

FATTY ACID PROFILING OF ZEBRAFISH OOCYTES TREATED WITH FIPRONIL

Submitted in partial fulfilment of the
requirements for the award of

Masters in Science

in

Medical Biotechnology and Clinical Research

by

VAISHNAVI.K (41101013)



**CENTRE FOR MOLECULAR AND NANOMEDICAL SCIENCES
INTERNATIONAL RESEARCH CENTER
SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

Accredited with grade 'A' by NAAC

JEPIAAR NAGAR, RAJIV GANDHI

SALAI, CHENNAI – 600119

MAY – 2023



SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

Accredited with "A" grade by NAAC

Jepplaar Nagar, Rajiv Gandhi Salai, Chennai –

600 119

www.sathyabama.ac.in



DEPARTMENT OF MEDICAL BIOTECHNOLOGY AND CLINICAL RESEARCH

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **VAISHNAVI.K (41101013)** and who carried out the project entitled **"FATTYACID PROFILING OF ZEBRAFISH OOCYTES TREATED WITH FIPRONIL"** under our supervision from December 2022 to May 2023.

For
Dr. R. Rajesh Kannan

Internal Guide

Dr. R.RAJESH KANNAN

Dr. A. Dayanandan

Head of the Department

Dr. A.DAYANANDAN

Submitted for Viva voce Examination held on 05-05-2023

S. John Pooj Samuel
05/05/23
Internal Examiner

[Signature]
External Examiner
5/5/23

DECLARATION

I **VAISHNAVI.K (41101013)** hereby declare that the project report entitled **"FATTY ACID PROFILING OF ZEBRAFISH OOCYTES TREATED WITH FIPRONIL"** done by me under the guidance of **Dr. R.RAJESH KANNAN** at **Sathyabama institute of Science and Technology, Chennai** is submitted in partial fulfilment of the requirements for the award of Master of science degree in Medical Biotechnology and clinical research.

DATE: 05/05/2023

PLACE: Chennai

Vaishnavi.k

SIGNATURE OF THE CANDIDATE

ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to Board of Management of SATHYABAMA for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

I convey my thanks to **Dr.A.Dayanandan**, Head of the Department, Dept. of Medical Biotechnology and clinical research for providing me necessary support and details at the right time during the progressive reviews.

I would like to express my sincere and deep sense of gratitude to my Project Guide **Dr.R.Rajesh Kannan** for his valuable guidance, suggestions and constant encouragement paved way for the successful completion of my project work.

I would also like to express my sincere thanks to the research scholars Mr. S. Nandha gopal and Mr.W.A.Carlton Ranjith , Centre for Molecular and Nanomedical sciences for helping me to carry out the project work

I wish to express my thanks to all Teaching and Non-teaching staff members of the Department of Medical Biotechnology and clinical research who were helpful in many ways for the completion of the project

ABSTRACT

Pesticides are typically applied to crops as acute applications, and residual effects of such intermittent exposures are not often characterized in developing fish. Fipronil is an agricultural pesticide that inhibits γ -amino-butyric acid (GABA) gated chloride channels. Fipronil residue has caused widespread concern around the world, especially after the recent “toxic eggs” event in seven European countries. To evaluate the effects of fipronil on vertebrates, we used zebrafish as an animal model. This study aimed to investigate the impact of fipronil exposure on the fatty acid composition of zebrafish oocytes. Oocytes were collected from adult zebrafish exposed to varying concentrations (10, 25, 50, 100 and 300 $\mu\text{g/lit}$) of fipronil for 72 hrs, and lipid extracts were analysed using gas chromatography-mass spectrometry.

Key words : Fipronil, pesticide, zebrafish oocytes, fatty acid, gas chromatography – mass spectrometry

CHAPTER NO	TABLE OF CONTENTS	PAGE NO
	TITLE	
	ABSTRACT LIST OF ABBREVIATION LIST OF TABLES LIST OF FIGURES	
1	INTRODUCTION	1
2	REVIEW OF LITERATURE	9
3	AIM AND SCOPE	13
3.1	AIM	13
3.2	OBJECTIVE	13
4	MATERIALS AND METHODOLOGY	14
4.1	MATERIALS	15
4.2	METHODOLOGY	16
4.2.1	CHEMICAL PREPARATION	18
4.2.2	GAS CHROMATOGRAPHY	20
4.2.3	SAMPLE PREPARATION	21
4.2.4	TISSUE PROCESSING	21
4.2.5	REFLUX PROCESS	22
4.2.6	GAS CHROMATOGRAPHY ANALYSIS	
5	RESULT AND DISCUSSION	
5.1	FATTY ACID PROFILING - CONTROL	23
5.2	FATTY ACID PROFILING-TREATED WITH 10µg/L FOR 24 HOURS	25
5.3	FATTY ACID PROFILING-TREATED WITH 10µg/L FOR 48 HOURS	26
5.4	FATTY ACID PROFILING-TREATED WITH 10µg/L FOR 72 HOURS	27
5.5	FATTY ACID PROFILING-TREATED WITH 25µg/L FOR 24 HOURS	28
5.6	FATTY ACID PROFILING-TREATED WITH 25µg/L FOR 48 HOURS	29
5.7	FATTY ACID PROFILING-TREATED WITH 25µg/L FOR 72 HOURS	30
5.8	FATTY ACID PROFILING-TREATED WITH 50µg/L FOR 24 HOURS	31
		32

5.9	FATTY ACID PROFILING-TREATED WITH 50µg/L FOR 48 HOURS	33
5.10	FATTY ACID PROFILING-TREATED WITH 50µg/L FOR 72 HOURS	34
5.11	FATTY ACID PROFILING-TREATED WITH 100µg/L FOR 24 HOURS	35
5.12	FATTY ACID PROFILING-TREATED WITH 100µg/L FOR 48 HOURS	36
5.13	FATTY ACID PROFILING-TREATED WITH 100µg/L FOR 72 HOURS	37
5.14	FATTY ACID PROFILING-TREATED WITH 300µg/L FOR 24 HOURS	
5.15	FATTY ACID PROFILING-TREATED WITH 300µg/L FOR 48 HOURS	
5.16	FATTY ACID PROFILING-TREATED WITH 300µg/L FOR 72 HOURS	
6	SUMMARY AND CONCLUSION	46
	REFERENCE	

LIST OF ABBREVIATION AND SYMBOLS:

µg/L – Micrograms per litre

Mg/L- Milligrams per litre

ppm – Parts Per Million

HR – Highest Residue concentration

% - Percentage

g/L – Gram Per Litre

NaOH – Sodium Hydroxide

NaCl – Sodium Chloride

H₂SO₄ – Sulfuric Acid

g – Gram

ml – Milli litre

mg – Milli gram

rpm – Rotation per minute

LIST OF TABLES:

Table no	Name of the table	Page no
1	Residue levels of fipronil in the market fruits and vegetables	5
2	Peak table for positive control	23
3	Percentage of fatty acid present in control	25
4	Peak table for zebrafish oocyte which is treated with 10µg/L for 24 hrs	26
5	Peak table for zebrafish oocyte which is treated with 10µg/L for 48 hrs	27
6	Peak table for zebrafish oocyte which is treated with 10µg/L for 72 hrs	28
7	Percentage of fatty acid present in 10µg/L of fipronil for 72 hrs	29
8	Peak table for zebrafish oocyte which is treated with 25µg/L for 24 hrs	30
9	Peak table for zebrafish oocyte which is treated with 25µg/L for 48 hrs	31
10	Peak table for zebrafish oocyte which is treated with 25µg/L for 72 hrs	32
11	Peak table for zebrafish oocyte which is treated with 50µg/L for 24 hrs	33
12	Percentage of fatty acid present in 50µg/L of fipronil for 24 hrs	34
13	Peak table for zebrafish oocyte which is treated with 50µg/L for 48 hrs	35
14	Peak table for zebrafish oocyte which is treated with 50µg/L for 72 hrs	36
15	Peak table for zebrafish oocyte which is treated with 100µg/L for 24 hrs	37
16	Peak table for zebrafish oocyte which is treated with 100µg/L for 48 hrs	38
17	Peak table for zebrafish oocyte which is treated with 100µg/L for 72 hrs	39
18	Peak table for zebrafish oocyte which is treated with 300µg/L for 24 hrs	40
19	Peak table for zebrafish oocyte which is treated with 300µg/L for 48 hrs	41
20	Peak table for zebrafish oocyte which is treated with 300µg/L for 72 hrs	42

LIST OF FIGURES:

FIGURE NO	TITLE OF FIGURE	PAGE NO
1	Zebrafish model – <i>Danio Rerio</i>	7
2	Fish treated in different concentration	20
3	Fish exposed to tricaine	21
4	Sample for gas chromatography-Mass spectrometry	22

CHAPTER 1 : INTRODUCTION

A new generation of pesticides have been widely employed to control pests and therefore, aid food production. Fipronil [5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1H-pyrazole-3-carbonitrile, $C_{12}H_4Cl_2F_6N_4OS$) is a derivative of the phenyl pyrazole insecticide, developed by Rhône-Poulenc after 1985 and primally produced by Bayer HealthCare LLC (Tingle et al., 2003). It was designed for a more specific range of applications, but is more persistent with soil and aqueous environments compared to conventional agrochemicals, such as organophosphates and carbamate (Gunasekara et al., 2007). Fipronil mainly targets insecticidal activity by blocking GABA receptors of insects, which disrupts the passage of chloride ion, leading to the inhibition of neuronal signalling, and eventually causing death (Cole et al., 1993); however, indiscriminate use of fipronil has begun to affect other non-target organisms, including mammals, which consume prey that have up taken contaminants through their skin or through ingestion of plants (Gibbons et al., 2015). Fipronil and its metabolites from oxidation (fipronil sulfone) and hydrolyzation (fipronil amide), which occur from exposure to sunlight, cause serious contamination around their production sites and the surrounding agricultural fields (Abraham and Gajendiran, 2019). With rising concerns about industrial pollution from insecticides, a decontamination process has been reported using bacteria for biodegradation (Kumar et al., 2012); however, pollutant residues are ubiquitous in the environment, including water and air. For environmental decontamination, a complete removal of fipronil is required by the clean-up of surrounding agricultural fields. Numerous institutions have focused on the environmental effects of fipronil and its metabolites as it has been detected in drains from the agricultural area (Simon-Delso et al., 2015). Fipronil was found to degrade quite slowly under anaerobic water and sediment conditions, exposing plants and the ground to fipronil residues for a considerable period of time. In the case of aquatic organisms, fipronil's effects on local fish were assessed and characterized by high toxicity, including growth retardation (Tingle et al., 2003). According to a geological survey in the U.S., the presence levels of fipronil in an urban area were inspected, and up to 0.1 $\mu\text{g/L}$ of

fipronil from surface waters was reported to have been released from the agricultural area; meanwhile, previous findings also reported the toxic approaches of fipronil with different concentrations, including ppb levels ($\mu\text{g/L}$) to ppm (mg/L), in diverse species including aquatic organisms, birds, and mammals (Gunasekara et al., 2007). Moderate water solubility, commonly leaching through the initial sites, could increase the risk potential of fipronil to the aqueous environment worldwide. It is hypothesized that pesticide runoff can accumulate over circumjacent environments, and even the lowest concentration of fipronil can contribute to contamination and finally generate higher toxicity for living organisms; a previous report has raised concerns regarding rapid climate change, including the increased erosion of soil, stating that heavy rainfall can promote pesticide mobilization which corresponds to the potential increase of the environmentally relevant concentration of pesticides, as compared to previously reported hazardous concentrations (Bonmatin et al., 2015). More specifically, as the potential of pesticide exposure occurrence increases, demonstration of the severe impacts of pesticides should be improved.

The toxicological risks of fipronil to nontarget organisms have been greatly reported in aquatic organisms (Overmyer et al., 2007). As more than 90% of pesticides are detected in surface waters and are persistent in sediments near agricultural areas, though insufficient to cover the range of fipronil in aquatic environments, widely performed analysis in laboratory ecosystem using non-targeted aquatic species represented chronic toxicity (Baird et al., 2013). Exposure to acute lethal doses of fipronil for several days leads to decreased reproductive rate, increased death rate, and doubling time in water flea (Stark and Vargas, 2005). Moreover, exposure to fipronil induces microenvironment stress and causes significant changes in metabolites, such as activated CYP isomers in liver; it also disrupts lipid metabolism by destroying the cellular membrane in zebrafish larvae (Yan et al., 2016). Furthermore, fipronil is released into the environment as a mixture of two enantiomers, which cause enantioselective toxicity with poor impairment recovery and increased mortality in the specific case of freshwater and marine organisms (Overmyer et al., 2007).

Vegetables and fruits have a significant role in the composition of our daily diet. However, the use of several pesticides has contaminated them (Mebdoua, Lazali, Ounane, Tellah, Nabi, & Ounane, 2017). It has been proven that the pesticide fipronil is extremely effective. Used to boost production by preventing plant diseases and insect pests in fruits and vegetables. However, according to Duhan, Kumari, and Duhan (2015), it could be harmful to human health. As a result, fipronil application was limited. However, the residue may still be found in the national monitoring efforts. Our study found that 0.2% and 0.8% of fruits and vegetables had fipronil, which was close to the report from the Nordic experiment across Southeast Asia. According to the researchers, there were 2 samples containing fipronil residue out of the total 584 samples analysed, meaning that the detection rate was 0.3% (Skretteberg et al., 2015). According to the study (Chowdhury, M.A.Z., Fakhruddin, A.N.M., Islam, M.N., Moniruzzaman, M., Gan, S.H., & Alam, M.K., 2013), there was only one fipronil positive sample out of locally consumed vegetables, meaning that the detection rate for fipronil was 0.5%. According to the findings of this study, the 0.1% detection rate of Fipronil in vegetables is supported by the 29 instances of vegetables with pesticide residue levels above the maximum residue limits reported by Wu et al. (2017) in 19786 vegetable samples. Top 9 positive detection rate performers were all vegetables with leaves. leafy vegetables absorb pesticides more readily due to their huge surface areas, thinner waxy covering, and higher number of stomata (Farha et al., 2018). Consequently, leafy vegetables Compared to fruits and root vegetables, demonstrated higher positive rates (Xu et al., 2018). Long-term exposure to pesticide residues in fruits and vegetables did not endanger the health of adults or kids. Children, on the other hand, are regarded as the most vulnerable group and constantly face more nutritional risks than adults. Therefore, Long-term exposure to pesticide residues in fruits and vegetables did not endanger the health of adults or kids. Children, on the other hand, are regarded as the most vulnerable group and constantly face more nutritional risks than adults. Therefore, According to Lozowicka et al. (2012) and Li et al. (2016), children ought to be a protected class.

Fruit consumption's short-term dangers from fipronil residues were a manageable concern for both adults and kids. While common beans, spring vegetables, and leaf lettuce presented unacceptable risks to both adults and children. In addition, children were at an unacceptable acute risk from mustard greens and spinach. The HQ as of green Chinese and garland chrysanthemum For youngsters, cabbages were close to 1. It suggested that children could be at risk for health problems.

Table.1 RESIDUE LEVELS OF FIPRONIL IN THE MARKET FRUITS AND VEGETABLES

F/V	SUBSTRATE	HR (mg/kg)
F	Litchi	0.0464
F	Dragon fruit	0.0262
F	Mango	0.0203
F	Kiwifruit	0.0211
F	Strawberry	0.0048
F	Grape	0.0056
F	Banana	0.0028
F	Apple	0.0097
F	Plum	0.0018
V	Spring vegetable	0.4857
V	Mustard greens	0.2189
V	Leaf lettuce	1.8644
V	Common bean	1.0026
V	Sweet potato leaves	0.0144
V	Spinach	0.0014
V	Mushroom	0.0274
V	Tomato	0.0014
V	Broccoli	0.0010
V	Cucumber	0.0051
V	cabbage	0.000015

F/V: Fruit/vegetable, HR: Highest residue concentration

Fipronil is a broad-spectrum pesticide that is frequently used in veterinary care and agriculture to control pests. Despite the paucity of research on the particular effects of fipronil on the reproductive system, several studies have indicated that it might be harmful to reproductive health. Fipronil exposure has been demonstrated to have a variety of effects on the reproductive system in animal studies. For instance, fipronil exposure has been linked to changed reproductive behaviour, decreased fertility, and alterations in hormone levels in rats and mice. Furthermore, fipronil intake during pregnancy has been connected to animal offspring's developmental defects. Additionally, there is some evidence to suggest that fipronil may harm human fertility. According to one study, women with higher While one study discovered that exposure to fipronil was linked to a decline in male sperm motility and count, another discovered that women with higher blood levels of fipronil had a higher risk of infertility. It is significant to emphasise that additional study is required to properly comprehend how fipronil affects the reproductive system, particularly in humans. But given the information at hand, it might be a good idea to minimise your exposure to pesticides like fipronil, especially if you're pregnant or trying to get pregnant (Chiu et al., 2018).

Fipronil is a broad-spectrum insecticide that belongs to the phenylpyrazole chemical family. It is a white crystalline powder with a molecular formula of $C_{12}H_4Cl_2F_6N_4OS$ and a molecular weight of 437.15 g/mol.

Melting point: 200-201°C

Boiling point: 473.3°C at 760 mmHg

Density: 1.477 g/cm³

Solubility: Slightly soluble in water (0.0033 g/L at 20°C), soluble in organic solvents such as acetone, methanol, and ethanol.

Vapor pressure: 1.5×10^{-5} Pa at 25°C

Log octanol-water partition coefficient (log Kow): 4.2-4.5

pH stability: Fipronil is stable under acidic and neutral conditions but is degraded under alkaline conditions.

Acidity and basicity: Fipronil is a weak acid with a pKa value of 0.05. It can also act as a weak base in some conditions.

Stability: Fipronil is stable under normal storage conditions. However, it can be degraded by sunlight, heat, and acidic or alkaline conditions.

Reactivity: Fipronil is not reactive towards most chemicals, but it can react with some reducing agents such as sodium borohydride and metal hydrides.

Hydrolysis: Fipronil can be hydrolyzed in acidic or alkaline conditions, forming a range of degradation products.

Redox potential: Fipronil has a redox potential of 1.2V, indicating that it can undergo oxidation and reduction reactions.

Chiral properties: Fipronil is a chiral molecule with two enantiomers. The S-enantiomer is more biologically active than the R-enantiomer

Genetic similarity to humans: Zebrafish share many genetic similarities with humans, with a genome that is approximately 70% identical to the human genome. This makes them a useful model for studying human genetic diseases and developmental processes.

Rapid development: Zebrafish embryos develop rapidly and are transparent, allowing for easy observation of development in real-time. This makes them an excellent model for studying developmental biology.

Small size: Zebrafish are small, making them easy and inexpensive to maintain in the laboratory. Their small size also makes them ideal for large-scale genetic and drug screening studies.

Regeneration: Zebrafish have a remarkable ability to regenerate damaged tissues, including heart tissue and spinal cord tissue. This makes them an excellent model for studying tissue regeneration and developing new regenerative therapies.

High fecundity: Zebrafish are highly fecund, with females capable of laying hundreds of eggs each week. This allows for large-scale genetic and developmental studies.

Easy to manipulate: Zebrafish are easy to manipulate genetically, making them a valuable tool for studying gene function and developing new genetic therapies.

Overall, zebrafish are a versatile and valuable model organism for studying a wide range of biological and biomedical processes. Their small size, rapid development, and genetic similarities to humans make them an excellent tool for advancing our understanding of human biology and disease.(Lieschke et al.,2007)



Fig.1 zebrafish – danio rerio

TAXONOMY

- Kingdom: Animalia
- Phylum: Chordata
- Subphylum: Vertebrata
- Class: Actinopterygii
- Order: Cypriniformes
- Family: Cyprinidae
- Genus: Danio
- Species: rerio

CHAPTER – 2 REVIEW OF LITERATURE

Wu et al. (2018) investigated the effects of fipronil on zebrafish oocyte maturation and fertilization. The researchers exposed zebrafish oocytes to different concentrations of fipronil for 24 hours and then measured oocyte maturation and fertilization rates. The results showed that fipronil exposure significantly reduced oocyte maturation and fertilization rates in a concentration-dependent manner.

Xing et al. (2019) investigated the effects of fipronil on zebrafish oocyte growth and development. The researchers exposed zebrafish to different concentrations of fipronil for 21 days and then measured oocyte growth and development. The results showed that fipronil exposure significantly reduced oocyte growth and development in a concentration-dependent manner.

Zhao et al. (2021) investigated the effects of fipronil on zebrafish oocyte apoptosis and mitochondrial function. The researchers exposed zebrafish to different concentrations of fipronil for 24 hours and then measured oocyte apoptosis and mitochondrial function. The results showed that fipronil exposure induced oocyte apoptosis and disrupted mitochondrial function in a concentration-dependent manner.

Chung-Hsin Wu et al. (2021) According to this research, oxidative stress, inflammation, and apoptosis may be induced by fipronil and its metabolites in the brains of aquatic vertebrates, leading to irreversible neuron loss. The findings suggest that fipronil toxicity may harm lateral hair cells and brain tissue in zebrafish by inducing oxidative stress, inflammation, and apoptosis, which may drastically lower the survival rate and result in impaired motility. As a result, aquatic species' sensory and motor systems may be compromised by fipronil poisoning. This impairment in the environmental setting can have a direct impact on the fish's capacity to flee or evade predators. The behavioural reactions of zebrafish exposed to fipronil poisoning may also provide insight into the validity of behavioural biomarkers used in the assessment of environmental risk.

Hanqing Xu et al. (2018) this study showed that high-dose fipronil could cause aberrant development and death in zebrafish larvae. Fipronil also continued to have severe harmful effects at environmentally relevant dosages by altering the molecular abundance of transcripts of some important regulatory proteins, like the AP-1 transcription factors, *in vivo*. *Midn* could potentially be chosen as a potential biomarker to reflect fipronil effects in zebrafish larvae at the transcriptional level because we observed a concentration-dependent response between the transcriptional level of *midn* and fipronil under environmental concentrations (0.5-50 g/L). Furthermore, we preliminary discussed the potential reasons for the accumulation of long-chain fatty acids using our data in conjunction with earlier metabolomic research.

However, additional study is required to determine whether Environmental exposures of fipronil or fatal amounts, like those found in "toxic eggs," will have similar deleterious consequences on human health, particularly those of infants. As a result, this results offer some useful data and information for assessing the risks to aquatic ecosystems and human health.

Hahyun Park et al.(2019) In this study, Zebrafish eggs that had been fertilised six hours post-fertilization (hpf) were exposed to fipronil for 72 hours in order to research the toxin's effects on environmental health. By giving zebrafish embryos different amounts of fipronil for 72 hours, the LC50 value was discovered. Zebrafish embryos were subjected to fipronil to explore the intricately elucidating mechanisms with risky end points of toxicity because they are effective vertebrate models for investigating developmental and genetic findings in toxicology research. Pathological changes such increased mortality, shorter body lengths, and decreased hatchability were supported by cell cycle arrest-related apoptosis. Furthermore, aberrant blood circulation contributed to the identified heart abnormalities, such as edoema and irregular pulse. Endothelial cells that express the gene eGFP were found to be present in damaged blood vessel forms in transgenic zebrafish models (fli1:eGFP and olig2:dsRED). In addition, neurogenic defects were discovered by observing oligodendrocytes and dsRED+ motor neurons. This study indicates fipronil accumulation in aquatic environments, coupled with its capacity to adversely affect critical zebrafish developmental processes including angiogenesis and neurogenesis throughout their early stages of development.

Ashley Eadie (2020) In this study, embryos of zebrafish (*Danio rerio*) were exposed to fipronil at various doses (0.02 g/L to 4000 g/L) for 48 hours (beginning at about 3 h after fertilisation, hpf). A portion of the fish were then moved to clean water for a 7-day depuration phase after this acute exposure. We postulated that fish would suffer later in life if they were exposed to fipronil in pulses at crucial stages of central nervous system development. Survival was decreased in embryos exposed to 2 g of fipronil/L or more after a 48 hour pulse exposure. However, there was no additional mortality during the depuration period, and there were no differences in body length or notochord length between controls and larvae 9 dpf (days post-fertilization). Additional tests were conducted at higher concentrations throughout 96 hours (up to 4 dpf) to clarify the teratogenicity and developmental consequences of fipronil (43.7 g/L to 4370 g/L). Fipronil significantly affected zebrafish development at these higher concentrations, 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 and otic lobes, and shortening of the lower jaw. We predicted that exposure-related molecular effects would still be present in depurated fish. Thus, 9 dpf depurated larvae treated initially for 48 h to one dosage of either 0.2 g/L, 200 g/L, or 2000 g/L fipronil were subjected to transcriptome profiling.

The expression of gene networks linked to omega-3 fatty acids and glycogen Fipronil exposure caused a decrease in metabolism in larvae at each of the three doses, indicating a disruption in metabolism. This study shows that following depuration, fipronil treatment in short pulses can impact the transcriptome networks for metabolism, circadian rhythm, and sensitivity to light in fish. and it is hypothesised that these chemical reactions account for the abnormal behavioural effects shown in the light-dark preference test.

Cui Wang et al.(2016) Fipronil has been shown to affect fish swimming behaviour in numerous studies. Here, we showed that newborn larvae's swimming speed can increase when exposed to fipronil during the embryonic stage. We assumed that this anxiety-like behaviour could encourage the use of fatty acids as an energy compensatory strategy. Fipronil may potentially contribute to the acceleration through its increased excitatory neurotransmitters and decreased inhibitory neurotransmitters. Moreover, it was shown that fipronil interfered with the branched amino acid synthesis pathway, which is necessary for the development of muscle. Our data, however, cannot be used to determine if additional neurotransmitter receptors besides GABA might be a new target for fipronil and, as a result, require more research. A metabolomics study is a sensitive and relatively recent method for understanding the mechanisms underlying chemically induced changes in metabolic toxicity research, particularly at the concentration that is significant to the environment

CHAPTER -3 AIM AND SCOPE

3.1 AIM :

To evaluate the changes in fatty acids for zebrafish oocyte which is treated with fipronil by gas chromatography

3.2 OBJECTIVE :

1. Zebrafish treated with fipronil in five different concentrations (10, 25, 50, 100 and 300 µl/lit)
2. Oocytes were dissected in four time intervals (24hrs, 48hrs, 72hrs and 1week)
3. Gas chromatography – lipid profiling
4. Concentration of lipids were observed and tabulated

CHAPTER – 4 MATERIALS AND METHODOLOGY

4.1 MATERIALS :

- Fish tank

SPECIMEN :

- Female Fish

CHEMICALS:

- Fipronil
- Tricaine methane sulfonate solution
- Methanol
- Chloroform
- 1 %sodium chloride
- 1% Methanolic sodium hydroxide
- 1 molar Methanolic Sulfuric acid
- Heptane

INSTRUMENTS:

Vortex

Centrifuge

Pipette

Rotary evaporator

Gas chromatography

4.2 METHODOLOGY:

4.2.1 CHEMICAL PREPARATION :

FIPRONIL :

It is bought from an agriculture pesticide dealer; It consist of 80% fipronil and rest 20% are solvents

Solvents :

sodium polycarboxylate(2.90%)

sodium dioctyl sulfosuccinate(1.90%)

silicone oil emulsion (0.30%)

sodium lignosulphonate(Q.S.)

PREPARATION:

1 gram of fipronil is dissolved in 1 litre of water

TRICAINE METHANE SULFONATE :

- Tricaine powder – 400mg
- Double distilled water – 97.9ml
- 1M Tris – 2.1ml

400 mg of tricaine powder is dissolved in 97.9ml double distilled water and 1M Tris

1% SODIUM CHLORIDE (NaCl)

- NaOH – 1g
- Distilled water – 100ml

1g of NaOH is dissolved in 100ml of distilled water

1% METHANOLIC SODIUM HYDROXIDE(NaOH)

- Methanol – 500ml
- NaOH – 5g

5g of NaOH is dissolved in 500ml of methanol

1M METHANOLIC SULFURIC ACID

- Methanol – 500ml
- Sulfuric acid – 27ml

27ml of Sulfuric acid dissolved in 500ml of methanol

4.2.2 GAS CHROMATOGRAPHY

PRINCIPLE:

gas chromatography (GC) is based on the separation of a mixture of compounds into individual components by partitioning them between a stationary phase and a mobile phase. The stationary phase is typically a solid support material coated with a thin film of a liquid phase or a polymer, while the mobile phase is an inert gas such as helium or nitrogen.

The separation occurs due to differences in the affinity of the components for the stationary and mobile phases. As the mixture is injected into the GC column, the mobile phase carries the sample through the column, and the components interact with the stationary phase to varying degrees. The components that have a higher affinity for the stationary phase will move more slowly through the column, while those with a higher affinity for the mobile phase will move more quickly.

As the components exit the GC column, they pass through a detector, which produces a signal that is proportional to the concentration of each component. The most commonly used detectors in GC are flame ionization detectors (FID) and mass spectrometers (MS).

The FID measures the concentration of compounds by detecting the ions that are generated when the sample molecules are burned in a hydrogen flame. The MS detector ionizes the sample molecules and separates them based on their mass-to-charge ratio, generating a mass spectrum that provides a unique fingerprint for each compound.

The data generated by the detector is analysed to identify and quantify the individual components in the mixture. GC is widely used in many fields, including environmental monitoring, forensic analysis, pharmaceuticals, and food and flavor analysis, due to its ability to separate and identify complex mixtures of compounds with high sensitivity and specificity.

COMPONENTS:

Carrier gas :

Helium, N₂, H₂, Argon are used as carrier gases.

Helium is preferred for thermal conductivity detectors because of its high thermal conductivity relative to that of most organic vapors.

N₂ is preferable when a large consumption of carrier gas is employed.

Carrier gas from the tank passes through a toggle valve, a flow meter, (1-1000 ml/min), capillary restrictors, and a pressure gauge (1-4 atm).

Flow rate is adjusted by means of a needle valve mounted on the base of the flow meter and controlled by capillary restrictors.

The operating efficiency of the gas chromatograph is directly dependant on the maintenance of constant gas flow.

Sample injection system:

Liquid samples are injected by a microsyringe with a needle inserted through a self-sealing, silicon-rubber septum into a heated metal block by a resistance heater.

Gaseous samples are injected by a gas-tight syringe or through a by-pass loop and valves.

Typical sample volumes range from 0.1 to 0.2 ml.

The separation column:

The heart of the gas chromatography is the column which is made of metals bent in U shape or coiled into an open spiral or a flat pancake shape.

Copper is useful up to 250°

Swege lock fittings make column insertion easy.

Several sizes of columns are used depending upon the requirements.

Stationary phase:

An infinite variety of liquid phases are available limited only by their volatility, thermal stability and ability to wet the support.

No single phase will serve for all separation problems at all temperatures.

Non-Polar – Paraffin, squalene, silicone greases, apeiron L, silicone gum rubber. These materials separate the components in order of their boiling points.

Intermediate Polarity – These materials contain a polar or polarizable group on a long non-polar skeleton which can dissolve both polar and non-polar solutes. For example. diethyl hexyl phthalate is used for the separation of high boiling alcohols.

Polar – Carbowaxes – Liquid phases with a large proportion of polar groups. Separation of polar and non-polar substances.

Hydrogen bonding – Polar liquid phases with high hydrogen bonding e.g. Glycol.

Specific purpose phases – Relying on a chemical reaction with solute to achieve separations. e.g. AgNO_3 in glycol separates unsaturated hydrocarbons.

Supporting material :

The structure and surface characteristics of the support materials are important parameters, which determine the efficiency of the support and the degree of separation respectively.

The support should be inert but capable of immobilizing a large volume of liquid phase as a thin film over its surface.

The surface area should be large to ensure the rapid attainment of equilibrium between stationary and mobile phases.

Support should be strong enough to resist breakdown in handling and be capable of packed into a uniform bed.

Diatomaceous earth, kieselguhr treated with Na_2CO_3 for 900°C causes the particle fusion into coarser aggregates.

Glass beads with a low surface area and low porosity can be used to coat up to 3% stationary phases.

Porous polymer beads differing in the degree of cross-linking of styrene with alkyl-vinyl benzene are also used which are stable up to 250°

Detector:

Detectors sense the arrival of the separated components and provide a signal.

These are either concentration-dependent or mass dependant.

The detector should be close to the column exit and the correct temperature to prevent decomposition.

Recorder:

The recorder should be generally 10 mv (full scale) fitted with a fast response pen (1 sec or less). The recorder should be connected with a series of good quality resistances connected across the input to attenuate the large signals.

An integrator may be a good addition.

4.2.3 SAMPLE PREPARATION :

female zebrafish was treated in five different concentration (10,25,50,100 and 300 μ l)



Fig.2 Fish treated in different concentration

Oocytes of the zebrafish were collected in four different time intervals i.e. 24hrs, 48 hrs and 72hrs)



Fig.3 Fish exposed to tricaine

4.2.4 TISSUE PROCESSING :

The tissue(oocytes) is homogenized with chloroform and methanol in 2:1 ratio

The homogenate is either filtered or centrifuged to recover the liquid phase

The solvent is washed with 1%NaCl solution , after vortexing for 1 min, the mixture is centrifuged at low speed (2000 rpm) to separate the two phases

The lower chloroform phase containing lipid is separated.

4.2.5 REFLUX PROCESS :

The chloroform phase which have been separated is evaporated under vacuum concentrator

After evaporation, add 15 ml of methanolic NaOH and stir it for 15 mins then leave the solution to cool

Add 15ml of methanolic H₂SO₄ and reflux it till the solution get cleared leave the solution to cool

Transfer the solution in 50ml tube ,add heptane 1ml and water 2ml mix it well

Then separate the upper layer and transfer to gas chromatography vial

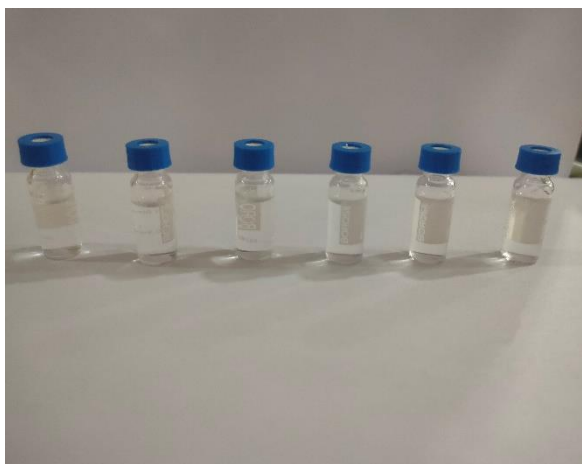


Fig.4 Sample for gas chromatography-mass spectrometry

4.2.6 GAS CHROMATOGRAPHY ANALYSIS

The injected sample is carried by a mobile phase (usually an inert gas such as helium or nitrogen) through a column containing a stationary phase (usually a polymer or a fused silica material coated with a thin layer of a stationary phase). The different components of the sample interact differently with the stationary phase, leading to their separation. As the separated components elute from the column, they pass through a detector that measures the concentration of each component in the sample. The output of the detector is recorded as a chromatogram, which is a plot of the detector response versus time. The peaks in the chromatogram correspond to the separated components in the sample, and the area under each peak is proportional to the concentration of that component.

CHAPTER -5 RESULT AND DISCUSSION

5.1 FATTY ACID PROFILING – CONTROL

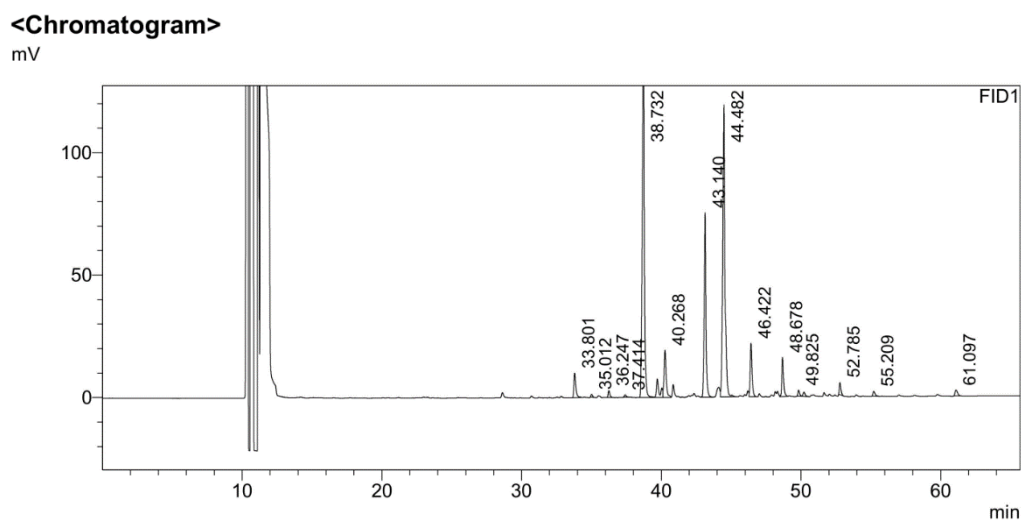


Table:2 Peak table for control

Peak#	Ret.Time	Area	Height	Name
1	33.801	92763	10029	Myristic Acid Methyl Ester
2	35.012	8313	1110	Myristoleic Acid Methyl Ester
3	36.247	23777	2845	Pentadecanoic Acid Methyl Ester
4	37.414	8921	958	Cis-10-Pentadecenoic Acid Methyl Ester
5	38.732	1596250	160200	Palmitic Acid Methyl Ester
6	40.268	196625	19218	Palmitoleic Acid Methyl Ester
7	43.140	654425	75250	Stearic Acid Methyl Ester
8	44.482	1307680	119092	Oleic Acid Methyl Ester
9	46.422	195585	21792	Linoleic Acid Methyl Ester
10	48.678	142660	16023	Cis-11-Eicosenoic Acid Methyl Ester
11	49.825	40037	2527	Cis-11,14-Eicosadienoic Acid Methyl Ester
12	52.785	40175	4911	Tricosanoic Acid Methyl Ester
13	55.209	21552	2023	Cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester
14	61.097	29416	2377	Cis-4,7,10,13,16,19-Docosahexaenoic Acid
Total		4358178	438355	

Table:3 Percentage of fatty Acid present in control

S.no	Name	Percentage(%)
1	Myristic Acid Methyl Ester	2.12
2	Myristoleic Acid Methyl Ester	0.19
3	Pentadecanoic Acid Methyl Ester	0.54
4	Cis-10-Pentadecenoic Acid Methyl Ester	0.20
5	Palmitic Acid Methyl Ester	36.62
6	Palmitoleic Acid Methyl Ester	4.51
7	Stearic Acid Methyl Ester	15.0
8	Oleic Acid Methyl Ester	30.0
9	Linoleic Acid Methyl Ester	4.48
10	Cis-11-Eicosenoic Acid Methyl Ester	3.27
11	Cis-11,14-Eicosadienoic Acid Methyl Ester	0.91
12	Tricosanoic Acid Methyl Ester	0.92
13	Cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester	0.49
14	Cis-4,7,10,13,16,19-Docosahexaenoic Acid	0.67

5.2 FATTY ACID PROFILING – TREATED WITH 10µG/L FOR 24HRS

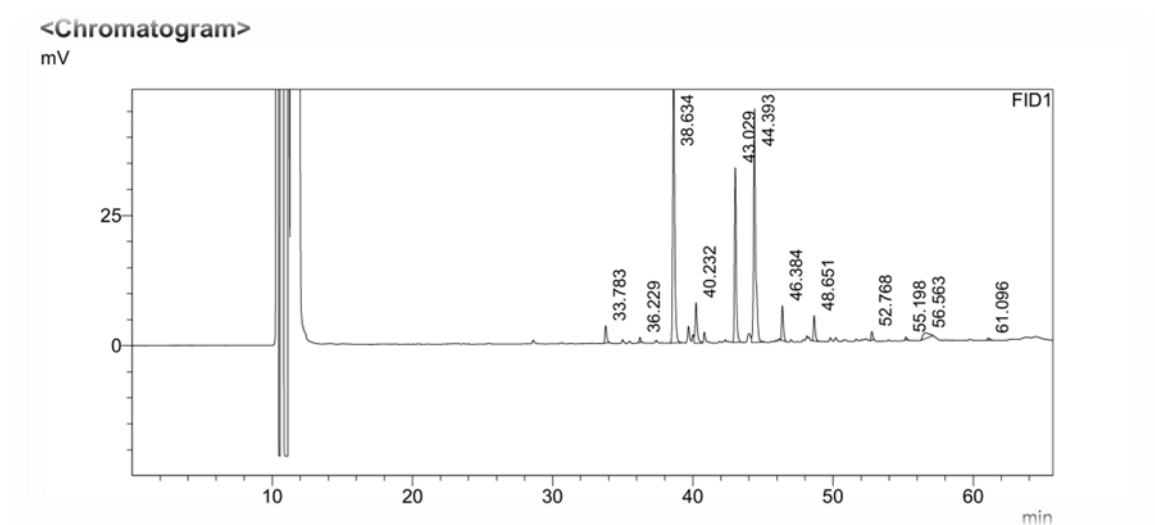


Table:4 Peak Table for zebrafish oocyte which is treated with 10µg/L of fipronil for 24 hours

Peak#	Ret.Time	Area	Height	Mark	Name
1	33.783	28818	3317	M	Myristic Acid Methyl Ester
2	36.229	6903	970	M	Pentadecanoic Acid Methyl Ester
3	38.634	554003	65464		Palmitic Acid Methyl Ester
4	40.232	78564	7754	SV	Palmitoleic Acid Methyl Ester
5	43.029	267570	33501		Stearic Acid Methyl Ester
6	44.393	445272	44755	SV	Oleic Acid Methyl Ester
7	46.384	55439	6687	M	Linoleic Acid Methyl Ester
8	48.651	40858	4854	M	Cis-11-Eicosenoic Acid Methyl Ester
9	52.768	13592	1684	M	Tricosanoic Acid Methyl Ester
10	55.198	4488	537	M	Cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester
11	56.563	35141	1147	M	Nervonic Acid Methyl Ester
12	61.096	5196	436	M	Cis-4,7,10,13,16,19-Docosahexaenoic Acid
Total		1535843	171105		

5.3 FATTY ACID PROFILING – TREATED WITH 10µg/L FOR 48HRS

<Chromatogram>

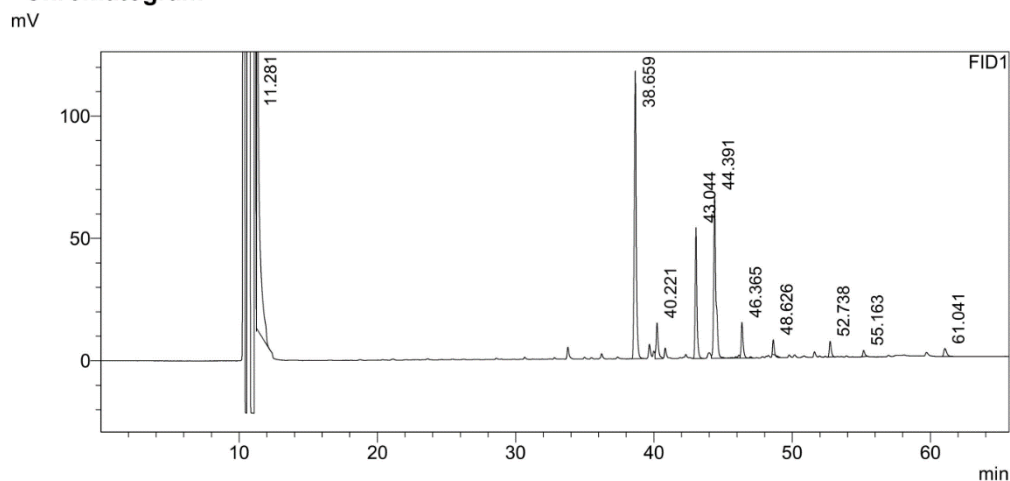


Table.5 Peak Table for zebrafish oocyte which is treated with 10µg/L of fipronil for 48 hours

Peak#	Ret.Time	Area	Height	Name
1	11.281	2267523	251674	Butyric Acid Methyl Ester
2	38.659	1121284	117549	Palmitic Acid Methyl Ester
3	40.221	154341	14561	Palmitoleic Acid Methyl Ester
4	43.044	460219	53433	Stearic Acid Methyl Ester
5	44.391	766392	66851	Oleic Acid Methyl Ester
6	46.365	139269	14577	Linoleic Acid Methyl Ester
7	48.626	38083	6042	Cis-11-Eicosenoic Acid Methyl Ester
8	52.738	62410	6364	Cis-11,14,17-Eicosatrienoic Acid Methyl Ester
9	55.163	27087	2538	Cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester
10	61.041	46075	3280	Cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester
Total		5082685		

5.4 FATTY ACID PROFILING – TREATED WITH 10µg/L FOR 72HRS

<Chromatogram>

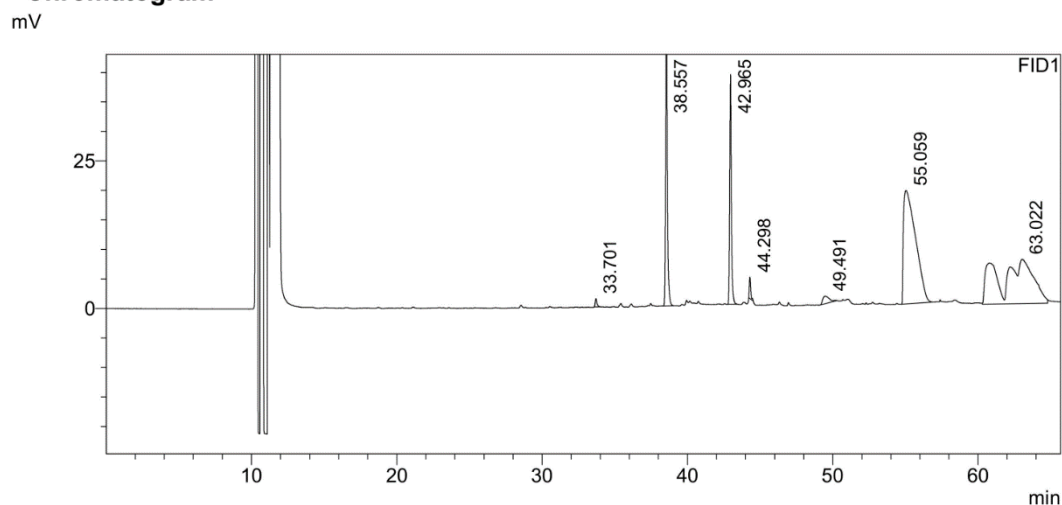


Table.6 Peak Table for zebrafish oocyte which is treated with 10µg/L of fipronil for 72 hours

Peak#	Ret.Time	Area	Height	Name
1	33.701	11612	1398	Myristic Acid Methyl Ester
2	38.557	419018	52005	Cis-10-pentadecenoic Acid Methyl Ester
3	42.965	305071	38933	Stearic Acid Methyl Ester
4	44.298	24599	3659	Elaidic Acid Methyl Ester
5	49.491	39820	1277	Heneicosanoic Acid Methyl Ester
6	55.059	1116477	19226	Lignoceric Acid Methyl Ester
7	63.022	1222534	7544	Cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester
Total		3139130	124040	

5.5 FATTY ACID PROFILING – TREATED WITH 25µg/L FOR 24HRS

<Chromatogram>

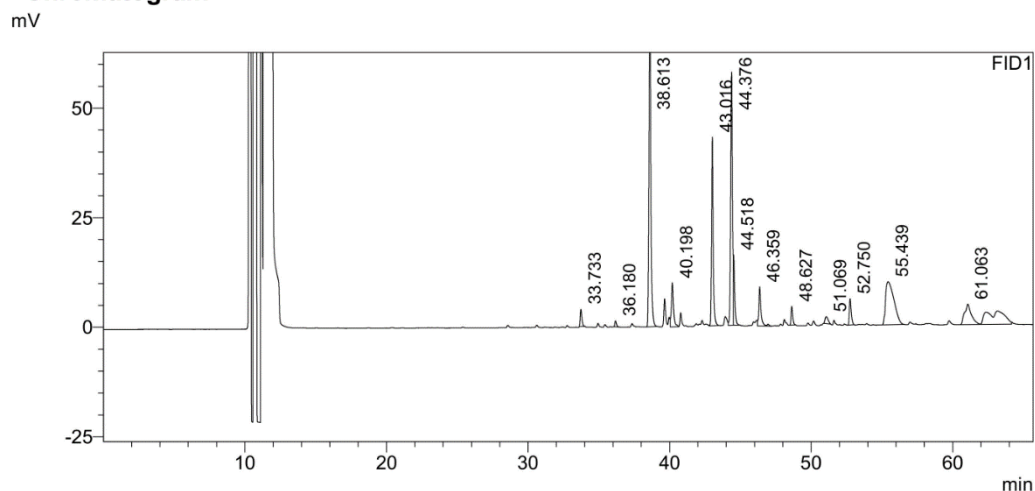


Table.7 Peak Table for zebrafish oocyte which is treated with 25µg/L of fipronil for 24 hours

Peak#	Ret.Time	Area	Height	Name
1	33.733	30962	3894	Myristic Acid Methyl Ester
2	36.180	11084	1368	Myristoleic Acid Methyl Ester
3	38.613	742242	85503	Palmitic Acid Methyl Ester
4	40.198	105082	10057	Palmitoleic Acid Methyl Ester
5	43.016	346504	43037	Stearic Acid Methyl Ester
6	44.376	491578	57727	Oleic Acid Methyl Ester
7	44.518	104849	16047	Oleic Acid Methyl Ester
8	46.359	95111	8932	Linoleic Acid Methyl Ester
9	48.627	34005	4261	Cis-11-Eicosenoic Acid Methyl Ester
10	51.069	24252	1604	Behenic Acid Methyl Ester
11	52.750	55888	6003	Cis-11,14,17-Eicosatrienoic Acid Methyl Ester
12	55.439	401295	9810	Cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester
13	61.063	415859	4640	Cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester
Total		2858710	252885	

5.6

FATTY ACID PROFILING – TREATED WITH 25µg/L FOR 48HRS

<Chromatogram>

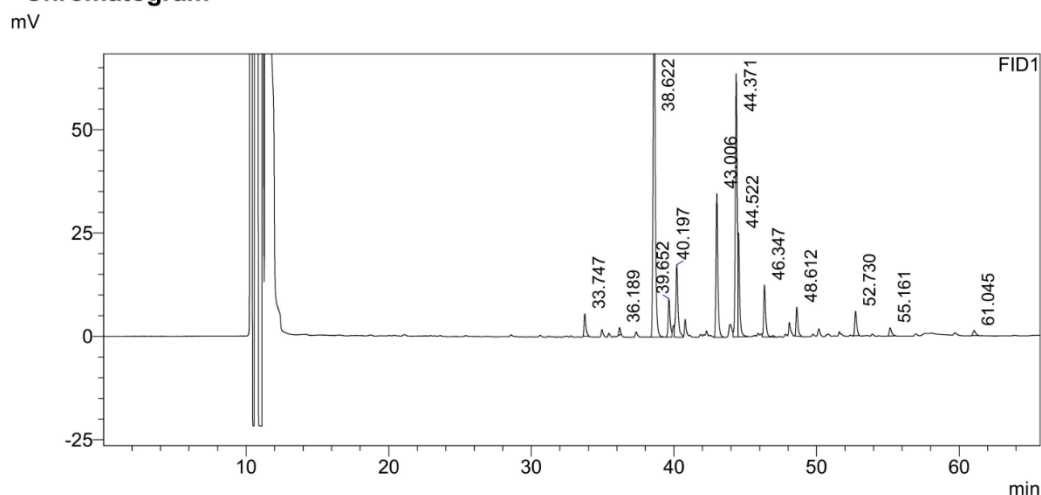


Table.8 Peak Table for zebrafish oocyte which is treated with 25µg/L of fipronil for 48 hours

Peak#	Ret.Time	Area	Height	Name
1	33.747	49322	5404	Myristic acid Methyl Ester
2	36.189	12569	1839	Pentadecanoic Acid Methyl Ester
3	38.622	916721	97535	Palmitic Acid Methyl Ester
4	39.652	89951	9300	Palmitoleic Acid Methyl Ester
5	40.197	196924	17567	Palmitoleic Acid Methyl Ester
6	43.006	301673	34710	Stearic Acid Methyl Ester
7	44.371	577011	63674	Oleic Acid Methyl Ester
8	44.522	196350	25143	Oleic Acid Methyl Ester
9	46.347	121809	12554	Linoleic Acid Methyl Ester
10	48.612	63011	6975	Cis-11-Eicosenoic Acid Methyl Ester
11	52.730	58331	5916	Cis-11,14,17-Eicosatrienoic Acid Methyl Ester
12	55.161	22571	1975	Cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester
13	61.045	16055	1196	Cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester
Total		2622299	283788	

5.7 FATTY ACID PROFILING – TREATED WITH 25µg/L FOR 72HRS

<Chromatogram>

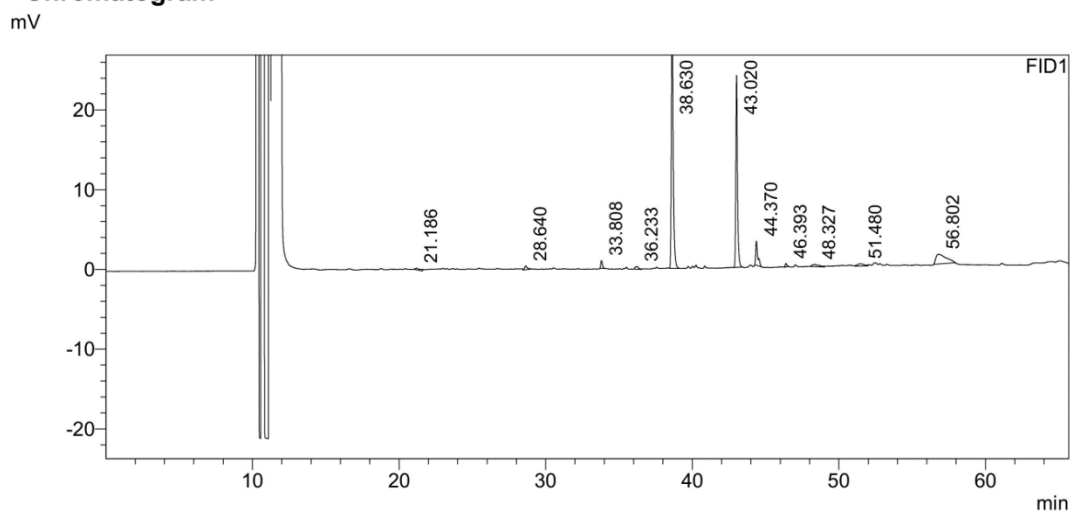


Table.9 Peak Table for zebrafish oocyte which is treated with 25µg/L of fipronil for 72 hours

Peak#	Ret.Time	Area	Height	Name
1	21.186	4848	194	
2	28.640	6246	463	Lauric Acid Methyl Ester
3	33.808	7948	993	Myristic acid Methyl Ester
4	36.233	5682	338	Pentadecanoic Acid Methyl Ester
5	38.630	290701	35656	Palmitic Acid Methyl Ester
6	43.020	188398	24073	Cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester
7	44.370	29445	3035	Oleic Acid Methyl Ester
8	46.393	3579	425	Linoleic Acid Methyl Ester
9	48.327	9649	280	Cis-11-Eicossenoic Acid Methyl Ester
10	51.480	9049	282	Cis-8,11,14-Eicosatrienoic Acid Methyl Ester
11	56.802	60045	1223	Nervonic Acid Methyl Ester
Total		615590	66962	

5.8 FATTY ACID PROFILING – TREATED WITH 50µg/L FOR 24HRS

<Chromatogram>

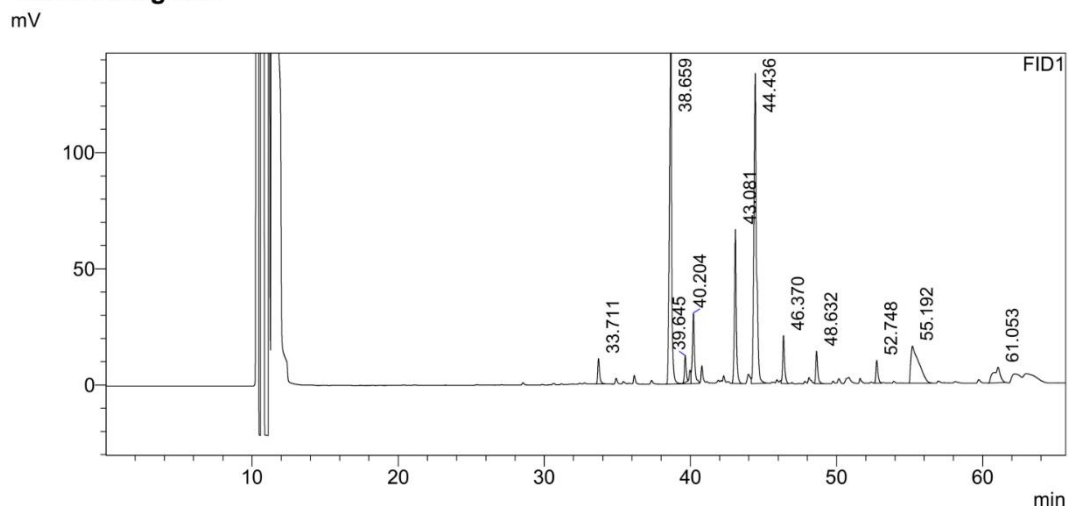


Table.10 Peak Table for zebrafish oocyte which is treated with 50µg/L of fipronil for 24 hours

Peak #	Ret.Time	Area	Height	Name
1	33.711	98726	10988	Myristic Acid Methyl Ester
2	38.659	1629986	156311	Palmitic Acid Methyl Ester
3	39.649	93064	11597	Palmitoleic Acid Methyl Ester
4	40.204	314331	30381	Palmitoleic Acid Methyl Ester
5	43.081	577235	66380	Stearic Acid Methyl Ester
6	44.436	1596851	133395	Oleic Acid Methyl Ester
7	46.370	190516	20695	Linoleic Acid Methyl Ester
8	48.632	128100	14122	Cis-11-Eicossenoic Acid Methyl Ester
9	52.748	94037	9818	Cis-11,14,17-Eicosatrienoic Acid Methyl Ester
10	55.192	602135	15975	Cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester
11	61.053	216608	6684	Cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester
		5541590	476348	

5.9 FATTY ACID PROFILING – TREATED WITH 50µg/L FOR 48HRS

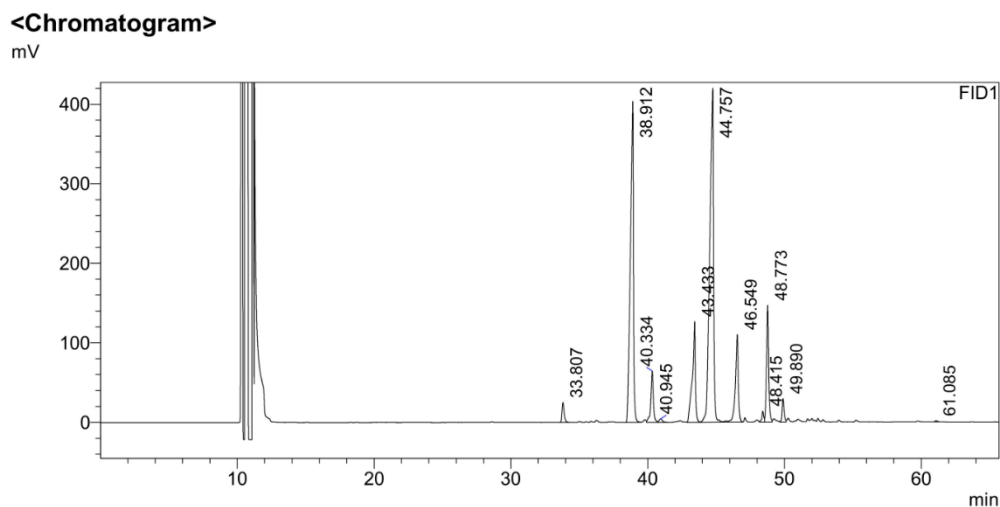


Table.11 Peak Table for zebrafish oocyte which is treated with 50µg/L of fipronil for 48 hours

Peak #	Ret.Time	Area	Height	Name
1	33.807	275181	25078	Myristic acid Methyl Ester
2	38.912	6241866	403516	Palmitic Acid Methyl Ester
3	40.334	834372	64548	Palmitoleic Acid Methyl Ester
4	40.945	70190	4276	Heptadecanoic Acid Methyl Ester
5	43.433	1938653	127043	Stearic Acid Methyl Ester
6	44.757	8039700	419948	Oleic Acid Methyl Ester
7	46.549	1604369	110503	Linoleic Acid Methyl Ester
8	48.415	132586	13884	Cis-11-Eicossenoic Acid Methyl Ester
9	48.773	1580081	146805	A-Linolenic Acid Methyl Ester
10	49.890	294088	29399	Cis-11,14-Eicosadienoic Acid Methyl Ester
11	61.085	18445	1470	Cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester
Total		21029530	1346470	

5.10 FATTY ACID PROFILING – TREATED WITH 50µg/L FOR 72HRS

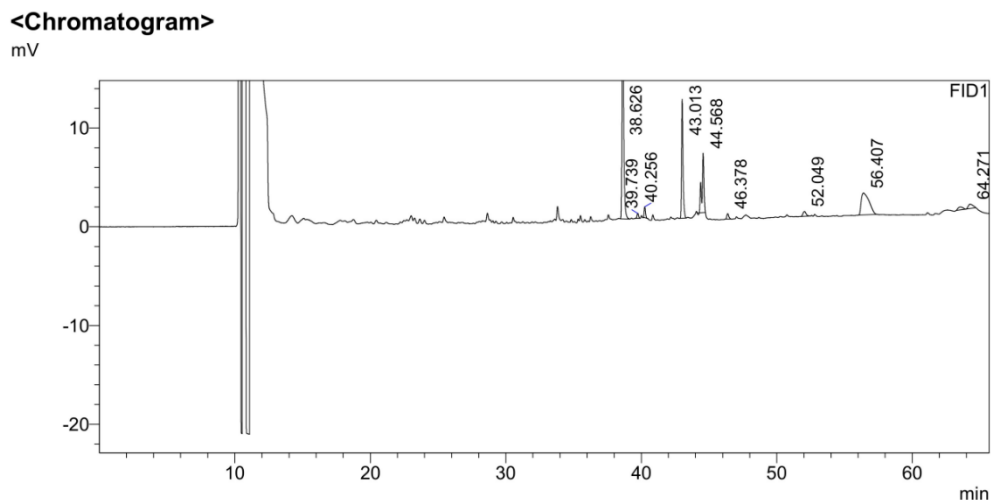


Table.12 Peak Table for zebrafish oocyte which is treated with 50µg/L of fipronil for 72 hours

Peak #	Ret.Time	Area	Height	Name
1	38.626	198061		Palmitic Acid Methyl Ester
2	39.739	3729	501	Palmitoleic Acid Methyl Ester
3	40.256	7725	1114	Palmitoleic Acid Methyl Ester
4	43.013	90198	12026	Stearic Acid Methyl Ester
5	44.568	62625	6001	Oleic Acid Methyl Ester
6	46.378	5392	548	Linoleic Acid Methyl Ester
7	52.049	8009	499	Cis-8,11,14-Eicosatrienoic Acid Methyl Ester
8	56.407	88881	2207	Nervonic Acid Methyl Ester
9	64.271	17928	457	Cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester
Total		482547	48229	

5.11 FATTY ACID PROFILING – TREATED WITH 100µg/L FOR 24HRS

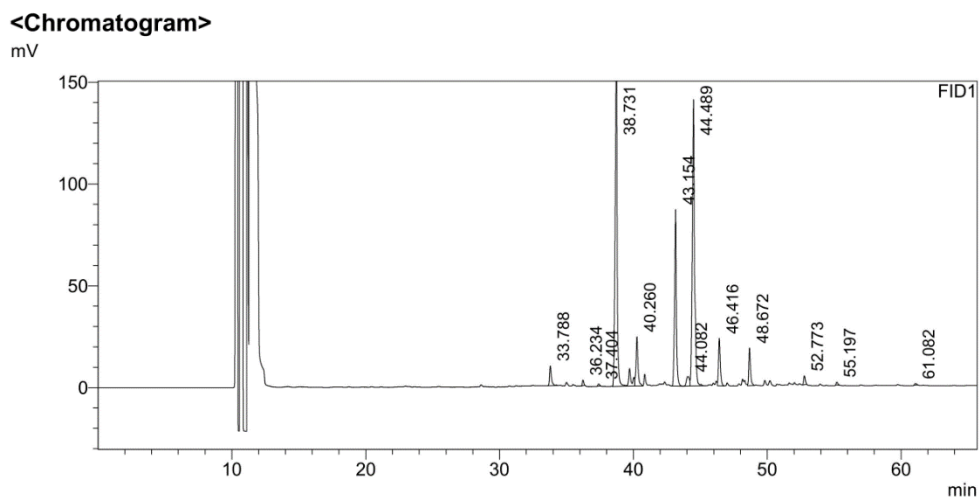


Table.13 Peak Table for zebrafish oocyte which is treated with 100µg/L of fipronil for 24 hours

Peak#	Ret.Time	Area	Height	Name
1	33.788	91424	9737	Myristic acid Methyl Ester
2	36.234	20177	2811	Pentadecanoic Acid Methyl Ester
3	37.404	9394	1115	Cis-10- Pentadecenoic Acid Methyl Ester
4	38.731	1807687	173230	Palmitic Acid Methyl Ester
5	40.260	252033	24192	Palmitoleic Acid Methyl Ester
6	43.154	791788	86579	Stearic Acid Methyl Ester
7	44.082	74818	4726	Elaidic Acid Methyl Ester
8	44.489	1630864	140544	Oleic Acid Methyl Ester
9	46.416	215142	23345	Linoleic Acid Methyl Ester
10	48.672	163508	18553	Cis-11-Eicosenoic Acid Methyl Ester
11	52.773	36083	4372	Tricosanoic Acid Methyl Ester
12	55.197	13544	1508	Cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester
13	61.082	9319	874	Cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester
Total		5115781	491587	

5.12 FATTY ACID PROFILING – TREATED WITH 100µg/L FOR 48HRS

<Chromatogram>

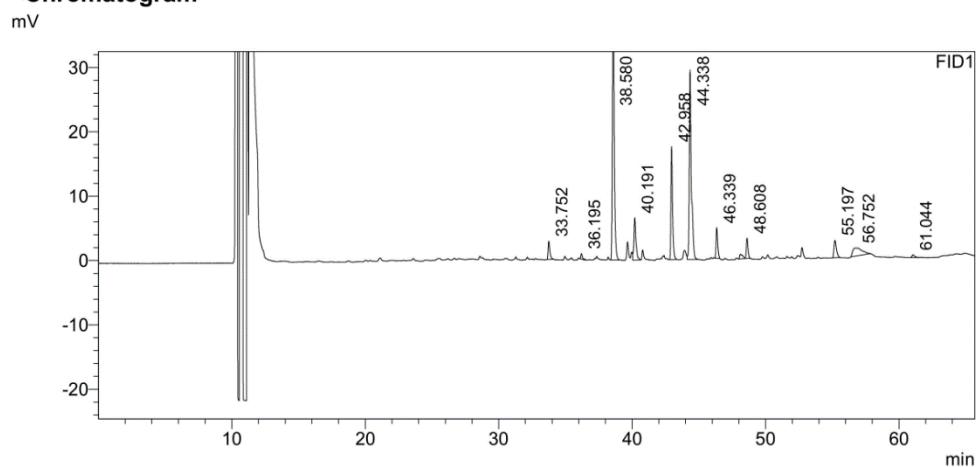


Table.14 Peak Table for zebrafish oocyte which is treated with 100µg/L of fipronil for 48hours

Peak#	Ret.Time	Area	Height	Name
1	33.752	25837	2811	Myristic acid Methyl Ester
2	36.195	7258	847	Pentadecanoic Acid Methyl Ester
3	38.580	406259	406259	Cis-10- Pentadecenoic Acid Methyl Ester
4	40.191	71980	6582	Palmitoleic Acid Methyl Ester
5	42.958	147849	17570	Stearic Acid Methyl Ester
6	44.338	316820	29409	Elaidic Acid Methyl Ester
7	46.339	40223	4765	Linoleic Acid Methyl Ester
8	48.608	39429	3178	Cis-11-Eicosenoic Acid Methyl Ester
9	55.197	33859	2678	Cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester
10	56.752	58782	1245	Nervonic Acid Methyl Ester
11	61.044	5892	409	Cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester
Total		1154188	116021	

5.13 FATTY ACID PROFILING – TREATED WITH 100µg/L FOR 72HOURS

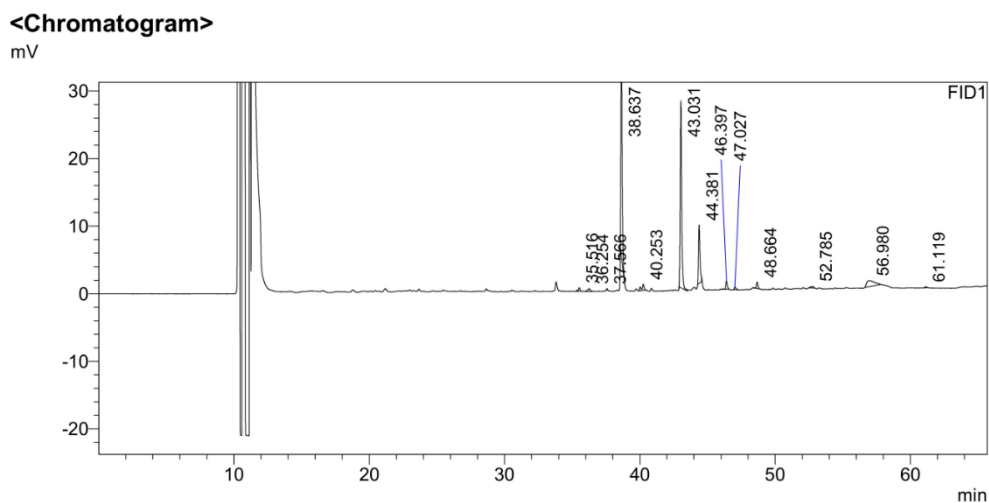


Table.15 Peak Table for zebrafish oocyte which is treated with 100µg/L of fipronil for 72hours

Peak#	Ret.Time	Area	Height	Name
1	35.516	4786	516	Myristic acid Methyl Ester
2	36.254	5590	421	Pentadecanoic Acid Methyl Ester
3	37.566	495	137	Cis-10- Pentadecenoic Acid Methyl Ester
4	38.637	233759	34318	Palmitic Acid Methyl Ester
5	40.253	10830	892	Palmitoleic Acid Methyl Ester
6	43.031	214159	27616	Stearic Acid Methyl Ester
7	44.381	63071	8631	Oleic Acid Methyl Ester
8	46.397	9626	1223	Linoleic Acid Methyl Ester
9	47.027	2781	373	Arachidic Acid Methyl Ester
10	48.664	8892	966	Cis-11-Eicosenoic Acid Methyl Ester
11	52.785	4160	272	Tricosanoic Acid Methyl Ester
12	56.980	35136	884	Nervonic Acid Methyl Ester
13	61.119	1933	186	Cis-4,7,10,13,16,19- Docosaehaenoic Acid Methyl Ester
Total		595219	76435	

5.14 FATTY ACID PROFILING – TREATED WITH 300µg/L FOR 24HOURS

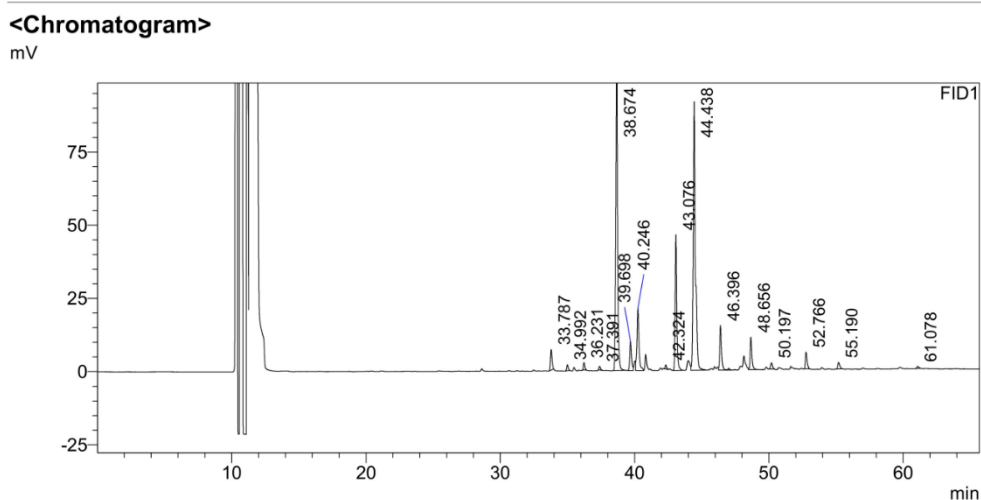


Table.16 Peak Table for zebrafish oocyte which is treated with 300µg/L of fipronil for 24hours

Peak#	Ret.Time	Area	Height	Name
1	33.787	55853	6966	Myristic acid Methyl Ester
2	34.992	16245	2004	Myristoleic Acid Methyl Ester
3	36.231	19094	2538	Pentadecanoic Acid Methyl Ester
4	37.391	10294	1250	Pentadecanoic Acid Methyl Ester
5	38.674	974097	108105	Palmitic Acid Methyl Ester
6	39.698	90541	9905	Palmitoleic Acid Methyl Ester
7	40.246	222308	21126	Palmitoleic Acid Methyl Ester
8	42.324	10363	1469	Cis-10-Heptadecenoic Acid Methyl Ester
9	43.076	384588	46308	Stearic Acid Methyl Ester
10	44.438	1020553	91679	Oleic Acid Methyl Ester
11	46.396	139631	15249	Linoleic Acid Methyl Ester
12	48.656	99587	11092	Cis-11-Eicosenoic Acid Methyl Ester
13	50.197	20899	2115	Cis-11,14-Eicosadienoic Acid Methyl Ester
14	52.766	51690	5680	Tricosanoic Acid Methyl Ester
15	55.190	22328	2220	Cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester
16	61.078	7431	695	Cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester
Total		3145499	328400	

5.15 FATTY ACID PROFILING – TREATED WITH 300µg/L FOR 48HOURS

<Chromatogram>

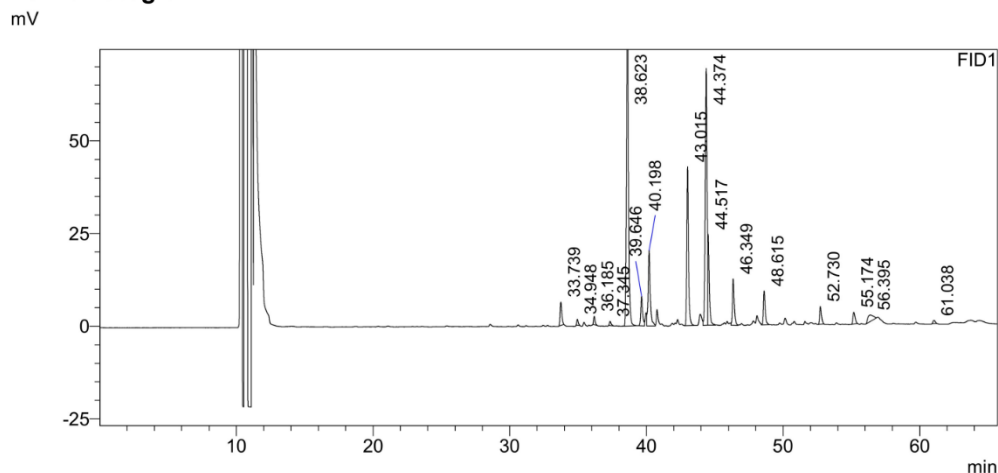


Table.17 Peak Table for zebrafish oocyte which is treated with 300µg/L of fipronil for 48hours

Peak#	Ret.Time	Area	Height	Name
1	33.739	54496	6317	Myristic acid Methyl Ester
2	34.948	12530	1626	Myristoleic Acid Methyl Ester
3	36.185	17604	2273	Pentadecanoic Acid Methyl Ester
4	37.345	10225	1136	Pentadecanoic Acid Methyl Ester
5	38.623	978857	106249	Palmitic Acid Methyl Ester
6	39.646	75700	7975	Palmitoleic Acid Methyl Ester
7	40.198	217527	20368	Palmitoleic Acid Methyl Ester
8	43.015	363501	42886	Stearic Acid Methyl Ester
9	44.374	620499	69277	Oleic Acid Methyl Ester
10	44.517	178171	24514	Oleic Acid Methyl Ester
11	46.349	118257	12542	Linoleic Acid Methyl Ester
12	48.615	84501	9152	Cis-11-Eicosenoic Acid Methyl Ester
13	52.730	46167	4806	Cis-11,14,17-Eicosatrienoic Acid Methyl Ester
14	55.174	35485	3149	Cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester
15	56.395	47246	1712	Nervonic Acid Methyl Ester
16	61.038	9919	866	Cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester
Total		2870685	314846	

5.16 FATTY ACID PROFILING – TREATED WITH 300µg/L FOR 72HOURS

<Chromatogram>

mV

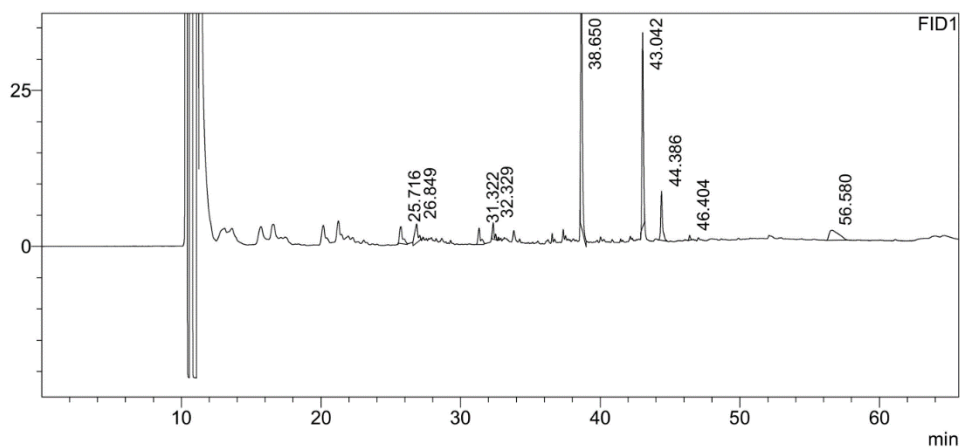


Table.18 Peak Table for zebrafish oocyte which is treated with 300µg/L of fipronil for 72hours

Peak#	Ret.Time	Area	Height	Name
1	25.716	41270	2692	Undecanoic Acid Methyl Ester
2	26.849	47615	3007	Undeconoic Acid Methyl Ester
3	31.322	30632	2600	Tridecanoic Acid Methyl Ester
4	32.329	23054	2655	Tridecanoic Acid Methyl Ester
5	38.650	322769	43840	Palmitic Acid Methyl Ester
6	43.042	207503	31037	Stearic Acid Methyl Ester
7	44.386	69500	7839	Oleic Acid Methyl Ester
8	46.404	5243	772	Cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester
9	56.580	76164	1615	Nervonic Acid Methyl Ester
Total		823749	96058	

Table.19 Percentage of fatty acids present in 10µg/L

S.NO	NAME	Percentage of fatty acid present in 24hrs(%)	Percentage of fatty acid present in 48hrs(%)	Percentage of fatty acid present in 72hrs(%)
1	Myristic acid methyl Ester	1.87	-	0.36
2	Pentadecanoic Acid Methyl Ester	0.44	-	13.34
3	Palmitic Acid Methyl Ester	36.07	22.06	-
4	Palmitoleic Acid Methyl Ester	5.1	3.03	-
5	Stearic Acid Methyl Ester	17.42	9.05	9.71
6	Oleic Acid Methyl Ester	28.99	15.07	-
7	Linoleic Acid Methyl Ester	3.60	2.74	-
8	Cis-11-Eicosenoic Acid Methyl Ester	2.66	0.74	-
9	Tricosanoic Acid Methyl Ester	0.87	-	-
10	Cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester	0.29	0.13	-
11	Nervonic Acid Methyl Ester	2.28	-	-
12	Cis-4,7,10,13,16,19-Docosahexaenoic Acid	0.33	0.90	38.94
13	Butyric Acid Methyl Ester	-	44.61	-
14	Cis-11,14,17-Eicosatrienoic Acid Methyl Ester	-	1.22	-
15	Cis-10-pentadecenoic Acid Methyl Ester	-	-	13.34
16	Elaidic Acid Methyl Ester	-	-	0.78
17	Heneicosanoic Acid Methyl Ester	-	-	1.26
18	Lignoceric Acid Methyl Ester	-	-	35.5

Table.20 Percentage of fatty acids present in 25µg/L

S.no	Name	Percentage of fatty acid present in 24hrs	Percentage of fatty acid present in 48hrs	Percentage of fatty acid present in 72hrs
1	Myristic Acid Methyl Ester	1.08	1.88	1.30
2	Myristoleic Acid Methyl Ester	0.38	-	-
3	Palmitic Acid Methyl Ester	25.96	2.40	47.59
4	Palmitoleic Acid Methyl Ester	3.67	7.50	-
5	Stearic Acid Methyl Ester	12.12	11.50	-
6	Oleic Acid Methyl Ester	17.19	12.0	4.82
7	Linoleic Acid Methyl Ester	3.32	4.6	0.58
8	Cis-11-Eicosenoic Acid Methyl Ester	3.18	1.10	1.07
9	Behenic Acid Methyl Ester	0.84	-	-
10	Cis-11,14,17-Eicosatrienoic Acid Methyl Ester	1.95	2.22	1.48
11	Cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester	14.0	0.86	-
12	Cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester	14.54	14.61	30.84
13	Pentadecanoic Acid Methyl Ester	-	0.47	0.93
14	Lauric Acid Methyl Ester	-	-	1.02
15	Nervonic Acid Methyl Ester	-	-	9.83

Table.21 Percentage of fatty acids present in 50µg/L

S.no	Name	Percentage of fatty acid present in 24hrs	Percentage of fatty acid present in 48hrs	Percentage of fatty acid present in 72hrs
1	Myristic acid Methyl Ester	1.78	1.30	-
2	Palmitic Acid Methyl Ester	29.41	29.6	-
3	Palmitoleic Acid Methyl Ester	5.67	3.96	1.60
4	Stearic Acid Methyl Ester	10.41	9.21	18.69
5	Oleic Acid Methyl Ester	28.81	38.23	12.97
6	Linoleic Acid Methyl Ester	3.43	7.62	1.11
7	Cis-11-Eicosenoic Acid Methyl Ester	2.31	0.63	-
8	Cis-11,14,17-Eicosatrienoic Acid Methyl Ester	1.69	1.39	1.65
9	Cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester	10.86	-	-
10	Cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester	3.90	3.95	3.97
11	Heptadecanoic Acid Methyl Ester	-	0.33	-
12	A-Linolenic Acid Methyl Ester	-	7.51	-
13	Cis-11,14-Eicosadienoic Acid Methyl Ester	-	1.39	-
14	Nervonic Acid Methyl Ester	-	-	18.41

Table:22 Percentage of fatty acids presents in 100µg/L

S.no	Name	Percentage of fatty acid present in 24hrs	Percentage of fatty acid present in 48hrs	Percentage of fatty acid present in 72hrs
1	Myristic acid Methyl Ester	1.78	2.23	0.80
2	Pentadecanoic Acid Methyl Ester	0.39	0.62	0.93
3	Cis-10- Pentadecenoic Acid Methyl Ester	0.18	35.19	0.083
4	Palmitic Acid Methyl Ester	35.33	-	-
5	Palmitoleic Acid Methyl Ester	4.92	6.23	1.81
6	Stearic Acid Methyl Ester	15.4	12.80	35.99
7	Elaidic Acid Methyl Ester	1.46	27.44	-
8	Oleic Acid Methyl Ester	31.87	-	10.59
9	Linoleic Acid Methyl Ester	4.20	3.48	1.61
10	Cis-11-Eicosenoic Acid Methyl Ester	3.19	3.41	1.49
11	Tricosanoic Acid Methyl Ester	0.70	-	0.69
12	Cis-5,8,11,14,17- Eicosapentaenoic Acid Methyl Ester	0.26	0.25	-
13	Cis-4,7,10,13,16,19- Docosahexaenoic Acid Methyl Ester	0.18	0.51	0.32
14	Nervonic Acid Methyl Ester	-	5.09	5.90
15	Arachidic Acid Methyl Ester	-	-	0.46

Table:23 Percentage of fatty acids presents in 300µg/L

S.no	Name	Percentage of fatty acid present in 24hrs	Percentage of fatty acid present in 48hrs	Percentage of fatty acid present in 72hrs
1	Myristic acid Methyl Ester	1.77	1.89	-
2	Myristoleic Acid Methyl Ester	0.15	0.43	-
3	Pentadecanoic Acid Methyl Ester	0.60	6.61	-
4	Palmitic Acid Methyl Ester	30.96	34.09	39.18
5	Palmitoleic Acid Methyl Ester	7.06	7.57	-
6	Cis-10-Heptadecenoic Acid Methyl Ester	0.32	-	-
7	Stearic Acid Methyl Ester	12.2	12.6	25.19
8	Oleic Acid Methyl Ester	32.4	21.61	8.43
9	Linoleic Acid Methyl Ester	4.43	4.11	-
10	Cis-11-Eicosenoic Acid Methyl Ester	3.16	2.94	-
11	Cis-11,14-Eicosadienoic Acid Methyl Ester	0.66	1.60	-
12	Tricosanoic Acid Methyl Ester	1.64	-	-
13	Cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester	0.70	1.23	-
14	Cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester	0.23	0.34	0.63
15	Cis-11,14,17-Eicosatrienoic Acid Methyl Ester	-	1.60	-
16	Nervonic Acid Methyl Ester	-	1.64	9.24
17	Undecanoic Acid Methyl Ester	-	-	5.78
18	Tridecanoic Acid Methyl Ester	-	-	3.71

DISCUSSION:

Fipronil is a broad-spectrum insecticide that is widely used in agriculture and veterinary medicine. Studies have shown that exposure to fipronil can have negative effects on the reproductive health of aquatic organisms, including zebrafish. Fatty acid profiling is a technique used to analyse the composition and distribution of fatty acids in biological samples. This technique can be used to investigate the effects of environmental stressors, such as exposure to fipronil, on the fatty acid profile of zebrafish oocytes. Wei et al. (2021) In this study, zebrafish were exposed to different concentrations of fipronil for 14 days. The researchers then extracted the oocytes from the fish and analysed their fatty acid profiles using gas chromatography-mass spectrometry (GC-MS). The results of the study showed that exposure to fipronil led to significant changes in the fatty acid profile of zebrafish oocytes. Specifically, the researchers found that exposure to fipronil resulted in a decrease in the levels of polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are important for reproductive health. The researchers also observed an increase in the levels of saturated fatty acids (SFAs) such as palmitic acid (PA) and stearic acid (SA), which are associated with negative health outcomes. This study highlights the importance of understanding the effects of environmental stressors on the reproductive health of aquatic organisms. The findings suggest that exposure to fipronil can have negative effects on the fatty acid profile of zebrafish oocytes, which could ultimately impact their reproductive success. Further research is needed to investigate the mechanisms underlying these effects and to determine whether similar effects are observed in other aquatic organisms.

In the journal *Ecotoxicology and Environmental Safety* investigated the effects of fipronil exposure on the fatty acid profile of zebrafish oocytes. The study exposed zebrafish to fipronil at a concentration of 0.05 µg/L for 21 days, which resulted in the accumulation of fipronil and its metabolites in the fish. The researchers then collected oocytes from the exposed fish and analysed the fatty acid profile. The results showed that exposure to fipronil led to significant changes in the fatty acid profile of zebrafish oocytes. Specifically, there was a decrease in the levels of polyunsaturated fatty acids (PUFAs) and an increase in the levels of saturated fatty acids (SFAs). PUFAs are important for reproduction in fish, as they are involved in the development of oocytes and embryos. SFAs, on the other hand, are typically associated with negative health effects, such as an increased risk of heart disease. The study concluded that exposure to fipronil can disrupt the fatty acid profile of zebrafish oocytes, which may have negative effects on reproduction and population dynamics. This finding highlights the need for further research on the potential reproductive toxicity of fipronil and other pesticides in fish and other aquatic organisms. In conclusion, the fatty acid profiling of zebrafish oocytes treated with fipronil has shown that exposure to this insecticide can lead to changes in the fatty acid profile, specifically a decrease in PUFAs and an increase in SFAs. These changes can have significant implications for reproductive processes and population dynamics.

CHAPTER-6 SUMMARY AND CONCLUSION:

Fipronil is a broad-spectrum insecticide commonly used in agriculture to control insects and pests. It has been reported to be toxic to aquatic organisms such as fish, including zebrafish. Fatty acid profiling is a technique used to analyse the fatty acid composition of a sample, which can provide information about the nutritional quality of the sample and the metabolic processes that occur within the organism. Fatty acids play critical roles in oocyte maturation, fertilization, and early embryonic development, and alterations in their composition can have significant consequences for reproductive success.

Results indicate that fipronil exposure altered the relative proportions of several fatty acids in zebrafish oocytes, including significant decreases in oleic acid, eicosenoic acid and eicosapentaenoic acid (EPA) and significant increases in docosahexaenoic acid (DHA). These findings suggest that fipronil may disrupt lipid metabolism and homeostasis in developing oocytes, with potential implications for reproductive success and embryonic development in exposed fish populations

REFERENCES:

- [1] C.C. Tingle, J.A. Rother, C.F. Dewhurst, S. Lauer, W.J. King, Fipronil: environmental fate, ecotoxicology, and human health concerns, in: *Reviews of environmental contamination and toxicology*, Springer, 2003, pp. 1-66.
- [2] A.S. Gunasekara, T. Truong, K.S. Goh, F. Spurlock, R.S. Tjeerdema, Environmental fate and toxicology of fipronil, *Journal of Pesticide Science*, (2007) 0706180001-0706180001.
- [3] L.M. Cole, R.A. Nicholson, J.E. Casida, Action of phenylpyrazole insecticides at the GABA-gated chloride channel, *Pesticide Biochemistry and Physiology*, 46 (1993) 47-54.
- [4] D. Gibbons, C. Morrissey, P. Mineau, A review of the direct and indirect effects of neonicotinoids and fipronil on vertebrate wildlife, *Environmental Science and Pollution Research*, 22 (2015) 103-118.
- [5] J. Abraham, A. Gajendiran, Biodegradation of fipronil and its metabolite fipronil sulfone by *Streptomyces rochei* strain AJAG7 and its use in bioremediation of contaminated soil, *Pesticide biochemistry and physiology*, 155 (2019) 90-100.
- [6] R. Kumar, B. Singh, V. Gupta, Biodegradation of fipronil by *Paracoccus* sp. in different types of soil, *Bulletin of environmental contamination and toxicology*, 88 (2012) 781-787.
- [7] N. Simon-Delso, V. Amaral-Rogers, L.P. Belzunces, J.-M. Bonmatin, M. Chagnon, C. Downs, L. Furlan, D.W. Gibbons, C. Giorio, V. Girolami, Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites, *Environmental Science and Pollution Research*, 22 (2015) 5-34.
- [8] J.-M. Bonmatin, C. Giorio, V. Girolami, D. Goulson, D. Kreutzweiser, C. Krupke, M. Liess, E. Long, M. Marzaro, E.A. Mitchell, Environmental fate and exposure; neonicotinoids and fipronil, *Environmental Science and Pollution Research*, 22 (2015) 35-67. Journal Pre-proof 24

- [9] J.P. Overmyer, D.R. Rouse, J.K. Avants, A.W. Garrison, M.E. DeLorenzo, K.W. Chung, P.B. Key, W.A. Wilson, M.C. Black, Toxicity of fipronil and its enantiomers to marine and freshwater non-targets, *Journal of Environmental Science and Health, Part B*, 42 (2007) 471- 480.
- [10] Mebdoua, S., Lazali, M., Ounane, S.M., Tellah, S., Nabi, F., & Ounane, G. (2017). Evaluation of pesticide residues in fruits and vegetables from Algeria. *Food additives & contaminants part B-surveillance*, 10(2), 91-98
- [11] Duhan, A., Kumari, B., & Duhan, S. (2015). Determination of residues of fipronil and its metabolites in cauliflower by using gas chromatography-tandem mass spectrometry. *Bulletin of environmental contamination and toxicology*. 94, 260–266
- [12] Skretteberg, L.G., Lyran, B., Holen, B., Jansson, A., Fohgelberg, P., Siivinen, K., et al. (2015). Pesticide residues in food of plant origin from Southeast Asia – A Nordic project. *Food Control*, 51, 225-235
- [13] Chowdhury, M.A.Z., Fakhruddin, A.N.M., Islam, M.N., Moniruzzaman, M., Gan, S.H., & Alam, M.K. (2013). Detection of the residues of nineteen pesticides in fresh vegetable samples using gas chromatography-mass spectrometry. *Food control*, 34, 457-465.
- [14] Wu, L.P., Zhou, X.L., Zhao, D.Y., Feng, T., Zhou, J., Sun, T., et al. (2017). Seasonal variation and exposure risk assessment of pesticide residues in vegetables from Xinjiang Uygur Autonomous Region of China during 2010-2014. *Journal of food composition and analysis*, 58, 1–9.
- [15] Farha, W., Abd El-Aty, A.M., Rahman, M.M., Jeong, J.H., Shin, H.C., Wang, J., et al. (2018). Analytical approach, dissipation pattern and risk assessment of pesticide residue in green leafy vegetables: a comprehensive review. *Biomedical Chromatography*, 32(1), e4134.
- [16] Xu, X.M., Li, L.Y., Huang, X.D., Lin, H., Liu, G.Y., Xu, D.H., et al. (2018). Survey of Four Groups of Cumulative Pesticide Residues in 12 Vegetables in 15 Provinces in China. *Journal of Food Protection*, 81(3), 377-385.

- [17] Lozowicka, B., Jankowska, M., & Kaczynski, P. (2012). Pesticide residues in Brassica vegetables and exposure assessment of consumers. *Food Control*, 25(2), 561-575
- [18] Li, Z.X., Nie, J.Y., Lu, Z.Q., Xie, H.Z., Kang, L., Chen, Q.S., et al. (2016). Cumulative risk assessment of the exposure to pyrethroids through fruits consumption in China - Based on a 3-year investigation. *Food and Chemical Toxicology*, 96, 234-243
- [19] Elwan MA, Richardson JR, Guillot TS, Caudle WM, Miller GW. Fipronil amplifies primary mouse microglial neurotoxicity in vitro. *Neurotoxicology*. 2011;32(6):902-911. doi:10.1016/j.neuro.2011.08.001
- [20] Slotkin TA, Levin ED, Seidler FJ. Comparative developmental neurotoxicity of organophosphates, carbamates, and nicotine. *Environ Health Perspect*. 2006;114(6):963-971. doi:10.1289/ehp.8700
- [21] Chiu YH, Williams PL, Gillman MW, et al. Association between pesticide residue intake from consumption of fruits and vegetables and pregnancy outcomes among women undergoing infertility treatment with assisted reproductive technology. *JAMA Intern Med*. 2018;178(1):17-26. doi:10.1001/jamainternmed.2017.5038
- [22] Froese R, Pauly D, editors. *Danio rerio*, Zebrafish. FishBase. 2021. <https://www.fishbase.de/summary/4523>. Accessed April 26, 2023.
- [23] Lieschke GJ, Currie PD. (2007). Animal models of human disease: zebrafish swim into view. *Nature Reviews Genetics*, 8(5): 353-367.L