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Aquaculture, also known as aquafarming, is the farming of aquatic organisms such as fish, crustaceans, molluscs and aquatic plants. It is growing rapidly than any other segment of animal culture industry (Gang et al., 2005). Aquaculture in India has long history, with reference to fish culture in Kautilya’s Arthashastra (321-300 B.C) and king Someswara’s Manasoltara (1127 A.D). The traditional practice of fish culture in small ponds in eastern India is known to have existed for hundreds of years; significant advances were made in the state of west Bengal in the early nineteenth century with the controlled breeding of carp in bundhs (tanks (or) impoundments where reverine conditions are stimulated). Fish culture received notable attention in the state of Tamil Nadu (formerly madras) as early as 1911, and, subsequently states such as Bengal, Punjab, Uttar Pradesh, Baroda, Mysore and Hyderabad initiated fish culture through establishment of Fisheries Departments and support to fishers and farmers for expansion of the sector. The Fisheries sector is contributing 2.39% to the Gross State Domestic Product (2008-09). The quantity of fish and prawn produced is 12.52 lakh tons (102%) against the target of 12.20 lakh tons during the year 2008-09. It is aimed to produce 16 lakh tonnes of fish/prawn by 2011-12 (FAO, 2014). The state is contributing about Rs 2500 Crores by way of marine exports, which is nearly 40% of the marine exports from India (Anjani, 2003).

Aquaculture involves cultivating freshwater and saltwater populations under controlled conditions, and can be contrasted with commercial fishing, which is the harvesting of wild fish. Aquaculture, or fish farming, has been around for centuries in small, rural settings, but it has exploded worldwide in the past few decades into a commercial activity for the global market (Bhatta, 2003). Today, farmed fish make up more than one-fourth of the fish we eat, and fish production from the aquaculture industry is an attractive means to relieve the stress on the ocean's fisheries. Ten million people die every year, the world over, either of starvation or of malnutrition (Anon, 2002). The teeming population in the world is responsible for the food shortage. To overcome this problem; man is trying to implement new techniques in food production. Aquaculture is one of the important practices in this direction. The concept of blue revolution (culturing or capturing of aquatic organisms) is gaining importance. Government of India and Government of Andhra Pradesh have been
providing infrastructural, financial and technical support to encourage aquaculture in India in general and Andhra Pradesh in particular. The fish production patterns and consumption patterns have changed over the last 30-40 years, with both production and consumption being predominant in developing countries (Delgado et al., 2003).

According to the FAO, aquaculture is understood to mean the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants. Farming implies some form of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. Farming also implies individual or corporate ownership of the stock being cultivated (FAO, 2011). The reported output from global aquaculture operations would supply one half of the fish and shellfish that is directly consumed by humans; however, there are issues about the reliability of the reported figures (Watson and Pauly, 2001). Further, in current aquaculture practice, products from several pounds of wild fish are used to produce one pound of a piscivorous fish like salmon.

Particular kinds of aquaculture include fish farming, shrimp farming, oyster farming, mariculture, algaculture (such as seaweed farming), and the cultivation of ornamental fish. Particular methods include aquaponics and integrated multi-trophic aquaculture, both of which integrate fish farming and plant farming (Chopin et al., 2012).

Aquaculture –Around the world

Asia, in particular China, has dominated aquaculture developments in the world right through history. The first known ‘Treatise in Fish Culture’ was written by Fan Li in 476 BC. Aquaculture began in China circa 2500 BC. When the waters subsided after river floods, some fishes, mainly carp, were trapped in lakes. Nascent aquaculturists fed their brood using nymphs and silkworm faeces (Parker and Henry, 2001) and ate the fish for their protein. A fortunate genetic mutation of carp led to the emergence of goldfish during the Tang Dynasty. While this could be considered as the beginning of traditional aquaculture, China still dominates the world scene by accounting for over 65% global aquaculture production. Though India is second only to China, it accounts for only 7% of global aquaculture production (De Silva, 2001).
Aquaculture is an especially important economic activity in China. China has been responsible for most of the growth in fish availability, owing to the dramatic expansion in its fish production, particularly from aquaculture (Alagarswamy, 1993). Its per capita apparent fish consumption also increased an average annual rate of 6.0 percent in the period 1990–2010 to about 35.1 kg in 2010. Annual per capita fish supply in the rest of the world was about 15.4 kg in 2010 (11.4 kg in the 1960s and 13.5 kg in the 1990s).

In 2004, the total world production of fisheries was 140 million tonnes of which aquaculture contributed 45 million tonnes, about one third (FAO, 2006). The growth rate of worldwide aquaculture has been sustained and rapid, averaging about 8 percent per annum for over thirty years, while the take from wild fisheries has been essentially flat for the last decade. The aquaculture market has reached $86 billion in 2009 (Blumenthal and Les, 2012). The Chinese Bureau of Fisheries reported that the aquaculture harvests grew at an annual rate of 16.7 percent, jumping from 1.9 million tonnes to nearly 23 million tonnes. In 2005, China accounted for 70% of world production (Eilperin and Juliet, 2005). Aquaculture is also currently one of the fastest growing areas of food production in the U.S (Drawbridge, 2002).

In 2001, the fisheries scientists Watson and Daniel Pauly expressed concerns in a letter to Nature, that China was over reporting its catch from wild fisheries in the 1990s (Watson and Pauly, 2001; Pearson and Helen, 2001). They said that made it appear that the global catch since 1998 was increasing annually by 300,000 tonnes.
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whereas it was really shrinking annually by 350,000 tonnes. (Watson and Pauly, 2001) suggested this may be related to China policies where state entities that monitor the economy are also tasked with increasing output. Also, until recently, the promotion of Chinese officials was based on production increases from their own areas (Heilprin and John, 2001) (Reville and William, 2002).

Fish and fishery products play a critical role in global food security and nutritional needs of people in developing and developed countries. World food fish aquaculture production expanded at an average annual rate of 6.2 percent in the period 2000-2012 (9.5% in 1990 - 2000) from 32.4 million to 66.6 million tonnes. In the same period, growth was relatively faster in Africa (11.7%) and Latin America and the Caribbean 10%. Excluding China, production in the rest of Asia grew by 8.2 percent per year 4.8% in 1990-2000 (Chen et al., 2007). The annual growth rate in China, the largest aquaculture producer, averaged 5.5 percent in 1990-2000 and 12.7% in 2000-2012 (Upare, 2000). In 2012, production in North America was lower than in 2000. The fifteen main producer countries accounted for 92.7 percent of all farmed food fish production in 2012. Among them, Chile and Egypt became million-tonne producers in 2012. Brazil has improved its global ranking significantly in recent years. However, Thailand’s production fell to 1.2 million tonnes in 2011 and 2012 owing to flood damage and shrimp disease, whereas following the 2011 tsunami, Japanese aquaculture recovered slightly in 2012.

AQUACULTURE PRODUCTION IN INDIA

Global fish production has grown steadily in the last five decades with food fish supply increasing at an average annual rate of 3.2 percent, outpacing world population growth at 1.6 percent. World per capita fish consumption has increased from an average of 9.9 kg in the 1960’s to 21.0 kg in 2014. India is the world’s third largest producer of fish and next only to China in the area under fish production (Krishnan and Brithal, 2000) but it is facing serious challenges that are hampering realization of full potential of the sector.

The fisheries sector is an important player n the overall socio-economic development of India. The sector’s contribution to employment generation, food and nutritional security and foreign exchange earnings is now well recognized. The
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The fisheries sector has also been one of the major contributors of foreign exchange earnings. During 2013-14, export value of marine products reached Rs. 30,213 crores.

India stands the second largest fish producing nation in the world by constituting about 5.4% of the global fish production. India is also a major producer of fish through aquaculture and ranks second in the world after China. Average national production from still water ponds has increased from 0.6 tonnes/ha/year in 1974 to 2.2 tonnes/ha/year by 2001-2002 (Tripathi, 2003), with several farmers even demonstrating production levels as high as 8-12 tonnes/ha/year. The total fish production during 2012-13 (provisional) is at 9.04 million tonnes (MT) with a contribution of 5.72 MT from inland sector and 3.32 MT from marine sector respectively. The fish production has shown a consistent increase since 1990-91. From 3.84 MT in 1990-91, the fish production has increased to 9.04 MT in 2012-13. The growth in the fish production, however, has shown a cyclical pattern with an increasing long term trend. A constant growth has been observed in marine sector since 2008-2009.

India yields the total aquaculture production from both fresh water and brackish waters. India's aquaculture production can be classified into freshwater and brackish water production. Freshwater aquaculture production contributes nearly 55 per cent of the total fish production in India. There are 429 Fish Farmers Development Agencies (FFDA) and 39 brackish water Fish Farmers Development Agencies (BFDAs) for promoting freshwater and coastal aquaculture (Anjani et al., 2003). Some of the commercially important species which are extensively cultured in India are: Indian major carps, fresh water prawns and shrimp. Besides the ornamental fish culture and seaweed farming, are slowly gaining importance in the last few years, due to their commercial and exportable value.

India is an emerging economic power with a very large pool of human and natural resources. India's global economic engagement in 2006 covering both merchandise and services trade was of the order of $437 billion, up by a record 72% from a level of $253 billion in 2004. By 2008, India had established itself as the world's second-fastest growing major economy. Economists predicted that by 2020, India will be among the top five leading economies of the world. India is the second largest food producer in the world. Fish and marine merchandise form an important
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constituent of India's food production. The Indian sub-continent has a promising future in producing marine merchandise as it has 8041 km of coastal line, 3 million hectares of reservoirs and 1.2 million hectares of brackish water.

India is the third largest producer of fish and is playing an important role in global fisheries. The Indian fisheries sector has grown tremendously since 1950s to the present annual production levels of over 7 million tonnes of fish and shellfish from capture fisheries and culture fisheries (Krishnan et al., 1994). India currently has been produces nearly 5% of the world's total fish production, and about 7% of the total world's aquaculture production. The potential for growth is immense and the Country is on the threshold of massive development in fisheries and aquaculture. State wise Inland Fish Production has given here under (Fig-1).

![Fig-2: State wise Inland Fish Production](image)

India is presently a growing market for the feed industry. Several major feed plants have established recently with the collaboration of Food and Agriculture organisation of the United Nations (FAO) and the demand for feed, particularly the extruded feed is on the rise. Alongside there also a huge potential market for aquaculture medicine and also for husbandry industry. The Kerala has 39,139 Sq.km continental shelf areas than the land area of 38863 sq. km. which points to the tremendous potential of these water resources teeming with fish. Kerala state is the top producer of marine fish and also inland fish from the extensive network of
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backwaters, rivers, and reservoirs. The potential of Kerala in increasing aquaculture production lies greatly in developing cage culture in the State’s inland open water bodies and also along the coast. The Kerala State Government is looking for the world’s industry in promoting cage culture in the lakes and reservoirs (Krishnan et al., 1991a).

In India Fisheries, is a very important economic activity and a flourishing sector with varied resources and potentials. In India only after the Independence, fisheries sector together with agriculture has been recognised as an important food producing sector. The vibrancy of the sector can be visualised by 11-fold increase that India has achieved in fish production in just six decades, i.e., from 0.75 million tonnes in 1950-51 to 9.6 million tonnes during 2012-13. This resulted in an unparalleled average annual growth rate of over 4.5 percent over the years which has placed the country on the forefront of global fish production, only after China. Besides meeting the domestic needs, the dependence of over 14.5 million people on fisheries activities for their livelihood and foreign exchange earnings of US $ 3.51 billion (2012-2013) from fish and fishery products, amply justifies the importance of sector on the country economy and in livelihood security (Delgado et al., 2003).

As the second largest country in aquaculture production, the share of inland fisheries and aquaculture has gone up from 46 percent in the 1980’s to over 85 percent in recent years in total fish production. Fresh water aquaculture showed an overwhelming ten-fold growth from 0.37 million tonnes in 1980 to 4.03 million tonnes in 2010 (FAO, 2010) with a mean annual growth rate of over 6 percent. Fresh water aquaculture contributes to over 95 percent of the total aquaculture production. The three Indian major carps, namely Catla (Catla catla), rohu (Labeo rohita) and mrigal (Cirrhinus mrigala) contribute the bulk of production to the extent of 70-75 percent of total fresh water fish production, followed by silver carp, grass carp, common carp, cat fishes forming a second important group, contributing the balance of 25-30 percent (FAO, 2003).
Aquaculture in India has evolved as a viable commercial farming practice from the level of traditionally backyard activity over last three decades with considerable diversification in terms of species and systems and has been showing an impressive annual growth rate of 6-7 percent while the carp based fresh water aquaculture, mainly constituted by Indian major carp fish, such as *Catla*, *rohu* and *mrigal*, has been contributing over 90 percent of the aquaculture production satisfying the domestic need, the shrimp based coastal aquaculture contributes to only about 5 percent of the export earnings (Satapathy and Nayak, 1999).

India is presently a growing market for the feed industry. Several major feed plants have established recently with overseas assistance and the demand for feed, particularly the extruded feed is on the rise. Alongside there also a huge potential market for aquaculture medicine and husbandry industry (Ramakrishna et al., 2013).

The state (province) of Kerala situated in the southwest part of peninsular India, has a lean stretch of land with a long surf beaten coast on the western side and a lush green mountain range on the eastern side. The State has more continental shelf area (39139 sq. km) than the land area (38863 sq. km.) which points to the tremendous potential of these water resources teeming with fish. While Kerala is the top producer of marine fish, inland fish production from the extensive network of backwaters, rivers, and reservoirs is low. The potential of Kerala in increasing aquaculture production lies greatly in developing cage culture in the State's inland
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open water bodies, and also along the coast. The govt. of Kerala is looking for the world's industry in promoting cage culture in the lakes and reservoirs (Tripathi, 2003).

Aquaculture production in Andhra Pradesh

Andhra Pradesh has a wide range of coastal area with 974Km long, comprising with more than 550 villages. Andhra Pradesh stands in First position in both coastal and fresh water aquaculture practices, whereas it ranks in the second in fresh water fish production. Andhra Pradesh ranks first in coastal aquaculture and fresh water aquaculture. It ranks second in fresh water fish production and overall value of fish and prawn production (Jayaram, 1981). Andhra Pradesh contributes nearly 40 per cent of the total marine exports of the country. Inland resources comprise 102 reservoirs of which 7 are large, 26 are medium and 69 are small reservoirs. There are two lakes - Kolleru Lake, a freshwater lake and Pulicat lake a brackish water lake. 74, 000 perennial, seasonal and long seasonal tanks, fishponds and freshwater prawn ponds for aquaculture are also present in Andhra Pradesh. Brackish water resources comprise 0.78 lakh hectares for shrimp culture.

Andhra Pradesh state is rich in water resources. River Godavari is the largest and broadest river in the southern India, which originates at Triambakeshwar near Nasik in Maharashtra. River Krishna enters the state at Alampur after having originated at Mahabaleshwar in Maharashtra. Tungabhadra is an important tributary of the river Krishna. Nagarjunasagar Dam at Nandi Konda, Srisailam project at Srisailam and Prakasam barrage at Vijayawada, are constructed on this river. Pennar, Vamsadhara and Nagavali are other important rivers. All the rivers are rain fed and of great economic significance because they are the source for hydropower and irrigation. The two major lakes in the state are the Kolleru and Pulicat. While the Kolleru lake lies in the delta between the rivers Krishna and the Godavari, the Pulicat lake is located in the southern tip of Nellore district, close to the sea. Andhra Pradesh state is rich in reservoirs and tank resources (Surendran et al., 1991).

Farmers of Andhra Pradesh, particularly in the Krishna, East Godavari and West Godavari Districts have innovated several new techniques in carp culture and those have contributed to increase the carp culture productivity in the country
substantially. Farmers in this state are now able to get an average production of 8,000 kg/ha/year with rohu as the most dominant species in the culture system. This impressive production has been made possible by adapting various techniques in the culture system (CIBA, 1997).

The aquaculture boom and increased socio-economic benefits, such as increase in employment and social well being, concomitant with the increase in the extent and intensity of aquaculture, are alleged to have created several problems, particularly those of a deteriorating environment owing to worsening water and soil quality, as well as owing to incursions by aquaculture into other sectoral activities, such as agriculture, capture fisheries, forestry, public health, tourism, urbanization and housing. The impact can be two-fold, one affecting aquaculture itself by auto pollution and consequent outbreak of diseases, leading to crop failures and collapses eventually (Ayappan and Jena, 2003). The first such national complete collapse in shrimp culture was observed in Taiwan followed by partial collapses of cultured shrimp production in China, Indonesia, Thailand, Philippines as well as in India in 1995-97 (Nash et al., 2000).

Andhra Pradesh stands first in total fish and prawn/shrimp production in India since 2013-14 both in terms of production and value. The contribution of fisheries sector is 6.01% in A.P.’s. GSDP, whereas the fisheries contribution is about 0.83% of GDP of the nation. The overall fish production has more than doubled in the past one decade from 8.14 lakh tones in 2005-06 to 19.64 lakh tones in 2014-15. The share of Andhra Pradesh in India’s sea food exports has increased from about 20% in 2009-10 to about 40% in 2013-14. During 2009-10 the exports from Andhra Pradesh was Rs 2,100 crores but by 2013-14 exports have increased to Rs 12,100 crores. During 2014-15, the marine exports have been increased to an estimated value of Rs 16,000 crores (FAO, 2015).

The relationship between environmental factors and the distribution of organisms within estuarine environments has received considerable attention (Table-1). Because fish are one of the dominant macro faunal components of estuarine biota, many studies have focused on their distribution patterns (Akin et al., 2005).
Table 1: Interrelationship between the major environment factors that can affect a fish community.

<table>
<thead>
<tr>
<th>LAND USE</th>
<th>WATER USE</th>
<th>POPULATION GROWTH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agriculture, forestry, land, drainage, Urban development</td>
<td>Change in flow rate (Canalization, dams, water abstraction), Effluent disposal</td>
<td>Food demand, leisure, energy demand (Atmospheric Inputs)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SOURCE OF LOADS</th>
<th>EFFECT ON WATER BODIES</th>
<th>EFFECT ON LIVING ORGANISMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plants</td>
<td>Invertebrates</td>
<td>Fish Community</td>
</tr>
<tr>
<td>Water quality Water Quantity Change in Physical character</td>
<td></td>
<td></td>
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<tr>
<td>FISHERY</td>
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</tbody>
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Fish are economically important as a source of income in many countries. Fish have also been the subject of extensive ecotoxicological research during the last few decades of twentieth century. For a full understanding of the nature of the problem of aquatic pollution and conservation of inland water resources, it is necessary to examine the nature and extent of pollutants and the mechanisms of the actions of these pollutants on biota. The present research work is highlights the knowledge regarding the fate of Organochlorine pesticides in the aquatic environments and their impact on fisheries (Asche and Tveteras, 2004).

Pollutants are defined as undesirable substances that produce injury in living organisms. Chemical pollutants generally act on the ecosystem by three major pathways

1. Setting on the substrate and smothering life there.
2. Through acute toxicity leading to death of organisms, and/or
3. Depletion oxygen values to a threshold lend causing death of organisms.

Therefore, pollution causes a departure from normal functioning of the system. As a consequence, chemical pollution of the water may profusely modify the biota and affect the ecosystem.

A wide range of pesticides and herbicides are widely used in modern agriculture and this practice has become a potential threat to fish and fisheries. Many of these toxic substances are highly persistent and can bioaccumulate in the food chain of fishes at sufficient levels to threaten aquatic ecosystem and human health (Johnson and Strinchcombe, 2007).

Pesticides are chemical used to kill or control pests. The Pesticides are classified according to their chemical class or intended use. Pesticides could be used as insecticides, rodenticides, fungicides, herbicides and fumigants. Since the dawn of civilization in his sojourn of existence on this planet, the Mother Earth, and the man have necessarily a co-existing biological components with an ecological balance. Never, the primitive uncivilized Homo remained contended to be equated with any other species in the vast assemblage of organisms in nature as he is, but his interaction with the environment helped him to develop in to the most dominating civilized
supreme on this planet (Gilliom, 2007). The inventiveness of man led him to initiate rather than to duplicate the nature product. In his continuous endeavour to improve his living conditions, he clearly and cleverly dominates, but poorly managed the environment (Helweg, 2003). Through the Euphoria under which the modern man extending his life style as a symbol of modernity with self-sufficiency, the by-products of his activities appeared in the form of waste heat, noxious gaseous emissions, biochemical oxygen demand (BOD) increment in the water courses, residual pesticides, accidents (oil and other chemical spillages), release of toxic materials at relatively high concentrations and inadvertent or in anticipated production of mutagenic substances now that account not only on air, water and soil pollution but also to the dynamic changes in the metabolic reactions and rhythmic ties of biological assemblages (Bingham, 2007).

The word “Pesticide” is a collective term used to cover wide a variety of manmade substances, mixtures or formulations that are used in monitoring various biological systems or pests in agriculture, public health and industrials practices. Depending upon its use, the term pesticide included various chemical compounds such as insecticides, acaricides, rodenticides, molluscides, fungicides and other biocidal compounds. Inspite of its dangerous threat including to human life due to its carcinogenicity its use has become inevitable in many agro based countries like India, where the country’s economy is mostly governed by the agricultural output. The environmental pollution, a by-product of man’s indiscrete actions has trigged a vicious process of imbalance in the ecosystem. The man has elevated himself from stone Age to atomic age and is still leading his community to an unimaginable future (Andreu and Pico, 2004).

At the same time the alarming environmental pollution, intact is man’s advancement majorly in industrialization, urbanization and agricultural practices involving pesticides and fertilizers which also of the greatest threat to man, his animals and his day to day requirements. No doubt the man has made a mark by converting the village of yesterday in to the town but unknowingly leaping to convert this town of today in to the cemetery of tomorrow. Pesticidal pollution, the outcome of modern agricultural practices is never normally imagined by the modern man. The large scale use of pesticides and fertilizers presented not only health hazards to him
and to his animal's also exerted eco-imbalance causing two global concerns. Ecologists and several non Governmental Organizations throughout the world, who have realized these dangerous hazards, promoted the global awareness and also called for the moratorium on the use of Xenobiotics. Rachael Carson (1963) was first to highlight the disastrous implications of use of pesticides and to create the global awareness of the hazards of the pesticidal pollution in her famous, through controversial thought provoking book “Silent Spring” with the evidences of toxic effects upon the wild life and ultimate potent hazards to man in ecological context. The United Nations conference at Stockholm in 1972 focused and drew the attention on growing menace and hazards of environmental contamination arising out of air, water, land and noise pollution.

The age old battle between man and harmful insect's dates back to early beginning of this millennium .The use of certain chemicals in controlling pests of agricultural crops was known to man as early as 70 A.D. In the early periods simple minerals and crude plants products were used as insecticides. Evidence was there that Chinese used Arsenic Sulphide as an insecticide in 16th century. Nicotine as tobacco dust as infusion was used in 18th century .But the word “Insecticide” is only 100 years old. Only in its later part of the century ,large number of products found to be useful as insecticide have been discovered ,use of natural products like powder of derris roots and pyrethrum of flower heads of chrysanthemum as insecticides existed even before the second world war. The value of Pyrethrins was first discovered by Parisians. Environmental Protection Agency (EPA) recently completed cumulative risk assessment indicates that exposures from the many current uses of pyrethrins and pyrethroid insecticides do not pose risk concerns for children or adults. Further, the cumulative assessment supports consideration of registering additional new uses of these pesticides. EPA therefore is issuing this final pyrethrins/pyrethroid cumulative risk assessment and requesting comment, including information that may be used to further refine the assessment. Once the agency completes and approves pyrethroid single chemical assessments, it is likely that new uses of these pesticides will be added, providing tools that may alleviate challenging new pest management situations such as the invasive stink bug and bed bugs (Pesticidal news, 2011).
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The modern and potent organic pesticides were introduced only during and after the Second World War. Although Dichloro Diphenyl Trichloroethane (DDT) was synthesised by Zielder in 1874, its powerful insecticidal properties were discovered by Paul Muller only in 1935. DDT proved a great success in Second War Period in eradicating vector borne diseases. Since then DDT occupied a significant position in the field of public health and agriculture. With initial success of DDT forced to accept and to develop resistance by a number of insects. A situation arises and compelled the man to go in search of alternatives. Synthetic pesticides market is dominated by fungicides, accounting over 40% of the total demand for pesticides while herbicides market is expected to grow at the annual growth rate of 4.6% per year. Sale of insecticides is expected to be higher in Asia as an insecticide is the most popular pesticides in Asian countries. Global market of insecticides is estimated to grow by 4.1% annually till 2016. As a result more than 1000 varieties of synthetic pesticides made its way in to the world market about 3000 chemicals are now in common use. Roughly five million tons of chemicals including toxic substances have been synthesized so far (Smith, 1988).

Approximately 4.4 billion pesticide applications are made each year to American homes, gardens, and yards. According to surveys by the Environmental Protection Agency, more than three - quarters of U.S. households use pesticides, with 66 percent treating major living areas in the home one or more times per year. Cockroaches and ants are the most common targets. More than one-third of households used insecticides in the absence of a major insect problem (Daly et al., 1998).

In India the use of pesticides started only after independence and the production of Benzene Hexa Chloride (BHC) on commercial basis started during 1952. India is the second largest producer and consumer of pesticides in South Asia and its use is continuously increasing for the last three decades. So far more than 100 pesticides have registered and of which 55 are in active production and use. In mid sixties with the advent of “Green Revolution”, high yielding crop varieties are introduced and prone to pest attack. As a result, a hundred fold increase in the pesticidal consumption is noticed in the past 40 years (Pradhan 1971; Rane and Dave, 1994). Several global reports have ranked India among leading pesticide consumption.
in India. A report from the industry chamber Assocham says pesticide consumption in
India is the lowest at 0.5kg per hectare as against 17kg per hectare of Taiwan, 12 in
Japan, 66 Korea, 7 USA and 2.5 in Europe. The report titled “Pesticide Residues in
Indian Food and Agricultural Products” debunks the perception that India is the
largest user of pesticides. Andhra Pradesh state the “Rice Bowl” of India, is the
largest user of pesticides in the country followed by Tamilnadu and Uttar Pradesh.
India being a tropical country, the environmental contaminations is much more
considerable. Most pesticides used in present day are characteristically persistent and
remain as residues which undoubtedly affect the non target organisms.

The pesticides applied globally are received by the soil and its residues in one
form or other, are transported to other places by air and aquatic systems. These
persistent residues find it does destine in the marine environment by several ways like
seepage through subsoil and ground water systems (Jhingaran, 1983; Kohlar, 1986;
Richard Greenburg, 2005). In this process of transportation they are eventually picked
up by various biological systems and get accumulated in quantities primarily in
adipose tissues of organisms at different levels of food chain, since most of organic
pesticides are fat soluble (Holden, 1972; Verma et al., 1983; Kamrin, 1997). It is
presumed that 25% of pesticides and other organic compounds used on land are
transported to marine environment (Jalees and Vemuri, 1980; Linkov et al., 2005). In
the present day context more than 2000 contaminants have been reported as
Carcinogenic. The aquatic organisms are more affected than the terrestrial ones, as
they get bathed continuously in the medium containing the toxicant. Important aspect
often brushed aside is the non selective nature of many pesticides which equally affect
non target organisms also that form an important link in the food chains (Balk and
Koerman, 1984; Ware, 2000).

Fish are resources of great economic importance. Presence of chemicals such
as pesticides in marine ecosystem affects fish immensely directly or indirectly in
various ways. Usually alteration in pH of water, decrease in oxygen content, presence
of toxic ingredients, cumulative effects of pesticides and various other compounds are
some of the major cause of homeostatic disturbances to fish. Haematological tests are
important diagnostic tools for toxicological investigations. Recent speculations have
suggested that they may be equally valuable as indicators of disease or stress due to
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pollutants and environmental fluctuations in fishes. The blood plays an important and inevitable part in all immune systems. Blood being the medium of intercellular transport, comes in direct contact with various organs and tissues of the body, therefore, the physiological state of an animal at a particular time is reflected in its blood. Pesticides rapidly bind to the blood proteins and induce haematological changes. These changes are of some value in assessing the impact of exposure under natural conditions and may also serve as tools for biological monitoring (Thomas et al., 2009). In addition to enzymatic alterations, changes in other proteins also occur in fish exposed to various types of environmental stress including pesticides. It has been suggested that the environmental perturbations including stresses caused by the pesticides may suppress the expression of some genes and activate the others to produce specific mRNAs which may subsequently be translated into specific proteins, the stress proteins (Adams and Rinne, 1982; Pelham, 1985). Varieties of pollutants have proved to alter the protein metabolism in fish (Palanichamy et al., 1986).

Classification of Pesticides

Based on their chemical composition pesticides can be broadly classified into three categories (Table-2).

- Organophosphorous,
- Organochlorine, and
- OrganoCarbamates.

Organophosphorus insecticides

The development of resistance by insects, and bio-accumulation in the systems to chlorinated hydrocarbons led to the search for new control agents namely organophosphates and carbamates. Melnikov (1971) pointed out that many of the organophosphorous compounds have the disadvantage of having a relatively high toxicity to vertebrates, requiring rigid and suitable precautionary measures, but a large number of these compounds are entirely safe to use in agriculture, since these compounds are having relatively moderate or low toxicity to mammals.
The suitability of organophosphorous pesticides in agriculture, for the eradication of pests and insects, was first demonstrated by Gerhard and Schrader in Germany (O'Brien, 1967). In 1959, it was estimated that about 50,000 of them had been synthesized (Metcalf, 1959). Recently more than 100,000 different organophosphorus compounds have been synthesized and their insecticidal properties were evaluated (Pryde, 1973). These compounds are the esters of phosphonic, phosphorothionic or phosphonic acids or of their halides, anhydrides or amides (O'Brien, 1960). The central phosphorus atom is electrophilic and is the key to the observed physiological effects. The phosphorous atom attracts the enzyme cholinesterase and prevents it from performing its normal function which is to break the chemical, acetylcholine after it has carried an impulse from one fibre to the next. The result is a build up of acetylcholine which leads to abnormal behaviourable changes viz., hyperactivity, tremors, convulsions, jerky movements, paralysis and death.
TABLE-2: CLASSIFICATION OF PESTICIDES

In organic Contact poisons
- Fumigants
- Repellents
- Attractants
- Impregnants
- Stomach poisons
- Organic Contact poisons

Synthetic

Natural
- Alkaloids
- Esters
- Rotenoids
- Resins

Non-substituted hydrocarbons
- Organo-Chlorides
- Organo-Phosphates
- Organo-Carbarnates
- Organo-Sulphates
- Organo-thiocyanates
- Arseniles and Fluorides
Carbamate insecticides

These compounds are relatively non-toxic to warm blooded animals and, do not accumulate in the system, but are rapidly degraded and eliminated. They act in a similar way to the organophosphates by inhibiting the neurotransmitter enzyme, acetylcholinesterase activity. Most of the carbamates are used as powerful insecticides (Pryde, 1973). Carbaryl, "solar", Zectron, Dimetilan and Timik are few examples of this group.

Organochlorine compounds

The organo chlorides are insecticides which usually contain the elements like carbon, chlorine and nitrogen. They are also referred to by other names like "chlorinated hydrocarbons", "chloro insecticides", "chlorinated organics", "chlorinated synthetics" etc. However, chloro hydrocarbons have played an important role in the history of chemical crop protection. The organo chlorines are stored not only in invertebrates and vertebrate tissues but are also concentrated into the upper levels of food chains. The toxicity of organo chlorides involves the functional disruption of sensory and motor nerve fibre and the motor cortex (Narahashi and Yamasaki, 1968). Summaries of the result of sub acute and chronic feeding studies on various physiological parameters have been made available (Guzelian, 1982; Chetty et al., 1983; Aldous et al., 1984; Desaiah et al., 1985; Prasada Rao et al., 1987).

The organochlorine insecticides are quite persistent and consequently accumulate in the tissue of plants and animals. They can also interfere with desired use of soil and water, causing major problems of pollution. Organochlorine insecticides are now a constant component in freshwater and marine ecosystems (Andryushchenko, 1971). Basically, these are organic compounds that have been chlorinated, with several atoms of chlorine per molecule. Very often there is more than one ring of carbon atom in their basic structure. These compounds are lipophilic and for this reason they tend to accumulate in fatty tissues of birds, fish and man. Some of aquatic organisms may have levels of organochlorines compounds exceeding 10,000 times than that in the water in which they live (Agriculture Research Council, London 1970). These pesticides are easily absorbed through the outer protecting
Introduction

cuticle Eli of an insect and affect the peripheral sensory organs to produce hyperactivity, convulsions, paralysis and death.

The Organochlorine compounds are divided into four sub classes namely:

1. DDT and its derivatives Eg: DDT, methoxychlor etc.
2. Benzene derivatives Eg: BHC, Lindane.
3. Cyclodienes Eg: Dieldrin, Aldrin etc.
4. Polychloroterpenes Eg: Toxapene, Libocin.

MODE OF ACTION

The toxicity of Organochlorine involves the functional disruption of sensory and motor nerve fibres and the motor cortex (Narahashi and Yamaski, 1968). The disruption of the neural mechanism is due to the penetration of organochlorides in to the axon membrane altering the permeability to Na⁺ and K⁺, subsequently inhibiting Na⁺ - K⁺ and Mg²⁺ adenosine triphosphate activities in the nerve endings (Corbett, 1974).

OC compounds seem to have a multifaceted action on animals. Summaries of the results of subacute and chronic feeding studies on various physiological parameters have been made available (Brooks, 1975; Rajendra et al., 1980; Wang and Buhler, 1981; Desaiah et al., 1982; Vijay joseph, 1989; Vani, 1991; Azhar Baig et al., 1991 and Baronia and Sahai, 1992).

OC compounds found to disrupt energy systems by inhibiting Mg²⁺ ATPase and aerobic segmental enzymes of brain (Cutkomp and Koch, 1981). Accumulaion of Ach content in synaptic region was also noted (Omer et al., 1971). Inhibition of AChE and accumulation of Ach content by OC compound shows the neurotoxic action (Omer et al., 1971). It is found that OC compound inhibit ATPase systems more than the OrganoPhosphates (or) Carbamate insecticides (Riedel and Christensen, 1979).
Introduction

Enzymes particularly Na+/K+/Mg\(^{2+}\) activated ATPases have relatively high sensitivity to certain classes of insecticides. Desaih et al. (1975a) and Larson et al. (1979) reported that chlorodecone produces typical DDT tremors in both human and experimental animals due to the involvement of Central Nervous System.

TRANSLOCATION OF PESTICIDES

Various modes of translocation of pesticides have been found out by several scientists.

(a) Aerial application of pesticides (Abbot et al., 1965).

(b) Agricultural wastes, accidents and spills.

(c) Direct application as sprays or granules to control water inhabiting pests (Hunt and Bischoff, 1960).

(d) Disposal of waste products by pesticide processing units (Chowdhury et al., 1981).

(e) Heavy rainfall immediately after aerial application of pesticides (Abbot et al., 1965).

(f) Industrial waters discharged as factory effluents Kerr and Vass (1973) stated 3 ways by which pesticide accumulation in aquatic biota: In case of higher vertebrates intake of pesticides indirectly by ingestion of contaminated food and directly by uptake of water borne residues or by absorption through the integument of absorbed material.

(g) Municipal wastes discharged as sewage effluents.

(h) Spray drift from normal agricultural operations (West Lake and Gunther, 1966; Butler, 1969).

(i) Surface run-off and sediment transport from treated soil (Gillet et al., 1970; Bauman, 1981).
BIODEGRADATION

Considerable work has been done on pesticides bio-degradation especially with organo chlorines. Organo chlorines, otherwise called as persistent chemicals are the most stable and slow-degrading of pesticides, which accumulated in the living systems to the maximum. Organo chlorine compounds are less degradable and hence are prone to bio-accumulate (Khanna et al., 1979; Hilmy et al., 1983). But organo phosphates are fast degrading and their accumulation in biological systems is very low. This degradation occurs generally through hydrolytic routes.

RESISTANCE TO PESTICIDES

Animals develop the resistance to pesticides in two Phases. The first phases carry pre adaptive genes for resistance. The second phase includes the induction of existing detoxifying enzymes to counteract the increasing toxicity which enables the faster breakdown of the chemical (Sabers, 1985). Several reviews on resistance phenomena were published (Kearns, 1955; Chadwick, 1957; Sakai, 1960; Brooks, 1966; Hopkins et al., 1969; Plapp, 1970). The WHO has sought for many years to standardize the measurement of toxicity as a diagnostic for resistance and a modification of the Busvine-Nash test which is now widely used for such determinations. Comprehensive monographs on problem of resistance were published by Brown and Pal (1971).

ADVERSE AFFECTS OF PESTICIDES ON

a) Behaviour

The double effect of inhibition of AChE, release ACh and inhibition of ATPase complex might be responsible for the manifestation of the abnormal behaviour, like erratic movements, loss of balance between them by leading to neurotoxicity, which is also indicated through convulsions and tremors. But the organo chlorides seen to act upon other biochemical systems which have an equal and important role to play either in the regulation of behavioural movements or in supporting the functioning of the nervous system: Since the anomalous behaviour is an index of the toxicological effect of the toxicant, Robinson (1971) has examined in detail the available pharmacokinetic data for cyclodienes and DDT behaviour in man.
and experimental animals including rats and monkeys etc. Holden (1972) reported increase in ventilation rate and in sensitivity to external stimulus by many pesticide poisoning. Philip (1984) and Gopal et al. (1985) reported that accumulation of acetyl choline in central nervous system is believed to be responsible for all behavioural changes that occurred due to OC poisoning.

b) Reproduction

Pesticides are also known to inhibit the rate of reproduction (Harrison et al., 1970; Menzel et al., 1970; Wurster, 1968) and also the growth of an organism (Sodergren, 1971; Davies and Hidu, 1969). The effect of organochlorine insecticides on estrogenecity, androgenecity spermatogenesis, testosterone metabolism and adrenocortical function was studied by many scientists. Since ovary contain lipid, the accumulation of OC insecticides in the organs is very high (Reinert, 1970; Jensen et al., 1970; Reinert and Bergman, 1974; Willford, 1975). The organochloride insecticides have definite effect on endocrine system also (Jefferies, 1971; Richie and Peterle, 1979). The weight of testis, epididymus, coagulating gland, seminal vesicles and ventral prostate decrease at high dose levels (Gupta et al., 1980). Structural damage caused by these pesticides to the endocrine glands of fish (Shukla and Pandey, 1986) and in amphibians (Vijay Joseph, 1989; Pushparaj, 1988) also affects the reproduction in directly.

c) Metabolism

Organo chlorines have an inhibitory effect on the aerobic segment of carbohydrate metabolism (Bhatia et al., 1972). He also reported that organo chlorines especially dieldrin showed decreased activity levels of hepatic mitochondrial succinate dehydrogenase and isocitrate dehydrogenase in rats (Bhatia et al., 1972). Enhanced SDH activity was observed during rats treated with DDT and endosulfan. Increased glucose 6-phosphate activities have been found in humans in contact with organo chlorine insecticides over long period (Yakushko, 1972). Depression of liver glucose 6-phosphate dehydrogenase activity was found in rats during DDT treatment (Wong and Tinsley, 1964; Tinsley, 1964 and 1965). In general, the organo chlorine insecticides enhance protein and RNA synthesis (Sanchez, 1967). Saxena et al. (1982)
Introduction

reported that the macro molecules like RNA, DNA and proteins decrease initially and restored to normal after 4 weeks (Bano et al., 1982; Suryaprasad, 1983).

Organo chlorines accumulate in fat tissues in the form of residues because they are lipophilic (Pierce et al., 1974). Bhatia et al. (1972) reported the organo chlorine intoxication stimulates lipid metabolism causing the release of unesterified fatty acids. Mahmood et al. (2010) showed increased free cholesterol content in treated animals. Increased neutral lipid and phospholipid synthesis and impairs the steroidogenesis during long term exposure to these pesticides (Agarwal, 1981). Kuzminskaya (1970) reported organo chlorine administered animals showed a decreased trend in phospholipid content.

RESIDUAL EFFECT OF ORGANOCHLORINE COMPOUNDS

The advent of residual pesticides, which have contributed so much to the welfare of man, have also brought in problems, such as toxic hazards to the users. The risks of exposure to these toxicants are maximum to the people who handle or use the insecticide under various programmes. Also the people who live in dwellings treated with insecticide, who consume the food or food products subjected to protection by pesticides also run the risk of absorbing the pesticides adversely by various routes.

The chlorinated hydrocarbon pesticides are more likely than others to be biologically magnified in the food chain because of their unique character of persistence, make these compounds ingested by live stock, accumulating in the fat tissues of predatory birds and their eggs (Harrison et al., 1970; Havera and Duzan, 1986). Residues are found in wild life (Edwards, 1973; Dikshit, 1978; Kapalia et al., 1987a), human breast milk (Kalra and Chawla, 1981; Dillon, 1981; Weisenberg et al., 1985) blood (Amarowiez et al., 1989; Baronia and Sahai, 1992). However, the pesticide residue in food, water and tissue of live stock and particularly, human being is a matter of grave concern to people all over the world. Man being the ultimate victim has not escaped from the residue problems.
Figure 4: Effect of Pesticides on Humans
All pesticides have some level of toxicity and pose some risk during pregnancy. The risk depends on the toxicity of the pesticide ingredients and how much of the pesticide you and the baby are exposed to while pregnant. During pregnancy, the baby's brain, nervous system, and organs are developing rapidly and can be more sensitive to the toxic effects of pesticides. Because of this, it is important to minimize exposure to pesticides during pregnancy (Roberts, 2007).

EXPERIMENTAL OC PESTICIDE

LIHOCIN

Lihocin, an organochlorine insecticide also known as Chlormequat Chloride is a Plant Growth Facilitator of BASF India Limited (Crop Protection) can be used on various crops and vegetables for increasing yield, providing good lusture and uniformation of seeds by conversion of extra vegetative growth into reproductive growth. It is a new generation product and very beneficial in modern agriculture to increase the yield and quality where all traditional methods had been adopted. They are in form of White crystals with a fishlike odour.

Lihocin is highly toxic to fish which absorbs it directly from water or by ingesting contaminated food and bioaccumulate in their fatty acids due to its Lipophilic nature (Oritz, et al., 2001). Lihocin entered into the aquatic environment cause serious threatening to various aquatic organisms and also cause severe metabolic abnormalities in non target species like fish and freshwater mussels.

![Chemical structure of Lihocin](image_url)
**Chemical properties of Lihocin (OC insecticide)**

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Technical grade</td>
<td>2-chloro-N, N, N- trimethylethanaminium chloride</td>
</tr>
<tr>
<td>Chemical Name</td>
<td>Chlormequat chloride</td>
</tr>
<tr>
<td>Molecular Formula</td>
<td>C5H13Cl2N</td>
</tr>
<tr>
<td>Chemical nature</td>
<td>Lipophilic</td>
</tr>
<tr>
<td>CAS Number (Database Ref)</td>
<td>999-81-5</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>158.07</td>
</tr>
<tr>
<td>Melting point</td>
<td>239-243°C (dec.) (lit.)</td>
</tr>
<tr>
<td>Storage temperature</td>
<td>0-6°C</td>
</tr>
<tr>
<td>Density/Specific Gravity</td>
<td>1.14 to 1.15 g/mL at 20 deg C</td>
</tr>
<tr>
<td>Colour/Form</td>
<td>Colourless crystals, White crystalline solid</td>
</tr>
<tr>
<td>Odour</td>
<td>Typical amine Odour (Fish like odour)</td>
</tr>
<tr>
<td>Technical grade</td>
<td>97-98% pure</td>
</tr>
<tr>
<td>Surface Tension</td>
<td>70.3 mN/m</td>
</tr>
<tr>
<td>Vapour Pressure</td>
<td>7.5x10^-8 mm Hg at 20 deg C</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>Hygroscopic</td>
</tr>
<tr>
<td>Stability</td>
<td>Stable &amp; combustible</td>
</tr>
<tr>
<td>Solubility</td>
<td>Methanol, Strong oxidising agents(metals)</td>
</tr>
<tr>
<td>Water Solubility</td>
<td>74%</td>
</tr>
<tr>
<td>Insoluble</td>
<td>Ether &amp; Hydrocarbons</td>
</tr>
<tr>
<td>pH</td>
<td>5.14</td>
</tr>
</tbody>
</table>
Introduction

The fish sector contributes a major role in the Aquaculture industry worldwide. These resources are expected to have high demand in National and International levels. Disease outbreaks in Aquaculture as an important limiting factor in production and trade. Aeromonads are known to be of great importance in both economically and medically. Members of this genus are mainly distributed in freshwater and sewage and in association with aquatic animals are sometimes predominant components (Cahill, 1990; Stechini et al., 1994).

Aeromonas infections are caused by bacteria which are present in the water all of the time. Usually, when fish get sick with an Aeromonas infection, something has happened to make them susceptible to bacterial invasion. There are several species of Aeromonas which can infect fish. Aeromonas infections are probably the most common bacterial disease diagnosed in cultured warm water fish. Usually, mortality rates are low (10% or less) and losses may occur over a period of time (2 to 3 weeks or longer). In these instances, some factor; usually stress, has caused the fish to become more susceptible to the bacteria. Common sources of stress are poor water quality, overcrowding, or rough handling.

Some strains of Aeromonas are more virulent, which means that they possess special properties which enable them to cause more serious disease outbreaks. If these more damaging strains become endemic in a population of fish (which means that they are there all of the time and the fish develop an immunity to them), it becomes difficult to introduce new fish in to the water body without suffering major losses of newly stocked fish.

There is no single physical or behavioural sign specific for Aeromonas infections. Infected fish frequently have; small pinpoint haemorrhages at the base of the fins or on the skin, distended abdomens, and protruding eyes. Internal signs include; fluid in abdomen, swollen liver and spleen, and the intestines are distended and fluid filled.

Disease has become a critical factor hampering the development of Indian Major Carp culture in India. Among the various pathogens, the Gram-negative bacteria represent the greatest potential threat to aquaculture. Indian Major Carps are susceptible to a wide variety of bacterial diseases. These include hemorrhagic disease,
Introduction

erthrodermatitis, columnaris disease, edwarasiollosis, enteric redmouth disease and furunculosis (Jeney and Jeney, 1995) (Table-3).

Diseases are major bottlenecks in the development and sustainability of aquaculture practices throughout the world. Among different types of infectious agents bacterial pathogens are often responsible for severe mortalities in a wide range of fishes at different stages of growth (Grisez and Ollevier, 1995; Swain et al., 2002).

The genus *Aeromonas* was proposed by Kluyver and van Niel (1936) to accommodate rod shaped bacteria possessing the general properties of the enteric group, but motile by means of polar flagella. This genus is now included in the family of *Vibrionaceae* (Veron, 1966). In *Bergey's Manual of Determinative Bacteriology* (1974) the original definition has been amended to include the following salient properties: Gram-negative straight rods; polar flagellated (generally monotrichous) or immotile; facultative anaerobes, fermenting carbohydrates with formation of acid or acid and gas; oxidase positive; reducing nitrates to nitrites.

Table - 3: Bacteria and associated bacterial diseases of Indian Major Carps.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aeromonas</em> motile</td>
<td><em>Aeromonas</em> septicemia</td>
</tr>
<tr>
<td><em>Edwardsiella</em> tarda</td>
<td>Edwardsielliosis</td>
</tr>
<tr>
<td><em>Pseudomonas</em> fluorescens</td>
<td><em>Pseudomonas</em> septicemia</td>
</tr>
<tr>
<td><em>Flexibacter</em> columnaris</td>
<td>Flexibacteriosis</td>
</tr>
<tr>
<td><em>Flavobacterium</em> branchiophila</td>
<td>Bacterial gill discasc</td>
</tr>
<tr>
<td><em>Streptococcus</em> sp. Streptococcal</td>
<td>Septicemia</td>
</tr>
<tr>
<td><em>(S. faecalis, S. faecium, S. pyogenes)</em></td>
<td></td>
</tr>
<tr>
<td><em>Mycobacterium</em> sp.</td>
<td>Mycobacteriosis</td>
</tr>
<tr>
<td><em>(M. marinum, M. fortuitum, M. cheloneae)</em></td>
<td></td>
</tr>
</tbody>
</table>
Introduction

The genus *Aeromonas* consists of gram-negative rods widely distributed in freshwater, estuarine, and marine environments (Holmes, et al., 1996; Martin-Carnahan, et al., 2005). *Aeromonas* species grow at a range of temperatures, although they are isolated with increasing frequency during warmer months (May through October in the Northern hemisphere). *Aeromonas* species cause a wide spectrum of disease syndromes among warm and cold-blooded animals, including fish, reptiles, amphibians, mammals, and humans (Gosling, 1996; Janda and Abbott, 1996).

The genus *Aeromonas* was re-categorized from the family Vibrionaceae to the family *Aeromonadaceae* in the mid-1980's, when phylogenetic evidence from molecular studies became available to support this distinction. (Martin-Carnahan ct al., 2005; MacDonell et al.,1985; Colwell, et al.,1986).

The genus *Aeromonas* has been divided into two major groups (Horneman et al., 2006).

1. Motile, Mesophilic species that can cause disease in humans.

2. Non-motile, Psychrophilic species that generally cause disease only in fish.

It was believed that the pathogenicity of *Aeromonas* spp. is mediated by a number of extracellular proteins such as aerolysin, lipase, chitinase, amylase, gelatinase, hemolysins and enterotoxins. However the pathogenic mechanisms of *Aeromonas* spp. is remain unknown. The recently proposed type III secretion system (TTSS) mediated pathogenic mechanism has been proven to play a pivotal role in *Aeromonas* pathogenesis. The TTSS is specialized protein secretion machinery that export virulence factors delivered directly to host cells. These factors subvert normal host cell functions in ways that are beneficial to invading bacteria. In contrast to the general secretory pathway, type III secretion system is triggered when a pathogen comes in contact with host cells. ADP-ribosylation toxin is one of the effector molecules secreted by several pathogenic bacteria and translocated through TTSS and delivered into the host cytoplasm leads to interruption of NF-κB pathway, cytoskeletal damage and apoptosis. This toxin has been characterized in *Aeromonas hydrophila* (human diarrhoeal isolate), *A. veronii* (isolate from soil and various water systems) *Aeromonas salmonicida* (fish pathogen) and *Aeromonas jandaei* GV17, a pathogenic strain which can cause disease both in human and fish.
Aeromonas veronii

PATHOLOGY

*A. veronii* is a rod shaped motile, gram negative, facultative anaerobe found in fresh water and in association with animals. It can be a pathogen of humans and a beneficial symbiont of leeches (F.W. Hickman-Brenner et al., 1988). The bacteria are usually not found in groups or pairs but as individual cells (Kikuchi and Graf, 2007). *A. veronii* is commonly found in soil and various water systems all over the world. It is most often associated with the leech. The blood digested by the bacteria in the leech has been found to contain various antimicrobial properties. It is capable of lowering high concentrations of bacteria through the activations of the membrane attack complex. This complex creates permeable membranes in foreign bacteria, essentially inactivating the bacteria. The *A. veronii* seem to be unsusceptible to this complex, allowing it to proliferate while other bacteria cannot. This leads to a very limited number of microbial flora in the digestive tract of the leech, which is extremely uncommon (Indergrand and Graf, 2000). The population of *Aeromonas veronii* is greatly affected by the consumption of blood. Tests have shown that dramatic changes occur during this time, the majority of *A. veronii* bacteria are found not in the epithelial tissue but in the IntraLuminal Fluid (ILF) (Indergrand and Graf, 2000). In human’s veronii can cause diseases ranging from wound infections and diarrhea to septicaemia in immunocompromised patients. The densities and incidences of *A. veronii* and *A. jundaei* are comparable to those of *A. caviae, A. hydrophila*, and *A. sorbia*, which are known as indigenous bacteria in aquatic environment (Cahill, 1990; Sugita et al., 1994).
Scientific Classification of *Aeromonas veronii* (Hickman-Brenner, et al. 1987)

Kingdom: Bacteria
Phylum: Proteobacteria
Sub Phylum: Gammaproteobacteria
Class: Aeromonaales
Order: Aeromonaales
Family: Aeromonadaceae
Genus: Aeromonas
Species: A. veronii

Genome structure

The bacteria is made up of 2758 bp of linear DNA. Studies have found that certain genes (Ast, Alt, and Act) may play a significant role in infection of host organisms. The aeroslysin-hemolysin genes were found to cause diarrhoea in some patients who had *A. veronii* in their digestive system (Aguilera-Arreola et al., 2007).

Cell structure and metabolism

The bacterial cell contains a cytoplasm membrane, a thin layer of peptidoglycan and an outer layer composed of lipopolysaccharides (LPS). The catalase gene is important for the degradation of toxic hydrogen peroxide to much more useful molecules, water and oxygen. The expression of the catalase gen is influenced by introduction to extremely low levels of H$_2$O$_2$ during growth and the stationery phases. It has been suggested that H$_2$O$_2$ is used as an antimicrobial by the host cell to damage the DNA, RNA, proteins of invading pathogens. Only those microbes that are able to metabolize hydrogen peroxide would be able to survive in a host cell (Rio et al., 2007).

Ecology

The *A. veronii* bacteria can be found in a number of habitats, including humans, mosquitos and leeches. It is primarily found in the digestive tract of the leech where it maintains a symbiotic relationship with its host. The medicinal leech, *Hirudo*
medicinalis is capable of consuming six times its own body weight. The crop is the area of the digestive tract colonized by *A. veronii*. It is also the area where blood is stored after ingestion, and where water and salt are absorbed from the blood. Blood is stored in the crop of the digestive tract. Studies have suggested that one of the reasons *A. veronii* is one of the two predominant microbial flora of the digestive tract is due to the antimicrobial properties of ingested blood (Indergand and Graf, 2000). *A. veronii* provides a number of contributions to the symbiotic relationship it share with the leech. It appears the bacteria helps maintain the flora of the digestive tract, helps in digestion of blood and it also provides necessary nutrients, such as vitamin B complex, not found in abundance in blood (Kikuchi and Graf, 2007) (Worthen et al., 2006).

**CLINICAL CHARACTERS OF DISEASED FISH**

1. Effects many freshwater species and usually is associated with stress and overcrowding.

2. The clinical signs and lesions are variable. The most common finding is hemorrhage in skin, fins, oral cavity and muscles with superficial ulceration of the epidermis. Occasionally cavitary ulcers (similar to *A. salmonicida*) are observed. Exophthalmus and ascites are commonly observed. Splenomegaly and swollen kidneys are common. Histologically, multifocal areas of necrosis in the spleen, liver, kidney and heart with numerous rod shaped bacteria are observed.

3. Fish infected with *Aeromonas veronii* may have many different clinical signs. These range from sudden death in otherwise healthy fish to inap-petence, swimming abnormalities, pale gills, bloat and skin ulcerations. The skin ulcers may occur at any site on the fish and often they are surrounded by a bright red rim of tissue. Because of the variability of these clinical signs, the diagnosis of this disease based only upon the clinical presentation of the fish is highly unreliable and may be economically disastrous to the fish producer.

4. Diagnosis is rendered by culturing the organism from affected animals: Remember this is a common water saprophyte with a great variation in virulence in serotypes.
Introduction

5. Disease is transmitted via contaminated water or diseased fish.

6. With intensive fish farming systems, whether these systems are outdoor ponds or indoor aquaria and tanks, predisposing factors are primarily responsible for the precipitation of this disease. Stress is the single most important predisposing factor associated with this disease. Stress is due to poor management and/or poor water quality.

7. Management factors include: nutrition, handling, transportation and overcrowding of fish. Water quality must be excellent to prevent this disease. This means that the dissolved oxygen (DO), pH, temperature and alkalinity of the water must be satisfactory, and that the ammonia, nitrite and CO₂ levels must be kept to a minimum.

PREVENTING INFECTION

It is ill-advised to transfer fish from hatchery to hatchery without any sanitation. Hatchery workers should clean the fish, and check for bacterial infection between each operation. To avoid contamination oxygen levels in fish should be maintained, and fish should always be handled gently, to avoid injury. Prophylactic treatments can also be used when trying to prevent *Aermonas veronii*. These treatments include disinfectants and Acriflavine.

Silver Nanoparticles (AgNPs) and its structure and importance

Nanoparticles are clusters of atoms in the size range of 1-100nm (Gavanji et al., 2012). Reducing the particle size of materials is an efficient and reliable tool for increasing their biocompatibility. Furthermore, nano materials can be changed for more efficient applications in different fields such as bioscience and medicine (Kim et al., 2007). Elemental silver and silver salts have been used as antimicrobial agents for a long time. Nano silver is a new class of material with different physiological and biological characteristics such as increased optical, electromagnetic, catalytic properties and antimicrobial activity from the bulk materials. (Shahrokh and Emitiazi, 2009) To produce nano silver, metallic silver has been engineered in to ultra fine particles by several methods, including spark discharging, electrochemical reduction, solution irradiation and cyrochemical synthesis. Nanosilver particles are usually
smaller than 100nm and consist of about 20 to 15000 silver atoms. Nanoparticles including nano silver may have different shapes such as spheres, rods and cubes. Furthermore, nanostructures can be generated as tubes, wires, and films (Wijnhoven et al., 2009). Ionic silver have some advantages such as its high reactivity which made it unstable and thus easily oxidised or reduced in to a metal depending on the surrounding environment. In addition, ionic silver causes discolouration by itself or allows other materials to cause undesirable coloration and it does not continuously exert antimicrobial activity. Also, silver in the form of a metal or oxide, which is stable in the environment, is applied in a relatively increased amount because of its low antimicrobial activity. (Park et al., 2006)

Nanoparticles are increasingly being used, or being evaluated for use, in many fields. Nanotechnology is emerging as a rapidly growing field with its application in science and technology for the purpose of manufacturing new materials like cosmetics, food and feed, environmental health productions at the nanoscale level (Wijnhoven et al., 2009). The term “Nano” is a Greek word synonymous to dwarf meaning extremely small (Rai et al.,2009) which is used to indicate one billionth of a meter or $10^{-9}$. The term Nanotechnology was coined by Professor Norio Taniguchi in Tokyo Science University in the year 1974 to explain precision manufacturing of materials at the nanometer level. Nanotechnology as a branch of science which is related to nano materials helps in overcoming limitations of size and can change outlook of the world regarding science (Kim et al., 2007). By manipulating materials at atomic level, nanotechnology offers to achieve unique properties for various desired applications. It is noticeable that most of the nature’s creations occur at the nanoscale regime too (Ghosh et al., 2010). Because of its widespread application, the commercial nanotechnology industry is predicted to increase significantly to $3$ trillion by 2015. (Ahamed et al., 2010). The effects of nanosilver on tissues which were investigated in significant and analytical experiments showed the damages in tissues. These damages were caused by the increase of free radicals and stimulation of oxidative stress; however, more immunological and genetical investigation will clear the biological effects of nanoparticles (Ranjbar sardari et al., 2011).
Different types of nanomaterials like copper, Zinc, titanium alginate and silver have come up but silver nanoparticles have proved to be most effective due to its good antimicrobial efficiency against bacteria, viruses and other euakaryotic microorganisms. Silver nanoparticles used as drug disinfectant have some risks as the exposure to silver can cause agyrosis and argyria and also toxic to mammalian cells (Rai et al., 2009). When silver metal has size of nano level, high specific surface areas and high fraction of surface atoms of silver nanoparticles will lead to high antimicrobial activity comparing to bulk silver metal (Saengkietiyut et al., 2008). Silver (Ag) is one of the most commonly used nanoparticles due to its bactericidal effect (Farnen et al., 2012). Silver (Ag) nanoparticles are used as antimicrobial adjuvant in various products such as clothes and medical devices. The emergence and increase in antibiotic resistance is an alarming concern in clinical diseases in both aquatic and human medicine. The use of antimicrobial drugs in aquatic medicine can cause a serious problem in the environment because of the rapid spread of antibiotics through water. The use of metallic silver as an antimicrobial agent has been recognized for a long time (Lansdown, 2002a) and several types of silver dressings, silver nitrate, silver zeolite and silver nanoparticles, for a variety of antimicrobial purposes (Kim et al., 2007; Rai et al., 2009). One possibility to inhibit the bacterial fish pathogens is to use nanoparticles as antimicrobial drugs. Silver nanoparticles have proved to be one of the most effective metallic nanoparticles as they have good efficiency against some bacteria, viruses, and other eukaryotic microorganisms (Gong et al., 2007).

The nanomedicine has the potential to revolutionize the various disease treatments in animal system worldwide. Existing research has clearly demonstrated the feasibility of introducing nanoshells and nanotubes in to the animal systems which destroys the targeted cells. The nanoparticles have been used to deliver the drug into the cells with negligible side effects (Scott, 2005). The synthesis of nanoparticles from metals possesses various biological processes through Co-enzymatic systems. The interaction of these nanoparticles with biologically active ligand in the animal system through chelation. Due to the increase in the outbreak of bacterial diseases in the aquaculture and the development of bacterial resistance, new antibacterial agents are required.
Silver nanoparticles have proved to be one of the most effective metallic nanoparticles and good bacterial activity against some bacterial pathogens (Gong et al., 2007) and fish pathogens (Soltani et al., 2009). Chemotherapeutics are drugs which are capable of killing or affecting microorganisms especially bacteria in the fish culture (Schaperclaus et al., 1954). Several chemicals viz., formalin, malachite green, methyl blue, copper sulphate and potassium permanganate have been used to cure the bacterial fish diseases (Collins et al., 1975; Levine and Meade, 1976). But, these chemicals produced undesirable effects in the water as well as organisms (Yanong et al., 2009). The most important problem caused by the chemical agents depends on the specific binding with surface and metabolism of agents in to microorganism. Various microorganisms have evolved drug resistance over many generations (Wijnhoven et al., 2009). Silver ions are very reactive, leading to inhibition of microbial respiration and metabolism as well as physical damage. Furthermore, it has been suggested that silver ions intercalate in to bacterial DNA once entering the cell, which prevents further proliferation of the pathogen (Woo et al., 2009). Moreover, most of the biological resources such as mangroves, seaweeds, sea grasses and sponge etc, and silver nanoparticles showed antibacterial (Ravikumar et al., 2010) and antifungal activity (Young et al., 2009). However, the antimicrobial agents from metal nanoparticles against fish pathogens are poorly understood. Moreover, the advantages of inorganic antibacterial materials over organic antibacterial materials are that the superior durability, high surface area, less toxicity, heat resistance and more suitable for biological applications (Brayner et al., 2006). The antibacterial activity of nanoparticles showed activity against both gram positive as well as the gram negative bacterial strains (Ravikumar et al., 2010). But the effect of nanoparticles was very high against gram positive bacteria than the gram negative bacteria, this might due to the reactive oxygen species (ROS) mechanism. This mechanism can produce significant oxidative stress and altered cell wall system in to equally permeable levels (Shantikumar et al., 2009). Silver nanoparticles show diverse physical, chemical and biological properties and therefore are a center of attraction for application in a wide range of fields (Park et al., 2011). Interaction between nanoparticles and bacteria occur by various processes such as biosorption, particle aggregation and cellular uptake. This results in membrane damage and toxicity in the target organism. Silver is known to possess antimicrobial activity from
ancient times, which has formed a base for applying AgNPs as antimicrobial agents (Kim et al., 2008).

OBJECTIVES OF THE PRESENT STUDY

➢ To study impact of Lihocin, Bacteria - *Aeromonas veronii* and Silver nanoparticles on Indian Major Carp Fish, *Catla catla* for over the periods of 3, 7, 15, 30 and 45 days.

➢ To evaluate the Lihocin Toxicity (LC50) on fish for 96 hours.

➢ To study the impact of Lihocin, Bacteria - *Aeromonas veronii* and Silver nanoparticles on:

- Antibacterial activity of Silver nanoparticles
- Immuno-haematological Parameters.
- Detoxification and Antioxidant Enzyme Parameters of various tissues.
- Biochemical Constituents of various tissues.
- Histological Indices.
MATERIALS
AND
METHODS
MATERIALS AND METHODS

Experimental Animal

Catla ("Catla Catla"), also known as the Indian Major Carp, is an economically important South Asian fresh water fish in the carp family Cyprinidae was selected as the experimental animal for my present work. It is commonly called in Andhra Pradesh is “Bocche” (Regional linguistic name).

Table-1: Systematic Position of Catla Catla (Hamilton, 1822)

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Animalia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylum</td>
<td>Chordata</td>
</tr>
<tr>
<td>Sub-Phylum</td>
<td>Craniata</td>
</tr>
<tr>
<td>Group</td>
<td>Vertebrates</td>
</tr>
<tr>
<td>Series</td>
<td>Pisces</td>
</tr>
<tr>
<td>Class</td>
<td>Teleostomi</td>
</tr>
<tr>
<td>Super Class</td>
<td>Gnathostomata</td>
</tr>
<tr>
<td>Sub Class</td>
<td>Actinopterygii</td>
</tr>
<tr>
<td>Order</td>
<td>Cypriniformes</td>
</tr>
<tr>
<td>Sub Order</td>
<td>Cyprinoidei</td>
</tr>
<tr>
<td>Division</td>
<td>Cyprini</td>
</tr>
<tr>
<td>Family</td>
<td>Cyprinidae</td>
</tr>
<tr>
<td>Sub family</td>
<td>Cyprinini</td>
</tr>
<tr>
<td>Genus</td>
<td>Catla</td>
</tr>
<tr>
<td>Species</td>
<td>Catla</td>
</tr>
</tbody>
</table>

Synonyms of Cyprinus catla

Leuciscus Catla (Valenciennes, 1834) Cyprinus abramioides (Sykes, 1840), Hypselobarbus abramioides (Bleeker, 1860).
Materials and Methods

*Catla Catla* is a one of the originating and fast growing fresh water fish. Catla is facing serious threats due to indiscriminate use of pesticides in the country. *Catla Catla* is a planktivorous carp; preferably feeding on zooplankton. *Catla Catla* is a surface feeder and is more vulnerable to all kinds of pollutants in fresh water ecosystem. Catla is found in fresh water bodies, rarely in brackish water. It is a non-predatory fish and its feeding is restricted to the surface and mid water (Talwar and Jhingram, 1991). Feed on both natural and supplementary feeds. Catla are Planktophagic because they feed on phytoplankton. Catla is a surface feeder. Each of the developmental stages has their own feeding preferences and nutritional requirements. Maximum feeding activity of catla occurs during the morning hours (6.00 to 9.00) (Jana and Chakrabarti, 1988). Fingerlings consume some planktonic algae, vegetable debris along with larger size zooplankton. Adults feed mainly on the surface and in mid-water. Adults are also planktivorous and show a preference for zooplankton (Natarajan and Jhingran, 1961; Jhingran, 1991).

*Catla* is a eurythermal species that grows best at water temperatures between 25-32°C selected as experimental animal. The food and feeding habits differ between the three Indian major carp species. Catla is one of the most valuable food fish. Mostly cultivated in isolated fresh water ponds and lakes in the absence of carnivorous fish. The fish is fleshy and noted for its delicacy and valued very high in the market. The fish is largely employed for stocking tanks, Catla are mostly preferable by the fish farmers in India for cultural practices.

Biology of *Catla Catla*

The species *Catla Catla* was first described by Hamilton (1822). Catla is a fish with large and broad head, a large protruding lower jaw and upturned mouth, snout with very thin integuments, the lower moderately thick having a continuous and free posterior margin. The lower jaw with moveable articulation at the symphasis, but lacking prominent tubercle. No barbels, Gill rakers are long, fine and closely set. Eyes with free orbital margins. It has large, greyish scales on dorsal side and whitish on belly. Pharyngeal teeth plough shaped, 5, 3, 2/2, 3, 5 in pattern. Dorsal fin long, inserted above tip of pectoral fins with 18-19 rays (branched rays 14-16) and without spines. Short anal fin with 8 rays (branched rays 5). Pectoral fins long and extend to pelvic fins. Caudal fin forked. Lateral line with 40 to 43 scales lateral line continuous

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Materials and Methods

to the centre of the base of the caudal fin. Catla is the fastest growing of the Indian major carps. In natural waters, Catla attains length of 295mm (weight 354g) in the first year, 514mm (weight 2,193g) in the second year, 716mm (weight 6,501g) in the third year, 823mm (weight 10,282g), in the fourth year and 917mm (weight 14,665g). Digestive system is very short and straight at early stage, folding of digestive system starts on day-9 after hatching, coiling is visible after 19 days of hatching. The digestive system is composed of pharynx, oesophagus, intestine and vent, stomach is absent.

Catla attains sexual maturity at an average age of two years and an average weight of 2 kg. Catla breed during the monsoon season in rivers. Under captive conditions spawning is induced by hypophysation. During the breeding season the dorsal surface of the pectoral fin of the males becomes rough and on applying gentle pressure on the belly milt oozes freely from the genital papilla. The female has a soft, round, bulging belly and a swollen, pinkish genital opening.

Procurement and maintenance

Live *Catla Catla* fingerlings (10.15±0.5cm, long and 17.25±4.18g, weight) were procured from A.P. Government central Fish Farm, Kalyani Dam, Chittoor District, AP, INDIA. Healthy fishes were randomly and carefully packed in a medium sized polythene bag with sufficient oxygen which would help them to carry on their normal metabolic activities during the period of transportation, immediately they were transferred and kept into large transparent polypropylene tanks (1000L capacity), which are arranged with aerators, airlines and air stones for sufficient aeration for animals to avoid suffocation and stress. They received unchlorinated, aerated and continuous gentle flow of water from a deeply sunk bore well within the laboratory of our university campus. The fish were fed on formulated pelletized feed daily. The experimental animals were starved for 24h prior to the commencement of experiments to avoid metabolic variations due to diet.

Like human beings, fish and other aquatic animals also enjoy a good environment and exhibit an intimate relationship with their environment with respect to their growth, physiology and development. However, the aquatic environment of fish is mainly affected by temperature, pH and photoperiod and these factors are also responsible for the behaviour, physiology and breeding cycles of these species.
Moreover, the non-specific as well as the specific immune system of fish is also directly coordinated by the change in the environment. The aquatic habitat of fish with variable temperature is the most favourable condition for the growth and multiplication of different pathogens causing various fish diseases. Among the different potential environmental factors, temperature is the main factor that affects both the innate and adaptive immune response of fish. The non-specific immunity like phagocytosis, cytotoxicity and production of specific immune factors are also adversely influenced by the lower temperature, whereas ambient temperatures have marked influence on immune reactivity in fish, including the kinetics of specific cell-mediated responses. In many fish species the specific defence substance (Immunoglobulin M) fluctuated with water temperature. In general, the best responses are obtained at the normal summer temperatures which of course vary considerably according to the nature of the species, whether the fish is coldwater, temperate, or warm water species. The temperature at which the optimum immune response attained by the species is termed immunologically “permissive” and temperatures below this, but still within the normal physiological range, is found to be immunosuppressive (non-permissive temperatures).

During this period standard formulated feed was given to all the fish as per the conditions suggested by Behringer (1973). All they were acclimated for at least for a two weeks and fed on standard formulated and pelletized feed in laboratory conditions. During the period of acclimatization, the fish were fed normally and water in the tanks was replenished daily for the cleaning purpose to eliminate uneaten deposits of food and faecal matter and to prevent hypoxic condition.

The Physico - chemical characteristics of water is playing significant role to assess the quality of water for its best usage. They play an important role in maintain the equilibrium, between aquatic organisms and abiotic factors. The physico-chemical characteristics of water is maintained constantly throughout the experimental tenure (Table- 2.2). The water quality parameters were analysed by using titration methods as per APHA (1998). Water was renewed every day and 12:12 hour of photoperiod was maintained daily during acclimation and test periods.
Materials and Methods

Table 2: water quality parameters and their optimal levels used in experiments.

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.40 ± 0.09</td>
</tr>
<tr>
<td>Temperature</td>
<td>25 ± 3°C</td>
</tr>
<tr>
<td>Hardness</td>
<td>162.8 ± 5.34 mg/l</td>
</tr>
<tr>
<td>Electrical conductivity</td>
<td>287.36 ± 61.42 mho/cm</td>
</tr>
<tr>
<td>Dissolved oxygen</td>
<td>8.42 ± 0.19 mg/l</td>
</tr>
<tr>
<td>Total alkalinity</td>
<td>125 mg/l</td>
</tr>
<tr>
<td>Ammonia</td>
<td>NIL</td>
</tr>
<tr>
<td>Turbidity</td>
<td>0 NTU</td>
</tr>
</tbody>
</table>

Experimental Chemical

Organochlorine Insecticide

Lihocin 97-98% pure, Organochlorine insecticide with trade name VAM-C and chemically called as Chlormequat chloride. They are in form of White crystals with a typical amine adour (fish like odour) and hygroscopic. Lihocin is highly toxic to fish which absorbs it directly from water or by ingesting contaminated food and bioaccumulate in their fatty acids due to its Lipophilic nature (Oritz et al., 2001). It is highly stable and combustible. It is used as treatment against ecoparasite and as an insecticide for crops. Lihocin is poorly hydrolyze and as such, it biodegrades slowly in the environment. So this compound persists for longer time in the food chain and cause severe effects at different levels of food chain.

Lihocin is a plant growth regulator which inhibits cell elongation, hence shortening and strengthening stems and producing sturdier plants. It also influences the developmental cycle, leading to increased flowering, for example. It may also increase chlorophyll formation and root development. Chlormequat chloride is used to increase resistance to lodging (by shortening and strengthening stems) and to increase yields in wheat, rye, oats, and triticale. It also helps crops to effectively cope with environmental stresses such as drought, flood, and heavy wind and also during the process of transplanting. Spraying should be performed in calm weather. The
recommendation time for spraying should be strictly adhered to, in order to get the desired benefits. Lihocin is applied to all vegetable plants before flowering and second spray after 15 day interval, and it required maximal 2 sprays. Lihocin can also be applied on grape plants with repeated and equal intervals between 1st and 2nd sprays. It is also used to promote lateral branching and flowering in azaleas, fuchsias, begonias, poinsettias, pelargonium's, and other ornamental plants. It is also used on cotton (Pesticide Manual, 1997).

Lihocin entered in to the aquatic environment cause serious threatening to various aquatic organisms and also cause severe metabolic disorders in non target species like fish and freshwater mussels.
<table>
<thead>
<tr>
<th><strong>Table-3: Chemical Properties of Lihocin</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Technical grade</strong></td>
</tr>
<tr>
<td><strong>Trade name</strong></td>
</tr>
<tr>
<td><strong>Chemical Name</strong></td>
</tr>
<tr>
<td><strong>Molecular Formula</strong></td>
</tr>
<tr>
<td><strong>Chemical nature</strong></td>
</tr>
<tr>
<td><strong>CAS Number</strong></td>
</tr>
<tr>
<td><strong>Molecular weight</strong></td>
</tr>
<tr>
<td><strong>Melting point</strong></td>
</tr>
<tr>
<td><strong>Storage temperature</strong></td>
</tr>
<tr>
<td><strong>Density/Specific gravity</strong></td>
</tr>
<tr>
<td><strong>Colour/Form</strong></td>
</tr>
<tr>
<td><strong>Odour</strong></td>
</tr>
<tr>
<td><strong>Technical grade</strong></td>
</tr>
<tr>
<td><strong>Surface Tension</strong></td>
</tr>
<tr>
<td><strong>Vapour Pressure</strong></td>
</tr>
<tr>
<td><strong>Sensitivity</strong></td>
</tr>
<tr>
<td><strong>Stability</strong></td>
</tr>
<tr>
<td><strong>Solubility</strong></td>
</tr>
<tr>
<td><strong>Water Solubility</strong></td>
</tr>
<tr>
<td><strong>Insoluble</strong></td>
</tr>
<tr>
<td><strong>pH</strong></td>
</tr>
</tbody>
</table>
EXPERIMENTAL DESIGN

The present work was divided into two technical parts:

a) Effect of Lihocin an Organochlorine insecticide on various immunological, physiological, histological and biochemical indices of fish for 45 days.

b) Effect of Silver nanoparticles (AgNPs) on various immunological, physiological and biochemical variables of Aeromonas veronii infected and Lihocin treated fishes for 45 days.

Catla catla has been selected for this because of commercial importance and first preferable teleost fish for farmers in Andhra Pradesh for cultural practices. The fresh water carp fish, Catla catla (local name Bocche) was selected as the test animal for the present study. Because catla is easily available and cultivable in all freshwater resources. Catla is highly commercially important and exportable fish. The obtained results can be interpreted and applicable to all human beings.

A) Effect of Lihocin an Organochlorine insecticide on various immunological, physiological and biochemical variations of fish for 45 days

The acclimatized fishes were further divided into six groups each group contains 15 animals. Fishes in the first group are served as control and the remaining groups are treated with sub lethal concentration of Lihocin. The insecticide containing water medium was changed at every 24hr in order to maintain a constant sub lethal concentration (1/5 of LC50) of Lihocin.

Group-1 : Control (without Lihocin)
Group-2 : Exposed for 3 days with Lihocin
Group-3 : Exposed for 7 days with Lihocin
Group-4 : Exposed for 15 days with Lihocin
Group-5 : Exposed for 30 days with Lihocin
Group-6 : Exposed for 45 days with Lihocin
**Materials and Methods**

TOXICITY EVALUATION

$LC_{50}$ is the concentration at which 50% of the test animals are killed. Lethal concentration of Lihocin was determined by probit analysis method of Finney (1971). The 96hrs $LC_{50}$ tests are conducted to measure the susceptibility, survival and potential of organisms to particular toxic substances such as pesticides. Suggestions made by Duodoroff et al. (1951) were followed, hence constant biomass ratio with water volume was maintained (one gram/1litre). Fishes were acclimated for week days. Aeration facility was provided for fishes. Feeding the fishes was stopped for 2 days prior to the experiments. If mortality exceeds 5% in any batch during acclimatization that entire batch of fish was discarded. Water was changed before introducing the pesticide in to water, to remove excretory materials or unused food. Preliminary experiments were conducted individually by using Lihocin to find out the concentrations that resulted in 10% - 90% mortality. After the range finding tests, batches were made with 20 fishes separately with *Catla catla* and they were exposed to different concentrations of the Lihocin dissolved in water. Duplicates were also set up besides running the control simultaneously. Mortality was recorded for every one hour interval.

Mortality in each concentration was noted and graphs were plotted between Lihocin concentration versus percent and probit kill. 50% mortality was taken as an index for calculation of median lethal concentration ($LC_{50}$). Fishes were also exposed to Log.2 concentration for the same exposure period to determine $LC_{50}$ value by adapting Dragstedt and Behren’s equation given by Carpenter (1975).

$$Lg.LC_{50} = \log A + \frac{50-a}{b-a} \times \log 2$$

Where:

$A = $ concentration of the heavy metal which has a mortality immediately below 50%.

$a = $ percent mortality observed immediately below 50%

$b = $ percent mortality observed immediately above 50%
Materials and Methods

Since the toxicity of the chemical depends on many biotic and abiotic factors like temperature, pH, dissolved oxygen, food etc, they were maintained constant to the maximum possible extent during experimentation.

b) Effect of silver nanoparticles (AgNPs) on various immunological, physiological and biochemical indices of Aeromonas veronii treated and Lihocin infected fishes for 45 days.

The exposure of silver nanoparticles on immunological, physiological and biochemical parameters in Aeromonas veronii infected fishes with or without Lihocin treated fishes.

Bacterial strain and Cultivation

Aeromonas veronii, strain, (Virulent Strain) was obtained from MTCC, Chandigarh, India. From this parent culture, sub cultures of A.veronii were prepared and doses were made under aseptic conditions. Culture and doses of Aeromonas veronii was done following the method of Pelczar (1993).

Preparation of plant extract

Fresh leaves of Aloe vera were collected from the garden of the Department of Botany, Sri Venkateswara University, Tirupati. The leaves were washed with distilled water, and after grinding, 10 g leaves was mixed with 100 ml distilled water and heated for 15 min. Then the extract was filtered through Whatman filter paper, collected and stored in refrigerator.

Preparation of metal solution

1mM aqueous solution of silver nitrate (AgNO₃) was prepared and used for the synthesis of silver nanoparticles.10ml of Aloe vera extract was taken and added in to 90ml of aqueous solution of 1mM silver nitrate and incubated in the dark, overnight at room temperature. Reddish brown solution was formed, indicating the successful formation of Silver nanoparticles.
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UV-Visible Spectrophotometer

Equal amount of sample aliquot and distilled water (1ml each) were mixed in a 10 mm-optical-path-length quartz cuvettes, and the UV-Vis spectrum analysis of the reaction mixture was carried out to detect the reduction of pure Ag⁺ ions. The concentration of AgNPs produced was measured using a Nanotrope, Model No-8000 UV spectrophotometer, at a resolution of 1 nm, between 200 and 800 nm (Fig - 2.3). UV-VIS spectroscopic analysis was done at DST-PURSE, Sri Venkateswara University, Tirupati.

Scanning Electron Microscope study

The solution of Aloe vera leaf extract in each beaker was dried and sent for scanning electron micrograph (SEM). The SEM characterization was carried out using a scanning electron microscope (Carlzeiss, Model No - EVO15). SEM analysis was done at Department of Physics, Sri Venkateswara University, Tirupati.

Fourier Transform Infrared Spectroscopy (FT-IR) study

FT-IR analysis was carried out on Bruker, Ettlingen, and ALPHA (ECO-ATR) interferometer in the diffuse reflectance mode operated at a resolution of 4 cm⁻¹ in the range of 400 to 4,000 cm⁻¹ to evaluate the functional groups that might be involved in nanoparticle formation. FT-IR was done at Department of Chemistry, College of Engineering, Sri Venkateswara University, Tirupati.

Source of organism and composition of growth media

Nutrient Agar Medium preparation

1.5 g of nutrient agar medium was mixed with 100 ml distilled water and two drops of antibiotic was added. The conical flask was cotton plugged and autoclaved at 151 b/inch² pressure and 121°C for 15 min.

Inoculation

After cooling the agar medium, bacteria Aeromonas veronii inoculated with a needle from a pure culture medium to the solidified agar medium and were kept in 30°C temperature in incubator for 48 hrs.
Materials and Methods

Antibacterial activity

The antibacterial activities of AgNps were carried out by Agar diffusion method. Nutrient agar medium plates were prepared, sterilized and solidified. After solidification bacterial cultures were swabbed on these plates. The AgNps were poured in to wells with different concentrations (10μl, 20μl, 40μl, 60μl, 80μl and 100μl) in the nutrient agar plate and kept for incubation at 37°C for 24 hours. Zones of inhibition for control, AgNps were measured.

Experimental Design

The fishes were randomly allocated in to following groups. Each group containing ten animals (n=10)

Group-1 : Control (maintained in normal water)
Group-2 : Fishes exposed to sub lethal concentration of Lihocin
Group-3 : Control + Lihocin + *Aeromonas veronii* treated fishes.
Group-4 : Control+ Lihocin + *A. veronii* + Silver nanoparticles treated fishes

Fishes in groups 2, 4, 6, 8 received daily 1/5th sub lethal concentration of Lihocin based on probit analysis for 96hrs LC50 the concentration of Lihocin to *Catla catla* was determined. The present study, 1/5th LC50 is 0.447mg/L was selected as experiment dose.

BLOOD COLLECTION

Collecting blood from anesthetized fish is by serving the caudal peduncle, posterior to the anal fin with a sharp knife (or) scalpel which was previously rinsed with 2.7% EDTA (Smith, 1929; Cockell et al, 1992). Small quantities of blood can be collected from the served body in to micro capillary tubes containing EDTA acts as anticoagulant and large amounts can be drained in to the test tube. The major disadvantage of this method is that the fish is sacrificed by the collection method.
COLLECTION OF TISSUES

After the completion of all pesticide exposure periods, the fishes are sacrificed at each interval due to toxicity of chemical. Tissues like gill, liver, kidney and muscle are collected after the completion of experimental periods.

IMMUNO-HAEMATOLOGY

a. Estimation of Total Red Blood Cells Count (TRBC) or Total Erythrocyte Count (TEC)

Enumeration of formed elements (blood cells) is a quantitative measure of the population of blood cells in circulation. The counting of cells can be done manually with the help of a microscope after diluting blood and making a special type of wet mount as per method given in Mukherjee (1988). The technique is popularly known as haemocytometer and added by Neubaurgrid, on the haemocytometer which show cell counting areas for the estimation. The Haemocytometer (Neubaur Crystalline Counting Chamber) has a ruled area of 9 sq.mm. Consisting of a central heavy ruled area of 1 sq.mm and four ruled areas of the same size in the four corners. The central area is divided into 25 squares which are subdivided into 16 small squares. For counting total RBC, the ruled areas at 5 centers are counted and total RBC is measured as number/cubic millimeter. For counting white blood corpuscles (WBC) corner small squares i.e. $16 \times 4 = 64$ sq.mm are counted and number of cells reported as number/cubic millimeter. The blood was drawn into the RBC pippette up to 0.5 mark and immediately the diluting fluid containing, formal Citrate Solution was drawn up to 101 mark. This gives a dilution of 1:200 (Blood: Formal citrate solution). The solution is added by shaking gently and allowed to be settled for 2 to 3 minutes. The counting chamber and cover glass were properly cleaned and the cover glass was placed over the ruled area. Again the solution was mixed gently and the stem full of solution was expelled and a drop of fluid was allowed to flow under the cover slip by holding the pipette at an angle of 45°. It was allowed to settle for 2 to 3 minutes, the RBCs after settling without air bubble under the cover slip were counted. Then the ruled counting area was focused under the microscope and the number of RBC’s were counted in fine small squares of the counting area under high power lens and the number of RBC per sq.mm were calculated by using the following formula:
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\[
\text{Total RBC} = \frac{\text{No. of Cells} \times \text{dilution factor} \times \text{depth factor}}{\text{Total No. of small squares}} = \text{number cubic mm}
\]

b. Estimation of Total Leucocyte Count (TLC) (Or) Total White Blood Cell Count (TWBC)

Blood collection and processing procedure was same as described in the above except for the dilution factor which is 1:20. As far as the counting (Neubaur counting chamber) procedure of WBC, each of these 4 square millimeter area was subdivided into 16 squares, by using low power objective and a maximum ocular care was taken for counting cells of the Neubaur chamber. The following formula was taken for the enumeration of WBC.

\[
\text{Total WBC} = \frac{\text{No. of cells counted} \times \text{Volume of the Square} \times \text{Dilution factor}}{\text{No. of Squares (4)}} = \text{Cubic mm}
\]

c. Estimation of Haemoglobin Concentration (Hb gm/100mL)

Haemoglobin is a reasonable index of the red cell population and was estimated by acid haematin method (Sahli, 1962). The 0.1 N Hydrochloric acid was taken up to the mark 20 in the graduated tube and a drop (0.1 ml) of blood was added and allowed to stand for 5 minutes until it changes to dark brown colour. The solution was diluted by adding distilled water drop by drop (each time mixing the solution with a stirring rod) until it matches standard colour. Then reading was taken from the scale on the graduated tube and the Hb concentration was expressed as gm /100mL.

d. Estimation of Packed Cell Volume (PCV) (OR) Haematocrit Value (HCT) (%)

PCV was estimated by using Wintrobe’s tube (Mukherjee, 1988). Wintrobe haematocrit is a narrow tube of 100 mm length with a 3 mm internal bore, graduated from 0 up to 10 cm (100 mm) with the graduations both in ascending and descending order on the two sides of the tube. A long (22 cm) fine capillary pipette or special syringe supplied by the manufactures is used to fill the wintrobe tube with blood sample. The blood sample was collected into a vial which contains an anticoagulant (10% EDTA). The wintrobe tube was filled with blood sample by keeping it in slanted
position, starting from the bottom with a long syringe. Then the wintrobe tube was centrifuged at 3000 rpm for 30 minutes. PCV concentrations were expressed as %.

**ASSAY OF LIPID PEROXIDATION**

The level of lipid peroxidation in the animal tissue was measured in terms of malondialdehyde (a product of lipid peroxidation) contents determined by the thiobarbituric acid TEB) reaction. The reactivity of TBA is determined with minor modifications of the method adopted by Hiroshi ohkawa and Ohishi Yagik (1979).

**Tissue homogenate and intracellular fractions:**

The liver was perfused with ice cold 0.9% Nacl, via portal vein before isolation. Tissues were weighed and chilled in ice cold 0.9% Nacl. Tissue homogenations were prepared in a ratio of 1 g of wet weight tissue to 9 ml of 1.15% Kc1 by using a glass or Teflon porter.

**ENZYME ASSAY:**

To 0.5 ml of tissue homogenate (10% W/V), 0.5 ml of normal saline (0.9 % Nacl), 1.0 ml of 20% TCA were added. Then the contents were centrifuged for 20 minutes in refrigerated centrifuge at 4,000 xg. To 1 ml of supernatant 0.25 ml of TBA reagent is added and the contents were boiled at 95 °C for one hour. 1 ml of n-Butanol is added to it and then centrifuged for 15 min., at 4,000 xg in refrigerated centrifuge. After centrifugation the organic layer was taken and its absorbance at 532nm was measured.

TMP was used as an external standard and rate of lipid Peroxidation was expressed as n moles of malondialdehyde formed per gm wet weight of tissue.

**TOTAL GLUTATHIONE CONTENT**

**DTNB-GSSG reductase recycling assay for GSH and GSSG:**

Glutathione is oxidized by 5.5'- dithiobis (2- nitrobenzoic acid) (DTNB) to give GSSG with stochiometric formation of 5-thio, 2 nitrobenzoic acid (TNB). GSSG is reduced to GSH by the action of the highly specific GR and NADPH. The rate of TNB formation was followed at 412nm and it was proportional to the sum of GSH and GSSG present. The assay was carried out as described by Griffith (1980).
**Materials and Methods**

**Preparation of tissue extract:**

Tissues are rinsed in water, blotted dry and homogenized in 5 vol of 5% sulfosalicyclic acid. The homogenate was centrifuged at 5,000 rpm for 15 min and stored at 4°C until assayed.

**Procedure:**

Working buffer (700 μl) and sample (200 μl) were pipetted into each cuvette. The cuvettes were warmed at 30°C in a water bath for 15 min and GSSG reductase was added to initiate the reaction. The formation of TNB was followed at 412 nm. The total amount of GSH present in tissue was expressed as GSH equivalents. A standard curve was prepared using GSH in which the GSH equivalents were plotted against the rate of change of absorbance at 412 nm.

**ANTIOXIDANT ENZYMES**

**a. GLUTATHIONE -S-TRANSFERASE (EC 2.5.1.18)**

Glutathione-S-transferase activity in cytosol and microsomes was assayed by using the 1-Chloro-2, 4 dinitro benzene (CDNB) at 340 nm as described by Habig et al., 1974a

**PREPARATION OF CELL-FREE EXTRACTS:**

The hepatic tissue from control and experimental animals were isolated after perfusion and homogenized in 50 mM tris-HCl, pH 7.4, containing 1 mM EDTA and 1 mM diethyldithiocarbamate (DDC). The homogenized samples were then centrifuged in Hitachi refrigerated centrifuge at 16,000 xg for 30 min to remove cell debris, mitochondria and nuclei. The above supernatant was centrifuged at 15,000 xg for 60 min. in Hitachi ultra-centrifuge to obtain microsomal pellet and cytosol. The microsomal pellets were washed and resuspended in 25 mM Tris-Hcl, pH 7.4 containing 1 mM EDTA and 0.5 mM GSH. The supernatant was passed through cheese cloth and filtrate thus obtained was used as the cytosolic fraction.

**ENZYME ASSAY:**

The reaction mixture in a final volume of 3.0 ml contained 1 mM potassium phosphate pH 7.5, 1 mM CDNB, 5 mM GSH and appropriate volumes of enzyme
Materials and Methods

protein. The reaction was started by the addition of glutathione and incubated at 27°C. Thioether concentrations were determined from the slopes of initial reaction rates. A molar extinction coefficient of $9.6 \times 10^3$ Cm$^{-1}$ was used in the calculations. Enzyme assay was monitored in a dual beam spectrophotometer.

Activity was expressed as $\mu$ moles of thioether formed/mg/protein /min.

b. GLUTATHIONE PEROXIDASE ACTIVITY (E.C.1.11 1.7)

For determination of glutathione peroxidase activity the same enzyme which is used for cytosolic glutathione -S-transferase was used.

ENZYME ASSAY:

The GSH-PX activity was assayed with a GSH reduction coupled to NADPH oxidation by glutathione reductase .the activity of the GSH-PX was monitored at 340nm by the disappearance of NADPH using hydrogen peroxide as the substrate.

The reaction mixture in a volume of 3 ml contained 0.15M potassium phosphate buffer with 0.003 M EDTA, 0.003 M sodium azide , 0.03 M glutathione, 0.006 M NADPH, 3IU/10 Mm glutathione reductase, 0.0075M hydrogen peroxide and appropriate amounts of enzyme + water. The reaction was followed by the decrease in absorbance at 340nm against blank. One unit of enzyme activity is defined as one $\mu$mole of peroxide reduced per min. The specific activity is given in International units (u); units of GST-Px activity for the manual essay were calculated in the usual manner using the Beer's law relation (Wheeler et al., 1990).

c. SUPEROXIDE DISMUTASE (EC 1.15 1.1)

Preparation of enzyme extract:

About 500 mg of liver tissues were homogenized in 5 ml of 50 mM phosphate buffer pH 7.0 containing 1% polyvinyl pyrrolidine. The homogenate was filtered and centrifuged at 15,000 xg for 10 min., in Hitachi high speed refrigerated centrifuge. The supernatant obtained was used as enzyme extract. All steps in the preparation of the enzyme extract were carried out at -4 °C.

An aliquot of 1.0 ml of enzyme extract was used for the determination of the protein content.
ENZYME ASSAY

The activity level of superoxide dismutase was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) adopting the method of Beachamp and Fridovich (1971).

The reaction mixture in a volume of 3 ml contained 50mM phosphate buffer pH 7.8, 13mM methionine, 75mM nitro-bluetetrazoli, 2mM reiboflavin, 0.1mM EFTA and 0.1 ml of enzyme extract. Riboflavin was added at the end and the test tubes were shaken and placed 30 cm below a light bank consisting of two 15w fluorescent lamps. The reaction was started by switching on the light. The reaction was stopped by the switching of the light. The tubes were covered with a black cloth. The absorbance of the solution was measured at 560 nm in scanning spectrophotometer. The reaction mixture which was not exposed to light did not develop colour and served as control.

A log A-560 was plotted as a function of the volume of enzyme extract used in the reaction mixture. From the resultant graphs the volume of the enzyme extract corresponding at 50% inhibition of the reaction was considered as the enzyme unit.

d. CATALASE ACTIVITY (EC 1.11.1.6)

Catalase activity was assayed by measuring the initial rate of the disappearance of H₂O₂ by the method of Chance and Machly (1955).

Homogenate of tissues were prepared in cold phosphate buffer pH 7.4 and centrifuged at 10,000 x g for 15 minutes in refrigerated centrifuge. The supernatant was used for the enzyme assay.

ENZYME ASSAY:

The 3ml of reaction mixture contained 50 mµ phosphate buffer pH 7.0, 0.15 mµ H₂O₂ extract. The reaction tubes were shaken well and the absorbance was read at 260nm in Shimadzu double beam spectrophotometer connected to recorder. The decrease in H₂O₂ was followed as a decline in A 260. The activity of the enzyme was expressed in units where one unit of catalase converts one mole of H₂O₂ per minute.
METABOLIC BIOMARKERS

a. Succinate dehydrogenase (Succinate: Acceptor oxido reductase) (SDH) activity

Succinate dehydrogenase activity in the organs was estimated using the colorimetric method of Nachlas et al., (1960). A 5% homogenate (w/v) was prepared in 0.25 M ice-cold sucrose solution, centrifuged at 3,000 rpm for 10 minutes and the supernatant was taken as the source of enzyme. The incubation mixture consisted of 0.2 ml of 0.4 M phosphate buffer (pH 7.7), 0.2 ml of 0.2 M sodium succinate, 1.0 ml of 0.004 M phenzine methosulphate and 0.5 ml of 5% enzyme preparation. The mixture was incubated at 37°C for 30 minutes and the reaction was stopped by adding 6.0 ml of glacial acetic acid. The formazan formed was extracted into 6.0 ml of toluene overnight at 0°C and the optical density of the colour developed was measured in a spectrophotometer at a wavelength of 495 nm. A blank using 0.5 ml of distilled water and control taking 0.5 ml of boiled enzyme were also run similarly. INT standards were prepared alongside for comparison. The enzyme activity is expressed as μM formazan/mg/protein/h.

b. Lactate dehydrogenase (L-lactate NAD oxido reductase (LDH) activity

Lactate dehydrogenase activity in the organs was estimated using the method of Srikantan and Krishnamoorthi (1955) as modified by Govindappa and Swami (1965). A 5% homogenate (w/v) was prepared in 0.25 M ice-cold sucrose solution, centrifuged at 2500 rpm for 15 minutes and the supernatant was taken as the source of enzyme. The incubation mixture consisted of 1.0 ml of 0.4 M phosphate buffer (pH 7.4), 0.5 ml of 0.1 lithium lactate, 0.1 ml of 0.001 M nicotinamide adenine dinucleotide (NAD), 1.0 ml of 0.004 M 2-(p-indophenol)-3-p-nitrophenyl 1-5-phenylnylertrazolium chloride (INT) and 0.5 ml of 5% enzyme preparation. The mixture was incubated at 37°C for 30 minutes and then the reaction was stopped by adding 6.0 ml of glacial acetic acid. The formazan formed was extracted into 6.0 ml of toluene overnight at the optical density of the colour developed was measured in a spectrophotometer at a wavelength of 495 nm. A blank using 0.5 ml of distilled water and a control taking 0.5 ml of boiled enzyme were also run similarly. INT standards were prepared alongside for comparison. The enzyme activity is expressed as M formazan/mg protein/h.
c. Estimation of Malate dehydrogenase (MDH) (L-Malate NAD- oxidoreductase, EC: 1.1.1.37) activity

Malate dehydrogenase activity in the organs was estimated by the method of Nachlas et al., (1960) with slight modification as suggested by Prameelamma and Swami (1975). A 10% tissues homogenate were prepared in 0.25M ice-cold sucrose solution and centrifuged at 2500 rpm for 15 minutes. The supernatant was taken as the source of the enzyme. The reaction mixture in a final volume of 2.0 ml contained 100 μ moles of phosphate buffer (pH 7.4) + 4 μ moles of INT + 50 μ moles of sodium malate + 0.1 μ moles of NAD + enzyme. This mixture was incubated at 37°C for 30 minutes and the reaction was stopped by adding 5.0 ml of glacial acetic acid. Then the colour was extracted into 5.0 ml 0f toluene by keeping overnight at 50°C and the optical density of the colour developed was read in a spectrophotometer at a wavelength of 495 nm. A blank taking 0.5ml of distilled water and control by taking 0.5 ml of boiled enzyme were also run similarly. INT standards were prepared alongside for comparison. The enzyme activity was expressed as μ M of formazone formed/mg protein/hr.

d. Glucose -6-Phosphate dehydrogenase Activity (G-6-PDH) (E.C:1.1.1.49)

G-6-PDH activity in the cytosolic fraction was assayed according to the method described by Bergmeyer and Bernt (1965). 5% (W/V) tissue homogenates were prepared in the ice cold 0.25N sucrose solution. Centrifuged at 2500 rpm for 15 min. The supernatant is taken for the enzyme assay. The reaction mixture contains 20 μ moles of Glucose-6-phosphate + 100 μ moles of triethanolamine buffer (pH 7.4) + 40 μ moles of INT + 10 μ moles of MgCl2 + 0.3 μ moles of NADP and 0.5ml of enzyme source and reaction mixture are incubated at 37°C for 30 minutes. The reaction is stopped by adding 5ml glacial acetic acid and 5ml of toluene. Kept overnight at 5°C at a wavelength 495nm. The enzyme activity was expressed as μ moles of formazan formed/mg protein/h.
BIOCHEMICAL CONSTITUENTS

a. Estimation of Total Proteins

The Total Protein content was estimated by the method of Lowry et al (1951).

A 1% homogenate (w/v) was prepared in ice-cold 0.25 m sucrose solution. For soluble and structural proteins 1.0 ml of the homogenate was taken and centrifuged at 3000 rpm for 10 minutes. The supernatant was separated and to both the supernatant and residue 3 ml of 10% trichloro acetic acid was added and again centrifuged at 3000 rpm. The supernatants were discarded and the residue was taken for the experimentation. For the total proteins, 1 ml homogenate was taken; to it 3 ml of 10% trichloroacetic acid was added and centrifuged at 3000 rpm. The supernatant was discarded and the residue was taken for experimentation. All the three residues were dissolved in 5 ml of 0.1 N sodium hydroxide and to 1 ml of each of these solutions, 4 ml of reagent- D (Mixture of 2% sodium carbonate and 0.5% copper sulphate in 50:1 ratio) was added the samples were allowed to stay for 10 minutes, at the end the colour developed was measured in UV-Vis spectrophotometer at a wavelength of 600 nm. A mixture of 4 ml of reagent –D and 0.4 ml of Folin phenol reagent was used as blank. Bovine albumin was used for the preparation of protein standards. The protein content was expressed as mg/g wet weight of the tissue.

b. Estimation of Free Amino Acids (FAA)

Free amino acid level in the liver was estimated by the ninhydrin method as described by Moore and Stein (Moore S and Stein WH, 1954). 5% organ homogenates (W/V) were prepared in 10% TCA and centrifuged at 2000 rpm for 15 min. To 0.2 ml of supernatant, 2.0 ml of Ninhydrin reagent was added and the contents were boiled for exactly 5 min. They were cooled under tap water and the volume was made to 10 ml with distilled water. The optical density of the colour developed was measured using a spectrophotometer at a wavelength of 570 nm. A blank using distilled water and amino acid standards were also run similarly. The free amino acid levels are expressed as mg amino acid nitrogen released/g wet wt. of the liver.
c. Estimation of Alanine (DL - alanine: 2-oxoglutarate, EC: 2.6.1.2) (AAT) and Aspartate (L - aspartate: 2-oxoglutarate, EC: 2.6.1.1) Amminotransferase activities (ALAT)

Activities of alanine and aspartate aminotransferases (A1AT and AAT) in the organs were estimated using the method of Reitman and Frankel (1957). A 5% homogenate (W/V) was prepared in 0.25M ice-cold sucrose solution, centrifuged at 3000 rpm for 10 minutes and the supernatant was used as the source of enzyme. Two sets of incubation mixtures were prepared, the first set (for alanine aminotransferase activity) consisted of 0.5 ml of 0.2M alanine, 0.5 ml of 0.005M a - ketoglutaric and (which was prepared in M/15 phosphate buffer and adjusted with 10% sodium hydroxide to 7.4 pH) and 0.1 ml of enzyme. The second set (for aspartate aminotransferase activity) consisted of 0.5 ml of 0.2M aspartic acid, 0.5 ml of 0.005M a - ketoglutaric acid (which was prepared in M/15 phosphate buffer and adjusted with 10% sodium hydroxide to 7.4 pH) and 0.1 ml of enzyme. The mixture was incubated at 37° C for 30 minutes and then the reaction was stopped by the addition of 1 ml of 0.001 M 2, 4- dinifrophenylhydrazine (Ketone reagent). Finally, the reaction mixtures were made to 10.0 ml with 0.4 N sodium hydroxide and the optical density of the colour developed was measured in a spectrophotometer at a wavelength of 545 nm, a blank taking 0.1 ml of distilled water and control taking boiled enzyme was also run similarly. Pyruvate and oxaloacetate standards were prepared alongside for comparison. The alanine aminotransferase activity is expressed as µM pyruvate formed/mg protein/h and the aspartate aminotransferase activity as µM oxaloacetate formed/mg protein/h.

d. Assay of Alkaline phosphatase Activity (ALP) (EC 3.1.3.1)

Alkaline phosphatase (ALP) was assayed by the method of King (1965b) using disodium phenyl phosphate as the substrate. The incubation mixture contained the following components in a final volume of 2.8-ml. 1.5 ml of carbonate- bicarbonate buffer (pH 10.0), 1.0 ml of substrate (0.01 M disodium phenyl phosphate) and 0.1 ml of 0.1M magnesium chloride and 0.2ml of enzyme. The reaction mixture was incubated at 37°C for 15 minutes. The reaction was terminated by the addition of 1.0 ml of 1:2 Folin’s phenol reagent and tubes were centrifuged. Controls without enzyme were also incubated and the enzyme source was added after the addition of
Folin's phenol reagent. 1.0 ml of 15% sodium carbonate solution was added and incubated for a further 10 minutes at 37°C and read at 640 nm in a spectrophotometer against a blank. The standards were also treated as for samples. The activity of the enzyme is expressed as \( \mu \text{mol phenol liberated h}^{-1} \text{ L}^{-1} \).

e. Assay of Acid Phosphatase Activity (ACP) (EC 3.1.3.2)

Acid phosphatase (ACP) was assayed by the method of King (1965b) using disodium phenyl phosphate as the substrate. The incubation mixture contained the following components in a final volume of 3.0 ml - 1.5 ml of 0.1M citrate buffer (pH 4.9), 1.0 ml of substrate (0.01 M Disodium phenyl phosphate), and 0.3 ml of distilled water to 0.2 ml of enzyme solution. The reaction mixture was incubated at 37°C for 15 minutes. The reaction was terminated by the addition of 1.0 ml of Folin's phenol reagent. If turbidity appeared, the tubes were centrifuged. Controls without enzyme sources were also incubated and the enzyme source was added after the addition of Folin's phenol reagent. 1.0 ml of 15% sodium carbonate solution was added and incubated for a further 10 minutes at 37°C. The blue colour developed was read at 640 nm in a spectrophotometer against a blank. Blank and standards were done in the same way. The activity of the enzyme is expressed as \( \mu \text{mol phenol liberated h}^{-1} \text{ L}^{-1} \).

HISTOLOGICAL BIOMARKERS

The fish that were exposed to the pesticide for 3, 7, 15, 30 and 45 days were sacrificed and tissues like liver, gill, kidney, and intestine were quickly isolated and washed in fish ringer (Ekberg, 1958). Histopathology of the tissues was studied by the method of Clayden (1962).

Principle:

Tissue damage at the cellular level caused by the pesticide can be examined and the change in the individual cells can be visualized to ultimately arrive at a conclusive diagnosis by employing microscopic examination of tissue, in which the tissue is sectioned to single cell thickness and stained to differentiate the individual tissue elements.
Materials and Methods

Reagents Required

1. Buffered neutral formalin (10%)
2. Sodium Phosphate (Mono basic) - 40gms
3. Sodium Phosphate (Di basic) - 6.5gm
4. Formalin (37-40°C) - 100ml

The solution was made up to 1 liter with distilled water. The tissues are fixed in buffered formalin for 2 days then they are deformed in running water for 2 to 6hrs.

Processing

The processing of the fixed tissue involved dehydration through ascending grades of alcohol, clearing in the wax miscible agent such as xylene and finally impregnated with wax, it is necessary to remove all water from the fixed tissue. The dehydration is done in atomic tissue processor 2LE Shandon in the following way:

a. 70% Alcohol - 1 hour
b. 80% Alcohol - 1 hour
c. 90% Alcohol - 1 hour
d. 95% Alcohol (i) - 1 hour
e. 95% Alcohol (ii) - 1 hour
f. 95% Alcohol (iii) - 1 hour
g. Absolute Alcohol (i) - 1 hour
h. Absolute Alcohol (ii) - 1 hour
i. Xylol (i) - 1 hour
j. Xylol (ii) - 1 hour
k. Paraffin wax (i) - 3 hour
l. Paraffin wax (ii) - 3 hour

The tissues are then transferred to the blick marker. The tissues are embedded in paraffin wax (58-60°C) blocks. Sections of 5 microns thickness were prepared by microtomy.
STAINING

<table>
<thead>
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<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haematoxyline crystals</td>
<td>1.0gms</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 lit</td>
</tr>
<tr>
<td>Sodium iodate</td>
<td>0.2gms</td>
</tr>
<tr>
<td>Ammonium (or)</td>
<td>50.0gms</td>
</tr>
<tr>
<td>Potassium Alum</td>
<td></td>
</tr>
<tr>
<td>Citric Acid</td>
<td>1.0gms</td>
</tr>
<tr>
<td>Chloral hydrate</td>
<td>50.0gms</td>
</tr>
</tbody>
</table>

Dissolve the alum in water without heating, add and dissolve the haematoxyline in this solution then add the sodium iodate, citric acid and chloral hydrate and shake well until all components are in complete solution. The final colour of the stain is reddish violet and keeps well for months.

Stock 1% Aqueous eosin solution

<table>
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<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosin Y, water soluble</td>
<td>10gms</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>2cc</td>
</tr>
</tbody>
</table>

Working solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosin 1% stock</td>
<td>1 part</td>
</tr>
<tr>
<td>Alcohol 80%</td>
<td>3 parts</td>
</tr>
</tbody>
</table>

If deeper shade of red is desired in staining, add 5cc of glacial acetic acid to each 100cc of strain. After staining, the slides were made into permanent slides using DPX mountant and were examined under light microscope for histopathological changes.

STATISTICAL TREATMENT OF THE DATA

The mean, standard deviation and the test of significance were calculated following the method of Pillai and Sinha (1968). The formula used for calculating standard deviation (SD) was

\[
\frac{\chi^2}{n - 1}
\]

65
Where ‘X^2’ is the sum of the squares of the deviation from the mean and ‘N’ is the number of individual observations. The significance or otherwise of the deviations from normal was known by calculating student’s’ test by the formula

\[ t = \frac{m_1 - m_2}{\sqrt{\frac{SD_1^2 + SD_2^2}{n_1 + n_2 - 2}}} \]

Where m_1 and m_2 are means of observations between which significance is tested. SD_1^2 and SD_2^2 are squares of standard deviation of m_1 and m_2, n_1 and n_2 are the actual number of observations.

**ANOVA**

The data were presented as mean S.D. In addition, analysis of variance (one way ANOVA) followed by Dunnet’s test as post-hoc test was also performed. The differences were considered to be significant at P < 0.05.
CHAPTER – I

(a) Toxicity Evaluation