

Chapter 1

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Chapter 1

Section 1.1 Introduction

1.1.1 Trends in Pesticide Usage and its Importance

Pesticides are an integral part of modern agricultural practices. Currently, agriculture feeds over 7 billion people worldwide; however, it has been estimated that to feed a projected 9–10 billion people by 2050, the world's food output must expand by 60–110% (Pardey et al., 2014). Pesticide is an umbrella term designated for a range of chemical substances or a mixture of substances used to prevent, destroy, repel, or mitigate pests (EPA, 2019). According to the UN Food and Agriculture Organisation (FAO), pests, weeds, and diseases decimate crops in developing nations by approximately 40%, while they are still in the fields and by 6-7% after harvest. In Africa, the pre-harvest loss is estimated to be approximately 15-20%, while in Asia, this accounts for about 20-30% (FAO, 2021). Pesticides have been employed to combat pest problems of about one-third of agricultural products. It is estimated that fruit production would decrease by 78%, vegetable by 54%, and the cereal by 32% without pesticide applications (Tudi et al., 2021). Owing to their indispensable action on pests, global use of pesticides has increased by 46% since 1996 to 2016 according to the FAOSTAT database (WHO, 2019). Despite a recent plateau, the average total use of pesticides increased by almost 50% during the last ten years compared to the 1990s. Around 2.7 million tons of active ingredients of pesticides are used in agriculture per year. Pesticide usage per area of crop land rose from 1.2 to 1.8 kg/ha in 2022. (FAO, 2022).

In terms of global pesticide use, India ranks third in Asia, after China and Turkey. According to Nayak and Solanki (2021), agriculture employs 70% of the country's workers and generates 18% of its overall GDP. But pests impact havoc on the production. The proliferation of crop pests greatly depends on climate, altitude, crop variety, harvesting methods, use of pesticides, biological control agents and economics of production (Muraleedharan, 1992). Monocultures of edible crops in fact provide a stable supply of food for pests. Infestations of arthropod pests on crops result in a loss of 45% of India's annual produce due to the country's tropical, humid climate, which favours the rapid proliferation of pests. The anticipated annual loss of agricultural products in India is 42.66 million USD (Subhash et al., 2017). Insecticides are the most used pesticides in India, followed by fungicides, herbicides, and bactericides. In fact, 51% of all

pesticides are insecticides (Nayak and Solanki, 2021). The use of pesticides has increased significantly in India during the past ten years, from 434 tonnes in 2000 to 62193 tonnes in 2021. According to the reports of the Government of India (GOI), 58720 tonnes of pesticides were consumed in India in 2022. According to data from the Agricultural Input Survey, the crops with the highest percentage of pesticide-treated land in 2011–12 were cotton (66.70%), arhar (64.74%), jute (53.27%), and rice (48.62%), while the maize (25.01%) being the lowest. Organophosphates, neonicotinoids, and pyrethroids are the three major groups of pesticides used in India (Nayak and Solanki, 2021; GOI, 2021).

West Bengal alone used 3630 tonnes of pesticide in 2021–2022 (GOI, 2022). Among the states/UTs of India, Maharashtra had the highest total pesticide consumption, followed by Uttar Pradesh, Punjab, and Haryana. Maharashtra and Uttar Pradesh account for 41% of India's pesticide consumption while West Bengal accounts for 6% of the total pesticide consumption (Nayak and Solanki, 2021). Paddy, tea, vegetables, fruits, and the cash crops like nuts are among the key crops cultivated in West Bengal (Khatun and Jamal, 2021). The most widely used pesticides in the state, after alpha-cypermethrin (46%), are methyl parathion (25.6%), imidacloprid (16.4%), dichlorvos (7.8%), and phorate (4.2%) (Banerjee et al., 2014). The bio-pesticides seem to be a better alternative to synthetic pesticides, but this account for only 9% of the total pesticide consumption in India (Nayak and Solanki, 2021; Mishra et al., 2020).

1.1.2 Effects of Pesticides on Non-Target Organisms

When used judiciously, pesticides are a boon to mankind, but indiscriminate usage of these toxic chemicals has led to several adverse impacts on the environment and human health. Unscientific and irrational use of pesticides in agricultural fields contaminates adjacent soil and the water bodies through surface runoff and pesticide drift. It further leads to unintentional exposure of non-target organisms like beneficial insects in the soil, bees, birds, and aquatic organisms, like fishes (Rajak et al 2023). Numerous typical foods and beverages, including prepared meals, water, wine, fruit juices, snacks, and animal feed, have been reported to have pesticide residues (Bajwa and Sandhu, 2014). The consumers of these food materials are at high risk of exposure to pesticides and poisoning. Despite agricultural advantages, these synthetic chemical compounds have the potential to cause pathogenic effects on non-target organisms ranging from invertebrates to vertebrates including human beings (Jeyaratnam et al., 1990; Stamati et al., 2016). No segment

of the population is completely protected against the exposure to the pesticides and the subsequent serious health issues, though a disproportionate burden, is shouldered by the people of developing countries and by high-risk groups in each country (WHO, 1990). Production workers, formulators, sprayers, mixers, loaders, and agricultural workers and farmers are among the high-risk populations exposed to pesticides directly or indirectly (Aktar et al., 2009; Dutta and Bahadur, 2019). Many of the pesticides have long half-lives and are generally stable that contaminate organisms in a food chain and bioaccumulate in the highest trophic levels (consumers) that may cause ecosystem disruption (Rana et al., 2022). Degraded, leached, or volatilized products of synthetic pesticides get absorbed into the air, surface water, groundwater, and soil and are often dispersed or transported from one site to another leading to environmental pollution and adversely affect non-target organisms even in areas that are not directly under pesticide application (Sharma et al 2019).

Studies around the world have reported the adverse effects of pesticide(s) contamination on non-target organisms (Beketov et al., 2013; Sharma et al., 2019). The EPA describes a non-target organism as ‘any organism’ for which the pesticide was not intended to control (Streibig and Green, 2017). Soil microflora is among the most affected organisms by indiscriminate pesticide applications in fields (Inglesfield, 1989). Water bodies contaminated with agro-pesticides have been observed to influence macro-invertebrates like Coleopterans, Gastrops, and Hemipterans as well as micro-invertebrates like zooplanktons (Merga and Brink, 2021; Leiss and Ohe, 2005). Entomophily is the transmission of pollen from plants including crops by insects. Bees are the primary pollinators in nature and are beneficial insects, but they have also been affected by chemical pollution due to pesticides (Bayo and Goka, 2014). A number of studies have documented bioaccumulation and toxicity of pesticides in higher animals, like fish, rodents, cattle, and humans (Desai and Parikh, 2014; Gibbons et al., 2015; Kumar et al., 2014; Mahmood et al., 2015; Naqvi et al 1993).

1.1.3 Impact of Pesticide on Freshwater Ecosystems

Pesticide contamination, a global problem, has been reported in freshwater ecosystems from different parts of the world. Lakes (Hladik et al., 2018), lagoons (Essumang et al., 2009), estuaries (Jabber et al., (2001), and the major river systems (Karlsson et al., 2020; Obidike et al., 2020; Ali et al., 2009) around the world are reported to be contaminated with argopesticides.

Most major rivers in the Indian subcontinent like Ganga (Shan and Parveen, 2021), Yamuna (Alam et al., 2017), Brahmaputra (Chakraborty et al., 2016), Kaveri (Gupta et al., 2022), Krishna (Patil et al., 2015), Ghagghar (Kaushik et al., 2010) and Tapi (Hashmi et al., 2020) have been shown to be contaminated by various pesticides. The Ganga and Hoogly in Southern part of West Bengal and Deomani, Changa, Manza, Tepu, Karola and Teesta in the Terai-Dooars region of northern part of West Bengal (Singh et al., 2015; Singh et al., 2017; Das, 2017; Mondol et al., 2018; Shah and Parveen, 2023; Dutta et al., 2023) have also been reported to be extensively contaminated.

Freshwater ecosystems are particularly affected by pesticide pollution as they serve as a natural sink. After application synthetic pesticides are degraded by microbial action, chemical reactions, and light. The degraded products are either vaporized into the atmosphere that is deposited by rainfall, absorbed into the soil and accumulated and leached into the groundwater, and finally due to surface runoff ultimately reach freshwater ecosystems like ponds, lakes, and rivers (Tudi et al., 2023, Merga and Brink, 2021).

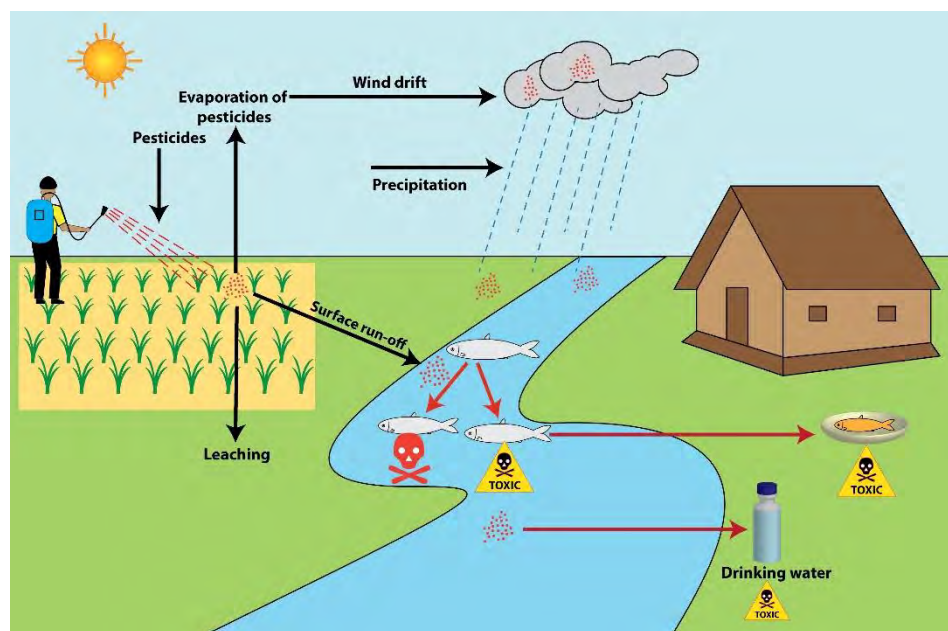


Figure 1: The pathways and environmental impact of pesticides used in agriculture.

Freshwater ecosystems are home to a wide variety of invertebrate and vertebrate organisms that are affected by pesticide pollution (Amenyogbe et al., 2020). Aquatic animals may accumulate pesticides in numerous ways, including directly ingesting the contaminated phyto and

zooplanktons, intake through their skin or gills (bioconcentration), consuming contaminated or polluted food (biomagnification), and ingestion of suspended particles, making them susceptible to the physicochemical changes in their environment (Rad et al., 2022).

1.1.4 A Multi-biomarker Approach to Detect Water Contamination/Pollution in Fish

Fish are good indicators of water contamination/pollution because they frequently exhibit behavioural changes in response to slight physicochemical changes of water, allowing them to adjust to those changes appropriately (Ullah et al., 2014; Srivastava et al., 2016). They also occupy diverse trophic levels in aquatic habitats, and because of their varying sensitivity, they are reliable indicators of contamination/pollution. Humans occupy the topmost place in the trophic level, and these toxicants/pollutants can reach humans through bio-accumulation in fish, thereby posing a risk to human health as well (Eskenazi et al., 2008). To enable early identification of contamination in the aquatic environment, fish biomarkers can be utilized as pollution indicators (Van der Oost et al., 2003). The word "biomarker" has historically been used to refer to any biochemical, physiological, or histopathological indicator of xenobiotic or chemical exposure or effect at the sub- or organismal level (Huggett et al., 1992).

Toxicity bioassays are particularly useful in measuring the extent of damage caused by a foreign substance on an organism's survival rate. The damage can be rapid and fatal, due to making the acute toxic effects at higher concentrations, or it can be chronic if concentrations of the toxicant are at low levels (Sprague, 1969). The degree of effect or potency can be defined by the median lethal dose/concentration LD₅₀/ LC₅₀, which is the dose/concentration required to kill or affect 50% of organisms relative to a maximum value (Streibig and Green, 1989). Bioassays in a large variety of fish species have been described to account for the toxic effect of pesticides on acute toxicity (Pickering and Henderson, 1966; Singh et al., 2017). A large number of studies have documented the sublethal effects of pesticides on fish species (Suchiang, 2021; Rohani, 2023). The sublethal effects of pesticides are of particular concern because of their long-term implications on the non-target organism. Repeated exposure to sub-lethal concentrations of pesticides has been associated with changes that reduce fish populations, decrease immunity to diseases, and decrease predator avoidance (Araújo and Blasco, 2019). These pesticide-induced changes include behavioural abnormalities, such as the frequency of surfacing, abnormal

opercular movement, loss of equilibrium or disorientation (Salunke et al., 2020); genotoxic effects, such as DNA damage and mutations (Ali et al., 2009, Obaikor et al., 2014), haematological changes (Pandey and Srivastava, 2007); physiological effects including those affecting biochemical processes and metabolisms, like enzyme functions (Benli and Celik 2021; Topal et al., 2015), histopathological effects on exposed organs, like gills, liver or brain (Qadir and Iqbal, 2016), reduced growth (Crosby et al., 2015), reproductive stress (Mohanty and Samanta, 2016) and reduced survivability. Such altered states of physiological, biochemical, genotoxic, and behavioural, activities can be used as good biomarkers for preliminary screening of toxicity of pesticides/toxicants.

Different groups of pesticides including organophosphates (chlorpyrifos and malathion), synthetic pyrethroids (cypermethrin, lambda-cyhalothrin), neonicotinoids (Imidacloprid, Thiamethoxam, Acetamiprid), have been shown to have genotoxic effects on fishes even at sub-lethal concentrations (Topal et al., 2018; Upadhyay, 2014). Most experiments show a dose and time-dependence increasing effect of the pesticides on the fish. Besides causing mortality, the genotoxicity reduces fish populations 'fitness' (i.e., growth, fertility, and fecundity) (Naqvi et al., 2016). Fishes in their early life stages are especially seen to be vulnerable and have been used extensively as sensitive species for toxicity evaluation (Byrne, 2012, Crosby et al., 2021).

There are several ways that different insecticides work. Insecticides irreversibly inhibit acetylcholinesterase, an enzyme that hydrolyzes the neurotransmitter acetylcholine in at the synapses of neuromuscular junctions and the brain cholinergic synapses. The organophosphorus pesticides, which make up 50% of all pesticides used in India, are known to be extremely neurotoxic (Yadav et al., 2015; Sobolev et al., 2022). In India, synthetic pyrethroids hold 19% market share (Yadav et al., 2015). Cypermethrin, the most widely used insecticide in India, extends the opening of sodium channels in the central nervous system, resulting in hyperexcitation and hypo-polarization of the neurons, which causes neurotoxicity and results in muscle deficits by adversely affecting the sodium chloride voltage-gated, calcium, potassium, and other channels. It has also been reported to modulate the activity of glutamate and acetylcholine receptors and adenosine triphosphatases (ATPs) and induce DNA damage and oxidative stress in target organisms (Singh et al., 2012). Neonicotinoids, on the other hand, are a new class of potent insecticides that occupy 24% of the global market, imidacloprid one of the

neonicotinoids being the largest-selling insecticide in the world (ENVIS Database). These neuroactive insecticides compete with acetylcholinesterase for binding to the nicotinic acetylcholine receptors (nAChRs) of the target organism (Li et al., 2022). As nAChRs regulate cholinergic synaptic transmission, continual activation of this receptor results in neurotoxicity, leading to mild to severe behavioural changes due to damage of neurons (Wolansky and Harill, 2008). Neonicotinoids have also been shown to reduce AChE activity by acting as an agonist to their post-synaptic nicotinic acetylcholine receptor, thereby leading to accumulation of acetylcholine in the cholinergic synapses and causing hyperstimulation (Saluke et al., 2020). Studies by Topal et al., (2016) on rainbow trout brain acetylcholinesterase (AChE) activity showed a significant decrease in AChE activity upon short-term exposure to chlorpyrifos. Enzyme inhibition was also reported to be relatively high at higher doses of pesticide exposure (Veeraiah et al., 2018; Zhang et al., 2021). In fish, a decrease in AChE activity causes acetylcholine accumulation within synapses, leading to the impairment of important functions such as swimming, feeding, and general behaviour (Gluszczak et al., 2006, Crosby et al., 2021). The neurotoxic effect of acute exposure to imidacloprid on freshwater fish, *Pethia conchonius* has been studied by Dutta et al., (2023) to reveal changes in behaviour like swimming patterns, initial hyperactivity, loss of equilibrium, bottom aggregation as well as mucus secretion that can be correlated to the decreased AChE activity in the brains of the fish as well as histopathological damages in the brain upon pesticide exposure.

Pesticides/Pollutants typically have direct impact on the target and non-target organisms at the molecular and cellular levels (Mir et al., 2014), altering cellular redox equilibrium and increasing the production of reactive oxygen species (ROS) resulting in oxidative stress (Lushchak et al., 2018). The antioxidant defense system eliminates the reactive oxygen species produced as by-product of metabolism under normal physiological circumstances. When antioxidant defenses are overwhelmed by prooxidant forces, it results in an imbalance or oxidative stress that causes oxidative damage such as lipid peroxidation, enzyme function disruption or inactivation, DNA strand breaks, and covalently binding to protein and nucleic acids (Ates et al., 2016). Thus, antioxidant enzymes play a crucial role in maintaining cell homeostasis (Doyotte et al., 1997). Antioxidant enzyme activity is thus also considered as an important biomarker for the assessment of oxidative stress caused due to xenobiotics like agrochemicals.

Pesticides can cause oxidative stress by raising steady-state ROS levels, promoting ROS-induced modifications of cellular components, interfering with essential homeostatic and regulatory functions, or weakening antioxidant defenses (Banerjee et al., 2001; Lushchak, 2011, Lushchak et al., 2018). Since ROS interacts with DNA in various ways, an increase in their steady-state levels might increase the likelihood of DNA damage, causing genotoxicity and a variety of mutations that can be detected by the assays, like single-cell gel Electrophoresis (SCGE) and Micronuclei (MN) assays (Franco et al., 2010).

Genotoxicity is a deleterious action that affects cell's genetic material affecting their cellular integrity (WHO, 1997; Bhatnagar et al., 2016). The genotoxic effects of environmental pollutants can be monitored using a broad range of *in vitro* as well as *in vivo* biomarker assays in different organisms including fishes. The techniques, like the Comet Assay or SCGE and MN tests are commonly used to assess DNA damage and nuclear abnormality as the effect of pesticides in fishes (Viera et al., 2016; Viera et al., 2018; Carrasco et al., 1990). SCGE is a microgel electrophoresis-based method that can be used to measure DNA damage in individual cells by the formation of a 'comet tail' formed of damaged and broken DNA moving towards an electric field distinctly from a 'comet head' (Collins, 2004). Micronuclei Test assesses the various types of nuclear abnormalities in erythrocytes, lymphocytes, buccal epithelial cells, etc. including blebs and notches in the nucleus and formation of detached small micronuclei upon exposure to the pesticide(s).

Developed by Singh et al., (1988), the comet assay, also known as alkaline single cell gel electrophoresis (SCGE), is a highly straightforward, quick, and accurate method for determining genotoxicity. For single cell gel electrophoresis, a variety of cell types from fishes, including erythrocytes, lymphocytes, gill, liver, and kidney, have been employed (Collins, 2004, Jiang et al., 2023, Olive and Banath, 2006). The comet assay is used to detect genetic damage in the form of DNA strand breaks. Cells with increased DNA damage display increased migration of DNA from the nucleus toward the anode. The DNA damage was quantified as % tail DNA, which indicates the extent of cellular damage (Imanikia et al., 2016). Genotoxic effects of pesticides on various freshwater fishes that are often commercially important or consumed by humans like *Labeo rohita* (Ghaffer et al., 2020), *Pethia conchonius* (Dutta et al., 2023), *Channa punctatus* (Pandey et al., 2016), *Danio rerio* (Benli and Celik, 2021), *Oreochromis mossambicus* (Naqvi et

al., 2016), *Cirrhinus mrigala* (Bhatnagar et al., 2016) have been reported from river systems around the world.

Micronuclei (MN) are formed from acentric chromosome fragments or whole chromosomes that lag at anaphase during nuclear division (Obiakor et al., 2014) (Fig 2).

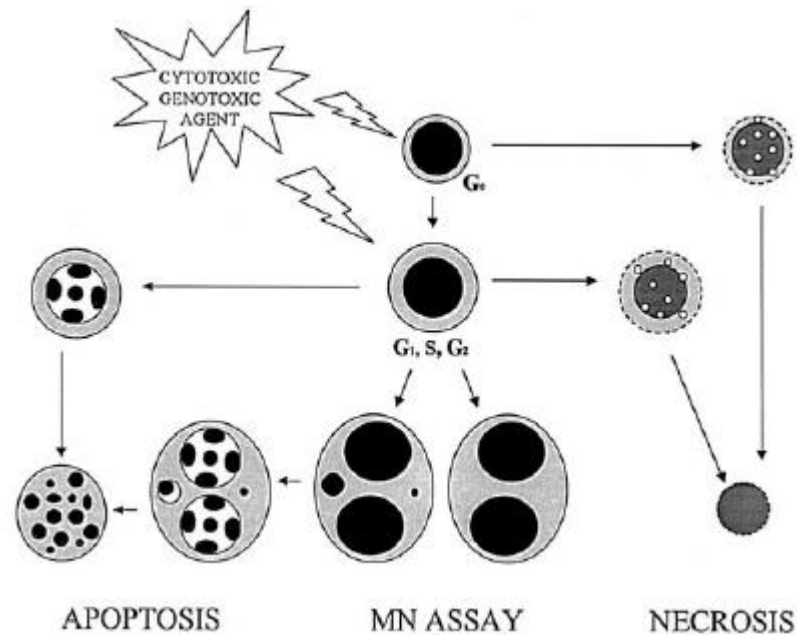


Figure 2: Effects of cytotoxic/genotoxic agent on cellular outcomes. (Fenech, 2000)

Micronuclei (MN) tests and nuclear abnormalities (notched, blebbed, lobbed, budding, fragmenting nuclei, and binucleated cells) are regarded as high-quality indicators of cytotoxicity because fish living in polluted waters have greater frequencies of MNs (Carasco et al., 1989). MN assay is commonly used for peripheral blood cells from organs like gills, hearts, kidneys, etc. (Carrasco et al., 1990). Bioaccumulation of pesticides as well as heavy metals, like Cu, Ni, Fe, Co, Mn, Cr, and Zn, was also shown to have a significant increase in three commercially important fishes, *Channa punctatus* (murrel), *Clarias gariepinus* (catfish), and *Labeo rohita* (carp) (Javed and Usmani, 2011). As fishes easily accumulate and retain these heavy metals and pesticides through submissive phenomena, therefore, pollutants in their environment can cause a severe genotoxic effect on them (Tahir et al., 2021).

Fish have highly developed biotransformation mechanisms that help them excrete toxicants like pesticides by transforming them into more water-soluble compounds (Beyer et al., 2010).

Pesticides are absorbed and then circulated through the fish's circulatory system until they reach the liver, where they are metabolized and finally excreted through the kidney (Maxwell, 2020). Pesticides move through Phase I (oxidation, reduction, and hydrolysis events) and/or Phase II (conjugation reactions) stages of the biotransformation process once they enter the hepatocytes, which are the main sites of metabolism of xenobiotics (Banerjee and Ramaiah, 2020). The toxic effect of pesticides has been particularly marked in Phase I antioxidant enzymes, including Cytochrome P450s, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST), or non-enzymatic Phase II conjugators such as glutathione (GSH), ascorbic acid, tocopherol, etc (Benli and Celik, 2021; Zhang et al., 2005; Yang et al., 2018). Since these enzymes are responsible for drug metabolism and biotransformation, these are effective biomarkers of pesticide toxicity in fish (Santana et al., 2022).

Xenobiotics are usually reduced by a reductase or reductant such as NADPH in a one-electron step to a xenobiotic free radical, producing ROS. These redox reactions produce superoxides at the expense of intracellular redox equivalents like NADH, glutathione, ascorbic acid, etc. (Lackner, 1988). Superoxide dismutase, glutathione peroxidase, peroxidase, and catalase are essential enzymes for detoxification of ROS in all organisms and are present in large quantities in fish tissues (DiGiulio et al., 1989). Catalase is a peroxisomal enzyme detoxifying hydrogen peroxide to molecular oxygen (O_2) and water (H_2O) while the effect of most pesticides is a general increase in detoxifying enzymes; some pollutants tend to inhibit CAT activity. High concentrations of Cu and $ZnSO_4$ have been shown to inhibit catalase activity in the liver, gill, and muscle cells of carps after 24 hrs of exposure (Radi and Matkovics, 1988). Superoxide dismutase (SOD) is a Cu-containing enzyme that catalyzes the conversion of superoxide anion radicals to hydrogen peroxide and water. Goldfish *Carassius auratus* exposed to 2,4-dichlorophenol (2,4-DCP) showed a general increase in activity of both hepatic SOD and CAT as early as 24 hrs of exposure (Zhang, 2005). Vierra et al., (2016) have also reported similar results on *Prochilodus lineatus* exposed to waters from streams in agricultural areas in Brazil, contaminated with heavy metals, herbicides, organochloride pesticides, and triazine. In most tissues, glutathione is the predominant thiol that acts as an antioxidant scavenging the ROS and as a cofactor for enzymatic processes, like conjugation of GST to the xenobiotics to form electrophilic molecules, which facilitates their reduction to simpler compounds (Lackner, 1988). The primary defense against

oxidative stress in cells is the metabolism of xenobiotics into simple compounds with intermediates linked to glutathione. Therefore, the ratio of GSH/GSSG is a good indicator of pesticide accumulation in fish (Yang et al., 2020). Sub-acute exposure to different pyrethroid pesticides has been shown to increase SOD, CAT as well as GST levels in the gills and liver of carp (Ensibi et al., 2013) and other fishes *Labeo rohita*, *O. niloticus* etc. (Ullah et al., 2014; Thenmozhi et al., 2011). Pesticides, however, may exert their toxicity by increasing ROS levels during the process of biotransformation by inhibiting antioxidant enzymes, or by the biosynthesis of these enzymes (Lushchak et al., 2018). A dose-dependent inhibition of the SOD, CAT, and GPX activity by deltamethrin has been reported in zebrafish. Also, it promotes lipid peroxidation, leading to developmental toxicity, such as body malformations (Parlak, 2018). Various studies show the effects of pesticide exposure on the antioxidant systems in different vertebrate models, including fishes (Bachattaet al., 2014; Vierra et al., 2016; Lushchak, 2011; Wang et al., 2016).

Pesticide poisoning has also been linked to failure to maintain oxidative equilibrium. According to Ayala et al., (2014), lipid peroxidation is a significant factor in the loss of cell function and cell membrane disruption under oxidative stress. Reduced cell integrity and functional alterations of receptors and enzymes are the results of lipid peroxidation (Fang et al., 2002). The bilayer of the membrane can change due to intermediates created by oxidative stress, which can also cause lipid peroxidation (Halliwell and Chirico, 1993). Due to the fact that malonaldehyde (MDA) is a by-product of lipid peroxidation, its elevated level in tissues can be utilized to assess the damage inflicted on by pesticide exposure (Soorya et al., 2013; Cong et al., 2020). The effectiveness of MDA as an indicator of oxidative stress has been demonstrated by several workers in different, organs, like gills, liver, and kidneys of fish and other vertebrates (Okhawa et al., 1979, Yang et al., 2020).

Histoarchitectural changes also represent an important tool in estimating the extent of toxic effects of pesticides/pollutants/toxicants, etc., in different organisms used for toxicological studies. Exposure of fish to sub-lethal or chronic levels of pollutants, including agropesticides, is likely to induce histopathological changes in tissues and organs, including gills, skin, liver, kidneys, as well as gonads (Rohani, 2023). For histopathological analysis, the skin and gills are favoured because they have vast surfaces that are in direct contact with the environment. The principal sites of xenobiotic metabolism and excretion, namely, the liver and kidneys,

respectively, are exposed to ingested pesticides and hence are also important for histological examinations (Bernet et al., 1999). Histopathological changes like lesions, vacuolization, necrosis, disruption of layers and integrity of membranes have been reported from these organs in fishes exposed to pesticides like dichlorvos (Velmurugan et al., 2009), chlorpyrifos (Farhan et al., 2021), diazinon (Banee et al., 2008), malathion (Bhart and Rasool, 2021), etc. The brain tissues were previously thought to be protected by the blood-brain barrier have also been shown to be affected by pesticides. The optic tectum, the part of the brain responsible for motor coordination in fish was, reported to be affected histopathologically exposed to the sub-lethal dose of Chlorpyrifos in *Channa punctacus* (Mishra and Devi, 2016) and imidacloprid in *Oreochromis niloticus* (Garawani et al., 2022) and *Pethia conchonius* (Dutta et al., 2024).

The presence of organophosphates (OPs) has been reported in invertebrates like stoneflies (*Claassenia* sp), mayflies (*Ephemerella* sp.), and caddis flies, (*Hydropsyche slosonae betteni*) that resulted in change in AChE levels (Day and Scott, 1990). Parsons (2010) has studied acute poisoning of birds ingesting rice from fields. Scavenging birds like hawks and vultures have long been known to be affected by pesticide contamination and bioaccumulation leading deaths (Shore et al., 2014). Biochemical disbalance and oxidative stress due to exposure to the sublethal dose of cypermethrin have been recorded in higher vertebrates including mammals like rats (Afolabi et al., 2019). Rabbits exposed to DDT and Sevin showed behavioural, reproductive, and morphological changes (Alban, 1967).

A major factor in the enzymatic control of xenobiotic metabolism or ROS inactivation is variations in gene expression. In fish, antioxidant responses are under the control of metabolic pathways, and changes in gene expression have an overall impact on these pathways (Ghelichpour et al., 2019). Responses to pollutants involve a cascade of gene interactions in addition to change in one or a few genes (Aardema and MacGregor, 2003). Various genes, namely Cytochrome *P450* family genes like *cyp1a*, *hsp70*, and *hsp90* are regarded as genetic biomarkers of oxidative stress and environmental pollution. *cyp450* family genes are necessary for the activation of cytochrome *P450*-dependent monooxygenases and aromatases that reduce micro-pollutants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls, polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (Lackner, 1988). HSPs are a set of highly conserved proteins that are often elevated during stress and assist in

folding and unfolding, aggregation, degradation, and transport of proteins that are caused by cellular stress (Ghelichpour et al., 2010). The changes in the expression of genes involved in antioxidant function in fish have also been used as a biomarker for pesticide contamination of water bodies (Jin et al., 2010). Different workers studied the changes in gene expression using mRNA from different tissues of pesticide exposed fishes and other vertebrates, like frogs (Gillardin et al., 2009), and rats (Chen et al., 2006). Housekeeping genes like β -actin that are not affected by pesticide contamination have been used as a standard to determine minute changes in the expression of target genes upon pesticide exposure (Megid et al., 2020; Nahas et al., 2017). ROS-scavenging enzymes, SOD and CAT are the first line of defense systems (Martínez-Alvarez et al., 2005), and *gpx* is an important antioxidant enzyme necessary for the conjugation of xenobiotics to prevent cytotoxicity through oxidative stress (Mukhopadhyay and Chattopadhyay, 2014). Pesticide-induced, down regulation of mRNA transcription of the defense genes in fish has been reported by different workers (Li et al., 2016; Gooncalak et al., 2017). Studies on Nile Tilapia (*Oreochromis niloticus*) from a pesticide-contaminated lake showed a general downregulation of *gst* and *vtg* genes in the liver (Nahas et al., 2017). Common carp exposed to indoxacarb showed an initial upregulation of *sod*, *cat* genes in the gills and kidneys, while downregulated in the liver (Ghelichpour et al., 2019). Xing et al., (2012) detected a significant decrease in the GST transcripts in common carp exposed to high concentrations of atrazine and chlorpyrifos. Downregulation of gene expression can be attributed to severe tissue damage (Liver) as reported by Megid et al., (2020) upon pyrethroid exposure in the fish, *Mugil capito*. The inhibition of transcription of *sod*, *cat*, *gst* and *gpx* has been attributed to several factors. Oxidative stress can change the activity of transcription machinery by changing the redox status of the cell. Oxidative stress can change the enzyme activity involved in the transcription or translation of mRNA. Accumulation of ROS may also damage cells irreparably and initiates apoptosis (Chen et al., 2011; Alak et al., 2017; Goncaalak et al., 2017). Thus, the activity of antioxidant enzymes depends on the susceptibility of exposed fish and the intensity and duration of pesticide exposure (Oruc and Usta, 2007), therefore, enzymatic activity and the gene expression can be good biomarkers that are extremely sensitive to environmental pollution, making them effective for evaluating the extent of contamination and toxicity.

Hence, a multi-dimensional approach with various genotoxic, antioxidant enzyme status as well as histoarchitectural indices have gained considerable interest in ecotoxicological researches and

are applicable in both field and laboratory studies to monitor the effect of pesticides on fish and other aquatic organisms (Zhang et al., 2005; Liang et al., 2013).

Section 1.2 Review of Literature

1.2.1 Pesticide Contamination of Freshwater Systems

The widespread utilization of pesticides to enhance agricultural output as well as industrial and domestic purposes leading to environmental pollution is a global issue. The application of pesticides has steadily risen since 1940s, when synthetic organic pesticides were first introduced in agricultural practices (Matthews, 2018). Asian and the South and Central American countries rank as the largest consumers of pesticides, experiencing a significant surge in pesticide usage over the past 25 years, primarily in the field of agriculture (FAO, 2019). When used appropriately, pesticides play a crucial role in safeguarding the seeds and crops from the unwanted insects, bacteria, fungi, rodents and weeds. Besides their intended effects, pesticides can also have adverse impacts on the environment, leading to soil and water contamination and bioaccumulation in non-target organisms (Amenyogbe et al., 2021). These consequences, over time, contribute to biodiversity depletion and in some instances, decline in crop yields. Freshwater ecosystems such as lakes, ponds, and the inland rivers function as natural reservoirs for surface runoff carrying pesticide residues from contaminated soil, irrigation systems, and leached groundwater. The contamination, bioaccumulation, and harmful impact of pesticides on the non-target organisms in the aquatic systems have been well-documented (Merga and Brink, 2021).

1.2.2 National Perspective

Pesticide contamination has been recorded in almost all major Indian rivers across the country (Table 1). The Ganga River Basin, one of the largest as well as most celebrated rivers in the country, covers 26% of India's land mass (861,404 sq. km) and naturally, the river passes through a large number of cities (36), towns (48), villages (thousands) and agricultural fields (Samanta, 2013). According to NGRBA, (2011) reports, about 2011 tons of pesticides accumulate annually in the Ganga River basin from various industrial establishments along the Ganga River basin including the chemical, fertilizer, and pharmaceutical industries, textile and paper mills, tanneries, oil refineries, electronic plants etc. as well as agricultural fields adjoining the river. An investigation conducted as early as 2001 has shown the residual concentration of five pesticides, namely, total-HCH, total-DDT, total-Endosulfan, Dimethoate, and Malathion in fish samples collected from various points of the river Ganga (Aktar et al., 2008). A survey undertaken in

Kanpur and the Northern India, has shown the presence of high concentrations of both organochlorine and organophosphorus pesticides in the surface and groundwater samples. High concentration of γ -HCH ($0.259 \mu\text{g L}^{-1}$) and malathion ($2.618 \mu\text{g L}^{-1}$) was detected in the surface water samples collected from the River Ganges in Kanpur. In the groundwater samples, dieldrin was also detected apart from γ -HCH and malathion. The pesticides γ -HCH, malathion, and dieldrin were present with a concentration of 0.900 , 29.835 , and $16.227 \mu\text{g L}^{-1}$, respectively (Sankararamakrishnan et al., 2003). Recently, Shah and Parveen (2023) reported the presence of 16 pesticides, including cypermethrin, methyl parathion, chlordane, heptachlor, methoxychlor, dichlorvos, and malathion, in the water of Ganga from three different regions of northern India, such as Upper Rishikesh, Middle Narora and Lower: Patna. Pesticide residues were also recorded in the tissues of fish found in Ganga due to bioaccumulation (Shah and Parveen, 2021).

Hooghly River is the lower stretch of the Ganga River that passes through West Bengal. Mondol et al., (2018) reported the presence of 16 pesticides, including 12 organochlorides, the most prevalent being δ - and β -HCH, and three organophosphates (phorate, methyl parathion, and monocrotophos) and one herbicide in the Hooghly River basin (Khuman et al., 2019).

The concentration levels and distribution patterns of persistent organochlorine pesticide (OCP) residues in both water and bed sediments collected seasonally over a two-year period in the Gomti River, a tributary of the Ganges was studied (Malik et al., 2009). Analysis of the samples from eight different sites showed various OCPs including aldrin, dieldrin, endrin, hexachlorobenzene (HCB), hexachlorocyclohexane (HCH) isomers, DDT isomers/metabolites, α and β endosulfan isomers, endosulfan sulfate, heptachlor and its metabolites, α -chlordane, γ -chlordane, and methoxychlor. In the river water and sediments, the total OCP residues ranged from 2.16 to 567.49 ng L^{-1} , and 0.92 to 813.59 ng g^{-1} , respectively (Malik et al., 2009).

The Brahmaputra is a river that crosses Northeast and Eastern India, and has been shown to contain polychlorinated biphenyls and organochlorine pesticides (Chakraborty et al., 2016). Water samples from 16 different sites of the river across North-East India showed Σ OCPs (the sum of organochlorine pesticides) present in an elevated concentration, ranging from 0.002 to $0.245 \mu\text{g L}^{-1}$ with a mean of $0.047 \pm 0.067 \mu\text{g L}^{-1}$ (Chakraborty et al., 2016). PDDTs (pentachlorodiphenyl sulfides) were also detected with significant concentrations, ranging from 0

to $0.225 \mu\text{g L}^{-1}$ (with a mean of $0.030 \pm 0.066 \mu\text{g L}^{-1}$), with o, p'-DDT exhibiting the highest concentration, while p, p'-DDD being the second highest pesticide (Chakraborty et al., 2016).

The Central Pollution Control Board (CPCB) in 2006 reported the presence of α and β endosulfan isomers in the Yamuna River in Delhi. During the pre-monsoon season, concentrations of Organochlorine pesticides (OCPs) ranging from 157.71 to 307.66 g L^{-1} were detected in the Yamuna River (Alam et al., 2017). This study also identified the presence of heavy metals like Fe, Cd, Ni, Pb, and Cr in the Yamuna basins, posing a risk of metal poisoning. Endrin aldehyde, Endosulfan sulfate, and DDT consistently had the highest percentage across all sampling sites and seasons. Total organochlorine pesticide levels ranged from $157.71 - 307.66 \text{ ng g}^{-1}$ in the pre-monsoon season to $195.86 - 577.74 \text{ ng g}^{-1}$ in the monsoon season and $306.9 - 844.45 \text{ ng g}^{-1}$ in the post-monsoon season (Pandey et al., 2011). A study was conducted on the Tapi, another major river in Central India that primarily flows through Gujarat, to detect pesticides in river water, sediment, and fish tissues. Gas chromatographic analysis of river water using flame ionization detector (FID), showed the presence of chlorpyrifos, methyl parathion, hexachlorocyclohexane (HCH), dichloro diphenyl trichloroethane (DDT), and endosulfan in water samples. Sediment samples showed endosulfan, DDT, and methyl parathion residues at 38.38 ng g^{-1} , 0.65 ng g^{-1} , and 0.77 ng g^{-1} , respectively, while Fish tissue samples exhibited the presence of endosulfan, chlorpyrifos, and methyl parathion at 101.28 ng g^{-1} , 0.392 ng g^{-1} and 3.49 ng g^{-1} , respectively (Hashmi et al., 2019). Many perennial rivers of Southern India, including Kaveri, Krishna, and Tamiraparani have been reported to be contamination with HCHs, DDTs, and endosulfan residues. Sediment samples from these rivers revealed 9.15 ng g^{-1} of HCHs and 158 ng g^{-1} DDTs. The water samples from Kaveri showed even higher concentrations, reaching up to $2,300 \text{ ng L}^{-1}$ for HCHs, $3,600 \text{ ng L}^{-1}$ for DDTs, and $15,400 \text{ ng L}^{-1}$ for endosulfan that indicated high pollution level (Patil et al., 2015; Gupta et al., 2022). In Godavari, one of the major rivers in Telangana, the surface water samples collected from 16 sites indicated the presence of Organochlorine Pesticides (OCPs) ranging from 3 to 70 ng/L , with an average of 20 ng L^{-1} for ΣHCH , while DDT was found to be ranging from non-detectable (ND) to 10 ng L^{-1} (average 3 ng L^{-1}), and 0.00 to 5 ng L^{-1} (average 1 ng L^{-1}) of endosulfans (Chakraborty et al., 2020). Furthermore, in the, surface water and sediment samples of Tamarapani River collected from 12 sampling stations over four seasons (pre-monsoon, monsoon, post-monsoon and summer) during 2008-2009, exhibited the

presence of various organochlorine pesticides, with varying pattern of contamination across different sampling seasons (Kumarasamy et al., 2012).

Pesticide contamination has also been observed in the rivers of North East India. Studies from this laboratory showed the presence of chlorpyrifos, dicofol, and ethion in the river Deomoni in the Terai region of the Darjeeling hills (Singh et al., 2015). Other rivers, such as Manza, Changa, and Tepu in the Dooars region have also been reported to be contaminated with pesticides (Singh et al., 2017; Das 2017).

Chilika Lake in India, the largest coastal lagoon in Asia and the world's second-largest, covering an area of 1100 km², has also been the subject of research regarding pesticide contamination. Between 2012 and 2016, multiple samples were analysed from three sites, namely Palur Bridge, Daya River Estuary, and Makara River. Approximately 25% of the water samples displayed the presence of organochlorinated (OC) pesticide residues among which HCH (α , γ & δ), DDD, DDE, and heptachlor were found at concentrations ranging from 0.025 to 23.4 $\mu\text{g L}^{-1}$, whereas the most prevalent organophosphates (OP) were chlorpyrifos (ranging from 0.019 to 2.73 $\mu\text{g L}^{-1}$) and dichlorvos (0.647 $\mu\text{g L}^{-1}$) (Nag et al., 2020).

Table 1: Pesticide contamination reported in major Indian river systems.

Sl. No.	Freshwater System/River	Pesticide/Contaminant Found	References
1.	Ganga	HCH, DDT, Endosulfan, Dimethoate, and Malathion γ -HCH, Dieldrin and Malathion Cypermethrin, methyl parathion, chlordane, heptachlor, methoxychlor, dichlorvos, malathion, nuarimol, tridemorph, atrazine, azinphosmethyl, binapacryl, azinphosmethyl, and dimethoate	Aktar et al., 2008. Sankararamakrishnan et al., 2003. Shah and Parveen, 2023.
2.	Hooghly	HCH, DDT, and Endosulfan. α -, β -, γ -, δ - and T- HCHs, 2,4' -DDE, 4,4' -DDE, 2,4' -DDD, 4,4' -DDD, 2,4' -DDT, 4,4' -DDT, α -endosulfan, β -endosulfan, T-endosulfans, Chlorpyrifos, Methyl parathion, Monocrotophos, Phorate, Atrazine and Butachlor	Khuman et al., 2019. Mondol et al., 2018.

3.	Gomti	Aldrin, dieldrin, endrin, HCB, HCH isomers, DDT isomers/metabolites, endosulfan isomers (α and β), endosulfan sulfate, heptachlor and its metabolites, α -chlordane, γ -chlordane, and methoxychlor.	Malik et al., 2009
4.	Brahmaputra	α -, β -, γ -, δ -HCHs, p,p'-DDD, o,p'-DDT, p,p'-DDT, Heptachlor, Aldrin, Dieldrin, Aldrin, α -Endosulfan, β -Endosulfan, PCBs and Pdl-PCBs	Chakraborty et al., 2016
5.	Yamuna	Organochlorine pesticides (OCP) and heavy metals like Fe, Cd, Ni, Pb, and Cr. 20 OCPS including Endrin aldehyde, Endosulfan sulfate, and DDT.	Alam et al., 2017 Pandey et al., 2017
6.	Gomti	Chlorpyrifos, methyl parathion, hexachlorocyclohexane (HCH), dichloro diphenyl trichloroethane (DDT), and endosulfan	Hashmi et al., 2019
7.	Kaveri	HCHs, DDTs, and endosulfan	Patil et al., 2015
8.	Krishna	HCHs, DDTs, and endosulfan	Gupta et al., 2022
9.	Godavari	HCHs, DDTs, and endosulfan	Chakraborty et al., 2020
10.	Tamarapani	DDTs, aldrin, dieldrin, cis-chlordane, trans-chlordane, and mirex while heptachlor, o,p'-DDE, dieldrin, o,p'-DDD, and mirex.	Kumarasamy et al., 2012
11.	Deomani	Chlorpyrifos, dicofol, and ethion	Singh et al., 2015
12.	Tista and Karola	DDT and PCBs	Das, 2015
14.	Chilika Lake	HCH (α , γ & δ), DDD, DDE, heptachlor, chlorpyrifos and dichlorvos	Nag et al., 2020

1.2.3 International Perspective

As pesticide contaminations and toxicity are global problems, studies suggest that more than 4 million tons of pesticides are used annually, with many water bodies around the world registering contamination well beyond their allowable limits (Syafrudin et al., 2021). The wastewater produced, containing pesticide residues, has detrimental effects on the human well-being, the ecosystem, and aquatic environments.

Several studies have been conducted in the neighbouring countries of India, including Pakistan, Bangladesh, and China, focusing on the presence of pesticides in their water systems (Uddin and Jeong, 2021; Rashid et al., 2022; Tan et al., 2021). Notably the Ganges-Brahmaputra-Meghna estuary serves as a significant drainage area, in Bangladesh. Jabber et al., (2001) reported the

presence of residues of DDT, aldrin, dieldrin, lindane, and heptachlor in various organs (muscle, liver, stomach, and egg samples) of the Ganges Perch, *Lates calcarifer* from the estuary. In Pakistan, analysis of water samples from Kabul River revealed the existence of six pesticides including chlorpyrifos, methomyl, carbaryl, carbofuran, triclosan, and caffeine in the domestic wastewater system., Significantly high content of $92 \pm 51 \mu\text{g L}^{-1}$ of caffeine originating from the high tea consumption area was found in the waste water system. The order of the other four pesticides, based on their concentrations, was found to be methomyl > chlorpyrifos > carbofuran > carbaryl. Methomyl, in particular, was detected in high concentrations (up to $8.3 \mu\text{g L}^{-1}$) causing high toxicity to water fleas (Saad et al., 2017). Indus River in Pakistan has been shown to have been contaminated with Persistent Organic Pollutants (POPs) (Ali et al., 2015). The concentration of POPs in the water ranged from 0.052 to $0.285 \mu\text{g L}^{-1}$ containing different OCPs in an order of PDDTs > PHCHs > heptachlor > chlordane > HCB > β -endosulfan. The POPs were found to range from 5.6–29.2 ng/g in the sediment samples, with the highest mean concentration recorded for PHCHs at $8.81 \pm 5.33 \text{ ng g}^{-1}$ (Ali et al., 2015).

In Guangzhou River of China, the water and sediment samples collected from three different environmental settings, namely agricultural, industrial, and unpolluted sites, during both wet and dry seasons revealed a total of 11 pesticides, five of which were present exceeding 100 ng L^{-1} . The most concentrated pesticides were dimethoate (1318 ng L^{-1}) in the surface water and quinalphos (328 ng g^{-1} dry weight) in sediments. Chlorpyrifos, acetochlor, and butachlor were the most commonly detected pesticides (Tang et al., 2018).

Ccancapa et al., (2015) reported Chlorpyrifos, diazinon, and carbendazim in the water samples collected from the Ebro River basin in Spain and demonstrated the detrimental impact of these pesticides on the first three trophic levels of aquatic life, which include fish, algae, and daphnia. Using a Risk Quotient (RQ) and ecotoxicological risk assessment, the organophosphorus and azole pesticides were shown to pose a significant risk to algae. Moreover, organophosphorus pesticides, benzimidazoles, carbamates, juvenile hormone mimics, and other pesticides were found to pose risks to invertebrates such as *Daphnia* sp. and the fish species (Ccancapa et al., 2015). Commonly used neonicotinoid pesticides in both agricultural and urban settings in this region are causing contamination in the Great Lakes, one of the world's largest freshwater systems. Hladik et al., (2018) have reported neonicotinoids, namely imidacloprid (53%),

clothianidin (44%), thiamethoxam (22%), acetamiprid (2%), and dinotefuran (1%) in the streams flowing into the Great Lakes, USA.

The African Catfish (*Clarias gariepinus*) and Nile Tilapia (*Oreochromis niloticus*) are highly favoured fish species in Egypt. As per a study conducted by Yahia and Elsharkawy (2013), fish samples from two different locations in the Nile River showed the presence of pesticide residues, e.g., trifluralin, hexachlorobenzene (HCB), polychlorinated biphenyls (PCBs), organophosphorus (OPs), and organochlorine (OCs) compounds. The presence of potentially harmful pesticides in the ecosystem poses high risk of health problems in consumers. Studies have reported high levels of organochlorine pesticides in water samples from the river Nile (Dahshan et al., 2016) which exceeded the World Health Organization's recommended limits (WHO, 1989). The study also showed OPs in these samples. Similarly, studies conducted on fish and water samples from four lagoons in Ghana, indicated contamination with pesticide residues (Essumang et al., 2009). The Chemu Lagoon displayed the highest pesticide contamination, contrasting with the Etsii Lagoon, which showed the lowest level of contamination compared to the Korle and Fosu Lagoon. The average total pesticide residues in water samples from these four lagoons, namely Chemu, Korle, Fosu, and Etsii, were 2.6384 mg L⁻¹, 0.4992 mg L⁻¹, 0.3045 mg L⁻¹, and 1.3629 mg L⁻¹, respectively. Furthermore, the average total pesticide residues in fish samples (*Sarotherodon melaanothern*) from the Fosu and Etsii lagoons were at 0.0155 mg kg⁻¹ and 0.0088 mg kg⁻¹ body weight, respectively (Essumang et al., 2009) Table 2 represents major river system, pesticide contamination and the references.

Table 2: Pesticide contamination reported in major freshwater systems around the world.

Sl. No.	Freshwater System/River	Contaminant/ Pesticide	Reference
1.	Ganges-Brahmaputra-Meghna estuary; Bangladesh.	DDT, aldrin, dieldrin, lindane, and heptachlor	Jabber et al., 2001
2.	Kabul River, Pakistan	Chlorpyrifos, methomyl, carbaryl, carbofuran, triclosan, and caffeine	Saad et al., 2017
3.	Indus River, Pakistan	PDDTs, PHCHs, heptachlor, chlordane, HCB and β -endosulfan	Ali et al., 2015
4.	Guangzhou River, China	11 pesticides including dimethoate, chlorpyrifos, acetochlor, butachlor and quinalphos.	Tang et al., 2018

5.	Ebro River Basin, Spain	Chlorpyrifos, diazinon, and carbendazim	Ccancapa et al., 2015
6.	Great Lakes, USA	Imidacloprid, clothianidin, thiamethoxam, acetamiprid, and dinotefuran	Hladik et al., 2018
7.	Nile River, Egypt	Trifluralin, HCB, PCBs, organophosphorus (OPs), and organochlorine (OCs) compounds of endrin, dieldrin, p, p'-DDD, p, p'-DDT, p, p'-DDE, Triazophos, Quinalphos, fenitrothion, Ethoprophos, chlorpyrifos, ethion, Fenamiphos, and pirimiphos-methyl	Yahia and Elsharkawy, 2013 Dahshan et al., 2016
8.	Chemu, Etsii, Korle and Fosu Lagoon, Ghana	2, 4' DDE, 4, 4' DDD, p-p' DDT, Propiconazol, Dichlorvos, Diazinon, Chlorpyrifos and Fenitrothion.	Essumang et al., 2009

1.2.4 Acute Toxicity Bioassay

Acute toxicity bioassay or Median Lethal Concentration 50 (LC₅₀) is one of the most effective methods to measure the toxic effects of a pollutant/toxicant on aquatic organisms. LC₅₀ is a statistical estimate of the concentration of toxic material in aqueous medium that kills 50 percent of the test species under experimental conditions during a specified time (Pickering and Henderson, 1966). Among different classes of pesticides, organophosphates are frequently used in agriculture because of their high insecticidal property, low mammalian toxicity, less persistence, and rapid biodegradability in the environment (Singh et al., 2009). In an acute toxicity test conducted on freshwater air-breathing catfish, *Heteropneustes fossilis* (Bloch), the LC₅₀ values for dimethoate were 3.38, 3.23, 3.08, and 2.98 mg L⁻¹ for 24, 48, 72, and 96 hrs, respectively. The fish displayed uncoordinated behaviour at higher concentrations of dimethoate (3.25 mg L⁻¹ and above), including irregular and jerky swimming, an attempt to jump out of the water, frequent surfacing and air gulping, decreased frequency of opercular movement, and abundant mucus secretion throughout the body (Pandey et al., 2009). Malathion, another widely used organophosphate, had an LC₅₀ value of 6.61 ppm at 96 hrs in *Channa punctatus*. The 96 hrs LC₅₀ of Malathion for *O. mossambicus* was determined to be 0.5925±0.0625 µg L⁻¹ (Subharaj et al., 2018), along with mild to extensive histopathological changes in the gills. The LC₅₀ of an organophosphate pesticide profenofos (*O*-4-bromo-2-chlorophenyl-*O*-ethyl *S*-propyl phosphorothioate) for the freshwater fish, *Channa punctatus* (Bloch) was found to be 2.68 µg L⁻¹ (Pandey et al., 2011). Estimated LC₅₀ values for dimethoate in the freshwater catfish,

Heteropneustes fossilis were 15.92 mg L⁻¹; 13.42 mg L⁻¹; 12.39 mg L⁻¹ and 11.34 mg L⁻¹ at 24, 48, 72, and 96 hrs of exposure, respectively (Srivastava et al., 2010). Cypermethrin a cyano-3-phenoxybenzyl ester of 2,2-dimethyl-3-(2,2-dichlorovinyl)-2,2-dimethyl cyclopropane carboxylate is a synthetic pyrethroid and a major pollutant present in agriculture and domestic runoff water that enter into the aquatic environment and pose adverse physiological effects on the aquatic organisms, especially fishes (Paravani et al., 2019). Chlorpyrifos is a widely used organophosphate insecticide. Singh et al., (2017) have determined the 96hour-LC₅₀ value of chlorpyrifos for Zebrafish, *Danio rerio* (Cyprinidae) to be 289 µg L⁻¹ and showed severe behavioural effects of the insecticide. Pyrethroids, including, cypermethrin modulate the opening of sodium channels in the central nervous system leading to hypo-polarization and hyper-excitation of the neurons thus causing neurotoxicity (Majumdar and Kaviraj, 2017). Fry of *Poecilia reticulata* exposed to different concentrations of pyrethroids, namely Cypermethrin, Lambda-cyhalothrin, and Deltamethrin for 96 hrs exhibited varying degrees of toxicity with median lethal concentration (LC₅₀) as 27.07 µg L⁻¹, 81.83 µg L⁻¹ and 31.51 µg L⁻¹, respectively (Salako et al., 2020). The reported 96hr-LC₅₀ values for the fish *Tinca tinca* exposed to various doses of deltamethrin were 6.77 and 0.63 ppm in fingerlings and larvae, respectively while the for deltamethrin 96-hour LC₅₀ for deltamethrin were 0.07 and 0.005 ppm, respectively (Hedayati et al., 2013). In a comparative study Babatunde et al., (2001) have demonstrated the 96hr-LC₅₀ of paraquat in *Oreochromis niloticus* to be 11,84 mg L⁻¹, while *Clarius gariepinus* exposed to 10 different pesticides revealed that λ-cyhalothrin was the most toxic with LC₅₀ value of 2.043 µg L⁻¹, and least toxic with a value of 10284.288 µg L⁻¹. Babutunde et al., (2021) have shown the relative toxicity in a decreasing order as follows: Lambda-cyhalothrin > Fipronil > Abamectin > cypermethrin > Deltamethrin > chlorpyrifos > carbofuran > dichlorvoss > dimethoate > paraquat.

Neonicotinoids bind to the post-synaptic nicotinic acetylcholine receptors (nAChRs) in insect brains, imitating the actions of the neurotransmitter acetylcholine (ACh). This results into prolonged activation and receptor inhibition, which ultimately cause neurotoxicity (Hladik et al., 2018; Tomizawa and Casida, 2011). The toxic effects of IMI on adult loaches (*Misgurnus anguillicaudatus*) have been studied and the LC₅₀ values were reported to be 167.7, 158.6, 147.9, and 145.8 mg L⁻¹ at 24, 48, 72, and 96 hrs, respectively, and its safety concentration (non-toxic) was 42.55 mg L⁻¹ (Xia et al., 2016). In this dissertation, Dutta et al., (2023) studied the effects of IMI on *Pethia conchoniis* and showed the LC₅₀ value of imidacloprid as 227.33 mg L⁻¹. A study

of combined toxicity of the insecticide imidacloprid (IMI), the herbicide acetochlor (ACT), and the fungicide tebuconazole (TBZ) on zebrafish showed that the 96-h LC₅₀ values of IMI, ACT, and TBZ were 276.84 mg L⁻¹, 1.52 (1.34–1.74) mg L⁻¹, and 8.16 (7.7–8.6) mg L⁻¹, respectively (Chang et al., 2020). The 24, 48, and 72-hour LC₅₀ values of imidacloprid for common carp embryos were estimated to be 12,708.8, 611, and 274.9 µg L⁻¹, respectively, and the 24,48,72, and 96-hour LC₅₀ values of imidacloprid for common carp larvae were estimated to be 93719, 21691, 2352.7, and 1292.6 µg L⁻¹, respectively, demonstrating a differential effect of the pesticide to different life stages of fish species (Islam et al., 2019). Hence, the toxic effects of pesticides have been widely studied to establish that these pesticides are harmful to non-target aquatic organisms, like fish.

1.2.5 Genotoxicity Study in Fish

Fish typically have adaptations to a wide range of changes in the aquatic environment, such as differences in salinity, temperature, and dissolved oxygen (Ali et al., 2022). On the other hand, fish suffer when xenobiotics are introduced into the aquatic environment. Because of their sensitivity, they are valuable model organisms for genotoxicological researches as well as bioindicators of pollution and contamination in aquatic environments, which change and degrade the ecosystem (Linde-Arias et al., 2008). The use of agro-pesticides combined with the discharge of sewage from cities and industries into water bodies, has led to the deposition of numerous novel organic compounds and harmful substances (Rad et al., 2022). Ecosystems and the people living in that environment are at risk of pathophysiological and genotoxic effects. Fish populations are burdened by mutations brought on by genotoxic agents (Hussain et al., 2017). Thus, development and the utilization of sensitive genotoxic assays are important for monitoring the effect of pesticides on aquatic organisms including fish.

1.2.6 Micronucleus Assay

The micronucleus (MN) assay is one of the most widely used, rapid and promising techniques for the analysis of cytogenetic damages. MN assay is the measurement of cytogenetic damages extremely useful for detecting pollution stress and load in aquatic ecosystems that may lead to the decline of specific species' population (Bolognesi, 2011). A single or extremely infrequently several supernumerary nuclear structures found in the cytoplasm different from the typical smooth, elliptically-shaped nucleus are referred to as micronuclei (Schmid 1976). A variety of

nuclear anomalies, in addition to micronuclei, including notched, blebbed, lobbed, blossoming, fragmenting, and karyorrhectic nuclei can be formed due to mitotic and meiotic defects. A "bleb" is defined as a relatively small evagination of the nuclear envelope still attached to the nucleus with a stalk, while a notch is a well-defined slit of uniform width extending to an appreciable depth into a nucleus (Carasco et al., 1900). MN assay is an ideal and cheap bio-monitoring technique that uses dividing cells from organisms to assess the genotoxic impact of pollutants/contaminants/pesticides in the field and in the laboratory conditions. Because micronuclei are more common in fish living in polluted waters, MN tests along with nuclear abnormalities are regarded as high-quality indicators of cytotoxicity (Mohanty and Samanta, 2016). The MN assay is frequently used to analyze the peripheral blood cells and organs, including kidneys, hearts, and gills. Ueda (1992) employed it for several somatic cell types as well as embryonic cells. Chlorpyrifos (O-diethyl O-3, 5, 6-trichloro-2-pyridylphosphorothioate) is one of the most commonly sold pesticides that has been found in surface waters in India. Ali et al., (2009) conducted the micronucleus test to investigate the acute genotoxic effects of chlorpyrifos in various fish tissues and have shown that the MN production is the result of induced DNA damages in freshwater teleost fish *Channa punctatus* (Kumar et al., 2010). Bioaccumulation of heavy metals like Cu, Ni, Fe, Co, Mn, Cr, and Zn has been shown to significantly increase the MN frequency in three commercially important fishes, *Channa punctatus* (murrel), *Clarias gariepinus* (catfish), and *Labeo rohita* (carp) (Javed and Usmani, 2011). Hussain et al., (2018) reported highest frequency for single micronucleus induction (50.00 ± 6.30), double micronucleus (MN) induction (14.40 ± 2.56), and even nuclear abnormalities (150.00 ± 2.92) in the fish specimens from the polluted experimental site of the Chenab River in Pakistan. The genotoxic effects of chemical compounds like mercury chloride and lead acetate were recorded in vivo using the micronucleus (MN) assay on acridine-orange (AO) stained peripheral blood erythrocytes, gill and fin epithelial cells of *Carassius auratus auratus* (Çavaş et al., 2008). Hussain et al., (2017) utilizing atomic absorption spectrophotometry showed that heavy metals, namely Cd, Cu, Mn, Zn, Pb, Cr, Sn, and Hg induced single, double micronuclei and nuclear abnormalities (NA), the frequencies of which were higher than single and double MNs in *Labeo rohita* from river Chenab in Pakistan. Ghaffar et al., (2015) studied the effects of butachlor in *Labeo rohita*, where significant increase in morphological and nuclear changes like pear shape erythrocytes, microcytes, tear shape erythrocytes, erythrocytes with micronuclei,

lobed, blebbed, and notched nuclei, and the cells with nuclear remnants has been observed. Other pesticides like malathion (S-(1,2-dicarboethoxyethyl) O, O-dimethyl phosphorodithioate) (Kumar et al., 2010), Carbosulfan (Nwani et al., 2010) have similar effects on the common freshwater teleost *Channa punctatus*. Vierra et al., (2018) reported an increase in the frequency of MN in the erythrocytes of the Neotropical fish *Prochilodus lineatus* exposed to imidacloprid for about 120 hrs with other oxidative damages. Dutta and Bahadur (2016) have reported significantly higher MNs and other nuclear abnormalities in the tea garden workers involved in insecticide handling, spraying tea leaf plucking compared to the control cohorts. There are several reports of MN assay in mouse, rats exposed to pesticides and human working in or exposed to environmental hazards (Hayashi et al., 1990, Holland et al., 2008). It is also used in cultured cells. So, MN assay is a rapid and cheap technique in genotoxicity assessment of xenobiotics.

1.2.7 Comet Assay

Comet Assay, also known as single-cell gel electrophoresis, developed by Singh et al., (1988) is a highly sensitive and cost-effective method for detecting genetic damage at the cellular level in alkaline (pH \geq 13) conditions. DNA breaks and alkaline labile sites can be quantified using this sensitive method for evaluating DNA damages in individual cells (Christofoleti, 2009). There are numerous reports of Comet assay based on in vitro and in vivo studies on fish (Hussain, 2018; Tice et al., 2000). Many cell types, such as erythrocytes, lymphocytes, gill, liver, kidney cells and cells cultured, can be used for single-cell gel electrophoresis (Collins, 2004, Jiang et al., 2023, Olive and Banath, 2006). As an indicator of genetic damage, DNA strand breaks can be identified with the comet assay. In the comet assay (SCGE), DNA migrates farther from the nucleus toward the anode in cells with greater DNA damages. The percentage of head and tail DNA has been used to quantify the DNA damage, signifying cellular damage (Imanikia et al., 2016). Ali et al., (2009) used the Comet assay to investigate the genotoxic effects of chlorpyrifos on Indian freshwater fish, such as *Channa punctatus*, in lymphocytes and gill cells. Even at sublethal doses of pesticide exposure, a time-dependent increase in DNA damage compared to the control groups was observed. Quinalphos is classified as a Class II pesticide by WHO as it is moderately harmful to humans. Genotoxic effects of sublethal doses of quinalphos on freshwater fish *Cyprinus carpio* from the South Indian River systems were also reported. The study showed a marked increase in DNA damage after treatment of Quinalphos for 7, 14, and 21 days, followed by decrease in the frequency after 9, 28, and 35 days (recovery phase) (Hemlatha et al., 2016).

Vieira et al., (2018) reported considerable DNA damage in the gill, kidney, and liver cells of South American fish *Prochilodus lineatus* exposed for 120 h to different IMI concentrations (1.25, 12.5, 125, and 1250 mg L⁻¹). It was also shown that the liver was the most affected organ presumably due to its role in the metabolism of the insecticide. Freshwater fish *Oreochromis mossambicus* exposed to three different concentrations (3ppm, 28ppm, and 56ppm) of arsenic (in the form of sodium arsenite (NaAsO₂)) for 48h, 96h, and 192 hrs revealed DNA damages in gill, liver, and blood cells in a concentration-dependent response showing heavy metal poisoning in fishes inhabiting in the polluted water (Ahmed et al., 2011). Sharma et al., (2007) studied the effect of Endosulfan, a widely used organochlorine pesticide by single-cell gel electrophoresis (comet assay) to determine the extent of single-strand DNA breaks in the freshwater teleost fish *Mystus vittatus* to show the genotoxic effect of the pesticide. Simoniello et al., (2009) have reported DNA damage in erythrocytes of *Prochilodus lineatus* due to exposure to different concentrations of Cypermethrin (0.300, 0.150, 0.075, and 0.000 µg L⁻¹) using comet assay. A number of studies have shown similar effects with other pesticides like Monocrotophos on *Tilapia mossambica* (Banu et al., 2001) and Malathion on *Channa punctacus* (Kumar et al., 2010). Large numbers of other chemicals used in day-to-day life have been reported to have adverse impacts on the organism. Industrial effluents, like boric acid (BA) and borax (BX) have been shown to have a genotoxic effect on *Danio rerio* in a concentration and time-dependent manner (Gulsoy et al., 2015).

Recently, our laboratory reported genotoxic effects on gill tissues of *Pethia conchonius* exposed to the sub-lethal concentrations (SLC) of Imidacloprid (Dutta et al., 2023) showing a gradual increase in %tail DNA and tail length (L tail) with decreasing head DNA with duration of exposure. Studies with mammalian hepatocytes have shown to induce genotoxicity by pesticides (Christofoletti et al., 2009). Earlier studies have shown genotoxic effects of cypermethrin in multiple organs (brain, kidney, liver, spleen) and tissues (bone marrow, lymphocytes) of mouse exposed to the pesticide (by intraperitoneal injection) (Patel et al., 2006). Leukocytes of floriculturists chronically exposed to various kinds of pesticides have been reported to have significant DNA damage using comet assay (Cadena et al., 2006). Thus, comet assay serves as a highly sensitive biomarker that evaluates the genotoxic potential of a chemical/xenobiotic/heavy metal/pesticide on the tissues of organisms and thus is widely used in ecotoxicological studies.

1.2.8 Biochemical marker Analysis

Globally, a variety of human activities, such as the production of wastewater effluent from households, industries, agriculture, aquaculture, and animal husbandry, are constantly contaminating aquatic bodies, such as rivers, lakes, ponds, and ultimately sea/oceans with various toxic xenobiotic compounds (Rana et al., 2020). As a result, these xenobiotics build up/accumulate in the fish's surroundings, and are consumed or ingested by them, having an array of negative effects. These include damage to the exposed cells at the molecular level that results in disruption of normal biochemical pathways and enzyme activities (Mir et al., 2014), leading to the imbalance of the cellular redox homeostasis that increases ROS and oxidative stress (Lushchak et al., 2018). The pesticide residues bioaccumulate in organisms with higher trophic levels, including humans. Thus, the consumption of fish from polluted environments poses a serious threat of health risks to humans, resulting into a wide array of diseases (Ray and Shaju, 2023)

1.2.8.1 Estimation of Antioxidant Enzyme Activity

Fish serves as an ideal model organism for ecotoxicological studies as they are particularly sensitive to the physiochemical changes in their environment caused by the introduction of xenobiotics in the form of sewage, industrial effluents, heavy metals, and agro-pesticides (Gibbons et al., 2015). The metabolism of xenobiotics into simpler compounds that can be easily excreted produce ROS that disrupts the normal oxidative balance in the cell (Lushchak et al., 2018). Pesticides cause ROS-mediated oxidative stress in fish (Yang et al., 2020). A change in the oxidative balance in the cell alters the activity of antioxidant enzymes that are usually up-regulated during oxidative stress. However, many pesticides also inhibit antioxidant enzyme activity that further intensify oxidative imbalance (Rohani, 2023). Pesticides like Chlorothalonil (Ahmed et al., 2016), Propanil (Tabassum et al., 2015) etc have shown inhibitory effects on antioxidant enzymes like SOD, GST, CAT and GPx. Similar studies have also been reported in invertebrates, rats, and human tissues (Sule et al., 2022). Common fish species like *Rita rita* and *Cyprinus carpio* procured from the river Ganga were analyzed for oxidative stress by Shah and Parveen (2023) using CAT, SOD, and GST enzymes analysis as well as histopathological changes. A significant increase in SOD, CAT, and GST activity was observed in the fish from the comparative more polluted downstream sites than the less polluted upstream riverine site.

Nwani et al., (2010) reported an increased level of SOD activity along with higher lipid peroxidation in *Cyprinus carpio* exposed to herbicide atrazine than control. Analysis of GPX, GST, SOD and CAT activities in the muscle, liver, and gills of *Notopterus notopterus* revealed a tissue-specific response for heavy metal poisoning in the river Mahanadi of Orissa (Mohanty and Samanta, 2016) and reported decrease of antioxidant enzyme activity in the muscle tissues, while in liver and gill tissues, the SOD and CAT activity increased as a result of oxidative stress; however, GPX activity was reversed. Majumdar and Kaviraj, (2017) reported the effects of cypermethrin on the hepatic enzyme (CAT, AChE, and alkaline phosphatase) activities of *O. niloticus*. In contrast to the CAT activity of the control group (13.37 ± 0.04), the cypermethrin-exposed group ($2.5 \mu\text{g L}^{-1}$) for 96 hr had a lower CAT activity (9.36 ± 0.08). Similarly, acetylcholinesterase and alkaline phosphatase levels significantly decreased in the same fishes. Fingerlings of *Labeo rohita* exposed to sublethal concentrations (0.02 mg L^{-1} ($1/5^{\text{th}}$ of LC_{50}) and 0.01 mg L^{-1} ($1/10^{\text{th}}$ of LC_{50}) of profenofos for a period of 21 days showed increased SOD and CAT activity at 7th day followed by a significant decrease on 14th and 21st day. Bojan et al., (2017) have reported significantly ($p > 0.05$) increased GST and LPO activity in the liver of the exposed group throughout the study period. Antioxidant defenses of the gold fish, *Carassius auratus*, exposed to 2,4-dichlorophenol for 40 days exhibited a steady increase in SOD and CAT activity in liver tissues, while GST was initially induced but decreased after the 20th day of pesticide exposure (Zhang et al., 2005). Enzyme extracts from the brain, gills, kidney, liver, and muscle of freshwater fish *Piaractus mesopotamicus* were examined for GST, CAT, GPx, and GR after exposure to endosulfan (ED), lambda-cyhalothrin (LC), and a combination of both for 96 hrs. Fish specimens exposed to ED showed a general increase in enzyme activity in brain, gill, and muscle tissues, whereas hepatic CAT and gill GST were reduced. On the other hand, the fish specimens exposed to LC showed tissue-specific effects (Bacchatta et al., 2014). A time-dependent increasing trend of antioxidant enzyme activity was also noted in the liver, brain, gills, and muscles tissue of *Tor putitora* exposed to Cypermethrin for 96 hrs and also reported behavioral and morphological changes along with extent of lipid peroxidation (Ullah et al., 2014). Thus, antioxidant enzymes like CAT, SOD, and GST are effective oxidative stress biomarkers and serve as a sensitive tool to evaluate the effect of environmental pollutants in fish that results in redox failure and oxidative stress in fish.

1.2.8.2 Estimation of Malonaldehyde Levels

Lipid peroxidation is one of the main causes of cells' losing their ability to operate under oxidative stress (Ayala et al., 2014). ROS ions, such as hydroxyl and hydroperoxyl radicals, can take electrons out of polyunsaturated fatty acids (PUFAs). This results in PUFA deprotonation at the double bond, which creates a lipid radical exposed to molecular oxygen, making it a reactive oxygen species (Fig 3). According to Halliwell and Chirico (1993), this reaction produces a lipid peroxide radical that oxidizes a neighboring PUFA to produce lipid hydroperoxide (LOOH) and a new lipid peroxide. Through beta-scission and hock cleavage, LOOH derived from LPO can further decompose into hundreds of secondary compounds, the aldehydes being the most notable harmful LPO secondary product (Fig 3) (Fritz and Petersen, 2011).

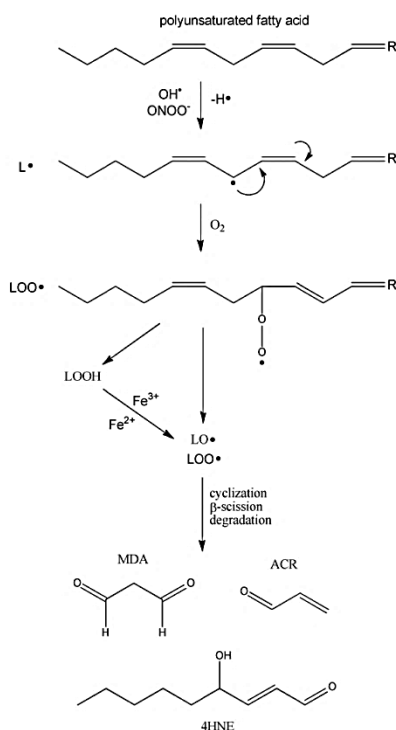


Figure 3: Lipid peroxidation of Polyunsaturated fatty acids (PUFAs) (Fritz and Petersen, 2011).

Malondialdehyde (MDA), among different aldehydes produced as secondary LPO products, is one of the most important ones. It has been shown that the MDA can serve as a useful biomarker of oxidative stress in a variety of tissues, including the kidney, liver, and gills (Okhawa et al., 1979, Ates et al., 2016). Vieira et al., (2016) studied many biomarkers in *Prochilodus lineatus* exposed to short-term *in situ* pesticide exposure in streams from agricultural areas in Southern

Brazil. Compared to untreated fish (control), the treated fish had higher MDA levels in their liver and also displayed higher level of DNA damage and erythrocytic nuclear abnormalities (ENAs) including MNi. Recent studies have shown elevated level of MDA in the adult *Danio rerio* exposed to sublethal doses of Dimethyl Phthalate as early as 24 hrs of treatment in a concentration dependent response (Cong et al., 2020). However, studies have also shown decreased levels of MDA upon exposure to certain pollutants, like diesel, is also an indicator of oxidative stress and suggests evidence of metabolism by antioxidant enzymes namely aldehyde dehydrogenase. (Gracia et al., 2017). Malonaldehyde levels in the liver, brain, and kidney of freshwater fish *Anabas testudines* exposed to sewage water for 21 days were found to be significantly higher than control groups indicating oxidative stress-induced damages in these tissues (Soorya et al., 2013). Pyrethroid pesticides like Deltamethrin, cypermethrin, and lambda-cyhalothrin also induce oxidative stress in the gills, liver, and muscles of fish elevating MDA levels leading to histopathological changes (Yang et al., 2020). Neonicotinoid pesticide, sulfoxaflor induced thiobarbituric reactive species (TBARS) levels by 36%, 32%, and 42% in *Danio rerio* at the end of 96 h of exposure of 0.87, 1.75, and 3.51 mg L⁻¹ sulfoxaflor (Benli and Celik, 2021). Male rats exposed to IMI showed a significant decrease in acetylcholinesterase, ATPase, and serum biochemicals such as creatine kinase, lactate dehydrogenase, sorbitol dehydrogenase, and alkaline phosphatase levels along with lipid Peroxidation in brain and liver tissues (Lonare, 2014).

1.2.8.3 Estimation of Acetylcholinesterase (AChE Activity)

Global pesticide sales are dominated by neuroactive pesticides such as synthetic pyrethroids and neonicotinoids. Imidacloprid is the most widely used pesticide at the moment, and synthetic neonicotinoids are the largest novel class of insecticides developed in the last 40 years (Tomizawa and Casida, 2011). Furthermore, acetamiprid, nitenpyram, and thiamethoxam—three additional neonicotinoids—have gradually gained importance as common pesticides (Demirci and Güngördü, 2020). Neonicotinoids bind to the post-synaptic nicotinic acetylcholine receptors (nAChRs) in insect brains, imitating the actions of the neurotransmitter acetylcholine (ACh). This results in prolonged activation and receptor inhibition, which is neurotoxic (Hladik et al., 2018; Tomizawa and Casida, 2011). Pyrethroids show neurotoxicity by altering the activity of sodium and potassium ion channels. Even at sub-lethal doses, these pesticides have been shown to alter

acetylcholinesterase activity, leading to altered behavioral states in the organisms (Salunke et al., 2020). Zebrafish exposed to IMI and thiamethoxam (THM) with a concentration ranging from 50 ng L⁻¹ to 50,000 ng L⁻¹ showed a general decrease in AChE activity along with a significant inhibition of transcription of key genes in γ -aminobutyric acid, dopamine and serotonin pathways such as *fosab* (FOS homolog), *glsa* (Glutaminase A), *grin1b* (Glutamate ionotropic receptor 1b), *gad1b* (Glutamic acid decarboxylase), *gad2*, *gabrg2* (Gamma-aminobutyric acid receptor), *gabral*, *slc6a1a* (Solute carrier gene family), *slc6a1b* and *abat* (aminobutyrate aminotransferase) suggesting modulation of the synaptic transmission in these fishes that can be correlated with the changes recorded in fish behaviour as well (Zhang et al., 2021). Exposure to different concentrations of cypermethrin caused significant visible behavioral changes and reduction in AChE activity in the fingerlings of *Labeo rohita*, like loss of equilibrium, hyperactivity, and mucous secretion, while fishes in the control group were free from such behavioural changes indicating that Cypermethrin has inhibitory activity to AChE (Tiwari et al., 2012). Topal et al., (2016) reported a significant decrease of AChE activity in the brain of rainbow trout exposed to 6.75 μ g L⁻¹ of chlorpyrifos for 72 and 96 hrs ($p < 0.05$), the reduction being almost half that of the control group. Neurotoxicity of IMI has also been documented in mature tilapia *Oreochromis mossambicus* (Salunke et al., 2020). Recently, Dutta et al., (2023) showed a significant decrease in AChE activity along with behavioural alterations in *Pethia conchonius* exposed to three sub-lethal concentrations of IMI. This can lead to disruption of synaptic transmission and affects the general behaviour of the fish as well. Similar results have been reported in other non-vertebrate organisms, such as crustaceans like aquatic *Daphnia magna* and terrestrial isopod *Porcellio scaber*, where cholinesterase activity along with other antioxidant enzymes was significantly reduced (Jemec et al., 2009). With the help of Ca²⁺ imaging experiments on human neurons, Loser et al., (2021) reported that imidacloprid metabolite-desnitro-IMI (DN-IMI) was found to be a potent agonist of important human nAChR subtypes $\alpha 7$, $\alpha 3\beta 4$, and $\alpha 4\beta 2$ (high-sensitivity variant) with similar potency as nicotine. Thus, acetylcholinesterase activity can serve as a sensitive biomarker for assessing the neurotoxic effects of xenobiotics in non-target aquatic organisms like fish.

1.2.9 Antioxidant Gene Expression Study in Fish

Oxidative stress can be triggered by ROS, such as hydroxyl radicals (HO•), superoxide anions (O₂•-), and perhydroxy radicals (HOO-), as well as Reactive Nitrogen Species (RNS), such as

nitric oxide (NO) (Wang et al., 2016). Inadequate antioxidant defense or an excess of free radicals can also result in the development of oxidative stress (Jin et al., 2020). Ecotoxicological tests for pesticides can also be done with more representable results at the level of gene expression to understand the molecular basis of upregulation or downregulation of enzyme activity upon pesticide exposure. Molecular studies showed a concentration and time-dependent differential regulation of antioxidant genes, *sod*, *cat*, *gpx*, and *gst* in liver of adult zebra fish exposed to Dimethyl Phthalate (DMP) Cong et al., 2020). Cong et al., also demonstrated that following 48 hrs of DMP treatment, a notable decrease in the expression of the *cat* gene was detected. After 24 hrs, low concentration of DMP did not significantly alter *cat* mRNA levels; however, after 48 hrs, SOD and GST transcription significantly increased. The *sod* and *gst* expression were down-regulated after 96 hrs, but *sod* mRNA expression was up-regulated after 24 and 48 hrs at both medium and high concentrations of DMP. Another study on fish from the river polluted with oil hydrocarbon spillage showed a general induction in the levels of *cat*, *sod*, *gpx*, *gr*, and *gst* expression in the skin, liver, and gills of *E. morio* and *S. canaliculatus* (Afifi et al., 2017). Ghelichpour et al., (2019) reported the effects of chronic exposure (21 Days) of common carp (*Cyprinus carpio*) to indoxacarb on immune, antioxidant, and stress gene expression for three different concentrations (0, 0.75, 1.5, and 3 ppm) and the control and assessed the expression of *il-1 β* , *il-8*, *il-10*, *tnf- α* , *ifn- γ* , *sod*, *cat*, *hsp70*, *igf-i*, and *igf-ii* in the liver, kidney, and gills. In general, exposure to low concentration of indoxacarb increased the transcription of these genes to deal with primary oxidative situations, however at high concentration a reverse situation was observed as a failure of oxidative homeostasis (Ghelichpour et al., 2019). The mRNA levels of *gst* and *cyp450* genes were examined in swordtail fish, *Xiphophorus helleri* upon exposure to Triclosan a common antimicrobial agent found in household sewage waters. The gene expression of *cyp1a* was inhibited at lowest dose (0.002 mg L⁻¹) but significantly induced at highest dose (1.25 mg L⁻¹) (Liang et al., 2013). Also, the expression of *gst* mRNA showed an increasing trend at doses of 0.86, 1.23, 1.76, and 2.52 mg L⁻¹ compared to the base levels of β -*Actin* housekeeping gene used as a standard (Liang et al., 2013). Another study on rainbow trout (*Oncorhynchus mykiss*) exposed to Deltametrin and azadirachtin showed a general downregulation of *sod*, *cat* and *gpx* genes along with the enzyme activity of the genes, which was also consistent with the increasing concentration of the pesticides (Gooncalak et al., 2017). However, *hsp70* and *cyp1a* expression was upregulated in the gill and liver tissues of the fish. Zebrafish exposed to IMI and

thiamethoxam (THM) with concentrations of 50 ng L⁻¹ to 50,000 ng L⁻¹ at 14 , 21, 28 and 35 days post fertilization (dpf) showed a change in locomotor activity including speed of movement and swimming behaviour along with significant changes in the transcription of the key genes of γ -aminobutyric acid, dopamine, and serotonin pathways showing a neurotoxic effect of the pesticides (Zhang et al., 2021). Thus, mRNA levels of genes, which encode *sod*, *cat*, *gpx* and *gst* antioxidant proteins amongst others can also be used as effective biomarkers for oxidative stress analysis in fish.

Section 1.3 Objectives of the Study

1. Survey and screening of pesticide contamination in the water collected from the selected sites of river Teesta.
2. To estimate the toxicity (LC₅₀) of the relevant pesticide(s) in the resident fish *Pethia conchonius* of river Teesta.
3. To assess the activity of enzymatic biomarkers in selected tissues of riverine and laboratory-reared *Pethia conchonius* exposed to relevant pesticide(s).
4. To study the extent of nuclear DNA damage in selected tissues of riverine and laboratory-reared *Pethia conchonius* exposed to relevant pesticide(s).
5. To study the expression of gene(s) related to antioxidant activity in selected tissues of riverine and laboratory-reared *Pethia conchonius* exposed to relevant pesticide(s).