Chapter 1

Introduction

1.1. GREEN REVOLUTION

Green revolution (GR), also known as the third agricultural revolution, are a set of technological advancements in the field of agronomy during late 1950's to improve and strengthen the agricultural setup in the developing nations. These research initiatives are attributed to Norman Borlaug, the "Father of Green Revolution", for developing disease resistant wheat in Mexico. Thereafter, this area of research was heavily funded by government agencies like Ford foundation and Rockefeller foundation in developing high varieties of wheat and rice. In addition to this, chemical fertilizers, pesticides, irrigation techniques and new methods of cultivation were introduced. These research developments were a set of practices that replaced traditional methods of farming which were transferred and adopted worldwide. Many countries became a witness to these improvements in agrobusiness and benefited from the GR work conducted by Borlaug.

1.2. THE INDIAN SCENARIO

India is an agriculture-based country, where more than 50 % of its total taskforce is employed by it (Madhusudhan, 2015). Not only it holds a strong position in the Indian economy but shares a great weight of approximately 23 % of India's total gross domestic product in 2016 (Ahluwalia, 2019). Being the largest employment source of the country, the contribution from agricultural sector has always declined since 1951 till date. Therefore, the development in the agriculture and allied sectors remains the most important objective of Indian planning commission since the pre-liberalization period. In the meantime, India was on the brink of mass famine in 1961 because of rapid expansion in population. Borlaug and colleagues were invited to address the situation by Dr. M. S. Swaminathan, the then minister of agriculture. Despite bureaucratic hurdles, Indian government collaborated with Ford foundation to import hybrid varieties of wheat and thus India began its GR by implementing these progressive techniques in agricultural setups (Sebby, 2010). Eventually the agricultural output augmented because of the prominent measures taken during the five-year plans and stable improvisations in GR practises (Datt and Sundharam, 2009).

The GR refers to agricultural advancements and technologies that washed off the soils during the late twentieth century in India. Modern practises, which increased the global productivity in agricultural set up, were adopted by the government to address famines, food shortages and rising population. Starting in 1960's, The Indian planning commission stressed upon rapid industrial development (Second five-year plan) maximizing the economic growth. The outcomes of these were seen in the consecutive five-year plans to make India self-reliant in terms of improved agricultural output. Since then, the agricultural expansion has always been an integral part of the nation's development. India's GR transformed the country's few fertile regions into actual breadbaskets, magnifying India's output of wheat and rice. The revolution brought new irrigation practises, hybrid seeds, fertilizers, insecticides, herbicides, and modernisation making farmers, the heroes of a self-sufficient India, with no dependency on foreign grain shipments further making the country free of starvation.

Although GR has been a unique event in the agricultural history of Independent India, it also has its own integrally deficient segments. Various demerits that came across are as follows (Pepper, 2008):

a) Inter-crop imbalance

The effect of GR was principally focussed on food-grains. Out of which, wheat and rice were the most benefitted ones while other crops like cotton, jute, sugarcane remain untouched. Subsequently, the cultivation became futile and went typically in favour of wheat and rice. The outcome of this unevenness was not good for the well-composed growth of Indian agriculture.

b) Increase in personal inequalities

It was observed that only the farmers with inherent acres of land were benefited the most during the revolution. They were financially stable to procure farm apparatuses, hybrid varieties of wheat and rice, chemical fertilizers and utilize their resources for modern irrigation practises to water the crops.

As against this, two-third of the farmers were with 2.5-7 acres of land and were falling below the poverty line. Due to their poor financial stature, they couldn't purchase these farming tools and were indirectly deprived of the benefits of GR technology. In short, it made the rich richer and rendered the poor resulting in wide-spread social and economic tensions (Bhalla and Chadha, 1982).

c) Regional disparity

GR technology raised discrepancies at inter and intra-regional levels pertaining to economic advancement. The areas which were better placed from agricultural point of view *viz.* Punjab, Haryana, Uttar Pradesh, Andhra Pradesh, Kerala and Tamilnadu benefitted more. The primary benefits were due to the distribution and application of pesticides, in addition to high yielding varieties of seeds (HYVs), advanced irrigation technologies and agrochemicals (Pradhan and Saluja, 1996). The eastern belt of India consisting of arid and semi-arid areas were tormented due to underdeveloped irrigation technology and inaccessibility of disease resistant high yield paddy strains and pesticides (Devi et al., 2017).

d) Environmental damage

One primary mission of the GR was to improve the production of Rabi and Kharif crops and the program required farmers to use pesticides and fertilizers to kill the pests and give extra nutrients to these high yielding plants including wheat and rice, to take advantage of efficient irrigation techniques, along with learning new management skills. Statistics show upsurge in the production of man-made chemicals *viz.* pesticides and fertilizers between 1960's and 1990's, which was the ultimate intention behind the revolution. Continuous and unscientific use of GR technology created a plethora of environmental problems like deforestation, increased levels of alkalinity and salinity in soil, reduction in soil fertility along with elevated soil erosion and diminished ground water resources. Monoculturing of crops in all seasons also increased incidents of human and livestock disease and vulnerability of the plants towards pests by making them develop resistance due to prolonged usage of pesticides (Chamala, 1990; Bala et al., 2010).

1.3. THE TOXIC CONSEQUENCES OF GREEN REVOLUTION

The Green revolution was 'green' (successful) in earlier days but the current scenario reveals the story of what happened behind the curtains. Rahman (2015) elaborated the negative impacts of over adoption of agricultural technologies and abrupt usage of pesticides. The epidemic use of fertilizers diminished the soil quality due to increased dependency on synthetic fertilizers than natural fertilizers such as animal manure. The genetically modified varieties of wheat and rice were vulnerable to the pests due to monocropping practices and led to increased dependence on pesticides. Further, their unceasing application in the fields developed resistance in the nuisance pests. In addition, the pesticides being adsorbed on the soil particles got washed off during the rains and got infiltrated in the ground water, thereby, polluting these water resources (Miller and Spoolman, 2014). These man-made chemicals have indirectly contaminated the human

populations and their ecosystems. The pesticides enter through various routes in the soil, which damages the niche for soil microbes and thereby the ecosystem (Figure 1.1). The disproportionate employment of pesticides to protect the crops caused the manufacturing industries to bloom in India, causing a radical production from 154 metric tonnes in 1954 to 88,000 metric tonnes in the year 2000 (Mathur et al., 2005).

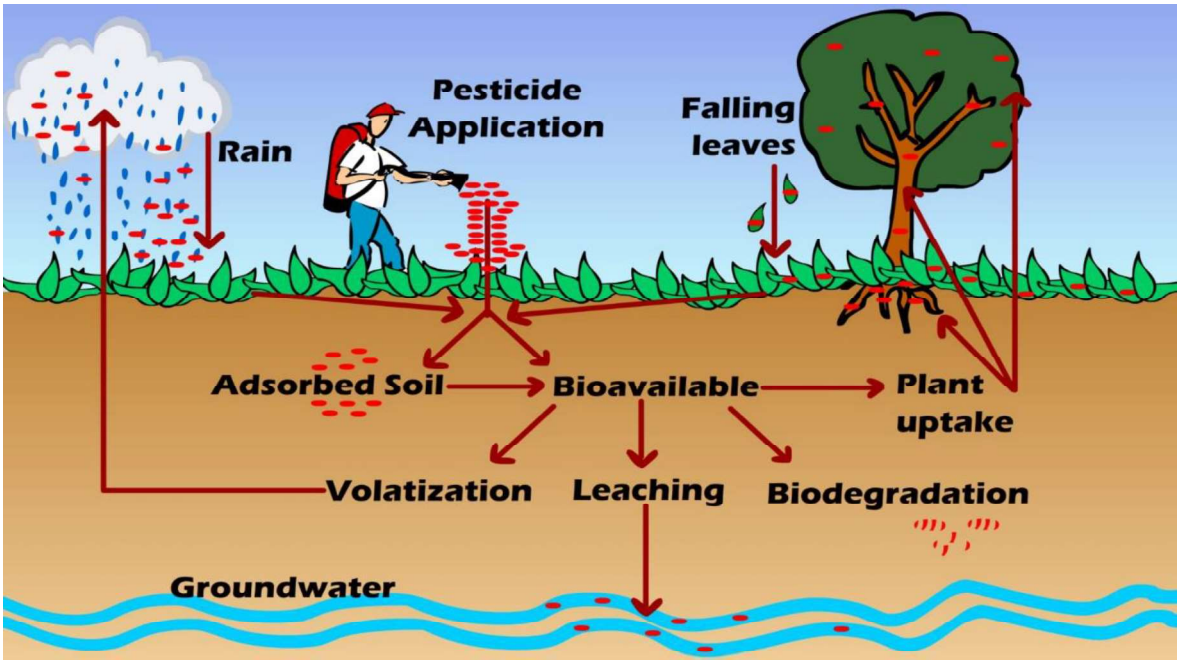


Figure 1.1: Overview of the pesticides entering the ecosystem through various pathways. Source: Langenbach, (2013)

Four decades after the so-called green revolution, Indian farmers are turning their backs on the modern agricultural techniques i.e. the use of pesticides, in favour of organic farming. The benefits of this GR movement were coupled with unanticipated harmful consequences which raised second thoughts about the change in farmers. Though pesticides have enhanced the economic potential in terms of food production and brought relief from vector borne diseases up to a certain extent, the shortcomings come in detrimental forfeits to man and his environment. One such example of the toxic manifestations of GR was observed in Punjab, a state which pioneered GR and was considered most favourable to implement and practice modern farming techniques (Rahman, 2015).

The studies conducted by Consultative Group on International Agricultural Research have stated the consequences of these over-adoption of farming practises and pesticides for the past 40 years taking Punjab into a state of socio-economic crisis (Liu et al., 2015). Over escalation of

agronomy led to heavy demand of water for irrigation and depleted the ground water levels at an alarming rate. Disproportionate use of nitrate-rich fertilizers aggravated the salinity and alkalinity of rich alluvial soil of Punjab state which has negligible slope (Shiva et al., 1991). The agricultural lands were becoming the centres of pesticide pollution that affected the human life especially of the farmers who came in contact directly or indirectly. PGIMER, a premier research institute in Chandigarh (Punjab, India) had carried out an elaborate study to comprehend relationship between indiscriminate use of pesticides with amplified occurrence of cancer in the whole region. Tirado and colleagues (2010), directed a study in 50 different villages of Punjab to check the levels of toxicity in water caused by these chemicals. The results were shocking as up to 20% of the samples showed presence of high levels of nitrates and were way above the safety limits established by W.H.O (Ahluwalia, 2019). A group of researchers at Punjabi University discovered DNA damage in 30% of Indian farmers who treated plants with herbicides and pesticides (Kaur et al., 2011). An additional study by Hazarika and Deka (2017) were in support of genotoxic and cytotoxic effects occurring to the ones exposed to pesticides. Several reports have confirmed the presence of heavy metals and pesticide in drinking water, soft drinks and a variety of food items (Johnson et al., 2006; Kar and Kar, 2008; Agrawal and Sharma, 2010; Mohammad et al., 2018). The state that lead the GR to make India a surplus country has been turned into another polluted area like Bhopal due to excessive use of chemicals.

Usage of such multiple varieties of chemicals for different purpose results in accumulation in the environment and the food chain, causing undue consequences towards different forms of life. The situation further worsens when the effects are silently passed on to the succeeding generations. One such report of poisoning due to pesticides in India came from Kerala in 1958, where about hundred people died after consuming wheat flour contaminated with parathion (Srinivas Rao et al., 2005). In addition, the endosulfan tragedy which happened in Kasaragod district of Kerala was another such unfortunate incident. It is considered by many experts in the field of pesticide toxicity as one of the world's worst pesticide disasters. Endosulfan, an organochlorine compound, acts as a human teratogen causing significant developmental anomalies in the offspring of pesticide intoxicated parents. The Plantation Corporation of Kerala (PCK), a public sector under the State Government, owned three cashew plantations, covering 4600 hectares in Kasaragod, sprayed endosulfan aerially in this area for 24 years (1976 to 2000), thrice annually (Devi, 2010). "If little is good, a lot more will be better": this saying being followed particularly in pesticides usage has created a havoc to non-targeted species, resulting

in several chronic, critical and life-threatening ailments in the populations residing in and around the plantations.

The Green Revolution hardly seems to have made much of an impact in terms of well-being, but widespread usage of these chemicals has given rise to several acute and chronic effects like behavioural change, renal failure, stillborn babies, birth defects, cancer, reproductive abnormalities etc (Sanborn et al., 2007; Brainerd and Menon, 2013).

1.4. PESTICIDE USAGE IN INDIA

Apart from fertilizers, pesticides played significant role in the agricultural production to meet the needs of growing population during the GR, but substantial amount of the harvest was always lost due to insect pests in the farms and during its storage. Therefore, to overcome such obstacles, pesticides became a prerequisite for a better agricultural yield. The Food and Agriculture Organization, categorized all those mixtures and substances under pesticides, which are employed to destroy, prevent or control any pests, which might be vectors of human or animal diseases or might be causing nuisance during storage, production, processing, transport or marketing of food, agricultural commodities and feed stuffs (Fischer et al., 2002). They could also be chemical derivatives, administered on domestic animals to control pests on their bodies.

The use of pesticides in India dates back to 1948 for controlling malaria and locust. Thereafter, a stable rise was observed in the production from 1966 after the introduction of GR technologies. Currently, India is the leading manufacturer of basic pesticides in Asia and ranks 12^{th} globally. Though India is leading in pesticide manufacturing, its consumption rate (Kg per hectare) is very low compared to other countries (Devi et al., 2017).

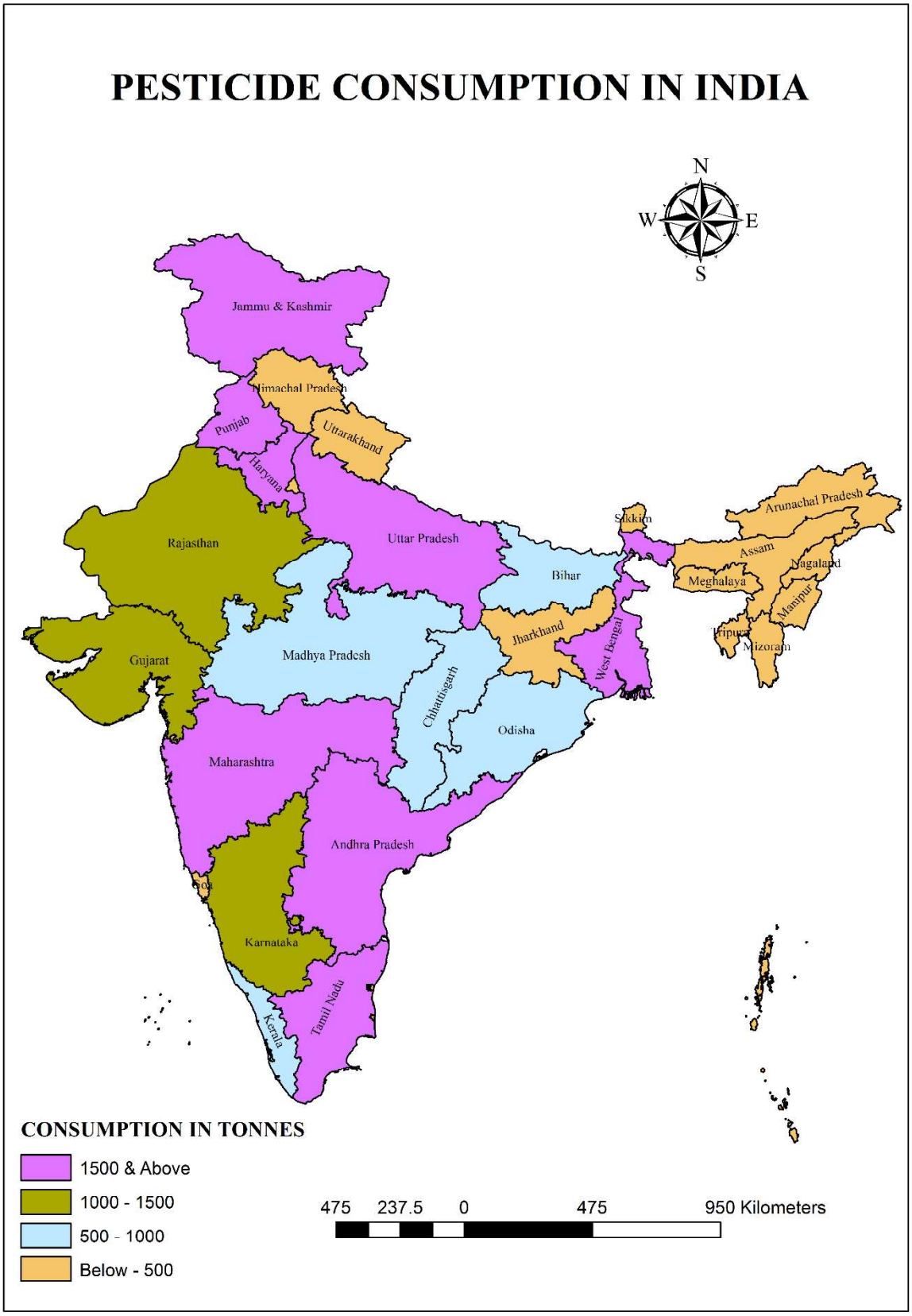


Figure 1.2: Intensity of pesticides usage in India. Adapted from: Devi et al., 2017

In addition to low consumption of pesticides approximately 600 g/ha in India, only the region's best suited for developing GR technologies show this range of consumption as compared to the other states in the country (Figure 1.2). Out of the total production in the world, Korea accounts for 16.6 %, 13.4 % by Italy, 10.8 % by Japan, 4.5 % is used by United States whereas the remaining developing countries consume 31% of pesticides (Devi et al., 2017) (Figure 1.3).

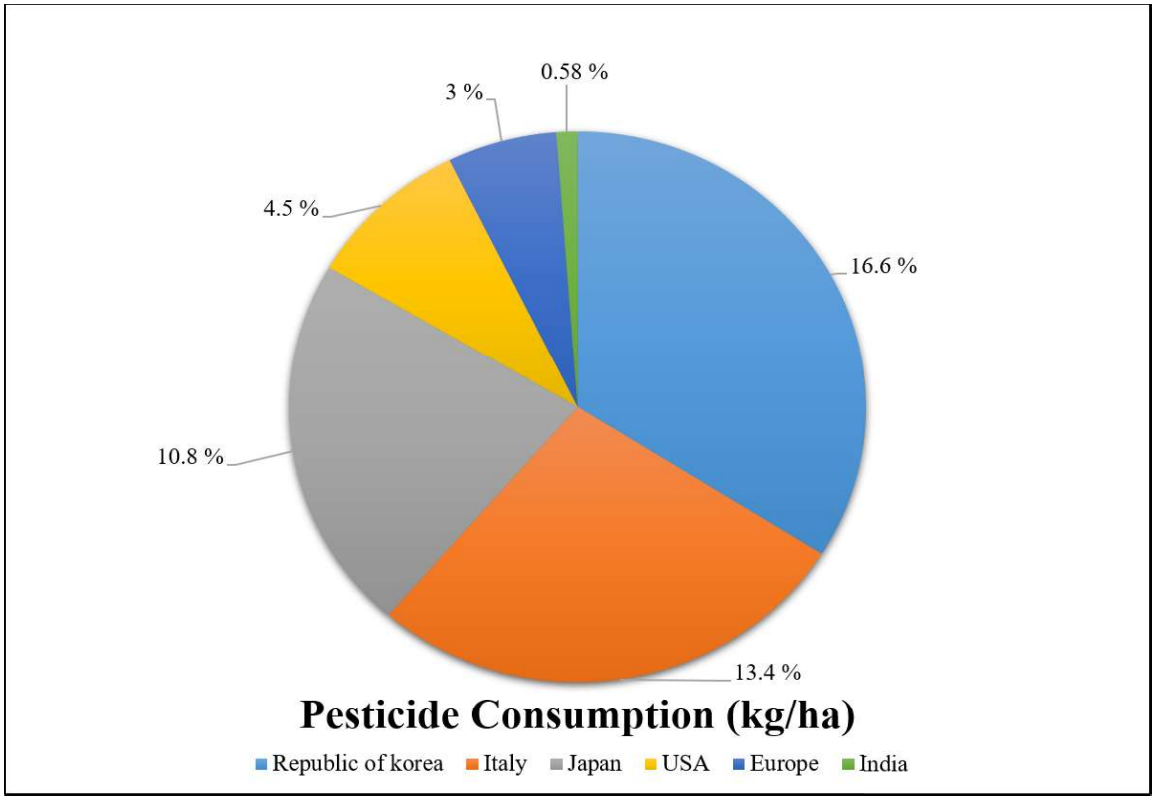


Figure 1.3: Image depicting the consumption of pesticides across the world (Devi et al., 2017)

The Indian Pesticide industry was only behind China in terms of production of 85,000 metric tonnes in the financial year 2011. India has been ranked as the fourth largest producer of agrochemicals after USA, Japan and China and twelfth in the world for the usage of pesticides. Among the four predominant classes of pesticides, insecticides hold a major share of 76% of the consumption in India (Figure 1.4), followed by fungicides (13%), herbicides (10%) and others (1%) (Mathur and Tannan, 1999; Abhilash and Singh, 2009; Dubey et al., 2011; Devi et al., 2017). Currently, approximately 256 pesticides are registered for use. Out of these, 4 are categorised under W.H.O. Class Ia, 15 in W.H.O. Class Ib and 76 are grouped as W.H.O. Class II pesticides, together constituting 40% of the total registered pesticides in India (Deshpande, 2017).

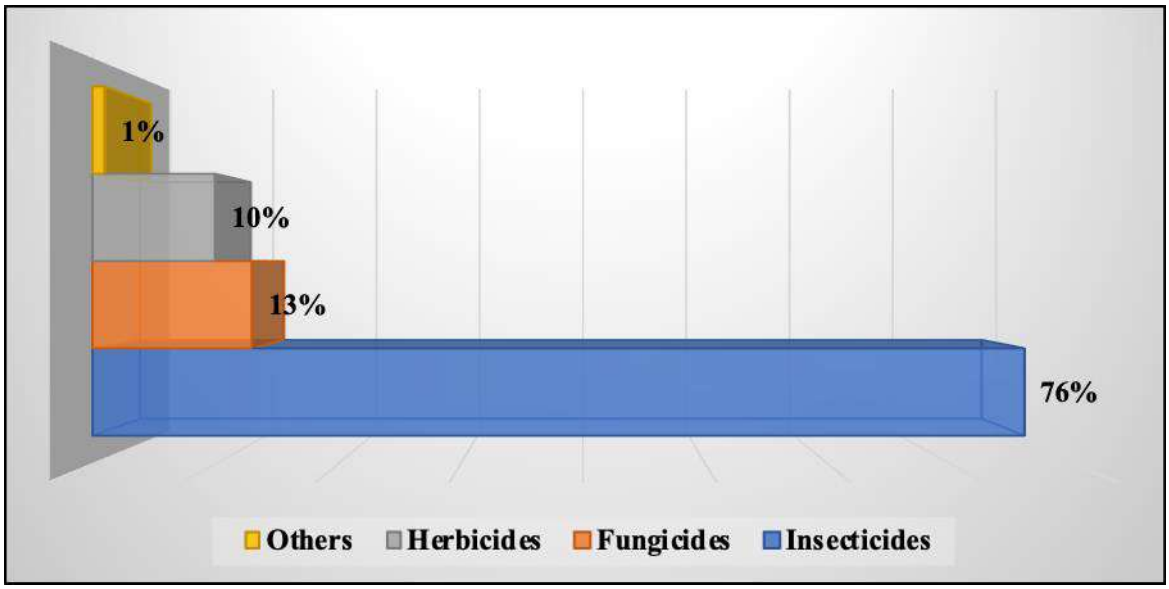


Figure 1.4: Image depicting the consumption pattern of types of pesticides in India. Adapted from: Aktar et al., 2009

1.5. TYPES OF PESTICIDES

Pesticides cover a wide range of chemical and biological compounds and can be organized in three ways 1) by the organism they target; 2) their chemical structure and 3) physical state (Sarwar, 2015). They can also be classified as inorganic, synthetic, microbial and biochemical pesticides. Some classes of pesticides are derived from plants such as pyrethroids, rotenoids, nicotinoid (Kamrin, 1997). Further, various pesticides can be classified based on their mechanistic action or methods of application. These chemicals are initially mass-produced as technical grade products and later they are converted into approved formulations in powder, emulsions or concentrates (Dureja et al., 1986; Chung et al., 2014). Here the chemicals are grouped based on their chemical structure. There are different families such as:

1. Organochlorines (OC)

Their principle use is for the annihilation of infectious vectors, mainly of intestinal sickness, dengue and jungle fever. They are additionally utilized in the cultivation of grapes, lettuce, tomato, horse feed, corn, rice, sorghum, cotton and wood, for protection against pests. In humans, organochlorine pesticides are reported to cause cancer, asthma, diabetes and growth disorders in children (Park et al., 2006). These substances or their metabolites demonstrate toxicity, fundamentally, at the level of focal sensory system by altering the electrophysiological properties of the nerves. They bring out modifications in Na^+ and K^+ streaming through the membrane of the nerve cell (Narahashi et al., 1992). This further results in the spread of multiple action

potentials for each stimulus (Kamrin, 1997), causing symptoms such as seizures, respiratory arrest and death due to acute poisoning (Tordoir and Van Sittert, 1994). DDT, Hexachlorocyclohexane etc. are the known examples of this family.

2. Organophosphates (OP)

These organic compounds containing phosphorous have found applications as pesticides against bugs and mites. They are utilized for vegetable products, grains, cotton and sugarcane. These compounds get absorbed rapidly through the skin, conjunctiva, gastrointestinal tract and lungs and gets metabolized by cytochrome P_{450} in the liver, generating more toxic metabolites than the parent compounds (Costa et al., 2008) In humans, they follow up on the focal sensory system by repressing acetylcholinesterase, a compound that tweaks the sum and levels of the neurotransmitter acetylcholine, disturbing the nerve drive by serine phosphorylation of the hydroxyl bunch in the dynamic site of the protein (Fukuto and Sims, 1971). The effects seen on central nervous system CNS are loss of reflexes, migraine, unsteadiness, sickness, writhing and extreme lethargy (Sulbatos et al., 1994; Perry et al., 1998; Paudyal, 2008).

3. Carbamates:

These are esters derived from N-methyl carbamic acid which are corrosive and produce clinical signs like organophosphates (OPs). The effects are reversible and less severe than OP. They are widely utilized as bug sprays, herbicides, fungicides and nematicides. The mechanism of toxicity of carbamates is comparable to that of OPs i.e. inhibiting acetylcholinesterase (AChE). However, this inhibition is short-lived and rapidly reversible, because of reactivation of the carbamylated enzyme by hydrolysis (Jokanovic, 2009). The sign and symptoms of carbamate intoxication are the same as observed following intoxication with OPs, and include miosis, urination, diarrhoea, salivation, muscle fasciculation, and CNS effects (Rosman et al., 2009).

4. Pyrethroids

They function mainly as insecticides, derived from pyrethrum extract obtained from chrysanthemum flowers, known as pyrethrins. These compounds used to decompose rapidly in light, so they were subsequently obtained synthetically. Soderlund (2002) with his co-workers reported their increased acceptance in the global market as potential insecticide because of the low mammalian toxicity, lack of environmental persistence, and low tendency to induce insect resistance. They follow up on the focal sensory system bringing about changes in the influx of Na^+ via the film of the nerve cell, making it extend its open time, causing persistent sodium current over the neuronal layer in both insects and vertebrates (He, 1994; Perry et al., 1998). These occasions can prompt neuronal hyperexcitation (Narahashi, 1996; Perry et al., 1998).

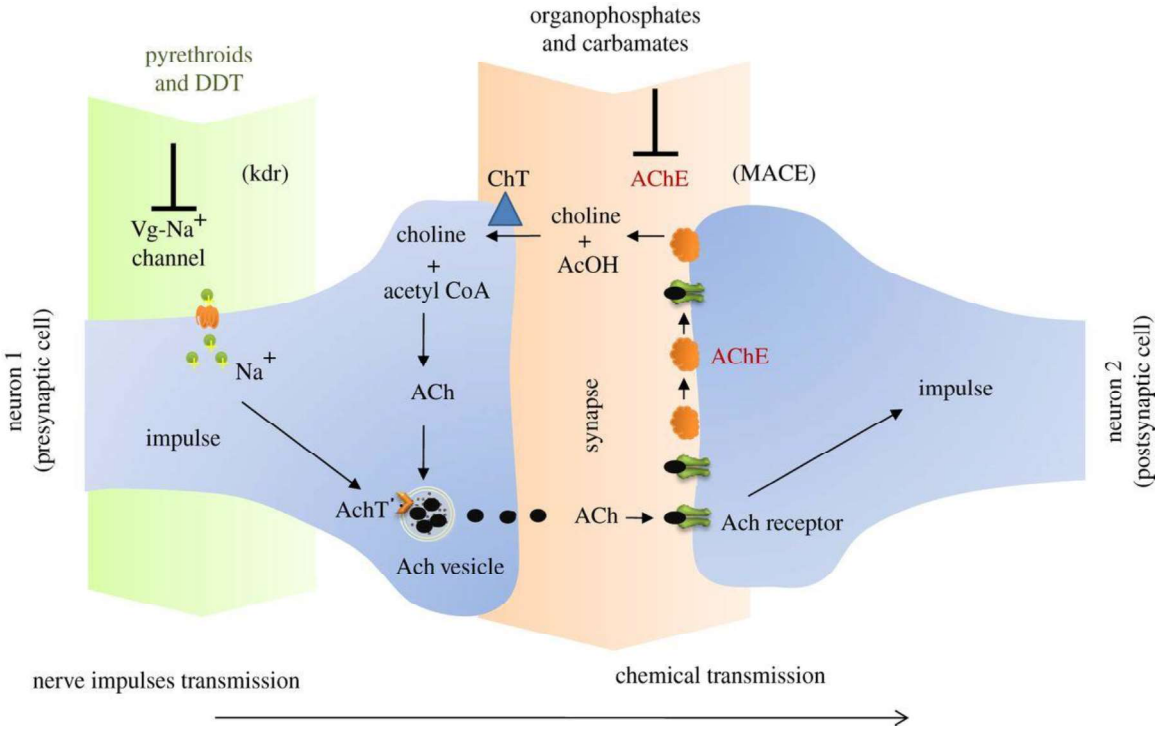


Figure 1.5: Mode of action of insecticides targeting voltage gated sodium channels and acetylcholinesterase. Source: Nauen (2006)

Furthermore, different pesticides, for example, triazine herbicides, phenoxy derivatives, benzimidazoles, bipyridyl mixes, ethylene dibromide, sulphur containing mixes are additionally utilized commercially. These insecticides are ideally designed to target specific species of organisms through a unique mode of inhibition (Figure 1.5). However, their specificity is often arguable. Ecobichon (2001) has briefed about the striking similarities between CNS of mammals and insects, making them highly sensitive to insecticides. The only difference lies is in the detoxification pathways taken up by the insects and the mammals. The way the target organism counteracts the insecticide is crucial as the consequences can be the release of more toxic compounds. Non-target species especially humans, are therefore more vulnerable to acute toxicity caused on exposure and therefore a great number of human poisonings and deaths are reported over the years. As a class, insecticides have higher acute toxicity towards nontarget species compared to other pesticides.

1.6. INSECTICIDES: A POTENTIAL THREAT TO NONTARGET ORGANISMS

Insecticides are one of the common contaminants found in soil, air and water. They can affect the living organisms ranging from plants to mammals. Once the toxicant is spread over the fields, it is not necessary that it would reach the target organism. Moreover, for the exposure to occur the toxicant spread over the area must overlap in time and space with the desired pests. The route of entry of these toxic compounds is generally via dermal layer or through inhalation. As the nontarget organisms get exposed to these insecticides, they try to cope up with their toxic effects. The liver enzymes detoxify these chemicals via hydrolysis, oxidation, reduction, or conjugation. Nevertheless, this biotransformation can also produce metabolites that are more lethal than their parent compound, a process termed as bioactivation. For instance, after detoxification of DDT, the metabolite DDE formed turns out to be more toxic to birds causing thinning of calcareous shells and also disrupting the calcium metabolism in the eggs (Levengood and Beasley, 2007).

The insecticides that highly solubilise in organic components are rendered in the fatty tissues and are eliminated at a very slow rate from the organism. Due to this bioaccumulation, the amount of these chemicals increase in the organisms and their concentration will undeniably get higher as compared to that spread in the environment (Akcha et al., 2012). These accumulated toxins reach the highest trophic levels in the food chain with far greater concentrations than the ones in the lower strata's and this phenomenon is termed as biomagnification. These chemicals are later transferred to the progeny of the topmost predators as they consume tissues with high fat ratio, which is being contaminated with the pesticides.

The classification of insecticides generally depends on whether they are persistent in the environment or not. Out of the four major classes we observed earlier, Organochlorines are the ones that accumulate in the environment whereas organophosphates, pyrethroids and carbamates are not persistent. These chemicals tend to accumulate in the environment and cause toxic effects, however, the non-persistent ones have much shorter shelf-life with very little or no bioaccumulation. Nonetheless, their outrageous usage in the agricultural set up still poses a major threat to the environment.

As mentioned earlier, these organophosphates and carbamates, act on acetylcholinesterase and eventually cause death of an organism. As this enzyme is present even in nontarget organisms, it allows the insecticides to reach insects, fishes, birds and even mammals. Both these class of chemicals are also known to affect the behaviour pattern in teleost fishes (Van Dyk and Pletschke, 2011). The chemicals belonging to organophosphorus class do not persist in the

atmosphere, however their unwarranted usage has effectively contaminated the soil layers, ground waters and they have subsequently entered the rivers. Vorkamp and Riget (2014) conducted a study where they detected traces of chlorpyrifos, an organothiophosphate insecticide, in the air and sea water of Arctic, representing the long-range transport of these toxicants. In addition, it is highly toxic to fish, and has led to their mortality in the waterways located near the treated fields (Deneer, 2000). Another study on Diazinon from organophosphate class, proven to be highly toxic to birds and fishes, and has been frequently observed in sewage treatment plants in urban and agricultural areas (Durmaz et al., 2006). Even secondary exposure to these chemicals have been proven to be lethal when Swainson's hawks got infected after they prayed up on grasshoppers sprayed with the monocrotophos as reported by Levengood and Beasley (2007).

Pyrethrins are non-persistent in nature and globally used as insecticides from agriculture upto household activities. Urban populations are more exposed to these compounds. They are specifically designed to target nervous system of arthropods and chordates. However, it is less toxic to mammals and especially humans (Pérez-Fernández et al., 2010). Ziram, an example of carbamate class, is best known for its toxicity to aquatic organisms. These carbamates function mainly as fungicides and rodent repellents (Kubrak et al., 2012; Padhye et al., 2013)

Organochlorines as stated earlier have a longer shelf-life and also bioaccumulate in the organisms. These compounds further get concentrated as they reach higher trophic levels in the environment and also spread over thousands of miles from their origin of contamination. Aldrin and dieldrin prove to be the best examples of organochlorines causing mortality in predatory birds (Levengood and Beasley, 2007). Another compound, Endosulfan, has been proved to be toxic to fishes and humans (Vorkamp and Riget, 2014). The ongoing practice of spraying of organochlorines and PCBs in India has posed a major threat to fresh and marine life as they elicit adverse effects on their reproductive and immunological functions (Bernardes et al., 2015).

Therefore, a large variety of pesticides are present in residual amount in soil, air, on surface and ground water across the nation. Their presence poses substantial hazards to the environment and especially to non-target organisms ranging from soil microflora, to plants, insects, fish, birds and humans. They may not cause acute toxicity at low concentrations but can definitely induce other kinds of damages, like genetic ailments and biological alterations in the long run and thus can reduce the organism's life span.

1.7. INSECTICIDES: A POTENTIAL TERATOGEN

Experimental embryology shows that disruption and perturbation caused by environmental factors produces detectable changes in the embryo's fate. Brent et al. (1990) documented various genetic and environmental factors (drugs, chemicals, viruses, hyperthermia or radiation, pesticides) which alter the normal development, commonly referred to as teratogens. The study of developmental flaws infested before conception due to these deleterious factors is called teratology. A teratogen, therefore, is known to cause structural and anatomical abnormalities, which are termed as congenital malformations. The first of such publication that came from Lenz and McBride in 1961 was for thalidomide which was primarily prescribed to pregnant women as a sedative to cure anxiety, insomnia, gastritis and tension, resulted into congenital malformation. Other effects included deformed eyes and hearts, deformed alimentary and urinary tracts, blindness and deafness (Gilbert, 2003). In the following year, the repercussions of pesticide usage were published in the book "Silent Spring" by Rachel Carson (Carson, 2009). The book documented how the pesticides with teratogenic potential had an impact over the environment and human health.

Many evidences have come forward indicating the serious threats posed by pesticides to various life forms and their ecosystem (Igbedioh, 1991; Forget, 1993; Sulbatos et al., 1994). The new generation insecticides *viz.* organochlorines, organophosphates, carbamates and pyrethroids, are designed to have a short half-life as well as less accumulation in the organisms. Despite of their benefits in wide range of activities, all the four classes of insecticides have been proven to be teratogenic and are found to cause serious intoxication symptoms (Jayaraj et al., 2016). The acute effects of pesticide exposure are irritation of skin and eyes, headache, and dizziness. The long-term effects of pesticides are associated with genotoxic risk as these compounds may damage DNA, by production of free radicals and causing mutations that lead to the development of cancer and adverse health effects (Alleva et al., 2018). Likewise, insecticides from organophosphorus, carbamates, and organochlorines are reported to act as endocrine disruptors and affect the function of hormones by blocking, mimicking, displacing, or acting to disrupt their natural roles in living species for e.g. DDT and its metabolites are among the most famous endocrine disruptors, affecting the reproductive system of mammals and birds (Bernardes et al., 2015). Infants, pregnant women and foetuses have been detected with trace amounts of insecticides in their body fluids (Barr et al., 2004; Bradman et al., 2005). Rull and colleagues (2006) have observed elevated risks of pesticides on inflicting teratogenic effects on human

populations. Similarly, reports on rodent and avian models also suggest developmental toxicity of the pesticides (Raees Ahmad, 2007; Slotkin, et al., 2008; Uggini et al., 2012).

Ngo and colleagues (2010) have reported the toxic effects of chemicals on the developing embryo and that too at a particular stage of development, where detoxification mechanisms have not been formed or are not fully functional. The teratogenic impact of such insecticides on living beings results into a variety of congenital structural malformations. It has been noted that approximately 10-15 % of developmental deformities are a result of the adverse effects of these environmental factors on prenatal development. Reports are in support of the exposures of insecticides during the developing period of the embryo results in adverse outcomes which may get expressed at early or later stages of life (Mai et al., 2012). This indicates that, approximately 1 in 250 infants developed structural defects due to environmental exposure to such toxicants. Further, a higher number of children elicited retardation in the growth and exhibited functional abnormalities resulting from non-genetic causes (Brent et al., 1990). The development of an organism is a sequential event and there are several check points or milestones to be achieved for the proper development. If any disturbance occurs during those stages, then it may lead to structural or physiological abnormalities. Moreover, protective mechanisms are not functional at embryonic stages and an acute exposure during the critical period of development may cause adverse effects (Slotkin, 2004). These toxicants induce developmental deformities through several mechanisms of cellular signalling and causing oxidative stress (Figure 1.6).

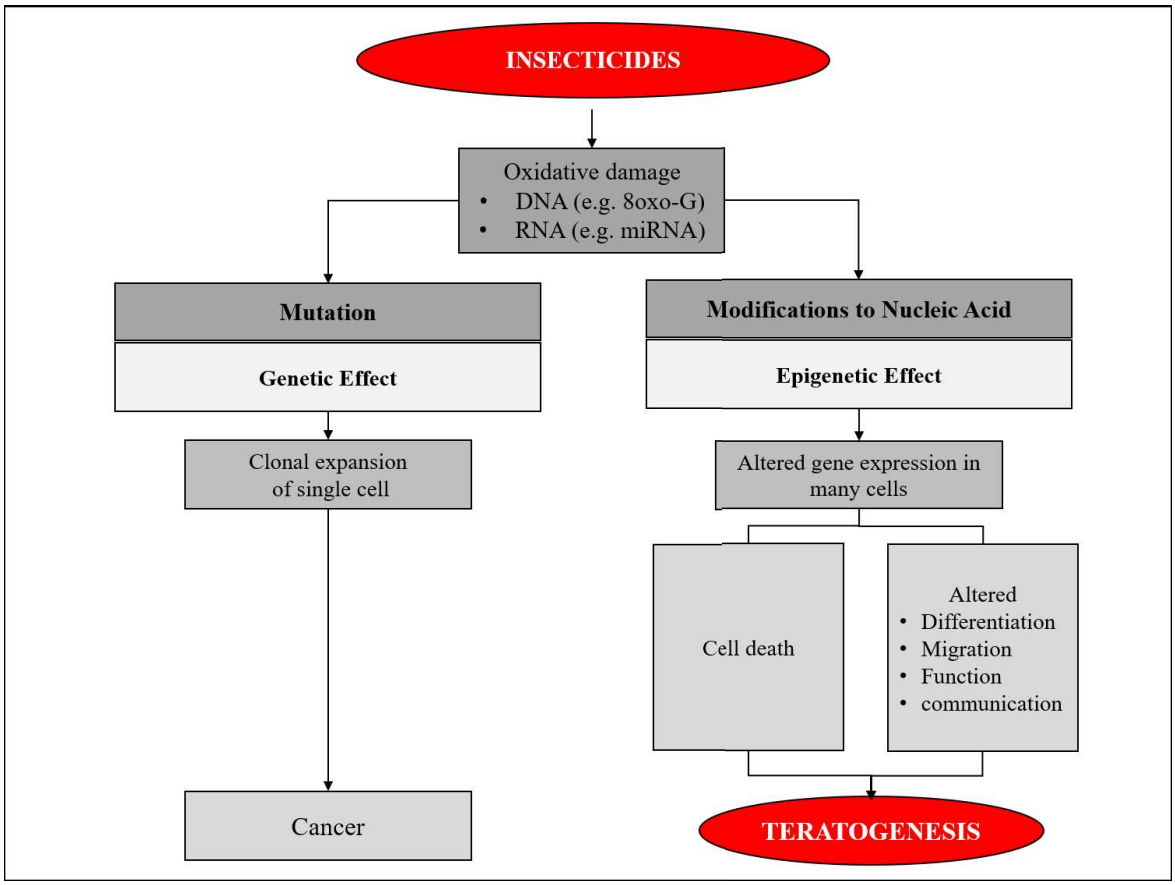


Figure 1.6: A schematic diagram representing oncogenic and teratogenic potential of insecticides. Adapted from: Moore et al., 2018

Although, pesticide usage has various merits, but the fact that the non-target species become susceptible to its negative effects is a matter of prime importance. Most of the insecticides alter the expression of genes as they are designed for being nervous and/or endocrine system disruptors. Most of the structural defects caused by teratogenic exposures occur during the embryonic period, which is when critical developmental events are taking place and the foundations of organ systems are being established. Therefore, it is mandatory to understand the early developmental processes which might probably give clues as to how the pesticide exposure can disturb the patterns of the embryonic development and lead to the malformations.

1.8. EARLY EMBRYONIC DEVELOPMENT

The process by which a fertilized egg undergoes sequential changes to achieve developmental milestones *viz.* 4-cell stage, 8-cell stage, blastula, gastrula, tissue formation, organ formation and maturation to form the embryo is called embryogenesis. These complex morphogenetic movements in the early developmental stages are carefully manoeuvred and are organised in a

strict spatiotemporal pattern. The embryonic development progresses take place in the following sequence.

1. After the fusion of male and female gametes, the zygote starts undergoing cleavage. The cells start dividing mitotically at a rapid rate and attains 16 cell hollow compacted ball termed as morula. Each cell in this stage is referred as blastomere. Further this develops into blastula and possesses a cavity, blastocoel.

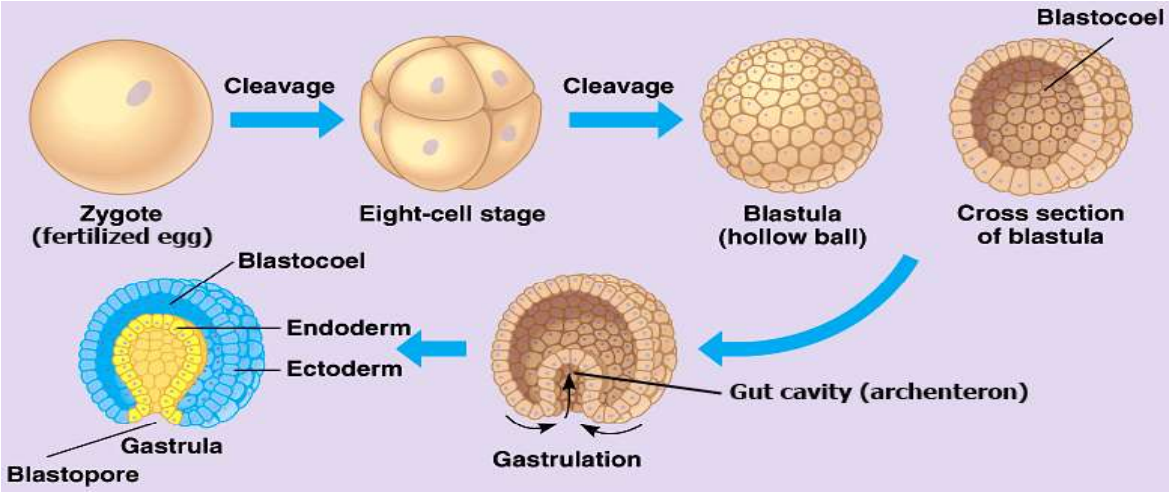


Figure 1.7: A representative diagram displaying development of a zygote up to gastrulation phase. Source: http://saalmoh17.blogspot.com/p/gastrulat.html

2. This event is followed by gastrulation (Figure 1.7) wherein single layered cells undergo cellular movements like invagination, evagination, ingression and involution, thereby landscaping a bilaterally symmetric three-layered structure, *viz.,* ectoderm, mesoderm and endoderm. Further, as the development proceeds these three germ layers undergo restructuring to craft the different organs in the body and this process is called organogenesis.

3. All the three layers interact with the surrounding cells and undergo cell migration and differentiation. The ectoderm gives rise to the skin and central nervous system. The endoderm forms the innermost layer of the embryo and further giving rise to the alimentary canal and the organs pertaining to respiration. The mesoderm becomes sandwiched between the ectoderm and endoderm and lead to formation of blood, heart, kidney, gonads, bones, and other connective tissues.

4. Followed by organogenesis, the organs finally start attaining functional maturity. Additionally, gametogenesis begins from this stage, but the production of gametes begin only after the organism accomplishes sexual maturity, usually after the birth (Gilbert, 2006).

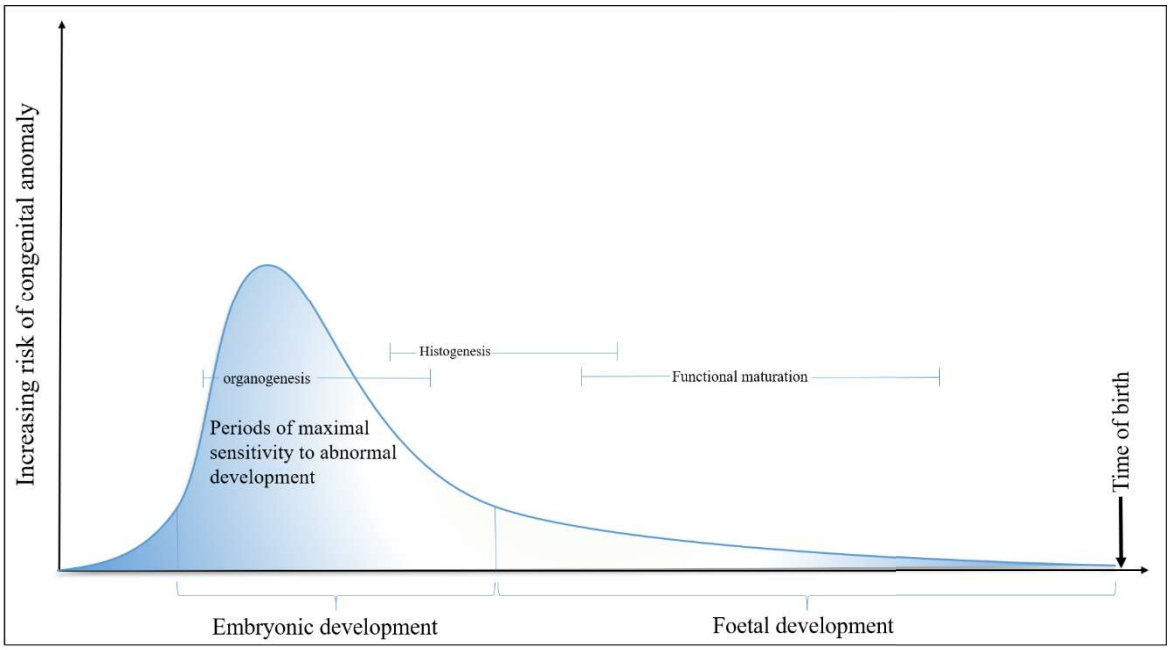


Figure 1.8: A diagrammatic representation of susceptibility of an embryo towards teratogen. Adopted from: Mcqueen, 2017

The time during embryogenesis when the embryo is exposed to a potential teratogen is crucial. The embryo is comparatively resilient to the teratogenic insults during the first few weeks of development (first 2 weeks in humans). Nevertheless, an acute exposure may result into death of the embryo. However, the embryo may not manifest any organ specific abnormality. The only clarification available for now is an assumption that early embryonic cells have not differentiated irretrievably, and that makes the viable cells to assume the functions of the dead cells. Among these stages, the embryo is highly susceptible during gastrulation and organogenesis phases. During organogenesis, the overall growth of the embryo is affected (Figure 1.8). The embryo is most susceptible to teratogenic exposure as they act in an organ-specific manner, affecting the organ system at their respective stages of development. The precise time at which the insult occurs, thus determines not only occurrence of malformation but also the specific spectrum of anomalies (Figure 1.9).

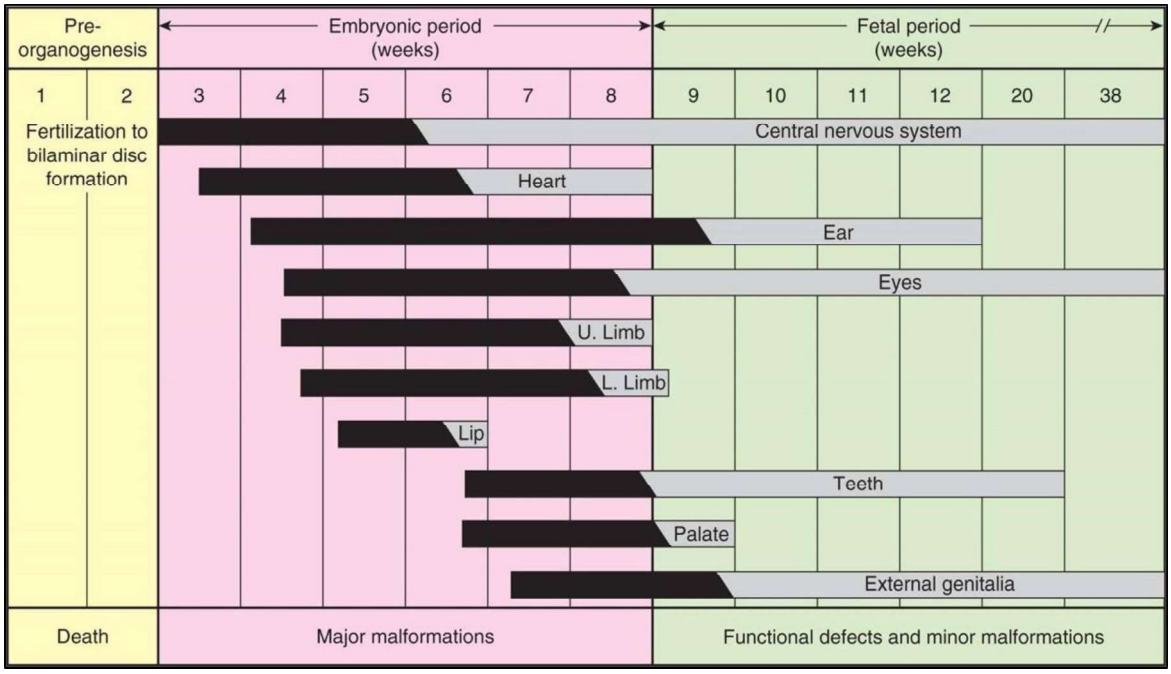


Figure 1.9: The periods showing greatest sensitivity to malformations to organs in human embryo are shown in the picture. The shaded areas are prone to higher susceptibility. Source: Moore et al., 2018.

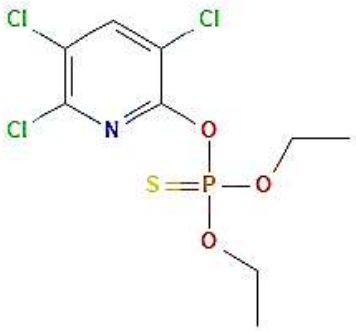
1.9. TEST SUBSTANCE SELECTED FOR THE CURRENT STUDY

These teratogenic effects at various developmental stages of the embryo can be explained under the light of various signalling cascades functioning herein. Hence, to investigate such ameliorative effects on the developing embryo, we selected a widely used combination insecticide, comprising of Chlorpyrifos (50%) and Cypermethrin (5%) which is commercially available as Anaconda505. This frequently used pesticide combination belongs to the class organophosphates (OP) and synthetic pyrethroids (PYR), which are available in many formulations. It is also reported that when applied together, the organophosphates enhance pyrethroids toxicity (Ray et al, 2000).

1.10. CHLORPYRIFOS

Chlorpyrifos is one of the frequently used chlorinated organophosphate compound. Its usage ranges from spraying on the agricultural fields to the residential usage in the form of containerized baits. It was first registered for usage in 1965 and manufactured by Dow Elanco in United States. The IUPAC nomenclature of Chlorpyrifos is O, O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate and is commercially manufactured by reacting 3,5,6-trichloro-2-pyridinol with diethylthiophosphoryl chloride (Muller, 2000).

1.10.1. CHLORPYRIFOS: PHYSICAL AND CHEMICAL PROPERTIES (NCBI database)

Empirical Formula	$C_9H_{11}Cl_3NO_3PS$
Chemical Abstracts Service (CAS) registry number	2921-88-2
Colour	colourless to white
Texture	crystalline solid
Odour	mild mercaptan-like
Melting point	41.5-42.5 °C
Boiling point	160 °C
Stability	decreases with increasing pH
Solubility	insoluble in water; soluble in most organic solvents
Vapour pressure	$1.87x10^{-5}$ mm Hg
Corrosivity	Corrosive to copper and brass
Decomposition temperature	Above 160 °C
Molecular weight	350.6 g/mol
Chemical structure	

1.10.2. CHLORPYRIFOS: MODE OF ACTION

Chlorpyrifos is a non-systemic and broad-spectrum insecticide which kills the pests by binding to the cholinesterase (ChE) enzyme and thereby affecting the nervous system (Smegal, 2000; Lotti and Moretto, 2005; Pung et al., 2006). Chlorpyrifos primarily acts as an acetylcholinesterase inhibitor and causes overstimulation of cholinergic receptors by releasing excess acetylcholine in the synaptic junction. After getting metabolised, it is converted into an

active metabolite chlorpyrifos-oxon, which binds irreversibly to the enzyme acetylcholinesterase and thereby, avoiding deactivation of acetylcholine during the nerve impulse. This accumulation of the neurotransmitter in the synapse leads to continuous neuronal firing, prolonged contraction of muscles, convulsions and may lead to death of an organism. Experiments carried out in rodent models have reported that, it alters serotonin signalling and hamper several serine hydrolase enzymes (Garcia et al., 2001; Eaton et al., 2008; Slotkin and Seidler, 2008). Moreover, it affects the cyclic AMP synthesis and compromise the activity of ATPase (Slotkin, 2004).

1.10.3 CHLORPYRIFOS: TOXICITY STUDIES

Though the mode of action of chlorpyrifos remains the same in non-targeted organisms, its toxic effects varies from species to species. Parenteral routes of chlorpyrifos exposure have been reported to be moderately toxic to humans, moderately to very highly toxic to birds and very highly toxic to freshwater fish and marine organisms (Fukuto, 1970; Kamrin, 1997; Reigart and Roberts, 1999; Lotti and Moretto, 2005).

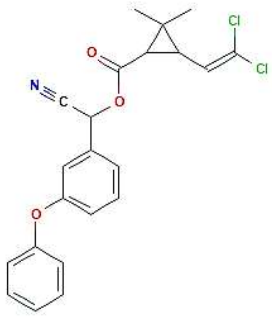
Reproductive and developmental defects occurring due to chlorpyrifos exposure have been studied in various animal models. In adult rats, acute and chronic exposure of chlorpyrifos have been reported to cause oxidative stress, developmental disorders, immunotoxicity and embryotoxicity. Behavioural defects such as delay in coordination and locomotor activity have also been reported by the many groups. (Yin et al., 2009, Ma et al., 2013). In addition, rat neonates were found to be more susceptible to chlorpyrifos even at a very low concentration (Smegal, 2000; Zheng et al., 2000). Morphological defects in brain, reduction in viable offspring number, decreased body weight were some of the adverse effects of chlorpyrifos exposure. Even in the avian model, low level exposure 60-125 ppm induced reduction in egg production, thinning of eggshells and increased death. All of these shortcomings might be due to impaired DNA, RNA and protein synthesis, increased programmed cell death, altered gene expression and cell differentiation processes (Whitney et al., 1995; Roy et al., 1998; Dam et al., 1999; Crumpton et al., 2000; Ricceri et al., 2003; Slotkin et al., 2008).

1.11. CYPERMETHRIN

Cypermethrin is from a class of synthetic pyrethroids which are chemically similar to pyrethrins (extract from chrysanthemum plants). These are designed to have long lasting effects compared to natural pyrethrins. Cypermethrin is a neurotoxic agent most probably acting through the central nervous system to cause repetitive nerve activity. It is readily absorbed from the gastrointestinal tract and minimally through intact skin (Jin and Webster, 1998). It was first

synthesized in 1974 (Mandal et al., 1986; WHO, 1989) and is widely used against pests to cotton, fruits, vegetables, for public health and in animal husbandry. The chemical structure of Cypermethrin is [(±)-α-Cyano-(3-phenoxyphenyl) methyl (±)-cis/trans-3-(2,2-dichlorovinyl)-2,2 dimethylcyclopropanecarboxylate]

1.11.1. CYPERMETHRIN: PHYSICAL AND CHEMICAL PROPERTIES (NPIC database)

Empirical Formula	$C_{22}H_{19}Cl_2NO_3$
Chemical Abstracts Service (CAS) registry number	52315-07-8
Colour	Yellowish
Texture	Semi-solid to liquid
Odour	Odourless
Melting point	60-80 °C
Boiling point	220
Stability	Stable in neutral and slightly acidic medium
Solubility	Insoluble in water, highly soluble in organic solvents
Vapour pressure	1.7X10-9 mm Hg
Corrosivity	Non-corrosive to metals
Decomposition temperature	Above 160 °C
Molecular weight	416.298 g/mol
Chemical structure	

1.11.2. CYPERMETHRIN: MODE OF ACTION

Pyrethrins are natural compounds extracted from the plant *Chrysanthemum cinerariaefolium*. These compounds tend to decay rapidly in presence of light, therefore, to overcome this disadvantage, synthetic pyrethroids came into existence. Cypermethrin is one example that belongs to a class of synthetic pyrethroids. This group of chemicals possess an acid and an

alcohol moiety with ester bond in the centre, that gets biotransformed via hydrolysis and oxidation reactions. The insecticidal potential is due to disruption of normal functioning of voltage-dependent Na^+ channels, required for efficient intercellular communication in the nervous tissue. This results in repetitive nerve action through prolongation of sodium permeability during the recovery phase of the action potential of neurons (Trainer et al., 1997).

Pyrethroid intoxication results from their potent effects on nerve impulse generation within both the central and peripheral nervous systems. Nearly all the nerve cells maintain a transmembrane potential or also called as resting potential around -70 mV. When the nerve impulse/action potential is generated, the neurons undergo a transient depolarisation driven by inward movements of Na^+ ions followed efflux of K^+ ions. These movements occur due to opening and closing of special ion channels that are embedded in the nerve cell membrane. The action potential is further propagated to the nerve endings where it releases chemical transmitters such as acetylcholine. The synthetic pyrethroids alter the properties of sodium channels by prolonging the Na^+ ion influx, in both vertebrate and invertebrate neuronal membrane (Eells et al., 1992). These chemicals bind to the receptor site of alpha subunit in the sodium channel and localises itself towards the cytoplasmic side of the membrane (Trainer et al., 1997). This delay in closure of sodium channels leads to depolarisation of the neuronal membrane that in turn leads to prolonged acetylcholine discharges. Moreover, it lowers the threshold potential that puts the nerve cell in hyperexcitation state (Narahashi et al., 1995). In addition, cypermethrin prevents the GABA receptor, causing excitability and tremors. It has been shown to inhibit active transport of ions in electron transport chain by deactivating ATPase enzymes. These disruptive activities might cause ionic imbalance that will disrupt the respiratory surfaces, indicating their toxic potential to marine life (Siegfried, 1993).

1.11.3. CYPERMETHRIN: TOXICITY STUDIES

Cypermethrin is a synthetic pyrethroid which is classified as ‘moderately hazardous” by (Mandal et al., 1986). It is a neurotoxic agent affecting the central nervous system. It majorly delays the sodium ion channel closure causing prolonged sodium ion influx and as a result continuous stimulation of the nerves (Vijverberg and Bercken, 1982; Chinn and Narahashi, 1986; Narahashi et al., 2001). Pyrethroids have been widely used because of their low toxicity to birds and mammals. However, aquatic life forms and especially fishes have been reported to be more susceptible to cypermethrin (Stephenson, 1982). El-Toukhy and Girgis (1993) and Siegfried (1993) have reported cypermethrin to inhibit ATPase and GABA receptors in fishes and aquatic

insects respectively. A dose-dependent apoptosis in the CNS of the tadpoles of the toad *Bufo arenarum* (Casco et al., 2006) and mice (Singh et al., 2011) has also been reported.

There is not much data available on reproductive abilities of the organisms but Cantalamessa (1993) observed developmental defects particularly in rat neonates. Incomplete development of the enzymes which metabolize cypermethrin can be considered as a critical factor in case of young ones. Several reports state occurrence of developmental delays in the rat young ones, when the females were exposed during their pregnancy period (Amer et al., 1993; Tateno et al., 1993; Hu et al., 2013). Chronic exposure to male rats resulted in increase in abnormal sperms. Abnormalities in bone marrow and spleen cells have also been reported (Amer and Aboul-ela, 1985). Moreover, cypermethrin exposure is known to have teratogenic effects on chick embryo (Anwar, 2003).

1.12. COMBINATION OF CHLORPYRIFOS AND CYPERMETHRIN

The organophosphates and pyrethroids have an ester bond in common in their chemical structure that makes it accessible to esterases, a class of enzyme involved in metabolising these compounds via oxidation, and hydrolysis reactions (Coats, 1990). Phosphoric triester hydrolases, paraoxonase and carboxylesterases are also reported in metabolising these insecticides. The major metabolic pathways in mammals transform the parent compound into metabolites with low toxicity. As seen in mammals, rapid metabolism of cypermethrin results in nonactive metabolites causing minimal toxicity and are excreted out of the body quickly (Kaneko, 2010). When organophosphates are simultaneously exposed with cypermethrin, the esterases get inhibited irreversibly, slowing down its activity to cleave ester bonds present within the insecticides (Iyyadurai et al., 2014). Thus, organophosphates and pyrethroids, show augmented combinatorial toxicity relative to exposure to a single pesticide (Nagar et al., 2018). Consequently, a mixture of these two insecticides was presented in the agricultural market as they show synergistic behaviour and are effective in controlling pests that have developed resistance to a single pesticide (Kostoff et al., 2018; Tiwari et al., 2019). The unwarranted usage of multiple insecticides has contaminated the niches of almost all living creatures and due to their variable utility in agricultural fields up to household activities, it is a matter of grave concern to select and study the effect of pesticides but in combinations. Moreover, because of the absence of detoxifying mechanisms in embryos, they are unable to bear the consequences of insecticides' toxicity. Also, previous reports have only highlighted the toxic effects on various animal models (adult stages) induced by either chlorpyrifos or cypermethrin only (Anwar, 2003; Tian et al., 2005; Palma et al., 2009; Xu et al., 2010; Shi et al., 2011; Chaudhary et al., 2017; Sumon et al.,

2017). Therefore, this combination insecticide needs to be critically studied for its combinational toxicity in an embryonic model.

1.13. MODEL FOR THE STUDY

The embryo of the chick holds the record to be the animal with a longest continuous history as an experimental model for studies in developmental biology (Jelinek, 1982; Needham and Hughes, 2015). Aristotle began the use of chicken embryos by dissecting open the eggs and studied the structural changes around 350 BCE. This model system has a significant contribution in understanding development of various organ systems, various aspects of cancer, immunology and more importantly provided insights of human development and diseases (Kain et al., 2014). The primary advantages of chick as an experimental model are the easy genetic manipulations possible in this system along with the well standardised cellular and tissue labelling methods known to biologists. As an added advantage, chick embryo holds multiple features, similar to the human systems (Stern, 2018).

There are numerous features of the avian embryo that makes it a suitable model. They are 1) it is phylogenetically closer to mammals with a short development time of 21 days; 2) it allows easy access to observe the embryo throughout its developmental period; 3) it provides an un precedented ease of microsurgery or manipulations in the embryo; 4) the amniotic egg has sufficient nutrition in the form of yolk and the embryo develops normally around 37 °C and 60-75 % air humidity without the requirement for dedicated facilities and apparatus.

The development of the chick embryo has been comprehended in 46 stages by Hamburger and Hamilton (1951) which are widely accepted for use in the scientific community. The developmental stages of the chicken embryo were based on their morphological and chronological age. This classification of avian embryos allows us to corelate with the human embryonic development of 40 weeks (Figure 1.10).

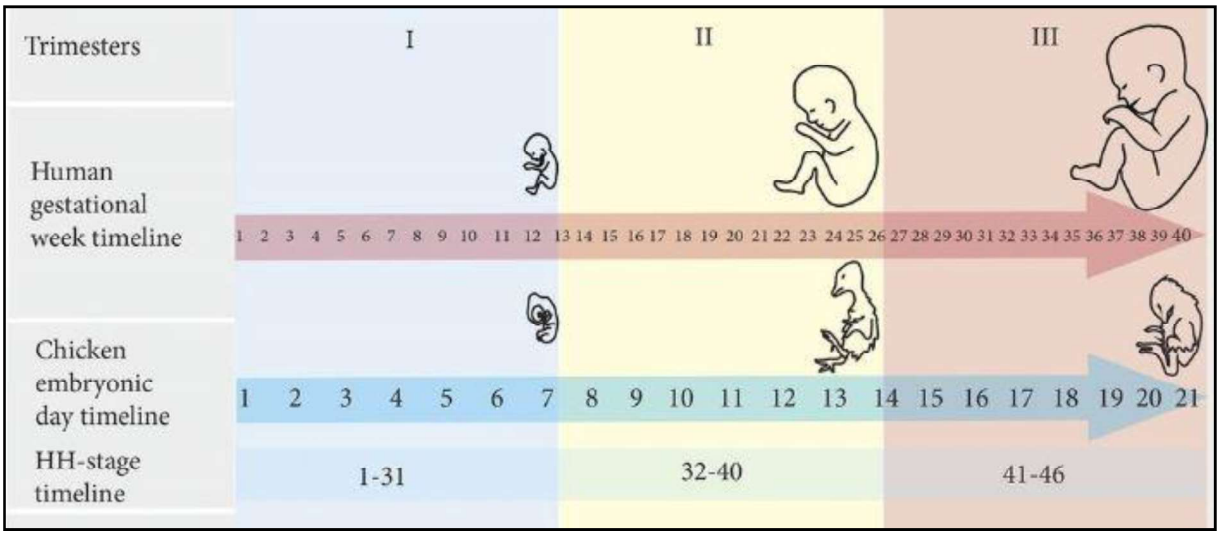


Figure 1.10: The developmental period of humans (40 weeks) has been compared with chicken (21 days) Source: Bjørnstad et al., 2015.

Subsequently chicken embryos played a pivotal role in understanding the development of nervous, cardiovascular and endocrine systems. Thus, making it possible to observe all the developing systems in one embryo, thereby reducing the number of animals in the study. Further, avian embryos have also been used for studying toxicant induced teratogenicity and developmental toxicity in the form of structural aberrations/malformed organs (Drake et al., 2006). Additionally, the chick embryo has also been in use for scrutinising the effects of toxicants that may alter the expression of genes that regulate differentiation and migration of cells in the central nervous system (Bjørnstad et al., 2015).

Even though rodent models provide a good clarification when it comes to understanding mammalian system, to study early developmental stages, a large number of offsprings are required for significant outcome. Moreover, in rodents, the sample size varies across the breeding and cannot be predicted, whereas fertilised eggs can easily be procured from the hatcheries in sufficient quantities. Also, in rodent models, to suffice the need of offsprings, additional matings have to be done which causes distress in the females and potentially large number of females are needed to be handled, manipulated and later killed. In case of chicken, a broader genetic variation can be obtained by taking a larger number of eggs from the poultry farms. It also excludes the mother from any experimental studies. Thus, the avian embryos are preferred above their mammalian counterparts in line with international ethical guidelines.

In this respect, the chick embryo serves as an appropriate and inexpensive model system to apply modern experimental tools to establish how a teratogen (here combination insecticide) interferes

with the mechanisms that underlie organogenesis and morphogenesis and ultimately leading to structural deformities.

1.14. OBJECTIVE OF THE STUDY

Against this background in our lab, experiments were conducted on chick embryo by exposing it to a combination insecticide (Ci) consisting of chlorpyrifos (50 %) and cypermethrin (5 %). The earlier study from the lab revealed that the Ci induced severe embryotoxic and teratologic manifestations in the axial and appendicular skeleton of the treated embryos. The abnormalities include craniorachischisis, microcephaly, hydrocephaly, agnathia, anophthalmia, umbilical hernia, micromelia, hind limb twist, sacral hygroma, drooping twist and kinky tail (Uggini et al., 2012). Subsequently, these toxic manifestations were also observed in the succeeding generation of the chicks (Khan et al., 2015). Largely the anomalies observed in both the studies were pertaining to defective neural tube and somite development. A careful observation of developmental events occurring in the early embryonic period in chick revealed that the insecticide hampers development during late phase of gastrulation and also during organogenesis. For instance, obstacles in the neural tube closure and defective migration of neural crest cells might have culminated in to craniorachischisis and other craniofacial malformations. The aforementioned anomalies were thought to be a result of deranged morphogenesis and altered upstream signalling mechanisms which regulate the developmental events in the embryo.

Furthermore, the organophosphates and pyrethroids are known to affect neurotransmission by inhibiting acetylcholinesterase. This constraint the embryonic development because acetylcholine is one of the transmitters that provide neurotrophic input, regulating the proliferation, differentiation, and migration of its target cells (Hohmann, 2003). Thus, at an early stage of cell development, this chemical messenger may stimulate the genes that regulate cellular processes ranging from replication to differentiation. Hence, any hindrance in the signalling pathways or inappropriate apoptosis during early embryonic development would be catastrophic to the embryo and later seen as skeletal deformities. Therefore, the present study was designed to understand the changes in the signalling pathways that regulate the major developmental events in the embryonic stages. This deviant signalling might have culminated into structural defects when the embryos were exposed to the combination insecticide.

In an effort to unearth the mechanism of Ci toxicity in the embryos, the studies were focussed on two major defects and presented in the following chapters:

Chapter 1: The ventral body wall defects: Signs of toxicity during H-H stage 13, 24 and 36 were noted, to understand somite development in the embryos. The embryos at above mentioned stages of development were examined for major signalling molecules and cell death. These results were supplemented with tissue architecture analysis.

Chapter 2: The neural tube defects: the signs of Ci toxicity were studied at early developmental stages (H-H stage: 13, 24 and 36) of the chick embryo. The study focussed on the signalling pathways regulating neural tube closure and patterning of the neural tube. In addition, deranged chondrogenesis was also studied at a molecular level and further supported by histopathological and biochemical evaluations.