INTRODUCTION

Soil and Microbes

Soil is a unique habitat which harbors a variety of microflora in addition to giving nutritional support to plants that sustain human life. The distribution of microorganisms in a typical soil profile has been described by Alexander (1971). There may be 1.5 million species of fungi and 1 million species of bacteria in the soil, but only 5 percent of them have been described (Tilak, 2000). One cubic meter of soil may house several hundreds of species of bacteria, actinomycetes, fungi and algae. These microorganisms bring about many chemical transformations such as sulfur transformations, iron and manganese transformations, N\textsubscript{2} mineralization, which are vital to soil fertility (Alexander, 1977). Soil fertility also depends largely on an adequate supply of inorganic nitrogen, phosphorus and potassium. The chemical forms of these nutrients that the plants can utilize are produced by microbes through mineralization of organic material. Thus the number, diversity and distribution of microorganisms reflect the overall productivity of the soil (Wainwright, 1978).

Factors affecting soil microbes

The presence of microorganism at a given place is influenced by a) its introduction, b) presence of associated organism (symbionts, hosts) favorable to its development, c) absence of organism (disease causing organism, pests, antagonists) so as to cause its extinction, and d) existence of physico-chemical environment favorable to its development.

Studies on the effect of pesticides on soil microbial community show that some pesticides stimulate the growth of microorganisms, but others have deleterious effects (Tu et al., 1976; Bromilow et al., 1996). Pesticides cause significant changes in microbial populations which are largely mediated through changes in soil pH. Endosulfan is highly toxic to soil enriching microorganisms that are responsible for maintenance of soil ecosystem (Singh et al., 2005; Vig et al., 2006).
Introduction

Ecologically, microbes are most essential for the restoration of contaminated environment. Microbial degradation is the main process affecting the environmental persistence of pesticides (Adhya et al., 1981). The process of bioremediation is an economical, versatile, environment friendly and efficient treatment strategy and a rapidly developing technology to degrade and/or detoxify chemical substances such as petroleum products, aliphatic and aromatic hydrocarbons, industrial discharges, pesticides and their metabolites and metals. With diversity in nature, microbes are capable of degrading variety of chemicals and toxic pollutants and as a result mitigating environmental contamination to a large extent.

Pesticides

Pesticides are substances used to control or kill insects, rodents, other such animals which can cause damage to crops. Since 1940, the use of pesticides has grown steadily at a rate of about 11% a year. These are toxic chemicals, which have been used extensively to control pests (Akhtar, 1985). Pesticides as a group of chlorine agent are used in plant protection, public health programs, household sprays and for fumigation of storage godowns. There are about 900 active chemicals which are used to make pesticides and while nearly all of them are environmentally hazardous to certain extent, only a few are highly toxic containing chlorinated hydrocarbon and are resistant to degradation. As per the reports of WHO, in developing countries, about 50,000 people are intoxicated and about 5,000 people die due to improper use of pesticides and other chemicals through the modern package of agricultural practices. In India, about 35,000 – 40,000 tonnes of hazardous chemicals are used each year. It is estimated that nearly 10,000 deaths occur annually due to use of chemical pesticides worldwide, with about three-fourths of these occurring in developing countries (Horrrigan et al., 2002). At present, India is the largest producer of pesticides in Asia with an annual production of 90,000 tons and ranks 12th in the world in the use of pesticides.

Sachan (1989) showed that India stands fourth in the consumption of pesticides. Use of pesticides in agriculture sector poses serious environmental and public health problems. Loss of aquatic and terrestrial biodiversity (Dutta et al., 2006), contamination of groundwater and agricultural produce, decrease in soil fertility and health hazards to agricultural workers are among the potential consequences of the use of pesticide in agricultural practices alone (Jayashree & Vasudevan, 2006). They enter into cyclic environmental processes such as
absorption by soil, leaching by water etc., and contaminate both lithosphere and biosphere. Owing to the interaction between lithosphere and biosphere, pesticides may enter the food chain and pose serious health hazards. Only a few of the pesticides get metabolically transformed and biodegraded (Dara, 2005).

Classification of Pesticides

Pesticides are classified into three major classes as **Insecticides** - designed to kill insects in crops; **Herbicides** - meant for killing weeds or undesirable vegetation; and **Fungicides** - toxic to moulds and other fungi. Based on the chemical groups, they are classified as a) Organochlorine compounds - DDT, HCH or Endosulfan, Lindane, Aldrin, Dieldrin, Dicofol, Endrin etc., b) Organophosphorus compounds - Malathion, Diazonin, Thion phorate, Dimethoate, Acephate phosphamidon, Monocrotrophos, Dichlorovas, Chlorpyrifos etc., c) Carbamates - Carbaryl, Aldicarb, Carbopropoxur, Benomyl (fungicide) etc., d) Hydroxy coumarin derivative (as rodenticide) - Warfarin, Flocoumafen etc., and e) Triazine derivative (as weedicides) - Simazine, Atrazine, Amitrole etc.

Table I: Insecticides manufactured and used in India

<table>
<thead>
<tr>
<th>Classification</th>
<th>Examples</th>
</tr>
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<tbody>
<tr>
<td>Organochlorine</td>
<td>DDT, HCH or Endosulfan, Lindane, Aldrin, Dieldrin, Dicofol, Endrin etc.</td>
</tr>
<tr>
<td>Organophosphorus</td>
<td>Malathion, Diazonin, Thion phorate, Dimethoate, Acephate phosphamidon, Monocrotrophos, Dichlorovas, Chlorpyrifos etc.</td>
</tr>
<tr>
<td>Carbamates</td>
<td>Carbaryl, Aldicarb, Carbopropoxur, Benomyl (fungicide) etc.</td>
</tr>
<tr>
<td>Napthalene</td>
<td>-</td>
</tr>
<tr>
<td>Aluminium phosphide</td>
<td>Celphos</td>
</tr>
<tr>
<td>Synthetic pyrethroids</td>
<td>Allethrin, pyrethrin, cypermethrin</td>
</tr>
<tr>
<td>Arsenic based insecticides</td>
<td>Arsenite, Paris green.</td>
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</tbody>
</table>

Source: Shukla et al., 1998
**Introduction**

**Permissible limit of pesticides**

The maximum permissible concentration of pesticides, according to European Economic Community (EEC) is fixed at $0.1\text{mgL}^{-1}$ for each pesticide and at $0.5\text{mgL}^{-1}$ for all pesticides taken together (Sharma, 2006).

**Fate of pesticides in soil**

Soil pollution is receiving greater attention due to its direct impact on public health. Once the pesticides are sprayed in the agricultural land, a part of it go either into the atmosphere via volatilization, or into surface water through surface runoff, or into the ground water via drift, deep down into soil. The residual pesticides may either be retained as such by certain soil or made available for degradation by the soil microorganisms which utilize the pesticides as sources of food. Soil types are shown to affect the persistence and retention of organochlorine pesticide residues (Edwards, 1966) and that DT50 (degradation time) rates of organochlorine pesticides vary from 4 to 30 years, depending on climatic and other environmental conditions.

Factors influencing the fate of pesticides in soil are, their movement in the soil, volatilization, uptake by plant, adsorption, chemical degradation, photochemical decomposition and microbial degradation. A report published by the Kerala State Council for Science and Technology and Environment (KSCSTE) concludes that Endosulfan is present in the soil and sediment samples even ten years after its aerial spray on cashew plantations in Kasargod area (Savvy, 2011b).

**Microbes in pesticide degradation**

The disappearance of pesticide is attributable to microbial activity. Many genera of heterotrophs use pesticides as substrates, either co-metabolizing the molecules or using them as nutrients. Species of *Agrobacterium, Arthrobacter, Bacillus, Clostridium, Corynebacterium, Flavobacterium, Klebsiella, Pseudomonas*, and *Xanthomonas* among the bacteria; *Alternaria, Aspergillus, Cladosporium, Fusarium, Glomerella, Mucor, Penicillium, Rhizoctonia* and *Trichoderma* among the fungi; and *Micromonospora, Nocardia* and Streptomycetes among the actinomycetes are shown to modify one or more of the synthetic chemicals (Alexander, 1977).
Microbial metabolism of insecticides

The common processes that microorganisms adapt to metabolise insecticides are

**Hydrolytic process:** It is a process carried out by the secretion of hydrolytic enzyme exogenously, as in the case of fungi. Eg: - Production of phenols, **Reductive system:** It is the process of reductive dechlorination where the reaction proceeds by replacing a chlorine atom on a non aromatic carbon with a hydrogen atom. Eg: - Conversion of DDT to TDE and DDE,

**Oxidation:** There are several oxidative reactions such as epoxidation of cyclodienes such as altruis and heptachlor to corresponding epoxies, oxidation of thioethers to sulphoxides and sulphones, oxidative dealkylation of alkyl amines, decarboxylation, etc. **Isomerization:** Isomerization reactions involve the conversion of γ-BHC to α-BHC, dieldrin to photodieldrin. In each of these cases, the isomers differ from the parent compound due to the difference between the positions of the chlorine atom in the benzene ring, **Dehydrochlorination:** This reaction involves simultaneous removal of hydrogen and chlorine from organochlorine insecticides. Typically the reaction takes place between the saturated chlorinated carbon and the adjacent hydrogen on the neighbouring carbon and **Conjugation:** Conjugation reactions include xylosylation, alkylation, acylation and can occur intra or extracellularly.

**Endosulfan**

Endosulfan-6,7,8,9,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3, benzodioxathiepine-3-oxide, is an organochlorine insecticide and acaricide (pesticide that kills ticks & mites) that comes under the cyclodiene subgroup. Introduced in the 1950’s, it emerged as a leading chemical used against a broad spectrum of insects (white flies, aphids, leaf hoppers, colarado potato beetles, cabbage worms etc.) and mites in agriculture and allied sectors. The half life of endosulfan varies from 60 days (α-endosulfan) to 800 days (β-endosulfan).
Figure I: Molecular structure of Endosulfan and its two stereoisomers.

Table II: The Physical properties of Endosulfan can be summarized as follows:

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>ENDOSULFAN</th>
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</thead>
<tbody>
<tr>
<td>Chemical formula</td>
<td>C_9H_6Cl_6O_3S</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>406.96 g</td>
</tr>
<tr>
<td>Water solubility</td>
<td>0.32 mg/L @ 22°C</td>
</tr>
<tr>
<td>Adsorption coefficient</td>
<td>12,400</td>
</tr>
<tr>
<td>Chemical class</td>
<td>Chlorinated hydrocarbon</td>
</tr>
<tr>
<td>Solubility in other solvents</td>
<td>Toluene and Hexane</td>
</tr>
</tbody>
</table>

Based upon the toxicity, the US Environmental Protection Agency (EPA) classifies Endosulfan as category-1b, highly hazardous and WHO classifies Endosulfan as category-II, moderately hazardous (Archita, 2006).
Regulatory process of Endosulfan in the world

In last 2 decades, many countries have recognized the hazards associated with the applications of these pesticides and have either stopped its production and/or banned its use. So far 81 countries have banned the use of endosulfan.

Endosulfan is banned in countries like Singapore, Germany, Indonesia, Iran, Japan, Korea, Pakistan, and Australia. India being one of the largest producers and consumers of endosulfan had not imposed ban or restriction on endosulfan till 2010 though a ban on endosulfan use in Kerala was imposed through court order since 2001. As per the Stockholm Convention held at Geneva during the year 2011, India had agreed to a global consensus to ban endosulfan but had asked for exemptions to continue for at least 5 years. So, use of endosulfan is banned after 5 years but conditional exemptions to use the pesticide are given to around 16 crops for 11 years (Savvy, 2011a). During November 2012, a joint experts committee had informed the Supreme Court that the use of endosulfan may be permitted for a period of 2 years in order to exhaust the available stock.

Trade name and formulations of Endosulfan

It was first introduced by Hoechst A.G.Company in Germany, under the brand name of “Thiodan”. Endosulfan is available in the market in a number of trade names. It is available as formulations of Emulsifiable Concentration (EC), Wettable Powder (WP), Ultra Low Volume liquid (ULV), Granules (G), Dust (D) and Smoke tablets. In India, it is marketed in different names.
Figure II: State wise consumption of Endosulfan in India

Source: Nair, 2011
Table III: List of trade names of Endosulfan in India

<table>
<thead>
<tr>
<th>Trade Name</th>
<th>Brand Name</th>
</tr>
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<tbody>
<tr>
<td>Agrosulfan</td>
<td>Malix</td>
</tr>
<tr>
<td>Endosulfan</td>
<td>Endomil</td>
</tr>
<tr>
<td>Endorifan</td>
<td>Thimul</td>
</tr>
<tr>
<td>Hildan</td>
<td>Thifor</td>
</tr>
<tr>
<td>Hexasulfan</td>
<td>Thinex</td>
</tr>
<tr>
<td>Krushi Endosulfan</td>
<td>Hildan</td>
</tr>
<tr>
<td>Thiodan</td>
<td>Endocide</td>
</tr>
<tr>
<td>Thiomex</td>
<td>Endocel</td>
</tr>
<tr>
<td>Vikas sulfan</td>
<td>Cyclodan</td>
</tr>
<tr>
<td>Pestical</td>
<td>Devisulfan</td>
</tr>
<tr>
<td>Endostar</td>
<td>Endosulpher</td>
</tr>
</tbody>
</table>

Of the total volume of endosulfan manufactured in India, three companies namely, Excel Crop Care, Hindustan Insecticides Ltd, and Coromandal Fertilizers produce 4,500 tonnes annually for domestic use and another 4,000 tonnes for export.

Exposure to Endosulfan

Endosulfan has been widely used to control bollworms, aphid, thrips, borers, white flies and hoppers on food crops; fruits like pear, peaches, apple; flowers like sunflower and other non food crops such as cotton, tobacco etc. One can be exposed to endosulfan by direct ingestion, smoking cigarettes made from endosulfan contaminated tobacco, drinking water contaminated directly or through run-offs, eating contaminated food, by breathing air sprayed with endosulfan, being in contact with contaminated soil, working at endosulfan production units etc.

Toxicological effects of Endosulfan

Endosulfan is acutely neurotoxic to both insects and mammals, including humans. Doses as low as 35mg/kg body wt, have been documented to cause death in humans and many cases of sub-lethal poisoning have resulted in permanent brain damage. It is shown to be a GABA-gated
chloride channel antagonist and a Ca^{2+}-Mg^{2+} ATPase inhibitor. Endosulfan is highly toxic and can be fatal if inhaled, swallowed or absorbed through the skin.

According to EPA, acute reference dose for dietary exposure to endosulfan is 0.015 mg/kg/day for adults and 0.0015 mg/kg/day for children. For chronic dietary exposure, the reference doses are 0.006 mg/kg/day and 0.0006 mg/kg/day for adults and children, respectively. Potentials to disrupt hormones and toxic effects on male reproductive system and developmental processes have been well documented.

The oral LD50 values ranging from 18 to 160 mg/kg in rats, 7.36 mg/kg in mice, and 77 mg/kg in dogs have been reported. The α-isomer is considered to be more toxic than the β-isomer. Stimulation of the central nervous system is the major characteristic of endosulfan poisoning. Symptoms of acutely affected humans include incoordination, imbalance, difficulty in breathing, gagging, vomiting, diarrhoea, agitation, convulsions and loss of consciousness. Blindness has been documented in some animals like cow, sheep and pig that grazed in a field sprayed with the compound. Several studies have demonstrated that endosulfan can also affect human development. Long term exposure to endosulfan has been found to affect the kidneys, parathyroid gland, liver and as well as the developing foetus.

Endosulfan exposure is linked to delay in sexual maturity among boys. The metabolites of endosulfan have also shown the ability to cause cellular changes. It is also reported that exposure to higher concentration of endosulfan may cause mutagenic effects in humans (Smith, 1991).

Endosulfan has been found in remote locations such as the Arctic ocean and as well as in the Antarctic atmosphere. The pesticide has also been detected in dust from the Sahara desert collected in the Caribbean after being blown across the Atlantic ocean. It has been shown that the compound is one of the most abundant organochlorine pesticides in the global atmosphere. According to EPA, the breakdown products of endosulfan are also toxic. The estimated half-life for the combined toxic residues (endosulfan and endosulfan sulfate) range from roughly 9 months to 6 years. EPA also concluded that endosulfan is a very persistent chemical which may stay in the environment including soil, for long period of time particularly in acidic condition. Endosulfan is known to be highly toxic to fish, birds, bees and wild life.
Biodegradation

Biodegradation is the process by which microorganisms are employed to rapidly degrade hazardous organic contaminants to environmentally safe level in soil, subsurface materials, water, sludge and residues. The microbial activity is enriched by the addition of nutrients. Microbes can acclimatize themselves to the toxic waste and thus new resistant strains develop naturally. Such strains can be used in pollution control and environmental protection.

The microorganisms use contaminants as food source and convert them into biomass and harmless by-products such as CO$_2$ and inorganic salts. Bioremediation has become an important tool to clean up the pesticide contaminated soil and water (Jilani & Khan, 2004). It is used to degrade contaminants that are sorbed to surfaces or dissolved in water rather than to degrade pure chemicals. As a result, the process is used in conjunction with other techniques in remediation of contaminated sites.

**Chemical properties influencing biodegradability**

The chemical properties that influence biodegradation of a compound may be listed as follows:

- a) Compound having hydroxyl and carboxyl groups,
- b) Hydrocarbons,
- c) Short chain unsaturated aliphatic and aromatic hydrocarbons,
- d) Polymerization,
- e) Hydrophobicity,
- f) Ring structure and number of rings,
- g) Branched structures,
- h) Non-alkyl groups (halogen, nitro, amino, sulfonates),
- i) Cross-linking with other molecules causing enzyme inaccessibility (e.g. lignin/cellulose),
- j) Volatization,
- k) Solubility.

**Characteristics of microbial metabolism**

Most of the metabolic activities in microorganisms are meant for the production of energy and the organic molecules can serve as sources of energy. Microorganisms undergo mutations to adapt to the chemicals that are initially toxic to them. In general, microbes alter the pesticide degradation process through several mechanisms such as:-

- a. **Enzymatic process:** Many microorganisms possess enzymes or enzyme systems that are capable of degrading specific pesticides. In most of the cases the pesticides are used as
the sources of essential elements like carbon, nitrogen, phosphorus, sulphur etc. and utilized in the microbial metabolism, leading to the degradation of the pesticide.

b. **Non-enzymatic process:** This process is achieved by promoting photochemical reactions. There are two ways by which the microorganisms can promote photochemical reaction. In the first case the microbial product can act as photo sensitizers by absorbing the energy from light and transmitting it to the insecticidal molecule. Secondly, microbial products can facilitate photo chemical reaction by acting as donors or acceptors. Insecticidal chemical are known to react with amino acid particularly with an –SH group to produce co-factors that help in the degradation of pesticide.

**Background of the study**

Environmental awareness has resulted in adopting suitable measures to preserve the environment from dangers of pollution and also to protect human health. Among the pollutants in the environment, pesticides are of major concern as they have been shown to affect almost every part of the human body. They persist in the environment and slowly enter human body through the food and water and accumulate in the body fat. Organochlorine pesticides like ‘Endosulfan’ having a slow decomposition rate, persists in the environment and accumulate in the upper trophic levels of the food chain. For the purpose of degradation of hazardous pollutants at their origin, suitable microbes capable of degrading specific pollutants could be used. Hence, identification and characterization of such microorganism is important to evaluate its potential to degrade the pesticide and its possible use in bioremediation, rendering the contaminants as less toxic or harmless.

**Significance and Impact of the study**

This study aims at throwing light on current status of the use of endosulfan in Karnataka and its effect on soil properties, in comparison with that of a Natural Farm. The study also proposes to describe the enrichment, isolation and characterization of bacterial species capable of bringing about the degradation of endosulfan which might lead to the bioremediation of water and soil contaminated with endosulfan residues.
Introduction

Objectives of the study

1. To evaluate endosulfan pesticide residues in the soil, at selected farms in Karnataka.

2. To isolate soil microbes from the selected area.

3. To screen the isolated microbes for their resistance to endosulfan pesticides.

4. To study the biodegradation of endosulfan by the isolated microbes.

5. To analyze the endosulfan metabolites produced by microbial degradation.

6. To create awareness among farmers on the possible effects of the use of endosulfan.