

IMMUNOHISTOCHEMICAL EVALUTION OF FIPRONIL-TREATED BRAIN OF ZEBRAFISH

Submitted in partial fulfilment of the requirements for the award of

Master of Technology degree in biotechnology

by

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DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF BIO AND CHEMICAL ENGINEERING

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

Accredited with Grade "A" by NAAC

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This is to certify that this Review Report is the bonafide work of **M. Yuvaraj** (Reg. No. **41810021**) who carried out the project entitled "**Immunohistochemical evaluation of pesticide induced degeneration in zebrafish**" under our supervision from **December 2022 to May 2023**.

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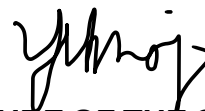
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DECLARATION

I, **M. Yuvaraj (41810021)** hereby declare that the Review Report entitled **“Immunohistochemical evaluation of fipronil-treated brain of zebrafish”** done by us under the guidance of **Dr. Jayshree Nellore (Internal)** and **Dr. R. Rajesh Kannan (External)** at **International Research Centre, Sathyabama institute of science and Technology** is submitted in partial fulfilment of the requirements for the award of Master of Technology Degree in Biotechnology.

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ABSTRACT

Fipronil a chemical pesticide which is commercially available and used in agricultural practices in day to day cultivation of vegetables and other crops which affecting the targeted organism of pests and also majorly affecting the non targeted organism and which leads to neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. This research is based on evaluation of fipronil causing neurodegeneration in central nervous system (CNS) by inducing the chemical to human model of zebra fish and identifying the Lethal concentration (LC50) value followed by histology of the brain of zebra fish. And majorly the tissue is undergoing immunohistochemical analysis resulting the interaction of antigen and antibody which will be evaluating the damages of tissue in CNS which is causing the disease, the analysis of total protein, acetylcholine assay and also behavioural study showing changes in movements of the treated fish like aggression, anxiety, surfacing and darting movements and irregular swimming patterns were noticed in the fish exposed to 300µl and also tissue damages were also observed. This study provides an evidence on the all possible mechanisms underlying the toxicity induced by fipronil, indicating that it is able to modulate CNS in zebra fish.

Keywords: Fipronil, Alzheimer's, Parkinson's disease, Neurodegeneration, Histology, Immunohistochemistry, Behavioural study.

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ABBREVIATIONS

GABA	Gamma-Aminobutyric Acid
TR	Thyroid Hormone Receptor
AD	Alzheimer's Disease
PD	Parkinson's Disease
MND	Motor Neurone Diseases
HD	Huntington's Disease
IHC	Immunohistochemistry
FFPE	Formalin Fix Paraffin Embedded
AR	Antigen Retrieval
LC 50	Lethal Concentration
PBS	Phosphate Buffer Saline
µg/l	Micrograms Per Liter

CHAPTER 1

INTRODUCTION

1.1 ORGANISM MODEL ZEBRAFISH (DANIO RERIO):

One of the most significant vertebrate model species in biomedical research is the zebrafish (*Danio rerio*). Since its inception as a tool for researching genetics and vertebrate development, the zebrafish model has evolved into a potent tool for investigating a variety of human disorders (Tonelli, *et al.*, 2020). In recent years, zebrafish (*Danio rerio*) has become a popular model organism in scientific research. A tropical freshwater fish native to South Asia's Himalayan area, including India, Nepal, Bhutan, Pakistan, Bangladesh, and Myanmar, the zebrafish lives in rivers (mostly the Ganges). Zebrafish share numerous physiological and genetic features in common with humans, including the innate immune system, musculature, and digestive tract. Additionally, 70% of human disease genes and zebrafish genes operate similarly. (Flores, *et al.*, 2020).

With the exception of the lungs, prostate gland, and mammary gland, most of the tissues and organs in humans and zebrafish are identical. Since zebrafish and humans share many traits, the cloning of mutant genes used to screen for certain abnormalities in zebrafish can be used as a model for human disease and to investigate underlying mechanisms. The first human disease discovered in zebrafish involved a blood condition with a specific impairment in haemoglobin production caused by a mutant gene called ALAS2 (Patton, *et al.*, 2021).

Zebrafish are inexpensive, tiny, and simple to raise and keep in big numbers. Zebrafish kept in laboratories are housed in custom aquaculture systems that are kept at 28.5°C with a regulated light-dark cycle. A gravid female zebrafish can naturally spawn hundreds of eggs per week due to their great fecundity. Both fertilisation and embryo development take place outside of the female body. Rapid embryonic development occurs; in under 24 hours, the single-celled zygote transforms into a transparent, motile embryo with a traditional vertebrate body plan. (Small, *et al.*, 2021)

Numerous characteristics of the zebrafish make it a suitable model for studying the biology and spread of human cancer. Between zebrafish and humans, a large number of the variables involved in tumour growth are substantially conserved. There are 26,206 protein-coding genes in the zebrafish genome. 71.4% of human genes were discovered to contain at least one zebrafish orthologue when compared to the human genome, while 82% of human disease-related genes did as well (Astell, *et al.*, 2020).

Zebrafish has been extensively discussed in other places for its general applicability as a model organism and for its application in the genetic and neuroanatomical investigation of larval behaviour. Even though they are more challenging to control than larvae, adult zebrafish exhibit a wide range of mature actions, which makes characterising them very alluring (Fitzgerald, *et al.*, 2021)

1.2 FIPRONIL:

The chemical pesticide class phenylpyrazoles includes the compound fipronil, also known as 5-amino-3-cyano-1-(2, 6-dichloro 4 trifluoromethylphenyl)- 4-trifluoromethyl sulfinyl pyrazole. It is one of the most often used broad-spectrum pesticides for controlling pests in both agriculture and non-agricultural settings. Contrary to carbamates and organophosphates, many insects have evolved resistance to fipronil's traditional insecticides. Compared to mammalian GABA receptors, fipronil has a higher affinity for binding to the GABA-regulated chloride channels found in insects. Fipronil disrupts GABA-gated channels, which results in neuronal excitement, paralysis, and death (Singh, *et al.*, 2021)

The relatively new pesticide fipronil can successfully control a variety of insects with fairly modest field treatment rates. This insecticide interferes with the normal functioning of GABA-gated channels, in contrast to more conventional insecticides like carbamates and organophosphates, to which some insects have developed resistance. It is a "new generation" pesticide because of this. The toxicity and environmental fate of fipronil, which is widely used worldwide, have been the subject of several studies (Abdollahdokht, *et al.*, 2022). Fipronil is routinely applied to crops in agriculture to prevent damage from swarming insects. Fipronil residues have been

discovered in non-targeted aquatic animals as well as everywhere else. A solid is fipronil. To control insects, ticks, lice, and mites on pets, use the pyrazole acaricide and pesticide fipronil.

In human studies most exposure cases (89%) resulted in minor, transient health consequences. The most prevalent symptoms were cutaneous (21%) and visual (44%), followed by gastrointestinal (28%), respiratory (27%), and neurological (50%) symptoms included headache, dizziness, and paresthesia. Exposures typically resulted through unintentional product spraying, splashing, or spilling or from poor ventilation of the treated area prior to re-entry. Human cell cultures were used in an in vitro cytogenetics assay to assess fipronil. At whatever dose level, there was no evidence of an increase in chromosome abnormalities. Fipronil did, however, cause DNA damage in human peripheral blood cells in an in vitro alkaline comet experiment (Bangert, *et al.*, 2019)

Where in animal studies rabbits, there was no evidence of skin irritation. In experiments with female rats, mortality did not occur until 4 days after treatment, and the clinical indications of toxicity did not peak in some animals until 2 days after treatment. When the observation period came to an end seven days following therapy, there were still some toxicity and body-weight loss symptoms present. Since these results suggested that the test substance could bioaccumulate, a 5-day trial with cumulative treatment was carried out in which groups of 4 female rats were given fipronil orally for a maximum of 5 days at a dose of 75 mg/kg bw/day. After the administration of 2 doses, clinical symptoms of neurotoxicity were evident, and 3 of 4 rats perished after the administration of 3 or 4 doses. The lone rat that survived the experiment showed anomalous behavioural reactions that continued for 6 days following the last injection, by which time it had gained the majority of its pretreatment weight back. From the sixth to the twentieth day of gestation, pregnant Wistar rats were given oral doses of 0.1, 1.0, or 10.0 mg/kg/day of a commercial product containing fipronil. Fipronil interfered with maternal hostility; aggression towards a male intruder decreased at the lowest dose but rose at the highest dose in the open field test, without altering with the dams' overall behaviour at either dose. An

examination of the histopathology produced no abnormalities. Fipronil delayed vaginal opening and altered the estrus cycle in pregnant rats, interfering with the development of the newborn female reproductive system without having any discernible effects on fertility. By employing CHO-K1 cells in in vitro reporter gene assays, the agonistic and antagonistic properties of fipronil and its metabolite, fipronil sulfone, were assessed. Both fipronil and fipronil sulfone displayed the same antagonistic oestrogen receptor alpha (ERalpha) activities but no agonistic estrogenic or antiestrogenic actions. Only fipronil sulfone demonstrated anti-thyroid hormone action in the thyroid hormone receptor (TR) assay. The photodegradation product (desulfinyl fipronil) of fipronil was subjected to *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, and TA1538 with negative results (De Barros, *et al.*, 2017).

Ecotoxicity studies say fipronil exposure affected the blood biochemistry and levels of sexual hormones in red-legged partridges (*Alectoris rufa*), while lowering cellular immune response, antioxidant levels, and carotenoid-based colouring. Aside from producing eggs with less antioxidants and kids with a worse cellular immune response, exposed pairings also had lower egg fecundity rates. Applying fipronil involves mixing the two enantiomers equally. After a 7-day subchronic treatment, larval fathead minnows (*Pimephales promelas*) showed signs of enantioselective toxicity, with racemate and (+) enantiomers showing higher toxicity than (-) enantiomers (Lopez-Antia, *et al.*, 2015).

1.3 NEURODEGENERATION AND ITS DISEASES:

Neurodegeneration is a most common term relates to nervous system which is the combination of two words Neuro meaning nerve cells, Degeneration meaning progressive damage. Mostly it damages central nervous system and its connections of mobility, strength, sensation, coordination and cognitions. Neurodegeneration is identified as the pivotal pathophysiological change in most brain-related disorders. Regardless of the incessant efforts by modern science to create a medical or surgical solution, the outcome has not been favorable. The term “neurodegenerative diseases” represents a large group of various diseases of the nervous system with

heterogenous clinical manifestation and diverse histopathological presentation. (Li, *et al.*,2022).

Neurodegeneration is associated with dysfunction of the synapse, neural network, and the deposition of physiochemically altered variants of proteins in the brain. Neurons are majorly defined as the functional unity of human brain which plays a vital role in terms of communications. Major examples of neurodegenerative diseases are as follows: Alzheimer's disease (AD), Parkinson's disease (PD), Prion disease, Synucleinopathies, Motor neurone diseases (MND), Huntington's disease (HD).

1.3.1 Alzheimer's disease

A neurodegenerative ailment known as Alzheimer's disease (AD), which greatly impairs social and vocational performance, is characterised by cognitive and behavioural impairment. It has a protracted preclinical period and a progressive course, and it is incurable. In Alzheimer's disease, plaques form in the cerebral cortex and other regions where thinking and decision-making occur, including the hippocampus, a structure located deep within the brain that aids in memory encoding. It is still unknown whether plaques are a direct cause of AD or if they are a byproduct of the AD process (David S, *et al.*,2021).

According to the Alzheimer's Association (2019), Alzheimer's disease (AD) dementia describes a specific age-related start and course of cognitive and functional deterioration that ultimately leads to death. When Alois Alzheimer first observed Auguste Deter, a 51-year-old woman with cognitive impairment, disorientation, delusions, and other behavioural problems in 1901, he first wrote about her case in 1906. In order to account for the use of biomarkers and the additional capacity to characterise preclinical phases of the disease, the clinical diagnostic criteria for AD dementia were updated in 1984 and further improved in 2011 and 2018.

1.3.2 Parkinson's disease

The impact of Parkinson's disease on society is significant. With over 6 million cases worldwide in 2016, this disease is a widespread ailment in terms of the number of

individuals it affects. The incidence and prevalence of this condition have significantly increased over the past 20 years, for causes that are still not fully understood. The impact of Parkinson's disease on an individual is significant. A degenerative disease's lifetime might last for decades, which is unique. The normal presentation for those who are affected involves a gradual progression with increasing handicap. The effects of Parkinson's disease on careers are likewise severe, with the majority reporting high levels of stress. Parkinson's illness imposes a growing socioeconomic impact on society. With more than 6 million cases worldwide, Parkinson's disease is the second most prevalent neurological condition. The prevalence of Parkinson's disease has increased by 2.5 times in the last 30 years, making it one of the main causes of neurological disability. Neural inclusions in the form of Lewy bodies and Lewy neurites, together with cell loss in the substantia nigra and other brain regions, are the pathological characteristic of Parkinson's disease. Parkinson's disease is categorised as a synucleinopathy because Lewy bodies are primarily made up of aggregated and misfolded α -synuclein species (Wenya, et al., 2020).

Over the past five years, clinical diagnostic criteria that are intended to improve Parkinson's disease diagnosis accuracy have been validated. A clear diagnosis cannot be made from the early stages due to tests or biomarkers, and clinical symptoms of this disorder can overlap with those of other neurodegenerative diseases. As a result, even when the disease has clinically fully developed, clinical diagnostic accuracy is still below ideal. Given that future disease-modifying medicines will have their best chance of success at this stage, prodromal illness identification is necessary. Finally, there is a need to more clearly distinguish the many subtypes of Parkinson's disease, as they require individualised treatment strategies due to their various clinical presentations, prognoses, and underlying disease mechanisms. Monogenic Parkinson's disease is the most prominent example, and there are currently clinical trials testing subtype-specific treatments for this condition (Anthony E., & Alberto J., 2018).

1.4 HISTOLOGY:

The study of tissues and their cellular makeup is called histology. Histology is related to anatomy and physiology since each tissue's structure is directly tied to how it performs. Similar to that, histopathology is the study of diseased tissues in the body. This is something that can be very helpful in making a diagnosis and figuring out how serious and how quickly a condition is progressing. Depending on the kind of tissue, the condition, and the stage of the disease, different disease processes affect tissues in different ways (Daniel P.,2010).

In order to understand the causes of disease and potential treatments, histology is extensively used in biomedical research. While this type of research may be carried out in a hospital laboratory, academic institutions, research institutes, and pharmaceutical companies are more likely to do it. The traditional image of a histopathologist is them looking through a microscope. In reality, most histology work consists of preparing tissues for microscopy, examining slices, and reporting the findings. However, a pathologist can frequently gain valuable information about a tissue without using a microscope. For instance, a person with multiple sclerosis has distinct lesions in their brain that are a few millimetres in size (regions of damage or injury). These are known as plaques, and a tissue sample clearly reveals them.

The Five Phases of Histological Slide Processing

1. Tissue Fixation: Fixing your tissue specimen is the first step in histology slide preparation. To avoid tissue autolysis and putrefaction, this critical stage in tissue preparation is necessary. The cellular tissue samples need to be put into fixative as soon as possible after collection for optimum results. Whereas there are many different types of fixatives, 10 percent formalin with a neutral buffer is used to fix the majority of specimens. The ideal formalin-to-specimen volume ratio ought to be at least 10:1 (10 ml of formalin per 1 cm³ of tissue, for example). Most tissues will be able to become effectively healed as a result within 24 to 48 hours. Formalin containers need to be properly labelled, capped, and leak-proof.

2. Transfer of Specimen to Cassettes

Following fixation, specimens are cut to size with a scalpel so they may fit into a tissue cassette with the proper labelling. Specimens are cut so they don't touch the cassette's edges and should not be large enough to fill the cassette. They must also be thin (preferably less than 4 mm thick), or they run the risk of becoming "waffled" when the cassette's lid is closed. The formalin-filled tissue cassettes are subsequently stored until processing starts.

3. Processing of Tissue

Typically, a paraffin block is used to process tissues into fine microscopic sections as follows: Dehydration is the process of removing both formalin and water from the tissue by submerging your specimen in progressively higher alcohol concentrations. Clearing, which involves removing the alcohol with an organic solvent like xylene to make room for the infiltration of paraffin wax. Embedding, in which the embedding agent, typically paraffin wax, is injected into the specimens. What has since been referred to as the "block" is created when a sizable block of molten wax composed of paraffin surrounds the tissue. After the block has solidified, a support matrix is created that enables very thin sectioning.

4. Tissue sectioning

Your tissue sample is now prepared to be divided into pieces and applied to a slide. To reveal the tissue, wax is scraped off the block's exterior. Before sectioning, blocks are placed on an ice tray or cold plate for ten minutes. Extremely thin ribbon-shaped tissue slices are cut from the block using a microtome. Although the microtome is able to be programmed to cut at various thicknesses, the majority of tissues are processed at a thickness of about 5 μ m.

5. Staining

When unstained, most cells are translucent and practically colourless. In order to create contrast to tissue slices, histochemical stains (usually hematoxylin and eosin) are utilised. This makes the structures of tissues more visible and easier to assess. To assist protect the specimen after staining, a cover slip is secured over the tissue

sample on the slide using optical grade adhesive (Gabriela Zimmermann Prado, *et al.*,2020).

1.5 IMMUNOHISTOCHEMISTRY

Using appropriately-labeled antibodies that attach selectively to their target antigens in situ, immunohistochemistry (IHC) integrates anatomical, immunological, and biochemical approaches to scan discrete components in tissues. IHC enables the excellent quality distribution and localisation of particular cellular elements within cells as well as their appropriate histological context to be seen and documented. IHC methodology allows for a variety of ways and combinations, but all of the stages are divided into two categories: preparation of samples and sample staining. A technique used in laboratories to utilise antibodies to look for specific antigens (markers) in tissue samples. Typically, an enzymatic agent or a dye with fluorescent properties is attached to the antibodies. The enzymatic agent or dye is made active once the antibodies attach to the antigen on the tissue specimen, allowing the antigen to be observed under a microscope. Diseases like cancer can be diagnosed via immunohistochemistry. It might also be employed to distinguish between various cancer kinds. Although the IHC concepts have been understood since the 1930s, the first IHC research was not disclosed until 1942. Pneumococcal antigens in tissue that was infected were identified by using FITC-labeled antibodies. Since that time, significant advancements have been made in the techniques for tissue fixation and sectioning, antigen/epitope retrieving, antibody conjugation, methodologies and reagents for immunostaining, as well as microscopy itself (Shino, *et al.*,2019). IHC is become a common yet crucial tool in medical and research laboratories as a result. IHC is utilised in the biological sciences, medication development, and the diagnosis of diseases. For instance, utilising certain tumour markers, doctors can utilise IHC to evaluate a tumor's stage and grade, as well as to identify the type of cell and place of source of a metastasis so as to locate the primary tumour. IHC is used to make a primary diagnosis or to confirm a diagnosis of a number of other non- neoplastic illnesses and disorders. IHC can be employed alone or in combination using other analytical techniques in a research setting to investigate a variety of

topics, including disorders such as healing of wounds, cell death and repair, and normal tissue and organ growth and development. By identifying the activity or elevation or decrease of disease biomarkers in targeted tissues and elsewhere, IHC is also utilised in medication development for evaluating drug efficacy. The immunostaining of tiny tissue sections that are affixed to separate glass slides is the basis of conventional IHC. A tissue microarray is a format in which numerous tiny sections can be put on a single plate for comparison examination. IHC slides are often made, handled, and stained by hand or in teams (David B., *et al.*, 2020).

However, high quantities preparation of samples and staining are now possible because to modern technology. Light or fluorescent microscopy can be used to view samples, and improvements in the previous 15 years have enhanced our capacity to take pictures, quantify multiparametric IHC information, and expand the gathering of that information through extensive screening. Frequently employed in anatomic pathology during surgeries for cell categorization and diagnosis, immunohistochemistry (IHC) uses antibodies directed against specific antigens in particular tissues and cells to make it easier to identify the variety of cell and organ of origin. Although the technique was initially developed on sections that were frozen and can also be applied to tissue embedded in plastic, it is most frequently used on formalin fix paraffin embedded (FFPE) tissue because it has the benefit of being adaptable to easy storage. Antigen retrieval (AR), primary antibody addition, secondary antibody addition that attaches the primary antibody, then detection reagent addition to localise the primary antibody are the successive processes in IHC. Antigen retrieval (AR), the initial step in IHC, includes pretreating tissue to extract antigens obscured by fixation to render them more amenable to antibody binding. Both the primary antibody and secondary antibody is where is directed towards the immunoglobulin of the organism from which this primary antibody originated, must be labelled in order to see the antigen antibody association under light microscopy. The primary antibody was quickly labelled and employed to the tissue using the direct method; however, this approach is less frequently used because it does not amplify the signal, necessitating higher antibody concentrations and the labelling of every primary antibody. The secondary antibody has been labelled in the indirect technique,

enabling signal amplification and application with a variety of primary antibodies. When performing IHC, there are numerous crucial processes. These involve handling the sample with care, fixing it appropriately, making a paraffin block, removing the antigen, choosing and making an antibody and reagents, incubating, washing, and counterstaining (Chung-Hsin, *et al.*,2021).

CHAPTER 2

REVIEW OF LITERATURE

2.1 FIPRONIL TOXICITY:

MicroRNA (miRNA) was a crucial component in the regulation of gene expression. There is no information, however, regarding the way the pesticide treatment changed the zebrafish (*Danio rerio*) miRNA expression. Zebrafish displayed diverse patterns of miRNA expression after being subjected to various chemicals. It was found that these substances were connected to the production of 21 miRNAs 96 hours after treatment. Fipronil, triazophos, the combination of the treatments caused differential expression in 14 of them; these treatments also modified the expression of 5 of them; and adjuvant treatments changed the expression of miR-29b and miR-738. Future research on the relationships between miRNAs and diseases brought on by chemicals may use miRNAs to show a distinct toxicological response that might be used as a toxicological biomarker (K. D & Singh., 2019).

Fipronil, a phenylpyrazole insecticide, is effective and used in many different sectors of the economy. Fipronil was especially reliable because it was understood that it only affected invertebrate species and not vertebrate ones like mammals. However, because fipronil blocks GABA receptors, that are present in both invertebrates as well as vertebrates, it may be dangerous to vertebrate animals. It was crucial to explain the detrimental impact of fipronil on vertebrates. Zebrafish (*Danio rerio*), which symbolise vertebrate animals, were used for this. This study used NMR-based metabolomics to show how fipronil ingestion in zebrafish altered their metabolism. According to a metabolic study, fipronil interfered with the ability of zebrafish to employ antioxidants for energy metabolism. Antioxidant capability was considerably diminished at high exposure dosages. Additionally, the results of metabolic tracking over time showed that zebrafish have reactions to lessen the excessive neuronal excitation after being exposed to fipronil (Yasmina K., *et al.*, 2021).

Environmental pollution caused by human settlement and business activity, which results in a range of polluted discharges reaching natural water resources, is the

reality of the modern world. In a number of locations and countries, agrichemical leftovers from plant production have been discovered. Among these agrochemicals, we looked into a glyphosate-based herbicide (GBH), a fipronil-based insecticide (FBI), or their combinations (GBH+FBI). Zebrafish who are exposed to between three and five mg/L of GBH spend the majority of their time at the top zone. We got to the conclusion that glyphosate-based herbicides and fipronil-based insecticides, as well as their mixtures, alter the behavioural patterns of zebrafish, which may have a big impact on the survival of the species as well as the food chain and the ecosystem.

The newly developed pesticide fipronil can successfully control a variety of insects with fairly modest field treatment rates. This insecticide interferes with the normal functioning of GABA-gated channels, in contrast to more conventional insecticides such as carbamates and organophosphates, to which some insects have developed resistance. It qualifies as a "new generation" pesticide because of this. The toxicity and environmental fate of fipronil, that is widely used worldwide, have been the subject of several studies. This work evaluates, summarises, and integrates significant toxicological and environmental destiny information from recent scientific publications and other literature to produce a full evaluation of fipronil. Japanese Society for Pesticide Science (Mohammad & Mohammad Saghir., 2022).

2.2 GROWTH DEVELOPMENTAL DEFECTS:

As part of the experiment, fish that were getting ready for the spawn and the actual spawns were exposed to three distinct concentrations of the experimental substance. After the exposure continued for 21 days, all three groups in the experiment including the control group experienced 100% survival. While the effects on reproductive parameters were evident even with the least concentration of fipronil employed, exposure to the maximum dosage dramatically decreased the spawn produced and also in fecundation and hatching percentages in comparison to the control group.

Although it is currently unclear whether or not the broad-spectrum chemical insecticide fipronil causes significant neurotoxicity in aquatic animals that are not its intended targets, as well as what molecular mechanisms underlie it. Only a few research have looked at the genetic mechanisms underlying the chiral herbicides'

excessively enantioselective toxicity. The epigenetic modifications caused by pesticides, notably DNA methylation, are another research need. R-fipronil caused more pronounced anxiety-like behaviours in zebrafish embryo and larval stages than S-fipronil did, including increased speed of swimming and improper photoperiodic locomotion. The MeDIP-Seq evaluation, along with the Gene Ontology analysis and KEGG, demonstrated that R-fipronil impacted five pathways of signalling (MAPK, Calcium signalling, Neuroactive ligand-receptor interaction, Purine metabolism, and Endocytosis) for a greater degree than S-fipronil by causing hypermethylation of a number of crucial neuro-related genes, while no significant alterations of global DNA methylation were observed on the two enantiomers. We concluded that the mechanism via that fipronil-induced enantioselective neurotoxicity exerted its enantioselectivity was most likely dysregulation of DNA methylation. The analysis of enantioselective biological impacts and pertinent underlying processes of chiral insecticide were also given new epigenetic insights by this research (Whasun Lim, & Gwonhwa Song., 2021).

2.3 FIPRONIL CAUSING NEUROTOXICITY:

A phenylpyrazole insecticide called fipronil has the potential to specifically block the gamma-aminobutyric acid receptor in insects. While fipronil is the most often used pesticide in aquatic habitats, there aren't many studies that have looked at its neurotoxicity for aquatic species' sensory and motor systems. We examined the impact of acute fipronil treatment on zebrafish (*Danio rerio*) survival rates, lateral line hair cell counts, and neurotoxicity. Heat maps and swimming trajectory speed and distance were also examined between zebrafish that received sham and fipronil therapies. To assess the expressions of proteins linked to oxidative stress, inflammation, apoptosis, neurodegeneration in the brain tissue of adult zebrafish with pseudo and fipronil treatments, Western blot analysis and immunohistochemistry were carried out separately. According to this findings, adult zebrafish who were exposed to fipronil had significantly lower survival rates and a shorter swimming distance. The findings revealed that embryos of zebrafish exposed to fipronil had considerably fewer hair cells in their lateral lines. Significant oxidative damage, inflammation, and apoptosis were found during the brain tissue of adult zebrafish that

was exposed to fipronil in histopathological and Western blotting studies. By damaging lateral hair follicles and tissue in the brain via oxidative stress, inflammation, and apoptosis, fipronil toxicity can affect the sensory and motor systems in zebrafish. This in turn leads to a much lower survival rate and impaired mobility. Zebrafish who receive fipronil poisoning should have their behavioural responses studied forenhancing our knowledge of the behavioural biomarkers' dependability in environmental toxicology risk assessment (Smitesh, *et al.*, 2019).

The majority of chemical control is required to maintain sugarcane, which is produced in Brazil to the highest level in the world. Two of the most frequently employed pesticides, the insecticide fipronil and the herbicide 2,4-D, have adverse impacts on the ecosystem. The goal of the current study was to explore the toxicological mechanisms of the herbicides DMA® 806 BR and Regent 800 WG (a.i. 2,4-D and fipronil, respectively). It specifically intended to ascertain if contamination by 2,4-D and fipronil can result in avoidance behaviour in the fish *Hyphessobrycon eques* (serpae tetra or mato-grosso) and *Danio rerio* (zebrafish). In addition, swimming behaviour and acetylcholinesterase action, a biochemical indicator of neurotoxicity, were taken into account while assessing the effects on fish (AChE). The swimming activity (distance traversed) and AChE were inhibited in *D. rerio* after exposure to fipronil. 2,4-D reduced the maximal swimming speed of *H. eques* while leaving AChE unchanged. Avoidance behaviour did not appear to be affected by the possible effects of contaminants on swimming behaviour or AChE activity. This study illustrated the significance of comprehending fish avoidance ability, swimming behaviour, and neurotoxic effects of pesticides, which can be beneficial as ecologically relevant tools for ecological risk assessment, in an incorporated and realistic setting involving exposure to pesticide-contaminated environments.

The phenylpyrazole insecticide fipronil particularly inhibits gamma-aminobutyric acid (GABA) sensors on insects. Despite the fact that fipronil is frequently used in or near aquatic ecosystems, little research has been done to determine how this neurotoxic affects aquatic species at vulnerable phases of development. We looked at the toxicological impact of fipronil on eggs and larvae using the zebrafish (*Danio rerio*) as an experimental model system. Embryos treated to fipronil at standard concentrations

in excess of 0.7mM (333 mg/l) demonstrated degeneration of the notochord, shortening across the rostral-caudal body axis, failed tails flips, and disorganised muscle contractions along the entire body axis in response to touch. To offset the degenerative effects of fipronil (1.1mM) exposure's 0.6mM degenerative effects, sodium channel blocker MS-222 was also administered. The notochord-specific effects of fipronil were phenocopied by exposure to 70mM strychnine, a glycinergic receptor antagonist. Contrarily, when gabazine was introduced to animals, it did not result in notochord degeneration but rather a hyperactive touch response. This indicates that fipronil affects a structurally similar glycine receptor subtype in fish, whereas being specifically made to target insect GABA receptors with low vertebrate toxicity. Fish spinal locomotor circuits are disrupted as a result. This suggests that vertebrates may have a novel and unanticipated method of fipronil poisoning (Ozge Nur & Guldeniz., 2020).

Both compounds completely inhibited each and every investigated glycine receptor subtype with IC₅₀ values that ranged from 0.2-2 mM, mirroring their potencies for vertebrate GABA receptors. According to molecular docking predictions, both lindane and fipronil connected with 6^l threonine receptors through bonds of hydrogen and hydrophobic interactions. Contrary to predictions, no evidence for lindane-lindane interaction on the 2^l level was found. We demonstrate that fipronil interacts to the anaesthetic binding region in a non-blocking manner and that lindane is a highly efficient pharmacological technique for determining the presence of b subunits of ab heteromeric glycine receptors.

Fipronil (FIP) is a widely used phenylpyrazole pesticide and veterinary drug in different parts of the world. FIP was the first insecticide to specifically target the GABA(c-aminobutyric acid) receptor. Compared to mammals, it is more specifically harmful to insects. However, there is mounting proof that FIP may have harmful consequences on humans and animals, including reproductive, cytotoxic, hepatotoxic, and nephrotoxic impacts on vertebrate and invertebrate organisms. This is a result of unintentional exposure, poor usage, or extensive use of FIP that contaminates soil and water. Oxidative stress has been scientifically linked to several FIP-induced toxicities within the past ten years. There has not yet been a thorough investigation

into the toxicity of FIP in relation to oxidative stress. Studies have been conducted to ascertain the association between reactive oxygen species (ROS) and oxidative stress, which are formed as a result of FIP therapy, and various types of toxicity. The metabolism of FIP, which involved several CYP450 enzymes and could result in oxidative stress, was also covered. The functions of various substances in avoiding it based upon their anti-oxidative activities were also summarised in order to better understand the function of oxidative stress on FIP-induced damage (Xiao, *et al.*, 2021).

2.4 BEHAVIOURAL CHANGES:

In agricultural areas, pesticides invariably make their way to water bodies through runoff or leaching. Due to the tremendous preservation of the nervous system across phyla, pesticides, despite having been created to be neurotoxic to insects, have negative effects on a variety of creatures. It is crucial to look into how pesticides affect non-target animals like fish in order to estimate their ecological consequences. We used zebrafish as a model to examine how various pesticide classes affect fish behaviour. We discovered the neural mechanisms underlying the resulting behavioural alterations, offering a previously unattainable perspective on how fish perceive these chemicals. We found that whereas zebrafish larvae do not react to other insecticides with a comparable method of action, they avoid diazinon and imidacloprid. Additionally, olfactory deprivation eliminated the aversive reactions, suggesting that fish could smell the pesticides. The hypothalamic regions that regulate the response to stress were among those areas with the greatest changes, according to an assessment of neural activity in 289 different brain regions. This finding suggests that the observed behavioural response is similar to responses to stimuli that compromise homeostasis, such as shifts in water chemistry. Our findings serve to clarify how pesticide exposure affects the ecosystem and can improve assessments of acute toxicity (Fabiele, *et al.*, 2019).

Children who are regularly exposed to chemical-based pesticides throughout their formative years may develop a multitude of ailments. A number of factors, such as parental ages, maternal smoking, labour and delivery issues, and early encounters

with poisons like pesticides, might contribute to autism spectrum disorder (ASD). This study examined the possibility of protective impact of vitamin C (Vit. C) from children's drugs on juvenile zebrafish for swimming skills, interpersonal behaviour, and oxidative stress related to the ASD model following 14 days of chronic exposure to the insecticide mixture fipronil (Fip) + pyriproxyfen (Pyr). In addition to reducing swimmingability and raising the oxidative stress markers SOD (superoxide dismutase), pesticides can cause problems with zebrafish behavioural patterns for 14 days following exposure. Glutathione peroxidase and malondialdehyde, or MDA. The vitamin C supplement markedly reduced the oxidative damage and neurotoxicity associated with the pesticide mixture. Through the use of juvenile zebrafish exposed to the harmful insecticide fipronil and pyriproxyfen over an extended period of time, this study provides possible in vivo evidence that vitamin C supplementation may mitigate the oxidative stress and damage to the brain brought on by those exposures (Yi, *et al.*, 2019).

Using zebrafish as a model organism to research how environmental toxins affect behaviour. Zebrafish are perfect for high-throughput behavioural investigations. Each day, hundreds of fertilised eggs can be removed from a single tank, and the synchronously growing offspring can then be placed on a culture vessel or multiwell plates to be exposed to environmental toxins. The zebrafish embryonic motor network has been effectively characterised at several levels, including behaviour, circuitry, and genes, providing a strong starting point for research into the mechanisms behind neurobehavioral toxicity. The use of three tests to check for behavioural abnormalities brought on by toxicants in embryonic zebrafish is reviewed and appraised critically. Because identical genes, neurotransmitters, and hormones regulate the early stages of brain development in vertebrate species, the mechanisms of neurological growth are well conserved (Madalina Andreea, *et al.*, 2020).

MicroRNAs (miRNAs), a family of tiny non-coding RNAs, may alter the expression of many protein-coding genes when an organism is subjected to an environmental chemical. We have previously demonstrated that fipronil treatment significantly reduced the expression of miR-155 in adult zebrafish (*Danio rerio*). Fipronil is 5- amino-1-[2,6-dichloro-4-(trifluoromethyl) phenyl]-4-[(trifluoromethyl) sulphonyl]- 1H-

pyrazole-3-carbonitrile. However, actual confirmation of the control of the predicted target gene of this miRNA, cyb561d2, an element of the cytochrome b561 (cyt b561) family implicated in transport of electrons, cell defence, and chemical stress, has not yet been made. In this study, we evaluated how fipronil affected the zebrafish miR- 155 and cyb561d2 genes.

The transcription of miR-155 in was downregulated in response to fipronil stimulation, although cyb561d2 was upregulated at both the protein and mRNA levels in a dose-dependent way. The dual firefly report assay demonstrated that miR-155 interacts with the 3' UTR of cyb561d2. The gene expression of cyb561d2 increased in both mRNA and protein quantities when spontaneous miR-155 was reduced by transfection using a miR-155 inhibitor, in contrast (Janiel K. Ahkin Chin., *et al*, 2020) . Our understanding of the molecular pathways behind toxicity following fipronil exposure has been improved by the findings, which also point to miR-155 as a potential novel toxicological biomarker for exposure to chemicals.

CHAPTER 3

AIM AND OBJECTIVE

3.1 AIM:

To evaluate the effects of fipronil on zebrafish brain by immunohistochemical study and behaviour changes.

3.2 OBJECTIVE:

- ✓ Determination of LC50 for Fipronil treated adult Zebrafish.
- ✓ Histological analysis of fipronil treated adult zebrafish brain.
- ✓ Immunohistochemical analysis of fipronil treated adult zebrafish brain.
- ✓ Total protein quantification and acetylcholine quantification of treated zebrafish.
- ✓ Behavioral responses of fipronil treated zebrafish.

CHAPTER 4

METHODS AND METHODOLOGY

4.1 ZEBRA FISH GROWTH MAINTENANCE:

The adult zebra fishes (*Danio rerio*) are taken in tanks. Each tank is provided with the required density of fishes per liters of water. In the total of required fishes per tank. A constant light and dark cycle is maintained as 10 hours of light exposed and 12 hours of dark exposed. The temperature of the room in which the fishes were kept is maintained for about 25° C (Peter, *et al.*, 2020)

4.2 LETHAL CONCENTRATION (LC 50) OF FIPRONIL TREATED FISHES:

Standard working solution of fipronil is made with 1 gram of fipronil in 1 liter of water which is used as pesticide in agricultural activity. And the process starts with formulation of fipronil and fish tanks for the treatment. The adult zebra fishes are taken in four tanks in order of different concentration for addition of fipronil. Each tank is provided with the 40 fishes per four liters of system water. And a control tank is kept without treating with fipronil. To standardize the concentration of fipronil the lethality is checked by calculating the LC50. The concentration of fipronil with least lethality is considered and fishes are exposed to fipronil treated tanks. The various concentrations 50µg/l, 100µg/l, 300µg/l of fipronil is added to the tanks respectively. And the treatment time period is one week. Where treated fishes are analyzed at different time intervals (Maria Cristina., *et al*, 2022). The concentrations and time interval is listed in the below table.

Table 4.2.1: Concentrations used for treatment

S.No	Fipronil	No. of fishes	Treatment period
1.	50µl	40	7 days
2.	100µl	40	7 days
3.	300µl	40	7 days
4.	Control	40	7 days

4.3 DISSECTION OF ZEBRAFISH BRAIN:

Four months old fishes were sacrificed by placing them in tricaine solution for 5 minutes. The head region of the fish is dissected. The skull was opened from the dorsal region of the head using fine forceps and needles. The brain was carefully dissected out without disturbing the tissues. The dissected brain of zebrafish is shown below

The dissected entire brain is washed 3 times in 1x Phosphate Buffer Saline (PBS) and analyzed under optical microscope to observe the morphology. The brain structures consist of forebrain and hindbrain were observed under microscope (Mehmet Ilyas, *et al.*, 2020).

4.4 HISTOLOGY OF ZEBRAFISH BRAIN

4.4.1 Tissue processing

Zebrafish brain was dissected out by whole fish is exposed in tricaine solution for 5 minutes were fish sacrifice and the skull is opened were brain is dissected out by fine needle and scalpel and the brain tissue is processed by the fixation of tissues in 10% formaldehyde overnight at room temperature. After overnight fixation the tissues is washed with distilled water and the dehydration process is done using series of ethanol in various concentration, xylene and different time intervals as described in Table 4.4.2. After processing of brain tissue, it is placed in paraffin wax which is made as blocks by melting it at 60°C and formulating it as blocks where the tissues is fixed in

right position to get clear cross sections of lock (Guihua, *et al.*,2019). The paraffin block is allowed to settle for 3 to 4 hrs.

Table 4.4.2: Tissue processing procedure

S.No	SOLUTION	PROCESSING TIME
1.	10% Formaldehyde	Overnight at 25 °C
2.	Washing (Distilled Water)	2 minutes
3.	70% ethanol	30 minutes
4.	80% ethanol	30 minutes
5.	90% ethanol	30 minutes
6.	100% ethanol	45 minutes
7.	Xylene	30 minutes

4.4.2 Tissue sectioning and embedding

After the block is allowed to set the tissue of durations of 4hrs, the blocked tissue is sectioned by placing it in the microtome. Where the sectioning is taken place. The block is kept on holder and blade is placed on the stand. The rotatory process of microtome which makes the sections of tissue embedded with wax in the form of blocks sliced in the blade and forming sections. The microtome sections the tissue with the thickness of 5 microns. The segments of sections in the forms of ribbons which placed on the warm water of temperature of 37°C which makes the segments wrinkles to be removed which is formed during slicing of tissue. Parallely the glass slides are coated with albumin and allowed to dry heat for 5mins on hotplate at 40°C. And the slide is taken out and dipped on the warm water were tissue is placed. Tissue is taken up by the glass slide coated with albumin. And the slide is air dried and conditioned to further process.

4.4.3 Hematoxylin and eosin staining

The dried sectioned tissue is rehydrated by series of ethanol and stained by H&E staining as given in the Table 4.4.3. Initially the dried sections of tissue will undergo rehydration by ethanol series followed by staining of hematoxylin and eosin with xylene at the end. The stained sections of tissue will undergo mounting process using DPX mounting, where drop of DPX is kept on the stained tissue region of the slide and the cover slip is applied over it. And the mounted sealed slides were air dried overnight before imaging. The histological sections of tissue mounted with stains will be imaged in optical stereo microscope (Karolina, *et al.*, 2020).

Table 4.4.3: Hematoxylin and Eosin Procedure

S.No	REAGENTS	PROCESSING TIME
1.	Xylene	15 minutes
2.	Air dry	2 minutes
3.	100% Ethanol	5 minutes
4.	90% Ethanol	5 minutes
5.	80% Ethanol	5 minutes
6.	70% Ethanol	5 minutes
7.	Rinsing (Distilled water)	3 minutes
8.	Air dry	2 minutes
9.	Hematoxylin	7 minutes
10.	Rinsing (Distilled water)	3 minutes
11.	Air dry	2 minutes
12.	Acid alcohol	3 dips
13.	Rinsing (Tap water)	3 minutes

14.	90% Ethanol	2 dips
15.	2% Eosin	1 dip
16.	90% Ethanol	3 minutes
17.	100% Ethanol	3 minutes
18.	Air dry	2 minutes
19.	Xylene	30 seconds
20.	Air dry	2 minutes

4.5 IMMUNOHISTOCHEMISTRY OF ZEBRAFISH BRAIN

4.5.1 Tissue processing:

Zebrafish brain was dissected out and the tissue fixation process is carried out by overnight incubation of tissue sample in 10% formaldehyde at room temperature. After the incubation, the tissue sample is washed with distilled water and dehydrated by series of ethanol of different concentrations at various time intervals as the process done for histology. After the process is done the tissue is kept in paraffin wax and allowed to settle for at least 3 hours (Ekrem, *et al.*,2023).

4.5.2 Tissue sectioning and embedding:

After the settlement of the waxed blocks containing the tissue was sectioned in rotator microtome with thickness of 5 microns. The sectioned ribbons spread in warm water to remove wrinkles created by the paraffin wax. And the tissue placed on the albumin coated glass slide as done in the process of histology (Elke, and Mario F., 2002).

4.5.3 Deparaffinization and rehydration:

The sectioned tissue glass slides are immersed in xylene for 10 minutes of 3 washes for total time of 30 minutes. After this process the slides are immersed in sequence of

ethanol concentrations 100%, 80% and 70%. Further carried with immersing the slides in two different charged of deionized water and rest in the water for 5 minutes.

4.5.4 Antigen retrieval:

HIAR is one of the antigen retrieval techniques. As they typically have a higher success rate for restoring antigenicity than an enzymatic method, HIAR using amicrowave or a pressure cooker is a popular method. Below are some examples of HIAR protocols using a pressure cooker or a microwave. It is important to follow the manufacturer's directions because other pressure cookers may utilise different procedures. It is emphasised that just one HIAR technique needs to be used—not the pressure cooker and the microwave. In this process were are using microwave for HIAR (Antonio, *et al.*, 2016).

Microwave oven

1. Submerge slides in 3% hydrogen peroxide for five minutes after deparaffinization and rehydration.
2. For five minutes, rinse slides with deionized water.
3. Microwave slides at 100 C for 5–10 minutes after dipping them in retrieval buffer (10 mM citrate, for example). Maintain a sufficient buffer level during the heating process.
4. Let slides cool at room temperature for 15 minutes.
5. Use deionized water to twice wash slides.
6. Phosphate buffered saline slides for five minutes.

4.5.5 Blocking with serum or universal blocking buffer:

Slides are dip in 0.1% TBS-Tween and it is treated by universal blocking buffer made of 1%BSA, 0.5% TritonX100, 0.05% Ampicillin and 0.01M PBS were solution is prepared and 3 to 5 drops of the universal blocking solution is applied on the slide for 15 minutes. Followed by rinsing with 0.1% TBS-Tween (José A., 2011)

4.5.6 Antibody application

1. A series of dilutions of a highly concentrated antibody are required to find the concentration that will result in the target antigen staining being the strongest and the background staining being the least. Typically, it is simplest to begin with the manufacturer's suggested dilution and add one dilution above along with a dilution below it. For instance, testing following dilutions on 1:200, 1:400, and 1:800 is likely to reveal the best signal to stain the background on one of the dilutions if the advised dilution is 1:400.
2. Apply 200 µL of diluted the primary antibody on slides and incubate for 80 minutes at room temperature (or for the amount of time recommended by the primary antibody, if applicable).
3. Use three distinct staining dishes and three dosages of 0.1% TBS-Tween for a total of three washings that last nine minutes.
4. Use a species-specific secondary antibody to coat slides as directed by the required conditions were the secondary antibody is added with stain of DAAP, which is providing the required condition for the staining process.
5. Use a new staining plate for each of the three washes, and wash for a total of 15 minutes in three separate applications of 0.1% TBS-Tween.

4.5.7 Mounting:

The immunohistochemical stained sections of tissue will undergo mounting process using DPX mounting, were drop of DPX is keep on the tissue region of the slide and the cover slip is applied over it. And the mounted sealed slides were air dried overnight before imaging. The immunohistochemical sections of tissue mounted with stains will be imaging in optical stereo microscope (Shino, *et al.*, 2019).

4.6 QUANTIFICATION OF TOTAL PROTEIN AND ACETYLCHOLINE

4.6.1 Total protein quantification:

The Dissected zebrafish brain is crushed in phosphate buffer and centrifuged at 3000 rpm for 10 minutes at 4 degrees Celsius which is process of homogenization. Supernatant is poured in well plate along with standards and BCA (bicinchoninic acid) is poured. The sample in well plate is mixed thoroughly and incubated at 37 degrees Celsius for 30 minutes in 'Multimode Plate Reader (Enspire)'. Then the sample is cooled at room temperature and absorbance is measured at 562nm on Multimode Plate Reader (Bisei, *et al.*, 2020).

4.6.2 Acetylcholine quantification

The Dissected zebrafish brain is crushed in phosphate buffer and centrifuged at 3000 rpm for 10 minutes at 4 degrees Celsius which is process of homogenization. Supernatant is poured in well plate and Ellman's reagent - DTNB (5,5'-dithiobis-2-nitrobenzoic acid) and Acetylcholine iodide is added. Then the absorbance is measured at 412nm in 'Multimode Plate Reader' (Shasha, *et al.*, 2021).

4.7 BEHAVIOURAL STUDIES

4.7.1 Treated behaviour assay

The major aim of the experiment is to measure the behavior changes of the fipronil treated adult zebra fish by measuring movements of the fish in the tank. The tank is made of glass of 15 x 10 x 30 cm and filled with system water at room temperature. The tank is light from below the tank of white light.

Fishes are placed on the tank and their movement is recorded for 10 minutes using video camera. The data is stored in AVI's format. If fish freezes for more than 30 seconds during the video recording period it should be excluded from final analysis. The recorded video is sent to TRACKER software where its tracks the fish movements in the tank and tracked path values are obtained which is stored in Ms excel for analysis. Parameters that can be analyzed the movement of fish throughout the tank. And this

process is done for the treated fishes of different concentrations and different time intervals 24 hrs, 48 hrs, 72 hrs and 168 hrs (J., *et al.*,2015).

4.7.2 Aggression behavioral assay

The aim of the experiment is to measure the aggression levels of the fipronil treated fishes are measured by their mirror image interaction. The aggression tank is made of glass where three walls of the tank are covered in white opaque material. The craft rubber is inert and does not distract the fish. A mirror placed outside of 4th wall, offset at an angle of 22.5 degrees. The tank is 15 x 10 x 30 cm and filled with system water at room temperature. The tank is lit from below infrared light and above white light.

Fishes are placed singly into the aggression setup and their reaction to the mirror is recorded for 5 to 10 minutes using video camera. The data is stored in AVIs format. Fish freezes for more than 30 seconds during recording then it should be excluded from the final analysis of results. The films should be analyzed blind and by two independent observers. Accuracy of the results can be judged using the pearson correlation. Results are copied to Ms excel for analysis. Parameters that can be compared include number of bites, pushes and tail thrashes directed against the fish in the mirror. And this process is done for the treated fishes of different concentrations and different time intervals 24 hrs, 48 hrs, 72 hrs and 168 hrs (Keith B.,2011).

4.7.3 Place preference boldness assay

The objective of the experiment is to measure the boldness by recording the amount of time spent on the non preferred side of a biased place of tank for preference. The tank is made up of glass with cover of color papers. One side of the tank is cover with white color and other side is brown color. Two black paper spots, 5 cm in diameter are placed in the middle of the white area of the tank. The tank is 15 x 10 x 30 cm and filled with system water and light from both below (infrared light) and above (white light).

A single adult fish is gently placed into the tank setup and its movement is video tracked for 10 minutes. The fish is been removed after tracked, this process is

repeated for the other entire group. Any fish freezes for a significant period during experiment are removed, since this will bias the amount of time spent on either side of the tank. The results are exported to Ms excel for analysis. Parameter that can be compared including the percentage of time spent on the either side of the tank and the total distance swim during the experiment. And this process is done for the treated fishes of different concentrations and different time intervals 24 hrs, 48 hrs, 72 hrs and 168 hrs (Lee David, *et al.*, 2012).

4.7.4 Novel tank

The main application of this experiment is to measure the amount of time spent on the upper and lower region of the novel tank. The tank is 15 x 10 x 30 cm and filled with system water at room temperature (25°C). Fish is introduced to novel tank and reaction is recorded for 10 minutes using video camera. The data is stored in AVIs. Fishes freezes for more than 30 seconds during process then it should be excluded. Results copied to Ms excel for analysis. Parameters that can be compared include the percentage of time spent on upper and lower region of the tank and total distances swim during the process. And this process is done for the treated fishes of different concentrations and different time intervals 24 hrs, 48 hrs, 72 hrs and 168 hrs (Farooq, *et al.*, 2012).

CHAPTER 5

RESULTS AND DISCUSION

5.1 FIPRONIL TREATED ZEBRA FISH AT VARIOUS CONCENTRATION AND ITS LC50 VALUE:

The various concentrations 50 μ l, 100 μ l, 300 μ l of fipronil is added to the tanks respectively. And the treatment time period is one week. Where treated fishes are analyzed at different time intervals.



Fig 5.1.1: Various concentrations of fipronil treated zebrafish

Lethal Concentration (LC 50)

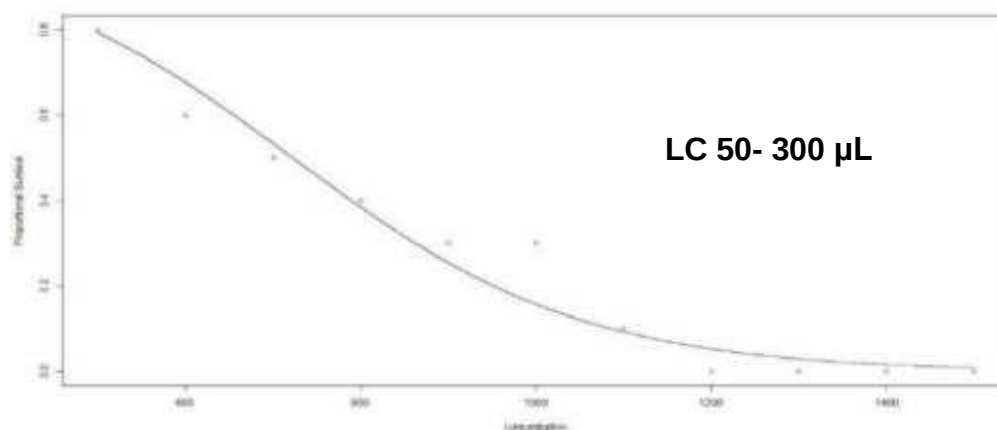


Fig 5.1.2: Lethal Concentration (LC 50)

5.2 HISTOLOGY ANALYSIS

The effect of the fipronil was analyzed in the condition of tissue damage. Different concentration of fipronil was induced to the zebrafish brain and its leads in the damage of brain tissues. The lower and higher dosage levels were determined as 50 μ L and 300 μ L respectively. The brain sections were stained by haematoxylin and eosin. The DPX mounted slides were observed under the light microscope. The histology analysis images are as follows:



Fig 5.2.1: Histology analysis process includes dissection of brain, fixation of brain, blocking of brain in paraffin wax



Fig 5.2.2: Histology analysis process includes sectioning of microtome, Slide preparation, removal of paraffin wax from tissue

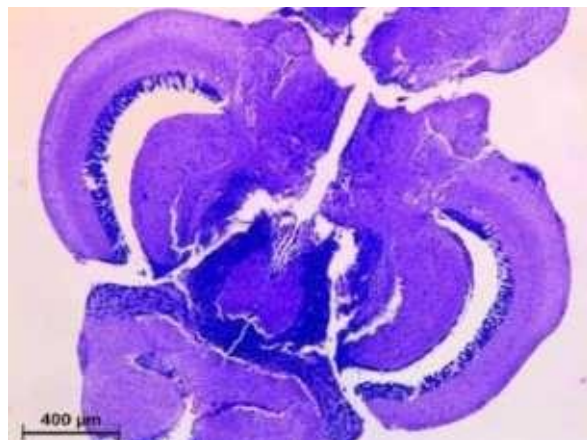


Fig 5.2.3 Histological image of zebrafish brain

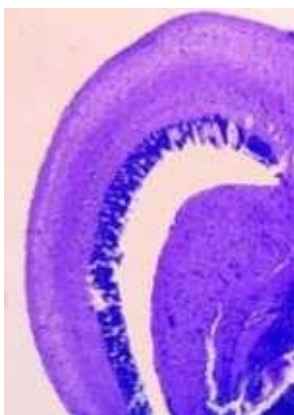


Fig 5.2.4 Histological image of optic tectum region of control fish in 24 hrs

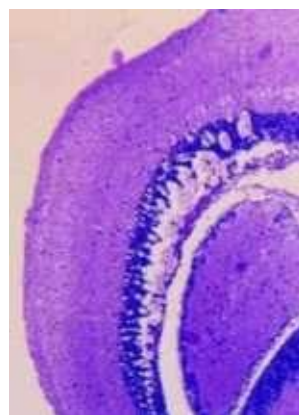


Fig 5.2.5 Histological image of optic tectum region of treated brain 50μg/L in 24 hrs

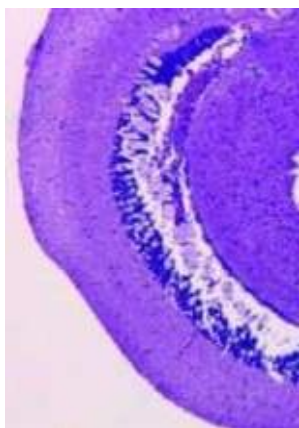


Fig 5.2.6 Histological image of optic tectum region of treated brain 100μg/L in 24 hrs

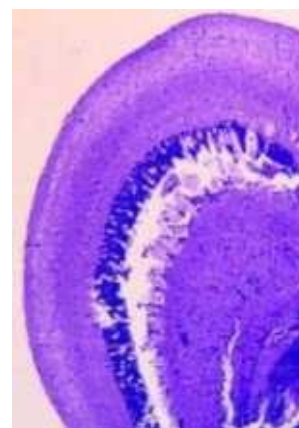


Fig 5.2.7 Histological image of optic tectum region of treated brain 300μg/L in 24 hrs



Fig 5.2.8 Histological image of optic tectum region of treated brain 50µg/L in 48 hrs



Fig 5.2.9 Histological image of optic tectum region of treated brain 100µg/L in 48 hrs

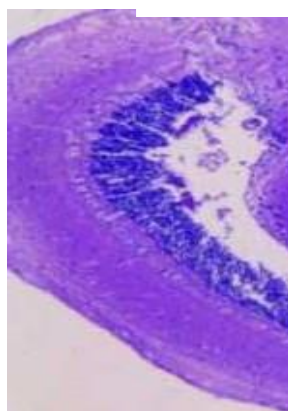


Fig 5.2.10 Histological image of optic tectum region of treated brain 300µg/L in 48 hrs

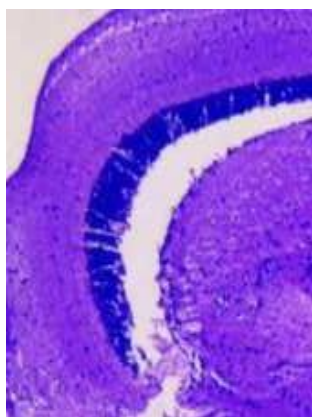


Fig 5.2.11 Histological image of optic tectum region of treated brain 50µg/L in 72 hrs

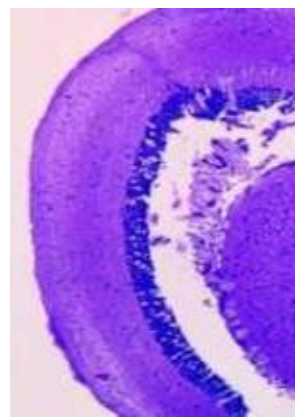


Fig 5.2.12 Histological image of optic tectum region of treated brain 100µg/L in 72 hrs



Fig 5.2.13 Histological image of optic tectum region of treated brain 300µg/L in 72 hrs



Fig 5.2.14 Histological image of optic tectum region of control brain in 168 hrs



Fig 5.2.15 Histological image of optic tectum region of treated brain 50µg/L in 168 hrs

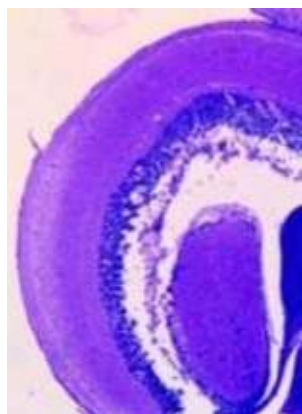


Fig 5.2.16 Histological image of optic tectum region of treated brain 100µg/L in 168 hrs



Fig 5.2.17 Histological image of optic tectum region of treated brain 300µg/L in 168 hrs

Interpretation of histological analysis in order of images shown above,

Fig 5.2.4: Control shows round, intact and round nuclei oligodendroglia cells in the central zone and deep white zone of optic tectum.

Fig 5.2.5: The lateral torus region shows no proper arrangement of cells and showing inflammation due to sudden shock of fipronil treatment.

Fig 5.2.6: At 100 ug/L, the pia matter looks intact suggesting no infiltration of inflammatory cells suggesting the less toxic nature of the fipronil at this concentration. Yet, the elongated nucleus in periventricular zone suggests the neuroinflammation happened from inside rather than outer inflammation. It may suggest the fipronil entry into brain didn't cause pia matter rupture but induced inflammation in granuloma cells.

Fig 5.2.7: Gliosis was observed in the deep white zone with elongated and mesh like structure. Due to inflammation, microglial cells have migrated thus leaving the deep white zone as mesh structure.

Fig 5.2.8: Mesh like structure observed in deep white zone suggesting glial cell movement towards pia matter. While comparing the day 1, day 2 fishes exhibit filtration of fipronil

Fig 5.2.9: Inflammation occurred at pia matter with superficial white and grey zone having infiltrates and inflammation. Elongated and moved oligodendroglia cells from central and deep white zone. Neurons are intact. Granule cells in the periventricular grey zone show no signs of inflammation, suggesting the infiltrate progressing towards the white matter from day 1.

Fig 5.2.10: Disturbed pia matter and clumped granule cells observed.

Fig 5.2.11: The 3rd day at 50ug/L concentration showed similar pattern observed in day 2 of 300ug/L, and pia matter seemed dissolved. Clumped granule cells and meshlike structure at superior white and grey zone. The neurons in optic tectum remains intact while inflammation triggered glial cell changes observed.

Fig 5.2.12: Granule cells at glial cells at the pia matter were completely destroyed due to the infiltration. Neurons and microglial cells were destroyed and no clear cell morphology observed.

Fig 5.2.13: Neurons and microglial cells were destroyed and no clear cell morphology observed.

Fig 5.2.14: Control shows round, intact and round nuclei oligodendroglia cells in the central zone and deep white zone of optic tectum.

Fig 5.2.15: No granule cells observed in pia matter. Infiltrated and mesh like structure observed all the four layers of optic tectum. Highly destroyed neurons and microglia observed. Clumped granules in grey area observed.

Fig 5.2.16: Clumped granules, damaged central zone and superficial white zone observed suggesting toxicity at higher level. The fish would have exhibited behavioral abnormality with this much damage both in white and grey matter.

Fig 5.2.17: Granuloma was significant in the grey matter with granules clumping together. This is the sign of extreme toxicity and inflammation. Neurons are intact.

Thus these inferences showing damage in optic tectum causing inflammation and destroy of neurons in highest concentration of 300µg/L comparative to other two concentrations and control.

5.3 IMMUNOHISTOCHEMICAL ANALYSIS

The brain sections listed as follow in which the sectioned tissues undergoes antigen retrieval followed by blocking with universal blocking buffer and which condition in addition of primary antibody in ratio of 1:500 dilution and also addition of secondary antibody in ratio of 1:1000 dilution in which the DAAP is added with secondary antibody and finally mounted with DPX mounting rest over night and analysing the results by using stereo microscope (Leica), and the imaging of tissues underwent immunohistochemistry are represented as follows ,

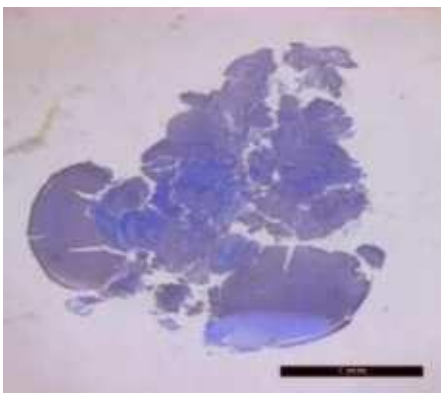


Fig 5.3.1 Immunohistochemical image of zebrafish brain



Fig 5.3.2 Immunohistochemical image of control fish in 24 hrs

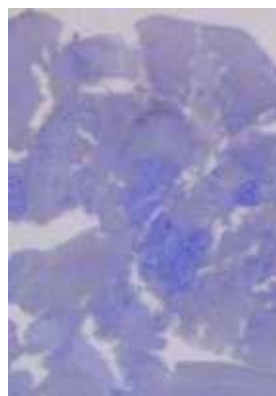


Fig 5.3.3 Immunohistochemical image of treated brain 50µg/L in 24 hrs

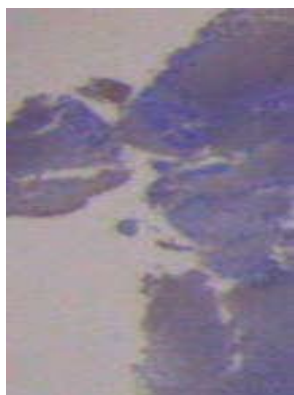


Fig 5.3.4 Immunohistochemical image of treated brain 100µg/L in 24 hrs



Fig 5.3.5 Immunohistochemical image of treated brain 300µg/L in 24 hrs

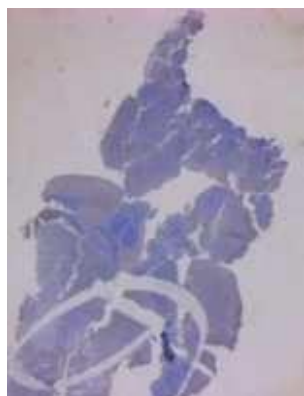


Fig 5.3.6 Immunohistochemical image of treated brain 50µg/L in 48 hrs

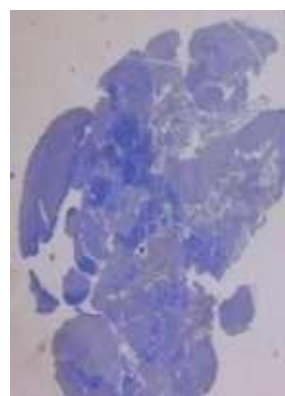


Fig 5.3.7 Immunohistochemical image of treated brain 100µg/L in 48 hrs



Fig 5.3.8 Immunohistochemical image of treated brain 300µg/L in 48 hrs



Fig 5.3.9 Immunohistochemical image of treated brain 50µg/L in 72 hrs

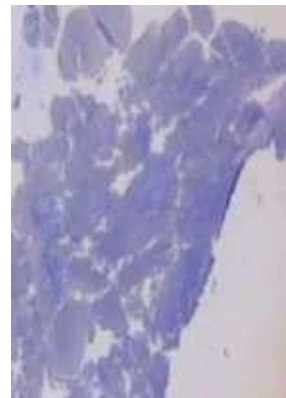


Fig 5.3.10 Immunohistochemical image of treated brain 100µg/L in 72 hrs



Fig 5.3.11 Immunohistochemical image of treated brain 300µg/L in 72 hrs

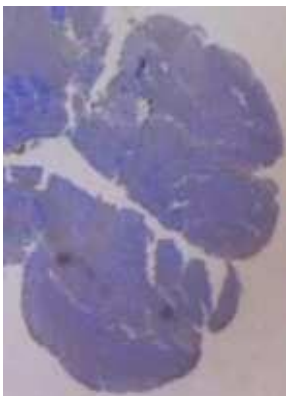


Fig 5.3.12 Immunohistochemical image of control brain in 168 hrs



Fig 5.3.13 Immunohistochemical image of treated brain 50µg/L in 168 hrs



Fig 5.3.14 Immunohistochemical image of treated brain 100µg/L in 168 hrs

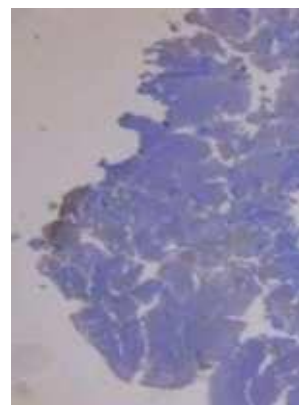


Fig 5.3.15 Immunohistochemical image of treated brain 300µg/L in 168 hrs

From the above images which represents the immunohistochemistry of fipronil treated zebrafish brain with comparison of control brain were antigen retrieved and followed by inducing primary antibody of Ab 1906 Anti-Apolipoprotein E antibody (Mouse Monoclonal Antibody) which is diluted in ratio of 1:500 and secondary antibody related to primary monoclonal antibody which is added in dilution of 1:1000 with DAAP which stains the tissues and forming the effective results in the images as shown above and finally its mounted with DPX mounting. And the images which represents as above shows the antigen is been identified in the epithelial cells were the ab 1906 antibody binds and resulting by the DAAP staining of brown color on the binding regions antigen and antibody in the tissues. This results that the antigens are

present on the epithelial cells which is causing high inflammation to the brain tissues which results in infectious encephalitis, which is define as infection which is caused in brain due to inflammation and this encephalitis leads in peoples were about 31 times more likely to develop Alzheimer's later in life, which is highest neurodegenerative disease. And thus the immunohistochemistry relieves that fipronil causing an sudden inflammation in the tissue of brain which is identified on the epithelial cells of the brain neural cells which is causing infectious encephalitis which results in neurodegenerative disease (Kimberly & Helena., 2018).

The tissues of treated fishes are showing antigens on the epithelial cells were antibody binds and resulting in high effective condition of damages on the cells of concentration in which 24 hrs concentration resulting effective condition on highest concentration which is compared with control which shows no binding of antigen to antibody hence the antigens are absent.

And similarly in 48 hrs comparative to the 24 hrs show slightly high damage of epithelial cells were the images resulting binding of antigen to the antibody in which second highest concentration also results effective results of ihc which shows the inflammation of the cells.

In 72 hrs the results are quite comparatively high were its been treated with past hours which have its impact highly on the highest concentration and slight lower in other concentration.

Finally in 168 hrs shows the condition of fipronil treated state were cells are been highly damaged due to inflammation caused by the chemical showing damages in the cells in condition of brown layer formed due to interation of antigen and antibody which shown in above images (Nickolay, *et al.*, 2014).

5.4 QUANTIFICATION OF ACETYLCHOLINE ENZYME

Total protein content in 24 hrs, 48 hrs, 72 hrs and 168 hrs were found below:

Table 5.4.1: Total Protein in 24hrs

Concentration	Total Protein(μ g)
Control	14.66666667
50 μ g/L	11.25
100 μ g/L	9.75
300 μ g/L	7.291666667

Table 5.4.2: Total Protein in 48hrs

Concentration	Total Protein(μ g)
50 μ g/L	11.33333333
100 μ g/L	10.625
300 μ g/L	7.208333333

Table 5.4.3: Total Protein in 72hrs

Concentration	Total Protein(μ g)
50 μ g/L	9.666666667
100 μ g/L	7.375
300 μ g/L	6.208333333

Table 5.4.4: Total Protein in 168hrs

Concentration	Total Protein(μ g)
Control	12.416666667
50 μ g/L	5.916666667
100 μ g/L	2.041666667
300 μ g/L	1.583333333

From the above tables the amount of Total protein is comparatively lesser in treated fishes and has steadily decreased in consecutive days. In which 24 hrs show total protein value of 7.29 in highest concentration and 11.25 in lowest concentration. Were in 168 hrs show total protein value of 1.58 in highest concentration and 5.91 in lowest concentration.

Acetylcholine content in 24 hrs, 48 hrs, 72 hrs and 168 hrs were found to be:



Fig 5.4.1: Acetylcholine Quantification in 24hrs

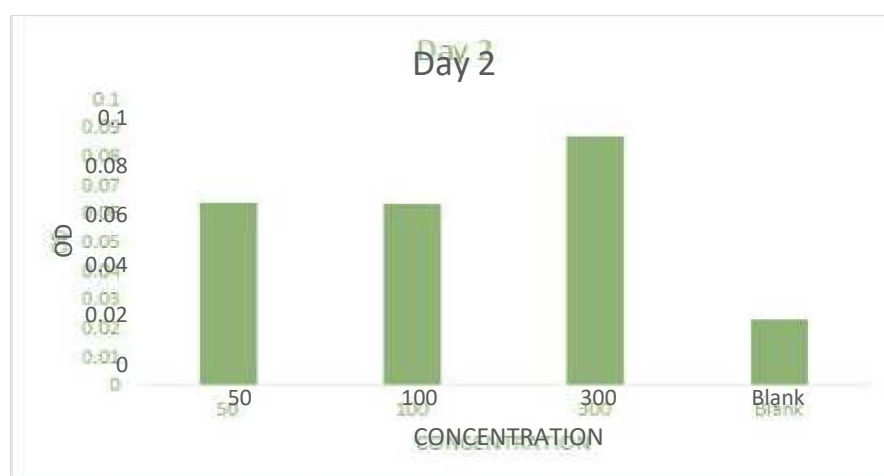


Fig 5.4.2: Acetylcholine Quantification in 48hrs

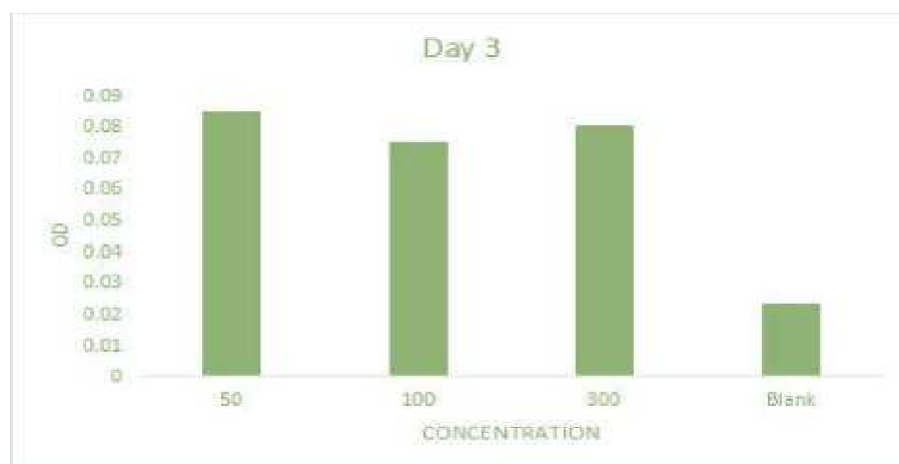


Fig 5.4.3: Acetylcholine Quantification in 72hrs

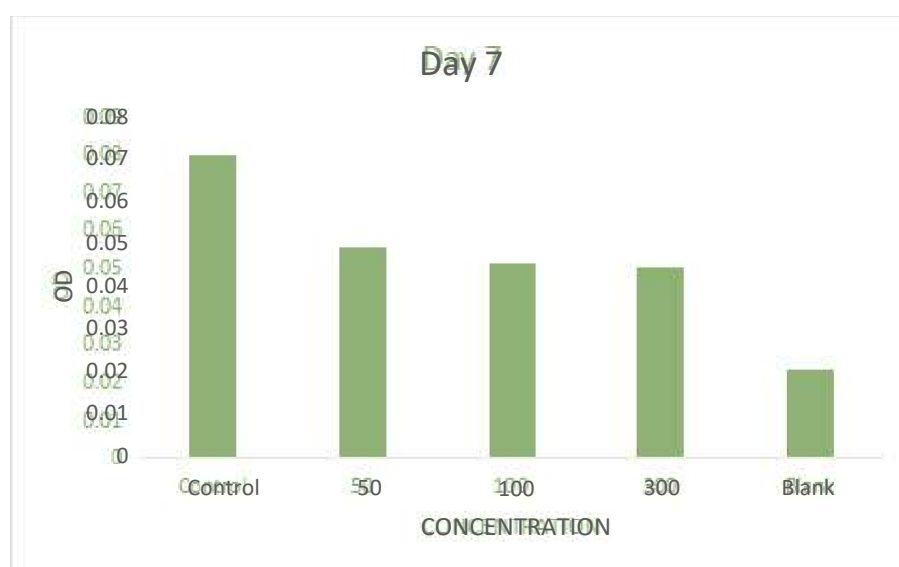


Fig 5.4.4: Acetylcholine Quantification in 168hrs

According to the graph, there was gradual decreasing of concentration of acetylcholine which resulting in the neuronal tissues affecting which leads in the degenerative diseases. Varying results were observed in each day which show gradual in acetylcholine values.

5.5 BEHAVIOUR STUDIES

The behaviour study of the process is done for the treated fishes of different concentrations and different time intervals 24 hrs, 48 hrs, 72 hrs and 168 hrs. And the data is graphical represented below.

5.5.1 Movement of treated and control fishes

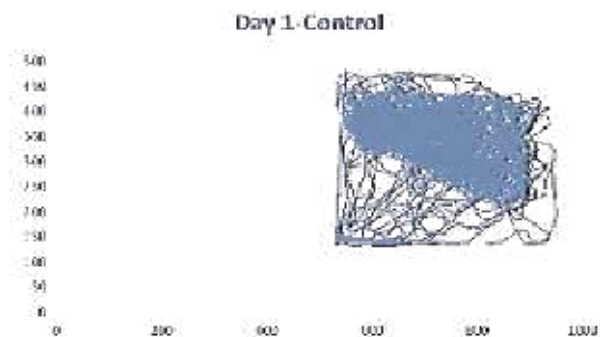


Fig 5.5.1: Movement of control fish in 24hrs

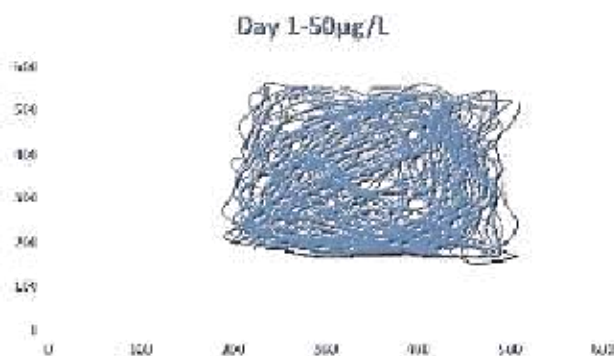


Fig 5.5.2: Movement of fish in 50 µg/L in 24hrs

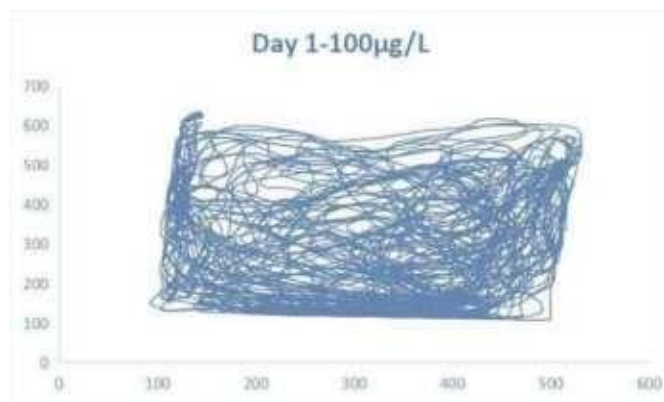


Fig 5.5.3: Movement of fish in 100 µg/L in 24hrs

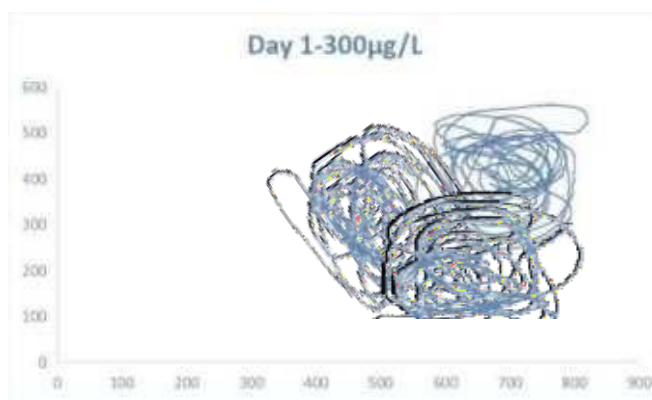


Fig 5.5.4: Movement of fish in 300 µg/L in 24hrs

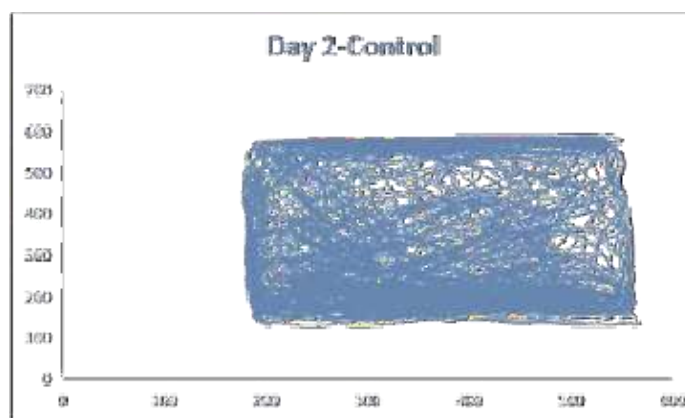


Fig 5.5.5: Movement of control fish in 48hrs

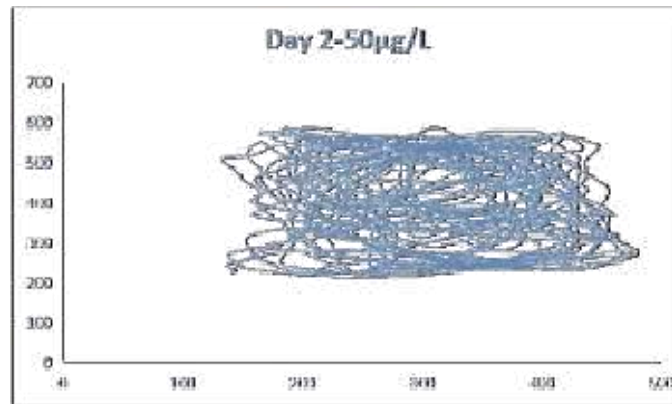


Fig 5.5.6: Movement of fish in 50 µg/L in 48hrs

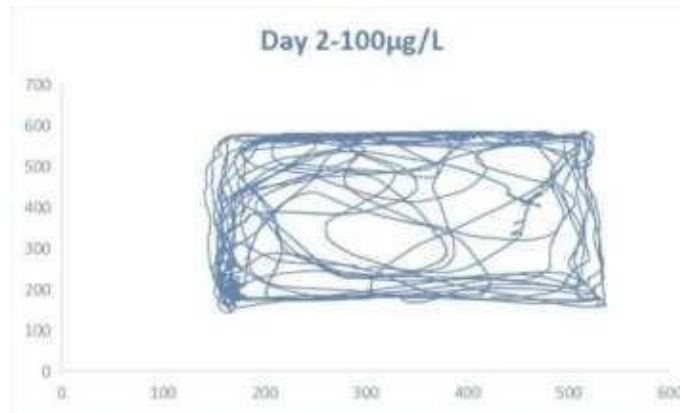


Fig 5.5.7: Movement of fish in 100 µg/L in 48hrs



Fig 5.5.8: Movement of fish in 300 µg/L in 48hrs



Fig 5.5.9: Movement of control fish in 72hrs

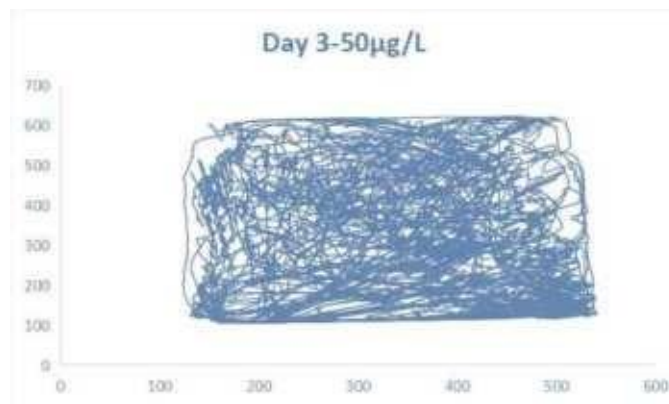


Fig 5.5.10: Movement of fish in 50 µg/L in 72hrs

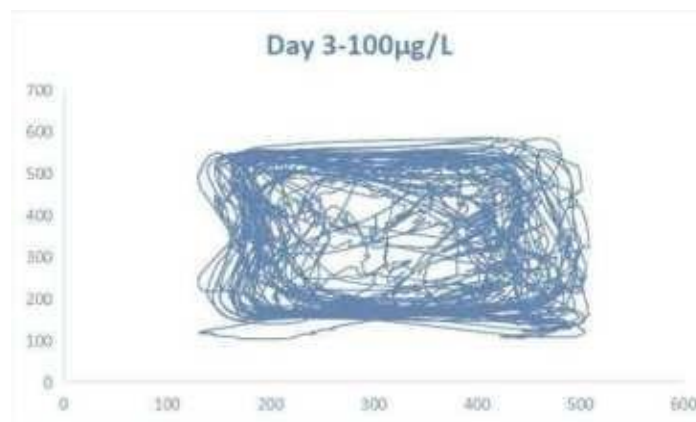


Fig 5.5.8: Movement of fish in 300 µg/L in 48hrs

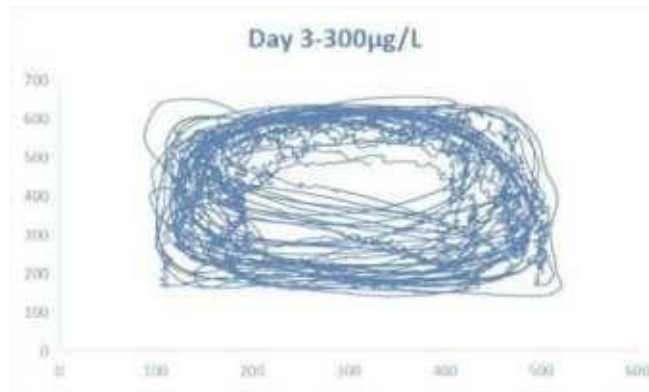


Fig 5.5.11: Movement of fish in 300 µg/L in 72hrs



Fig 5.5.12: Movement of control fish in 168hrs

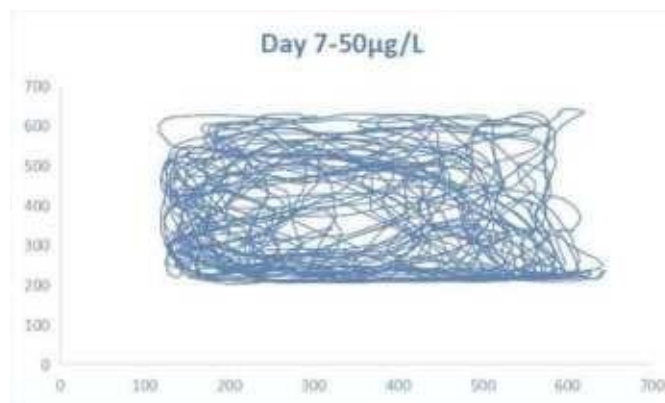


Fig 5.5.13: Movement of fish in 50 µg/L in 24hrs

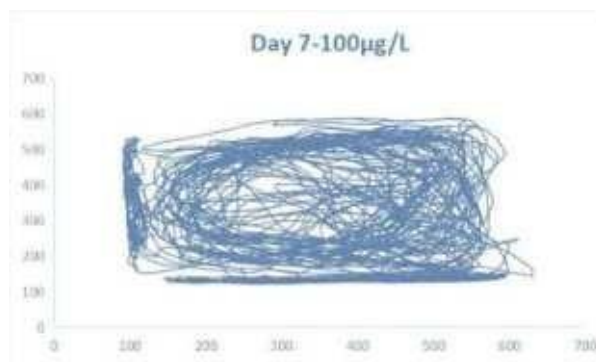


Fig 5.5.14: Movement of fishes in 100 µg/L in 168hrs

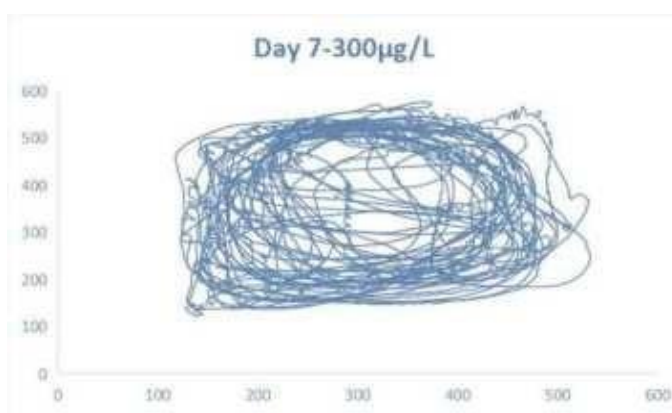


Fig 5.5.15: Movement of fishes in 300 µg/L in 168hrs

From the above graph represents that as the concentration of Fipronil increases, fishes explored and travelled lesser in the tank of 15 x 10 x 30 cm, which is compared from Day 1, 2, 3 respectively and after 5 days the fishes gradually started to explore and travel a little more than the day of treatment but it does not equal to control fishes.

5.5.2 Relative distance of treated and control fishes

Relative distance is defined as total distance covered by the treated fishes which is represented below refers relative distance of treated fishes are comparatively lower than control.

Different time intervals of 24hrs, 48 hrs, 72 hrs and 168hrs were analysed in which 24 hrs lower concentration shows relatively low than that of control fish. And other two

concentration gradually decrease in the distance covered. In 48 hrs all the treated concentration shows relatively low in comparison to the control. In 72 hrs treated concentration shows gradual increase from lower to higher but comparatively lower to the control. In 168 hrs second highest concentration of treatment gradual increased than that of other two concentration which show that fishes start to travel and move places than initial treated days but it not equals with control. The graphical representation of relative distance of treated in compared with normal fish (Cameron, *et al.*, 2015).



Fig 5.5.16: Relative distance of treated and control fishes in 24hrs

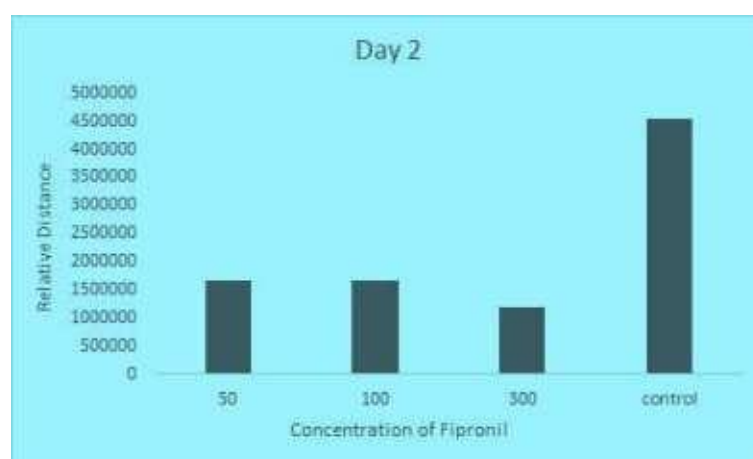


Fig 5.5.17: Relative distance of treated and control fishes in 48hrs

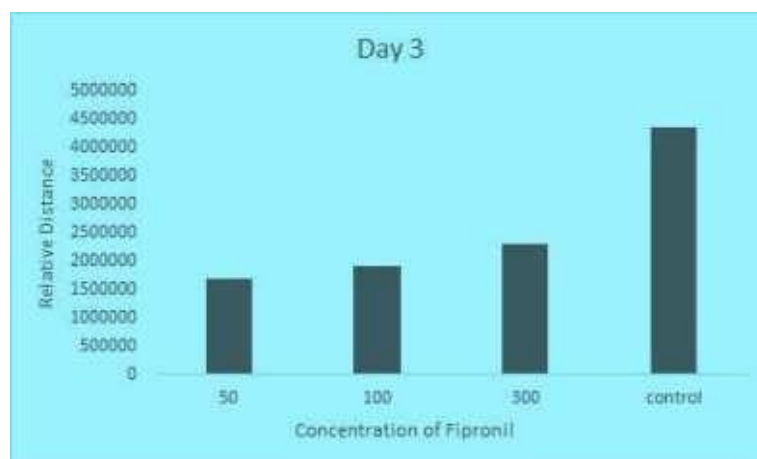


Fig 5.5.18: Relative distance of treated and control fishes in 72hrs



Fig 5.5.19: Relative distance of treated and control fishes in 168hrs

5.5.3. Aggression behavioural assay:

Aggression is a complex suite of behaviours serving a number of adaptive purposes. Fish use aggression to protect offspring, monopolize resources such as food, territory, mates and also establish dominance hierarchies. Aggression can be measured in the laboratory by recording the interaction using two free swimming fish or by mirror induced stimulation. In this study aggression was measured by mirror induced stimulation.

Untreated control zebrafish has shown the conventional behaviour response toward the mirror. The fipronil treated zebrafishes were shown the prominent response based on the concentration. The highest concentration of fipronil treated zebrafish shows less aggressive from 24 hrs to 72 hrs and slight increase in 168 hrs and lowest concentration of fipronil treated zebrafish shows high aggression towards self- replicate mirror image but the middle concentration shows partially similar response like control towards the mirror.

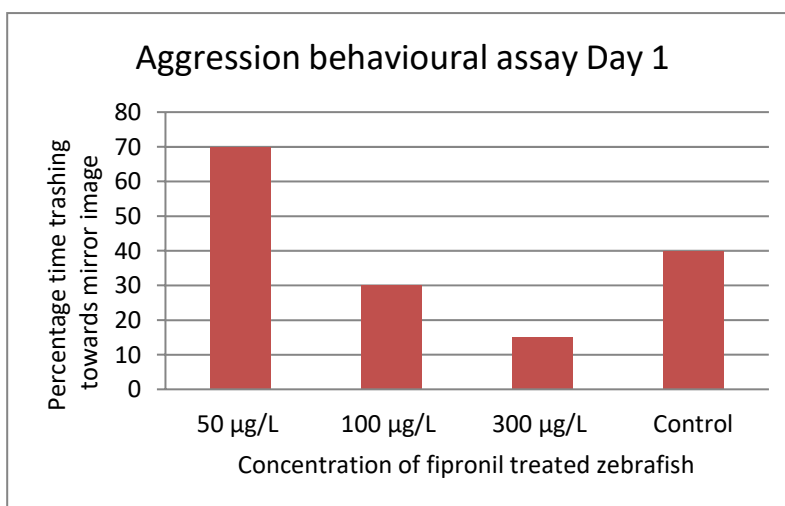


Fig 5.5.20: Aggression behavioural response of fipronil treated and control fishes in Day 1

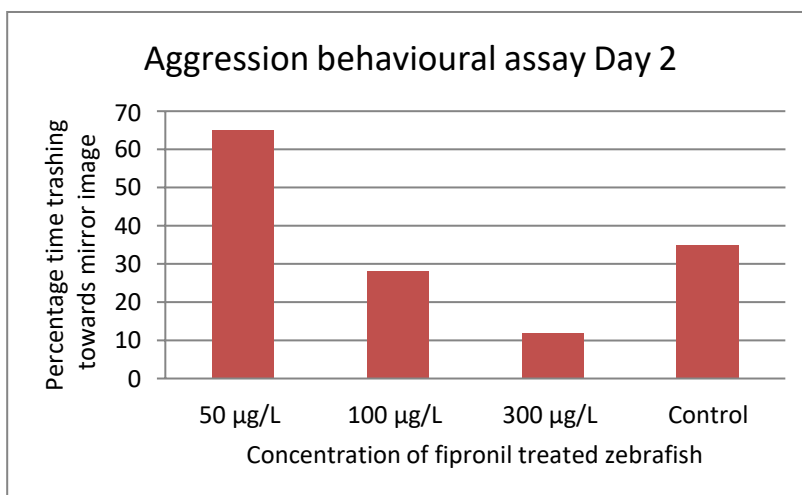


Fig 5.5.21: Aggression behavioural response of fipronil treated and control fishes in Day 2

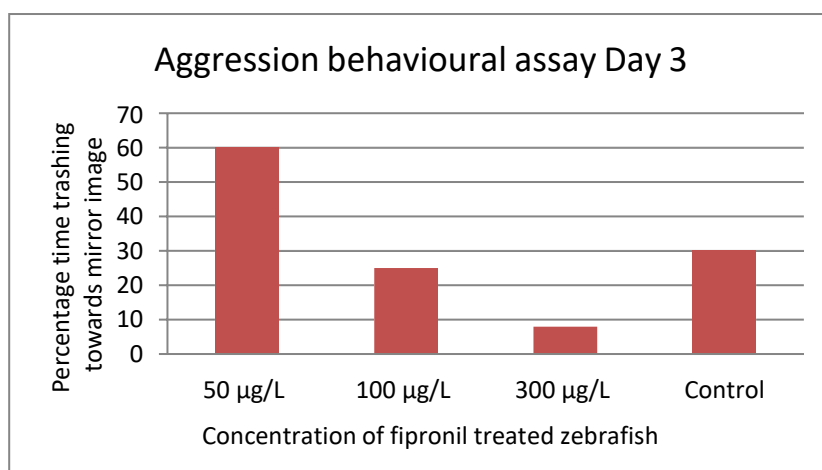


Fig 5.5.22: Aggression behavioural response of fipronil treated and control fishes in Day 3

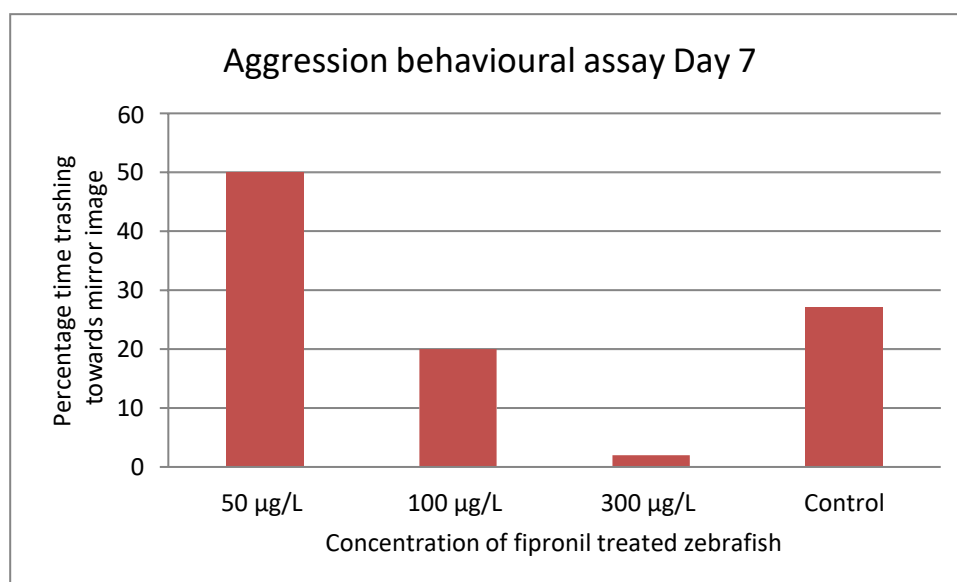


Fig 5.5.23: Aggression behavioural response of fipronil treated and control fishes in Day 7

5.5.4. Place preference boldness assay:

In the Place preference boldness, fish show preference for an environment that has previously been associated with fipronil thus indicating the positive reinforcing qualities of the substance. In this study the control untreated fishes were chosen the

dark area but after 5 mins fishes became adaptive to the environment with anxiety. 100µg/l and 300µg/l fipronil treated zebrafishes were spend closely 76% of the time in dark region of the tank. Anxiety levels were elevated in 100µg/l and 300µg/l concentrations.

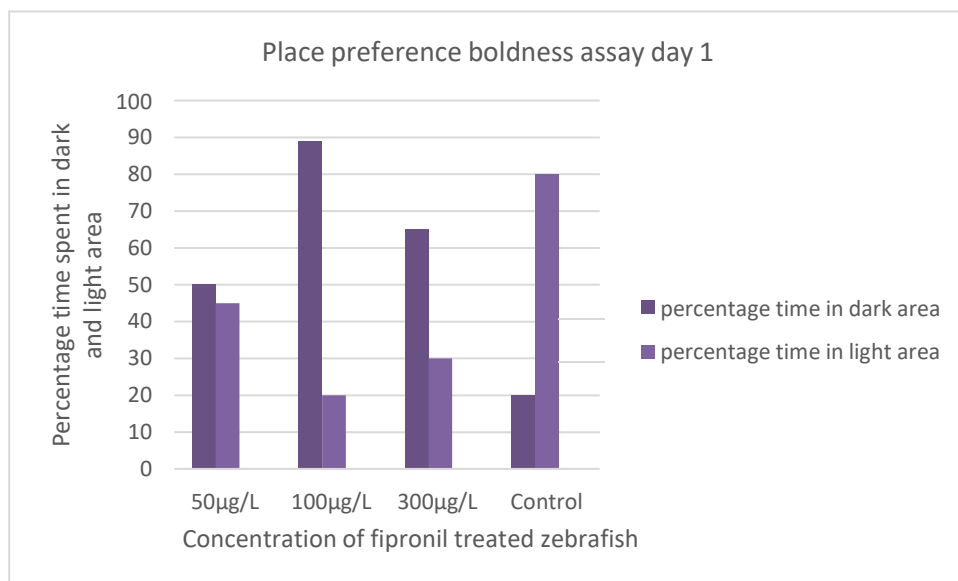


Fig 5.5.24: Place preference boldness assay of fipronil treated and control fishes in Day 1

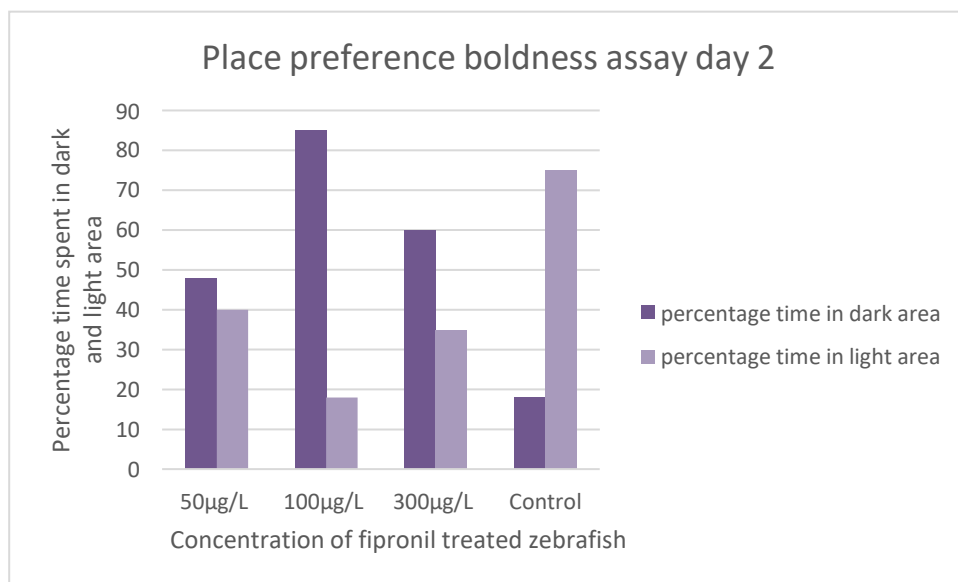


Fig 5.5.25: Place preference boldness assay of fipronil treated and control fishes in Day 2

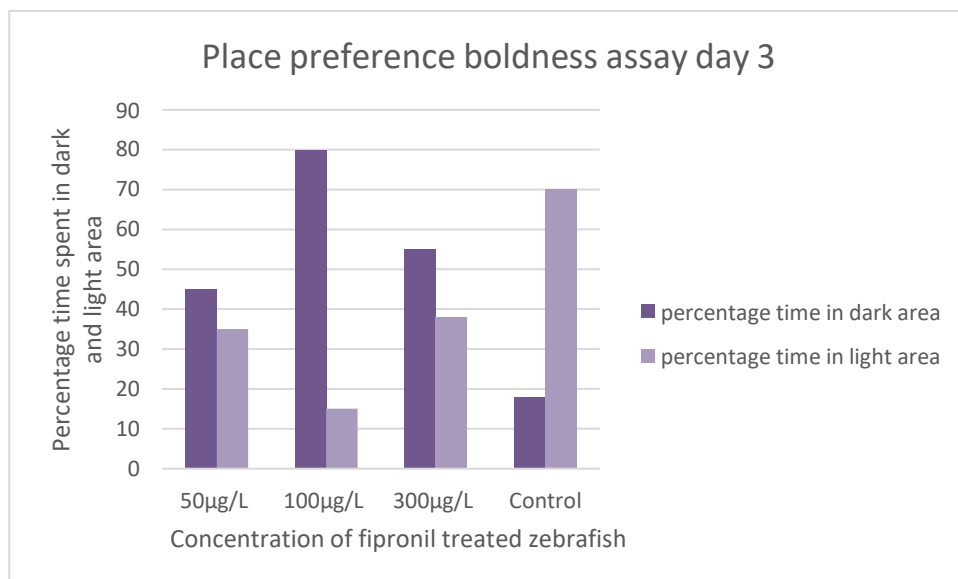


Fig 5.5.26: Place preference boldness assay of fipronil treated and control fishes in Day 3

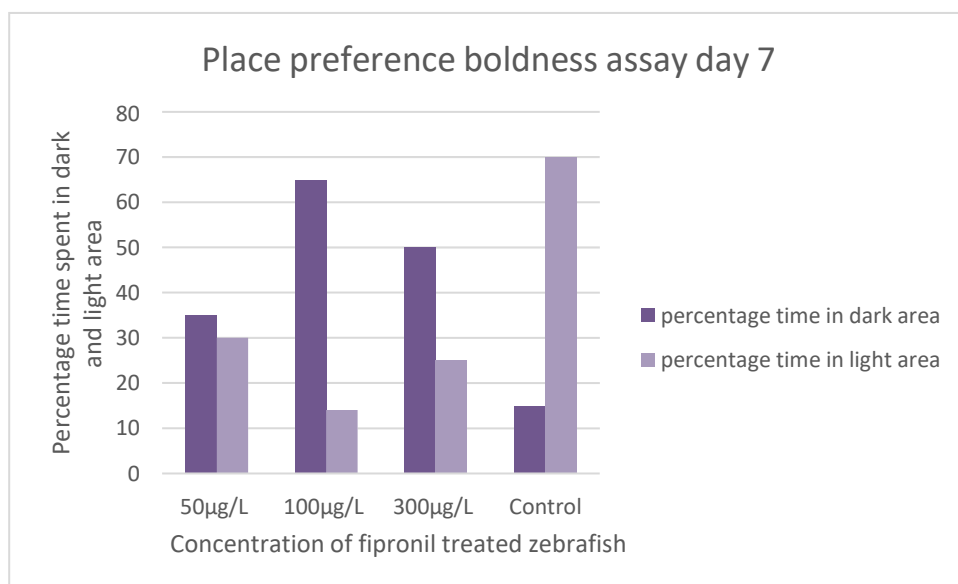


Fig 5.5.27: Place preference boldness assay of fipronil treated and control fishes in Day 7

5.5.5 Novel tank assay:

The novel tank assay is most commonly used to assess zebrafish anxiety paradigms. This test exploits the natural tendency of zebrafish to seek refuge when exposed to a novel environment. In this process, the fish initially dives to the bottom of the tank and remains there until it feels safe enough to explore the rest of the tank and gradually explores the upper zone of the tank. The time spent in the bottom dwelling, the time elapsed in the upper part of the tank, erratic and velocity of the movements have been interpreted as indices of anxiety. This assay is based on the different regions of the tank of top and bottom in which control fishes remain 65% in bottom and 35% in top zone. The highest concentration of fipronil treated fishes were hang around the upper zone of the novel tank. Where the lowest concentration stayed equally in bottom as well as upper zone of the tank and middle concentration fishes remained 85 % in upper zone and 15% in bottom of the tank. This result directs the anxiety level is gradually increasing when the fipronil concentrations were increased.

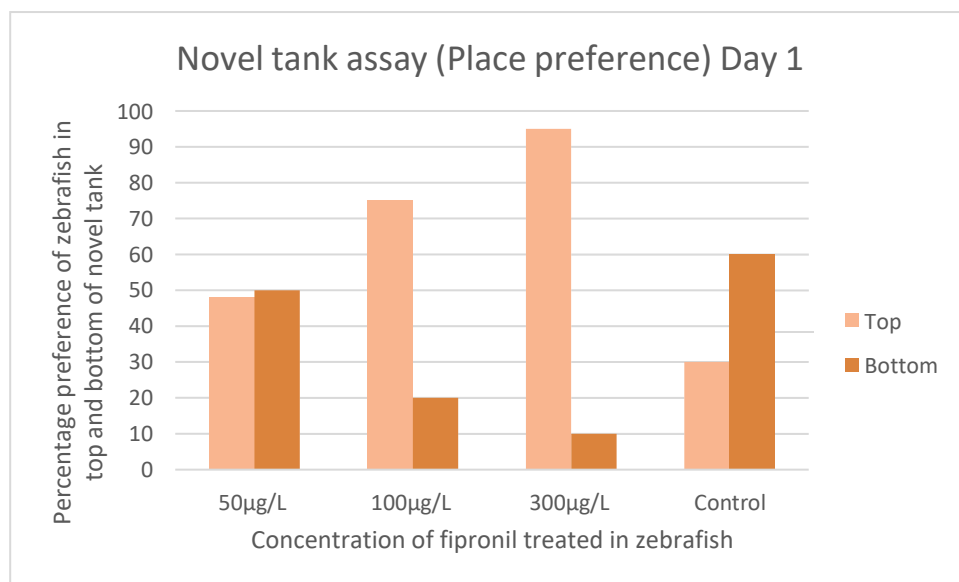


Fig 5.5.28: Novel tank assay of fipronil treated and control fishes in Day 1

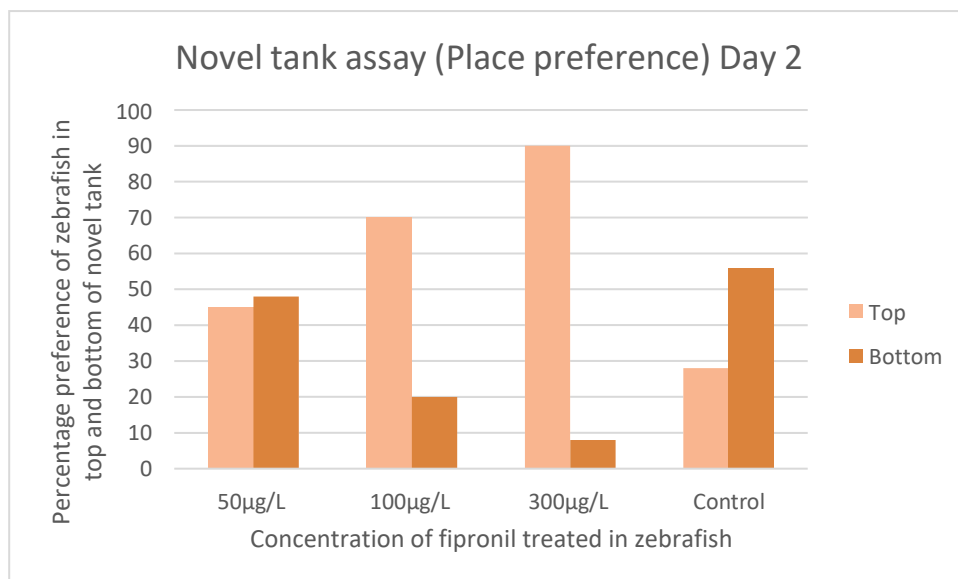


Fig 5.5.29: Novel tank assay of fipronil treated and control fishes in Day 2

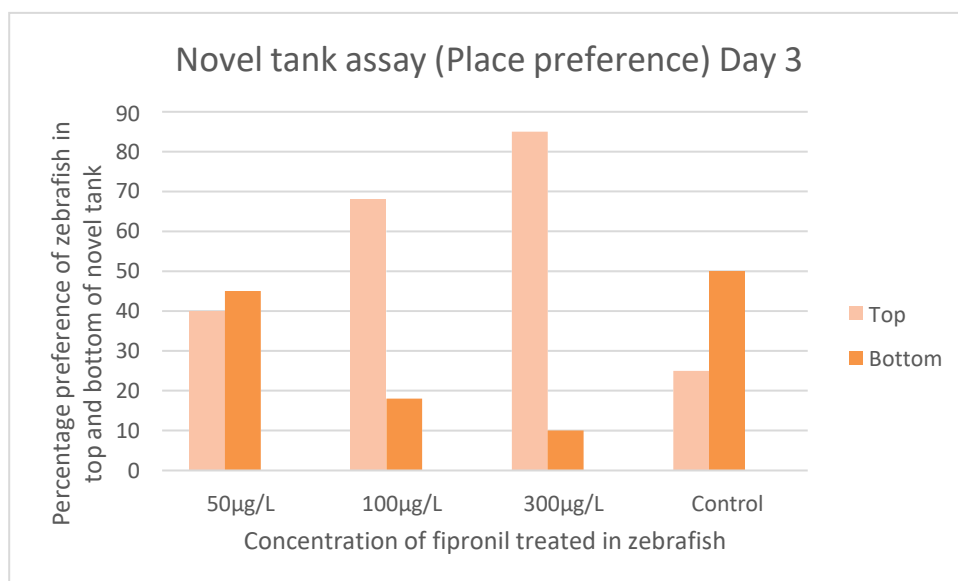


Fig 5.5.30: Novel tank assay of fipronil treated and control fishes in Day 3

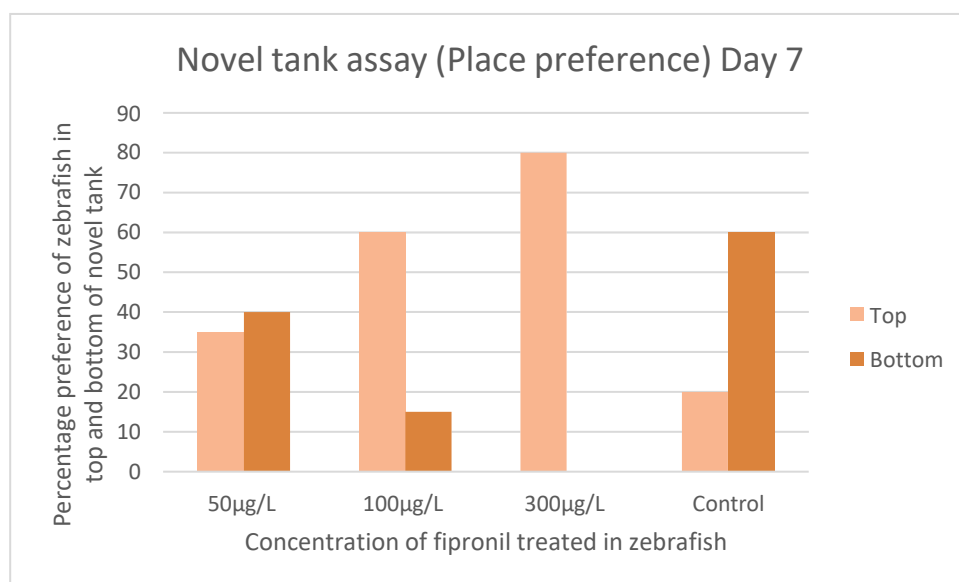


Fig 5.5.31: Novel tank assay of fipronil treated and control fishes in Day 7

CHAPTER 6

SUMMARY AND CONCLUSION

Fipronil is widely used as pesticides in agriculture practices. A toxic chemical pesticide called fipronil was developed as a safer substitute for traditional pesticides like organophosphates. However, more recent research has revealed the harm it does to organisms other than the ones it is intended to kill. When fipronil is metabolized, it releases metabolites that are further toxic than the original compound. Both of these fipronil's metabolites are extremely persistent and take a long time to break down. Fipronil is used to kill insects in agriculture and urban areas, but it also causes genetic changes in non-target organisms, which has a detrimental effect on non-target species, the food chain, and the ecosystem. Zebrafish were used as a model system, which monitored and handled easily for several experiments. The experiments were planned carefully to meet the objectives to get a better understanding about the neuronal effects of fipronil using histology, immunohistochemistry and behavioral study. The lethal concentration of fipronil was calculated as 300 µg/l in zebrafish. Different concentration was observed in the zebrafish brain. A defined tissue damage was observed in the optic tectum of brains concentration of 300 µg/l in zebrafish. The behavioural responses also revealed as elevated in the level of anxiety as the concentration increase. However, it shows that damages in brain tissue which leads to the high cause of neurodegenerative disease.

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