DNA Damage and Histopathological Alterations in Muscles and Gill Tissues of *Danio rerio (H.)* Induced by Combined Exposures of Imidacloprid and Endosulfan

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ISLAMABAD, PAKISTAN

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In

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Certificate of Approval

It is to certify that the research work presented in this thesis, entitled "**DNA Damage and Histopathological Alterations in Muscles and Gill Tissues of *Danio rerio* (H.) induced by Combined Exposures of Imidacloprid and Endosulfan**" was conducted by Ms. Barzah Muazzam (02311511017) under the supervision of ~~Prof.~~ Dr. Mazhar Iqbal Zafar. No part of this thesis has been submitted else for any other degree. This thesis is submitted to the Department of Environmental Sciences, in the partial fulfillment of the requirements for the degree of **Master of Philosophy** in the field of Environmental Science, Quaid-i-Azam University Islamabad, Pakistan.

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Dedicated

To

My Sweet and Loving Parents

Mama G, Abu G and my Husband

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LIST OF ABBREVIATIONS

ALA	Alkali Labile Sites
ADI	Acceptable daily intake
BSA	Bovine serum albumin
CAT	Catalase
DMS	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DDTs	Dichlorodiphenyltrichloroethane
EFSA	European Food Safety Authority
ESF	Endosulfan
EDTA	Ethylenediaminetetraacetic acid
EU	European Union
GPx	Glutathione peroxidase
HCB	Hexachlorobenzene
LMA	Low melting agarose
LPO	Lipid peroxidation
MRL	Maximum residual limit
MDA	Malondialdehyde
NMA	Normal melting agarose
NBT	Nitrobluetetrazoliumchloride
nAChR	Nicotinicacetylcholine receptor
ROS	Reactive oxygen species
SDS	Sodiumdodecylsulphate
SOD	Superoxide dimutase
SCGE	Single cell gel electrophoresis
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TBA	Thiobarbituric acid
USEPA	United States of Environmental Protection Agency

Abstract

Pesticide residues in environment pose significant harmful impacts on terrestrial and aquatic ecosystems. In the present study the combined exposure of imidacloprid and endosulfan on Zebrafish in terms of oxidative stress and DNA damage in liver and histological alterations in gills and muscles and impacts on antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), lipid peroxidation product malondialdehyde (MDA) were evaluated. Zebrafish were distributed in four different aquariums: control group without pesticide exposure (F1), Treatment 1 (T1), Treatment 2 (T2) and Treatment 3 (T3) and were exposed to constant imidacloprid concentration in all three treatments (1ppm) and increasing concentrations of endosulfan from 0.5μg/L in T1 to 0.1μg/L in T2 and 1μg/L in T3. Fish sampling was done after 7, 14 and 21 days of exposure. Dose and time dependent decrease in superoxide dismutase (SOD) and catalase (CAT) activity was observed after 21 days of exposure while the lower concentrations of pesticides induced SOD and CAT activities after early exposure to reduce the oxidative stress. Substantial increase of 98.6, 70.2 and 22.6% was observed in SOD activity in T1, T2 and T3 as compared to control on day 7 while a significant decline of 55.1% was observed in T3 after 21 days of exposure. Similarly, 47.4% increase in CAT activity was observed after 14 days of exposure. Malondialdehyde (MDA) levels increased significantly in the groups having the highest concentrations of pesticides at all sampling intervals. 36.8 and 66.2% increase in MDA content in T2 and T3 after 21 days of exposure was observed as compared to control. This study reveals the genotoxic potential of endosulfan and imidacloprid. DNA damage was measured through comet assay and increased DNA damage was observed in T3 after 21 days of exposure as compared to all treatments and control groups. Histopathological alterations in gills and muscles were dose and time dependent but the morphological changes in gills were more significant than muscles. Prolonged exposures induced epithelial lifting, pronounced hyperplasia, fused secondary lamellae, narrowed water, shortening of secondary lamellae and sloughing of lamellae in gills. Splitting of muscle bundles and sloughing of gills was randomly observed in groups having highest concentrations of pesticides (T3) after prolonged exposure of 21 days. The present study provides data regarding the combined exposure of imidacloprid and endosulfan to zebrafish.

Introduction

1.1 Pesticides: A Menace to Aquatic Fauna

Pesticides usage has become necessary in developing countries where agriculture is considered to be the backbone of economy. During the last two decades, the worldwide production and application of pesticides has amplified (Tišler *et al.*, 2009). The worldwide usage of pesticides is about 2 million tonnes/year, about 45% of pesticides are used in Europe only, USA 25% and rest of the world consumes 25% (De *et al.*, 2014). The extensive pesticides application for pests control has brought the pollution of various environmental systems (Al-jubouri, 2015). Runoff, leaching, direct spillage and wind drift are the major routes which contribute to addition of pesticides in waterbodies (Anju *et al.*, 2010). Contamination of water is mainly caused by runoff of pesticides used in agriculture sector which seems currently a major issue worldwide. From pesticides application on agricultural lands mostly non-target organisms are affected and It also causes soil and water contamination (Margni *et al.*, 2002). These compounds are associated with various environmental threats as they resist biological, chemical and physical degradation and can cause acute and chronic illnesses and besides causing toxicity to non-target organisms (Chow *et al.*, 2013).

To apprehend better the toxic effects of pesticides, information about target mechanism, metabolic pathways, chemical relations and toxic kinetics is required (Hernández *et al.*, 2013). Pesticides cause acute as well as chronic poisoning of fish and may impair their vital organs. They are known to cause metabolic, physiological and structural changes in different organs. Direct accumulation of toxic pollutant occurs from contaminated water and indirectly via food chain. Accumulation of pesticides in tissues of fish is exposure and concentration dependent, other factors like temperature, hardness, salinity of water and fish metabolism also affect the accumulation. Pesticides are accumulated by absorption through gills and their concentrations in gills reflect the pesticide levels in water where fish species resides (Napit, 2013). Action of various pesticides can cause formation of a particular enzymes that lead to toxication and demise at a cellular level, stated as necrosis

(Bhuvaneshwari *et al.*, 2015). Environmental pollutants like pesticides induce reactive oxygen species (ROS) in organisms; these excessive ROS may cause oxidative damage of deoxyribonucleic acid (DNA) i.e. breakage of DNA strands in the zebrafish liver. Oxidative stress, i.e. destruction of oxidant-antioxidant balance can lead to the formation of free radicals which may cause modifications in antioxidants enzyme systems. The enzymes induced by oxidative stress includes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Oruc *et al.*, 2004)

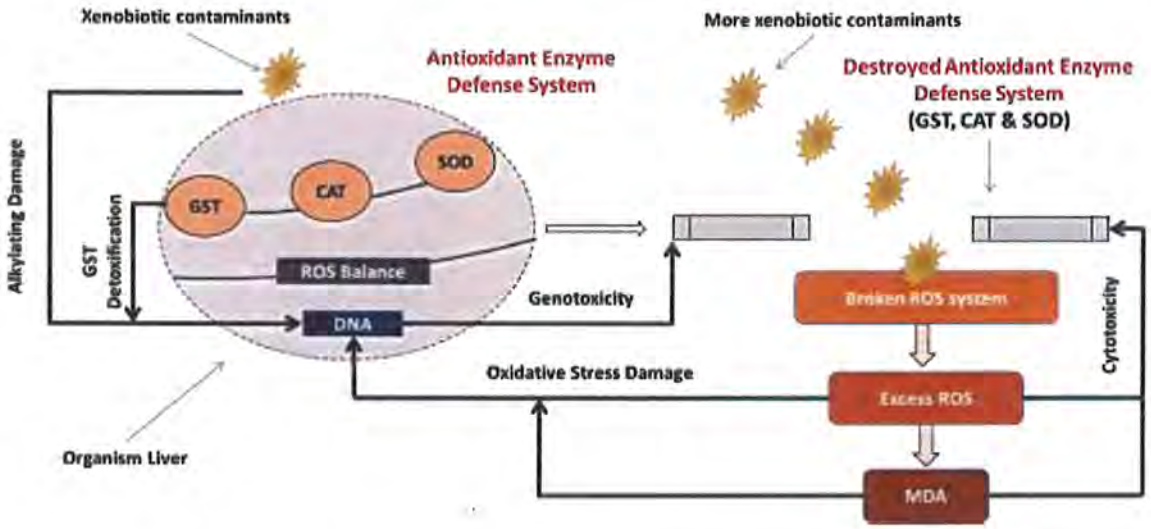


Figure 3.1: Effect of xenobiotic chemical on SOD, CAT, GST, ROS, MDA and DNA damage. This conceptual figure of antioxidant defensive system of enzymes is taken from Ge *et al.* (2015).

Oxidative stress and antioxidant levels are supposed to be good indicators of aquatic pollution. Biochemical responses are known as biomarker of exposures induced in organisms due to environmental stress. There is a balance in the production and elimination of reactive oxygen species (ROS) in organisms. The antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) can eliminate the excessive ROS from the organisms, when the equilibrium among formation and exclusion of ROS is disturbed by the external pollutant, excessive production of ROS leads to oxidative stress, lipid

peroxidation and cellular death (apoptosis). Malondialdehyde (MDA) will be formed as a result of lipid peroxidation and will provide toxic stress to cells (Ge *et al.*, 2015).

1.2 Pesticide Usage Trends in Pakistan

Pakistan is the 2nd largest consumer of pesticides among the south Asian countries. The use of pesticides in Pakistan was started in 1952 with the introduction of an aerial spraying on crops such as, cotton, rice and sugarcane (Khan *et al.*, 2002) About 254 tonnes of pesticides were imported in 1954 (Feenstra *et al.*, 2000). During green revolution in early 1960's and late 1970's, large quantities of pesticides were imported from Europe for malaria, locust and pest control. During mid-1960s, the pesticides consumption reached over 7000 tons per annum and then increased to the level of 16,226 metric tons in 1976–77 (Anwar *et al.*, 2013). But later on, it has been reduced to 25000 mt tonnes in 2006 (SP-2007). Dichlorodiphenyltrichloroethane (DDTs) was also being produced locally in Nowshera KPK, Lahore, and Karachi but after promulgation of pesticide ordinance 1971 and 1973, its use and production was banned due to its mutagenic and carcinogenic effects and manufacturing units were demolished (Eqani *et al.*, 2012) (Ahad *et al.*, 2010). Between 1989 and 1993, twenty two pesticides including DDTs, Eldrin and Dieldrin were banned in Pakistan; later on hexachlorobenzene (HCB), endosulfan and heptachlor have also been banned (Jabbar *et al.*, 1993). In 1985, subsidies on pesticides were withdrawn and sales import and distribution of pesticides was transferred to private sector which grew from this business (Ahad *et al.*, 2010) and about five-fold increase in pesticide consumption was seen in just one year (Tariq *et al.*, 2004). Pakistan does not have any capacity of manufacturing pesticide after demolishment of pesticide factories. Unregistered and unregulated pesticides are being imported as generic i.e. by their chemical formulae; such pesticides are technical grade as they arrive. They are modified during formulation by the scores of formulators and importers, including some landlords, who have jumped in to take the advantage of the provisions in the import policy (Beg, 2005).

1.3 An overview of Endosulfan and Imidacloprid

1.3.1 Endosulfan

Endosulfan is an organochlorine insecticide that because of its wide production, usage, release and resistance to degradation is considered to be the most persistent of all halogenated hydrocarbons (Ritter *et al.*, 1995). It is applied on variety of fruits, vegetables and cereals and also used as wood preservative. It was developed in 1954 and since its development it is one of the widely used insecticides around the world (Miller, 1999). From 1950's to 2000, its worldwide usage is estimated about 338 kilotons of which, 113 kilotons was from India only, whereas its usage in USA was 26 kilotons (Li and Macdonald, 2005). The ADI for ESF in humans was about 0.006 µg/kg for a 60 kg adult drinking 2 liters of water regularly for period of two years (WHO, 2004). Its acceptable limit in feed is 1 mg/kg in EU (Thorstensen, 2014). Despite being banned in the year 2011 in developing nations, endosulfan is still being used in South Asian countries as it is cheap to produce and remain highly effective due to its broad-variety, therefore the developing countries cannot afford to ban these obsolete pesticides (Sankararamakrishnan *et al.*, 2005). Endosulfan has been categorized as moderately hazardous (WHO, 2004). Its residues remain in the environment for longer period and unveils its effects on surrounding areas and also affects many non-target organisms, particularly aquatic organisms like modulation of antioxidant systems in fish liver could be induced (Shao *et al.*, 2012). It enters the surroundings of agricultural areas either through atmospheric transport and deposition or agricultural runoff (Wan *et al.*, 2005). Endosulfan has also been detected in fish from remote lakes of U.S., Canada, and Europe where it has reached through atmospheric transport and deposition (Ackerman *et al.*, 2008). Generally, it is found to be very toxic to fish as it is reported that concentrations of 0.50 to 0.75 µg/L has negative impacts on fish species (Alamdar *et al.*, 2014) and causes chronic effects including genotoxic, reproductive, developmental and transgenerational effects (Watts, 2012). The lethal concentration value (LC_{50}) of fish for endosulfan is in the range between 1-100 µg/L (Hose *et al.*, 2003). Its half-life in water with anaerobic conditions varies between 35-187 days (ATSDR, 2000). According to (Stockholm Convention, 2004) a chemical is considered as being persistent in water if its half-life is

greater than two months; so endosulfan is also persistent under some conditions like as in more acidic conditions. For Zebrafish, endosulfan's 96-h LC_{50} value is reported 0.1µg/L (Tiwari and Ansari, 2014), 96-h LC_{50} values of endosulfan for Rainbow trout and Striped bass are 1.2µg/L and 0.31µg/L (Mayes and Barron, 1991).

1.3.2 Imidacloprid

Imidacloprid is a chloronicotinyl nitroguanidine insecticide. It is a neonicotinoid insecticide which impacts nervous system of insects either through contact or ingestion (Sjöqvist, 2014). It basically acts as a nicotinic acetylcholine receptor (nAChR) agonist and causes impairment of central nervous system (Ge *et al.*, 2015). It is the 2nd most widely used pesticide in the world (Ansoar-Rodríguez et al., 2015). US EPA has classified it as highly toxic to aquatic invertebrates US EPA, (2002). In 2010, about 20,000 tonnes of imidacloprid was produced in the world (CCM international, 2011). Various studies have reported that imidacloprid is stable to hydrolysis at environmentally relevant pH values but undertakes photolytic degradation rapidly (Ge *et al.*, 2015; (Tišler *et al.*, 2009). As it has been extensively used therefore its elevated concentrations are expected in aquatic environment (Tišler *et al.*, 2009). Majority of the water monitoring studies have reported detectable imidacloprid concentrations ranged from 0.001 to 320 µg/L (Morrissey *et al.*, 2015). It is also used on rice, maize, fruit, vegetables, sugar beet, cotton and turfs. (Hassanzadeh *et al.*, 2012).

In Pakistan, rice samples from rice growing areas of Punjab province contain high residues of imidacloprid that are above the maximum residual limit (MRL) (Niaz *et al.*, 2016). Freshly recommended maximum residual limit (MRL) by European Food Safety Authority (EFSA) for imidacloprid in rice is 1.5 µg/ g (Niaz *et al.*, 2016). Imidacloprid toxicity against fishes varies from species to species but in general it is slightly toxic to fish. Very less information regarding the toxicity of imidacloprid against fish is available. Acute toxicity of imidacloprid for fish is considerably high over 80ppm while juvenile fish is more sensitive US EPA (1992). Many histopathological alterations in kidney and liver of *Labeo rohita* exposed to 120 mg/L of imidacloprid were observed. Degenerative

changes were mostly observed hepatic necrosis, enlargement in lumen of renal tubules, necrosis and inflammatory cells proliferation were observed (Qadir and Iqbal, 2016). Imidacloprid had adverse effects on reproduction and hatchability of embryos of Common carp. Embryos viability was decreased as the concentration of imidacloprid increased in the experimental groups (Tyor and Harkrishan, 2016).

Due to its high toxicity to bees and its negative impact on apiculture businesses in many countries, it is becoming an emerging issue regarding research point of view. (Sanchez-Bayo and Goka, 2014). Many environmental studies carried out on this pesticide have indicated that imidacloprid can be detected in the soil and can be carried by storm, rain and runoff through which it can move into irrigation canals, streams, rivers, lakes and can leach into the ground water.

Imidacloprid affects the nervous system by influencing the post-synaptic nicotinic acetylcholine receptors. In nervous system, electrical impulse is generated when neurotransmitter binds with the receptors on the dendrites of neighboring nerve cells, imidacloprid mimics the shape of neurotransmitter and will act on various nicotinic acetylcholine receptors which causes nerves to fire and will lead to tremors, convulsion and death in insects (Krämer and Mencke, 2001).

1.4 Zebrafish: An appropriate toxicity Indicator

For monitoring aquatic toxicology, fish is considered as a suitable indicator because of its ability to accumulate toxins in its tissues directly from water or indirectly through its diet (Patel *et al.*, 2016). Zebrafish (*Denio rerio*) has appeared as a distinguished vertebrate model for studying genetics and development in past few decades because of its number of favorable characteristics like small body size, rapid development, optical transparency during early development and its genetic resemblance to humans (Lawrence, 2007). Zebrafish is a tropical fresh water fish that belongs to minnow family (cyprinidae) and its native to Himalayan regions (Lawrence, 2007). It is mainly found in south and west of peninsular India, city of Bangalore, and beyond India, Pakistan and Nepal, and in east Bangladesh and Myanmar (Engeszer *et al.*, 2007). Shallow, slow moving streams,

irrigation ditches, rice fields, man-made fish ponds, seasonal still pools that are formed along stream sides during the monsoons are its habitat (Lawrence, 2007). The water of these streams and pools is normally clear, but after rain it can get turbid. It occurs mainly in rice paddies, drainage ditches and stock ponds. It is reported in environmental conditions of temperatures between 12–39 °C, pH levels in range 5.9–9.8, and salinity between 0.01–0.8 (Parichy, 2015).

1.4.1 Location of Zebrafish in Pakistan

On the basis of freshwater fishes, Pakistan is categorized into five geographical provinces:

- Hindukush Karakoram Himalayan province
- Abasinh Kashmir Province
- Yaghistan Province
- Mehran Province
- Gedrosian Province

Zebrafish is located in Mehran province and 50% area of Kashmir and Pakistan is included in this area. Indus plain, adjoining hills and vale of Peshawar comes in this area. In southwest region of this area, climatic conditions vary from marine type to subtropical type with moderate temperatures having cold winters and hot summers in other parts of this province (Mirza, 1994)

1.5 Gentoxic and histopathological effects of pesticides on aquatic organisms

Several studies have been conducted previously on genotoxic and histopathological effects of pesticides on aquatic organisms. Effects of atrazine exposure on induction of oxidative stress and changes in gene expression of liver and ovary samples of Zebrafish were studied. Antioxidant enzyme activities (e.g. SOD and CAT) in addition to the glutathione (GSH) and malondialdehyde (MDA), in the liver altered considerably. mRNA levels for the genes encrypting like Cu/Zn–SOD, Mn–SOD, CAT, and GPx, were up-regulated expressively in the liver section when Zebrafish were exposed to different

levels (0.3, 3, 30 and 90 µg/L) (Jin *et al.*, 2010). Similarly, effects of Cypermethrin exposure on hepatic oxidative stress and DNA damage were also studied (Jin *et al.*, 2011) in which levels of hepatic mRNA were upregulated for the genes encrypting antioxidant proteins like Cu/Zn-SOD, Mn-SOD, CAT, and GPx. Tail moment and tail length observed in comet assay were also increased with ascending concentration levels; it was observed that high DNA mutilation was occurred even at small levels (Diekmann *et al.*, 2004). In a study by (Zodrow *et al.*, 2004) effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure on histopathology of Zebrafish demonstrated lipidosis, hepatocyte hypertrophy and a decrease hepatocyte nuclei number per field (Zodrow *et al.*, 2004).

Effects of endosulfan on antioxidant enzymes, reactive oxygen species and DNA damage in Zebrafish were studied by (Shao *et al.*, 2012) at different endosulfan concentrations (0.01, 0.1, 1, and 10 µg/L). In this study, Low endosulfan concentrations (0.01µg/L) caused a minor increase in activity of SOD and CAT, which retained the ROS level quiet stable. Increased endosulfan concentration (10 µg/L) caused undue ROS fabrication which surpassed the capability of the cellular antioxidants and exhausted the CAT and SOD enzyme (Shao *et al.*, 2012). In a study by (Blahová *et al.*, 2013), the concentration of oxidized lipids amplified in groups that were exposed to pesticide, atrazine at 30 and 90 µg/L as compared to control. Glutathione *S*-transferase activity declined in the group with the maximum concentration in comparison to the control group. A substantial decline was witnessed in activity of catalase in all experimental sets as compared to control group (Blahová *et al.*, 2013). In a study by Han *et al.* the toxic effects of strobilurin fungicide azoxystrobin on Zebrafish were examined in which reactive oxygen species were stored in excess in the livers of Zebrafish and superoxide dismutase activity was repressed expressively in male Zebrafish. An increase in glutathione-*S*-transferase activity was also witnessed after 21st day. DNA damage enlarged in a concentration-dependent manner and lipid peroxidation (LPO) occurred (Yadav *et al.*, 2016). Histopathological alterations were observed when Zebrafish was exposed to heavy metals and organochlorine pesticides for a period of 14 days. Damages such as dilation and cramming of blood sinusoids, cytoplasmic vacuolation and necrosis were observed in

liver tissues. Whereas pronounced hyperplasia and well defined necrosis was observed in gills (Bhuvaneshwari *et al.*, 2015). Similarly, in another study toxicity of various imidacloprid doses on Zebrafish after 7, 14, 21 and 28 days of exposure were assessed. High imidacloprid levels 1.25 mg/L and 5 mg/L increased ROS levels in the zebrafish liver on 21st day of exposure, MDA content was increased. The activities of SOD and GST amplified during early exposure but at the end of exposure duration they ultimately suppressed. whereas CAT levels decreased during initial exposure (Ge *et al.*, 2015).

Genotoxicity is mainly defined by measuring the alterations in DNA or mRNA, these alterations could be in the form of damage to genome or they can induce changes in genome by changing the genes. For assessing genotoxicity, the most promising technique developed in recent years to examine DNA damage in single cells is comet assay also known as single-cell gel electrophoresis (SCGE) assay is used in most of the studies (Ritter and Knebel, 2009). Pesticide induced ROS mutilation occurs when reactive oxygen species with a free oxygen interact through oxidation with nucleic acids, lipids, proteins due to which conformation change is induced in the structure and charge of that particular nucleic acid or protein that alters its properties disrupt its function or reading frames. DNA repair mechanism fixes the damaged DNA, but often this damage lead to mutagenesis and cancer (Thorstensen, 2014)

1.6 Comet Assay

Comet assay is the most suitable, fast, and consistent technique for the assessment of single or double DNA strand disruptions, it can also detect the labile sites and late repair sites in eukaryotic cells. Under highly alkaline conditions during electrophoresis comet like structures can be detected by fluorescence microscopy. Higher strength of comet tail represents the higher DNA damage (Collins, 2004). Its a simple and visual technique for the evaluation of DNA breakage in cells . The main working principle of comet assay is that the electrophoresis will elongate the super coiled duplex DNA containing strand breakage. Alkali labile sites (ALS) as a single strand breaks will appear and unwinding of duplex DNA takes place under highly alkaline conditions. Busted ends of DNA molecule

will appear in the form of comet and they will move towards anode in electrical field. Comet formation is based on the migration of DNA from head towards tail, as the damage increases tail becomes enlarged. Cells are fixed in agarose. Slides are immersed in a lysing solution for the formation of nucleoids that contains super coiled loops of DNA attached to nuclear matrix. Loops having breakage will be unable to find their supercoiling and they will freely move towards anode. More the migration of DNA towards tail higher will be the comet length and higher would be the DNA damage. Comet assay has a wide range of applications in genotoxic studies, biomonitoring and environmental monitoring to control the pollution (Collins, 2004)

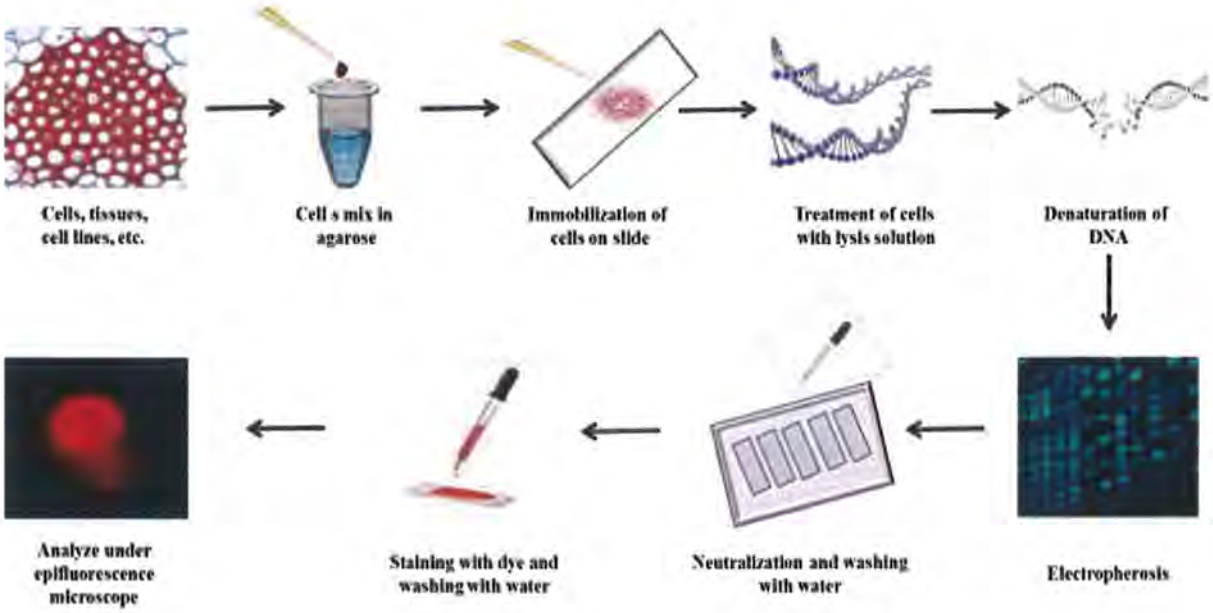


Figure 1.4: Schematic diagram of Comet Assay (Osman *et al.*, 2014).

Comet assay is famous because it is a simple, sensitive, low cost, time effective and an efficient method and it automatically scores the comets by image analysis software (Osman, 2014). Comet assay was used to detect the DNA mutilation in the mixing erythrocytes of Common carp to evaluate the genotoxic effect of disinfectants treated surface water ; Comet assay immediately detected the induced DNA damage in circulating erythrocytes (Buschini *et al.*, 2004)

1.7 Histology as Biomarker of Aquatic Pollution

Histological examination appears as a sensitive factor and is critical in describing alterations in cells of target organs, like gonads, liver and gills (Dutta, 1996). In histopathology, provides us with the knowledge about the functionality of different organs. Organ and Tissue damage can reduce fitness growth and survival of the individual and can lead to reduced reproductive success or increased vulnerability to pathological agents. Tissue lacerations, their frequency and intensity is influenced by the concentrations of insecticides and the exposure time to which fishes are exposed (Banaee, 2012)

Liver is the organ that is affected by the contaminants the most as it is concerned with detoxification and biotransformation (Camargo and Martinez, 2007). Gills are associated with respiration and play an important role in the detention and transmission of metal toward inner parts through blood transport. Gills are respiration sites and are involved in osmoregulation (Fernandez and Mazon, 2003). Because of large surface area they are susceptible to pollutants in water therefore, are considered as a appropriate water contamination indicator, also the examination of pesticide effect on fish has a indicative importance in appraising the effects of pesticides on human health condition. Histopathological biomarkers are assumed to be the important indicators of fish health and reflect the exposure effects of various anthropogenic contaminants (Hinton *et al.*, 1992). Various studies have reported histopathological alterations in different tissues of fish exposed to different chemicals including pesticides. But no such study has been conducted analyzing the genotoxic and histopathological effects of mixture of endosulfan and imidacloprid on Zebrafish. Hence, the current study intended to investigate the genotoxicity, histopathology and oxidative stress induced by individual and combined toxicity of endosulfan and imidacloprid in tissues of organs like muscle, liver and gill of Zebrafish but due to lack of resources we investigated only the combined toxicity of endosulfan and imidacloprid.

1.8 Problem Statement

Being a developing country with agro-based economy, the life line and development of Pakistan depends upon sustainable agricultural practices. In order to maximize its yield, the overuse of pesticides is a broadly accepted practice which in turn is resulting in excessive residues in environmental media and risking exposure to biotic components. Pesticides are assumed as major group of chemicals from agricultural runoff which pollute water bodies besides pesticides from industrial discharge. As in Pakistan, there is scarcity of water so it requires comprehensive management practices. Aquatic organisms, including fish, accumulate pollutants like pesticides and heavy metals from water directly or through food chain (Sasaki *et al.*, 1997). Zebrafish is native to rivers of Pakistan, India and Bangladesh (Moretz *et al.*, 2007) Population of Zebrafish in Pakistan is very rare now and the main reason of its decline is habitat loss, environmental degradation, pollution, drainage of wetlands and global warming. Zebrafish can be used as bioindicator to assess the pesticide pollution in aquatic environment as pesticides cause number of toxicological effects to fish species like histopathological alterations, skeletal deformities, reduced reproductive ability and genotoxic effects.

1.9 Objectives

Following are the objectives that have been focused in the current study:

- To evaluate the potential effects of mixture of Imidacloprid and Endosulfan on genotoxicity of Zebrafish liver by the application of Comet Assay
- To study the histopathological changes induced in, gill tissues and skeletal muscles of Zebrafish by the combined exposure of Imidacloprid and Endosulfan
- To determine the oxidative stress induced by imidacloprid and endosulfan in Zebrafish liver tissues

Material and Methods

2.1 Experimental Outline

In present study, 240 adult Zebrafish (*D.rerio*) of mixed sex were purchased from a commercial fish supplier (Fish Harbour) and transported to the Chemical Stress Ecology Laboratory in Department of Environmental Sciences, Quaid-i-Azam University, Islamabad. Prior to the experiment, fishes were acclimatized in laboratory conditions in fiber glass aquariums having dimensions of 30×30×30 cm (L×W×H) for two weeks. Tap water was used for the whole experiment. During acclimatization, water was changed on a daily basis. 30 individuals were placed in each aquarium and 10 individuals at each sampling interval from each treatment and from control group were dissected . In this experiment, three different concentrations of endosulfan and imidacloprid in following treatments, i.e. T1, T2 and T3 were administered to Zebrafish. After 7, 14 and 21 days of experiment fishes were sampled , biochemical analysis, estimation of DNA damage in liver tissues and histopathological alterations were examined in gills and muscles of Zebrafish in order to estimate the effects of pesticide on the given species. The fishes were fed twice a day with commercially available dry flakes (2% of their body weight). The feed was not given to fishes a day before sampling. Water quality parameters were checked daily which included temperature, pH and dissolved oxygen and ensured that they remained in the optimum range. The fishes were maintained under conditions as specified by Diekmann *et al.*, (2004a), with 12:12 h light regiment, 26 ±1 °C temperature, oxygen saturation above 70 %, and pH fluctuating from 7.4 to 8.1. The acclimatized healthy fishes both male and female were selected randomly for the experiment. Behavior and swimming pattern were observed regularly. Control groups showed normal behavior while abnormal swimming behavior was observed with increasing concentration of pesticides and exposure time in different treatment groups.

2.2 Preparation of Endosulfan and Imidacloprid solutions

Stock solutions were prepared from technical-grade endosulfan and imidacloprid. For endosulfan stock solution 25 mg endosulfan was dissolved in 50 ml of analytical grade

acetone. From stock solution, three dosing solutions of concentrations 10000 µg/L , 5000 µg/L and 1000 µg/L were made. From each dosing solution 1.5 ml was dissolved in 14998.5 ml of water to make three treatment levels of 0.5, 0.1, and 1µg/L and this last dilution contained only 0.05% acetone.

For Imidacloprid stock preparation, 100 mg of imidacloprid was dissolved in 1L of distilled water then 150ml of stock was added in each treatment (T1, T2, T3) level to maintain the concentration of 1ppm in three treatments. Pesticide concentrations were conserved by changing the water after two days.

2.3 Experimental design

Healthy and uniform sized fishes (132 males and 103 females) with an average weight of 0.38g and average length of 4.5cm were selected and evenly distributed into 4 glass aquaria (30 × 30 × 30 cm) (L×W×H). The experiment was conducted in duplicate. 1 glass aquarium was assigned as F1 (control group) while other three aquaria were assigned as three treatment groups T1, T2 and T3. All aquariums were fitted with air stones and heaters for maintaining temperature and dissolved oxygen. Fish were acclimatized for two weeks prior to the experiment. Water quality parameters (pH, temperature, dissolved oxygen and ammonia) were regularly observed and maintained within a fixed range. After two weeks of acclimatization chronic exposure of pesticides was given to Zebrafish. Control group was exposed to acetone only. Treatment 1 (T1) was exposed to 0.1 µg/L of endosulfan, Treatment 2 (T2) was exposed to 0.5 µg/L and Treatment 3 (T3) was exposed to 1 µg/L of endosulfan while the imidacloprid concentration was maintained 1mg/L in all these three treatments. Experiment was conducted for 21 days and behavior of fish was monitored regularly. Water of aquariums was changed after every two days and pesticide concentration level was maintained by dosing after two days. Fish were sampled at day 7, 14 and 21. Ten fishes at each sampling were sacrificed from each treatment group and control group. Before dissection, the fishes were anesthetized on ice. Liver tissues were obtained by dissecting the fish on ice box. Tissue samples were kept on ice during preparation and later kept at -80°C until further analysis.

2.4 Homogenate preparation

Liver tissues were homogenized in 50 mM ice-cold potassium phosphate buffer pH 7.0 in a Teflon tissue homogenizer. Centrifugation of homogenate was done at 10,000 rpm at 4° C for 10 minutes. For analyzing enzyme activity and protein determination, supernatant was instantly used.

2.5 Biochemical Study

Biochemical study involves protein estimation, Thiobarbituric acid reactive substrates (TBARs) and activities of antioxidant enzymes SOD and CAT in Zebrafish liver tissues.

2.6 Protein Assessment

Protein contents of liver tissues were estimated by Sigma Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as a standard. Stock solution was prepared by mixing 25 ml methanol, 0.05 g comassive, 50 ml H_3PO_4 and 100ml distilled water. Stock was kept in dark at 4°C. Stock solution was mixed with distilled water in ratio of 1:4 after cooling to prepare working solution, then 2900µL of working solution and 100µL of protein extract was mixed and mixture was incubated for 10 minutes at room temperature. Absorbance of the reaction mixture was measured at 595 nm using UV-spectrophotometer (A-25 auto analyzer, Biosystems). For standard curve generation different dilutions (0.2M, 0.6M, 1M, 1.2M) of BSA were made. Finally the level of protein was measured using bovine serum albumin curve.

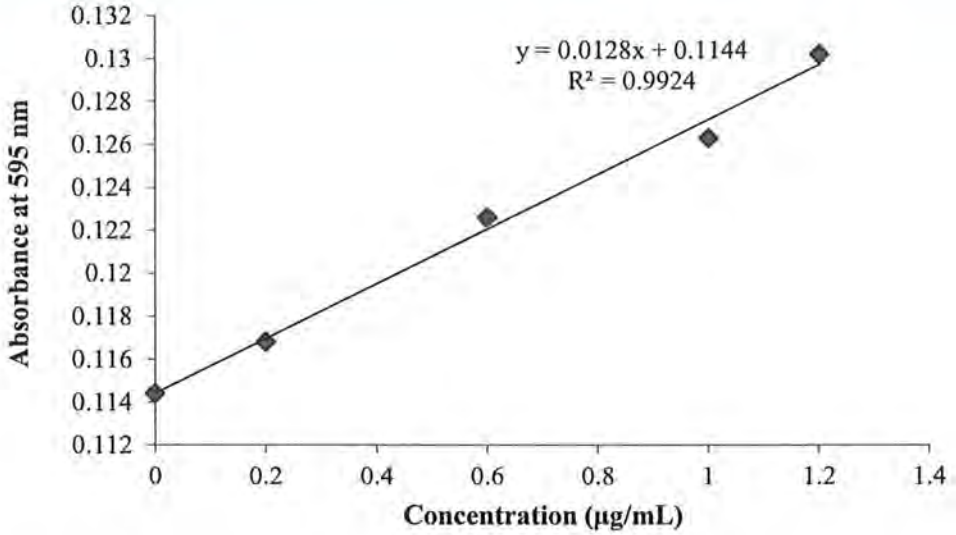


Figure 2.5 : BSA curve for estimation of total protein in tissue sample

2.7 Superoxide dismutase (SOD) Activity

For estimation of SOD activity, protocol of (Shao *et al.* 2012) was followed. Reaction mixture of 3ml was prepared that contained 50 mM pH 7.8 phosphate buffer, 100 µM ethylenediaminetetraacetic acid (EDTA), 130 mM methionine, 750 µM nitrobluetetrazolium chloride (NBT), 20 µM riboflavin, and 50 µL enzyme supernatant was knocked up and irradiated with a 4000 lx fluorescent lamp for 20 min. The absorbance of the reaction mixture was measured using UV-visible spectrophotometer at 560 nm wavelength. One unit of SOD activity (U) was termed as the enzyme quantity necessary to cause inhibition of 50 % of nitrobluetetrazolium chloride photoreduction rate. The results were expressed as U/mg of protein.

2.8 Catalase (CAT) Activity

CAT was measured according to the method described by (Claiborne, 1985).. Mixture for assay was prepared by adding 0.5 ml of 5.9 mM H_2O_2, 1 ml of 50 mM potassium phosphate buffer and 0.1ml of sample (tissue homogenate). Mixture was incubated at 37°C for 15 min and absorbance was noted at 240nm. Catalase activity was expressed in Umol/min/mg protein unit.

2.9 Measurement of Malondialdehyde MDA content

MDA contents were determined using methods used by Zhang *et al.* (2003). Thiobarbituric acid (TBA) assay was used. 200 µL supernatant was added in reaction mixture that contained 0.2 mL of 8.1% sodium dodecyl sulfate (SDS), 1.5 mL of 20% acetic acid (pH 3.5), 1% thiobarbituric acid (TBA) and 1 mL of water, resulting solution was retained in water bath for 60 minutes at 90°C. Mixture was then cooled and centrifugation was done at 3000 rpm for 15 min, absorbance of the supernatant was read at 532 nm. The malondialdehyde content was observed as the content of thiobarbituric acid-reactive substances nanomoles per milligram of protein. In each assay mixture without tissue homogenate sample was considered as blank.

2.10 Comet Assay

Single-cell gel electrophoresis (SCGE) was used to assess the DNA damage. Comet assay was piloted to investigate the DNA strand breaks in Zebrafish livers. Cell suspensions were prepared using the method described by Ge *et al.* (2015).

2.10.1 Reagents Preparations

Following reagents were prepared for carrying out comet assay:

a. **Low melting agarose (LMA):** By dissolving 250mg of low melting agarose (LMA) in 50ml of 1% PBS, 0.5% LMA was prepared. LMA was then placed in refrigerator, but before use its temperature was raised to 37°C using water bath

b. **Normal Melting Agarose:** 1% normal melting agarose (NMA) was prepared by dissolving 500 mg of NMA in 50 mL of distilled water and heating it.

c. **Lysing solution:** Lysis solution consisted of 1.46 g of NaOH (2.5M), 37.2 g of EDTA (100mM) and 1.2 g of Trizma base (100mM). Distilled water was added to raise volume upto 890 ml. pH was maintained at 10.0 by addition of HCl or NaOH and was kept at room temperature. Final lysing solution was prepared by mixing 10% dimethyl sulfoxide (DMSO) and 1% Triton X with previously prepared solution. Prior to slide preparation, this solution was kept in refrigerator for 30 minutes,

d. **Electrophoresis buffer:** It was prepared by mixing 0.5 ml of EDTA (200mM) and 30 ml of NaOH; distilled water was added in this mixture and volume was raised upto 1000 ml. pH was maintained at level >13.

e. **Neutralization buffer:** 1000 ml distilled water and 48.5g of 0.4 M tris were mixed and pH was adjusted at 7.5 by concentrated HCl and buffer was left at room temperature. Buffer was formed by mixing 48.5 g tris (0.4M) in 1000 ml dH_2O, pH was then adjusted at 7.5 by using concentrated HCl and was kept at room temperature.

f. **Staining Solution:** It was composed of 10X ethidium bromide (stock-30 µg/mL). It was prepared by the addition of 50 ml dH_2O in 10 mg of ethidium bromide.

g. **PBS buffer:** (Mg^{2+}, Ca^{2+}): PBS (Phosphate buffer saline) buffer was prepared in 990mL of distilled water and volume was raised up to 1000 mL. pH was adjusted at 7.4 while keeping the solution at room temperature.

2.10.2 Slides preparation

For sterilization purpose, slides were dipped in methanol and then exposed to flame. Mixture of LMA (200 µL) and cell suspension was pipetted onto frosted slides containing layer of 100 µL normal melting agar. After solidification of layer, another layer of low melting agar (85 µL) was added on the slide. When layer of LMA solidified then slides were submerged in lysis solution. While being immersed in solution, slides were refrigerated for 2 hours and were kept in dark conditions.

2.10.3 Electrophoresis

After two hours of refrigeration, slides were taken out from lysis solution and then retained in horizontal gel apparatus. Buffer was poured in gel tank in order to soak slides in freshly prepared buffer. Power of 24 volts was supplied for 30 minutes and slides were left for unwinding of DNA. Afterwards slides were taken out and treated with neutralizing buffer. Similar steps were repeated twice. 80 µL of 1X ethidium bromide was utilized for staining and slides were covered with cover slips.

2.10.4 Slides visualization

For detection of DNA damage, fluorescent microscope (Nikon) (40X) was used. CASP 1.2.3.b software was utilized for estimation of extent of DNA damage. Migration of DNA from head to tail was observed and 50-100 cells were under observation in each sample. Comparison between amount of migration per cell, number of cells with increased migration, viability and ability of migration between damaged cells was made.

2.11 Tissue Histology

Histology of gill tissues and skeletal muscles of Zebrafish was carried out in order to evaluate the potential effects of imidacloprid and endosulfan induced in the above mentioned tissues.

a. **Fixation:** Muscles and gills of Zebrafish were secured in 4% paraformaldehyde for 24 h at 4 °C which was prepared using 10 mM phosphate-buffered saline (PBS). Tissues were then rinsed twicely with 10 mM PBS.
b. **Dehydration:** After fixation tissues were further rinsed and processed in an ascending sequence of alcohol (70%, 80%, 90%, 95%, and 100%) and were then cleared in xylene.
c. **Embedding:** Afterwards tissues were transferred in melted paraffin wax within a boat, bubbles were removed from the wax and then wax was allowed to solidify. Paraffin embedded tissues were mounted on wooden blocks with the help of melted wax. Thin sections of 7µm were cut using microtome (Griffitt *et al.*, 2008).
d. **Slide preparation:** Tissue ribbons were stretched by fixation on previously prepared clean albumenized glass slides, were placed on (Fisher) slide warmer at 60°C. For complete stretching slides were placed over night in an incubator.
e. **Staining and microscopy:** Slides were hydrated using a sequence of reducing ethanol and slides were then run through a series of histological stains haematoxylin and eosin. Afterwards slides were examined under light microscope at 10X and 40X.

2.12 Statistical analysis

For estimating the differences between three treatments, i.e. T1, T2 and T3, different statistical analysis tools were employed. As the experiment was done in duplicate, so in order to get representative value of each treatment, arithmetic mean and standard deviation of each group was estimated. Means were graphically represented in the form of bar graphs and standard deviation was shown as error bars to demonstrate range of data. All quantitative data was subjected to analysis of variance (ANOVA) to estimate significance of difference among different treatments. For statistical analysis, Microsoft Excel and SPSS software were used.

RESULTS

In the present study, antioxidant enzyme activities superoxide dismutase (SOD), catalase (CAT), lipid peroxidation product malondialdehyde (MDA) and DNA damage by comet assay was measured in Zebrafish liver tissues while histopathological alterations induced by the combined exposure of imidacloprid and endosulfan were examined. The results and interpretation of the results are described in this section.

3.1 SOD Activities

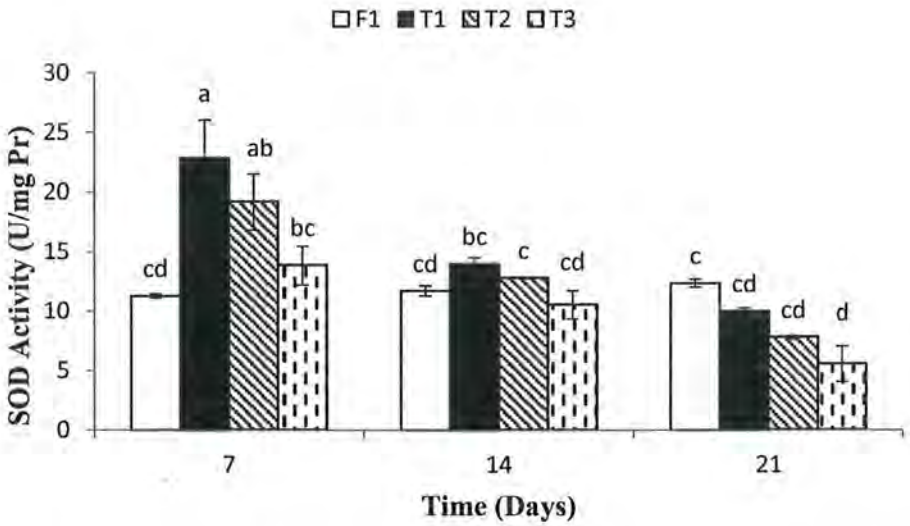


Figure 3.1: SOD concentrations in liver tissues of Zebrafish: F1 represents control group without pesticide, T1 represents treatment 1 having the lowest concentration of endosulfan (0.5µg/L) and 1ppm imidacloprid, T2 represents treatment 2 having endosulfan concentration (0.1µg/L and 1ppm imidacloprid, T3 represents treatment 3 having highest concentration of endosulfan (1µg/L) and 1ppm imidacloprid. Bars denoted with different alphabets were significantly different at $p < 0.05$ following least significance difference test.

SOD activity was observed to be the highest after 7 days of experiment for all three treaments as shown in Fig.3, by increase in exposure time, SOD activity for all treatments declined, indicating negative effect of pesticide pollution on SOD activity of Zebrafish

livers. Fishes in control (without pesticide exposure) demonstrated constant SOD activity of 12 U/mg Pr (protein) exhibiting consistent enzyme activity without toxicant. Comparison among treatments indicated reduction in enzyme activity with increase in pesticide concentration; the ascending trend of SOD activity is as follows i.e. T3<T2<T1 for all three days of sampling as shown in Fig.3. On 7^{th} day, SOD activity of all three treatments was higher than control; with temporal increase in exposure. On 14^{th} day enzyme activity of all three treatments did not show any significant difference as compared to control. Similar results were observed on the 21^{st} day of sampling for treatment T1 and T2, while T3 activity decreased as compared to control.

Significant increase was observed in SOD activity on the first week of exposure in liver tissues of Zebrafish as compared to control. In comparison to control, substantial increase observed in SOD activity in treatments T1, T2 and T3 was of 98.6, 70.2 and 22.6% on day 7, while T2 and T3 had less SOD activity as compared to T1 on day 7 but this difference was not found to be significant , however SOD activity on day 14 was greater in T1 and T2 by 19.5 and 9.3% respectively, as compared to control but there was no significant difference among all three treatments in comparison to control . On day 21, SOD activity was inhibited in all three treatments as compared to control but no significant decrease was observed in T1 and T2 as compared to control; whereas T3 activity showed significant decline of 55.1% with respect to F1.

3.2 CAT Activities

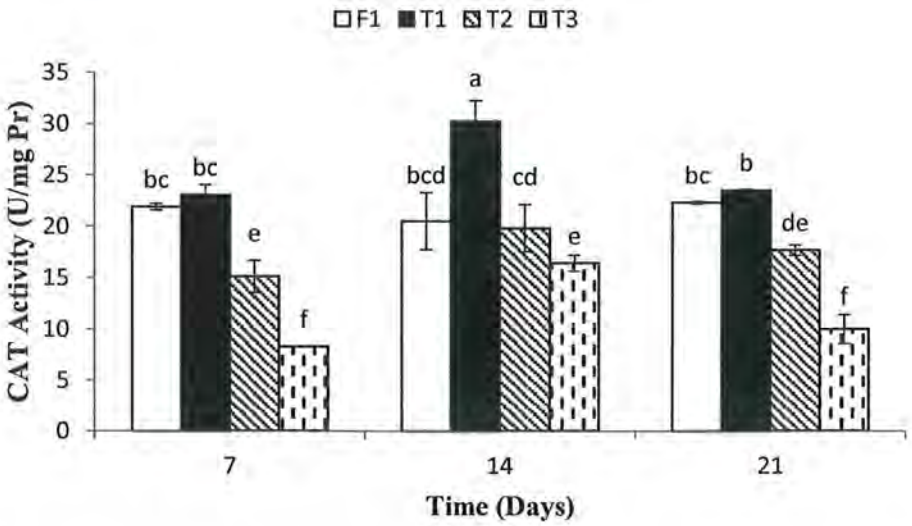


Figure 3.2: Catalase activity in liver tissues of Zebrafish: F1 represents control group without pesticide, T1 represents treatment 1 having lowest concentration of endosulfan (0.5µg/L) and 1ppm imidacloprid, T2 represents treatment 2 having endosulfan concentration (0.1µg/L and 1ppm imidacloprid, T3) represents treatment 3 having highest concentration of endosulfan (1µg/L) and 1ppm imidacloprid. Bars denoted with different alphabets were significantly different at $p < 0.05$ according to least significance difference test.

Highest catalase activity in liver of zebrafish was observed on day 14 of sampling, and decline in catalase activity was observed after day 21 of sampling. Control treatments showed enzyme activity in range of 20.5-22.3U/mg Pr (protein), indicating the normal level of catalase in Zebrafish in given environmental conditions. In comparison to control, catalase activity of T1 was similar to control level on day 7 of sampling, while it increased on day 14 in treatment 1 and decreased again to the level of control on day 21. But for T2 and T3, significant decrease in catalase activity was observed as compared to control on day 7 , similarly on day 14 catalase activity decreased in T2 and T3 but decrease was not significant, after 21 days of exposure level of catalase activity decreased again and (T3) showed significant decrease as compared to control shown in fig. 3.2. This shows that as the fish was exposed to pesticides the antioxidant enzyme catalase activity increased after early exposures to reduce the oxidative stress, but as the pesticide

concentration and exposure duration increased (CAT) activity decreased because it fails to eliminate the excessive reactive oxygen species (ROS).

There is no significant difference in CAT activity in T1 on day 7, just a slight increase of 5.3% as compared to control was observed. While in T2 and T3 as the concentration of endosulfan is increased from 0.5µg/L to 0.1µg/L and 1µg/L with the constant level of imidacloprid (1ppm), CAT activity significantly decreased by 30.9 and 62.1% in comparison to control on day 7 of sampling. Induction of CAT activity was observed on day 14 in T1 having lowest concentration of endosulfan 0.5µg/L and consequently having catalase activity greater than other treatments; it is 47.4% greater than those observed in control. Whereas, T2 showed no difference from control and T3 showed 19.9% decrease as compared to control. Whereas activity of CAT on day 21, significantly decreased in T1 and T3 as compared to the treatments in 14th day of sampling. On day 21, T1 and control F1 showed similar catalase activity, whereas, in comparison to F1, T2 and T3 showed significant difference of 22.7 and 55.3% respectively, as shown in Fig. 4.

3.3 MDA Content

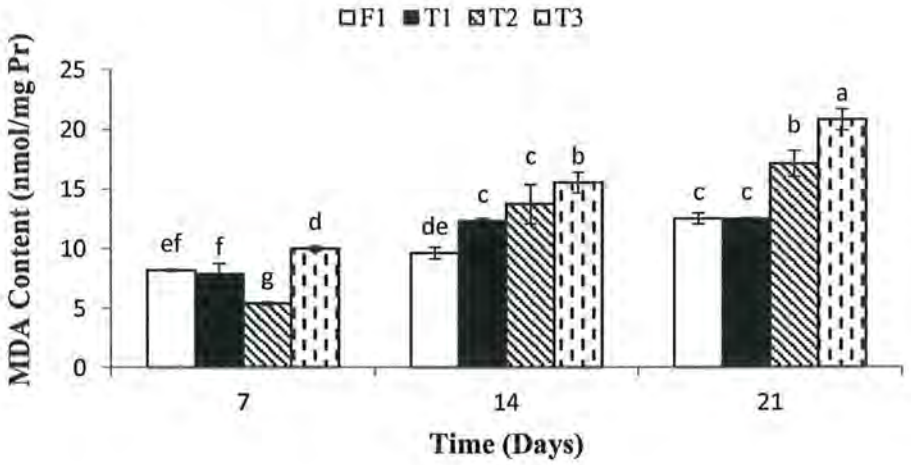


Figure 3.3: Malondialdehyde (MDA) content in liver tissues of Zebrafish: F1 represents control group without pesticide, T1 represents treatment 1 having lowest concentration of endosulfan (0.5µg/L) and 1ppm imidacloprid, T2 represents treatment 2 having endosulfan concentration (0.1µg/L and 1ppm imidacloprid, T3 represents treatment 3 having highest concentration of endosulfan (1µg/L) and 1ppm imidacloprid. Bars denoted with different alphabets were significantly different at $p < 0.05$ according to least significance difference test.

MDA content showed the opposite trend to catalase and superoxide dismutase activity; malondialdehyde (MDA) content showed time-dose dependent relationship and it was highest in treatments with maximum concentrations (1μg/L endosulfan and 1ppm imidacloprid) of pesticides. Higher (MDA) content was observed in treatment 3 (having higher concentration of pesticides) at all sampling intervals(Day 7, 14 and 21). The variation in MDA content followed this trend, i.e. T2<T1<T3 on day 7, while day 14 and 21 showed increase in MDA content with increase in pesticide concentration therefore, T1<T2<T3 this trend was followed in all three treatments.

The time dependent increase was observed in the MDA content of Zebrafish liver tissues, MDA content increased on highest T3 and middle T2 concentrations on day 21. At the start of experiment, i.e day 7 of sampling , highest MDA content was observed in T3, it was 22.1% higher as compared to F1, while T1 showed no significant difference in comparison to control and T2 was 34.1% lower than control, as shown in Fig. 5. On day 7, significant increase was observed in T3 while T2 showed a significant decrease in MDA content as compared to control. On day 14, MDA level increased in T1, T2 and T3 by 55.9, 154.3 and 55.2% as compared to similar treatments on day 7 of sampling. T3 showed highest MDA content on day 14 and it was significantly different from T1, T2 and from control. A significant increase was observed in treatment 3 on day 21, T2 also showed a significant increase in MDA as compared to control on day 21. T2 and T3 showed significant increase of 24.5 and 33.8% respectively as compared to these treatments on day 14. On day 21, control and T1 showed no significant difference, while, T2 and T3 showed 36.8 and 66.2% increase in MDA content as compared to control shown in Fig. 5.

Table 3.1 : DNA damage induced by imidacloprid and endosulfan in Zebrafish livers

Treatment		Comet Length	Head Length	Tail Length	% DNA in Head	% DNA in Tail	Tail Moment	Olive Tail Moment
Day 7	F1	47±12.8^{a}	44±12.7^{a}	3±0.001^{b}	99.99±0.005^{a}	0.004±0.005^{c}	0.0001±0.0001^{b}	0.0009±0.0009^{d}
	T1	54±31.1^{a}	51±31.1^{a}	3±0.004^{b}	99.29±0.9^{a}	0.701±0.9^{c}	0.0212±0.03^{b}	0.11±0.13^{d}
	T2	63.5±47.4^{a}	57±42.4^{a}	6.5±4.9^{ab}	97.79±1.5^{ab}	2.209±1.5^{bc}	0.106±0.009^{b}	0.399±0.08^{cd}
	T3	66.5±40.3^{a}	59±39.6^{a}	7.5±0.7^{ab}	94.5±3.82^{abc}	5.498±3.82^{abc}	0.399±0.25^{b}	1.09±0.014^{bc}
Day 14	F1	38±5.7^{a}	35±5.7^{a}	3±0.02^{b}	99.9±0.002^{a}	0.007±0.002^{c}	0.0002±0.0006^{b}	0.002±0.0002^{d}
	T1	63±21.2^{a}	60±21.2^{a}	3±0.006^{b}	99.7±0.27^{a}	0.344±0.27^{c}	0.0103±0.008^{b}	0.12±0.11^{d}
	T2	83±7.07^{a}	75±11.3^{a}	8±4.24^{ab}	97.4±0.06^{ab}	2.62±0.06^{bc}	0.211±0.12^{b}	0.92±0.09^{bc}
	T3	105±66.5^{a}	96±57.9^{a}	9±8.5^{ab}	93.1±4.96^{bc}	6.93±4.96^{ab}	0.413±0.14^{b}	1.39±0.17^{b}
Day 21	F1	51±4.24^{a}	48±4.2^{a}	3±0.003^{b}	99.9±0.006^{a}	0.012±0.006^{c}	0.0004±0.0002^{b}	0.003±0.002^{d}
	T1	49.5±36.1^{a}	45±33.9^{a}	4.5±2.12^{b}	94.8±0.97^{abc}	5.185±0.97^{abc}	0.223±0.067^{b}	0.74±0.35^{bcd}
	T2	59.5±17.7^{a}	51±16.9^{a}	8.5±0.7^{ab}	94.5±2.42^{abc}	5.468±2.42^{abc}	0.46±0.16^{b}	1.33±0.244^{b}
	T3	83.5±2.1^{a}	70±7.07^{a}	13.5±4.95^{a}	90.1±5.14^{c}	9.859±5.14^{a}	1.46±1.18^{a}	2.68±0.94^{a}

3.4 DNA Damage and Olive Tail Movements observed via Comet Assay

Comet assay was used to detect the genotoxicity induced by imidacloprid and endosulfan in liver tissues of Zebrafish. Comet length gradually increased in all three treatments (T1, T2 and T3) at all sampling intervals as compared to control. Similarly tail length also increased in all treatments as compared to control at all sampling intervals. As the concentration of pesticides in the treatment groups increased percent DNA in head was decreased because of DNA damage and the migration of damaged DNA towards anode. This migration of damaged DNA is the cause of tail formation. Control groups of all three sampling intervals showed the same value of percent DNA in head which is 99.9% which shows intact DNA. Percent DNA in tail increased as the pesticide concentration increased besides pesticide concentration time period of pesticide exposure also plays a significant role in rise of DNA damage. Percent DNA in the tail was highest in treatment three (T3) after the exposure of 21 days of pesticide as compared to day 14 and day 7. Percent DNA in head followed this trend T1>T2>T3 while percent DNA in tail followed this trend T1<T2<T3 in all treatments at all sampling intervals.

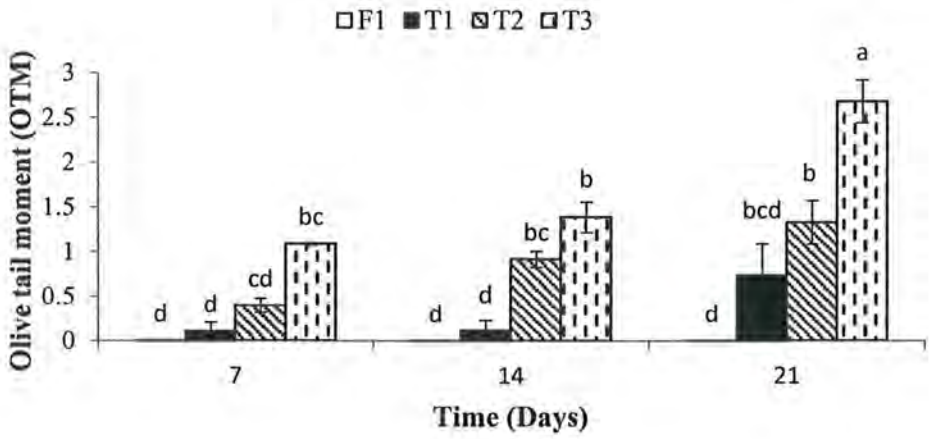


Figure 3.4: Olive tail moment in liver tissues of Zebrafish: F1 represents control group without pesticide, T1 represents treatment 1 having lowest concentration of endosulfan (0.5µg/L) and 1ppm imidacloprid, T2 represents treatment 2 having endosulfan concentration (0.1µg/L and 1ppm imidacloprid, T3 represents treatment 3 having highest concentration of endosulfan (1µg/L) and 1ppm imidacloprid. Bars denoted with different alphabets were significantly different at $p < 0.05$ according to least significance difference test.

Olive tail moment increased in all treatments at all sampling intervals as compared to control. Highest olive tail moment was observed in T3 after 21 days of exposure. Increased concentration of endosulfan and exposure time played a significant role in enhanced olive tail moment. The variation of olive tail moment in Zebrafish liver was in order T1<T2<T3. Olive tail moment increased on highest concentrations T3 at all sampling intervals. After 7,14 and 21 days of exposure there was no significant difference observed between control groups and treatment 1, similarly no significant difference was observed between T2 and T3 after 7 and 14 days of exposure but a significant increase of olive tail moment was observed in T3 after the exposure of 21 days of pesticides so the prolonged exposure of pesticides can induce DNA damage in aquatic species.

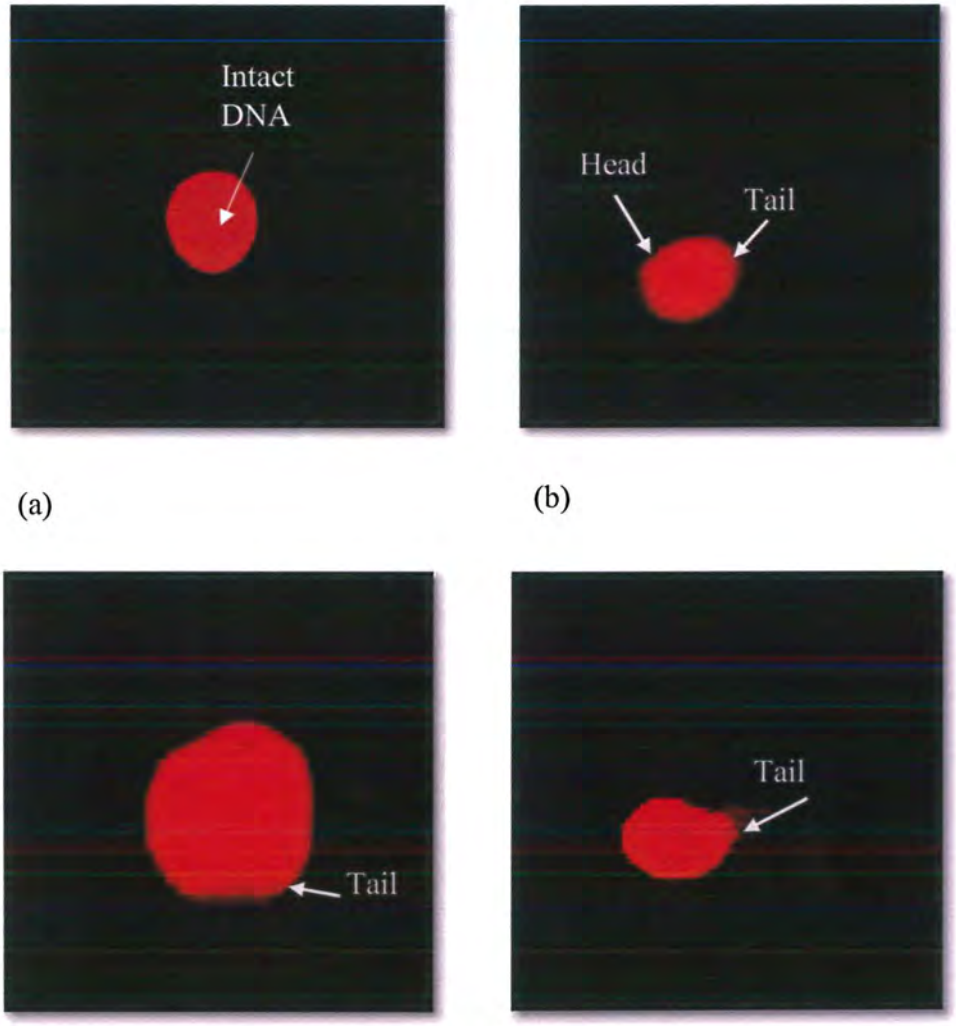


Figure 3.5: Fluorescence photomicrographs of pesticides induced DNA damage in liver tissues of Zebrafish after 7 days of exposure (a) control group, (b) treatment 1 (0.5µg/L endosulfan and 1ppm imidacloprid), (c) treatment 2 (0.1µg/L endosulfan and 1ppm imidacloprid), (d) treatment 3 (1µg/L endosulfan and 1ppm imidacloprid).

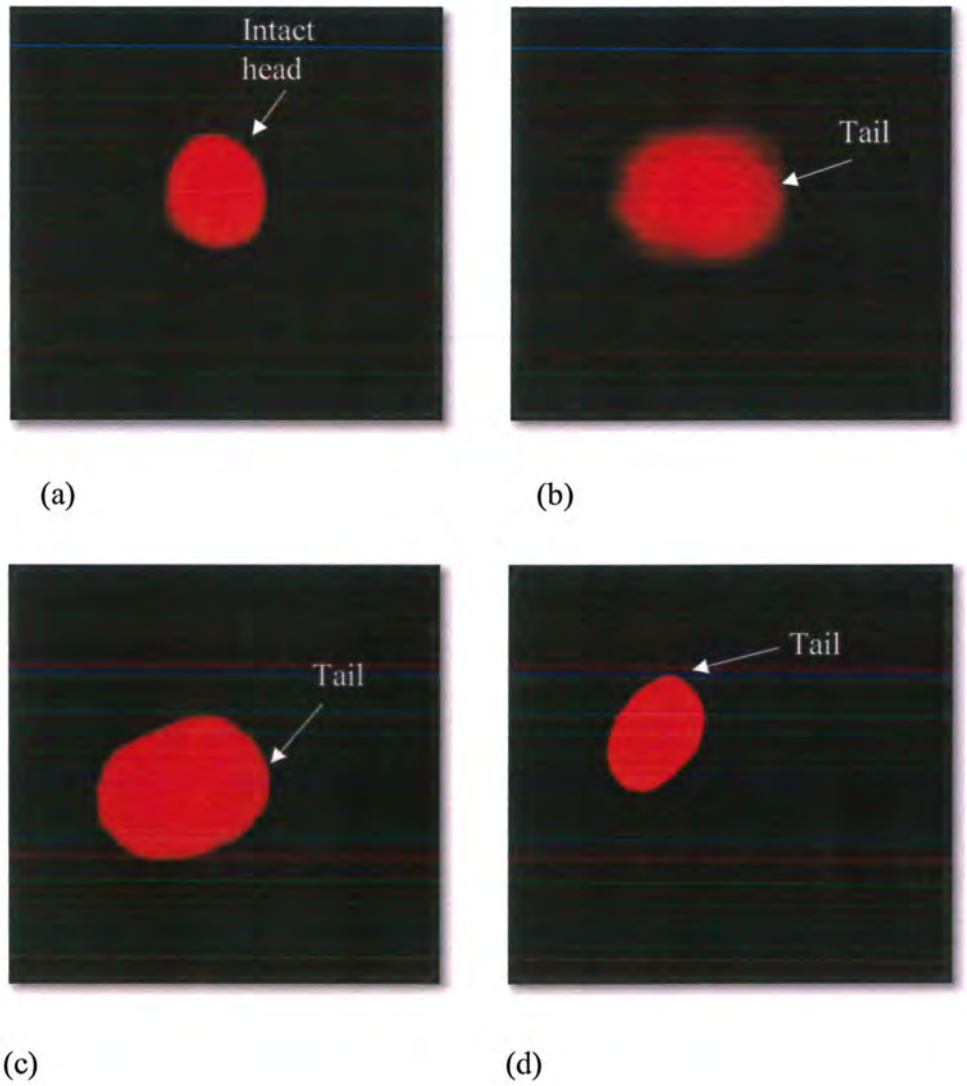


Figure 3.6 : Fluorescence photomicrographs of pesticides induced DNA damage in liver tissues of Zebrafish after 14 days of exposure. (a) control group, (b) treatment 1 (0.5µg/L endosulfan and 1ppm imidacloprid), (c) treatment 2 (0.1µg/L endosulfan and 1ppm imidacloprid), (d) treatment 3 (1µg/L endosulfan and 1ppm imidacloprid).

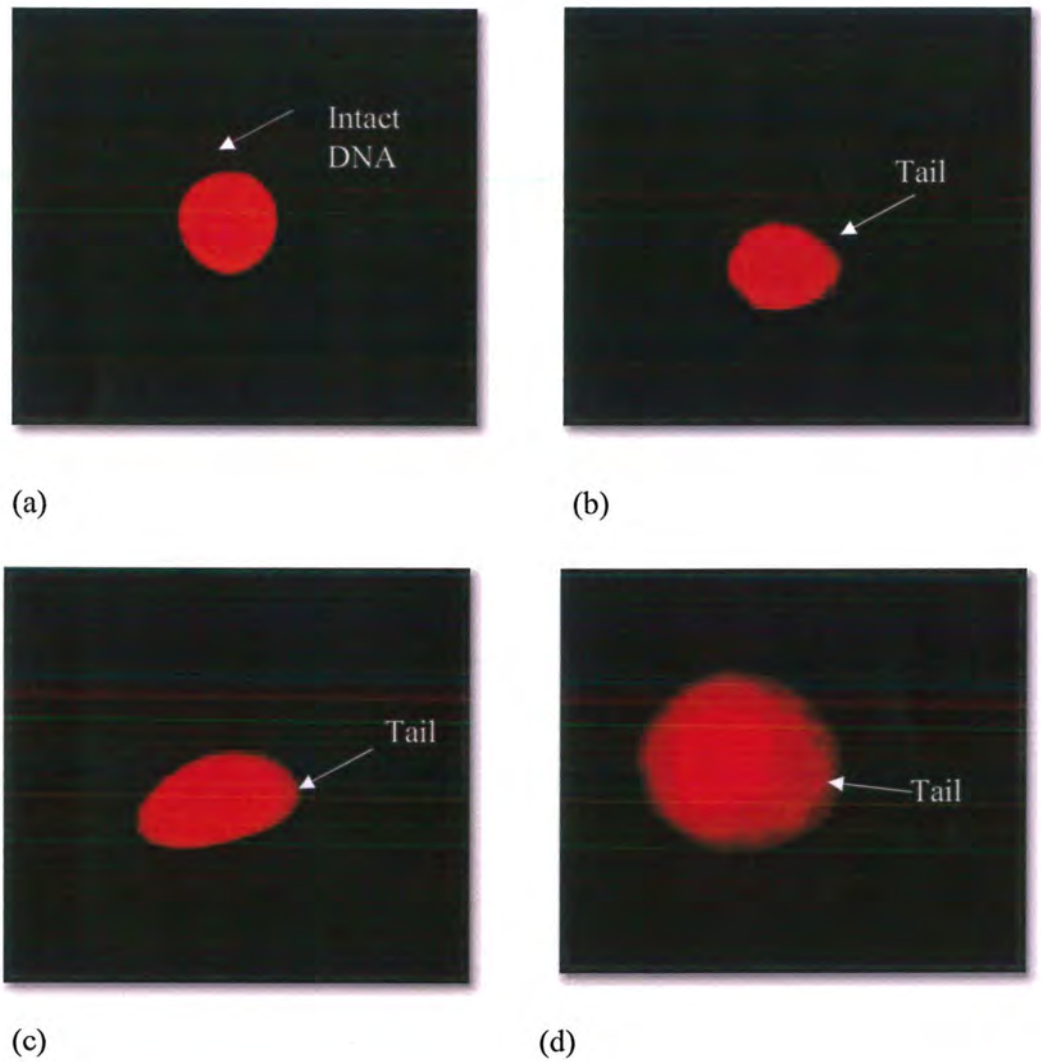


Figure 3.7: Fluorescence photomicrographs of pesticides induced DNA damage in liver tissues of Zebrafish after 21 days of exposure. (a) control group , (b) treatment 1 (0.5µg/L endosulfan and 1ppm imidacloprid), (c) treatment 2 (0.1µg/L endosulfan and 1ppm imidacloprid), (d) treatment 3 (1µg/L endosulfan and 1ppm imidacloprid).

3.5 Histological Changes in Gills and Muscles

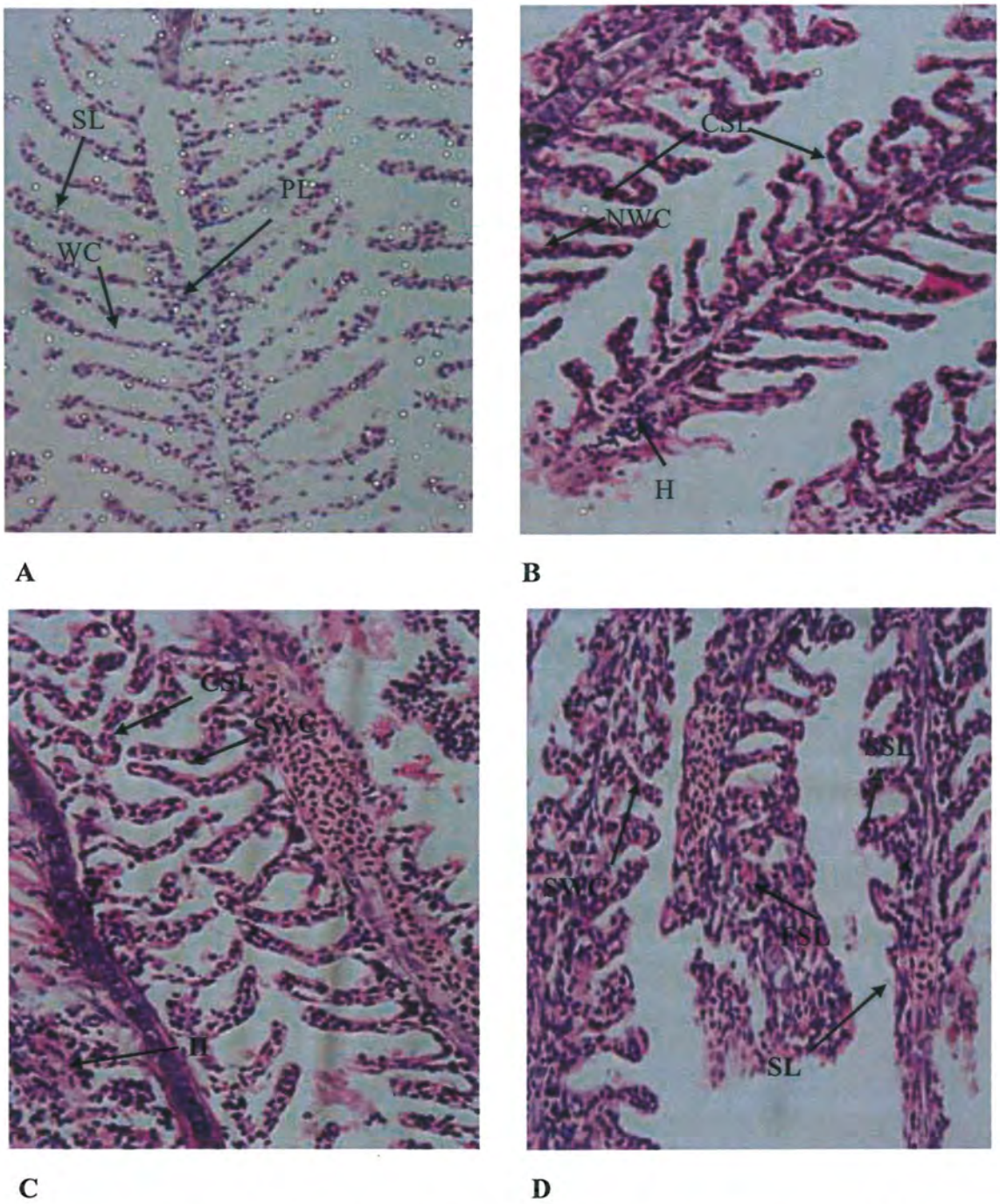
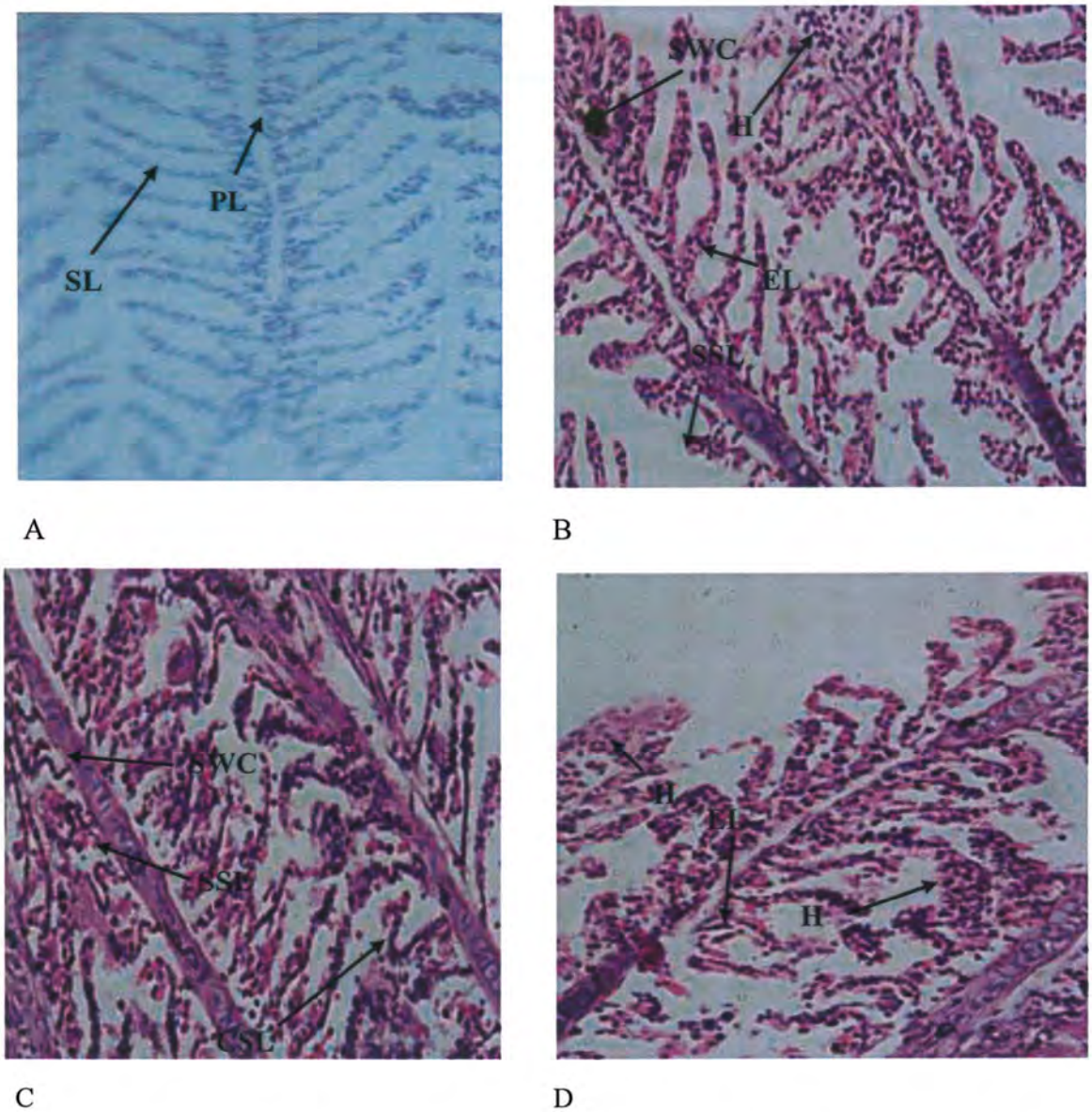


Figure 3.8: Photomicrographs of gill sections of Zebrafish H and E stained, 40X magnification after 7 days of exposure. (A) control group (SL) Secondary lamellae, (PL) Primary lamellae, (WC) Water channel; (B) Group having lowest concentration of pesticide (0.5µg/L endosulfan, 1ppm imidacloprid) (CSL) Curling of secondary lamellae, (NWC)

Narrowed water channels, (H) Hyperplasia. (C) Group having medium concentration of pesticides (0.1µg/l endosulfan , 1ppm imidacloprid) (H) Hyperplasia , (SWC) Shortening of water channels, (CSL) Curling of secondary lamellae (D) Group of fishes having highest concentration of pesticide (1µg/L endosulfan, 1ppm imidacloprid), (SL) Sloughing of secondary lamellae , (SSL) Shortenting of secondary lamellae, (SWC) Shortening of water channels , (FSL) Fusion of secondary lamellae.



A B

C D

Figure 3.9: Photomicrographs of gill sections of Zebrafish H and E stained, 40X magnification after 14 days of exposure. (A) control group, (B) treatment 1, (C) treatment 2, (D) treatment 3. H represents hyperplasia, CSL represents curling of secondary lamellae, SSL stands for shortening of secondary lamellae, EL represents epithelial lifting and SWC represents shortening of water channels.

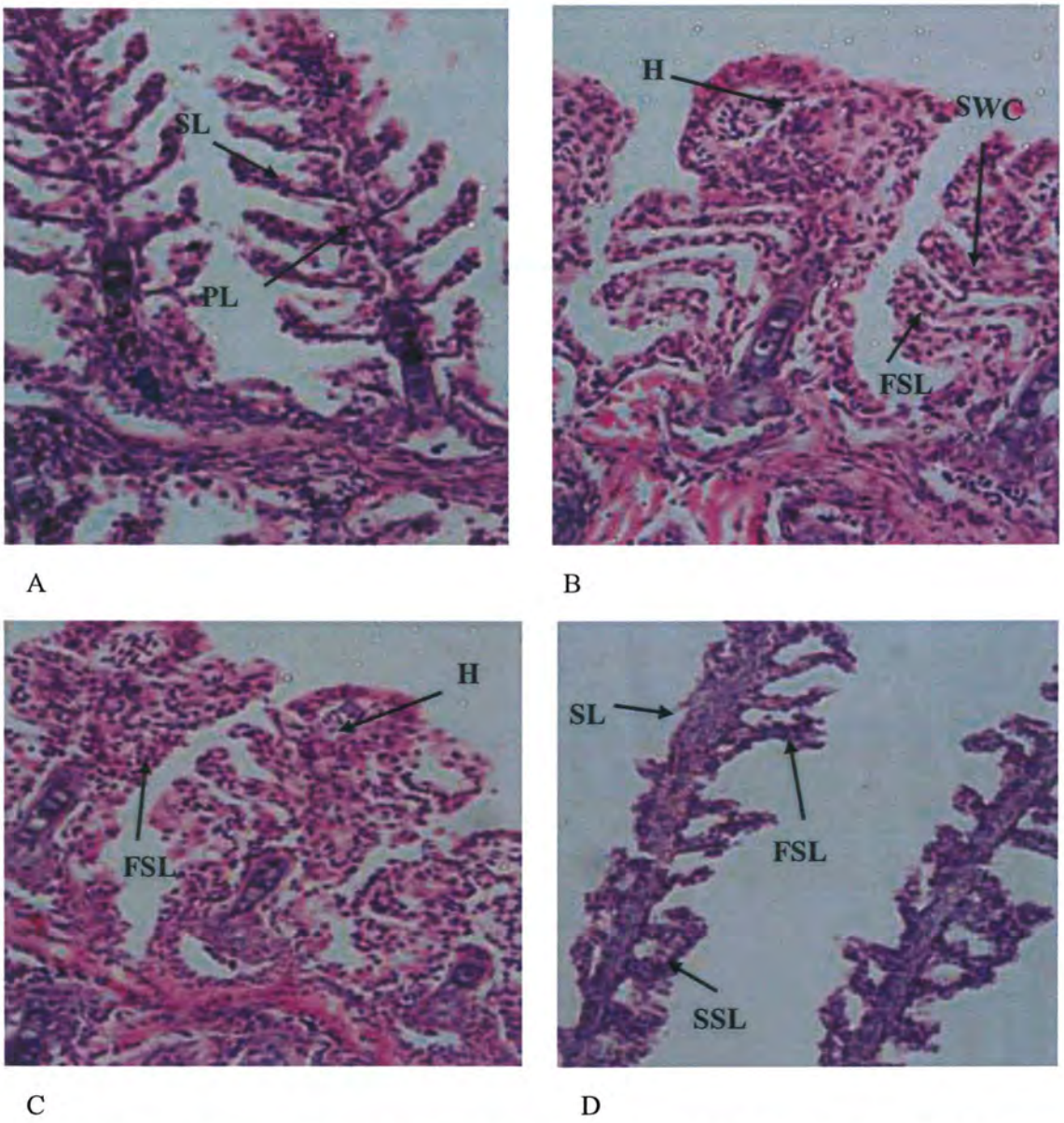


Figure 3.10 : Photomicrographs of gill sections of Zebrafish H and E stained, 40X magnification after 21 days of exposure, (A) Gills of control group, (B) Gills of T1, (C) Gills of T2, (D) Gills of T3. Sl stands for sloughing, EL represents epithelial lifting, H represents hyperplasia, FSL represents fusion of secondary lamellae, SSL represents shortening of secondary lamellae.

The basic structure of gills of Zebrafish consisted of filaments of primary lamellae, from these lamellae secondary lamellae projects out, known as respiratory lamellae that is surrounded by two cells thick epithelial layer (Bhuvaneshwari *et al.*, 2015). Rigid mass of

cartilaginous tissue is present in the core of primary lamellae. In inter lamellar epithelium chloride and mucous cells are frequently present (Nowak, 1992). Gaseous exchange, transport of ions, uptake and excretion of various pollutants or xenobiotics are some major functions of gills (Banaee *et al.*, 2013). Xenobiotic exposure through water is not the only cause of histopathological alterations in gills but gill damage could also be caused by the uptake of contaminated food. Histopathological alterations in gills could be in the form of epithelial lifting, hyperplasia, necrosis, inflammation and hypertrophy, all these lesions could reduce the diffusion of oxygen in blood (Heath, 1995). Histopathological alterations were more pronounced in the treatments having higher concentration of pesticides, exposure time also played a significant role in alteration of the gills and morphological changes were more intense after prolonged exposure of pesticides. Epithelial lifting was commonly observed after early exposure of pesticides which increases the sensitivity of lamellae as the continuity of epithilia becomes no longer. Because of the discontinuity of squamous epithelium fusion of adjacent secondary lamellae was observed as a result of hyperplasia, besides narrowed water channels and shortening of secondary lamellae were also observed at all treatments at sampling intervals. After the exposure of 21 days of pesticide more intensed morphological changes were observed. More intensed hyperplasia was observed in a treatment group having medium concentration of pesticides (0.1µg/L endosulfan and 1ppm imidacloprid) and this huge increase in number of cells fused all the adjacent secondary lamellae which narrowed the water channels while in treatment 3 having highest concentrations of endosulfan (1µg/L endosulfan and 1ppm imidacloprid) after 21 days of exposure significant reduction in primary and secondary lamellae occurs and a higher degree of sloughing was observed that shows that a constant exposure of pesticides leads to the deleterious effects.

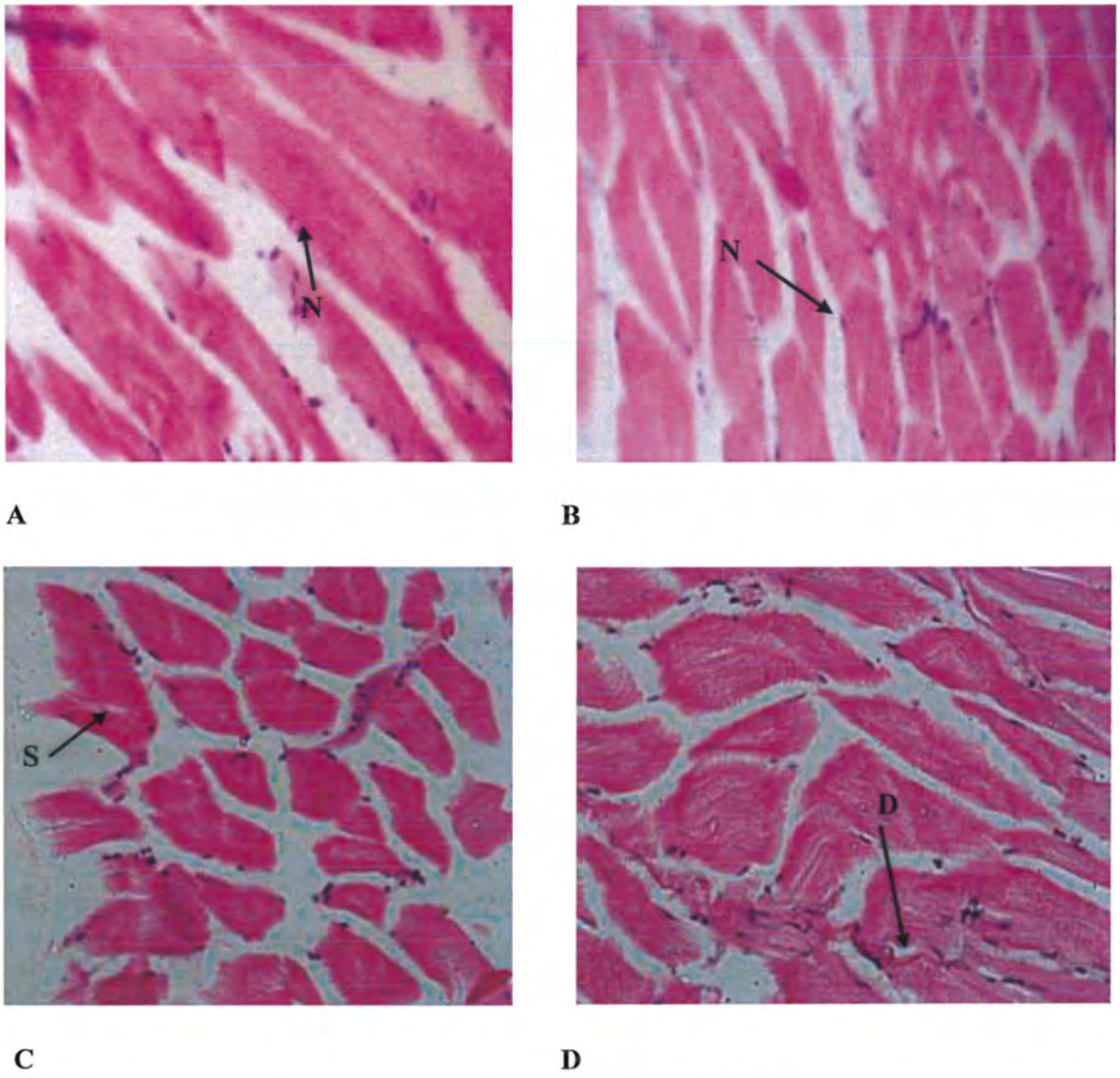


Figure 3.11: Photomicrographs of Zebrafish muscles after 7 days of exposure at 40X magnification. (A) muscles of Zebrafish in control group having normal structure with nucleus located at periphery of fibers, (B) muscles of T1 after 7 days of exposure, (C) muscles of T2, (D) muscles of T3, N represents nucleus located at periphery of muscle fibers and D represents degeneration of muscle bundles.

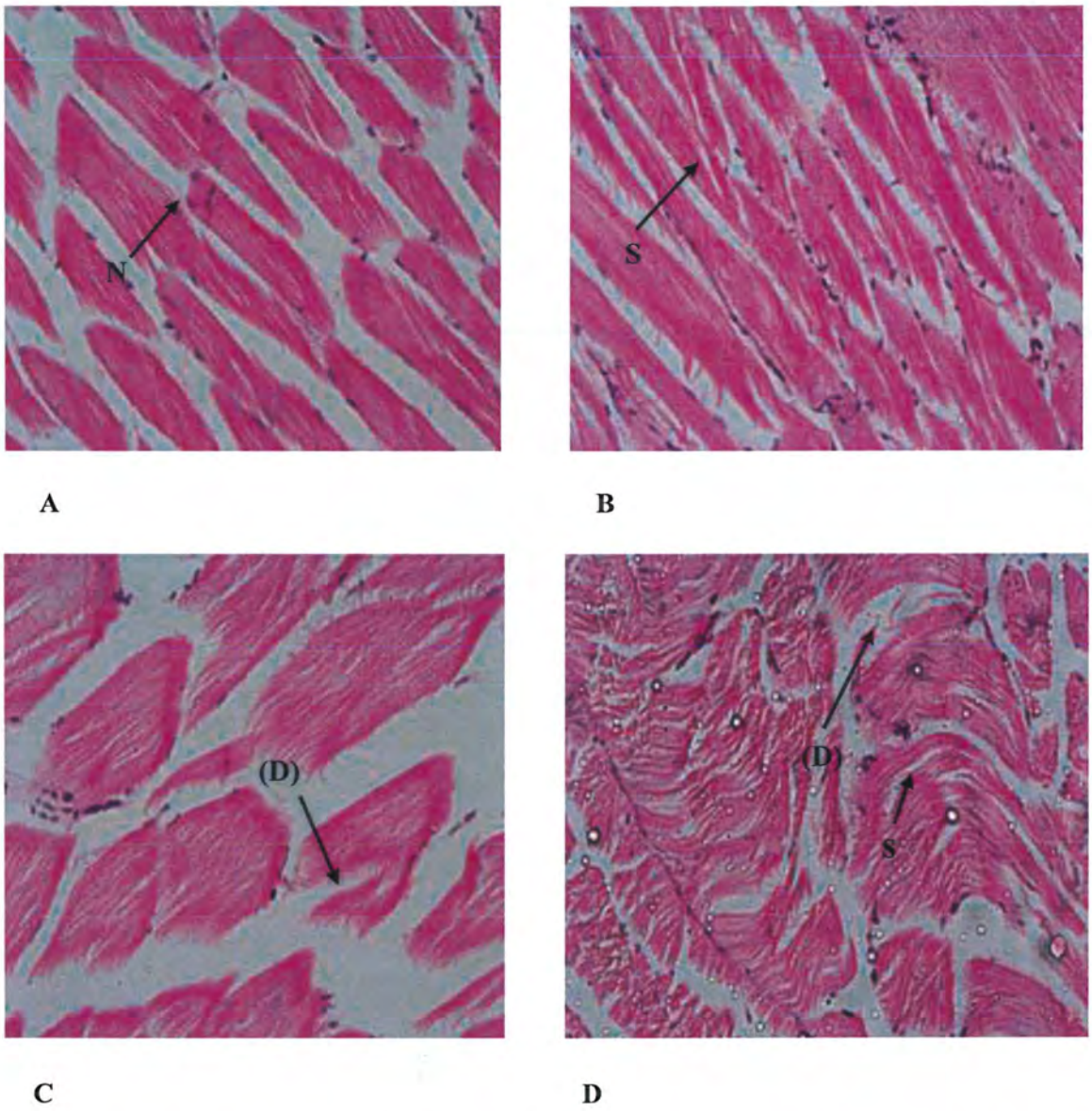


Figure 3.12 : Photomicrograph of Zebrafish muscles after 14 days of exposure (A) muscles of Zebrafish from control group, (B) muscles of Zebrafish from treatment 1 , (C) muscles of Zebrafish from T2 , (D) muscles of Zebrafish from T3 , (D) represents degeneration of muscle bundles , S represents splitting of muscle fibers.

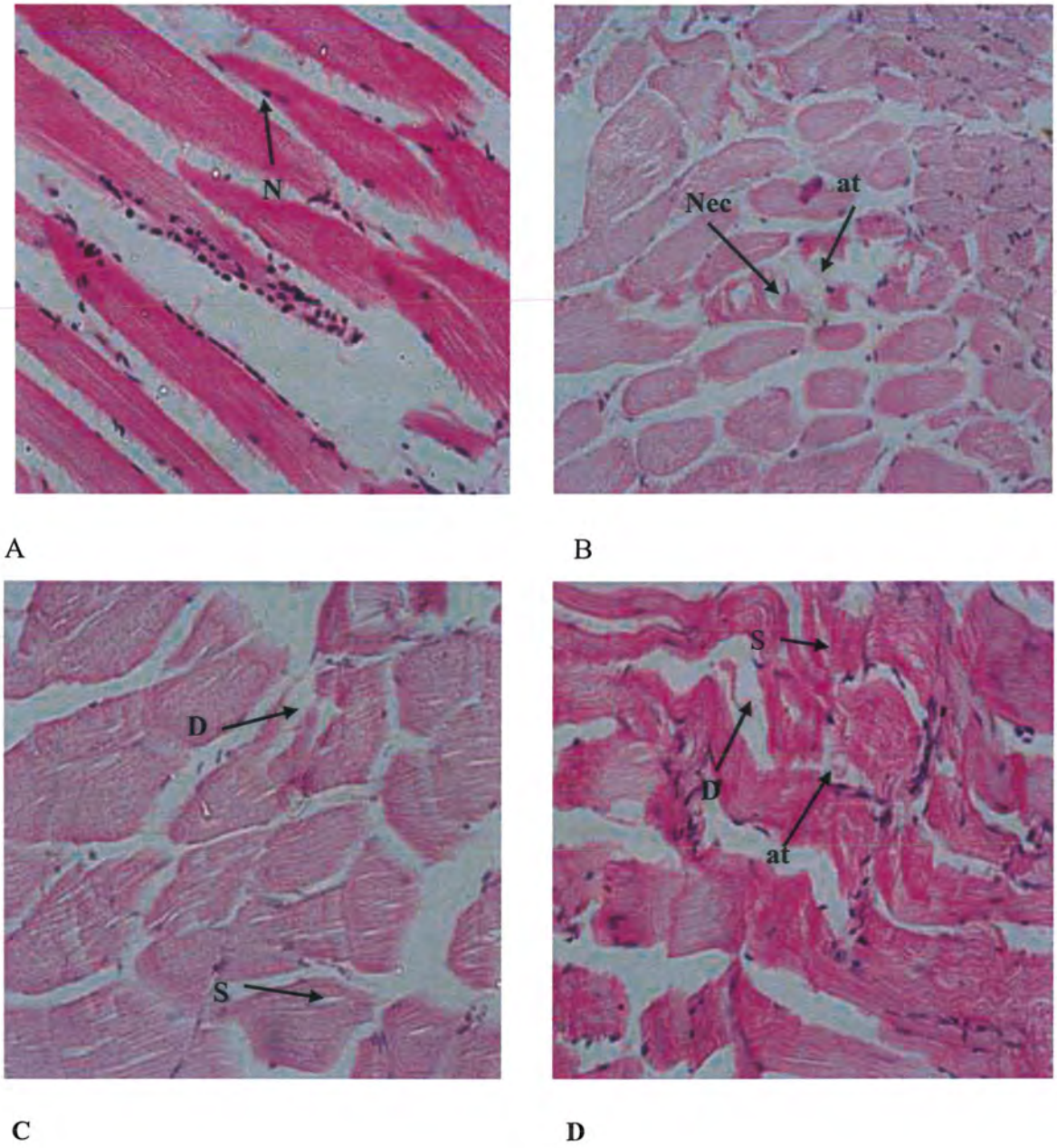


Figure 3.13: Photomicrographs of Zebrafish muscles after 21 days of exposure, (A) represents the muscles of Zebrafish from control group, (B) muscles of Zebrafish in T1, (C) muscles of Zebrafish in T2, (D) muscles of Zebrafish in T3 , S represents splitting of muscle fibers, D represents degeneration of muscle bundles, and (Nec) represents necrosis and (at) represents atrophy.

In the present study muscles of control groups at all sampling intervals showed normal morphology with the nucleus at the periphery of fibers. No significant alterations in the muscles of Zebrafish were observed in T1 after the exposure of 7 days of pesticides while mild splitting and degeneration of muscle bundles was observed in T2 and T3. After 14 and 21 days of exposure quite significant changes were observed in muscles. Splitting of muscle fibers was observed in T1 and degeneration of muscle bundles was observed in T2 while in T3 splitting and degeneration of muscular bundles both were observed after 14 days of exposure. After prolonged exposure of 21 days of pesticides (imidacloprid and endosulfan) severe muscle damage was observed in all three treatment groups while the control group showed normal muscles. In T1 atrophy and necrosis were observed in muscle bundles. Splitting , degeneration of muscle bundles was observed in the muscles of T2 ,while in T3 not only degeneration and splitting of muscle bundles was observed, but atrophy of muscles was also observed after exposure of 21 days.

Discussion

4.1 Antioxidant Enzymes

The current study was conducted to assess the dual effects of imidacloprid and endosulfan on Zebrafish in terms of oxidative stress and morphological responses of muscles and gills. In ecotoxicology, antioxidants are widely discussed because they play significant role in maintaining cell's homeostasis. Oxidative damage is a toxicity mechanism induced by pollutants in aquatic organisms (Santos *et al.*, 2004). This stress is assessed by measuring reactive oxygen species (ROS), antioxidant enzymes and malondialdehyde (MDA) content. Toxic chemicals are continuously being released into environment because of industrial, agricultural and domestic activities (Patel *et al* ., 2016). Pesticides enters to the aquatic ecosystems through many pathways runoff, leaching, direct spillage and wind drift (Köhler and Triebskorn, 2013), these pesticides are absorbed by the aquatic organisms specially fishes and their long term exposure causes adverse toxic health effects. Due to increased pesticide toxicity in aquatic ecosystems, mostly fish is effected as result of oxidative stress, highly reactive oxygen species are produced that causes oxidative damage to the to the cellular bodies. These highly reactive intermediates are scavenged by the natural defensive systems in the form of antioxidant enzymes, These antioxidant enzymes do not allow the excessive ROS production since their basic function is to maintain a balance between production and elimination of ROS but if the antioxidant enzymes fails to maintain this balance oxidative stress occurs (Zhou *et al.*, 2013); (Han *et al.*, 2016); (Ge *et al.*, 2015). Superoxide dismutase (SOD) and catalase (CAT) are two most important enzymes of antioxidant defense systems and they have ability to capture hydrogen peroxide and superoxide anions to protect the organism from oxidative stress (Han *et al.*, 2016). Significant oxidative stress induced by imidacloprid and endosulfan in livers of Zebrafish was observed in the present study. In the present study superoxide dismutase (SOD) activity was greatly inhibited by the increased pesticide exposure. Highest pesticide concentration and increased exposure time showed significant inhibition in SOD activity (day 21). Early exposures showed increased SOD activity (day 7 and 14) as compare to

control. During early exposures SOD activity may increased to resist the oxidative stress caused by the pesticide exposure , Decreased SOD activity below than control in all treatments after 21 days of exposure of pesticide may be explained by the fact that oxidative stress increased so much that SOD level was not enough to resist that stress. These results are in agreement with the outcomes published by (Ge *et al.*, 2015); (Shao *et al.*, 2012) where SOD activity increased during early exposures of pesticides in order to protect the organisms from adverse oxidative stress by eliminating the excessive ROS produced as a result of pesticides exposure while inhibition of SOD activity after prolonged exposure of toxicant was observed because of excessive ROS production and adverse effects of ROS on antioxidant enzymes production while catalase activity increased at lower concentrations (0.01µg/L ,0.1µg/L) of endosulfan as compare to control while at higher concentrations of endosulfan (1µg/L, 10µg/L) whereas CAT activity decreased significantly in comparison to control, these findings are in accordance with our results. Slight increase in CAT activity at lowest concentration (T1) of pesticide after 7 days of exposure, then significant increase after 14 days of exposure and then decrease in T1 after 21 days of exposure as compare to (T1) on day 14 shows that early exposure of pesticides induce CAT activity to reduce or balance the excessive ROS production by converting hydrogen peroxide into water and molecular oxygen but after the prolonged exposure and higher concentration of pesticides induce oxidative damage (Shao *et al.*, 2012). Similar results were observed in a study performed by (Han *et al.*, 2016) where SOD activity was notably increased in all treatment groups as compare to control on day 7 while after 14 and 21 days of exposure of azoxystrobin to Zebrafish SOD activities decreased significantly in liver as compare to control this is because increase in oxygen free radicals due to constant exposure of pesticide for long duration reduced SOD and the active sites of this antioxidant enzyme becomes inactivated and enzyme dysfunction occur because of oxidation process (Butterfield *et al.*, 1998). Pollutant concentration, duration of exposure and specie type are three most important factors on which antioxidant enzyme activity depends (Elia *et al.*, 2002). Reduced SOD activity was observed in brain and kidney of Zebrafish exposed to atrazine and clorpyrifos, as the concentration of atrazine, clorpyrifos and ATR/CPF combination

increased SOD activity reached to its lowest levels which shows the negative impact of pesticides alone and in mixture to Zebrafish and these observations are in accordance with our findings while CAT activity also followed the same pattern of decrease in activity as the pesticide concentration increased along with exposure time (Xing *et al.*, 2012). Increase in CAT activity at low doses of pesticides may represents the compensatory mechanism against oxidative stress. Findings of CAT activity are consistent with the results of (Rosety *et al.*, 2005). Where CAT activity was decreased by the exposure of malathion pesticide. Significant increase in SOD and CAT activities was observed in livers and kidneys of *Channa punctatus* exposed to thermal power plant effluents (Javed *et al.*, 2016) increased level of antioxidant enzymes in liver and kidneys of *Channa punctatus* was observed so that the oxidative stress caused by the thermal effluents could be reduced these findings are in agreement with the results obtained after early exposure of pesticides in our study.

4.2 Effects of pesticides imidacloprid and endosulfan on MDA content

MDA content indicates the level of lipid peroxidation, measuring MDA indicates the extent of severe damage to the cell membranes. Environmental pollution can be measured using MDA as a biomarker of pollution. Elevated MDA levels were observed in female Zebrafish livers when exposed to atrazine, dose dependent increase was observed in MDA content about 1.8 fold and 2.1 fold higher than control group (Jin *et al.*, 2010). These findings are in accordance with our results where highest increase in MDA content was observed in groups having highest concentration of pesticides. Excessive ROS leads to the inhibition of antioxidant enzymes which leads to lipid peroxidation and MDA is the end product which is measured to evaluate the extent of oxidative stress. Similar results were observed in a study performed by (Han *et al.*, 2016) where increased MDA contents were observed in Zebrafish livers exposed to middle and highest concentrations of fungicide azoxystrobin, significant increase in MDA content was induced at highest concentration of azoxystrobin (100µg/L) in both male and female Zebrafish after 21 days of exposure which is exactly in accordance with our findings. Exposure of pesticide mixtures also induce the same changes in MDA levels as the trends observed in case of exposure of pesticides alone. Higher concentrations of pesticides (atrazine and

clorpyrifos alone and in mixture) induced significant increase in MDA level of brain and kidneys of Common Carp (Xing *et al.*, 2012) these results are in accordance with our findings and shows that exposure of elevated levels of pesticides induce peroxidative stress in tissues of different species. Similar results were observed in a study performed by (Ge *at al.*, 2015) where no significant increase in MDA levels was observed on day 7 and 14 at low concentrations of imidacloprid while a significant increase in MDA was observed after exposure of 21 and 28 days at higher concentration and these results are relevant to our findings.

4.3 DNA Damage

DNA damage is quite a suitable biomarker in toxicological studies assessed through comet assay. As an initial damage comet assay identifies strand breakages, alkali labile positions and late repair sites (Yong *et al.*, 2013). Oxidative stress and lipid peroxidation leads to the production of alkyl free radicals which are also accountable for DNA damage. Tail length and percent DNA in tail gradually increased as the concentration of pesticides increased and the significant increase in olive tail moment was observed after 21 days of exposure of highest concentration of pesticides (imidacoprid and endosulfan). These results are in agreement with the results published by (Ge *et al.*, 2015) where dose dependent DNA damage was observed by imidacloprid exposure to Zebrafish, time dependent relationship was observed in olive tail moment and it increased significantly after prolonged exposure of imidacloprid. DNA damage may be induced by biochemical processes, lipid peroxidation products, pesticide concentration and oxygen free radicals. Significant increase in olive tail moment (OTM) was observed by the increasing concentration of endosulfan in both male and female Zebrafish (Shao *et al.*, 2012) , which is in accordance with the results of present study. Endosulfan induced genotoxicity in cultured hepatic cells in the form of DNA adducts but high endosulfan mediated genotoxicity was observed in rats and human cells (Dubois *et al.*, 1996) so in the present study we may infer that at high endosulfan concentration formation of DNA adducts could be the cause of DNA damage. In comet assay migration of DNA takes place towards anode in the presence of electric field and the extent of DNA damage is evaluated by quantifying the migrated DNA away from nucleus as damaged DNA

particles move towards anode. Significant increase in olive tail moment was observed in Zebrafish hepatopancreas exposed to atrazine, dose dependent response in DNA damage was observed (Zhu *et al.*, 2011) these results shows similarity with the data obtained from current study. Oxidative stress and DNA damage are closely related , as a result of oxidative stress antioxidants fails to detoxify the intermediate products formed by the toxin exposure. Oxidative stress induces the production of excessive ROS and lipid peroxidation which leads to DNA damage by strand break and nucleotide modification particularly by increasing guanosine content (Bennett, 2001). DNA strand breaks were induced in Zebrafish liver by the exposure of azoxystrobin which is a fungicide, as the concentration of azoxystrobin and exposure time was increased DNA damage was enhanced in term of nucleotide bases modifications (Han *et al.*, 2016). Tail length is an important parameter to investigate the magnitude of DNA damage as the damaged DNA migrates from head towards tail, tail length will increase. In our present study tail length increased as the concentration of pesticides and exposure time increased because of increased migration of damaged DNA, these results are in agreement with the study published by (Javed *et al.*, 2016) where tail length of liver cells of *Channa punctatus* exposed to thermal effluents is significantly higher than the control cells.

4.4 Histology of Zebrafish gills and muscles

Pathological changes in gills can be used as an indicator of water pollution. Hyperplasia, lifting of epithelia, fusion of secondary lamellae and narrowed water channels were observed as the lesions produced by the result of pesticides exposure. Results of present study are in accordance with the study presented by (Nowak, 1992), where hyperplasia and lifting of lamellar epithelium and lamellar hypertrophy was observed in gills of Cat fish having residues of endosulfan. Results presented by (Bhuvenashwari *et al.*, 2015) are in accordance with our findings where same alterations were produced when Zebrafish was exposed to mixture of organochlorine pesticides (aldrin, dieldrin, heptachlor, mirex, HCH and DDE), splitting of muscle fibers and degeneration of muscles were observed in the muscles of Zebrafish exposed to organochlorine pesticides, heavy metals (Cd, Pb, Ni) and mixture of both, similarly in gills fusion of secondary lamellae, shortening of secondary lamellae and hyperplasia were commonly observed which is in accordance

with our results. Marked alterations in gill epithelia of *Clarias gariepinus* were induced at higher concentrations of herbicide glyophosate, severe hyperplasia, epithelial disintegration and fusion of secondary lamellae after 14^{th} and 28^{th} day interval are in agreement with results of this study. Histopathological damage in gills of Rainbow strout induced by chronic exposure of diazinon at concentration of 0.1mg/L and 0.2mg/L are in accordance with our findings where dilation in blood capillaries, hyperplasia and swelling of epithelium in secondary lamellae, necrosis and shortening of secondary lamellae were observed (Banaee *et al.*, 2013). Lesions in gill tissues may interfere with osmoregulatory dysfunction and respiratory disorders . This uplifting of lamellar epithelium and fusion of secondary lamellae could be the adaptations of gill tissues in order to reduce the contact between pollutants by reducing the surface area of gills. Splitting of muscle fibers, disintegration of muscle bundles, focal area of necrosis , vacuolar degeneration in muscle bundles were the histopathological alterations observed in *Tilapia Orechromis mossambicus* exposed to textile dyes (Sripriya, 2014) Degeneration, splitting, necrosis and atrophy were observed as histopathological alterations in muscle tissues of Tilapia exposed to three different concentrations of textile dyes (0.5, 1 and 1.5ppm) for 21 days these findings are similar to our results.

Conclusion

This is the first study to our knowledge to investigate the combined toxicity of endosulfan and imidacloprid on Zebrafish. Both these pesticides induced oxidative stress in liver of Zebrafish and which then induced lipid peroxidation that leads to DNA damage. The pesticides stimulated the antioxidant enzyme activities CAT and SOD at low concentrations, but high concentrations of pesticides and prolonged exposure duration decreased the activity of both enzymes. As the antioxidant enzyme activity decreased malondialdehyde (MDA) content increased which enhanced the DNA damage because of the excessive reactive oxygen species. MDA content increased with increased exposure time and pesticide concentration which demonstrated increased lipid peroxidation in cellular bodies. Similarly a substantial increase in DNA damage was noticed after 21 days exposure of pesticides. Histolpathological alterations in gills and muscle tissues, marked lesion in gills in the form of epithelial lifting, merging of secondary lamellae, hyperplasia, restriction of secondary lamellae , narrowed water channels were observed specially in high dose groups while in the muscles splitting of muscle bundles, necrosis was randomly observed. Its very important to study the pesticides interaction with aquatic species specially fish as many pesticides coexist and mixtures of pesticides are usually detected in the natural environment. All the negative effects of pesticides should be taken into concern while using these agrochemicals for pest control in agricultural fields surrounding the freshwater ecosystems.

Recommendations

Oxidative stress, DNA damage and histopathology of fish organs can contribute as useful bioindicators to assess the quality of aquatic ecosystems by future researchers . Its very important to study the pesticides interaction with aquatic species, particularly fish as many pesticides coexist and mixtures of pesticides are usually detected in the natural environment.

Additional work is required to be carried out to understand the mechanism of pesticides toxicity to non target organisms on the basis of the data presented in this study. All the negative effects of pesticides should be taken into concern while using these agrochemicals for pest control in agricultural fields surrounding the freshwater ecosystems. Pesticide exposure to the species like Zebrafish will allow us to understand the toxicity mechanisms in human beings also because it has a genetic resemblance to human beings.

In this project we have examined the chronic toxicity of Zebrafish in a bioassay by exposing Zebrafish to pesticides mixture which offers a prompt and squat means to expose the nature of induced contaminants as well as the resistance of the specie. The effect of these pesticides against Zebrafish can be evaluated separately and then mixtures of pesticides can be used to understand that how mixtures of pesticides can affect the antioxidant defense systems of organisms.

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