results/ Percentage removal
1.	Sojinu *et al*	2012	Assessment of organochlorine pesticides residues in higher plants from oil exploration areas of Niger Delta, Nigeria	Organochlorine pesticides (OCPs) α–HCH, β–HCH, γ–HCH (lindane) and δ–HCH DDT and its derivatives	Phytoremediation (*P. purpureum N. laevis, C. colocynthis, N. bisserata, M. esculenta, Z. mays, M. indica, M. pudica*)	GC-MS	High concentration of OCPs obtained in the *P. purpureum*
2.	Al-Baldawi *et al*	2015	Phytodegradation of total petroleum hydrocarbon (TPH) in diesel-contaminated water using Scirpus grossus	Total petroleum hydrocarbons (TPH)	Phytoremediation (*Scirpus grossus*)	Scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX) analysis	The degradation of total petroleum hydrocarbons (TPH) was obtained up to 81.5%, 71.4%, and 66.6% for 0.1%, 0.175%, and 0.25% diesel (v/v) treatments, respectively
3.	Abhilash *et al*	2013	Remediation of lindane by Jatropha curcas L: Utilization of multipurpose species for rhizoremediation	Lindane (Gamma-Hexachlorocyclohexane)	Phytoremediation (Rhizoremediation) Jatropha curcas L.	Gas-Chromatograph equipped with Ni Electron Capture Detector (GC-ECD) HPLC	Lindane concentrations in soil were reduced up to 89, 82, 77 and 72% with respect to the applied lindane amounts

Endosulfan belong to group of organochlorine pesticide widely used in agronomy. Typically, it was used as pesticide for crops like fruits, cotton, vegetables, tobacco, sugarcane, and tea. Furthermore, it was used for the purpose of wood preservation (Rice *et al.,* 1997; Antonious *et al.,* 1998). ATSDR (Agency for Toxic Substances and Disease Registry) included endosulfan in the list of persistent toxic pollutant in 2001 and enlisted as POP (persistent organic pollutant) by the Stockholm Convention in 2011. The persistent nature of endosulfan in soil and marine environments has been reported under various settings (Singh and Singh, 2014). Thus, presence of endosulfan remains in soil samples, represents a constant source of environmental contamination despite being banned by legislation (Jia *et al.,* 2010). Mitton *etal.* (2016) carried out a study to evaluate the potential of tomato, sunflower, soybean and alfalfa species to remediate the soil polluted by endosulfan. Soils spiked with endosulfan were used to grow all the species for 15 and 60 days. The results indicated that after 60 days, sunflower accumulated highest concentration of endosulfan in roots and leaves. Sunflower plants appeared to be a promising future for phytoremediation of endosulfan.

Sandoval *et al.,* (2011) conducted a research study discover a potential way to bioremediate the endosulfan contaminated soils. The experiment conducted with two plant species of the same genus: *Ocimum basilicum L. and Ocimum minimum L.* Small plants were transplanted into soil which was spiked with endosulfan. Both the species were not affected by endosulfan, grew well and produced seeds. After 90 days, no differences in endosulfan concentrations were found between soil with or without *O. minimum*. Whereas, within 30 days there was a significant difference between the endosulfan concentration in the soil with *O. basilicum* and in the soil without plants. *O. basilicum* reduced endosulfan concentration up to 37%. As a result, *Ocimum basilicum* appears to be a suitable candidate for phytoremediation of endosulfan-polluted soils.

Castro *et al.,* (2013) studied the phytoremediation of soil contaminated with hexachlorocyclohexane (HCH), using *Cytisus striatus* and bacterial inoculants with varied organic matter content. *Cytisus striatus* reduced the concentrations alone by 20% and 8% respectively, but with the inoculation of the two strains, displays an enhanced HCH reduction in two soils: 53% and 43%.The combination of the two selected strains are very suitable for carrying out future experiments in the soils contaminated by HCHs.

Spinacia oleracea L (Spinach) was used to assess the phytoextraction and dissipation of lindane. The plant was grown in the lindane contaminated soil with different concentrations (5, 10, 15 and 20 mg kg-1) and reaped after 10, 30 and 45 days. The results showed clearly that with increasing exposure time and increased lindane concentration in soil, Spinach can accumulate lindane more effectively. After harvesting, the plant was analyzed for lindane concentration. The results were quite surprising as spinach reduced the lindane from soil and accumulated it in its roots, shoot and leaves. The lindane concentration was reduced up-to 81%. Moreover, it can also dissipate lindane from soil. Hence, Spinach seems to be an ideal plant for the remediation of soils contaminated by lindane (Dubey *et al.*, 2014).

As a conclusion from all the reviewed studies, it can said that plants can indisputably be considered as green livers, which acts as a sink for environmental contaminants. Phytoremediation is a promising technology that aims to provide an economically feasible and safe treatment for contaminated sites. A number of plants are being identified to be used in the phytoremediation of organochlorine pesticide

RESEARCH METHODOLOGY

3.1 Sampling

The HCH was purchased and two plant species were selected that had potential to degrade POPs (HCHs). Uncontaminated soil samples were collected from field of National Agricultural Research Centre (NARC). The samples were air dried, ground and sieved with 2 mm pore size sieve.

Table 2: Treatments of HCHs

Control soil			Spiked soil with 5 mg kg-1 HCHs			Spiked soil with 15 mg kg-1 HCHs			Spiked soil with 25 mg kg-1 HCHs		
P2T1R1	P2T1R2	P2T1R3	P2T2R1	P2T2R2	P2T2R3	P2T3R1	P2T3R2	P2T3R3	P2T4R1	P2T4R2	P2T4R3
P3T1R1	P3T1R2	P3T1R3	P3T2R1	P3T2R2	P3T2R3	P3T3R	P3T3R	P3T3R	P3T4R1	P3T4R2	P3T4R3

The soil samples were spiked with 5, 15 and 25 mg kg-1 HCHs. After spiking, the samples were stored in polythene bags and kept for aging for 15 days (Park *et al.*, 2011). All the treatments were replicated thrice.

3.2 Physico-chemical analysis of soil

Physico-chemical analysis of soil samples was carried out before and after the spiking. The analysis are as follows:

3.2.1 Soil moisture (SM)

10 g soil was weighed and put in a petri plate, then weighed again and oven-dried at 105 °C for 24 hours in a digital oven to remove moisture contents. SM was calculated by using the formula.

$$\textbf{Soil Moisture (\%)} = \frac{\textbf{Loss of weight in soil samples}}{\textbf{Weight of oven dried soil}} \times 100$$

3.2.2 Soil texture

Soil texture was determined by using hygrometer (Bouyoucos, 1962).

3.2.3 pH

The pH of the soil was measured by using multi meter of model MM 40 + (APHA, 2005).

3.2.4 Electrical Conductivity

Multi-meter (MM 40 + model) was used for the measurement of electrical conductivity (Muhammad *et al.,* 2008).

3.2.5 Total organic carbon (TOC)

Total organic carbon analysis was carried out using the titration method of Walkley (1947). One gram of soil was used to transfer to a conical flask and 10 ml of Normal solution of potassium dichromate with 20 ml concentrated Sulphuric acid was added. The solution was shaken and it was allowed to stand for 30 minutes. 200 ml distilled water and 10 ml orthophosphoric acid was added and then the solution allowed to cool. 10-15 drops of

Diphenylamine indicator were added and then the solution was titrated against freshly prepared 0.5M ferrous ammonium sulphate solution, until the colour changes from violet blue to green.

$$\% \text{ Oxidizable Organic Carbon} = \frac{[V\,blank - V\,sample] \times 0.3 \times M}{Wt}$$

$$\% \text{ Total Organic Carbon} = 1.334 \times \% \text{ Oxidizable Organic Carbon}$$

3.2.6 Soil organic matter

Soil organic matter was analyzed by titration method of Walkley (1947). Soil Organic Matter is calculted using the following formula.

$$\% \text{ Organic Matter} = 1.724 \times \% \text{ Total Organic Carbon}$$

3.2.7 Microbial Biomass Carbon (MBC)

Microbial biomass carbon was calculated by improved method of rapid microwave [mw] irradiation and extraction method (Islam and Weil, 1988).

The MBC was calculated as:

$$\mathbf{MBC\ (mg\ C\ kg\ soil^{-1}) = (MWC_{ext} - C_{ext}) \times 2.64}$$

3.3 Pot Experiments

The experiments were carried out at the growth chamber of Department of Environmental Sciences. The selected plant species including two grass species (*Cynodon dactylon* and *Lolium multiflorum*) were planted in the pots and replicated three times. To study the HCHs dynamic activity in soil especially in rhizosphere and plant potential towards the remediation of these pollutants, the 60 days experiment was designed in which three concentrations of HCHs were selected. Every treatment was replicated three times. All the samples were freeze dry below –

30 degree centigrade. After 60 days, plants were harvested and all the samples were treated properly for further analysis...

3.4 Plant Growth Parameters:

After harvesting, the following growth parameters were observed

- Root and shoot length were measured by means of common meter rod in centimeters (cm).
- Electrical balance was used to measure fresh and dry weight in grams (g).

3.5 HCHs analysis

3.5.1 HCHs analysis in soil

Soil extraction was done by transferring 5g soil samples in to 100 ml Teflon tubes then mixed with 10 ml of Di chloromethane and then each sample was extracted for 20 minutes in a sonication, then samples were centrifuged at 4000 rpm for 5 minutes and the supernatants were collected. The extract was eluted with 1 and 2 ml mix of n-hexane: dichloromethane (v/v 50:50) and the supernatant was extracted for HCHs and dried by sparging with N_2, Solid residues were re dissolved in 1 ml of acetonitrile and run on the GC-MS with ECD detector (Li-Hong *et al.*, 2006, Rhind *et al.*, 2013).

3.5.2 HCHs analysis in plant

The plant material was extracted in water bath for 20 min in ethyl acetate. The extracts were passed through silica gel packed column. Elution was concentrated under reduced pressure, dried under nitrogen stream, and then eluent was re-dissolved in 1ml of acetonitrile for analysis by GC- MS (Li-Hong *et al.*, 2006, Rhind *et al.*, 2013).

3.6 Quality Assurance

For the assurance of quality control, standard and sample recovery was maximized. One standard and one sample replica were analyzed after every 10 samples to ratify the results of GC-MS analysis.

3.7 Instrumental Analysis

For the analysis of hexachlorocyclohexane samples were run on Gas chromatography, mass spectrometry combined with Electron capture detector GC-MS-ECD (Malik *et al.,* 2011).

3.8 Statistical analysis

The statistical analysis was carried out with SPSS, version 18.0; exploratory data analysis was carried out (multivariate analytical techniques). Other analysis including comparison of means (parametric tests, t test, ANOVA) was carried out.

RESULTS AND DISCUSSION

The soil was collected from field of National Agricultural Research Centre (NARC). The soil was analysed for physicochemical parameters. Soil physical properties are in table 3. The texture of soil is sandy clay loam with 18% moisture.

4.1 SOIL ANALYSIS

Difference was observed in soil parameters before and after the spiking of soil with HCHs. Variations were observed in all the physicochemical properties of the soil at the start and the end of the two months experiment.

4.1.1 pH

Figure 2 represents the variation of soil pH among different treatments. The pH of the soil spiked for two weeks was 7.68, 7.47, 7.43 and 7.30 respectively in all the four treatments (T1, T2, T3, and T4). After planting Rye grass for 60 days pH shows a decreasing trend and reached to 6.77, 7.37, 6.87 and 7.17 among the four treatments (fig. 2a). Similarly, the pH of the soil in which Bermuda grass was planted also shows a decreasing trend from 7.68, 7.47, 7.43 and 7.30 to 6.87, 7.17, 7.37 and 7.10 in each of the respective treatment (fig 2b). The **pH** of the soil after pot experiment has decreased as compared to the spiked soil. This decrease is certainly due to degradation of hydrocarbons by microorganisms and enzymatic activities of plants (Akpan and Ekpo, 2006).

4.1.2 Electrical Conductivity

Fig. 3 shows the variation in electrical conductivity (EC) of soil among different treatments (T1, T2 T3, and T4) after spiking and then after plantation in the soil. The two types of grasses were planted for 2 months after the spiking of soil with different concentration of

HCHs. EC of the soil with different treatments was 250.1 µS cm-1, 249.5 µS cm-1, 249.3 µS cm-1 and 249.1 µS cm-1 respectively. After 60 days of experiment, the soil in which Rye grass was planted showed a significant decrease in EC. The lowest values of EC were 179.0 µS cm-1and 158.4 µS cm-1shown by T3 and T4, while T1 and T2 showed comparatively higher values but lower than the initial values i.e. 240.5 µS cm-1and 202.4 µS cm-1 (fig. 3a). Figure 3b shows the variation in EC among different treatments in which Bermuda grass was planted. The EC of the soil planted by Bermuda grass showed similar variation as of the Rye grass soil. The EC values decreased and finally after 2 months the lowest values were 185.1µS cm-1 and 165.1 µS cm-1, shown by T3 and T4. While maximum values were observed in T1 and T2 (231.2 µS cm-1 and 212.1 µS cm-1). As value of T1 has not decreased much because, it does not contain any pesticide.

Overall electrical conductivity showed a decreasing trend through the course of experiment. The reason behind this decrease is organic matter mineralization. During organic matter mineralization process ions releases and increase the electrical conductivity Moreno *etal.* (1999). But, in the present study the plants might have taken up the salts or ions from the soil after mineralization, resulting in a decrease in electrical conductivity of the soil.

4.1.3 Organic Matter (OM)

Soil organic matter among different treatments in soil samples is shown in fig.4. Initially the organic matter in the spiked soil was T1: 1.15%, T2: 1.16%, T3: 1.28% and T4: 0.99%. The spiked soil was then planted with Rye grass and Bermuda grass for 2 months, but it shows a decreasing trend after 2 months of experiment. Fig. 4a shows the variations in organic matter of the soil treatments in which Rye grass was planted (0.50%, 0.75%, 0.44% and 0.62%). The organic matter of the soil treatments in which Bermuda grass was planted reached to 0.56%, 0.51%, 0.60% and 0.57% (fig. 4b).

Organic matter did not show much difference (from control) after spiking with HCH for two weeks, but it showed a decreasing trend after 60 days of pot experiment. Research studies reported that the mineralization rate of organic matter in soils spiked with pesticides is usually higher compared to the control. This showed that growth and activities of heterotrophic microorganisms are stimulated by biodegradation of pesticides which caused the mineralization of organic matter and biologically transform other plant nutrients in soil, resulted in lowering the concentration of organic matter in soil (Bhuyan *et al.*, 1993; Debnath *et al.*, 1994; El-Shahaat *et al.*, 1987; Murthy *et al.*, 1991; Rangaswamy and Venkateswarlu, 1993).

4.1.4 Total Organic Carbon (TOC)

Figure 5 represents the total organic carbon concentration among different treatments after spiking in comparison with treatments after pot experiment. Initially the concentration of total organic carbon was 0.67%, 0.67%, 0.74% and 0.57% in the respective treatments. After 2 months of pot experiment, a significant decrease in the TOC content was observed among all the treatments. Fig. 5a is showing the variations in the TOC in the soil treatments which are planted with Rye grass for 2 months. A significant decrease in the TOC was observed (0.29%, 0.43%, 0.27% and 0.36%) after two months of pot experiment as compared to the initial concentration. Similarly, the soil in which Bermuda grass was planted also showed a decreasing trend in TOC concentration among all the treatments; 0.33%, 0.30%, 0.35% and 0.33% (fig. 5b).

In previous studies it was reported that pesticides have a stimulatory effect on mineralization process. Sukul (2006) studied the effect of metalaxyl (fungicide) which showed a significant decrease in total organic carbon content in soil during 0–30 days of incubation. Similarly, Das and Mukherjee (1994, 1998, 2000) also reported that the mineralization rate of organic C in soils treated with different pesticides (BHC, representing the organochlorine group of insecticide) was higher compared to the control resulted in decrease of organic C in soil.

4.1.5 Microbial Biomass Carbon (MBC)

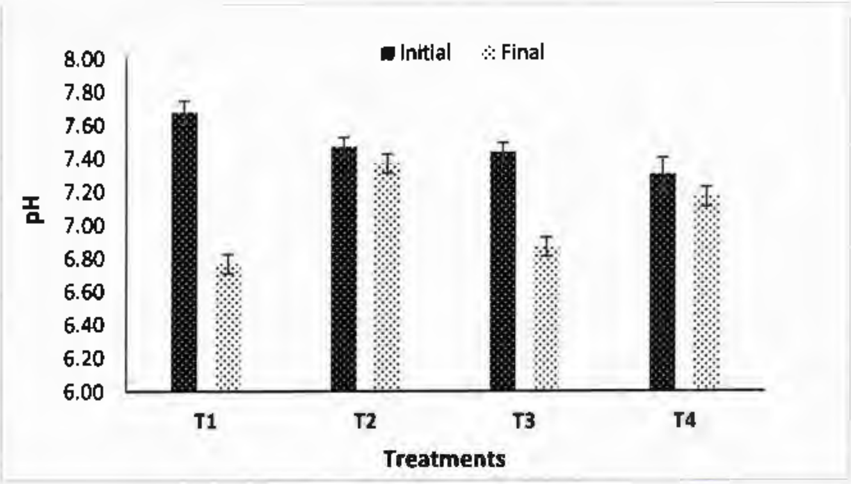
Microbial biomass carbon showed significant variations from the initial values to the final ones. Initially, the MBC of soil spiked for two weeks was T1: 0.85%, T2: 0.83%, T3:0.47%, T4: 0.83%. But after 2 months of pot experiment, the MBC showed an increasing trend in all the treatments and reached to 0.92%, 0.88%, 1.37% and 1.07% in the soil samples which were planted with Rye grass (fig. 6a). Similarly the soil planted with Bermuda grass also showed an increasing trend of MBC from the initial values and reached to 0.88%, 0.88%, 1.73% and 1.95% respectively (fig. 6b). Moreover, the treatments T3 and T4 from both the plants showed a relatively higher MBC level.

During the course of experiment microbial biomass carbon tend to increase after the two months of experiment. The reason behind this increase is certainly the pesticide (HCH). Pesticides make an interaction with microorganisms of soil and affect their metabolic activities (Singhand Walker, 2006). Sometimes these pesticides undergo to change the physical, chemical and biological behaviour of soil microorganisms. Microbial biomass carbon is an essential indicator of microbial activities (Schultz and Urban, 2008).Various recent studies revealed that pesticides can have adverse impacts on soil microbial biomass and soil respiration (Pampulha and Oliveira, 2006; Zhou *et al.*, 2006). And this increase in respiration indicates the higher growth of microbial population (Haney *et al.*, 2000; Wardle *et al.*, 1994) which leads to increase microbial biomass carbon.

Table 3: characteristics of soil sample

Soil type	Sandy clay loam
Silt (%)	16
Clay (%)	19.8
Sand (%)	64.2
Colour	Brown
Moisture (%)	18%

a.



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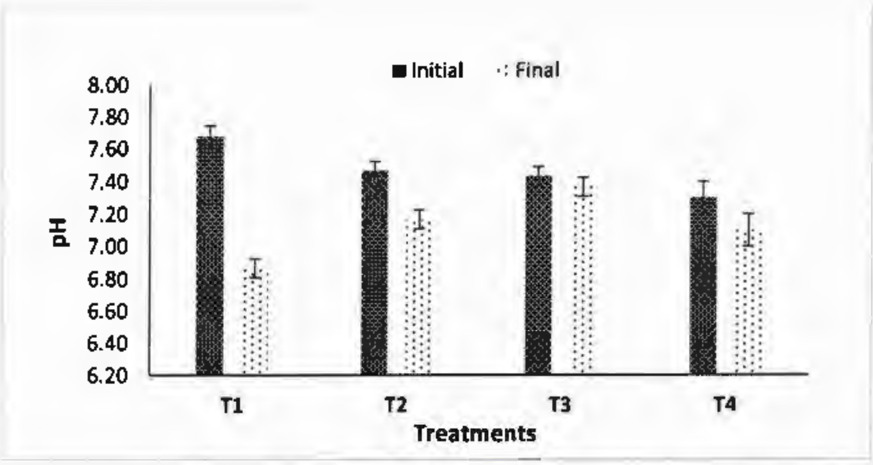
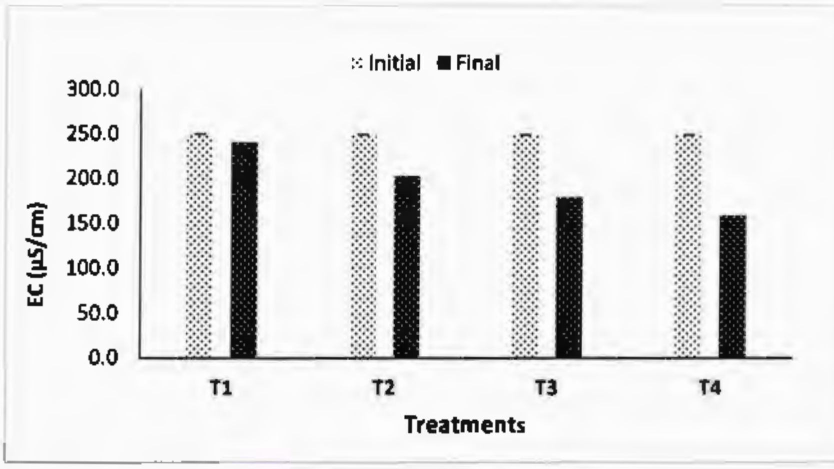


Figure 2: Variation in soil pH among the different treatments after spiking for two weeks in comparison with soil planted for 2 months. a: Rye grass (P2), b: Bermuda grass (P3), T1: control soil with no pesticide; T2: soil spiked with 5mg/kg HCHs; T3: soil spiked with 15mg/kg HCHs and T4: soil spiked with 25mg/kg HCHs

a.



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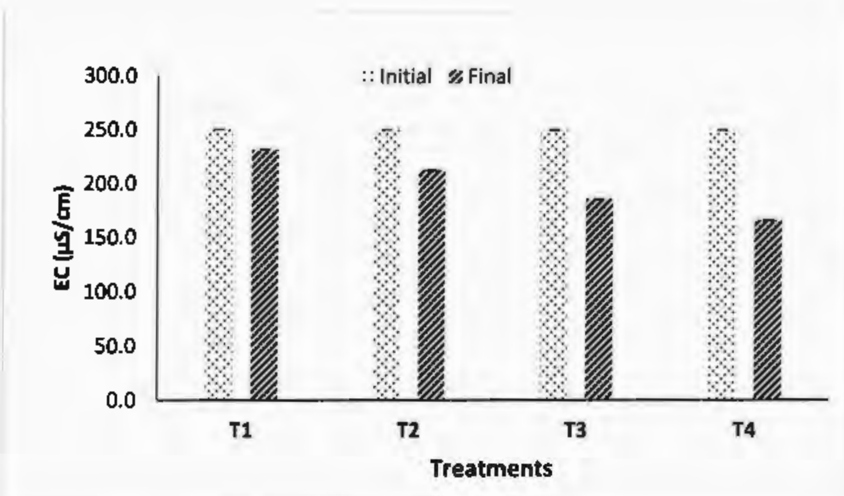


Figure 3: Difference in EC of soil among different treatments after spiking for two weeks in comparison with soil in which plants were grown for 2 months. a: Rye grass (P2), b: Bermuda grass (P3), T1: control soil with no pesticide; T2: soil spiked with 5mg/kg HCHs; T3: soil spiked with 15mg/kg HCHs and T4: soil spiked with 25mg/kg HCHs.

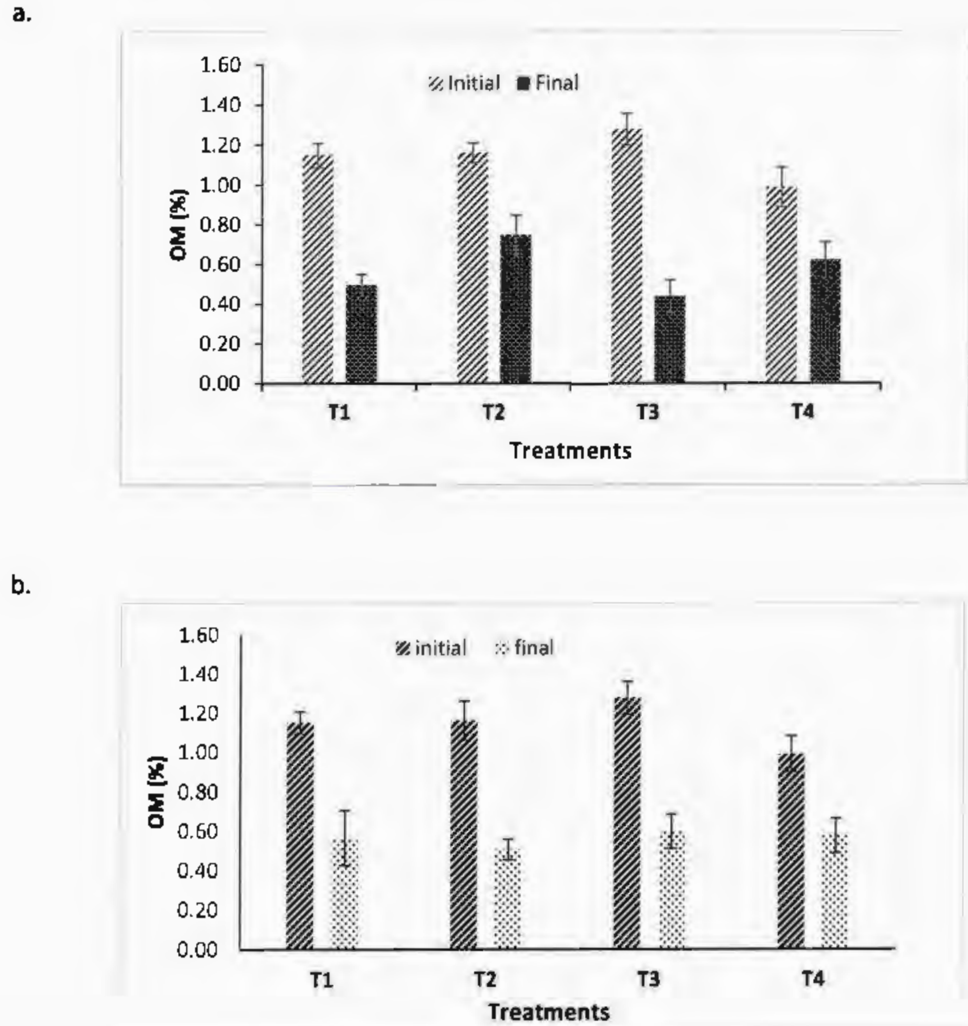
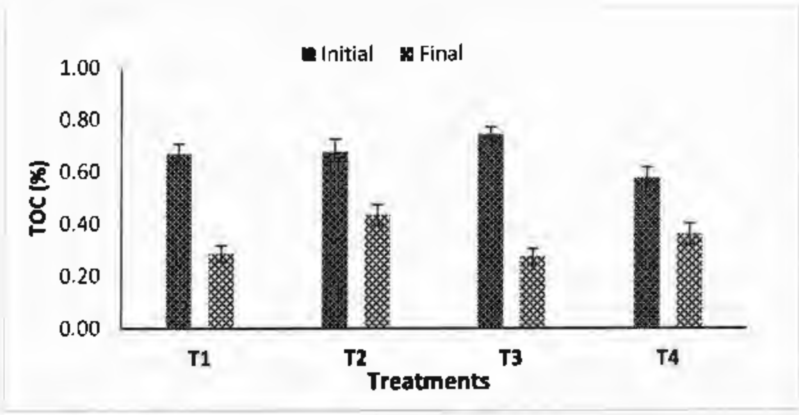


Figure 4: Content of Soil Organic Matter among different treatments in the soil. a: Rye grass (P2), b: Bermuda grass (P3), T1: control soil with no pesticide; T2: soil spiked with 5mg/kg HCHs; T3: soil spiked with 15mg/kg HCHs and T4: soil spiked with 25mg/kg HCHs.

a.



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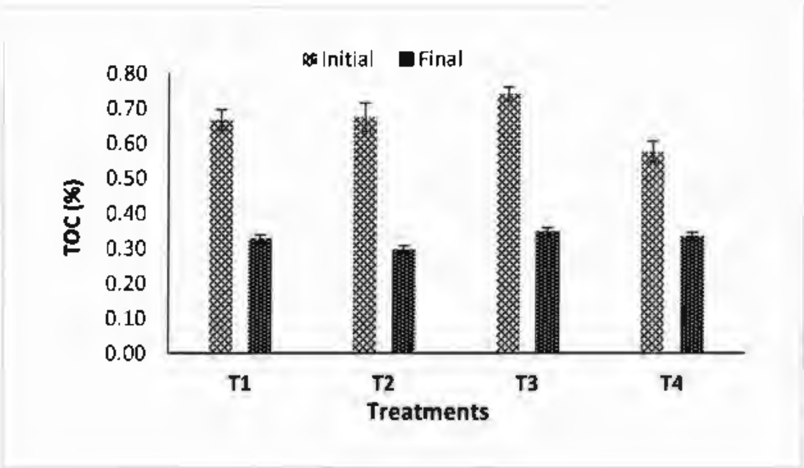
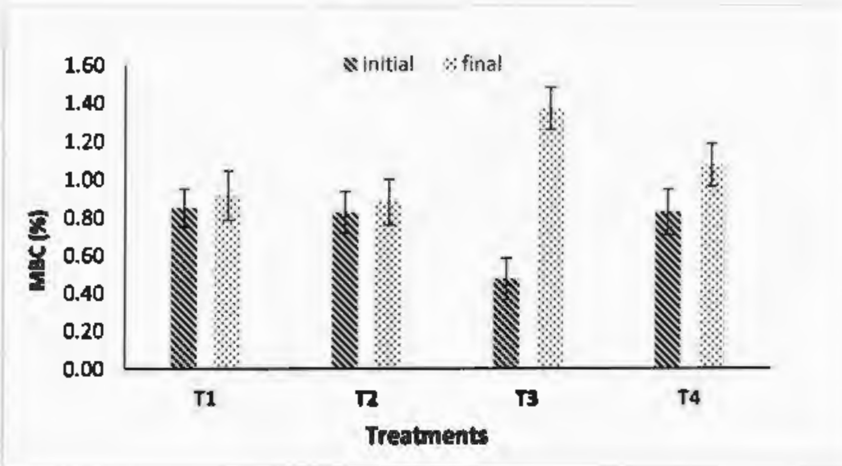


Figure 5: Total organic carbon content among different treatments in the soil samples. Difference in total organic carbon of soil among different treatments after spiking for two weeks compared with the soil in which grasses were planted for 2 months. a: Rye grass (P2), b: Bermuda grass (P3), T1: control soil with no pesticide; T2: soil spiked with 5mg/kg HCHs; T3: soil spiked with 15mg/kg HCHs and T4: soil spiked with 25mg/kg HCHs.

a.



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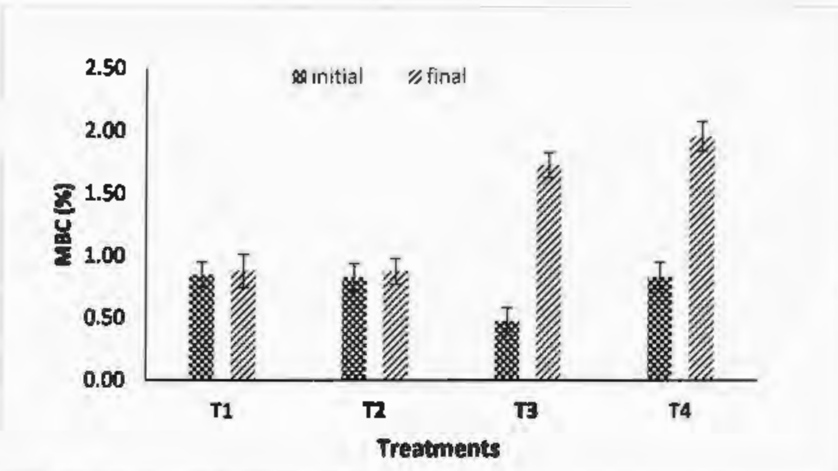


Figure 6: Microbial Biomass Carbon content among different treatments. a: Rye grass (P2), b: Bermuda grass (P3), T1: control soil with no pesticide; T2: soil spiked with 5mg/kg HCHs; T3: soil spiked with 15mg/kg HCHs and T4: soil spiked with 25mg/kg HCHs.

4.2 POT EXPERIMENT

Uncontaminated soil samples were collected from field of National Agricultural Research Centre (NARC). The soil samples were spiked with 5, 15 and 25 mg kg-1 HCHs. Then these samples were stored in polythene bags for 15 days for aging. Then the physicochemical characteristics of soil were studied and selected plant species including two grass species (Cynodon dactylon and Lolium multiflorum) were planted in the pots. To study the HCHs dynamic activity in soil and plant potential towards the remediation of these pollutants. After 60 days, plants were harvested and all the samples were treated properly for further analysis.

4.2.1 Plant Growth Parameters

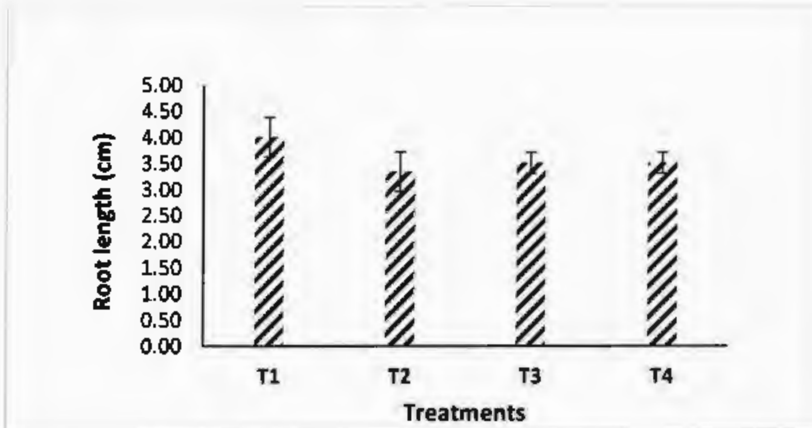
Initially the root length of Rye grass was 2cm and Bermuda grass had root length of 5cm.With the passage of time experiment progressed, the plants started to grow and biomass also increased in all the treatments. The difference was clear between the plants grown in HCH spiked soil and the control soil. The plants grown in the uncontaminated soil showed faster growth than those grown in the contaminated soil. Moreover, root length showed a slight difference between the planted grown in different treatments. Fig. 7a shows the root length and difference of growth among the treatments of Rye grass. The root length of Rye grass reached from 2cm to 4.00 cm, 3.33 cm, 3.50cm and 3.50cm in the respective treatments. Initially the root length of Bermuda grass was 5cm and reached up to 10.10 cm, 9.33 cm, 7.67cm and 7.83cm (fig. 7b).

At the start of the experiment the plants had shoot length of Rye grass was 8 cm and of Bermuda grass was 6 cm. As the experiment progressed, the shoot height increased in all the treatments but the one planted in the control soil has the highest shoot length as compared to the others. Rye grass showed an increase in shoot height from 8cm to 13.11 cm, 10.33 cm, 12.33 cm and 10.50 cm in the respective treatments (fig. 8a). On the other side, shoot height of Bermuda grass increased from 6 cm to 9.81cm, 8.50 cm, 8.74cm and 8.67 cm (fig. 8b).

Plant biomass, is the sum total of root and shoot dry weight per pot. The plant biomass varied in different treatments as well of the two plants. Fig. 9a shows the fresh weight and then dry weight of Rye grass. It is clear that the total biomass of Rye grass is more in the control treatment (T1: uncontaminated) as compared to the other treatments (contaminated). On the other side, the results are similar and it is evident that the total biomass of Bermuda grass is highest in the control treatment and lowest in the treatment T4 which is spiked with 25 mg/kg HCHs (fig. 9b).After oven drying the plants, the plant biomass decreased accordingly.

The root and shoot length of all the plants increased with the passage of time. But, there is a difference between control and spiked treatments. The plants grown in control samples have relatively higher root and shoot heights than the plants which were grown in the spiked soil. This negative effect of HCH contamination on the growth of Rye grass and Bermuda grass was obvious because of the toxicity of HCHs. The plant biomass varied in different treatments as well of the two plants. Generally the plant biomass was more in the control samples as compared to the treated/spiked samples. Dubey *et al.,* 2014 reported that at concentrations 15 and 20 mg kg^{-1} of lindane (γ-HCH), the values of dry biomass, root size, and foliage were declined as compared to their control values. This reduction in these parameters of Spinach can be associated to the noxiousness of the lindane.

a.



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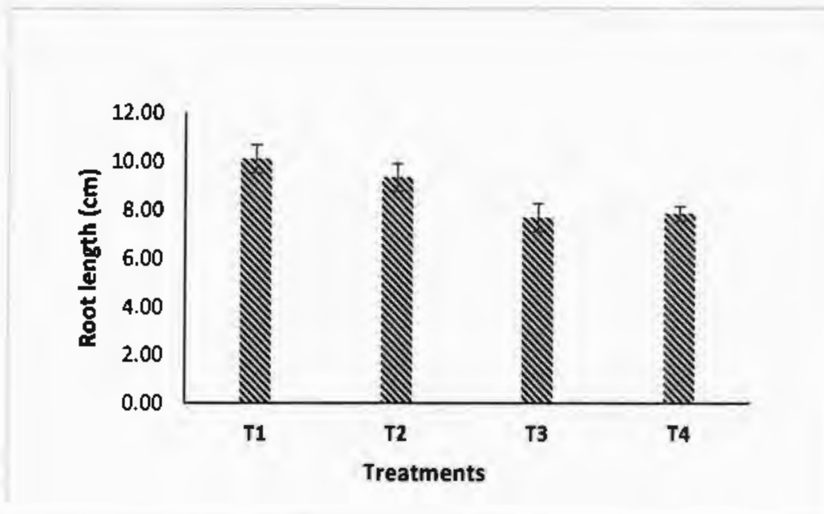
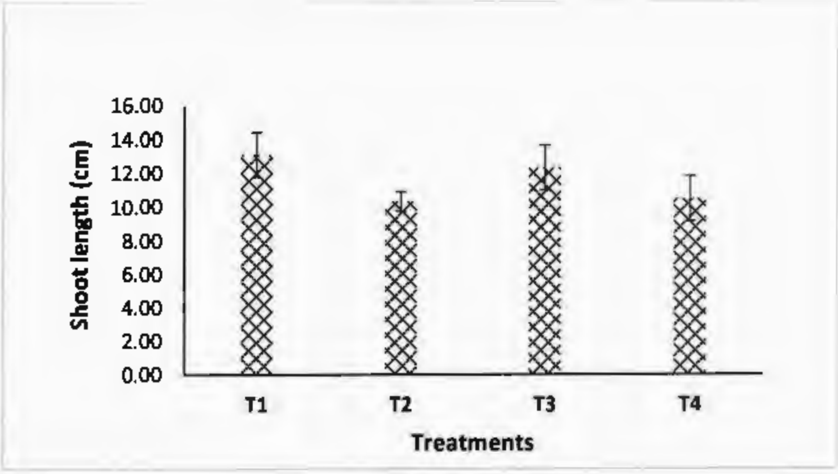


Figure 7: Root Length of the plants which were planted in the spiked soil for 2 months. a: Rye grass (P2), b: Bermuda grass (P3), T1: control soil with no pesticide; T2: soil spiked with 5mg/kg HCHs; T3: soil spiked with 15mg/kg HCHs and T4: soil spiked with 25mg/kg HCHs.

a.



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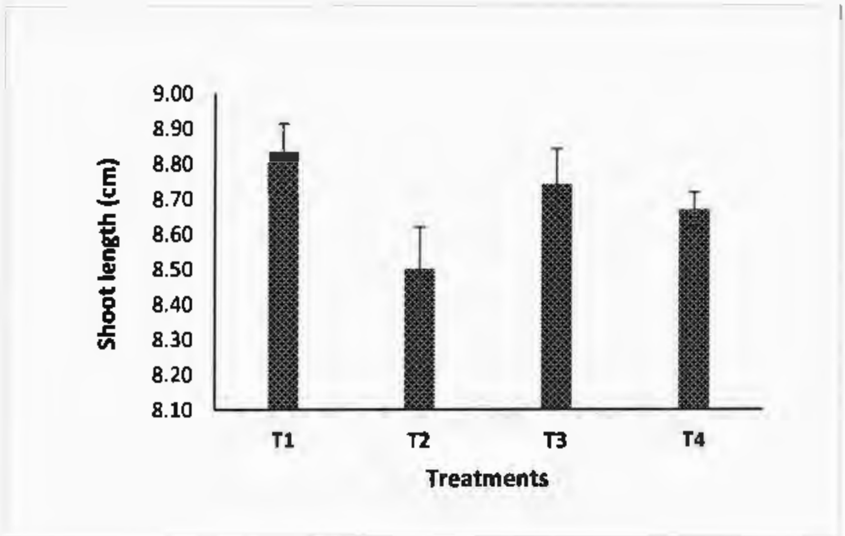
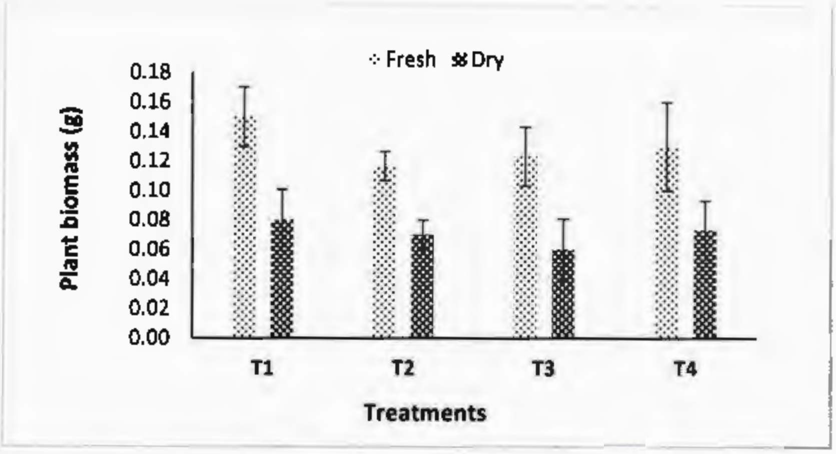


Figure 8: Shoot length of the plants which were planted in the spiked soil for 2 months. a: Rye grass (P2), b: Bermuda grass (P3), T1: control soil with no pesticide; T2: soil spiked with 5mg/kg HCHs; T3: soil spiked with 15mg/kg HCHs and T4: soil spiked with 25mg/kg HCHs.

a.



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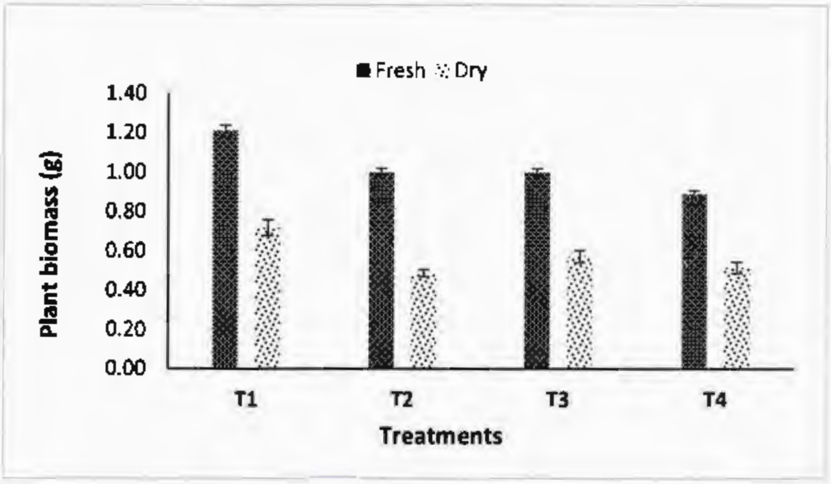


Figure 9: Plant biomass of the plants which were planted in the spiked soil for 2 months and the difference between fresh and dry weight of the plants a: Rye grass (P2), b: Bermuda grass (P3), T1: control soil with no pesticide; T2: soil spiked with 5mg/kg HCHs; T3: soil spiked with 15mg/kg HCHs and T4: soil spiked with 25mg/kg HCHs.

4.3 HCHs ANALYSIS

Soil was first spiked with different treatments of HCHs and then after 15 day of aging, the soil was planted with two grass species and replicated thrice. After 60 days, the plants were harvested and analysed for HCHs concentration. Soil and plants were analysed separately for HCHs. Initially the treatments were T1: control, T2: 5mg/kg, T3:15mg/kg and T4: 25mg/kg but after 60 days of experiment, the concentration of HCHs in soil has changed significantly and up-taken by plants.

4.3.1 HCHs concentration in soil

Figure 10 shows HCHs concentration in soil after spiking in comparison with HCHs concentration after 60 days of experiment. The difference is very obvious in initial and final HCHs concentration in soil in all the treatments. The concentration of HCHs has decreased significantly in all the treatments. While there is a slight difference in the two sets of samples due to presence of different plants. Fig. 10a shows the change in HCHs in soil in the presence of Rye grass which is a little more than the other. Fig. 10b shows the difference in HCHs in soil in the presence of Bermuda grass. Initially, the HCHs concentrations were T1: control, T2: 5mg/kg, T3:15mg/kg and T4: 25mg/kg and finally reduced to 0.00 mg/kg, 2.01mg/kg, 9.97mg/kg and 21.36mg/kg respectively in the presence of Rye grass. On the other hand, the HCHs concentrations in the presence of Bermuda grass decreased to -0.03mg/kg, 2.56mg/kg, 10.38mg/kg and 19.85mg/kg in the respective treatments.

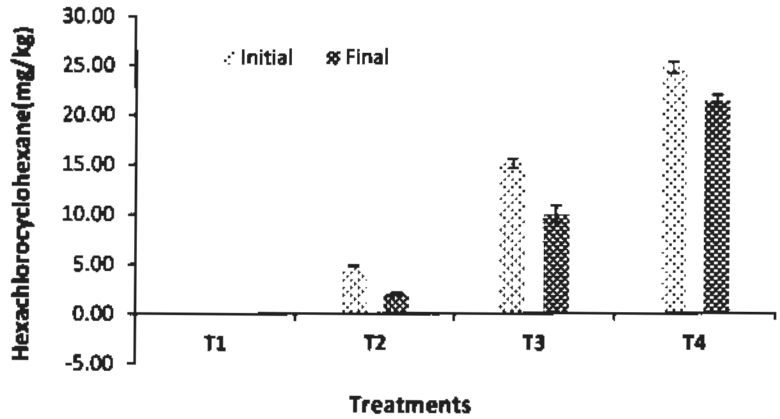
In general, the HCHs concentration has decreased in all the treatments. The reason of this decrease is the presence of plants. The dissipation of lindane was calculated in different treatments in the presence of Jatropha. Results showed a substantial difference in the dissipation of lindane in presence and absence of plant in the soil. After an experiment of 300 days, the concentration of lindane in different treatments decreased to 89, 82, 77 and 72% as compared to the applied lindane amounts respectively, however the dissipation rate was 52, 49, 45, 40% in the soil which was not planted (Abhilash *et al.*, 2013).

4.3.2 HCHs concentration in plants

Figure 11 shows uptake of HCHs by plants grown in HCHs spiked soil for 60 days. The decrease of HCHs concentration in soil is due to the presence of Rye grass and Bermuda grass. The uptake of HCHs from soil by rye grass is 0.00mg/kg, 1.13mg/kg and 2.26mg/kg 2.42mg/kg (fig. 11a). While uptake of HCHs by Bermuda grass is shown in fig. 11b which is 0.00mg/kg, 0.98mg/kg, 2.89mg/kg and 3.15mg/kg. There is a slight difference between the uptake of HCHs by Rye grass and Bermuda grass in different treatments. HCHs uptake by Rye grass is more than the other in T2 while, in T3 and T4 HCHs uptake is more by Bermuda grass.

The results shows a similarity with the previous studies. Previously experiments with HCHs showed a rapid dissipation of these compounds by several plants. Dubey *et al.*, 2014 reported that Spinach played a major role in the degradation of lindane (γ HCH) from soil as compared to the non-vegetated soil. *Cytisus striatus* species along with two strains of bacteria showed a good future for the phytoremediation of HCH-contaminated soils (Castro *et al.*, 2013). Pereira *et al.*, 2010 carried out a study to select candidate plant species for phytoremediation of HCH contaminated soils. Nine plant species of economic and/or agricultural interest were grown in a soil contaminated with a diverse mixture of the main HCH isomers. The results shown that plants responded differently to the uncontaminated soil and the soils containing HCH. Generally, all of the species showed signs of stress due to the presence of HCH, although to different levels. Out of all the nine species, three species (Hordeum vulgare L., Brassica sp., Phaseoulus vulgaris L.) displayed the capability of neglecting the negative effects of HCH, and shown resistance to a certain level, as their biomass production was not significantly affected by the HCH. Therefore, these three plant species can serve as suitable option for phytoremediation purposes.

a.



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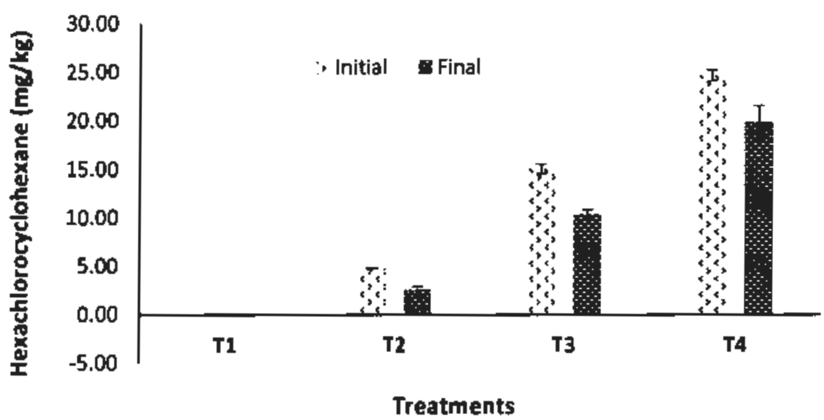

Figure 10: HCHs concentration in soil among different treatments in the presence of Rye grass (a) and Bermuda grass (b), T1: control soil with no pesticide; T2: soil spiked with 5mg/kg HCHs; T3: soil spiked with 15mg/kg HCHs and T4: soil spiked with 25mg/kg HCHs.

CONCLUSION

Remediation of polluted soils with HCHs is much needed in the current situation, as they are banned and included in Stockholm list of POPs. However, phytoremediation has been widely proven for the cleaning of several organic and inorganic pollutants, but only a small number of flora is reported so far, for the phytoremediation of HCHs in the literature. Therefore, it is much needed to explore more species for the remediation of HCHs. Hence, the present study was designed to explore the phytoremediation potential of two grass species (*Cynodon dactylon* and *Lolium multiflorum*) for the reclamation of HCHs contaminated soils. These two species possess qualities such as (i) grow quickly and (ii) not much care is needed make them desirable species for the phytoremediation of HCHs. The results revealed that the selected species can remediate and accumulate the HCHs from polluted soil. Moreover, they can also dissipate the HCHs from soil. Thus, these two species can serve as a better options for phytoremediation of HCHs contaminated soil, but their accumulation and dissipation potential can be raised by appropriate agronomic activities.

RECOMMENDATIONS

- ✓ Phytoremediation is a sustainable technology in the clean-up of polluted soil and water however it is still in the early stages and is not completely understood and applied at the field level in Pakistan.
- ✓ More research in this field is required to explore new species for the phytoremediation of HCHs,
- ✓ Pakistan have a lack of resources to apply conventional technologies, especially in large contaminated sites. Experiments should be done in the laboratory and field level to discover the potential of flora from Pakistan for phytoremediation of HCHs.
- ✓ Relevant authorities should take steps for the promotion of this research field as soil and water contamination is a terrific problem of Pakistan.

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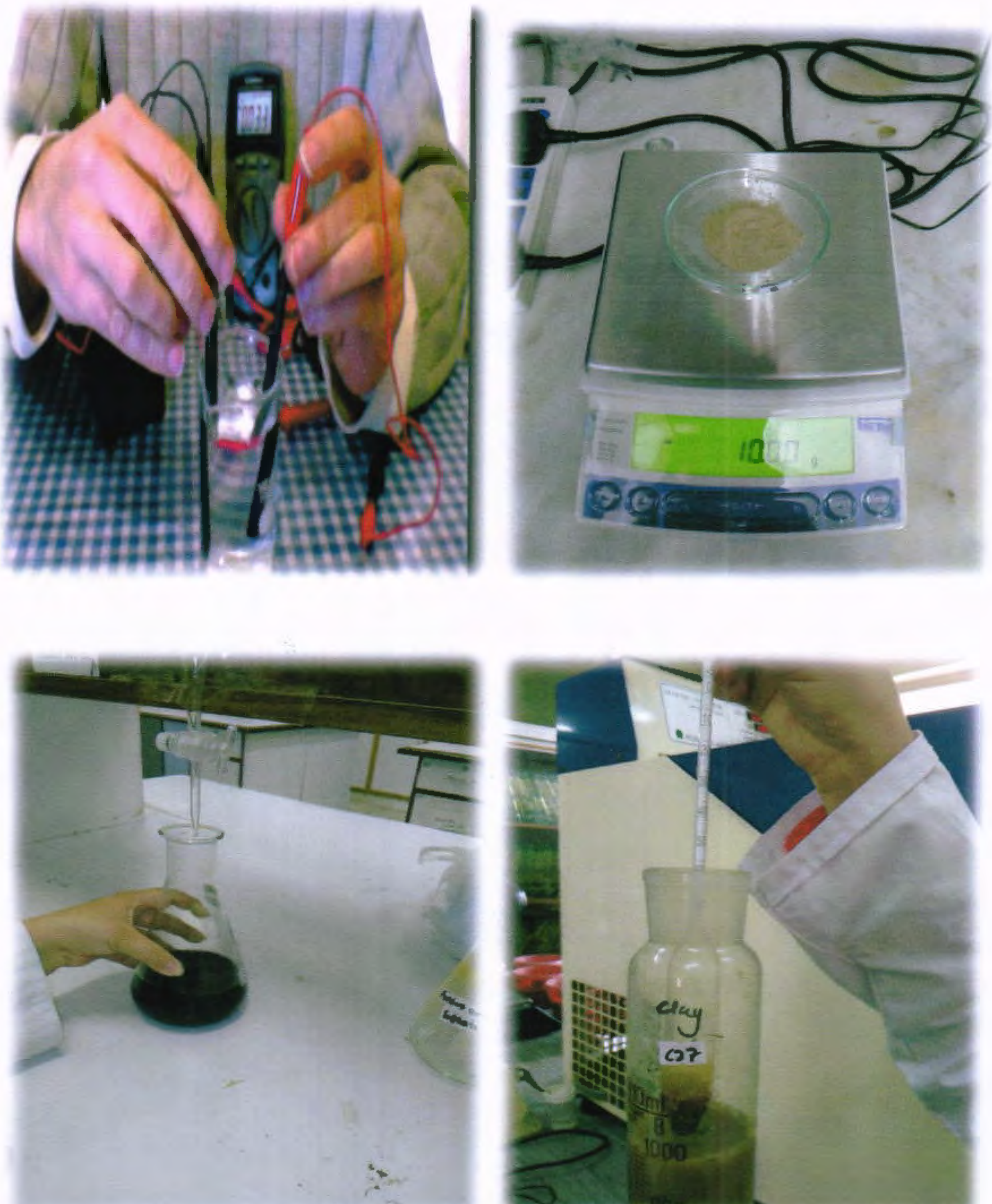
Annexure A

Soil collection, drying, grinding and sieving

Annexure B



Physico-chemical analysis of soil

Annexure C

Plants germination and transplantation

Annexure D

Sample preparation for HCHs analysis