

BIOCHEMICAL EVALUATION OF BRAIN IN FIPRONIL TREATED ZEBRAFISH

Submitted in partial fulfillment of the requirements for the award of
Bachelor of Technology degree in Biotechnology
by

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**DEPARTMENT OF BIOTECHNOLOGY
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SATHYABAMA

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

Accredited with Grade "A" by NAAC

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **S. GANESH (39230016)** and **M. SANJAY (39230039)** who carried out Project entitled "**BIOCHEMICAL EVALUATION OF BRAIN IN FIPRONIL TREATED IN ZEBRAFISH**" under our supervision from September 2022 to May 2023.

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ABSTRACT

Fipronil was thought to be an alternative to the classical insecticides like organophosphate which were both harmful to non-target organism. But it has been shown to have neurotoxic effects in various animal models. In this study, we investigated the biochemical effects of fipronil on the brain of zebrafish. Zebrafish were exposed to fipronil at concentrations of 50, 100, and 300 µg/L for 7 days, and the effects on brain biochemistry were evaluated using histology, gas chromatography, and acetylcholine assay and behaviour tests. Our results show that there are changes in neurotransmitter levels and this indicates potential disruption of the normal functioning of the nervous system. With time the neurons and glial cells are affected greatly and are completely destroyed in some regions of brain in day 7. Total protein quantification showed a concentration-dependent decrease in protein content in the brain. Behavioral testing using the open tank test showed a decrease in exploratory behaviour and movement speed in fish exposed to fipronil.

Keywords: Fipronil, zebrafish, brain, oxidative stress, neurotransmitters, glial cells, histology, gas chromatography, total protein quantification, open tank test.

TABLE OF CONTENTS

S.NO	CONTENTS	Page No.
1	List of Abbreviations	iv
2	List of Figures	v
3	List of Tables	ix
4	1: Introduction	1
5	2: Literature survey	3
	2.1 Developmental Toxicity	3
	2.2 Behavioural Changes	6
	2.3 Metabolism, Chemical pathways and Gene expressions	9
6	3: Aim and Scope	13
7	4: Methods and Materials	14
	4.1 Experimental Animals And Fipronil	14
	4.2 Fipronil Treatment	14
	4.3 Histology	14
	4.4 Lipid Profiling In GC-FID	16
	4.5 Quantification Of Acetylcholine Enzyme	19
	4.6 GC-MS (Gas Chromotography-Mass Spectrometry)	22
	4.7 Behaviour Studies	22

8	5: Results and Discussion	23
	5.1 Histology of Zebrafish Brain	23
	5.2 Quantification of Acetylcholine Enzyme	27
	5.3 Behaviour Studies	30
	5.4 GC-FID	35
	5.5 GC-MS	44
9	6: Summary and Conclusion	58
10	7: References	59

LIST OF ABBREVIATIONS

Gamma-aminobutyric acid	(GABA)
Nuclear magnetic resonance	(NMR)
Glyphosate-based herbicide	(GBH)
Fipronil-based insecticide	(FBI)
Hours post-fertilization	(HPF)
Glutathione S-transferases	(GSTs)
7-ethoxycoumarin O-deethylase	(ECOD)
Carboxylesterases	(CarEs)
Digital gene expression	(DGE)
Acetylcholinesterase	(AChE)
Cytochrome P450	(CYP450)
Methylated DNA Immunoprecipitation Sequencing	(MeDIP-seq)
Kyoto Encyclopedia of Genes and Genomes	(KEGG)
Paraformaldehyde	(PFA)
Lethal Concentration	(LC)
Bovine Serum Albumin	(BSA)
Bichoninic acid	(BCA)
Tricaine Methanosulfonate	(MS-222)

LIST OF FIGURES

Figure no.	Figure name	Page no.
Fig 2.1.	Avoidance vs Fipronil	8
Fig 2.2	Flow chart of response	9
Fig 4.1	Fish exposed to MS-222	15
Fig 4.2	Wax cubes containing Zebrafish Brain	16
Fig 4.3	Tissue sectioned in Microtome	16
Fig 4.4	Brain ground in Chloroform	18
Fig 4.5	Methanol and chloroform layers after centrifuge	18
Fig 4.6	Sample for GC FID	18
Fig 4.7	Gas Chromatography with Flame Ionization Detection (GC-FID). GC 2010 Plus.	18
Fig 4.8	Graph of Standard (BSA+PB)	20
Fig 4.9	EnSpire Multimode Plate reader	21
Fig 4.10	Quantification of Acetylcholine Enzyme	21
Fig 4.11	Samples for Acetylcholine enzyme	21
Fig 4.12	GC-MS(Gas Chromatography Mass Spectroscopy)	22
Fig 5.1	Histology 24 hrs – 50 µg/L	23
Fig 5.2	Histology 24 hrs – 100 µg/L	23
Fig 5.3	Histology 24 hrs – 300 µg/L	23
Fig 5.4	Histology 24 hrs – Control	23
Fig 5.5	Histology 48 hrs – 50 µg/L	24
Fig 5.6	Histology 48 hrs – 100 µg/L	24
Fig 5.7	Histology 48 hrs – 300 µg/L	24
Fig 5.8	Histology 72 hrs – 50 µg/L	25
Fig 5.9	Histology 72 hrs – 100 µg/L	25

Fig 5.10	Histology 72 hrs – 300 µg/L	25
Fig 5.11	Histology 168 hrs – 50 µg/L	26
Fig 5.12	Histology 168 hrs – 100 µg/L	26
Fig 5.13	Histology 168 hrs – 300 µg/L	26
Fig 5.14	Histology 168 hrs - Control	26
Fig 5.15	Acetylcholine Quantification in 24hrs	28
Fig 5.16	Acetylcholine Quantification in 48hrs	29
Fig 5.17	Acetylcholine Quantification in 72hrs	29
Fig 5.18	Acetylcholine Quantification in 168hrs	29
Fig 5.19	Movement of control fish in 24hrs	30
Fig 5.20	Movement of fish in 50 µg/L in 24hrs	30
Fig 5.21	Movement of fish in 100 µg/L in 24hrs	30
Fig 5.22	Movement of fish in 300 µg/L in 24hrs	30
Fig 5.23	Movement of control fish in 48hrs	31
Fig 5.24	Movement of fish in 50 µg/L in 48hrs	31
Fig 5.25	Movement of fish in 100 µg/L in 48hrs	31
Fig 5.26	Movement of fish in 300 µg/L in 48hrs	31
Fig 5.27	Movement of control fish in 72hrs	32
Fig 5.28	Movement of fish in 50 µg/L in 72hrs	32
Fig 5.29	Movement of fish in 100 µg/L in 72hrs	32
Fig 5.30	Movement of fish in 300 µg/L in 72hrs	32
Fig 5.31	Movement of control fish in 168hrs	33
Fig 5.32	Movement of fish in 50 µg/L in 168hrs	33
Fig 5.33	Movement of fish in 100 µg/L in 168hrs	33
Fig 5.34	Movement of fish in 300 µg/L in 168hrs	33
Fig 5.35	Relative distance of treated and control fishes in 24hrs	34

Fig 5.36	Relative distance of treated and control fishes in 48hrs	34
Fig 5.37	Relative distance of treated and control fishes in 72hrs	34
Fig 5.38	Relative distance of treated and control fishes in 168hrs	34
Fig 5.39	GC-FID 24hrs-Control Chromatogram	35
Fig 5.40	GC-FID 24hrs-50µg/L Chromatogram	35
Fig 5.41	GC-FID 24hrs-100µg/L Chromatogram	35
Fig 5.42	GC-FID 24hrs-300µg/L Chromatogram	36
Fig 5.43	GC-FID 48hrs-50µg/L Chromatogram	36
Fig 5.44	GC-FID 48hrs-100µg/L Chromatogram	36
Fig 5.45	GC-FID 48hrs-300µg/L Chromatogram	37
Fig 5.46	GC-FID 72hrs-50µg/L Chromatogram	37
Fig 5.47	GC-FID 72hrs-100µg/L Chromatogram	37
Fig 5.48	GC-FID 72hrs-300µg/L Chromatogram	38
Fig 5.49	GC-FID 168hrs-Control Chromatogram	38
Fig 5.50	GC-FID 168hr-50µg/L Chromatogram	38
Fig 5.51	GC-FID 168hr-100µg/L Chromatogram	39
Fig 5.52	GC-FID 168hr-300µg/L Chromatogram	39
Fig 5.53	GC-MS 48hrs-Control Chromatogram	44
Fig 5.54	GC-MS 48hrs-50µg/L Chromatogram	44
Fig 5.55	GC-MS 48hrs-100µg/L Chromatogram	44
Fig 5.56	GC-MS 48hrs-300µg/L Chromatogram	45
Fig 5.57	GC-MS 72hrs-50µg/L Chromatogram	45
Fig 5.58	GC-MS 72hrs-100µg/L Chromatogram	45
Fig 5.59	GC-MS 72hrs-300µg/L Chromatogram	46
Fig 5.60	GC-MS 168hrs-50µg/L Chromatogram	46

Fig 5.61	GC-MS 168hrs-100µg/L Chromatogram	46
Fig 5.62	GC-MS 168hrs-300µg/L Chromatogram	47

LIST OF TABLES

Table no.	Table name	Page no.
Table 4.1	Amount of reagents required	19
Table 5.1	Total Protein in 24hrs	27
Table 5.2	Total Protein in 48hrs	27
Table 5.3	Total Protein in 72hrs	28
Table 5.4	Total Protein in 168hrs	28
Table 5.5	Changes in fatty acid concentration at different time intervals for fipronil treated brain at 50ug/L(GC-FID)	40
Table 5.6	Changes in fatty acid concentration at different time intervals for fipronil treated brain at 100ug/L(GC-FID)	41
Table 5.7	Changes in fatty acid concentration at different time intervals for fipronil treated brain at 300ug/L(GC-FID)	42
Table 5.8	GC-MS 50µg/L	48
Table 5.9	GC-MS 100µg/L	51
Table 5.10	GC-MS 300µg/L	54

1. INTRODUCTION

The current world population is almost 8 billion people, and to feed everyone, we would need to produce around 3.7 billion metric tons of food annually.

The amount of food produced is greatly affected by pests and different kinds of plant diseases. Weeds, pests, and various plant diseases are responsible for over 30% of losses in the three crucial crops that feed humans: wheat, maize, and cotton. According to projections, food production will need to expand by 70% by 2050 to meet the demand of 9.1 billion people. To boost crop output, one of the essential elements of contemporary agriculture is the use of pesticides. Many pesticides have unique modes of action and selective toxicity. But some pesticides affect many non-target organisms including humans.

Fipronil [5-amino-3-cyano-1-(2, 6-dichloro 4 trifluoromethylphenyl)- 4-trifluoromethyl sulfinyl pyrazole] belongs to the chemical pesticide class phenylpyrazoles. It is the most widely used broad-spectrum pesticide that is used to control both agricultural and non-agricultural pests.

Fipronil is a broad-spectrum insecticide that is widely used in agriculture and household products. It has been shown to have neurotoxic effects in various animal models, including mammals, birds, and insects. However, the potential neurotoxicity of fipronil on non-target organisms, including zebrafish, has not been fully explored. Zebrafish are a popular model organism for studying the effects of chemicals on the nervous system due to their well-characterized brain anatomy and behavior, as well as their sensitivity to neurotoxicants.

Fipronil mode of action differs from the classical insecticide carbamates and organophosphates, and many insects have developed resistance to these classical insecticides. Fipronil has a greater binding affinity to insect's GABA-regulated chloride channels than the GABA receptors of mammals. Fipronil interferes with g-aminobutyric acid (GABA)-gated channels and causes neural excitation, paralysis, and death.

Initially, fipronil was thought to be a better alternative for classical insecticides, but now researches show that fipronil affects non-target organisms in various ways. Here we will analyze the effects of different concentrations of fipronil on the lipids and proteins present in the brain of zebrafish.

In this study, we aim to evaluate the biochemical effects of fipronil on the brain of zebrafish. We will use a variety of tests to assess changes in brain biochemistry, including histology, gas chromatography, and biochemical assays. We will also use behavioral testing to assess the effects of fipronil on zebrafish behavior.

Histology is an important technique for assessing the structural changes in the brain caused by exposure to toxicants. In this study, we will examine brain tissue from fipronil-treated zebrafish to detect any changes in brain structure that may indicate neurotoxicity.

Gas chromatography is a powerful technique for identifying and quantifying small molecules, including neurotransmitters and other signaling molecules. In this study, we will use gas chromatography to analyze the brain tissue of fipronil-treated zebrafish to detect any changes in neurotransmitter levels or other small molecules that may indicate neurotoxicity.

Biochemical assays, including assays for oxidative stress markers and antioxidant enzyme activities, will provide important information about the effects of fipronil on the cellular and molecular processes in the brain of zebrafish.

Behavioral testing, including tests for anxiety-like behavior and motor activity, will provide insight into the effects of fipronil on zebrafish behavior and may help to identify specific behavioral deficits that result from exposure to fipronil.

Overall, this study will provide important information about the potential neurotoxicity of fipronil on non-target organisms, specifically zebrafish. The results of this study may have important implications for the use of fipronil in agriculture and other settings.

2. LITERATURE SURVEY

2.1 DEVELOPMENTAL TOXICITY

2.1.1 Effect of Fipronil on the developmental stage

Zebrafish embryos that were six hours post-fertilization (hpf) were exposed to fipronil for 72 hours to determine the LC50 value. Additionally, it was found that it caused an increase in mortality, a reduction in body size, and a reduction in hatchability were supported by cell cycle arrest-related apoptosis. Additionally, improper blood circulation was the source of the cardiac problems that were seen, such as edema and irregular pulse. Disrupted blood vessel forms were shown by endothelial cells in transgenic zebrafish models. Additionally, motor neurons and oligodendrocytes research revealed neurogenic abnormalities.

Fipronil also caused other abnormalities, such as a twisted spine and an uninflated swim bladder, which were dose-dependent, the presence of saturated fatty acids and alterations in the major bile acid production pathway in the chemical-related group were signs of liver damage (*Hahyun Park et al., 2019*).

2.1.2 Effect of Fipronil on reproduction parameters

Fish that were preparing to spawn and the actual spawn were subjected to three different concentrations of the test drug as part of the experiment. Both the three experimental groups and the control group had 100% survival after the exposure lasted for 21 days. While exposure to the highest dosage of fipronil significantly reduced the spawn obtained as well as the fecundation and hatching percentages, relative to the control group, the impacts on reproductive parameters were visible even at the lowest concentration of fipronil used (*Lu Yan et al., 2016*).

2.1.3 Pulse embryonic exposure to fipronil

Fipronil was administered to zebrafish (*Danio rerio*) embryos at different concentrations during 48 hours (beginning at around 3 hours after fertilization, or hpf). These dosages ranged from 0.02 g/L to 4000 g/L. A portion of the fish was then moved to clean water for a 7-day purging period after this acute

exposure. We postulated that pulse exposure to fipronil at crucial times for central nervous system development would harm fish later in life. Embryos exposed to 2 g or more of fipronil/L after a 48-hour pulse exposure had a lower survival rate (*Hanqing Xu et al., 2018*).

The larvae at 9 dpf (days post-fertilization) showed no additional mortality during the depuration period, nor did they differ from controls in terms of body length or notochord length. To better understand fipronil's teratogenicity and developmental impacts, further studies were conducted at larger doses over a longer period of time (96 h, up to 4 dpf). These higher concentrations of fipronil had a significant impact on zebrafish development, and the following morphometric and teratogenic effects were seen in fish at 4 days of pregnancy: shortening of the body, swelling of the yolk sac and pericardium, shortening of the midbrain, shortening of the optic lobes, and shortening of the lower jaw (*Yi Qian et al., 2017*).

It was predicted that exposure would have left behind molecular consequences in depurated fish. Therefore, transcriptome profiling was done on 9 dpf depurated larvae that had been given a single dosage of either 0.2 g/L, 200 g/L, or 2000 g/L fipronil and exposed to it for 48 hours at a time. In larvae exposed to any of the three amounts of fipronil, the expression of gene networks linked to the metabolism of glycogen and omega-3 fatty acids was reduced, indicating metabolic disturbance (*Hainzl et al., 1988*). Transcriptomics also showed that fipronil inhibited gene networks involved in photoperiod sensing, circadian rhythm, and light-dark adaption. These results led us to examine fish using a Light-Dark preference test for changed behavioral responses. When compared to controls, larvae were exposed to more than 200 g fipronil/L as embryos made fewer trips (20–30% fewer) to the dark zone. Further evidence that fipronil reinforced dark avoidance behavior, a sign of anxiety, was shown in the fact that larvae spent less time in the dark zone than did controls. The results of this study show that a brief pulse of fipronil can alter the transcriptome networks for metabolism, circadian rhythm, and response to light in fish after depuration. It is hypothesized that these molecular responses are connected to the abnormal behavioral effects seen in the light-dark preference test (*Anca Boaru et al., 2013*).

2.1.4 Transcriptome network data in larval zebrafish

In both residential and agricultural settings, the phenylpyrazole insecticide fipronil is utilized. Run-off and water systems close to where the herbicide has been sprayed have been found to contain fipronil. The herbicide causes the central nervous system to become overexcited by antagonistically acting on gamma-aminobutyric acid receptors. Fipronil is hazardous to fish at relatively large doses, although the processes behind the toxicity are poorly known throughout the embryonic stages (Bownik A. *et al.*, 2021).

A single dose of fipronil was applied to zebrafish embryos for 48 hours, approximately 3–4 hours after fertilization. Transcriptome and behavioral investigations were carried out after a 7-day depuration period (Carla M. Stehr *et al.*, 2006). With varied dosages of fipronil (0.2 g, 200 g, and 2 mg fipronil/L), transcriptomics identified brain processes as those that expressed differently. With exposure, gene networks connected to, among other routes, myelination, neural tube development, brain stem response, innervation, nerve regeneration, and astrocyte differentiation were changed. Fipronil exposure also disrupts miRNA-related processes and exposed larval fish showed an increase in genes linked to miRNA processing. These studies outline potential processes linked to zebrafish neurological effects as they mature. This is crucial because it is unclear how early exposure to pesticides like fipronil affects how organisms and the central nervous system operate later in life (Ashley Eadie *et al.*, 2020).

2.1.5 Notochord Degeneration and locomotor defects in Embryos and Larvae

Gamma-aminobutyric acid (GABA) receptors in insects are specifically inhibited by the phenylpyrazole pesticide fipronil. Although fipronil is often utilized in or close to aquatic habitats, there isn't much research that has examined this neurotoxin's effects on aquatic animals throughout vulnerable life stages. In response to touch, embryos exposed to fipronil at nominal concentrations at or above 0.7mM (333 mg/l) showed notochord degeneration, shortening along the rostral-caudal body axis, unsuccessful tail flips, and uncoordinated muscle contractions along the body axis. The lack of reciprocal inhibitory neurotransmission by glycinergic commissural interneurons in the spinal

cord is consistent with this phenotype, which closely mirrors that of accordion-class zebrafish locomotor mutants (*Robiul Islam et al., 2011*). The patterns of gene and protein expression for markers particular to notochord development were unaltered by fipronil, supporting the idea that notochord degeneration may be brought on by aberrant mechanical stress from muscular tetany(*Al Badran et al., 2019*).

Additionally, the sodium channel blocker MS-222 was added to the fipronil (1.1mM) exposure to counteract the degenerative effects (0.6mM). Exposure to 70mM strychnine, a glycinergic receptor antagonist, phenocopied the effects of fipronil on the notochord. The powerful GABA antagonist gabazine, on the other hand, caused notochord degeneration but just a hyperactive touch response when it was exposed to animals. This implies that fipronil inhibits a structurally comparable glycine receptor subtype in fish while being particularly designed to target insect GABA receptors with minimal vertebrate toxicity. This disrupts the development of spinal locomotor pathways in fish. This indicates an unexpected and maybe new fipronil toxicity mechanism in vertebrates (*Ashley Eadie et al., 2020*).

2.2 BEHAVIOURAL CHANGES

2.2.1 Effects of Glyphosate and Fipronil based agrochemical

When zebrafishes were exposed to Glyphosate-based herbicide (GBH) and Fipronil-based insecticide (FBI), they had behavioral changes, and many zebrafishes were found to spend time on the top zone in the water body rather than being in the bottom zone. Being in the top zone posed a great threat to this species by predators. By this, we can understand the effect these agrochemicals can bring on a non-target species and indirectly on the food chain and the environment (*Fabiele da Costa Chaulet et al., 2019*).

2.2.2 Enantiomers of fipronil

The effect caused by enantiomers of fipronil differs greatly. When compared to S-fipronil, video tracking revealed that R-fipronil caused more severe anxiety-like behavior in zebrafish embryonic and larval stages, including increased

swimming speed and dysregulated photoperiodic locomotion (*Krishnan et al., 2018*).

The MeDIP-Seq analysis, along with Gene Ontology and KEGG, showed that R-fipronil disrupted five signaling pathways (MAPK, Calcium signaling, Neuroactive ligand-receptor interaction, Purine metabolism, and Endocytosis) to a greater extent than S-fipronil through the hypermethylation of several crucial neuro-related genes, whereas no significant alterations of global DNA methylation were observed on the two enantiomers (*Amrith S. Gunasekara et al., 2007*).

2.2.3. Avoidance behavior

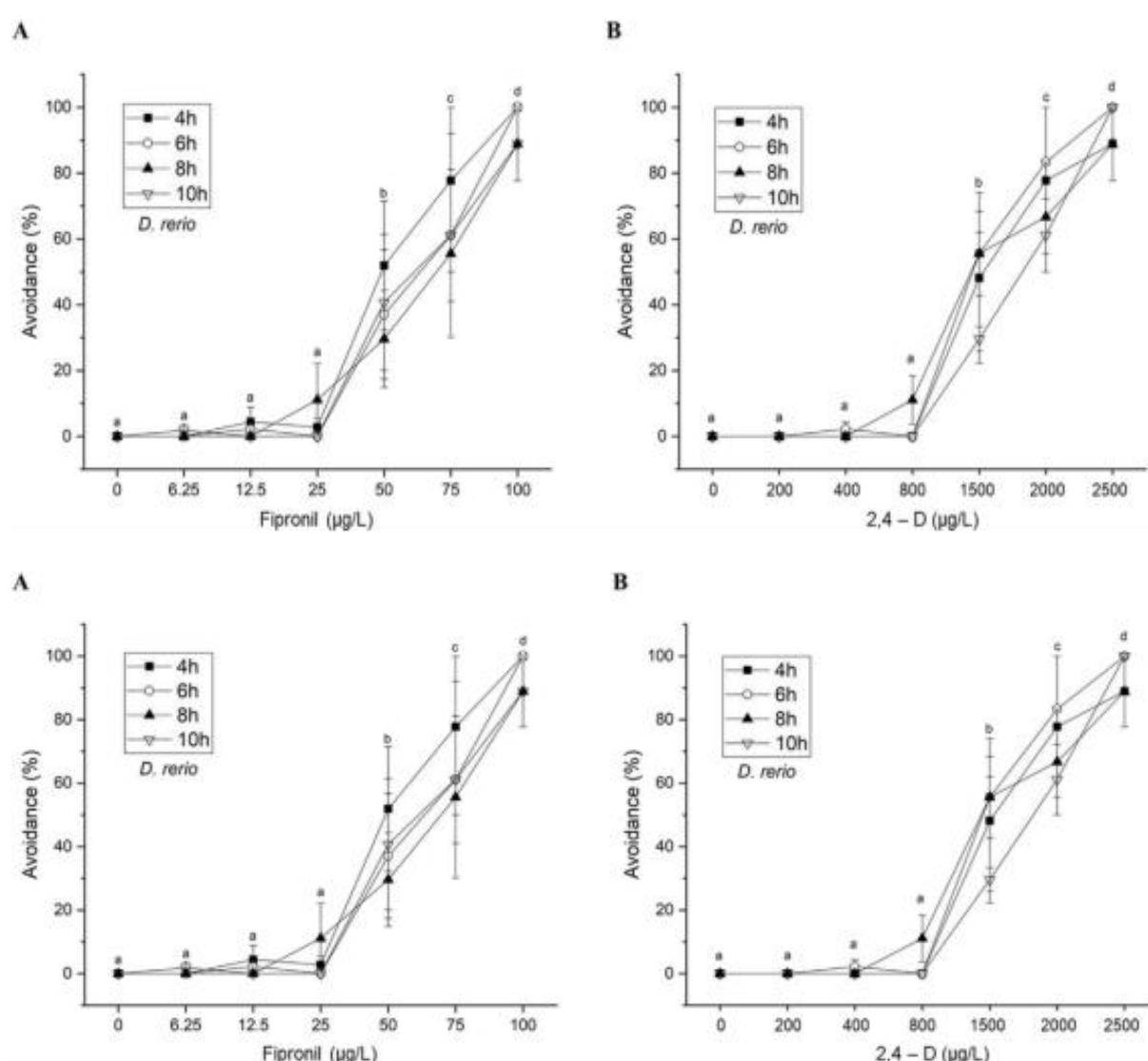
Brazil is the world's top producer of sugarcane, a crop whose upkeep is mostly reliant on chemical control. The environmental effects of two of the most widely used pesticides, the insecticide fipronil, and the herbicide 2,4-D, are a cause for worry. Through an integrative approach, the current study sought to investigate the toxicity mechanisms of the pesticides Regent® 800 WG (i.e. fipronil) and DMA® 806 BR (i.e. 2,4-D). Specifically, it sought to determine whether contamination by fipronil and 2,4-D can cause avoidance behavior in the fish *Danio rerio* (zebrafish) and *Hyphessobrycon eques* (serpae tetra or mato-grosso).

Additionally, acetylcholinesterase activity, a biomarker of neurotoxicity, and swimming behavior were taken into consideration while analyzing the impacts on fish (AChE). *D. rerio* avoided the greatest concentrations of the two substances in avoidance studies using pesticide gradients, but *H. eques* only avoided the maximum concentration of 2,4-D. When *D. rerio* was exposed to fipronil, the swimming behavior (distance traveled) was decreased and AChE was suppressed. *H. eques*' swimming (maximum speed) was impacted by 2,4-D, while AChE was unaffected. The potential impacts of pollutants on swimming behavior and AChE activity did not seem to have an impact on avoidance behavior (*Yi Qian et al., 2019*).

2.2.4. Sensory and motor systems

A phenylpyrazole insecticide called fipronil has the potential to specifically block gamma-aminobutyric acid receptors in insects. Although fipronil is the most often used pesticide in aquatic habitats, there isn't much research that

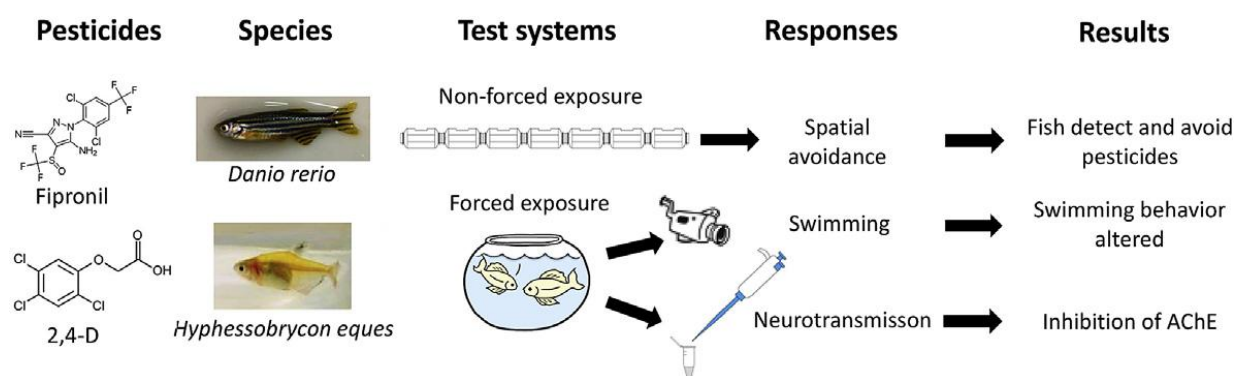
has looked at its neurotoxicity for aquatic species' sensory and motor systems. The effects of acute fipronil exposure on zebrafish (*Danio rerio*) survival rates, lateral line hair cell counts, and neurotoxicity, and the Heat maps and swimming trajectory speed and distance were also examined between zebrafish that received fipronil treatments (S Lee et al., 2018). To assess the expressions of proteins linked to oxidative stress, inflammation, apoptosis, and neurotoxicity in the brain tissue of adult zebrafish with fipronil treatment. Western blotting and immunohistochemistry were carried out independently. According to our findings, adult zebrafish exposed to fipronil had considerably lower survival rates and a shorter swimming distance (Chung-Hsin Wu et al, 2021).



Source: <https://doi.org/10.1016/j.chemosphere.2020.127972>

Fig:2.1: Avoidance vs Fipronil

The findings revealed that zebrafish embryos exposed to fipronil had considerably fewer hair cells in their lateral lines. Significant oxidative damage, inflammation, and apoptosis were found in the brain tissue of adult zebrafish exposed to fipronil in histopathology and Western blotting studies. By damaging lateral hair cells and brain tissue via oxidative stress, inflammation, and apoptosis, fipronil toxicity may impair sensory and motor systems in zebrafish. This in turn leads to a much lower survival rate and decreased mobility (*Raquel Aparecida Moreira et al., 2020*).



Source: <https://doi.org/10.1016/j.chemosphere.2020.127972>

Fig:2.2: Flow chart of response

2.3 METABOLISM, CHEMICAL PATHWAYS, AND GENE EXPRESSIONS

2.3.1 Gene Expressions

The phenylpyrazole insecticide fipronil is efficient and utilized in a variety of industries. Fipronil was particularly trustworthy since it was known to only affect invertebrate species rather than vertebrate ones, including mammals. Fipronil, however, poses possible dangers to vertebrate animals since it inhibits GABA receptors, which are found in both vertebrates and invertebrates. Clarifying the negative effects of fipronil on vertebrates was thus important. Vertebrate creatures represented by zebrafish (*Danio rerio*) were employed for this purpose. The zebrafish were treated to fipronil at concentrations of 5, 25, and 50 g/L for 12, 24, and 72 hours. 12 hours and 24 hours of extra time points were created in the exposure test to carefully evaluate the hazardous process. Metabolomics based on nuclear magnetic resonance (NMR) is a method

for identifying metabolic changes caused by external stimuli in an organism(*Park H et al., 2019*).

This work employed NMR-based metabolomics to demonstrate the metabolic alterations produced by fipronil consumption in zebrafish. Fipronil interfered with zebrafish's capacity to use antioxidants for energy metabolism, according to a metabolic study. At high exposure concentrations, there was a notable reduction in antioxidant capacity. Additionally, the findings of metabolic monitoring over time revealed that following fipronil exposure, zebrafish had responses to reduce excessive nerve excitation (*Xingxing Wang et al., 2014*).

In the control of gene expression, microRNA (miRNA) is an essential player. In a study, it was found that after being exposed to fipronil for about 96 hours many miRNAs had different expression patterns. However, there is no information on how the pesticide treatment affected the expression of the miRNA in zebrafish (*Danio rerio*). MiRNAs may exhibit a unique toxicological response that might be employed as a toxicological biomarker and may take a different path in future studies of their interactions with miRNAs connected to illnesses caused by chemicals (*Sujin Lee et al., 2020*).

2.3.2 GABA receptors and NMR

Zebrafish (*Danio rerio*) detoxifying enzymes (carboxylesterases (CarEs), glutathione S-transferases (GSTs), and 7-ethoxycoumarin O-deethylase (ECOD)) were studied to determine the acute toxicity of fipronil and its sublethal effects. According to the findings, fipronil's 24-hour LC50 for zebrafish was 220.4 mg/L. Fipronil doses below the fatal threshold did not significantly alter CarEs activity. At the measured concentrations, there was no appreciable difference between the GST activities in the liver and muscle tissues and those in the control. The GST activities in the brain and gill tissues were considerably lower at a concentration of 4 mg/L than they were at a concentration of 2 mg/L. The findings imply that CarEs and GSTs were inadequate biomarkers for assessing the effects of fipronil in *D. rerio*. In comparison to the control, a considerable increase in ECOD activity was seen in the brain, gill, liver, and muscle tissues (*Philip B. Bedient et al., 2005*).

Moreover, following treatment with sublethal doses of fipronil in the range of 2–20 µg/L, the dose-dependent responses of the ECOD activity were seen. The findings showed that ECOD could work as a biomarker for the effects of fipronil in zebrafish (*Haihua Wu et al., 2014*).

2.3.3 MicroRNA-155 Targets cyb561d2

When an organism is exposed to an environmental chemical, microRNAs (miRNAs), a family of short noncoding RNAs, may modify the expression of several protein-coding genes. Exposure to fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl) phenyl]-4-[(trifluoromethyl) sulphonyl]- 1H-pyrazole-3-carbonitrile) dramatically decreased the expression of miR-155 in adult zebrafish (*Danio rerio*). The control of this miRNA's anticipated target gene, cyb561d2, a member of the cytochrome b561 (cyt b561) family involved in electron transport, cell defense, and chemical stress, hasn't yet been experimentally verified. Upon stimulation with fipronil, the expression of miR-155 was downregulated, but cyb561d2 was increased at the mRNA and protein levels in a dose-dependent manner.

The dual luciferase report test showed that miR-155 interacted with cyb561d2 3' - untranslated regions (3' -UTR). In contrast, when endogenous miR-155 was decreased by transfection with a miR-155 inhibitor, the expression of cyb561d2 rose in both mRNA and protein levels. The findings enhanced our knowledge of the molecular mechanisms behind toxicity after fipronil exposure and suggest miR-155 as a possible new toxicological biomarker for chemical exposure (*Hannian Huang et al., 2014*).

2.3.4 Oxidative stress

As a broad-spectrum phenylpyrazole insecticide and veterinary medication, fipronil (FIP) is extensively used across the globe. The first insecticide to target the γ-aminobutyric acid (GABA) receptor was FIP. It is more selectively toxic to insects than to mammals. But there is growing evidence that FIP could have several toxic effects on animals and people, including neurotoxic, hepatotoxic, nephrotoxic, reproductive, and cytotoxic effects on vertebrate and invertebrate species. This is because of accidental exposure, improper use of FIP, or widespread FIP use that contaminates water and soil (*Silva MH et al., 2016*).

Over the last ten years, oxidative stress has been implicated in several FIP-induced toxicities. The toxicity of FIP in connection to oxidative stress has not yet been extensively reviewed. Reactive oxygen species (ROS) and oxidative stress are produced as a consequence of FIP therapy, and studies have been done to determine their relationship to different forms of toxicity. It was also discussed how FIP was metabolized, which required several CYP450 enzymes and might lead to oxidative stress (Xu Wang et al., 2016).

2.3.5 Vitamin C attenuates oxidative stress and behavioral abnormalities

Several diseases may develop in children who are exposed to synthetic pesticides regularly throughout their early years. Autism spectrum Disorder (ASD) may be brought on by several factors, including parental age, maternal smoking, delivery problems, and exposure to poisons like pesticides throughout infancy. The purpose of this study was to assess the potential protective effects of vitamin C (Vit. C) from children's supplements on juvenile zebrafish for swimming abilities, social behavior, and oxidative stress associated with ASD model after 14 days of chronic exposure to insecticide mixture fipronil (Fip) + pyriproxyfen (Pyr).

Pesticides may interfere with zebrafish social behavior for 14 days after application, as well as reduce swimming abilities and increase the oxidative stress indicators SOD (Superoxide dismutase), GPx (Glutathione peroxidase), and MDA (Malondialdehyde). The oxidative stress and neurotoxicity of the pesticide combination were dramatically reduced by the vitamin C supplement. This work offers potential in vivo proof that vitamin C supplementation might lessen the oxidative stress and brain damage caused by long-term exposure to the insecticides fipronil and pyriproxyfen in juvenile zebrafish (Madalina Andreea Robea et al., 2020).

3. AIM AND SCOPE

- To analyse the effects of Fipronil on Zebrafish brain with varied concentrations.
- To assess the damage done by Fipronil to Zebrafish brain anatomy.
- To observe the Zebrafish brain Lipids under the influence of Fipronil.
- To perform Gas chromatography- mass spectrometry and Gas Chromatography- Flame Induced Detection
- To perform Total Protein Quantification.
- To perform Acetylcholine enzyme Quantification.
- To perform Open Field test.

4. MATERIALS AND METHODS

4.1 EXPERIMENTAL ANIMALS AND FIPRONIL

Actively moving Adults or juveniles of zebrafish (total length, 2.0 ± 1.0 cm, weight, 0.19 ± 0.09 g) were purchased from a local fish supplier. The fish were acclimatized for 2 weeks to laboratory conditions in a 90-L glass aquarium. During the acclimatization period, the fish were fed dry food thrice a day at fixed times. The feces and uneaten food were siphoned out once a day and 50% of reconstituted water were exchanged daily. The water in the glass aquarium was aerated and heated. The water temperatures ranged from 23 to 25 C, with a pH of 7.1 ± 0.5 and dissolved oxygen content of 7.3 ± 0.5 mg/L during the period of acclimation. The natural photoperiod of 13:11 L:D was maintained. To reduce the amount of excreted products in the test tanks, feeding was stopped 24 h prior to the commencement of acute bioassay tests. All animals were healthy; a mortality rate of less than 2% was observed in the stocks used during the acclimation period.

4.2 FIPRONIL TREATMENT

1 gram of Fipronil was dissolved in 1 liter of distilled water. LC50 value was calculated as 700 $\mu\text{g/l}$. Although viability was steeply decreased at between 650 $\mu\text{g/l}$ to 850 $\mu\text{g/l}$ of fipronil, most of the morphological abnormalities showed a plateau effect at 650 $\mu\text{g/l}$ to 850 $\mu\text{g/l}$. Fipronil was added at different concentrations to a different group of fish, the concentrations were 50 $\mu\text{g/l}$, 100 $\mu\text{g/l}$, and 300 $\mu\text{g/l}$. Fipronil (80% WG), other components are Sodium Polycarboxylate: 2.90%, Sodium dioctyl sulfosuccinate: (85%) 1.90%, Silicone Oil emulsion: 0.30%, Sodium lignosulphonate: Q.S.

4.3 HISTOLOGY

Treated zebrafish were anesthetized using tricaine and immediately euthanized by decapitation, and brains were dissected and put in

Paraformaldehyde (PFA 4%). After 24 hours of incubation Tissue processing is performed. The tissue is washed in distilled water for 1 minute, then the tissue is kept in 70% alcohol for 30 minutes. Followed by 80% and 90% every 30 minutes. Then it is transferred to 100% and kept for 45 minutes. Paraffin wax is melted at 60°C and maintained at this temperature for 30 minutes. Then the wax is poured into a mold and the tissue is kept in it in a particular position. The wax block was allowed to settle at least for 3 hours. After 3 hours the block is ready and the slides are prepared. The block is sectioned in microtome (LEICA RM2125RTS) and the ribbons were placed on the slide (with adhesive, that is albumin and glycerol) containing 1ml of distilled water, then the slide is dried for 2 minutes. Then the slide is kept in Xylene for 15-20 minutes (until the wax was removed). Then the slide was kept in 100% alcohol for 2-5 minutes. Followed by 90%, 80%, 70%, and distilled water for 2-5 minutes. Then it was air dried and kept in hematoxylin for 5-15 minutes and washed in distilled water and air dried again for 2 minutes. Then the slide was dipped 2-3 times in acid alcohol and washed in tap water and air-dried for 2 minutes. The slide was dipped in 2% eosin and then immediately dipped in 90% alcohol for 1 minute, air dried, and kept in Xylene for 30 seconds. Finally, the slides are air dried, a single drop of DPX mountant was placed on the slide, and the tissue was covered by a cover slip. After 24 hours the slide is visualized under the microscope.



Fig 4.1: Fish exposed to MS-222



Fig 4.2: Wax cubes containing Zebrafish Brain



Fig 4.3: Tissue sectioned in Microtome

4.4 LIPID PROFILING IN GC-FID (GAS CHROMATOGRAPHY – FLAME IONIZATION DETECTION)

The brain was homogenized in chloroform and methanol. Then the sample was kept in the vortex for 2 minutes and then 1% NaCl was added. Chloroform, methanol, and NaCl are added in the ratio of 2:1:1. Then the sample was spun for 2 minutes, and chloroform along with lipids was separated from methanol. The chloroform layer was pipetted out and heated in a round bottom flask until it evaporates. Then 15 ml of methanolic NaOH was added, and the

round bottom flask was connected to the condenser, and heated for 20 minutes (this method is called saponification) followed by methanolic sulphuric acid and heated for 20 minutes (this method is for esterification). The sample was transferred to an Eppendorf tube and 2 ml and heptane and 1 ml of water are added to the sample. The sample was gently shaken and kept to settle for 2 minutes. The thin heptane layer was pipetted out and transferred to GC vial. The sample in the vial is injected into the column through the septum along with the mobile phase (we have used Helium as the mobile phase). The column in the chamber is maintained at the temperature of 150-300°C and the temperature at the injection region is maintained at 20-50°C higher than that of the column, this allows rapid volatilization of the compounds and after the sample gets volatilized the sample was passed through a column where the separation occurs. Separation occurs based on the interaction of molecules between the mobile phase and the stationary phase. The less volatile molecule interacts more with the stationary phase and moves slowly, while the more volatile molecule interacts more with the mobile phase and moves faster. The detection is done with the help of the detector- FID (Flame Ionization Detector). FID consists of Hydrogen and Oxygen cylinders to ignite a flame and when the sample reaches the flame it gets ionized. The two electrodes across the flame detect the ions in the form of a current which is amplified and detected by the computer. As the sample is detected the computer gives a peak for the retention of the sample, the area under the peak gives information about the concentration of the sample.



Fig 4.4: Brain ground in



Fig 4.5: Methanol and chloroform

Chloroform

layers after centrifuge



Fig 4.6: Sample for GC-FID



***Fig 4.7: Gas Chromatography with
Flame Ionization Detection
(GC-FID). GC 2010 Plus.***

4.5 QUANTIFICATION OF ACETYLCHOLINE ENZYME

Nine different standards were made by adding Bovine Serum Albumin (BSA) and Phosphate Buffer (PB) according to the given table in Eppendorf tubes.

Table 4.1: Amount of reagents required

PROTEIN(μ g)	BSA(ml)	PB (ml)
2000	1	0
1500	0.75	0.25

1000	0.5	0.5
750	0.375	0.625
500	0.25	0.75
250	0.125	0.875
125	0.0625	0.9375
25	0.0125	0.9875
0	0	1

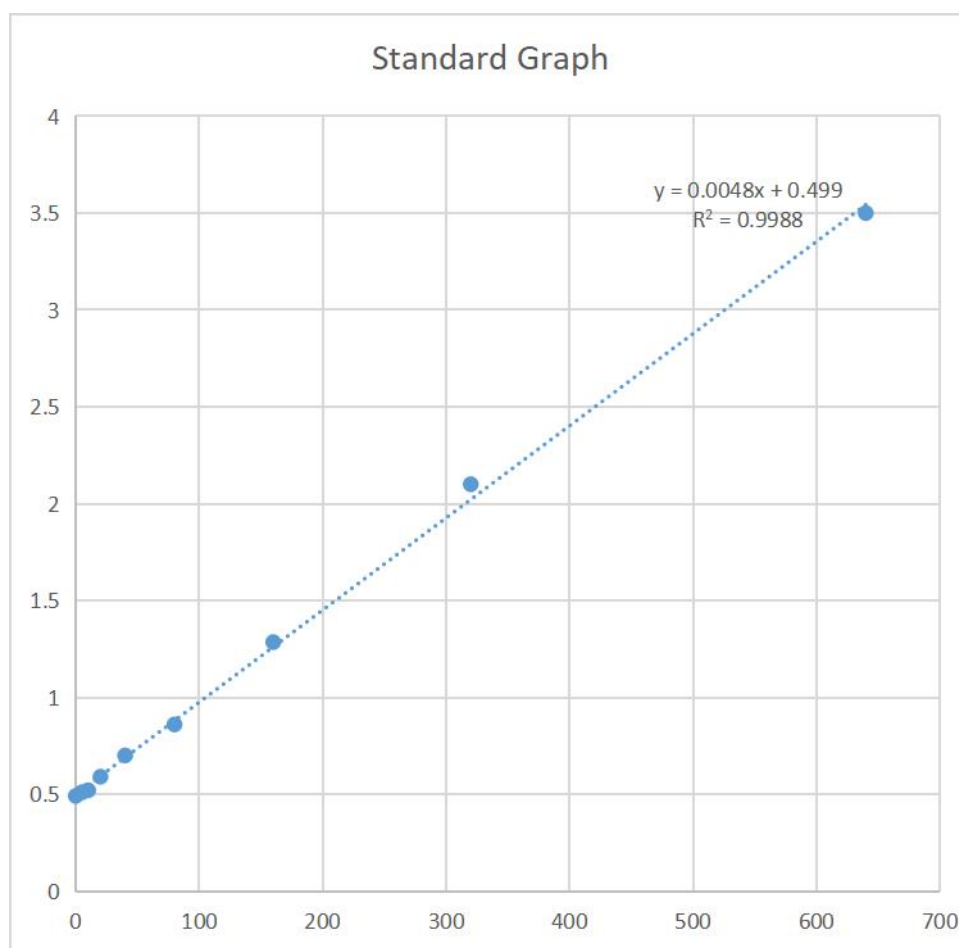


Fig 4.8: Graph of Standard (BSA+PB)

The samples were prepared by anesthetizing of zebrafish, followed by euthanized by decapitation of zebrafish. The brains were taken out and homogenized using a

mortar in 1ml chloroform. The contents were transferred to Eppendorf tubes and they were centrifuged at 3000rpm for 10 minutes at 4°C. BCA(A) and BCA(B) were prepared in the ratio of 50:1. Standards and unknown samples were added to the 96 well plate along with the BCA(A+B) in the ratio of 1:8. The well plate was then kept in EnSpire multi-mode reader and absorbance was read at 562nm. 40µl of the three unknown samples were added in the 96 well plate, followed by 10µl of DTNB(Elman's Reagent) and 2µl of Acetylcholine iodide. The well plate was then kept in EnSpire multi-mode reader and absorbance was read at 412nm(*Miroslav Pohanka et al., 2011*).



Fig 4.9: EnSpire Multimode Plate reader

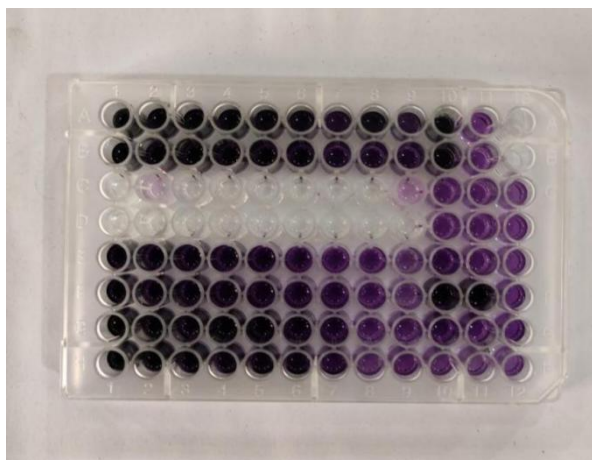


Fig 4.10: Quantification of Acetylcholine Enzyme



Fig 4.11: Samples for Acetylcholine enzyme

4.6 GC-MS (Gas Chromatography-Mass Spectrometry)



Fig 4.12: GC-MS (Gas Chromatography-Mass Spectrometry)

In GC-MS the sample is detected using mass spectrometry. The sample from GC is bombarded with an electron beam for ionization and the ionized sample reaches the mass analyzer, multiple electrodes affect the trajectory of the sample. The electrodes across the flame detect the ions in the form of a current which is amplified and detected by the computer. The computer detects the

concentration of the sample based on the ratio of mass and charge of a sample. As the sample is detected the computer gives a peak for the retention of the sample, the area under the peak shows' information about the concentration of the sample.

4.7 BEHAVIOR STUDIES

The open-field test is performed on zebrafish. Open field test uses horizontal distribution in a novel environment as a validated measure of 'anxiety-like' behaviors in adult zebrafish. Open field test utilizes zebrafish's instinctive behavior to seek protection in novel environments. When the fish is introduced to a novel environment the fish will stay in a corner for some time and observe the surrounding and slowly start moving across the tank.

5. RESULTS AND DISCUSSION

5.1 HISTOLOGY OF ZEBRAFISH BRAIN

5.1.1 Day 1

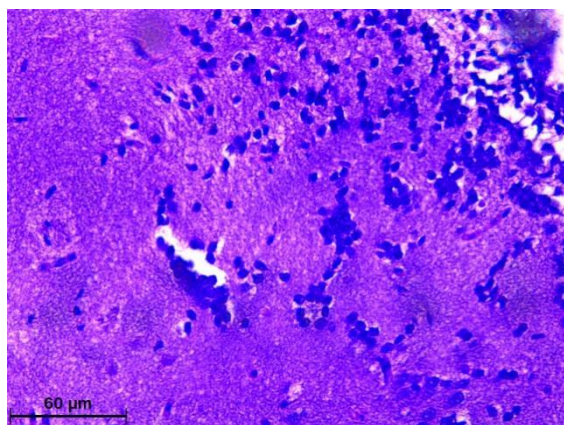


Fig 5.1: 24 hrs – 50 µg/L

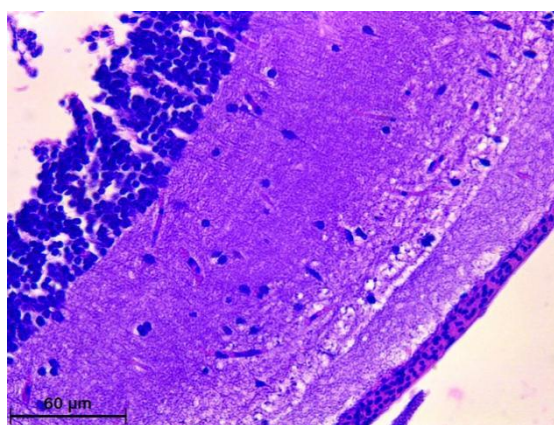


Fig 5.2: 24 hrs – 100 µg/L

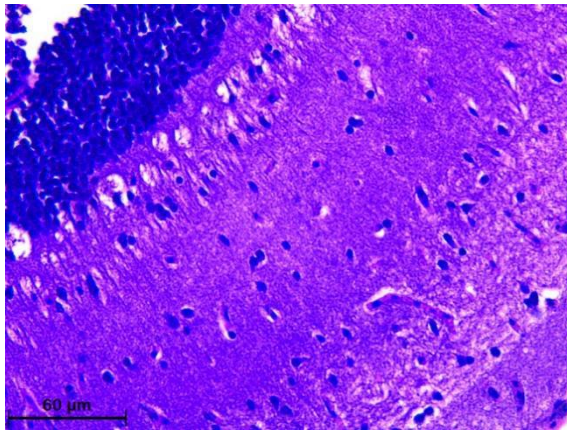


Fig 5.3: 24 hrs – 100 µg/L

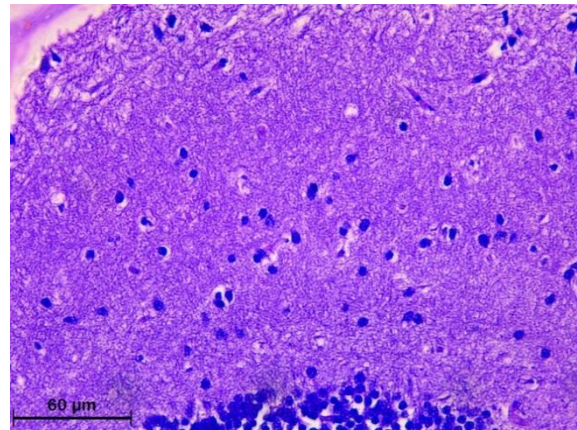


Fig 5.4: 24 hrs – control

Fig 5.1: The lateral torus region shows no proper arrangement of cells and showing inflammation due to sudden shock of fipronil treatment.

Fig 5.2: 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 5.3: 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 5.4: Control shows round, intact and round nuclei oligodendroglia cells in the central zone and deep white zone of optic tectum.

5.1.2 Day 2

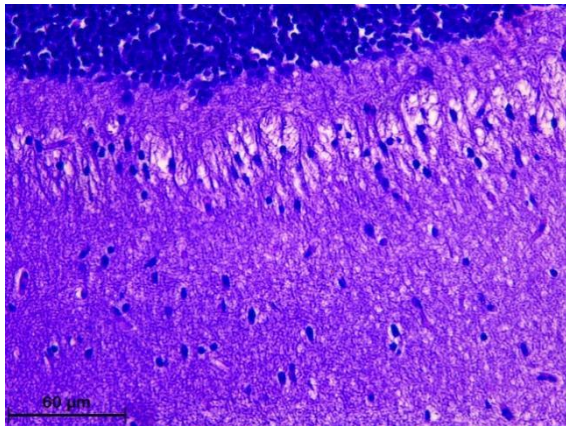


Fig 5.5: 48 hrs – 50 µg/L

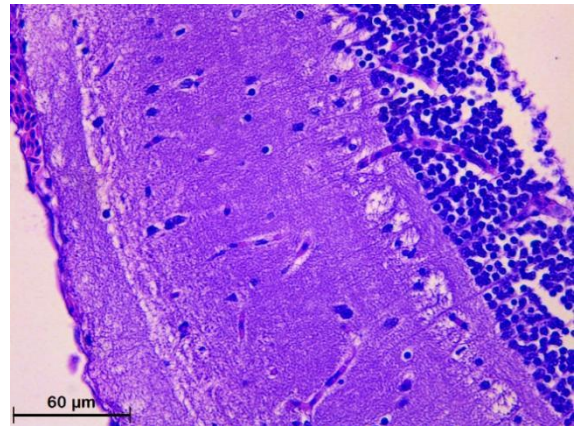


Fig 5.6: 48 hrs – 100 µg/L

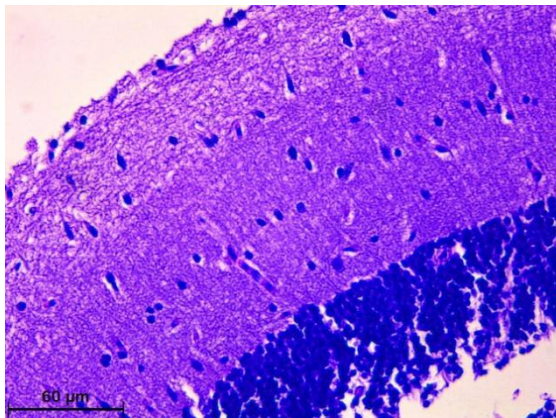


Fig 5.7: 48 hrs – 300 µg/L

Fig 5.5: 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 5.6: 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 shows no signs of inflammation, suggesting the infiltrate progressing towards the white matter from day 1.

Fig 5.7: Disturbed pia matter and clumped granule cells observed. It suggests the neurotoxic nature of fipronil at 300ug/L concentration on 2nd day.

5.1.3 Day 3

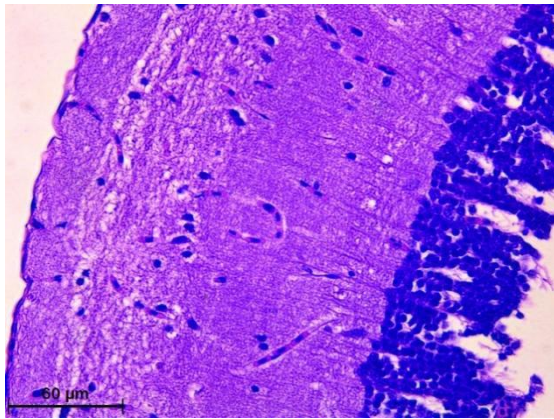


Fig 5.8: 72 hrs – 50 µg/L

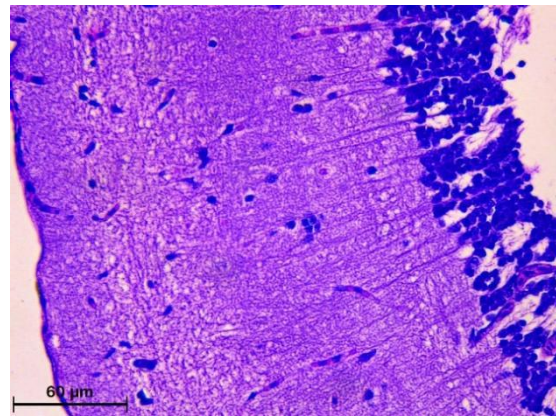


Fig 5.9: 72 hrs – 100 µg/L

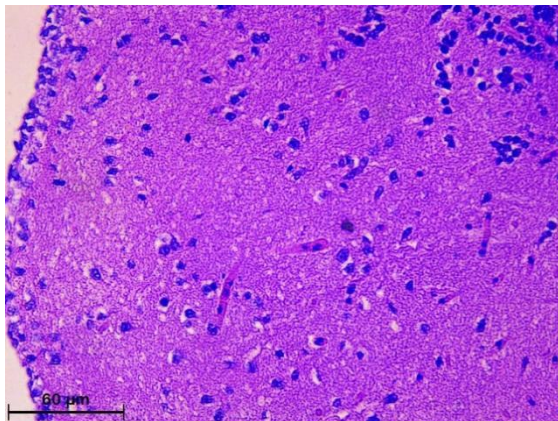


Fig 5.10: 72 hrs – 300 µg/L

Fig 5.8: 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 mesh like structure at superior white and grey zone. The neurons in optic tectum remains intact while inflammation triggered glial cell changes observed.

Fig 5.9: 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 5.10: The 3rd day at 300ug/L concentration showed similar pattern observed in day 3 of 100ug/L.

5.1.4 Day 7

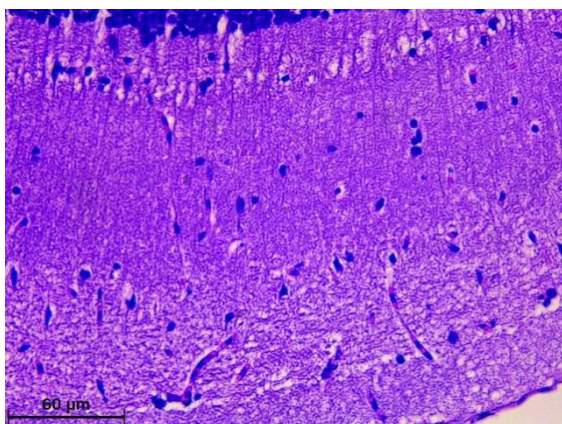


Fig 5.11: 168 hrs – 50 µg/L

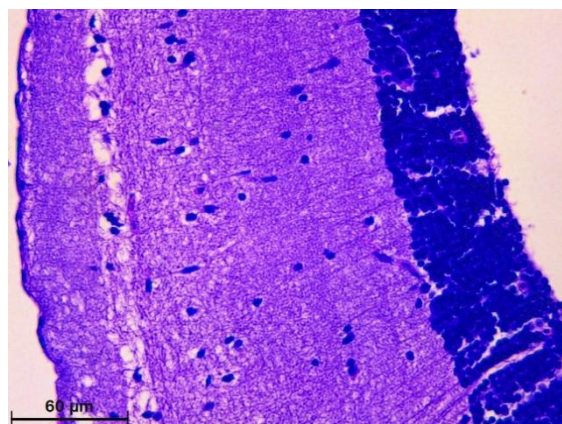


Fig 5.12: 168 hrs – 100 µg/L

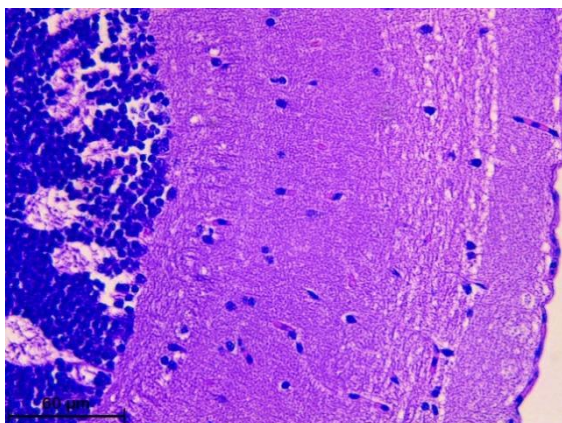


Fig 5.13: 168 hrs – 300 µg/L

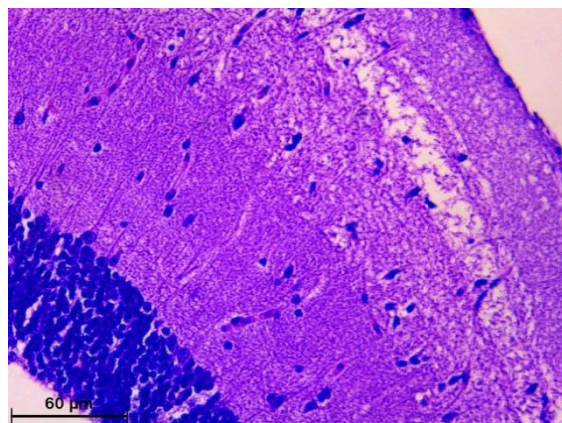


Fig 5.14: 168 hrs – control

Fig 5.11: 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 5.12: 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 5.13: Granuloma was significant in the grey matter with granules clumping together. This is the sign of extreme toxicity and inflammation. Neurons are intact.

5.2 QUANTIFICATION OF ACETYLCHOLINE ENZYME

Total protein content in 24 hrs, 48 hrs, 72 hrs and 168 hrs was found to be:

Table 5.1: 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 5.2: 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 5.3: 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: 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 we infer that the amount of Total protein is comparatively lesser in treated fishes and has steadily decreased in consecutive days

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

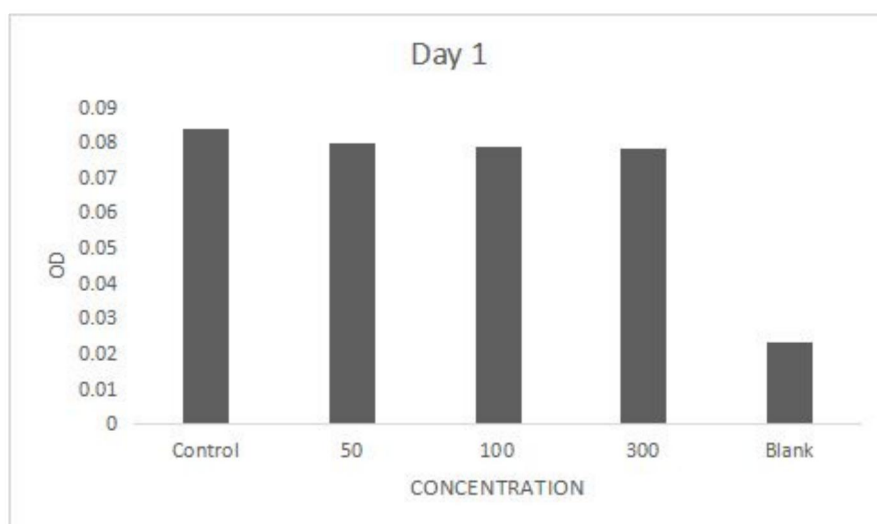


Fig 5.15: Acetylcholine Quantification in 24hrs

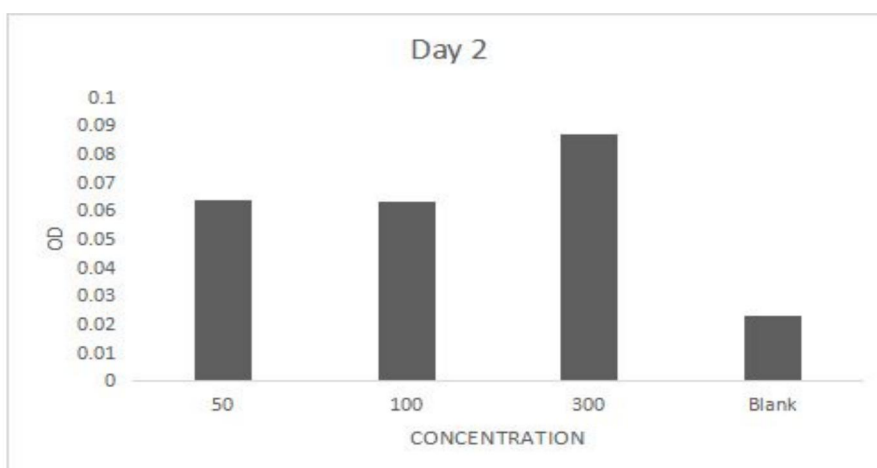


Fig 5.16: Acetylcholine Quantification in 48hrs

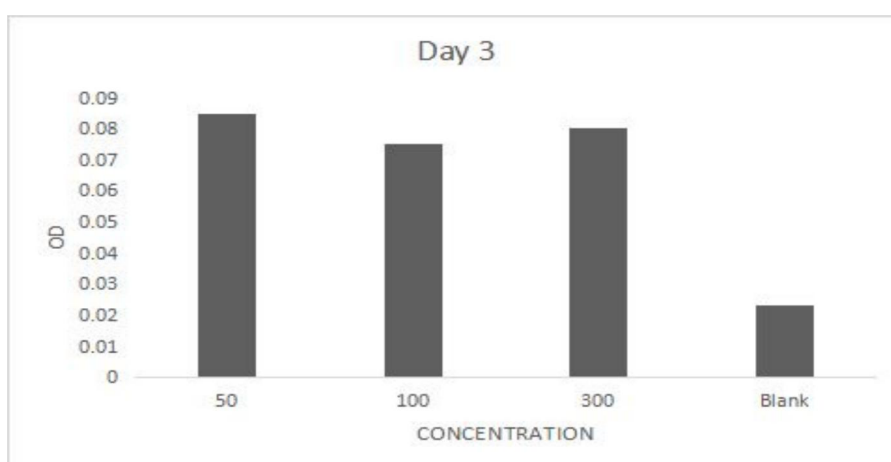


Fig 5.17: Acetylcholine Quantification in 72hrs

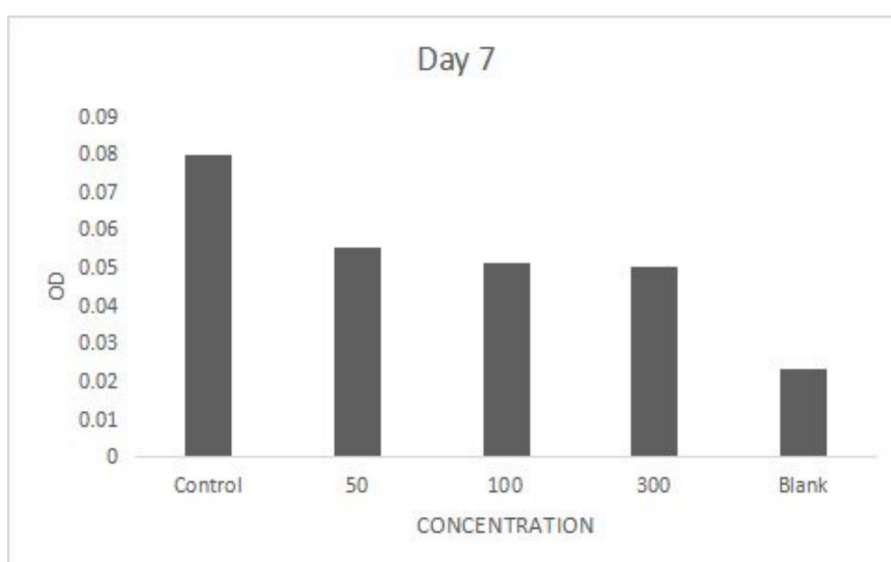


Fig 5.18: Acetylcholine Quantification in 168hrs

According to the graph, there was no increasing or decreasing trend found. Varying results were observed in each day.

5.3 BEHAVIOR STUDIES

5.3.1 Movement of treated and control fishes

5.3.1.1 Day 1

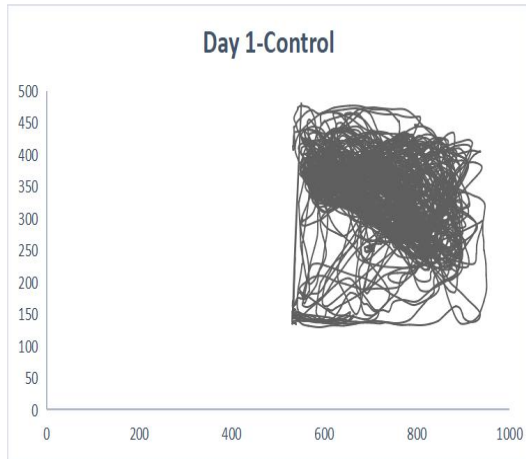


Fig 5.19: movement of control fish in 24hrs

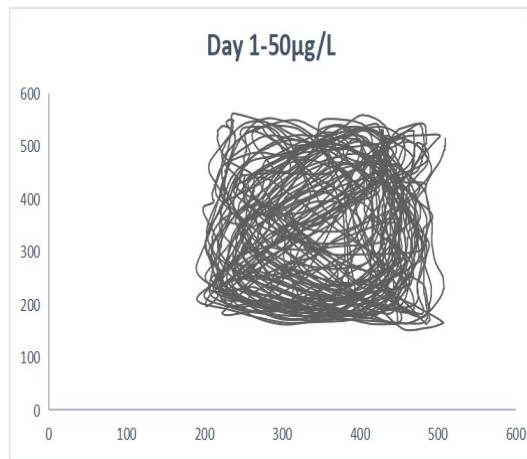


Fig 5.20: movement of fish in 50 µg/L in 24hrs

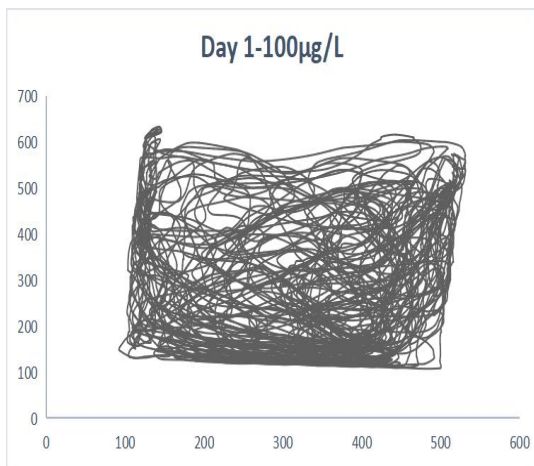


Fig 5.21: movement of fish in 100 µg/L in 24hrs

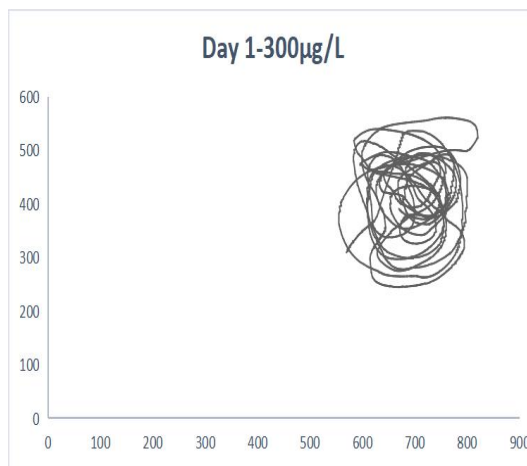


Fig 5.22: movement of fish in 300 µg/L in 24hrs

5.3.1.1 Day 2

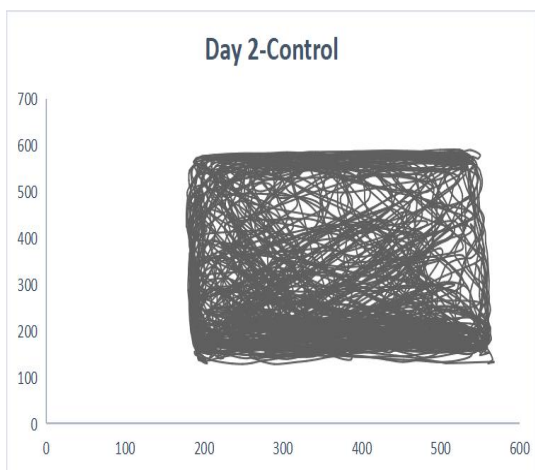


Fig 5.23: movement of control fish in 48hrs

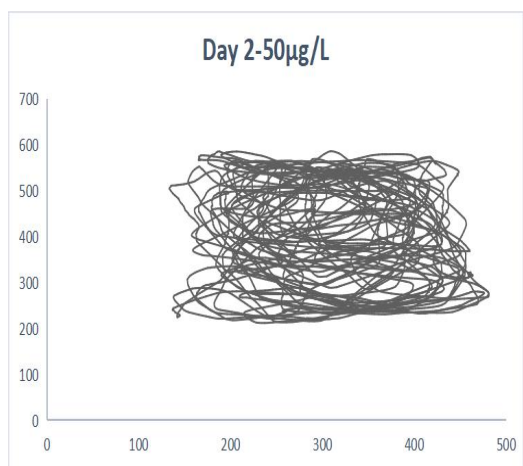


Fig 5.24: movement of fish in 50 µg/L in 48hrs

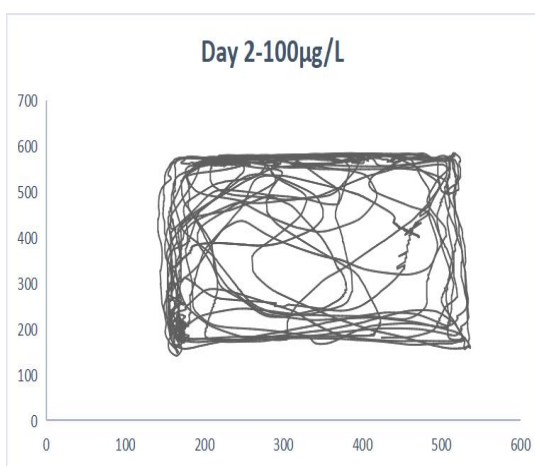


Fig 5.25: movement of fish in 100 µg/L in 48hrs

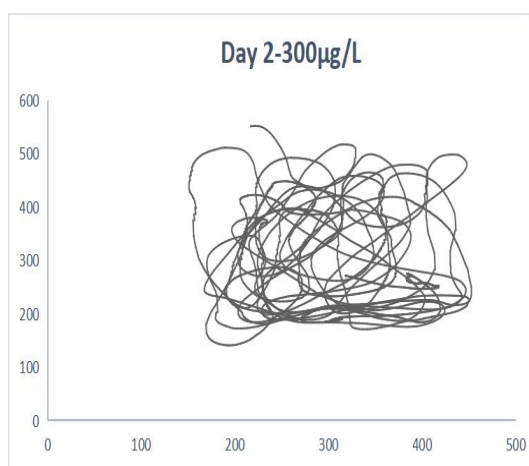


Fig 5.26: movement of fish in 300 µg/L in 48hrs

5.3.1.1 Day 3

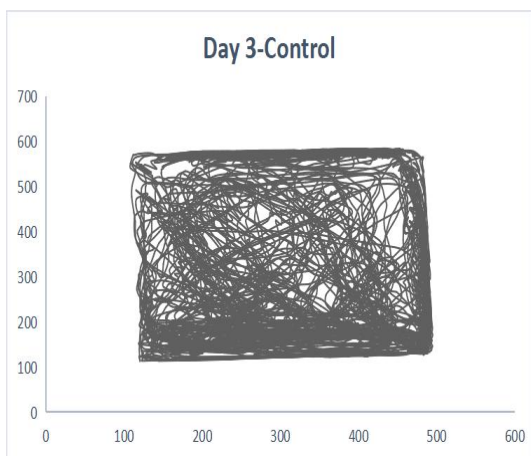


Fig 5.27: movement of control fish in 72hrs

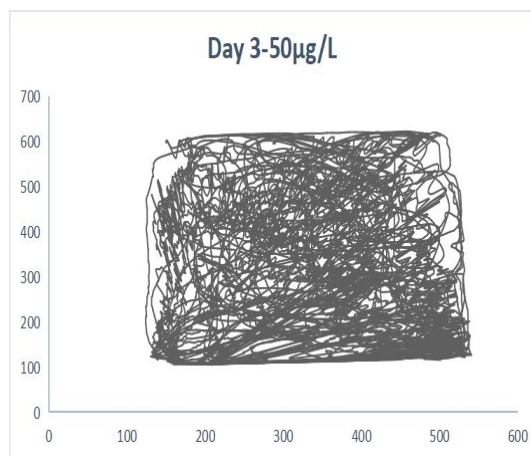


Fig 5.28: movement of fish in 50 µg/L in 72hrs

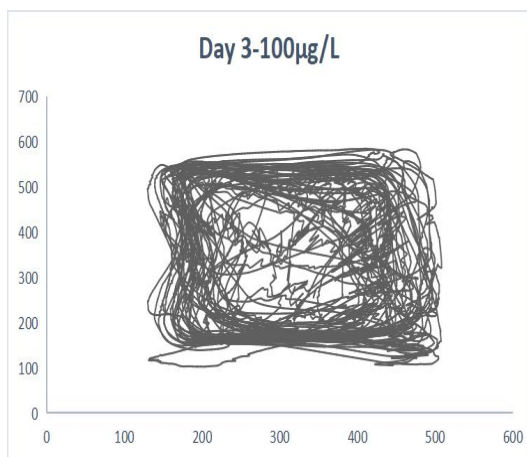


Fig 5.29: movement of fish in 100 µg/L in 72hrs

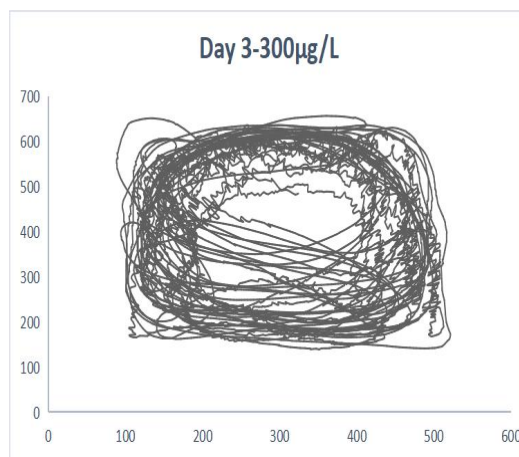


Fig 5.30: movement of fish in 300 µg/L in 72hrs

5.3.1.1 Day 7

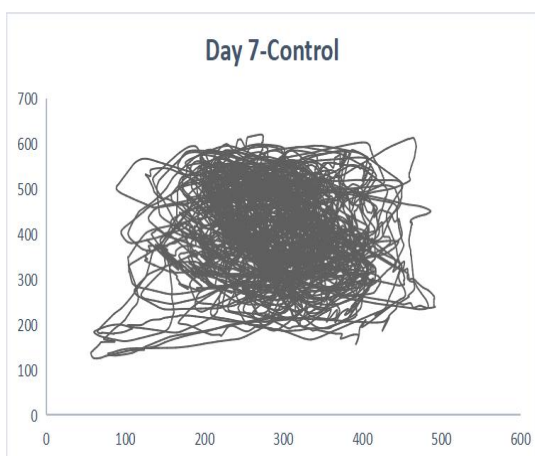


Fig 5.31: movement of control fish in 168hrs

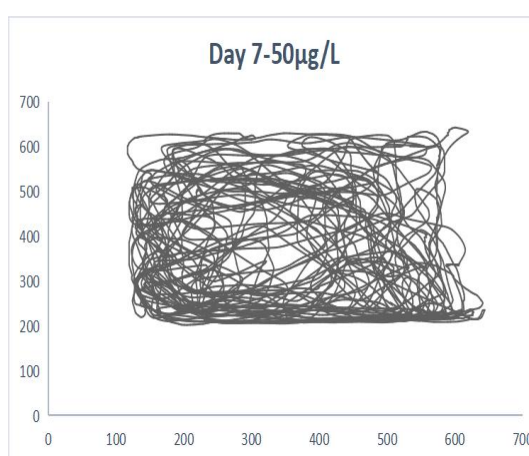


Fig 5.32: movement of fish in 50 µg/L in 24hrs

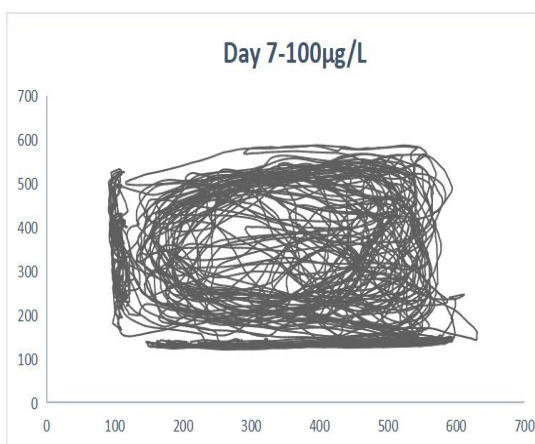


Fig 5.33: movement of fishes in 100 µg/L in 168hrs

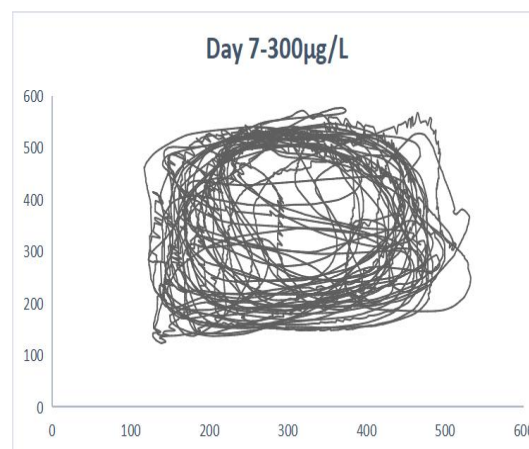
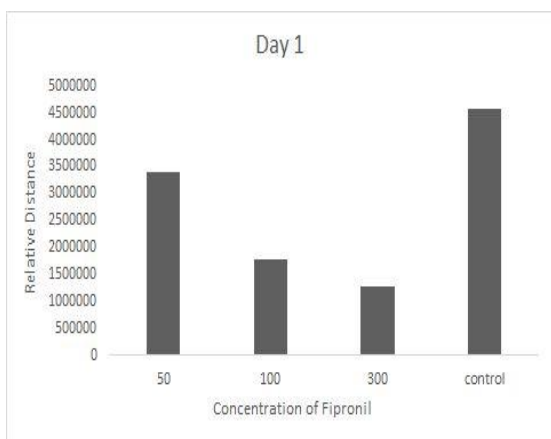


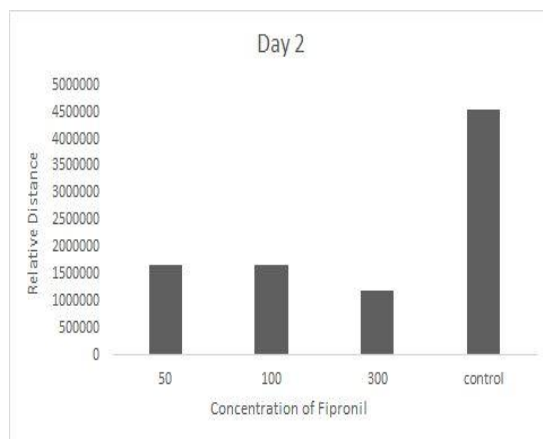
Fig 5.34: movement of fishes in 300 µg/L in 168hrs

From the above graph we infer that as the concentration of Fipronil increases, fishes explored and travelled lesser. And after 5 days the fishes gradually started to explore and travel a little more than before but not as equal as control fishes.

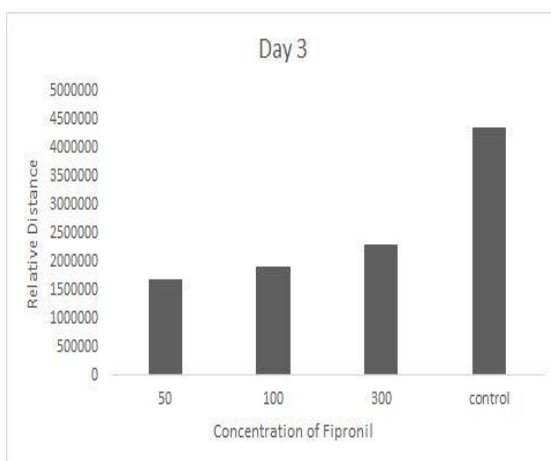
5.3.2 Relative distance of treated and control fishes



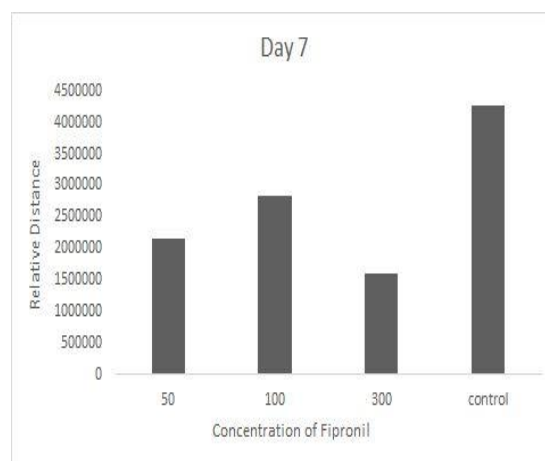
***Fig 5.35: Relative distance
of treated and control
fishes in 24hrs***



***Fig 5.36: Relative distance
of treated and control
fishes in 48hrs***



***Fig 5.37: Relative distance
of treated and control
fishes in 72hrs***



***Fig 5.38: Relative distance
of treated and control
fishes in 168hrs***

Relative distance of control fishes was found to be double the amount of average of treated fishes. Relative distance of treated fishes was found to be low at first three days and slightly increased after five days.

5.4 GC-FID

5.4.1 Day 1

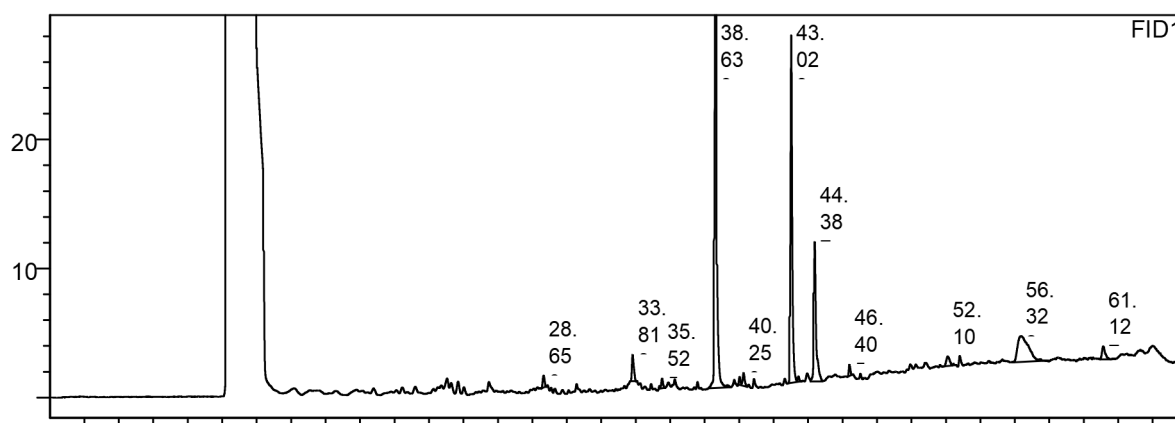


Fig 5.39: 24hrs-Control Chromatogram

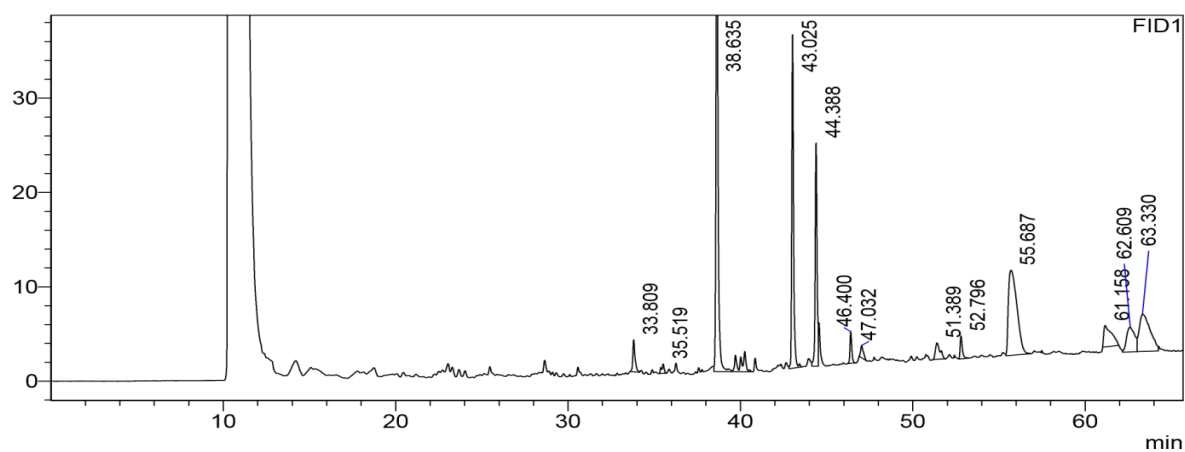


Fig 5.40: 24hrs-50µg/L Chromatogram

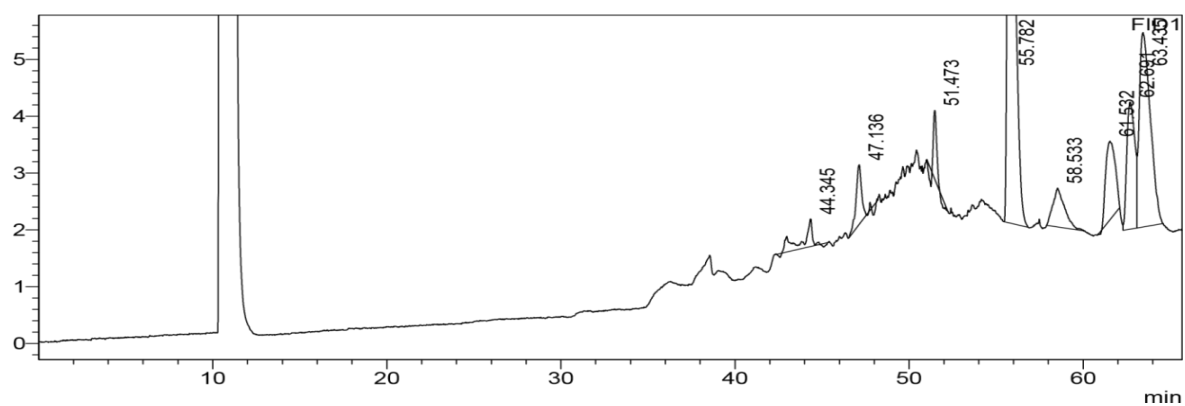


Fig 5.41: 24hrs-100µg/L Chromatogram

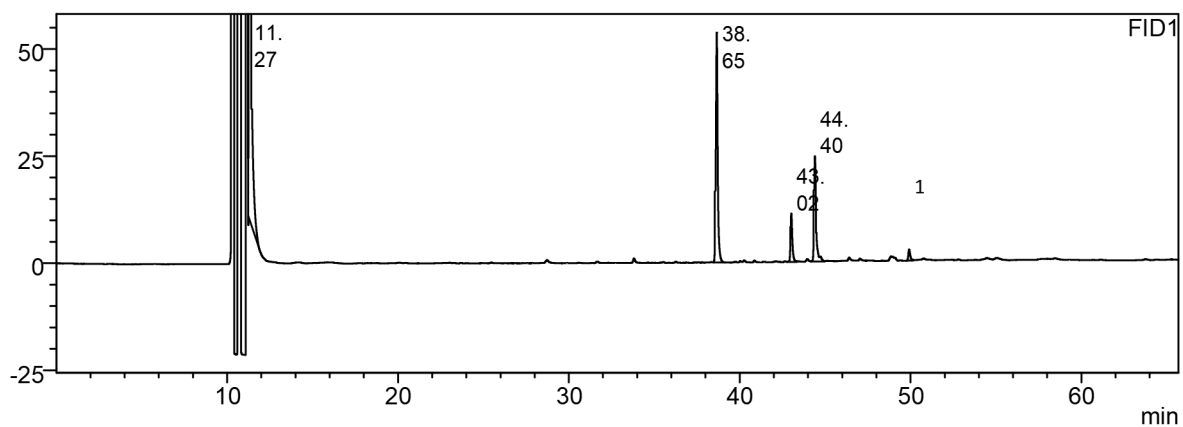


Fig 5.42: 24hrs-300µg/L Chromatogram

5.4.2 Day 2

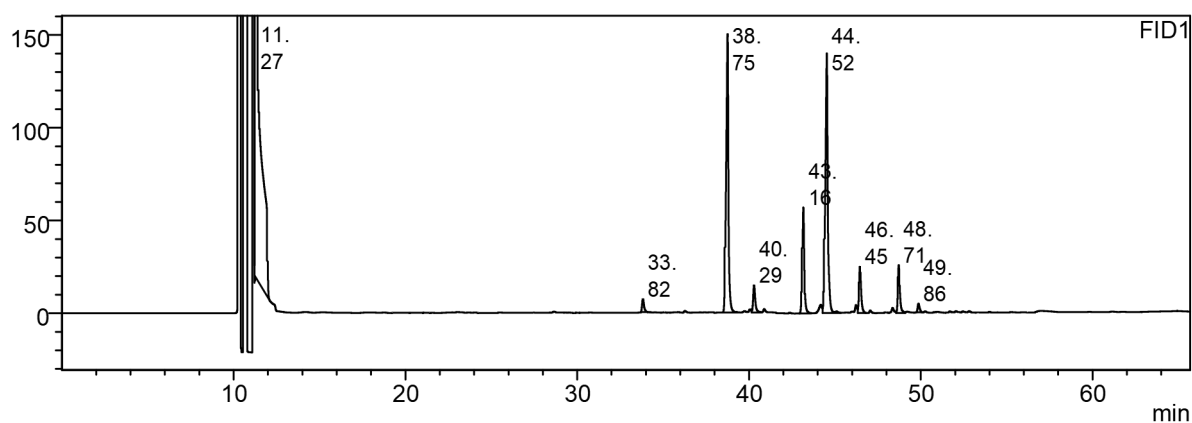


Fig 5.43: 48hrs-50µg/L Chromatogram

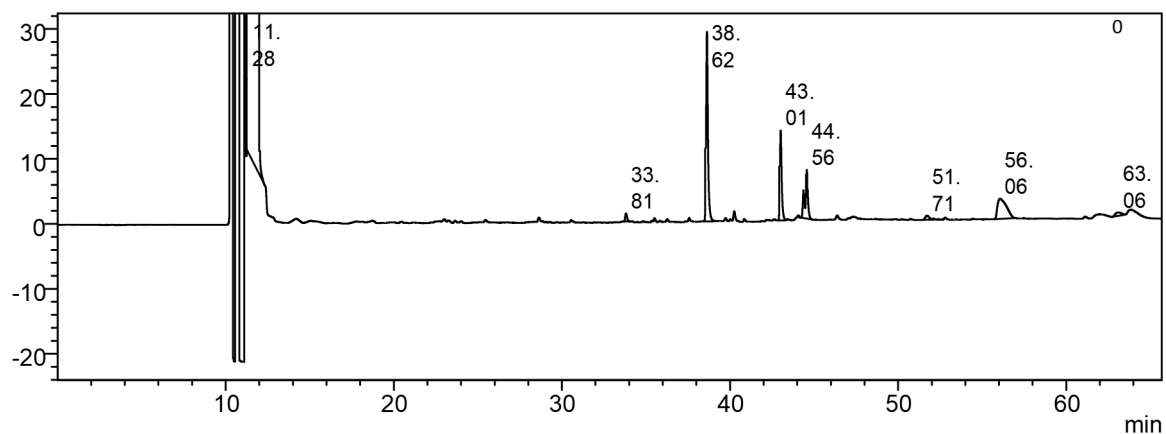


Fig 5.44: 48hrs-100µg/L Chromatogram

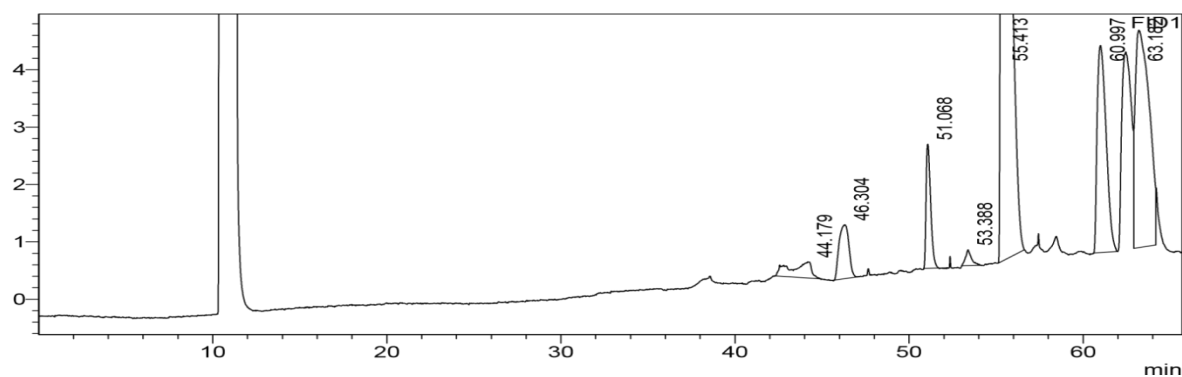


Fig 5.45: 48hrs-300µg/L Chromatogram

5.4.3 Day 3

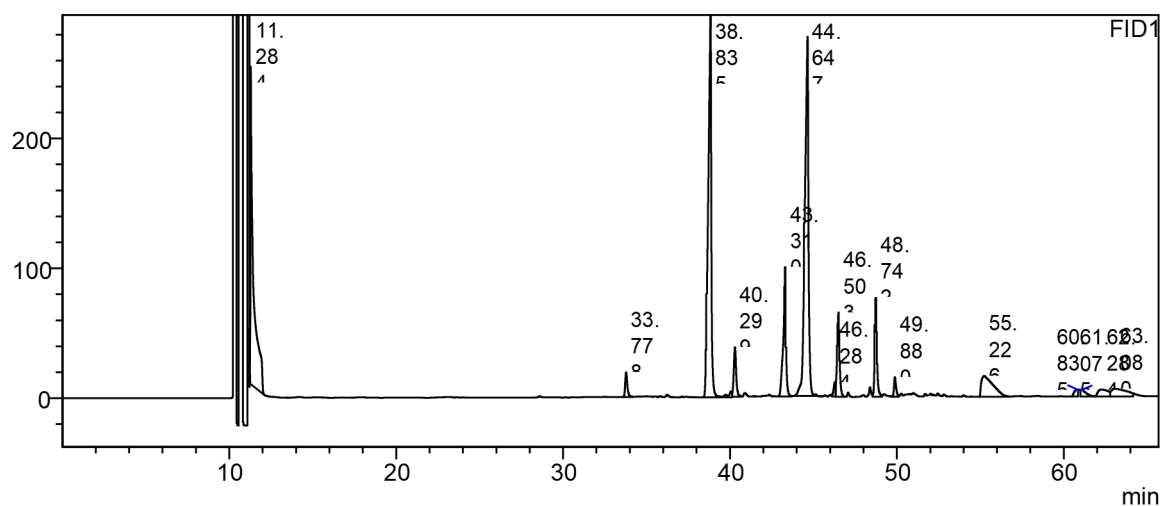


Fig 5.46: 72hrs-50µg/L Chromatogram

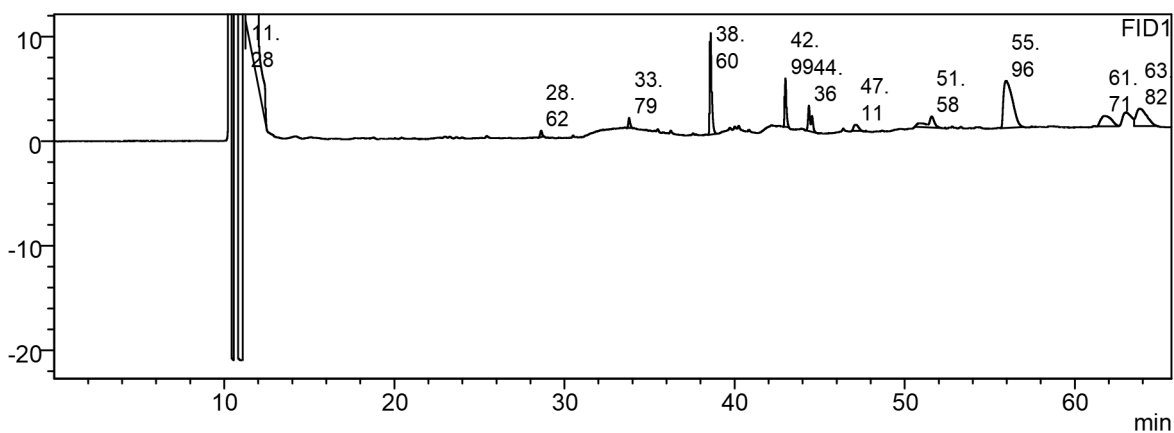


Fig 5.47: 72hrs-100µg/L Chromatogram

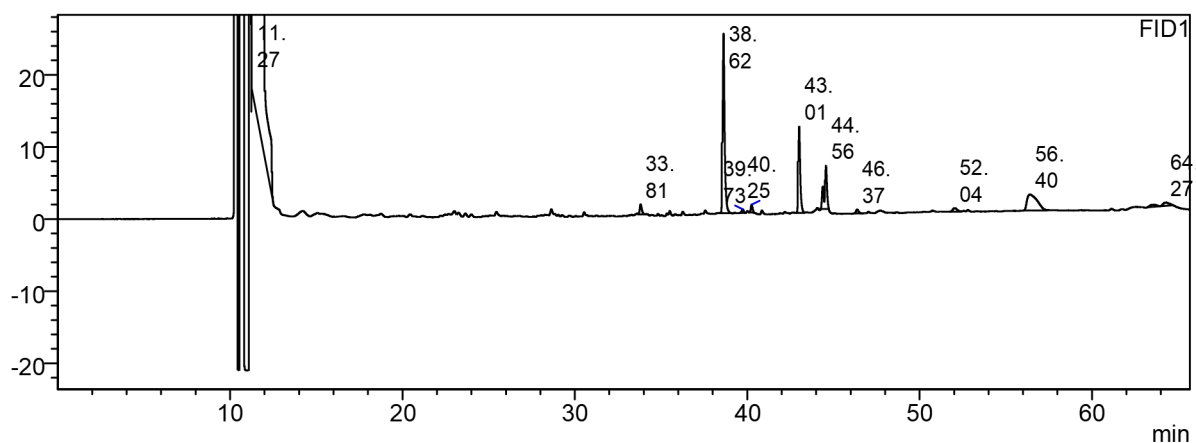


Fig 5.48: 72hrs-300µg/L Chromatogram

5.4.4 Day 7

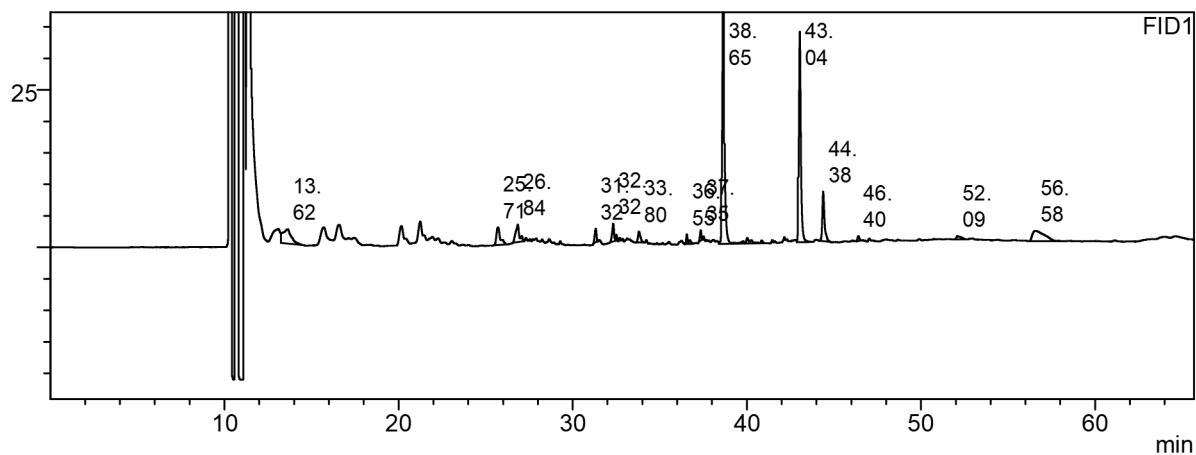


Fig 5.49: 168hrs-Control Chromatogram

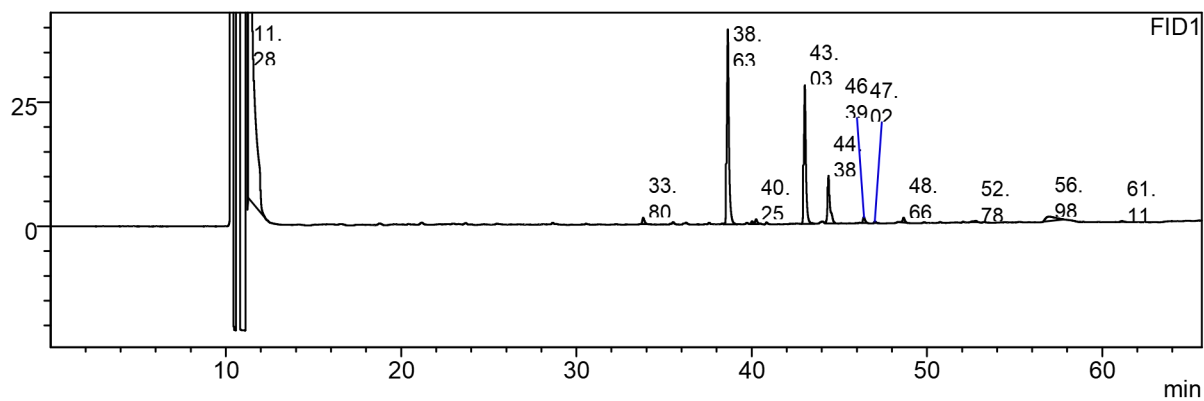


Fig 5.50: 168hr-50µg/L Chromatogram

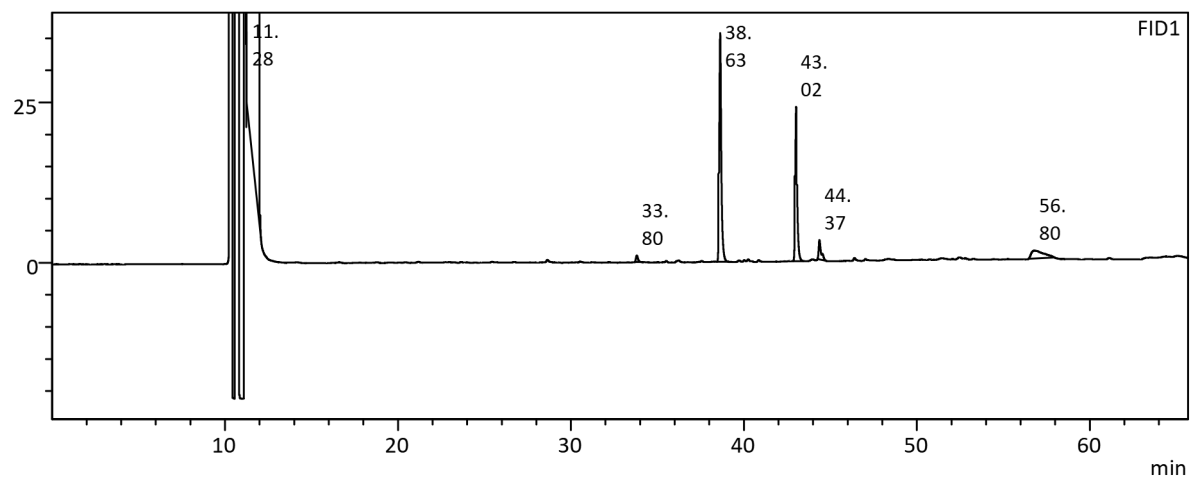


Fig 5.51: 168hrs-100µg/L Chromatogram

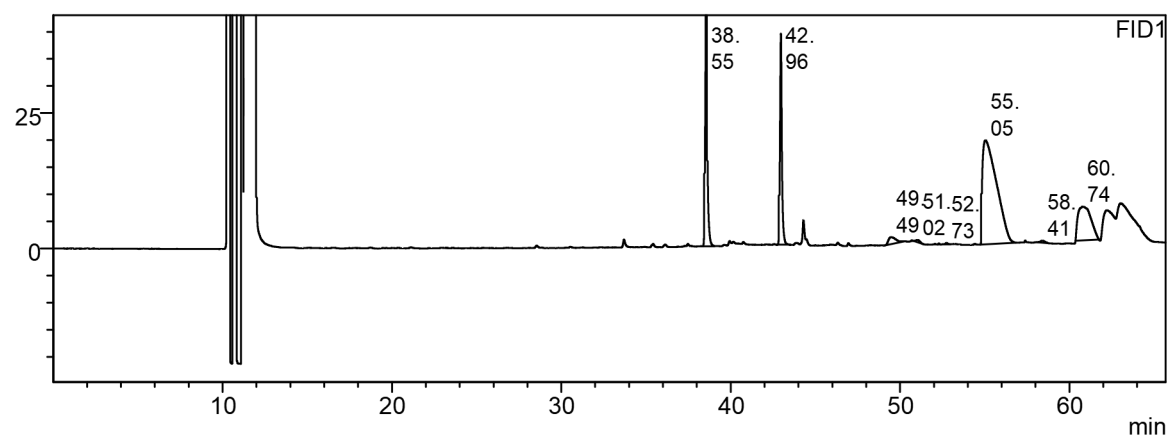


Fig 5.52: 168hrs-300µg/L Chromatogram

5.4.5 Concentration 50µg/L

Table 5.5: Changes in fatty acid concentration at different time intervals for fipronil treated brain at 50ug/L

S.No	Name	Area%			
		24hrs	48hrs	72hrs	168hrs
1	A-Linolenic Acid Methyl Ester	—	2.63	4.41	—
2	Arachidic Acid Methyl Ester	0.88	—	—	0.09
3	Behenic Acid Methyl Ester	1.92	—	—	—
4	Butyric Acid Methyl Ester	—	51.52	18.71	74.44
5	cis-11,14-Eicosadienoic Acid Methyl Ester	—	0.47	0.84	—
6	cis-11-Eicosenoic Acid Methyl Ester	—	—	—	0.31
7	cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester	19.67	—	0.72	0.06
8	cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester	18.42	—	4.93	—
9	Linoleic Acid Methyl Ester	1.42	2.66	4.3	0.34
10	Myristic Acid Methyl Ester	1.8	0.73	1.08	0.37
11	Myristoleic Acid Methyl Ester	0.62	—	—	—
12	Nervonic Acid Methyl Ester	—	—	—	1.24
13	Oleic Acid Methyl Ester	10.57	17.33	26.39	3.28
14	Palmitic Acid Methyl Ester	28.27	17.31	24.29	11.39
15	Palmitoleic Acid Methyl Ester	—	1.58	2.45	0.38
16	Stearic Acid Methyl Ester	15.07	5.72	7	7.88

17	Tricosanoic Acid Methyl Ester + Methyl cis-5,8,11,14-eicosatetraenoic acid methyl ester	1.29	–	–	0.14
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5.4.6 Concentration 100µg/L

Table 5.6: Changes in fatty acid concentration at different time intervals for fipronil treated brain at 100ug/L.

S.No	Name	Area%			
		24hrs	48hrs	72hrs	168hrs
1	Arachidic Acid Methyl Ester	3.67	–	0.26	–
2	Butyric Acid Methyl Ester	–	90.75	89.85	91.64
3	cis-11-Eicosenoic Acid Methyl Ester	–	–	–	–
4	cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester	8.58	–	0.89	–
5	cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester	–	0.22	–	–
6	cis-8,11,14-Eicosatrienoic Acid Methyl Ester	1.77	0.16	0.7	–
7	Elaidic Acid Methyl Ester	2.74	–	–	–
8	Lauric Acid Methyl Ester	–	–	0.1	–
9	Linoleic Acid Methyl Ester	–	–	–	–
10	Myristic Acid Methyl Ester	–	0.15	0.15	0.11
11	Nervonic Acid Methyl Ester	–	1.88	3.51	0.87
12	Oleic Acid Methyl Ester	–	1.45	0.62	0.42

13	Palmitic Acid Methyl Ester	—	3.68	1.6	4.21
14	Palmitoleic Acid Methyl Ester	—	—	—	—
15	Stearic Acid Methyl Ester	—	1.68	0.68	2.73

5.4.7 Concentration 300µg/L

Table 5.7: Changes in fatty acid concentration at different time intervals for fipronil treated brain at 300ug/L.

S.No	Name	Area%			
		24hrs	48hrs	72hrs	168hrs
1	Behenic Acid Methyl Ester	—	4.46	—	0.29
2	Butyric Acid Methyl Ester	67.5	—	89.24	—
	cis-10-Pentadecenoic Acid Methyl Ester	—	—	—	18.79
3	cis-11,14-Eicosadienoic Acid Methyl Ester	0.86	—	—	0.11
4	cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester	—	14.75	0.39	14.95
5	cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester	—	49.05	—	—
6	cis-8,11,14-Eicosatrienoic Acid Methyl Ester	—	—	0.17	—
7	Elaidic Acid Methyl Ester	—	2.58	—	—
8	Heneicosanoic Acid Methyl Ester	—	—	—	1.78
9	Lignoceric Acid Methyl Ester	—	—	—	50.58

10	Linoleic Acid Methyl Ester	–	4.19	0.11	–
11	Myristic Acid Methyl Ester	–	–	0.23	–
12	Nervonic Acid Methyl Ester	–	–	1.93	0.28
13	Oleic Acid Methyl Ester	9.12	–	1.36	–
14	Palmitic Acid Methyl Ester	18.77	–	4.32	–
15	Palmitoleic Acid Methyl Ester	–	–	0.08	–
16	Stearic Acid Methyl Ester	3.72	–	1.96	13.68
17	Tricosanoic Acid Methyl Ester + Methyl cis-5,8,11,14-eicosatetraenoic acid methyl ester		0.73	–	

We can infer from the tables that the area percentage of Stearic acid was more than 24.96% in control fishes, but less than 8% in treated fishes. In the case of Palmitic acid, the area percentage for control fishes was 36.04%, but less than 20% in treated fishes. Whereas the area percentage of Oleic acid decreased steadily as time progressed in treated fishes and no difference in area percentage is found for Linoleic acid.

5.5 GC-MS

5.5.1 Day 2 Control

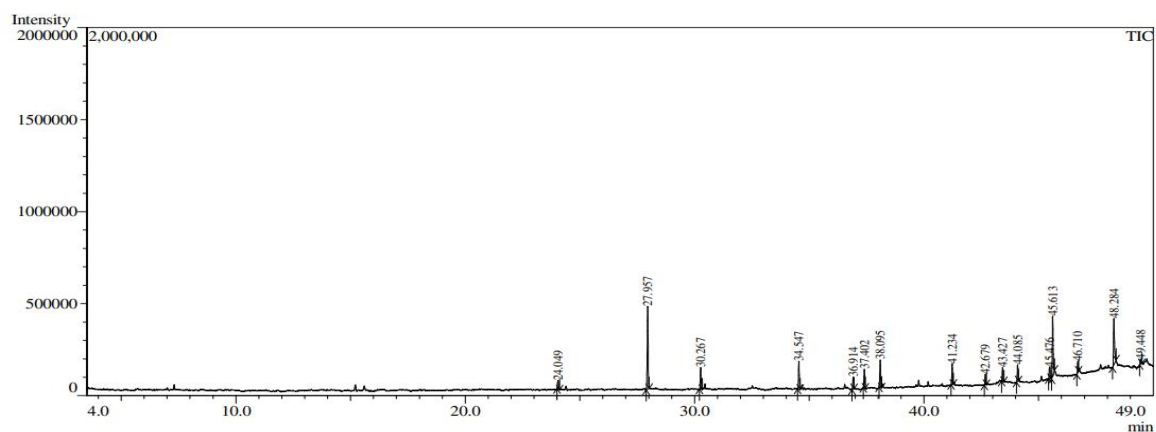


Fig 5.53: 48hrs-Control Chromatogram

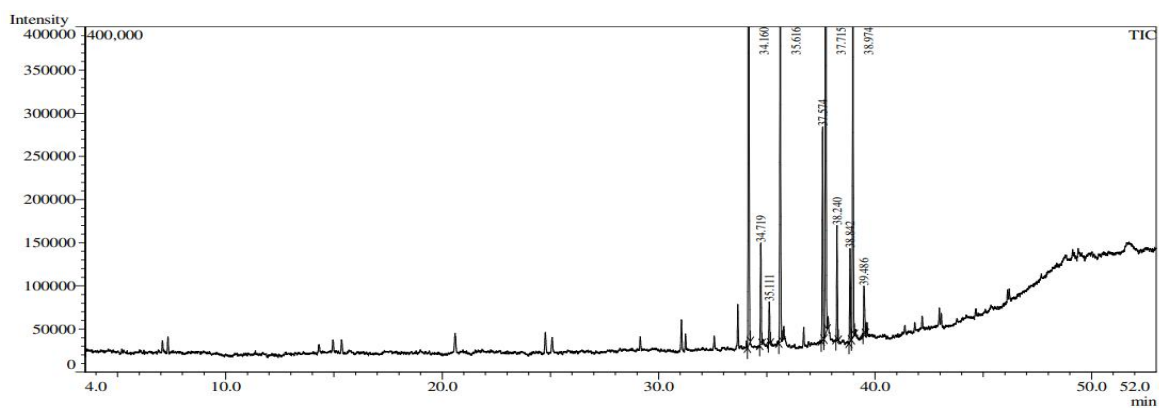


Fig 5.54: 48hrs-50µg/L Chromatogram

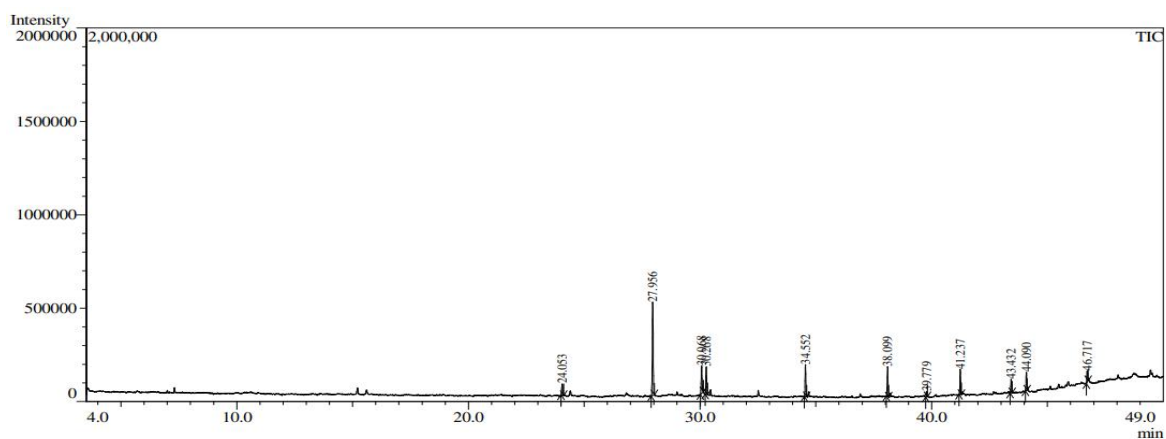


Fig 5.55: 48hrs-100µg/L Chromatogram

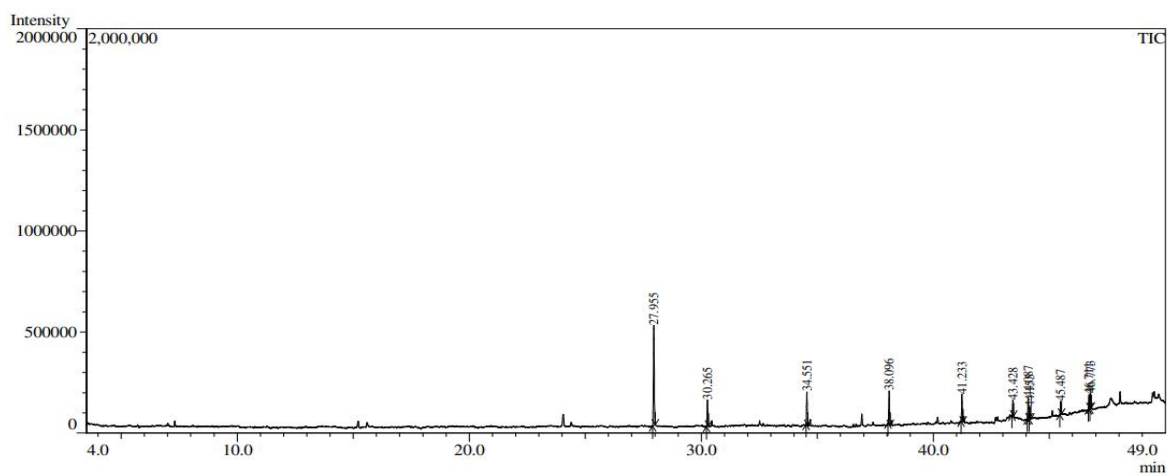


Fig 5.56: 48hrs-300µg/L Chromatogram

5.5.2 Day 3 Control

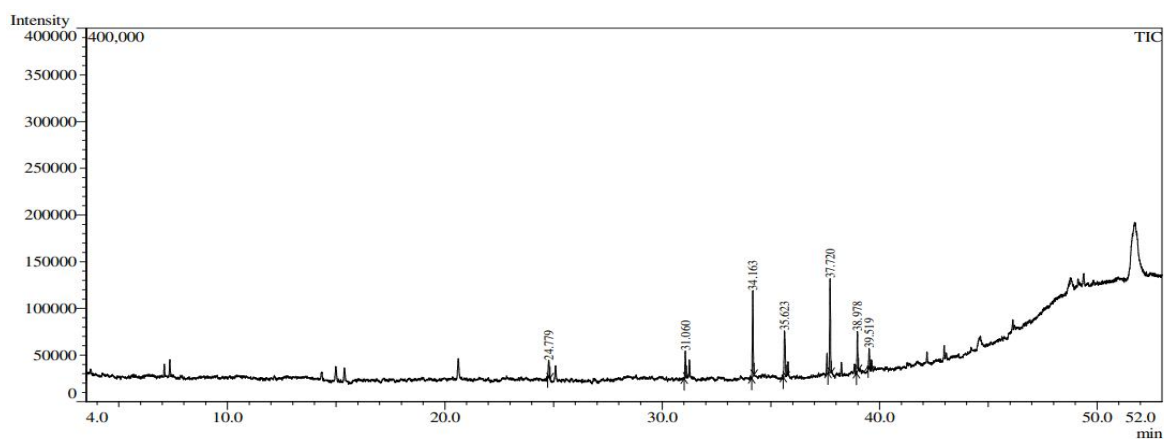


Fig 5.57: 72hrs-50µg/L Chromatogram

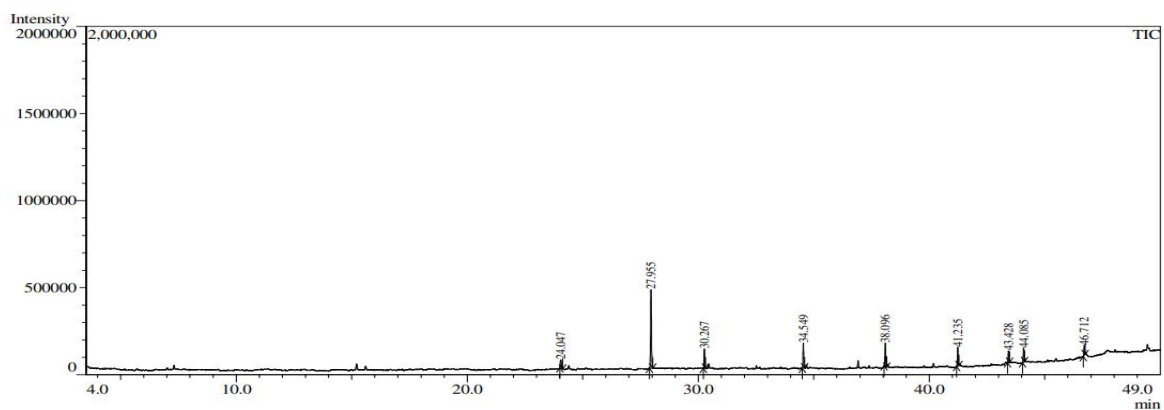


Fig 5.58: 72hrs-100µg/L Chromatogram

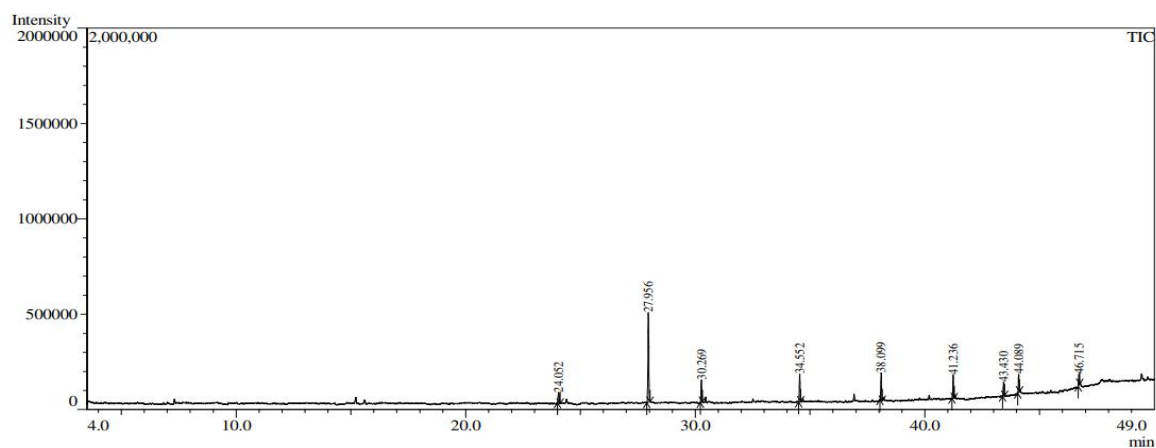


Fig 5.59: 72hrs-300µg/L Chromatogram

5.5.3 Day 7 Control

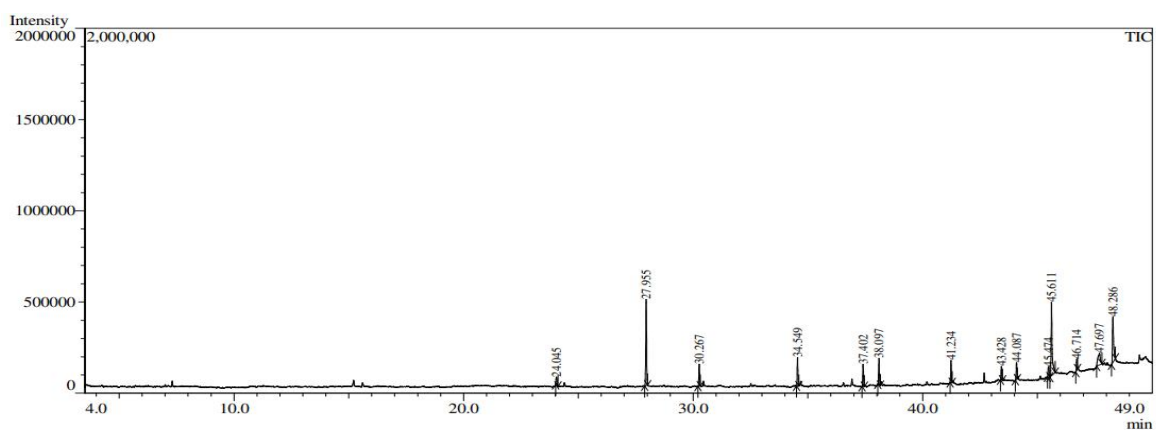


Fig 5.60: 168hrs-50µg/L Chromatogram

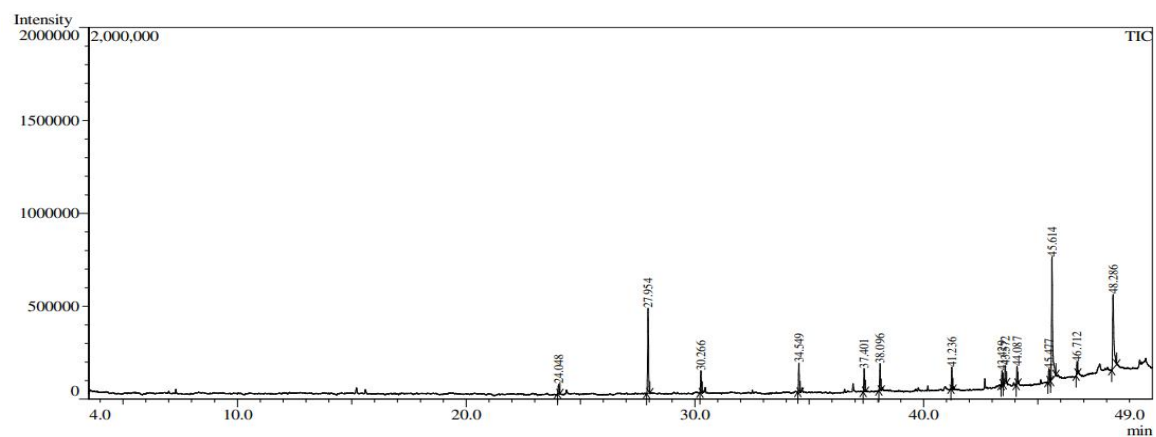


Fig 5.61: 168hrs-100µg/L Chromatogram

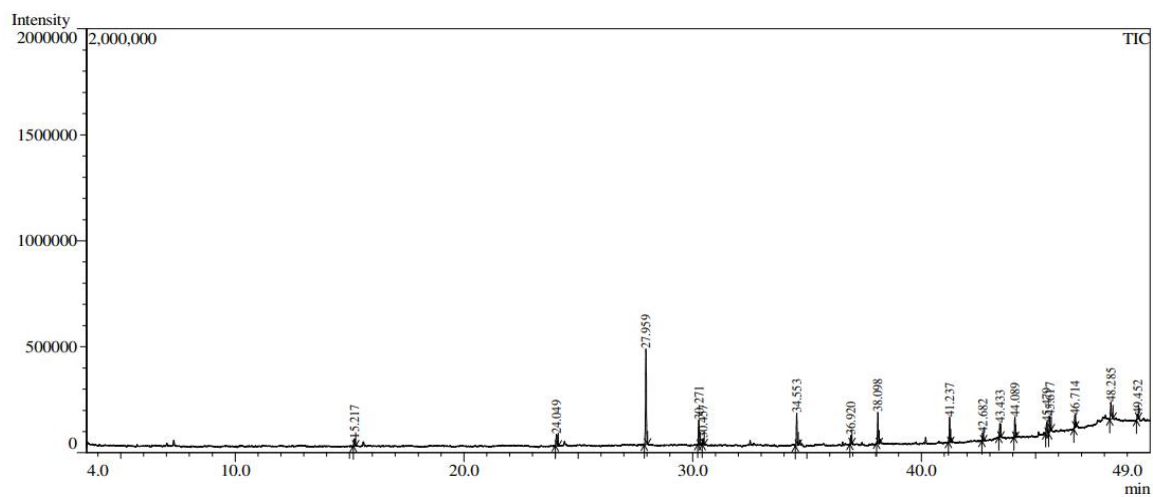


Fig 5.62: 168hrs-300µg/L Chromatogram

5.4.8 Concentration 50µg/L

Table 5.8: Concentration 50µg/L

S.NO	NAME	Control	48hrs	72hrs	168hrs
1	1,2-Benzenedicarboxylic acid, butyl octyl ester	-	1.21	-	-
2	17-Pentatriacontene	1.27	-	-	-
3	1-EICOSANOL	6.68	-	5.32	4.38
4	1-Heptacosanol	4.83	-	-	2.13
5	1-Nonadecene	6.53	-	7.13	5.62
6	1-Tetradecanol	-	-	-	4.77
7	2-methylhexacosane	-	-	-	-
8	3-OCTADECENE, (E)-	2.71	-	-	2.26
9	7-Tetradecene	-	-	-	-
10	9-EICOSENE, (E)-	-	-	-	-
11	9-Octadecenamide, (Z)-	-	-	-	-
12	9-OCTADECENOIC ACID (Z)-, ETHYL ESTER	-	12.86	-	-
13	9-OCTADECENOIC ACID (Z)-, METHYL ESTER	-	-	-	-
14	Acetic acid, trifluoro-, undecyl ester	-	-	-	-
15	Behenic alcohol	-	-	-	-
16	Cholesterol	-	-	-	7.70
17	Dibutyl phthalate	4.31	-	-	4.38
18	Docosyl heptafluorobutyrate	-	-	-	-
19	Docosyl trifluoroacetate	-	-	-	-

20	DOTRIACONTANE	-	-	-	-
21	Eicosyl trifluoroacetate	-	-	-	-
22	ETHYL (9Z,12Z)-9, 12- OCTADECADIENOATE	-	2.88	-	-
23	Ethyl Oleate	-	-	11.05	-
24	Heneicosane	-	-	-	-
25	Heneicosyl trifluoroacetate	-	-	-	-
26	Heptadecane	-	-	-	-
27	HEXADECANOIC ACID, 1-(HYDROXYMETHYL)- 1,2-ETHANEDIYL ESTER	2.31	-	-	-
28	Hexadecanoic acid, 2-hydroxyl- 1-(hydroxymethyl)ethyl ester	14.90	-	-	-
29	HEXADECANOIC ACID, METHYL ESTER	2.80	25.94	-	-
30	HEXADECANOIC ACID, ETHYL ESTER	-	12.63	18.29	-
31	Hexadecanoic acid, 1-hydroxyl- 1-(hydroxymethyl)ethyl ester	-	-	-	20.59
32	Hexadecanoic acid, 2-hydroxyl- 1-(hydroxymethyl)ethyl ester	14.90	-	-	-
33	HEXADECANOIC ACID, 1-(HYDROXYMETHYL)-	2.31	-	-	-

	1,2-ETHANEDIYL ESTER				
34	Methyl stearate	-	3.33	-	-
35	Methyl10-trans,12-cis-octadecadienoate	-	6.58	-	-
36	n-Pentadecanol	5.36	-	5.82	-
37	n-Tridecan-1-ol	-	-	-	-
38	O O'-BIPHENOL, 4,4',6,6'-TETRA-T-BUTYL-	3.19	-	-	2.74
39	Octacosanol	2.39	-	-	3.39
40	Octadecanoic acid, 2,3-dihydroxypropyl ester	13.92	-	-	13.74
41	Octadecanoic acid, 2-hydroxy-1,3-propanediyl ester	-	-	-	-
42	Octadecanoic acid, ethyl ester	-	1.83	-	-
43	9-OCTADECENOIC ACID (Z)-, METHYL ESTER	-	12.86	-	-
44	Phenol, 2,4-bis(1,1-dimethylethyl)-	22.23	-	-	20.53
45	Tetracontane	-	-	-	-
46	Trifluoroacetic acid,n-tridecyl ester	-	-	-	-

5.4.9 Concentration 100µg/L

Table 5.9: Concentration 100µg/L

S.NO	NAME	Control	48hrs	72hrs	168hrs
1.	1,2-Benzenedicarboxylic acid, butyl octyl ester	-	-	-	-
2.	17-Pentatriacontene	1.27	-	-	-
3.	1-EICOSANOL	6.68	9.50	8.32	-
4	1-Heptacosanol	4.83	3.34	6.22	3.56
5	1-Nonadecene	6.53	9.76	10.94	4.83
6	1-Tetradecanol	-	-	-	-
7	2-methylhexacosane	-	-	-	-
8	3-OCTADECENE, (E)-	2.71	-	5.23	-
9	7-Tetradecene	-	4.94	-	-
10	9-EICOSENE, (E)-	-	-	-	-
11	9-Octadecenamide, (Z)-	-	-	-	2.91
12	9-OCTADECENOIC ACID (Z)-, ETHYL ESTER	-	-	-	-
13	9-OCTADECENOIC ACID (Z)-, METHYL ESTER	-	1.72	-	-
14	Acetic acid, trifluoro-, undecyl ester	-	-	-	2.03
15	Behenic alcohol	-	-	10.10	-
16	Cholesterol	-	-	-	-
17	Dibutyl phthalate	4.31	-	-	3.78
18	Docosyl heptafluorobutyrate	-	-	-	-
19	Docosyl trifluoroacetate	-	-	-	1.85
		51			

20	DOTRIACONTANE	-	-	-	-
21	Eicosyl trifluoroacetate	-	-	-	-
22	ETHYL (9Z,12Z)-9,12-OCTADECADIENOATE	-	-	-	-
23	Ethyl Oleate	-	-	-	-
24	Heneicosane	-	-	-	-
25	Heneicosyl trifluoroacetate	-	-	4.08	-
26	Heptadecane	-	10.41	-	-
27	HEXADECANOIC ACID, 1-(HYDROXYMETHYL)-1,2-ETHANEDIYL ESTER	2.31	-	-	-
28	Hexadecanoic acid, 2-hydroxyl-1-(hydroxymethyl)ethyl ester	14.90	-	-	27.99
29	HEXADECANOIC ACID, METHYL ESTER	2.80	-	-	-
30	HEXADECANOIC ACID, ETHYL ESTER	-	-	-	-
31	Hexadecanoic acid, 1-hydroxyl-1-(hydroxymethyl)ethyl ester	-	-	-	-
32	Hexadecanoic acid, 2-hydroxyl-1-(hydroxymethyl)ethyl ester	14.90	-	-	27.99
33	HEXADECANOIC ACID, 1-(HYDROXYMETHYL)-	2.31	-	-	-

	1,2-ETHANEDIYL ESTER				
34	Methyl stearate	-	-	-	-
35	Methyl10-trans,12-cis- octadecadienoate	-	-	-	-
36	n-Pentadecanol	5.36	9.00	8.96	3.87
37	n-Tridecan-1-ol	-	-	-	-
38	O O'-BIPHENOL, 4,4',6,6'-TETRA-T- BUTYL-	3.19	4.00	5.29	2.13
39	Octacosanol	2.39	7.53	-	2.84
40	Octadecanoic acid, 2,3-dihydroxypropyl ester	13.92	-	-	20.48
41	Octadecanoic acid, 2-hydroxy-1,3- propanediyl ester	-	-	-	-
42	Octadecanoic acid, ethyl ester	-	-	-	-
43	9-OCTADECENOIC ACID (Z)-, METHYL ESTER	-	1.72	-	-
44	Phenol, 2,4-bis(1,1- dimethylethyl)-	22.23	34.50	40.85	16.90
45	Tetracontane	-	-	-	-
46	Trifluoroacetic acid,n- tridecyl ester	-	-	-	-

5.4.10 Concentration 300µg/L

Table 5.10: Concentration 300µg/L

S.NO	NAME	Control	48hrs	72hrs	168hrs
1.	1,2-Benzenedicarboxylic acid, butyl octyl ester	-	-	-	-
2.	17-Pentatriacontene	1.27	-	-	-
3.	1-EICOSANOL	6.68	9.79	10.63	7.69
4	1-Heptacosanol	4.83	8.07	8.46	6.65
5	1-Nonadecene	6.53	9.87	10.72	8.85
6	1-Tetradecanol	-	8.41	-	-
7	2-methylhexacosane	-	3.80	-	-
8	3-OCTADECENE, (E)-	2.71	-	-	4.56
9	7-Tetradecene	-	-	-	2.61
10	9-EICOSENE, (E)-	-	-	8.73	-
11	9-Octadecenamide, (Z)-	-	-	-	-
12	9-OCTADECENOIC ACID (Z)-, ETHYL ESTER	-	-	-	-
13	9-OCTADECENOIC ACID (Z)-, METHYL ESTER	-	-	-	-
14	Acetic acid, trifluoro-, undecyl ester	-	-	-	-
15	Behenic alcohol	-	-	-	-
16	Cholesterol	-	-	-	-
17	Dibutyl phthalate	4.31	-	-	-
18	Docosyl heptafluorobutyrate	-	4.45	-	-
19	Docosyl trifluoroacetate	-	-	-	-
20	DOTRIACONTANE	-	4.42	-	-
21	Eicosyl trifluoroacetate	54	-	6.42	-

22	ETHYL (9Z,12Z)-9,12-OCTADECADIENOATE	-	-	-	-
23	Ethyl Oleate	-	-	-	-
24	Heneicosane	-	-	-	1.65
25	Heneicosyl trifluoroacetate	-	-	-	-
26	Heptadecane	-	-	-	-
27	HEXADECANOIC ACID, 1-(HYDROXYMETHYL)-1,2-ETHANEDIYL ESTER	2.31	-	-	2.13
28	Hexadecanoic acid, 2-hydroxyl-1-(hydroxymethyl)ethyl ester	14.90	-	-	4.26
29	HEXADECANOIC ACID, METHYL ESTER	2.80	-	-	2.31
30	HEXADECANOIC ACID, ETHYL ESTER	-	-	-	-
31	Hexadecanoic acid, 1-hydroxyl-1-(hydroxymethyl)ethyl ester	-	-	-	-
32	Hexadecanoic acid, 2-hydroxyl-1-(hydroxymethyl)ethyl ester	14.90	-	-	4.26
33	HEXADECANOIC ACID, 1-(HYDROXYMETHYL)-1,2-ETHANEDIYL ESTER	2.31	-	-	2.13

34	Methyl stearate	-	-	-	-
35	Methyl10-trans,12-cis-octadecadienoate	-	-	-	-
36	n-Pentadecanol	5.36	-	-	-
37	n-Tridecan-1-ol	-	-	5.98	-
38	O O'-BIPHENOL, 4,4',6,6'-TETRA-T-BUTYL-	3.19	4.51	4.96	3.53
39	Octacosanol	2.39	-	3.70	4.87
40	Octadecanoic acid, 2,3-dihydroxypropyl ester	13.92	-	-	6.10
41	Octadecanoic acid, 2-hydroxy-1,3-propanediyl ester	-	-	-	3.50
42	Octadecanoic acid, ethyl ester	-	-	-	-
43	9-OCTADECENOIC ACID (Z)-, METHYL ESTER	-	-	-	-
44	Phenol, 2,4-bis(1,1-dimethylethyl)-	22.23	36.85	40.39	29.16
45	Tetracontane	-	3.36	-	-
46	Trifluoroacetic acid,n-tridecyl ester	-	-	-	7.09

We infer from the tables that there is no difference in area percentage of "Hexadecanoic acid", it remained stable. The area percentage of "Octadecanoic acid" was very less in fishes treated in 100µg/L and 300µg/L, comparatively Control fishes and fishes treated in 50µg/L had higher area percentage. The area percentage of control and 100µg/L for "Heptacosanol" was more or less the same, while area percentage for fishes treated in 50µg/L was less than control and above the control for fishes treated in 300µg/L. In the case of "O O'-BIPHENOL, 4,4',6,6'-TETRA-T-BUTYL-" the area percentage decreases day by day gradually. The area percentage of "Phenol, 2,4-bis(1,1-dimethylethyl)-" increased on 72hrs and gradually decreased to normal levels. As concentration increased, the area percentage was also seen to be increased in "1-EICOSANOL".

6.SUMMARY AND CONCLUSION

In conclusion, the present study provides evidence that exposure to fipronil can lead to significant changes in the brain biochemistry of zebrafish. The results show that there are changes in neurotransmitter levels and this study suggest that fipronil has the potential to disrupt the normal functioning of the nervous system.

These findings are consistent with previous studies that have reported similar effects of fipronil on oxidative stress and neurotransmitter levels in different animal models. For instance, a study by Sivakumar et al. (2019) reported that fipronil caused alterations in neurotransmitter levels in the brain of rats.

The results of this study have important implications for the use of fipronil in agriculture and public health. Fipronil is widely used as an insecticide in agriculture and veterinary medicine, as well as in the control of pests in residential areas. The findings of this study suggest that exposure to fipronil can lead to neurotoxic effects in aquatic organisms, which can have implications for the health of aquatic ecosystems. Furthermore, the potential for fipronil to cause oxidative stress and disrupt neurotransmitter levels in humans and other animals cannot be ruled out.

In conclusion, this study highlights the need for further research into the potential neurotoxic effects of fipronil and other pesticides, as well as the need for better regulation and control of the use of these chemicals.

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