# INTRODUCTION

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**Pesticides**

Food factories catering to human consumption are depended on agricultural plants. Various methods have been employed for bettering crop growth and yield and meeting the ever increasing demand for food. Pesticides occupy unique position among chemicals to meet ever increasing demand for agricultural products. The discovery of DDT in 1939 by Paul Mueller (Dhaliwal *et al.*, 2000) had ushered in a new era in pest control, by means of chemical control. The chemical control became popular due to immediate and spectacular effects. Pesticides mean any substances or mixture of substances intended for preventing, destroying, repelling or mitigating any pest, including vectors of human and animal disease, unwanted species of plants or animals causing harm during, or otherwise interfering with, the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products or animal foodstuffs, or which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies (Parmar and Dureja, 2004).

Pesticide consumption in developing countries is approximately 20 per cent of the world's usage as compared to 80% of the developed countries (Joia, 2001). The consumption in India is 570 g/hectare against 2500 g/hectare in USA, 3000 g/hectare in Europe and 12000 g/hectare in Japan (Singhal, 2000). In India, the pesticide production increased from 6600 MT in 1966 to 61000 MT in 1988-89 (Dhaliwal *et al.*, 2000). The predominant classes of pesticides used in India (during 2000-01) were insecticides, accounting for 61 per cent of total consumption, followed by fungicides (19%) and herbicides (17% ; Figure 1) and maximum amount is consumed by cotton (45%), followed by rice (22%; Figure 2).

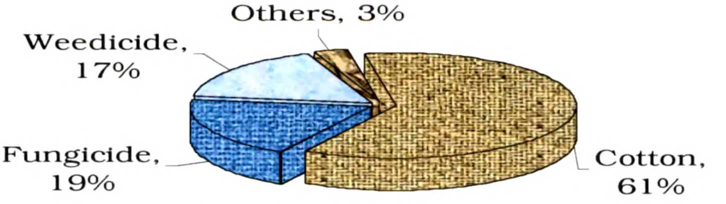



Figure 1. Composition of Pesticide Usage in India (Krishna *et al.*, 2003).

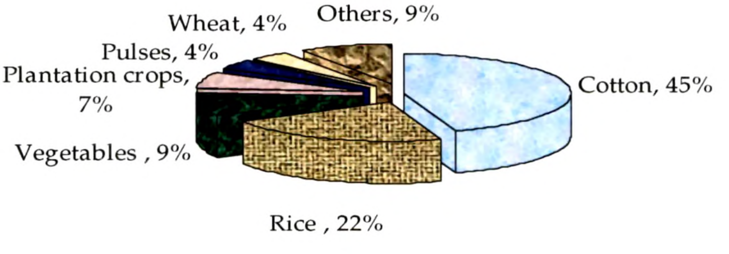



Figure 2: Pesticide Consumption by Different Crops in India (Krishna et al., 2003).

Pesticides are heterogeneous class of chemicals whose action is to kill or control unwanted organisms in a selective manner. However, not many are highly selective being toxic to many non target species i.e., including human being and environmental organisms via various mechanisms. Their unconditional application or misuse in developing countries is posing a serious threat to human health. Nevertheless, nearly 80 % of world pesticide consumption is in developed countries; incidence of pesticide poisoning is 13 times higher in the developing countries and India accounts for 1\3 of pesticide poisoning cases in the world (Dhaliwal *et al.*, 2000). As per world health organization estimate, pesticides poison 3 million people every year, and kill 200,000, most of them belonging to developing countries (Down to Earth, October 15, 2004). Pesticide market of developed countries is dominated by herbicides, which tend to posses lower acute toxicity than insecticides. Contrarily, predominant class of pesticides used in most of the developing countries is insecticides, posing higher level of risk. Organophosphates and carbamates are the major compounds in the class of insecticides and are responsible for higher percentage of worldwide pesticide poisoning (Wahid, 2004).

Large scale or indiscriminate use of pesticides, made great impact on environment i.e., pest resistance, resurgence of pests, effect on non target organisms and pesticide residues in food commodities. All India coordinated research on pesticide residues had revealed that 60% of food commodities were found to be contaminated with pesticide residues, out of which 14% contained residues above the maximum residue limit (MRL) as compared to 21% contamination with only 2% above MRL on world-wide basis (Agnihotri, 1999). The problems of pesticide residues in India resulting from unregulated use are quite alarming. Pesticide residues mean any specified substances in food, agricultural commodities, or animal feed resulting from over use of pesticides. The term includes any derivatives of pesticides such as conversion products, metabolites, reaction products and impurities considered being of toxicological significance (Gill, 2001).

In 2003, Centre for Science and Environment (CSE) had made big issues in national news papers by showing presence of pesticides in soft drinks like Coca cola, Pepsi, and even in Bisleri water bottles (The Times of India

Online, 2003). The existence of these products really worries pollution research biologists. Who is the originator of this variety of pollutants? It is not because the cola companies add them to their drink. It is because the water used by them contains pollutants. The ground reality lies in ground water contamination by pollutants. There is definitely a desperate need to ensure that harmful pollutants do not contaminate groundwater supplies. Much of the groundwater pollution is blamed on intensive farming practices based on an increase in the use of chemical fertilizers and pesticides. Studies at Cornell University have established that 99.9% of the pesticides sprayed (whether in developed or the developing countries) go into the environment, with only 0.01% of pesticides reaching the target pest (Sharma, 2003).

Chlorinated hydrocarbons, the first generation insecticides have been used as insecticides for a long time in agricultural practices. Later on, their use declined and this group of chemicals was replaced by organophosphates. Organophosphorus compounds (OP) were first synthesized in 1854 but their remarkable toxicity was not recognized until the 1930s. The first synthesized OP pesticide , tetraethyl pyrophosphate (TEPP), was developed in Germany before world War II to replace the highly toxic and lipid soluble botanical insecticide, nicotine (Savolainen, 2001). Following World War II, with the capture of Schrader's research record (Chambers *et al.*, 2001) interest in organophosphate (OP) insecticides grew rapidly and have dominated the market for the last four decades. Although all of the early chemicals were effective and were quickly accepted as insecticides, they were also highly toxic to mammals. The use of these chemicals in place of chlorinated hydrocarbons is attributed to their higher biodegradability and low persistence in the environment (Srivastava and Dumka, 2001). However, their unconditional or indiscriminate use led to food commodities being contaminated and, accidental poisoning of man and animals has raised alarm.

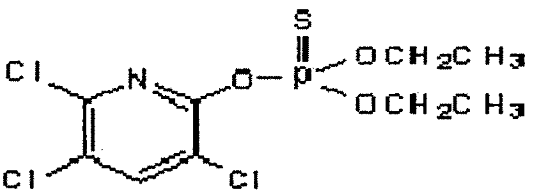
Anticholinesterase organophosphorus compounds (OP's) are derivatives of phosphonic acid or their sulfur-containing analogs, notably phosphorothioic acid, phosphonothionic, phosphordithioic, or phosphonodithioic acids. Phosphonic acid or its derivatives do not generally inhibit AChE activity. OP's which inhibit AChE activity usually have two alkyl groups and a third group (the leaving group), that is often an aryl group or a heterocyclic group. The leaving group is more susceptible to hydrolysis than the alkyl groups. Typically, OP's with the P=S configuration have little or no inhibitory action on AChE unless they have been activated through enzymatic or nonenzymatic oxidative desulfuration to the corresponding oxon that contains the P=O configuration. Such conmpounds are termed indirect inhibitors of AChE, and include many important insecticides used in the world such as chlorpyrifos, malathion and parathion. In terms of OP chemistry, most OP's are poorly water soluble, have a high oil-water partition coefficient and a low vapor pressure. In alkaline conditions, they are hydrolyzed to water soluble products that are generally considered to be nontoxic (Savolainen, 2001).

**CHLORPYRIFOS**

Chlorpyrifos [*O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate] is a broad-spectrum organophosphorus pesticide that, over the last 3-4 decades, has become extensively used in agriculture, horticulture, termite control and indoor pest control. It exhibits its toxic action on mammals/vertebrates/ insects by inhibiting cholinesterase enzymes. The Dow Company of USA originally developed it for root dip treatment in rice cultivation (TIFAC, 2003). Chlorpyrifos is used in a formulated form as a broad spectrum insecticide for the control of Coleoptera, Diptera, Homoptera and Lepidoptera in soil or on foliage in a wide range of crops.

Chlorpyrifos is widely used for ant control since the phasing out of organochlorine pesticides. It is known to provide efficacious ant control in citrus, cucurbits, mangoes, custard apples and pineapples. It has both good contact action and a degree of residual action against a range of ant species (The NRA Review, 2000). There appears to be no satisfactory alternatives to chlorpyrifos for ant control in fruit trees.

Chlorpyrifos is the phosphorothioate OP with the P=S ester configuration.




Chlorpyrifos

Chlorpyrifos binds strongly to soil or disperses into the atmosphere following application. Limited quantities may enter aquatic environments with spray drift or run-off, and will mainly partition to sediment where slow to moderate degradation occurs. Atmospheric persistence appears limited, while residues in soil are degraded at a moderate rate by chemical and microbial processes (The NRA Review, 2000).

Consistent with its properties, chlorpyrifos is very much an occasional contaminant of surface waters, but can reach high levels on occasions. The use pattern is of main concern with respect to high level surface water contamination as in termite protection, which involves generally higher rates of application than agricultural treatments. As a broad spectrum insecticide, chlorpyrifos is very highly toxic to a range of insects, including beneficials. A very high toxicity is also evident in aquatic arthropods, under both laboratory and field situations. Fish kills have been reported where aquatic contamination is high, with termiticide treatments a common cause particularly, if followed by heavy rain (The NRA Review, 2000).

Maximum residue limit (mg/kg) for chlorpyrifos in different food commodities are as follows (Handa *et al.*, 1999).

| **Food commodities** | **Maximum Residue Limit (mg/kg) for Chlorpyrifos** |
|---|---|
| Food grains | 0.05 |
| Milled food grains | 0.01 |
| Fruits | 0.5 |
| Potatoes and onions | 0.01 |
| Cauliflower and cabbage | 0.01 |
| Other vegetables | 0.2 |
| Meat and poultry | 0.1 |
| Milk and milk products | 0.01 |
| Cotton seed | 0.05 |
| Cotton seed oil | 0.025 |

Due to its high usage in garden, horticulture and agriculture practices and subsequent higher risk associated with children, on January 25, 2002, U.S. EPA (United States Environmental Protection Agency) had issued cancellation order for pesticide products containing chlorpyrifos registered by six companies in USA (Jayakumar, 2003).

Chlorination is commonly used for treatment of domestic water supplies. The study conducted by Wu and Larid (2003) regarding abiotic transformation of chlorpyrifos to chlorpyrifos oxon in chlorinated water raised a caution about the safety in domestic use of chlorpyrifos products. This finding is more relevant to toxicologists due to abiotic transformation of chlorpyrifos to chlorpyrifos oxon. Chlorpyrifos oxon is about 1000 times more toxic than chlorpyrifos.

**Heavy Metals**

The heavy metals are a group of elements between copper and mercury on the periodic table of the elements having atomic weights between 63.546 and 200.590 and specific gravities greater than 4.0. Organisms require trace amounts of some heavy metals, including cobalt, copper, iron, manganese, molybdenum, vanadium, strontium, and zinc, but excessive levels can be detrimental. Other heavy metals such as mercury, lead and cadmium have no known vital or beneficial effect on organisms, and their accumulation over time in the bodies of mammals can cause adverse toxic effects.

Food and environmental contamination by heavy metals are very common and, numerous reports are available for their excessive concentration in food commodities. Metals and chemicals associated with mine dumps, mill tailings and irrigation of agricultural land by not properly treated sewage water are of serious threat to environment (Zou *et al.*, 2004; Pandey *et al.*, 2004). They pollute ground water and soil and finally result in uptake by vegetation and successive bioaccumulation in the food chain. Metals occur in all foodstuffs. Toxicologist's/Scientist's concern is on presence of toxic metals, which include lead, cadmium, arsenic and mercury. The toxic metal content of foods is influenced by many factors ranging from environmental conditions during growth to post-harvest handling, processing, preparation and cooking techniques. For example, metal content increases in some commodities grown in contaminated soils and/ or conditions while post-harvest handling steps such as washing generally remove metal contaminants. Cooking may reduce metal content although some foods can absorb metals if the cooking water is contaminated. Metals used in food processing equipment or food packaging material may contribute to food contamination. Contamination may also occur during kitchen preparation and storage (Morgan, 1999).

**Lead**

The nonessential metal, lead occurs naturally as a sulfide in galena. It is a soft, bluish-white, silvery gray, malleable metal with a melting point of 327.5 °C. Elemental lead reacts with hot boiling acids and is attacked by pure water. The solubility of lead salts in water varies from insoluble to soluble depending on the type of salt. Lead also forms salt with organic acids, such as lactic and acetic acids, and stable organic compounds such as tetraethyl and tetramethyl lead (Davidson, 1994).

Lead has been in use by mankind for over about 6000 years, and solely as a result of anthropogenic activities it has become the most ubiquitous toxic metal. Hippocrates was probably the first of the ancients to recognize lead as the cause of colic. Lead's toxicity was recognised and recorded as early as 2000 BC (World Environment Day, 2001). Lead is a natural element that is persistent in water and soil. Most of the lead in environmental media is of anthropogenic activities. The mean concentration is 3.9 µg/L in surface water and 0.005 µg/L in sea water. River sediments contain about 20,000 µg/g and coastal sediments about 100,000 µg/g. Soil content varies with the location, ranging up to 30 µg/g in rural areas, 3000 µg/g in urban areas, and 20,000 µg/g near point sources. Human exposure occurs primarily through diet, air, drinking water, and ingestion of dirt and paint chips

(Davidson, 1994). In 1999, a blood lead survey of 23,000 children and adults in 7 cities in India, revealed that 53% of children under 10 years of age have levels of 10 μg/dL or higher, the current U.S. limit (Clark *et al.*, 2004).

India was one of the early civilizations, which took to metallurgy, and the use of lead in various forms has prevalent for 3 millenia. In recent years considerable concern has been expressed regarding human risks due to environmental lead in India and, several epidemiological and environmental monitoring surveys, experimental studies and case reports have appeared, suggesting the gravity of the problem. Monitoring of lead and other heavy metals has been undertaken under an Integrated Environmental Programme on Heavy Metals (IEPHM), Ganga Action Plan, and Rajiv Gandhi Drinking Water Mission (World Environment Day, 2001) and heavy metals in Indian environment by ITRC (ITRC, 2000). Several studies in India have revealed the existence of lead pollution in air, water and food commodities. Major lead consumption sources and environmental contamination by lead based industries in India are given below.

**Lead (Pb) Consumption in India** (World Environment Day, 2001)

| **Lead Consumption in India** | | | | | |
|---|---|---|---|---|---|
| Batteries | Cables | Paints | Sheets/Pipes | Industrial alloys | Miscellaneous |
| 42% | 22% | 10% | 10% | 8% | 8% |

**Environmental Contamination by Lead Industries** (World Environment Day, 2001).

| **Environmental Contamination by Lead Industries** | |
|---|---|
| **Sample** | **Maximum range of Pb concentration measured near industries** |
| Soil | 200-3454 mg/g |
| Grass | 145-1048 mg/g |
| Jawar | 15.81 mg/g |
| Air | 0.5- 120 mg/m$^3$ |
| Blood (children) | 23.4 – 43.47 mg/dL |
| Urine (children) | 45.8 – 169.4 mg/L |

Possible sources of lead exposure in India (World Environment Day, 2001).

- Contaminated soil and dust
- Mining
- Cooking and storage vessels containing lead (tinned polish);cans
- Ceramic pottery with painted lead glaze
- Country liquor, beverages
- Food adulterant in ice cream, tobacco and tobacco products
- Toys, pencils
- Cosmetics
- Herbal medicine
- Paints, pigments
- Industrial effluents; disposal
- Water pipes
- Contaminated aquifers: mining, smelting, processing, recycling
- Occupational exposures: silver jewelry making; battery breaking and manufacturing; welding; repairing automobile radiators, etc.

The growing popularity of traditional Indian remedies necessitates a crucial evaluation of risks associated with their use. The finding of lead in ayurvedic medicines alerts us about their usage. Keen *et al.* (1994) reported that an Indian patient with hepatitis was found to have lead poisoning and the source was traced to ethnic remedies he had been taking for diabetes. The lead contents of some of the medicinal plants used in remedies are as follows (World Environment Day, 2001).

| **Levels of Lead in Ayurvedic Medicines** | | | | | | |
|---|---|---|---|---|---|---|
| **Serial N°** | **Medicine** | **Pb (mg/g)** | | **Serial N°** | **Medicine** | **Pb (mg/g)** |
| 1 | Saptamrut loh | 5.12 | | 8 | Arogyavardhini | 63.20 |
| 2 | Keshar gugal | 2.08 | | 9 | Sankhvati | 13.00 |
| 3 | Punarvadi gugal | 1.99 | | 10 | Brahmivati | 27500.0 |
| 4 | Trifla gugal | 4.18 | | 11 | Chyavanprash | 7.30 |
| 5 | Ghasard | 16,000 | | 12 | Trivanga bhasma | 261200.0 |
| 6 | Bala goli | 25 | | 13 | Diabline bhasma | 37770.4 |
| 7 | Kandu | 6.7 | | 14 | Hepatogaurd | 0.4 |

In 2003, a research project (Marshal and Agrawal, 2003) funded by the United Kingdom Department for International Development (DFID) has reported that consumers in Delhi are purchasing vegetables with high levels of heavy metals. The vegetables studied for their presence of lead, zinc and cadmium included spinach, cauliflower and okra. The main cause for concern in terms of contamination of vegetables in Delhi by heavy metals relates to lead. 72% of 222 samples of spinach which contained lead concentrations that exceeded the Indian Prevention of Food Adulteration Act (PFA) permissible limit of 2.5 mg/kg.

The study conducted by Dey and Dwivedi (2000) revaled excees amount of lead concentration in poultry egg samples. The majority of samples had Pb concentrations that exceeded normal background level of 0.020 μg/g. They ranged between 0.142 and 0.936 μg/g. And it was also observed that the mean Pb concentration recorded in their study was higher than the concentrations found in hen's eggs from Germany, Canada, Taiwan, China, and Finland.

Clark *et al.* (2004) reported that an estimated 10% of the lead in India is reported to be used in making paint. In 1999, they evaluated 24 new paints available in India and found that four had lead concentrations exceeding 0.5% lead by weight (the U.S. limit for paint in housing) and one contained more than 10% lead.

The need for lead in India is continuously growing. According to the report of Down to Earth (Down to Earth, Nov 15, 2004) the annual demand for lead is about 160,000 tonnes, half of which is met through imports. In 2002-2003, as much as 31,282 tonnes of lead scrap made its way to India as against 16,345 tonnes in 1997-98.

There is large database on subclinical neurotoxic effects of lead in children. Neuropsychological impairment and cognitive (IQ) deficits are sensitive indicators of lead exposure; both neuropsychological impairment and IQ deficits have been the subject of cross-sectional and longitudinal studies in children. Studies reported IQ score deficits of four points at blood lead levels of 30-50 µg/dL and one to two points at levels of 15-30 µg/dL among 75 black children of low socioeconomic status. Children living in the Boston, Massachusetts area have been studied up to the age of 10 years. Cognitive performance scores were negatively correlated with blood lead in the younger children in the high lead group (greater than or equal to 10 µg/dL), and improvements were noted in some children at 57 months as their blood lead levels became lower. Whereas, measures of IQ and academic performance in 10-year-old children showed a 5.8-point deficit in IQ and an 8.9-point deficit in academic performance as blood lead level increased by 10 µg/dL within the range of 1-25 µg/dL (Davidson, 1994). Extensive literature on postnatal lead exposure and children's neurobehavioral development suggests that there is no clear threshold for neurotoxic effects of lead in children (ATSDR, 2005).

**Pesticide and Heavy Metal Combination**

Pesticides and heavy metals as food contaminants have raised serious threat to human and other organisms due to pollution boom during the last 4-5 decades. Pesticides in food are predominantly residues from their application on different steps/stages of growth and processing of agricultural products, whereas, heavy metals contaminate food at various stages along the food production line, starting from agricultural lands to food processing. Due to large scale use of these two kinds of chemicals, their common existence everywhere throughout food chain is inherent. To reduce risks associated with dietary intake of pesticides and heavy metals, international agencies like European Union have made some risk estimations based on acceptable daily intake for pesticides and tolerable daily intake for heavy metals (Barstad, 1978; Nasreddine and Parent-Massin, 2002).

Toxicity of chemicals is influenced by number of factors. The presence of two chemicals at a given time leads to various interactions depending on the environment i.e., both inside the living organisms and also outside the biotic environment. Most of the interactions are so complex and obscure, that they remain unidentified. Some foreign chemicals have distinct or well recognized interactions in the body, and in some instances the mechanism of these interactions have been identified. There are mainly four types of interactions namely; additive, antagonistic, potentiating and synergistic. The latter two kinds of interactions worry Toxicologists much more. The intrinsic capacity/ toxicity is determined by the dose. The dose of a chemical required to produce toxicity may vary dramatically when it interacts with other chemicals (Rozman *et al.*, 2001).

Whether interactions will be of any practical importance depends on its degree and on the chance a person or organism may have simultaneous exposure to adequate amounts of two interacting compounds. The organophosphorus pesticides form chelating complexes with heavy metals. The chance of humans being simultaneous exposed to these two different classes of chemicals is higher in recent days. In India, there are many acres of agricultural lands irrigated by sewage water/ effluent/or water supplied with high concentration of heavy metals. The usage of effluents for agricultural purposes and contamination due to heavy metals has been reported by many authors (Gupta and Mitra, 2000; Lark *et al.*, 2002;

Adhikari and Gupta, 2002). Pesticides are used very often to control pests. Pesticide residues deposited on the surface have tendency to penetrate into the tissues of plants (Rao and Rao, 2000). As a consequence, both heavy metals and pesticides enter different tropic levels of food chain through uptake by plants. The agricultural lands irrigated by sewage water/ effluent/or water supplied with high concentration of heavy metals and pesticides enter different tropic levels of food chain through uptake by plants. Recent report by Bansal (2004) revealed higher concentration of heavy metals and pesticides in sewage irrigated soils of Aligarh district of UP.

The presence of chlorpyrifos and lead in food commodities is very common. Available literature on chlorpyrifos revealed its adverse effects on nervous system mainly inhibiting cholinesterase enzyme with more risk to children and immature animals. Lead is a well known developmental neurotoxicant and its systemic toxicity is also well established. However, no work has been published on their combined action when they are simultaneously challenged to test systems. Agency for Toxic Substances and Disease Registry (ATSDR) scientists in collaboration with mixture risk assessors and laboratory scientists, have developed approaches for the assessment of the joint toxic action of chemical mixtures. ATSDR (December, 2004) asked for data/investigations on interaction profile for chlorpyrifos, lead, mercury, and methylmercury. Chlorpyrifos, lead and mercury/methylmercury were chosen as the subject for their interaction profile because of the likelihood of co-exposure and because of concerns about neurological effects in children co-exposed to these chemicals. No pertinent health effect data or physiologically-based pharmacokinetic (PBPK) models were identified for the complete mixture.

Due to their (chlorpyrifos and lead) common occurrence in day today life, the present investigation was designed to evaluate:

1. combination effect of chlorpyrifos and lead on neurobehavioural aspects by single and nonlethal high dose levels
2. combination effect of chlorpyrifos and lead at the biochemical level by single and nonlethal high dose levels
3. combination effect of chlorpyrifos and lead on neurobehavioural aspects by repeated subchronic dietary exposure
4. delayed or persistence effect of chlorpyrifos and lead combination on neurobehavioural aspects after repeated dose (subchronic) 90-day oral exposure followed by 28 days post treatment period
5. combination effect of chlorpyrifos and lead at the biochemical level by repeated dose subchronic dietary exposure
6. delayed or persistence effect of chlorpyrifos and lead combination on biochemical parameters after repeated dose (subchronic) 90-day oral exposure followed by 28 days post treatment period

To correlate behavioural findings and clinical pathology findings with morphological changes at cellular level, histopathology of required organs were considered to evaluate;

7. single dose effect of chlorpyrifos and lead combination at cellular level
8. effect of repeated subchronic dietary exposure of chlorpyrifos and lead combination at cellular level