Chapter 1

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Chapter 1

Section 1.1 Introduction

Agriculture holds a paramount importance in India, often called an agricultural country, due to its central role in the country's economy, culture and livelihoods (Abhilash and Singh, 2009). India's agriculture sector is the second-largest in terms of agricultural land area globally. It provides employment for approximately half of the country's population and contributed 18.3% to India's GDP in the 2022-23 (GOI, 2023). Staple crops like rice, wheat, pulses, and millets are among the extensively cultivated crops, ensuring a steady supply of food to meet the dietary requirements of billions of Indians. Pesticide use in Indian agriculture is deeply connected with the country's food security and agricultural sustainability (Shetty, 2018; Koli and Bhardwaj, 2018). To ensure consistent and increased crop yields and protect the agricultural output from the damage by pests, diseases, and weeds, Indian farmers have increasingly turned to synthetic chemical pesticides as one of the important tools in their agricultural practices. The adoption of pesticides in India began in the mid-20th century and has since witnessed a steady growth (Abhilash and Singh, 2009). These chemical formulations are designed to mitigate crop losses and maximize agricultural productivity. They serve as a critical component used by farmers to combat the myriad challenges that can affect crop health and yield. Pesticides are chemical compounds used in agriculture to control the pest infestation including insects, rodents, fungi and unwanted weeds (Russel, 2005). A vast majority of the population in India (56.7%) is engaged in agriculture and is, therefore, at risk of exposure to pesticides affecting the general health (ILO, 1996). Different health hazards which includes immune suppression, hormone disruption, diminished intelligence, reproductive abnormalities, cancer, and other chronic illness are associated with occupational exposure to pesticides (Lander eta al., 2000; Priyadarshi et al., 2001; Alavanja et al., 2004; Ji et al., 2001; Kamel and hoppin, 2004; Dutta and Bahadur, 2019). In India, the primary categories of pesticides used for pest control are insecticides, fungicides, and herbicides. After 2009-10, both the total and per hectare pesticide consumption in the country rose significantly. In 2014-15, the pesticide consumption reached 0.29 kg/ha, which is nearly 50% higher than in 2009-10. The recent surge in pesticide use is mainly due to the increased application of herbicides, as the cost of manual weed control has gone up because of rising agricultural wages (FICCI, 2015).

In 2021-22, pesticide consumption in West Bengal was 3,630.02 metric tonnes (GOI, 2022). Maharashtra had the highest total pesticide consumption, followed by Uttar Pradesh, Punjab, and Haryana (Nayak and Solanki, 2021), while West Bengal ranked 5th in total pesticide use during the same period (GOI, 2023). Despite this, India's pesticide use per hectare is considerably lower than that of China (13.06 kg/ha), Japan (11.85 kg/ha), Brazil (4.57 kg/ha), and other Latin American countries (FAOSTAT, 2017). In India, pesticide manufacturing primarily targets insects and fungicides, followed by herbicides and rodenticides. However, the share of insecticides has decreased from over 70% in 2003-04 to 39% in 2016-17, while the shares of fungicides, herbicides, and rodenticides have been steadily increasing (Subash et al., 2018). The rise in fungicide use is largely due to their applications in fruit and vegetable crops (FAOSTAT, 2017).

Fungicides are compounds used to control pathogenic fungi to prevent crop damage and fungal infection in crops (Russel, 2005). In order to ensure global food security, fungicides are often used to control fungal infestations (Strange et al., 2005) as they are a major threat to crop production (Fisher et al., 2012). In agricultural landscapes, fungicides are mainly used on fruits and vegetables and account for over 35% of the global pesticide market share (Research and Markets, 2014). This includes both synthetic and organic fungicides, which make up about 60% of all fungicides (Eurostat, 2021). In wine-growing regions, fungicides represent more than 90% of all pesticide applications (Mailly et al., 2017). The use of fungicides is expected to rise due to climate change, the development of fungicide resistance, and the emergence of invasive fungal species (Fisher et al., 2012; Hakala et al., 2005; Boxall et al., 2009). Major fungicide groups in the U.S. include dithiocarbamates, chloronitriles, demethylation inhibitors (DMIs), and strobilurins, which make up approximately 65%, 12%, 7%, and 6% of the total synthetic fungicides used, respectively (USGS, 2016). In Sweden, fungicides are the second largest group of crop protection agents after herbicides (Gustafsson et al., 2010). Globally, about four million tonnes of pesticides are used annually, with herbicides accounting for 56%, insecticides 19%, fungicides 25%, and other types like rodenticides and nematicides (FAO, 2018). In India, about 33%, of fungicides are used compared to 51% and 16% of insecticides and herbicides, respectively (FAO, 2018). According to several reports, Sulphur, mancozeb, Carbendazim, Propiconazole, etc. is extensively used as fungicides in agriculture as well as in the tea gardens of India (Gurusubramanian et al., 2008; Nayak and Solanki, 2021).

Fungicides can be applied either to seeds or directly onto crops. Some fungicides are systemic, meaning they can be absorbed into plant tissues, providing protection against pests and pathogens (McCornack and Ragsdale, 2006; Seagraves and Lundgren, 2012). When applied to seeds, fungicides are effective against soil-borne pathogens but may persist at low concentrations for several months within the plant or the rhizosphere (Bonmatin et al., 2015; Thompson, 2010; Nettles et al., 2018). For direct applications, using fungicides on three-dimensional crops such as trees and vine branches can significantly increase drift distances due to the greater nozzle height, raising the risk of fungicides being transported to nearby aquatic systems (Lefrancq et al., 2013). The risk is also increased as fungicides, unlike most other pesticides, are often applied as frequently as 10 times per season to target pests of grapes, apples, watermelons and peaches (Reilly et al., 2012; kousik et al., 2017). This disparity in consumption enhances the likelihood of chronic exposure of aquatic ecosystems to low concentrations of fungicides. Althoμgh fungicides are extensively employed, fungal diseases still account for 7-24% of yield losses in commodity crops. This can be attributed to the development of resistance to regularly used fungicides (Fisher et al., 2012; Oerke, 2006), which can partly be due to the development of resistance to commonly used fungicides (Avenot and Michailides, 2007; Brent and Hollomon, 2007; Van Den Bosch et al., 2011). Fungal pathogens can become resistant to fungicidal treatment depending on the pathogen genome and mode of action within a couple of years (Avenot and Michailides, 2007; Ma et al., 2003). This scenario could quickly lead to overuse of different types of fungicides.

Fungicides are moderately lipophilic and have a moderate to high potential for adsorption to organic carbon. As a result, fungicides can adsorb to sediments and organic surfaces in aquatic systems, as reported in several field studies (Kronvang et al., 2003; Castillo et al., 2000; Smalling et al., 2013). Similar to highly lipophilic pesticides like pyrethroid and organophosphate insecticides, the amount of adsorbed fungicides is positively correlated with the organic carbon content in particle complexes (Smalling et al., 2013). Despite their lipophilic nature, fungicides still exhibit moderate to high mobility in the soil and pore water matrix

(Reilly et al., 2012), which is highlighted by their frequent detection in water. Additionally, fungicides are moderate to highly persistent in water, with a median 50% dissipation time (DT_{50}) of 5 days, also known as the half-life. In soil, the DT_{50} is 54 days, with half-lives similar to those of herbicides and insecticides (Zubrod et al., 2019).

Frequent applications of fungicides, along with their moderate to high environmental persistence and mobility, raise concerns about the chronic exposure of biota in agricultural and urban water bodies (Knabel et al., 2014; Wightwick et al., 2012). When fungicides are applied to crops, they can adhere to soil particles. During rain or irrigation, these soil particles, along with the attached pesticides, can be washed into nearby water bodies. This surface runoff may lead to the direct contamination of aquatic ecosystems (Aamlid et al., 2020). The occurrence of pesticides has been reported in various Indian rivers, namely Krishna and Godavari (Reddy et al., 1997), Hindon (Ali et al., 2008), Gomti and Ganga (Singh et al., 2008), Yamuna (Kaushik et al., 2009) and the Bay of Bengal (Rajendran et al., 2005). In USA alone, various studies showed that more than 90 percent of water and fish samples from major streams contaminated by one and more often by several pesticides (Bortleson and Davis, 1995; Kole et al; 2001; Battaglin et al., 2011).

However, surface water exposure to fungicides has received less attention compared to insecticides and herbicides (Knabel et al., 2014). Nonetheless, various field studies have documented the presence of fungicides in both agricultural and urban surface waters worldwide (Zubrod et al., 2019). Little information are available on fungicide contamination in ponds and lakes, however, fungicide concentrations up to 6μg/L and 97μg/kg in the water and sediments have been reported in ponds and lakes, respectively in Northern Turkey (Yurtkuran and Saygi, 2013). According to several reports, chloronitriles, DMI (i.e., triazoles), and strobilurin fungicides constituted about 41% of all observations pertaining to pesticides. For dithiocarbamates, in contrast, only limited observations are available. Carboxamide and organophosphate fungicides were frequently detected, primarily in North America and Asia, respectively. In Europe, fungicides had a higher median concentration (0.96 μg/L) than herbicides (0.063 μg/L) and insecticides (0.034 μg/L) (Stehle and Schulz, 2015). Some fungicides have been found at concentrations exceeding 1 μg/L in streams during base flow, and combined concentrations of several dozen μg/L during surface runoff in the Layon catchment in

France (Lefrancq et al., 2017; Rabiet et al., 2010). In several European countries, propiconazole, boscalid, and carbendazim were among the most frequently detected pesticides (Schreiner et al., 2016). Detection frequencies in stream water and sediments often exceed 75% and can reach up to 96% in agriculture-dominated catchments (Smalling et al., 2013a, b; Ramussen et al., 2015; Lefrancq et al., 2017). Studies have also reported the presence of fungicides in streams from agricultural regions in other countries namely, Australia (Wightwick et al., 2012), China (Cao et al., 2015), Sweden (Tomlin, 2000), Germany (Berenzen et al., 2005), Thailand (Satapornvanit et al., 2004) and Malaysia (Latiff et al., 2010). Fungicides are detected in agricultural streams throμghout the year (Nanos et al., 2012) but the highest concentrations in stream water occur during the growing season (Wightwick et al., 2012) and the highest concentrations in the stream-sediments occur during the post-harvest season (Smalling et al., 2013a). This sμggests that fungicides can persist in stream systems during periods of frequent use, leading to prolonged exposure at low concentrations both locally and potentially downstream, due to sediment immobilization.

In India, Hexaconazole was found at a concentration of 0.048–0.2 μg/L in the groundwater of Bhandra district of Maharashtra (Agarwal et al., 2015). Tridemorph, binapacryl and nuarimol were found in variable concentrations along different stretches of the river Ganga. In another report, metaxyl was found at 0.083 ng/L concentration in the ponds of the Hooghly River Basin, West Bengal (Mondal et al., 2018). As very little information has been found related to fungicide contamination in Indian rivers, this study aims to detect the presence of fungicides in the river water and evaluate its adverse effects on resident fish.

In the present study, river Teesta has been selected which flows throμgh different regions including northern part of West Bengal also called North Bengal. North Bengal is characterized by its varied landscape, which includes the Gangetic plains, floodplains, and the foothills of the eastern Himalayas. The diversity in the terrain and climate has given rise to a wide range of agricultural practices, each adapted to suit the specific conditions of the area. One of the prominent forms of agriculture in North Bengal is rice cultivation. The fertile Gangetic plains are ideal for paddy cultivation, and rice is a staple crop for both local consumption and commercial purposes (Lepcha et al., 2020). In addition to rice, North Bengal also excels in the cultivation of tea. The region is renowned for its lush tea gardens, particularly in places like Darjeeling hills, the Terai and the Dooars (Bhutia, 2016). Other than these, different vegetables are also grown in the agricultural fields. River Teesta is one of the major rivers of this region. The mighty Teesta originates in the Himalayas in Sikkim, India and flows throμgh the Indian states of West Bengal and Sikkim before entering Bangladesh and eventually emptying into the Bay of Bengal. In North Bengal, it flows throμgh Darjeeling, Kalimpong, Jalpaiguri and Coochbehar. This river is an important water source for irrigation and hydropower generation in India and Bangladesh (Chaudhury et al., 2015). It also plays a vital role in the economy and ecology of the region (Mullick et al., 2011).

The ichthyofaunal diversity of this region is unique from the zoogeographical point of view (Acharjee and Barat, 2013). But major threats to riverine biodiversity of this region are suspected due to the indiscriminate use of pesticides and fertilizers in agricultural fields adjacent to the banks of Teesta, over-exploitation, flow modification and destruction or degradation of habitat (Acharjee, 2013). In spite of possible risk of fungicide contamination in this river due to the presence of widespread agricultural fields on the river bank, no study has been carried out to detect the presence of pesticides in the water of Teesta as well as the adverse effects of pesticides on its resident aquatic organisms. Our preliminary screening of water has shown the presence of fungicides in the water of River Teesta. Despite the extensive use of fungicides and their potential ecotoxicological risks to non-target aquatic organisms, their environmental fate and effects have been significantly less studied compared to insecticides and herbicides (Kohler and Triebskorn, 2013). Therefore, river Teesta has been selected to evaluate the adverse effects of fungicides on its resident fish in this study.

The extensive use of fungicides for agricultural and non-agricultural purposes has resulted in the accumulation of their toxic residues in various ground water as well as surface water sources. Even raw drinking water has been shown the presence of fungicides (Szekacs et al., 2015). Many studies have shown that fungicides are highly toxic not only to fish but also to other organisms within the food chain (Taxvig et al., 2008; Capriglione et al., 2011; Schmidt et al., 2016; De la Paz et al., 2017). Long-term exposure to fungicides induces physiological, behavioural, haematological, and histopathological alterations, biochemical changes, immune suppression, hormone disruption, reproductive abnormalities and cancers (Pinho et al., 2013; Gao et al., 2016; Du et al., 2019; Souders II et al., 2019; Cao et al., 2019; Li et al., 2019; Kumar et al., 2020). Recent studies have documented the potential impacts of fungicides on amphibians (Hooser et al., 2012) and the other non-target macroinvertebrates like *Daphnia magna* (Rasmussen et al., 2012) and macro-invertebrate communities (Liess and Ohe, 2005; Schäfer et al., 2012). Field investigations, mesocosms, *in vivo* (zebrafish and human lymphocytes), and *in vitro* studies have confirmed that various fungicides possess genotoxic, teratogenic, and endocrine-disrupting effects (Bony et al., 2008; Taxvig et al., 2008; Sisman and Türkez, 2010; Orton et al., 2011). After application, the accumulation of fungicides in the water body by surface run-off can cause DNA damage interacting with DNA in the aquatic organisms (Li et al. 2018; Jiang et al. 2018, 2019a, 2019b; Wang et al. 2018). Nowadays, the populations are at high risk of exposure to pesticides due to environmental contamination as a result of intentional/irrational usage (Kapka-Skrzypczak et al., 2011). Reports sµggest that Pesticides used in agriculture have led to increased risk for two million people worldwide (Scholz, 2012).

The acute toxicity bioassay or the LC_{50} value offers a quantitative measure of toxicity allowing researchers to compare the relative toxicity of different substances. LC stands for "Lethal Concentration" which refers to the concentration of a chemical that kills 50% of the test animals. This study helps in the safety evaluation and assesses the potential impact of toxic chemicals on the ecosystem. By measuring the extent of toxicity of a specific substance to various organisms, researchers can evaluate the potential ecological risks and design appropriate strategies for environmental protection (Arome and Chinedu, 2013). The findings obtained from acute toxicity testing are utilized as a reference for determining the appropriate dosage for long-term toxicity studies and other investigations that encompass the utilization of animals (Maheshwari and Shaikh, 2016). Several studies have reported the sub-lethal effects of fungicides on fish and other aquatic organisms (Sisodia et al., 2016; Zhu et al., 2015; Li et al., 2018; Jiang et al., 2018; Wang et al., 2018). It is important to evaluate the effects of fungicides at low sub-lethal concentrations to detect the damage at early stages as repeated sub-lethal exposure can lead to higher damages and decimate an entire community or ecosystem.

The assessment of genotoxicity in fish after exposure to pesticides is a critical area of research that examines the possible genetic damage caused by pesticide contamination to aquatic organisms (Bolognesi, 2014). Genotoxicity, the capacity to cause damage to an organism's genetic material, is a pivotal aspect of pesticide toxicity that has far-reaching implications for both the individual organism and the broader ecological balance of aquatic ecosystems (Phillips and Arlt, 2009). Understanding the genotoxic effects of pesticides on fish populations is of paramount importance not only for the conservation of aquatic biodiversity but also for the protection of human health, as fish are a vital source of nutrition for many communities worldwide. Fishes are excellent experimental organisms for mutagenic and/ or carcinogenic studies of potential contaminants present in water samples since they are capable of metabolizing, concentrating and storing water-borne pollutants. Moreover, fishes often respond to toxicants like higher vertebrates. Hence, they can be used to screen the chemicals that are potentially teratogenic and carcinogenic in humans (Al-Sabti and Metcalfe, 1995). Genotoxins in fishes cause malignancies, reduced growth, abnormal development, and reduced survival of embryos, larvae, and adults, ultimately affecting the economy of fish (Srivastava et al., 2016; Shah and Parveen, 2020; Akpoilih, 2013). Run-off of pesticide-contaminated water into the aquatic bodies can have a serious impact on the fish population and in turn human lives throµgh biomagnifications, human being the top consumer in the trophic level (Joseph and Raj, 2010).

To bring forward the threats faced by fishes due to insecticides/fungicides/pollutants/ xenobiotic exposure, this study investigates the genotoxicological responses of fungicides in *Pethia conchonius,* also known as rosy barb, which is a member of the family Cyprinidae and is native to rivers and fast-flowing streams of South Asian countries including Afghanistan, Pakistan, Nepal, India and Bangladesh. It is also a popular ornamental fish throµghout the world (Kupren et al, 2008; Prusiñska et al, 2009). This fish has been widely used in the fields of toxicology (Gill et al. 1990), genetics (Varadi et al. 1995), development biology (Kumar et al., 2020) and behavioural study (Kirankumar et al. 2003; Bhattacharya et al. 2005). In addition, reports showed that *Pethia conchonius* has a high sensitivity to pesticides (Xiao et al. 2007). It is, therefore, becoming a potential experimental fish for biological and biotechnological researches. Being easy to maintain in the laboratory condition, with high food value and high abundance in this region, *Pethia conchonius* was therefore, selected as the experimental fish for this study.

The development of sensitive biomarkers for the detection of genotoxic effects in fish has gained importance due to the growing concern over the presence of genotoxins in the aquatic environment, (Hayashi et al., 1998). The micronucleus assay, developed by Schmidt (1975), is an extensively used sensitive tool for *in vivo* and *in vitro* screening of mutagenic and genotoxic chemicals/pollutants in the environment. This assay basically involves the assessment of nuclear abnormalities (NA) (da Silva and Fontanetti, 2006; Rivero-Wendt et al., 2013) and due to its simplicity, is one of the most applicable techniques to identify genomic alterations in animals (Bolognesi and Hayashi, 2011). The occurrence of micronuclei in fish blood cells has been used as an important tool for monitoring genotoxicity in aquatic environments as well as *in vivo* experiments in the laboratory conditions (Ayllon and Garcia-Vazquez, 2000). The formation of nuclear abnormalities, such as blebbed, notched and lobbed nuclei as described by Carrasco et al. (1990), have been reported in fish erythrocytes as a result of exposure to environmental and chemical contaminants (Muranli and Güner, 2011). An increase in MN frequency has been reported as a consequence of exposure to various compounds found in the aquatic environment (Al-Sabti and Metcalfe, 1995; Minissi et al., 1996; Chaudhary et al., 2006; Klobucar et al., 2010; Lemos et al., 2007; Cavas and ErgeneGozukara, 2003, 2005).

The comet assay or single-cell gel electrophoresis (SCGE) (Singh et al. 1988), a simple and versatile method has been widely used to detect DNA damage in the individual cells. It is based on the capacity of negatively charged fragments of DNA to be pulled throμgh an agarose gel under an electric field, appearing like a ‘comet.’ It was first introduced by Ostling and Johanson in 1984. Singh et al. (1988) later modified it as Alkaline Comet Assay (Fairbairn et al., 1995). Comet Assay became very popular in the last two decades and probably is one of the most used assays for the assessment of DNA damage nowadays. It has gained popularity as this assay is less time-consuming and can be performed with any type of eukaryotic cell (Hartmann et al., 2003). This assay can be used to detect DNA damages caused by double-strand breaks, single-strand breaks, alkali labile sites, oxidative base damage, and DNA cross-linking with DNA or protein. The Comet Assay is also used to monitor DNA repair by living cells (McKelvey-Martin, 1993).

The combination of comet assay and the MN test allows the detection of both the subtle repairable effects of genotoxic agents and established mutagenicity. Numerous authors have successfully used the micronucleus test (MN) and comet assay. In addition to providing a quick molecular response, they examined the impact on a more ecologically relevant biological scale (Anderson and Wild, 1994; Kurelec, 1993; Depledge, 1994; Diekmann et al., 2004). Fish are highly suitable organisms to perform these two assays as their erythrocytes provide an easily accessible source of nucleated cells and they have been demonstrated to be sensitive to a wide range of pesticides (Ali et al., 2009; Konen and Cavas, 2008; Grisolia, 2002).

The antioxidant enzymes are often used as biomarkers in aquatic organisms, especially in fish for early detection of harmful effects as well as chronic damage caused by exposure to pesticides (Van der Oost et al., 2003; Osman et al., 2010). Antioxidants are compounds that are involved in the effective scavenging of free radicals and in suppressing the actions of reactive oxygen species (ROS). Some of such antioxidants, including glutathione, ubiquinol, and uric acid, are produced during normal metabolism in the body (Shi et al., 1999). However, there are several enzyme systems within the body that scavenge free radicals, some of the principle micronutrient antioxidants including vitamin E (α-tocopherol), vitamin C (ascorbic acid), and Beta-carotene must be supplied in the diet (Levine et al., 1996). In most situations, pesticides can directly affect organisms at cellular and molecular levels (Downs et al., 2001), disrupting the cellular redox homeostasis and increasing the generation of reactive oxygen species (ROS) and oxidative stress (Zhang et al., 2003; van der Oost et al., 2003; Ubani-Rex et al., 2017). During normal metabolic processes in aerobic cells, the ROS, such as superoxide anion and hydroxyl radicals are generated, particularly by mitochondrial oxidative phosphorylation, which can lead to oxidative stress on the cells and cause damage to them (Zhang et al., 2004). As a result of oxidative damage, hemolysis, muscle degradation, nervous system impairment, metabolic degradation, and even cell death can occur (Zhang et al., 2004). Antioxidant systems protect cells against the attack from either exogenous or endogenous sources of ROS (Lushchak, 2011; Singh et al., 2011). Fish possess both enzymatic namely, superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) and non-enzymatic antioxidants, such as reduced glutathione and vitamin E in mammals for detoxification of free radicals (Droge, 2002; Lushchak, 2011). The antioxidant enzymes SOD, CAT and GST can be used as biomarkers of pesticide-induced oxidative stress in the aquatic organisms (Borkovi et al., 2005). SOD catalyzes the dismutation of superoxide radicals into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) and thus presents an important defense mechanism against superoxide radical toxicity (Younus, 2018). Catalase acts to dissociate hydrogen peroxide (H_2O_2) into molecular oxygen (O_2) and water (H_2O) (Olson et al., 2019) Catalase is the second most abundant enzymatic antioxidant after superoxide dismutase, which attenuates the level of ROS that ubiquitously accompany pathological disorders such as aging, cataract, cancer, nutritional deficiency, atherosclerosis, and diabetes (Vendemiale et al., 1999). Glutathione S-transferases (GSTs) are multifunctional enzymes that are responsible for the metabolism and detoxification of both physiological substances and xenobiotics including pesticides, carcinogens and reactive intermediates (Sheehan et al., 2001). The primary activity of GSTs is catalyzing the thiol addition of the reduced glutathione to organic compounds throµgh their electrophilic centers to produce more water-soluble conjugates which would facilitate their easy elimination. GST also combats oxidative stress damage by GSH-dependent peroxidase activity and conjµgate reactive α-β-unsaturated aldehydes produced during lipid membrane peroxidation (Hayes and Pulford, 1995). The α-β-unsaturated aldehydes are highly reactive and genotoxic which are formed as a result of the beta-scission of lipid alkoxy radicals which are produced from the reaction of carbon-centered lipid radicals with molecular oxygen during oxidative stress (Esterbauer et al., 1992; Schauer et al., 1990).

Lipid peroxidation (LPO) is another common consequence of oxidative stress. A lipid radical (L•) is generated when certain ROS, such as hydroxyl and hydroperoxyl radicals, extract electrons from polyunsaturated fatty acids (PUFAs). This leads to deprotonation at the double bond of PUFAs and the introduction of molecular oxygen. As a result of this process, a lipid peroxide radical (LOO•) is formed, which oxidizes a nearby PUFA, resulting in a lipid hydroperoxide (LOOH) on LOO• and a new L• (Halliwell and Chirico, 1993). LOOH resulting from LPO can further decompose into hundreds of secondary products with aldehydes as the most prominent deleterious secondary products (Fritz and Petersen, 2013). Among the different secondary products of LPO, the malondialdehyde (MDA), propanal, hexanal, acrolein, and 4-hydroxynonenal (4-HNE) are main products. Their negative effects on biological systems have been extensively studied in human cell lines and rats (Abd EL, 2012; Fritz and Petersen, 2013).

Malondialdehyde (MDA) is the most frequently used biomarker for lipid peroxidation which represents the secondary lipid peroxidation product with the thiobarbituric acid reactive test. MDA is the final product of lipid peroxidation. The concentration of MDA is a measure of free radical damage to lipids (Moore and Roberts, 1998). MDA is one of the most commonly used biomarkers for oxidative stress due to its ease of measurement in biological samples (Draper and Hadley, 1990; Giustarini et al., 2009; Tsikas, 2017). Elevated level of MDA and lipid peroxidation (LPO) have been widely reported in various fish species exposed to environmental pollutants, both under natural conditions and in controlled experiments (Cheng et al., 2021; Dragun et al., 2017; Felício et al., 2018; Li et al., 2018; Mohanty and Samanta, 2016; Sehonova et al., 2019; Singh et al., 2019; Sumi and Chitra, 2019; Zhang et al., 2017). Consequently, measuring antioxidant enzyme activity and MDA level is a common and effective method for assessing oxidative stress caused by fungicides (Oliveira et al., 2009; Tagliari et al., 2004; Zhang et al., 2012).

Another way to measure fungicide-induced oxidative stress is to quantify the transcript abundance of genes, including *sod, cat* and *gpx*. The transcripts of *sod, cat*, and *gpx* encode the corresponding antioxidant enzymes, which cope with excess free radicals (Zhang et al., 2016). The induction of downstream phase II metabolic enzymes and antioxidant proteins/enzymes offers cellular protection under oxidative stress (Osburn et al., 2008). Antioxidants such as N-acetylcysteine (NAC) can hinder antiapoptic genes (McGowan et al., 1996), sμggesting that excessive oxidative stress may induce cell apoptosis. Apoptosis is a natural mechanism that removes the undesired/ damaged cells from the body and plays an important role in development and tissue homeostasis (Burke, 2017). Pro-apoptotic genes such as bcl2 associated X protein (bax), anti-apoptotic genes like b-cell lymphoma/leukemia-2 (bcl2), and the apoptotic proteins downstream of the cascade, for example, apoptotic protease activating factor-1 (apaf1), caspase 3 (casp3), caspase 9 (casp9) are the most common genes involved in the mitochondrial apoptotic pathway. The bcl2/bax ratio regulates the release of mitochondrial cytochrome c. The cytochrome c interacts with apaf1, which directly results in the activation of casp9 and casp3, inducing apoptosis (Hildeman et al., 2003). The regulation of apoptotic genes especially bcl-2 and caspases including caspase-9 and caspase-3 (casp3a) have already been reported during the analysis of apoptosis in *C. elegans*, *Drosophila* and mammals (Ahmad et al., 2018; Teng et al., 2019; Zhang et al., 2022; Li et al., 2019). Therefore, several studies have been conducted to get insight into the alterations of antioxidant and apoptotic gene expression levels due to fungicide exposure (Liu et al., 2013; Han et al., 2016; Jiang et al., 2019). These multidisciplinary approaches have been focused on advancing our knowledge of the intricate relationship between fish genetics and fungicides. By understanding the mechanisms of genetic damage, assessing the consequences for fish populations and exploring the implications for human consumers, this study aims to provide valuable insights into the potential environmental and health impacts of fungicide(s) used in aquatic ecosystems. This knowledge can be helpful in conservation efforts, regulatory decisions, and public health recommendations regarding fish consumption.

Section 1.2 Review of Literature

Fungicides are chemical compounds used to control or kill/prevent pathogenic fungi or their spores, which cause serious damage to crops. Fungicides are used both in plants and animals (Haverkate et al., 1969) to prevent pathogenic parasitic fungi in agriculture and to treat fungal infections in animals including human to treat infections/ spores (Russel, 2009). Due to surface run-off or drift the fungicides can contaminate soil and water bodies etc. In aquatic system, fungicide contamination can adversely affect the health of aquatic organisms by disturbing various important metabolic and physiological mechanisms (Ali et al., 2021) and also cause lethal toxicity in exposed organisms (Pazhanisamy and Indra, 2007). Fungicide toxicity can induce harmful impacts, such as mutagenesis, carcinogenesis, hematological, endocrinological, reproductive and histopathological complexities (Aktar et al, 2009; Maurya and Malik, 2016). Fungicide-induced toxicity also result in several complexities including behavioral changes (Boran et al., 2012, Stoyanova et al., 2015; Nataraj et al., 2023), histopathological alterations (Raibeemol and Chitra, 2016; Chamarthi et al., 2014; Velmurµgan et al., 2006), genotoxic impacts (Han et al., 2016; Crupkin et al., 2021), hemato-biochemical and hormonal imbalnce (Baliarsingh et al., 2023), as well as oxidative stress (Liu et al., 2013; Guo et al., 2024) in different groups of animals,, such as aquatic invertebrates, fish, rodents and human. The genotoxic as well as oxidative stress-related alterations due to fungicide(s) exposure will be discussed in detail herein.

1.2.1 Mode of action of different fungicide groups

Different fungicide groups along with chemical fungicides under each group and their mode of actions have been shown in the Table 1:

Table 1: Classification of fungicides and their mode of actions*.

Fungicide groups	**Substances**	**Mode of Actions**
Demethylation inhibitor	Cyproconazole, difenoconazole, epoxiconazole, metconazole, myclobutanil, penconazole, propiconazole, tebuconazole, tetraconazole	Block the cytochrome P450 monooxygenases (CYP)-mediated step responsible for fungal ergosterol production and thus cell wall synthesis.
Strobilurin	Azoxystrobin, kresoxim-methyl, pyraclostrobin, trifloxystrobin	Inhibits the electron transfer from cytochrome b to cytochrome c1 in the fungal mitochondrial membrane.
Dithio-carbamate	Propineb, zineb, maneb, thiram, and mancozeb	The dithiocarbamates get metabolized to isothiocyanates which react with vital thiol compounds in the fungal cell.
Benzimidazole	benomyl, carbendazim, chlorfenazole, cypendazole, debacarb, dimefluazole, fuberidazole, mecarbinzid, rabenzazole, thiabendazole	Benzimidazole fungicides inhibit microtubule assembly in fungal mitosis
Chlorothalonil		Chlorothalonil attacks fungal cell at several sites, inhibiting various enzymes and other metabolic processes in fungi and disrupting spore germination.

***NB: Adopted from** (Stenersen, 2004)

1.2.2 Occurrence of fungicides in the surface water

New generation fungicides developed since 1990 onward are systemic and improved for protection of crops from fungal infection (Gandhi et al., 2019). With the rising use of fungicides, environmental contamination has become more common globally. After fungicides are applied to agricultural fields, they can persist in the air, water, or soil and eventually enter aquatic environments throµgh runoff and soil leaching (Liu et al., 2015; Aamlid et al., 2021). Environmental assessments have found fungicides in surface water, groundwater, and water in paddy fields worldwide. In paddy fields, fungicide residues exceed 100 mg/L, while in other aquatic systems, reach up to 10 mg/L (Suárez-Serrano et al., 2010). As the use of fungicides in crop protection increases, there is growing concern about their potential impact on aquatic biodiversity as they eventually enter water systems (Sun et al., 2015; Garanzini and Menone, 2015; Edwards et al., 2016).

Local and regional field studies have recorded the extensive and global presence of fungicides in both agricultural and urban surface waters. There are several reports which show the presence of fungicides along with other pesticides in various water bodies around the world including India (Calamari et al. 1995; Kishimba et al. 2004; Chakraborty et al. 2016; Ali et al. 2016; Mitra et al. 2019; Nag et al. 2020; Hashmi et al. 2020; Ramírez-Morales et al. 2021).

1.2.2.1 Worldwide

North America: Propiconazole, a triazole fungicide used to prevent powdery mildews, rusts, leaf spots, scabs and blights has been detected as 0.15–13 µg/L in a banana plantation drainage river in Costa Rica (Castillo et al. 2006). A large number of fungicides namely, chlorothalonil (9.8-76.0µg/L), azoxystrobin (5.7-27.4µg/L), pyraclostrobin (1.5-13.7µg/L), trifloxystrobin (0.1-2.1µg/L), Propiconazole (13.2-42.3µg/L), tebuconazole (14.2-45.7µg/L), tetroconazole (4.0-10.3µg/L) and myclobutanil (3.3-16.0µg/L) have been recorded in the cropped areas of Indiana (Deb et al., 2010). Battaglin et al. (2011) reported the presence of azoxystrobin (0.163µg/L), chlorothalonil (0.031µg/L), propiconazole (0.291µg/L), pyraclostrobin (0.031µg/L), tebuconazole (0.053µg/L), tetroconazole (0.047µg/L) and trifloxystrobin (0.029µg/L), while the mean concentration of azoxystrobin, pyraclostrobin and boscalid were found as 30.6ng/L, 15.2ng/L and 22.6ng/L, respectively (Reilly et al., 2012) in different regions of the USA. In the Rainwater Basin Wetlands of Nebraska, the most prevalent fungicides, propiconazole, trifloxystrobin, pyraclostrobin and azoxystrobin were estimated to be 1.16µg/L 0.33µg/L, 1.61µg/L and 2.47µg/L, respectively (Mimbs et al., 2016).

South America: Captan, a fungicide of the group phthalamide was reported at a concentration of 0.78 µg/L in the surface water of an irrigated farming area in the region of Guaira country, Sao Paulo, Brazil, (Filizola et al., 2005). The Azoxystrobin fungicide was shown to contaminate the freshwater ecosystems in Argentina, ranging from 0.01 to 0.06 µg/L (Corcoran et al., 2020).

Europe: In several European countries, propiconazole, boscalid, and carbendazim are the most frequently detected pesticides and commonly occurred in mixtures with 2−3 herbicides (Zubrod et al., 2019)**.** Berenzen et al. (2005) reported the presence of azoxystrobin, epoxiconazole, kresoxim-methyl and tebuconazole at a concentration of 29.7 µg/L, 2.7 µg/L, 0.4 µg/L and 9.1 µg/L, respectively in a stream in an agricultural environment in Germany. In one report, Liess et al. (2005) have shown the presence of Kresoxim-methyl (0.41 µg/L), epoxiconazole (0.40 µg/L), azoxystrobin (0.46µg/L), propiconazole (0.60µg/L), fenpropimorph (0.20µg/L) and tebuconazole (0.56µg/L), while Schriever et al. (2007) reported the presence of azoxystrobin (1.43µg/L), epoxiconazole (0.52 µg/L), fenpropimorph (0.28 µg/L), kresoxim-methyl (0.46µg/L) and tebuconazole (0.85µg/L) in the streams of Braunschweig, Germany. Bereswill et al. (2012) have detected Folpet (1.1 µg/L), boscalid (0.56 µg/L), azoxystrobin (0.15µg/L), myclobutanil (0.30 µg/L), metrafenone (0.27 µg/L), dimethomorph (0.60 µg/L) and iprovalicarb (0.17 µg/L) fungicides in the vineyard streams of Germany. Tebuconazole, propiconazole and carbendazim were shown to be comparatively than the maximum residual limit which ranged from 11ng/L, 6.0ng/L and 94ng/L, respectively in the river Rhine in Koblenz, Germany (Wick et al., 2010).

In France, carbendazim along with the other fungicides are predominantly used in the agricultural area detected at 156 µg/L in Morcille catchment (Costa et al., 2019; Rabiet et al., 2010), whereas, tebuconazole, carbendazim, azoxystrobin, dimetomorph and procymidone were detected with mean concentrations of 0.2±0.3 µg/L, 0.1±0.2 µg/L, 0.08±0.09 µg/L, 3.4µg/ L and 1.3 µg/L, respectively in Lyon catchments. Van De Steene and Lambert (2008) detected propiconazole concentrations of 0.17–0.24 µg/L and 0.012–0.14 µg/L in influent and effluent of

Belgian pharmaceutical companies, respectively. In another report, clotrimazole was found from 1 to 31 ng/L in various sites of the Warta River in Poznan County (Poland) (Zgoła-Grzeskowiak et al.,2016). Casado et al. (2013) reported the presence many other potent fungicides, namely Fluconazole, ketoconazole, miconazole and clotrimazole in wastewater samples in Spain at a concentration up to 200ng/L. Fluconazole and clotrimazole were also found in the lakes of Switzerland at concentrations between 10 and 110ng/L. Propiconazole and tebuconazole were detected with concentrations of tebuconazole (1-10ng/L) being generally lower than those of propiconazole (4-40ng/L) (Kahle et al., 2008).

Azoxystrobin (0.1µg/L) was found in the four Danish experimental field sites (Jorgensen et al., 2012), while it was detected at 0.3 µg/L in streams and above 1 µg/L in water samples from agricultural regions in Sweden (Han et al. 2016). Captan, which is used to control black rot, early and late blight, and downy mildew, was detected in the river water of Greece with maximum concentrations of 260 ng/L (Konstantinou et al., 2006).

Australia: To protect crop from different fungal infections, a wide range of fungicides namely, azoxystrobin (0.02µg/L), cyproconazole (0.39µg/L), difenoconazole (0.03µg/L), fenarimol (0.2µg/L), iprodione (2µg/L), mycobutanil (0.24µg/L), pyraclostrobin (0.05µg/L), pyrimethanil (0.27µg/L), tebuconazole (0.03µg/L) and trifloxistrobin (0.08µg/L) were reported from the Horticultural-Production Catchment in Southeastern Australia (Wightwick et al., 2012) while, Oliver et al. (2012) showed the presence of fenarimol and propiconazole ranging from 0.08-10.0 µg/L and 0.02-0.53 µg/L, respectively in the Mt. Lofty regions of Australia.

Asia: The fungicides such as Clotrimazole, ketoconazole, tebuconazole, propiconazole were found up to concentrations of 4ng/L, 1ng/L, 3ng/L and 6.6ng/L, respectively in the rivers of Guangzhou (Huang et al., 2010), while, 34 mg/L of azoxystrobin was detected in another river near a company in Shanghai, China (Wang et al. 2009). In one report, the residue of azoxystrobin was detected in the paddy field water at the concentration of 0.183 mg/L, 0.035 mg/L and 0.022 mg/L after 10, 21 and 28 days of application of 506.25 g/ha of azoxystrobin, respectively in the Hangzhou province of China (Xie and Gong 2013). Chen et al. (2015) reported the presence of Triclosan (28.8ng/L), fluconazole (52.8ng/L), carbendazim (48.8ng/L), methylparaben (21.8ng/L), whereas, Guo et al. (2017) found 17.24µg/L of pyraclostrobin in the paddy fields of South China. Carbendazim (0.5 μg/L) was also detected in the receiving water around the agricultural fields after the application of 14.7 kg/ha pesticides, sμggesting a high risk for the ecosystem (Carazo-Rojas et al., 2018).

Chatupote and Panapitukkul (2005) have reported the presence of carbendazim (4.5 μg/L) in the Rattaphum catchment of Thailand. Pradhan et al. (2022) have shown contamination of Tengi River by different fungicides namely, tebuconazole, propiconazole and difenoconazole with a concentration of 512.1ng/L, 4493.1ng/L and 1620.3ng/L, respectively in Malaysia. Recently, the presence of different fungicides like, boscalid (0.022μg/L), iprodione (0.047μg/L), metalaxyl (0.030μg/L) and pyrimethanil (0.735μg/L) have been reported in the agriculturally influenced reservoir and its tributaries in Nepal (Acharya et al. 2023).

Table 2: Occurrence of different groups of fungicides in the agricultural fields and water bodies around the world.

Fungicide groups	Fungicide Names	Continents	References
Strobilurin	Azoxystrobin, kresoxim-methyl, pyraclostrobin, trifloxystrobin	Africa, Asia, Europe, North America, Australia	Berenzen et al. 2005; Bereswill et al. 2012; Liess et al. 2005, Magali et al. 2016; Maillard et al. 2012; Neumann et al. 2003, Rabiet et al. 2010; Wightwick et al., 2012
Triazole	Cyproconazole, difeno-conazole, epoxiconazole, metconazole, myclo-butanil, penconazole, propiconazole, tebuco-nazole, tetraconazole,	Asia, Africa, Europe, North America, South America, Australia	Diepens et al. 2014; Milhome et al. 2015; Berenzen et al. 2005; Liess et al. 2005; Magali et al. 2016; Neumann et al. 2002; Diepens et al. 2014
Benzimidazole	Benomyl, carbendazim, thiabendazole	Africa, Europe, North America, South America	Stehle et al. 2016; Rabiet et al. 2010; Readman et al. 1997; Sancho et al. 2010; Palma et al. 2004

Carboxamide	Boscalid, captofol	Europe, North America	Bereswill et al., 2012; Papadakis et al., 2015; Reilly et al., 2012; Smalling and Orlando, 2011
Chloronitrile	Chlorothalonil	Asia, Europe, North America	Sangchan et al. 2012; Lambropoulou et al. 2004
Dicarboximide	Iprodione, procymidone	Africa, Europe, North America,	Ludvigsen and Lode, 2001; Smalling and Orlando, 2011
Phthalamide	Captan, folpet	Africa, Asia, Europe, North America	Abbassy et al. 1999; Oh, 2009; Angelidis et al. 1996; Martinez et al., 1996

1.2.2.2 India

Very few studies have been carried out on the fungicide contamination of water, soil and air and their genotoxic effects in the different groups of animals including fish. During the 80s of the last century, Sneha and Bhimte (2012) reported the hexaconazole contamination at a concentration of 0.048–0.2 µg/L in the groundwater of Bhandara district of Maharashtra. Tridemorph, binapacryl and nuarimol were found up to concentrations of 0.092 µg/kg, 0.091 µg/kg and 0.084 µg/kg in the sediments along stretches of the river Ganga (Shah and Parveen, 2023). In a report, Mondal et al. (2018) showed metaxyl (0.083 ng/L) in the ponds of the Hooghly River Basin, West Bengal. Recent studies have shown the presence of fungicides, such as, carbendazim, azoxystrobin, fenamidone, pyraclostrobin, iprovalicarb, hexaconazole, kresoxim-methyl, triadimefon, penconazole in the sediment samples of river Godavari at a concentration of 5.6±0.01, 2.03±0.02, 4.07±0.01, 10.08±0.1, 7.07±0.01, 4.51±0.05, 3.62±0.1, 10.64±0.03 and 4.87±.03 ng/g, respectively (Manzoor et al., 2023).

1.2.3 Acute Toxicity Bioassay

Median lethal concentration 50 or LC_{50} is the exposure concentration of a toxic substance which causes death to half of the test animals. The LC_{50} gives a measure of the immediate or acute toxicity of a chemical/xenobiotic in the strain, sex, and age group of a particular animal species being tested. Acute toxicity bioassay is one of the most popular methods to measure the toxic effects of chemicals in aquatic organisms (Kokkali and van Delft, 2014).

Aquatic invertebrates: Aquatic invertebrates including cladocerans, copepods, amphipods, and mollusks are crucial to aquatic ecosystems, and research has examined the toxicity of fungicides to these organisms. Studies have consistently demonstrated that fungicides are highly toxic to aquatic invertebrates (Lin et al., 2012; Janaki Devi et al., 2013; Flores et al., 2014).

The Acute toxicity of pyraclostrobin to *Daphnia magna* at 96 h-LC_{50} was shown to be relatively low with a value of 14 mg/L (Ochoa-Acunna et al., 2009), whereas, the 96 h-LC_{50} values varied from 20-25 mg/L for pyraclostrobin and trifloxystrobin, respectively for amphipod crustacean, *Hyalellaazteca* (Morrison et al., 2013). Echeverría-Saenz et al. (2018) estimated that the acute toxicity of azoxystrobin for *Daphnia magna* from the River Madre de Dios was 2.1 mg/L. Sublethal effects of fungicides have also been studied in aquatic invertebrates. For example, exposure to azoxystrobin at concentrations of 0.026 mg/L was found to impact both respiration and reproduction in *D. magna* (Warming et al., 2009). Copepods and cladocerans have been shown to be highly sensitive to azoxystrobin, with their reduced abundance when exposed to concentrations ranging from 3 to 33 mg/L in both brackish and freshwater mesocosms (Gustafsson et al., 2010; Zafar et al., 2012). In a study, the *D. magna* embryos exhibited early developmental arrest, abnormalities in the cephalic body regions and antennae, and curved shell spine following acute exposure to 20 mg/L kresoxim-methyl, 0.6 mg/L pyraclostrobin and 0.4 mg/L trifloxystrobin (Cui et al. 2017). In the chronic toxicity test, arrays of adverse effects of strobilurins were observed such as, delayed time to first brood, low frequency of neonates per female, decreased numbers of broods, as well as reduced molting rate and body length at even lower concentrations than acute toxicity (Cui et al., 2017). Mollusks appear to be less sensitive to strobilurins compared to crustaceans. Ali et al. (2020) recently reported a higher 96-hour LC_{50} value of 790 mg/L for azoxystrobin in mollusks. Another study estimated the chronic EC_{50} for azoxystrobin to be greater than 28 mg/L for the survival, biomass, and reproduction of Lampsilis siliquoidea (Kunz et al., 2017). In L. luteola, exposure to 400 mg/L of azoxystrobin led to oxidative stress, apoptosis, and both histological and genotoxic changes in the hemolymph and digestive glands (Ali et al., 2020). Additionally, the 96-hour LC_{50} values for pyraclostrobin were 70.7 mg/L for Lymnaea stagnalis juveniles and 197.5 mg/L for adults. Pyraclostrobin is toxic to freshwater mussels (*Lampsilis siliquoidea*) at median effective concentrations (EC_{50}s) below 50 mg/L (Bringolf et al., 2007). Chronic sub-lethal toxicity tests revealed that exposure to 20 mg/L of pyraclostrobin significantly reduced the feeding rate. Additionally, *L. stagnalis* exposed directly and maternally to 30 mg/L of pyraclostrobin showed decreased hatching success, with longer hatching times for the pyraclostrobin-exposed egg masses (Fidder et al., 2016). These findings sµggest that strobilurin fungicides have detrimental effects on invertebrates such as cladocerans, copepods, and amphipods, both in acute and chronic exposures, even at environmentally relevant concentrations.

Demethylation inhibitor (DMI) fungicides, which include chemical groups such as triazoles and imidazoles, share a common mechanism of action: they inhibit the cytochrome P450 monooxygenases (CYP) involved in fungal ergosterol production and cell wall synthesis (Stenersen, 2004). While DMI fungicides generally exhibit acute toxicity to invertebrates only at high concentrations, sub-lethal effects such as changes in food processing, reduced energy reserves, and decreased growth and reproduction have been observed even at very low concentrations. Difenoconazole was reported to be lethal to shrimps *M. lanchesteri* at a concentration of 1.313 mg/L, where 5% of the population was affected by an LC_{50} value of 2.91 mg/L (Ahmad et al., 2022). In a study, the LC_{50} values of propiconazole and difenoconazole to *Procypris merus* at 96 h were reported to be 11.3 and 31.2 mg/L, respectively (Chen et al. 2022), while the 48 h LC_{50} value for difenoconazole was shown to be 0.97 mg/L in *Daphnia magna* (Herrera et al., 2023).

Another group, Benzimidazole fungicides inhibit microtubule assembly in fungal mitosis (Stenersen, 2004). Carbendazim, a benzimidazole fungicide, was reported to be moderate to highly toxic to most of the 11 invertebrate species tested by van Wijngaarden et al. (1998) among which the flatworms, oligochaetes, amphipods, and cladocerans being most sensitive. The severity of carbendazim toxicity considerably varied in different organisms, such as the 96h

LC_{50} of carbendazim was 460 μg/L in *Daphnia magna* (Canton, 1977) and 370 μg/L, in *Oncorhynchus mykiss* (Palawski and Knowles, 1986), whereas, the acute toxicity assay showed that carbendazim was moderately toxic to the earthworm *Eisenia foetida* at 14 day-LC_{50} of 8.6 mg/kg dry soil (Huan et al., 2016). In a recent study, Li et al., (2020) reported the 24-h LC_{50} value of 0.867 mg/L for carbendazim for *C. elegans*, which was relatively very toxic. Acute exposures of triclosan and carbendazim to *Daphnia magna* resulted in 48h-LC_{50} values of 856.8 mg/L and 87.6 mg/L, respectively, indicating that triclosan is less toxic than carbendazim (Silva et al., 2015).

Amphibia: The decline of amphibian population is a critical threat to global biodiversity, and pesticide pollution is seemed to be one of the major factors. Several studies investigating the impact of strobilurin fungicides on amphibians have reported mortality at concentrations relevant to field applications. Pyraclostrobin caused over 50% mortality in juvenile *Bufo cognatus* at the corn label application rates and led to 100% mortality in tadpoles at one-tenth of the corn label rate (Belden et al., 2010). The strobilurin fungicides inhibit development and produced malformations in amphibians at environmentally relevant concentrations (Hooser et al., 2012; Hartman et al., 2014). Li et al. (2016) reported the 48 h-LC_{50} values ranging of 0.61-196.59 mg/L for four strobilurins, namely, azoxystrobin, picoxystrobin, pyraclostrobin and trifloxystrobin in *Xenopus tropicalis* embryos. Investigations also showed the lethal and teratogenic effects of strobilurins on tadpole at species level (Junges et al., 2012; Wu et al., 2018). Additionally, sub-lethal concentrations of trifloxystrobin have the potential to alter the prey behavior i.e. reduced predation rates, thus indirectly altering predator-prey (eel-tadpole) interactions, which may be a sign of adverse effects on the neuronal system. The fungicide, folpet, an organochlorine phthalimide, is used as a protective, broad-spectrum fungicide against leaf spot diseases in grape vines. The 96h LC_{50} value of folpet for *Xenopus laevis* was estimated to be 2.18mg/L (Adams et al., 2021). The 120h LC_{50} values of Maneb, a dithiocarbamate fungicide were 1.966 mg/L and 0.332 mg/L for *Bufo bufo* and *Pseudepidalea viridis,* respectively (Gürkan and Hayretdağ, 2015).

Fish: Several studies have reported the toxicity of fungicides in fish. Most complete dataset for fungicide-induced toxicity in fish species has been derived from the widely used zebrafish (*Danio rerio*) model. This model is advantageous for chemical testing because of its rapid development and well described chemically induced teratogenesis (Wang et al., 2021). Sancho et al. (2010) reported toxic effect of tebuconazole, a triazole fungicide in zebrafish using lethal median bioassay. Earlier studies have shown that *D. rerio* is less sensitive to tebuconazole compared to *O. mykiss* (4.4 mg/L) and *Lepomis macrochirus* (5.7 mg/L) (Tomlin 2000). Others have also shown high tebuconazole toxicity to the fish *Cyprinus carpio* (Binsha and Binitha, 2014) and the silver catfish, *Rhamdia quelen* (Kreutz et al., 2008). The 96 h LC_{50} values of tebuconazole were 2.37, 7.2 and 1.13 mg/L in the fish *Cyprinus carpio* (Toni et al., 2011), *Cirrhinus mrigala* (Subbiah et al., 2020) and *Carassius auratus* (Tofan et al., 2023), respectively.

Based on the LC_{50} , the strobilurin fungicides can be classified as highly or moderately toxic to fish species, as LC_{50} values range from <100 to 1000 mg/L (Zubrod et al., 2019). There are several studies measuring acute toxicity for zebrafish at different life stages, spanning embryos, larvae, juveniles and adults at 3h post fertilization (hpf), 3 days post fertilization (dpf), 1 and 3 months post fertilization (mpf), respectively (Li et al., 2018; Jiang et al., 2018, 2019a, 2019b; Wang et al., 2018). According to these studies, mortality rates in larvae appears to be the highest compared to the other life stages. Jiang et al. (2018) reported the 96h LC_{50} values for azoxystrobin in larval, embryonic, juvenile and adult stages of zebrafish, viz. 0.777 mg/L, 0.810 mg/L, 0.987 mg/L and 1.08 mg/L. In different studies, Mu et al. have shown the toxic effects of difenoconazole and azoxystrobin in the zebrafish larvae, embryos and adults, (Mu et al. 2013, 2015). Acute toxicity assays of Captan (Zhou et al., 2019), carbendazim (Andrade et al. 2016), Kresoxim-methyl (Jiang et al., 2019a) and Pyraclostrobin (Jiang et al., 2019b) fungicides have been shown to be moderate to highly toxic to different developmental stages in zebrafish. Recently, Li et al. (2022) reported 96 h-LC_{50} value of 289.8μg/L of Kresoxim-methyl in zebrafish. Maneb was moderately toxic to zebrafish embryos with a 96h-LC_{50} value of 1.14 mg/L (Cao et al., 2019). Dicarboximide fungicides, Procymidone and iprodione, generally used in grapes and strawberries to prevent fungal infection, exhibited moderate toxicity to adult zebrafish with LC_{50} of 2.00 mg/L, 5.70 mg/L at 96h, respectively (Lai et al., 2021).

Genotoxic effects of triazole fungicide, propiconazole were analysed in zebrafish and Indian major carp *Labeo rohita* (Henriques et al. 2021; Hemalatha et al. 2016). Recently, Nataraj et al. (2023) determined the 96 h-LC_{50} value (4.5 mg/L) of difenoconazole in the fish *Labeo rohita* more or less similar to other species.

Another widely used fungicide captan belonging to the group phthalimide is applied on fruits and vegetables to prevent oomycetes (Stenersen, 2004) which contaminates the water bodies and adversely affect the aquatic organisms. Mayer and Ellersieck (1986) found the 96 h-LC_{50} value of captan was 0.26 mg/L for brown trout, whereas in rainbow trout was 0.38 ± 0.13 mg/L (Boran et al., 2012). Rico et al. (2011) reported that Amazonian fish appeared to be slightly less sensitive to carbendazim than temperate fish with LC_{50} values ranging between 1.64 and 4.23 mg/L, and Amazonian invertebrates were found to be significantly more resistant than their temperate counterparts, with LC_{50} values higher than 16 mg/L. The 96 h LC_{50} value for milkfish *Chanos chanos* exposed to carbendazim was 11.5 μg/L (Palanikumar et al., 2014), while for *Oncorhynchus mykiss* was 0.370 mg/L (Palawski and Knowles, 1986). The 96h-LC_{50} for thiram was reported to be 0.66 mg/L in *Cyprinus carpio* (Farsani et al., 2015).

There are a large number of studies showing wide range of toxicity of different fungicides in different fishes, such as mancozeb in *Oreochromis mossambicus* (Saha et al., 2016) and *O. niloticus* (Ibrahim et al., 2023); trifloxystrobin in bluegill, while less toxic to propiconazole (USEPA, 2007). Pyraclostrobin has been shown to be toxic to bluegill (*Lepomis macrochirus*), with a median lethal concentration (LC_{50}) of 11 mg/L (BASF, 2008) as well as to *Ctenopharyngodon idella* (Babu et al., 2019) and *Labeo rohita* (Anitha and Rathnamma, 2016). The acute toxicity bioassay reported the highly toxic nature of folpet and Folpan in *O. mykiss* (Adama, 2015).

Other Vertebrates: Larson et al. (1976) reported that high doses of mancozeb and, particularly, maneb were teratogenic in rats. Specifically, doses of 730 mg/kg for mancozeb and 770 mg/kg for maneb caused hemorrhages or malformations in the fetuses of pregnant rats. Additionally, dithiocarbamates have been associated with mutagenic and embryotoxic effects, including stillbirths, reduced survival rates, skull deformities, and shortening or absence of tails (Pilinskaia, 1970). Thiram has been reported to cause moderate acute oral toxicity in birds and reduced egg production when hens were fed at a concentration of 35μg/ml in their diet (Waible et al., 1995).

Effect on human:

Fungicides pose potential environmental and health risks (Goswami et al., 2015). Increasing use of fungicides in agriculture has led to environmental disruptions (Beketov et al., 2013) and health hazards, especially for those directly exposed (Wilson et al., 2001). Ethylenebisdithiocarbamate (EBDC), a widely used organic sulfur fungicide, can be toxic to humans when ingested, causing eye and upper respiratory tract inflammation, and allergic skin reactions (Laborie and DEdieu, 1964; Goswami et al., 2018). Sensitivity to dithiocarbamate fungicides can increase after alcohol consumption. Agrochemicals have been associated to significant risks, such as potential impacts on the human endocrine and immunological systems, as well as the development of cancer (Bhandari, 2014). EBDC fungicides decompose under high temperatures and oxidative conditions to form ethylenethiourea (ETU), which is known to be carcinogenic and goitrogenic (Graham and Hansen, 1972; Innes et al., 1969; Ulland et al., 1972). Laboratory studies by the National Research Council (NRC) have highlighted serious cancer risks, particularly for children exposed to chemical residues in processed food, with increased danger from ETU production when these fungicides are exposed to heat or when residues are present in cooked produce (McEven and Stephenson, 1979). Other fungicides tested for cancer risk by the NRC include captafol and chlorothalonil. Captafol has been reported to cause skin irritation and rashes in workers, while mercury compounds, used as seed dressers for seed-borne diseases, are highly persistent and toxic, posing risks throµgh mercury release. The World Health Organization (WHO) classifies these compounds as highly hazardous due to their persistence and potential to cause dermatitis, allergies, and permanent nervous system damage (Gujral, 1987). Some mercury compounds have been banned in various countries due to these risks. Elemental sulfur used in agriculture can lead to pollution hazards throµgh inhalation and direct consumption of fungicides on treated berries. Inhalation of pesticides during spraying also contributes to exposure. Direct exposure to fungicides can cause health issues, including dermatitis from maneb and streptocyclin, and skin irritation from captan, with residues found on workers' hands even after wearing gloves (Sharma and Kaur, 1990; Fenske, 1989). Groundwater contamination has been reported, with detectable levels of ETU in potato field wells (Neil and Williams, 1988) and minimal risk of chlorothalonil contamination in New Jersey (Winnet et al., 1990). Copper-based fungicides used in bananas have rendered soil infertile in Costa Rica (Thrupp, 1991), and oxidation of elemental sulfur has led to significant vegetation decline in lodgepole pine stands (Kennedy et al., 1988). According to WHO, in developing countries about

3,000,000 cases of pesticide poisoning and 220,000 deaths occur annually (Lah, 2011), with 2.2 million people at increased risk of exposure (Hicks, 2013). Studies over the past two decades have linked pesticide exposure to neuronal disorders, degenerative diseases, fetal growth effects, congenital anomalies, and cancer (Asghar et al., 2016). In Cameroon, metalaxyl and beauchamp have been associated with throat irritation, headaches, fatigue, skin and eye irritation, and difficulty breathing, with more cases of nose irritation (Sonchieu et al., 2018).

1.2.4 Genotoxicity Study

Genotoxicity refers to processes that alter the structure, information content, or segregation of DNA in a cell (Phillips et al., 2009). Thus, genotoxicity assay include tests that assess induced damages to DNA. Evaluation of genotoxicity is an important and early step in the safety assessment of chemicals for industrial development and regulatory purposes. The genotoxicity endpoint is frequently used in environmental monitoring in the field to describe the exposure of aquatic species to pollutant mixtures. Genotoxic indicators of toxic events and chromosomal instability include nuclear buds, micronuclei, and nucleoplasmic bridges (Luzhna et al., 2013; Fenech and Morley, 1985). In the laboratory, the genetic damages can be measured easily using a number of techniques and parameters of which the most widely used tests are Micronucleus and Comet assay.

1.2.4.1 Micronucleus Assay

A micronucleus (MN) assay is a test used in toxicological screening of potential genotoxic compounds. The assay is widely acknowledged as one of the most effective and dependable methods for detecting genotoxic carcinogens. This test is based on the formation of number of MN in treated cells (Ng et al., 2010; Fenech, 2008). In this test, MNs are formed during anaphase from chromosomal fragments or whole chromosomes that are left behind when the nucleus divides (Figure 1).

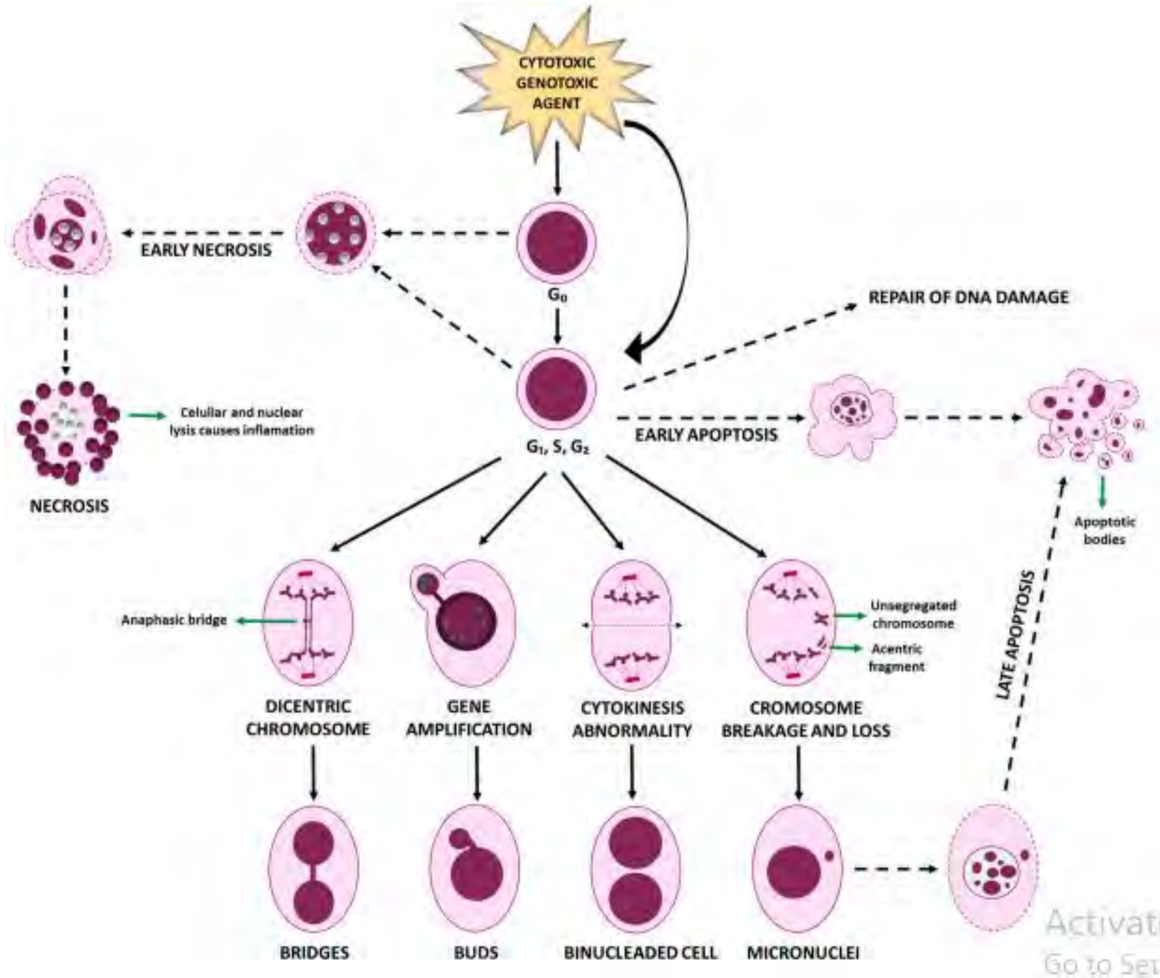

Figure 1: Schematic diagram of micronucleus (MN) and other nuclear abnormalities (NA) formation (Canedo et al., 2021)

The micronuclei assay in fish has demonstrated practical in vivo methods for genotoxicity testing and the possibility for in situ water quality monitoring (Al-Sabti and Metcalfe, 1995; Cavas and Gozukara., 2005; Nwani et al., 2010). The fish *Clarias batrachus* upon exposure to fungicide propiconazole showed increased MN frequency with increasing time and dose compared to control (Srivastava and Singh, 2015). The fungicide Propamex causes nuclear abnormalities in erythrocytes of goldfish *(Carassius auratus*) which decrease with decreasing dose (Vllasaku et al., 2017). Lizard (*Podarcis sicula*) exposed to 1.5% Thiophanate-methyl for 30–40 days showed a marked rise in the frequency of micronuclei with the extended exposure period (Capriglione et al., 2011). Bony et al. (2010) reported increased number of micronuclei upon acute and chronic exposure to azoxystrobin in the liver and germ cells of *Danio rerio.* Boscalid has higher frequency of DNA damage than the control after 20 hours of exposure to human peripheral blood mononuclear cells (Cobanoglu et al., 2019). A mixture of boscalid and pyraclostrobin has been reported to induce micronuclei formation in human lymphocytes whereas, pyraclostrobin (0.751gm/L) increased the frequency of nuclear buds (Çayır et al., 2014). The formation of micronuclei in proliferating lymphocytes was significantly increased in cells exposed to Signum, boscalid, and pyraclostrobin. Additionally, there was a notable rise in nucleoplasmic bridge formation in proliferating lymphocytes exposed to Signum and pyraclostrobin (Çayır et al., 2016). Genotoxicity measured in peripheral blood was evidenced in *Jenynsia multidentata* by the increase of micronuclei frequency when fish were exposed to 5, 10 and 100 μg/L carbendazim, while increased nuclear abnormalities (NA) were found at 0.05, 0.5, 5, 10 and 100 μg/L of carbendazim (Gotte et al., 2020). Micronuclei assay results have shown increased abnormality with increasing doses of carbendazim exposed to milkfish *Chanos chanos* (Palanikumar et al., 2014). Srivastava and Singh (2013) reported the induction of chromosomal aberrations in catfish (*Clarias batrachus*) after short-term exposure (24–96 h) to 11.43 and 22.87 mg/L of mancozeb, whereas, mancozeb induces DNA strand breaks in *Anguilla anguilla* blood cells with a low concentration of 2.9µg/L (Marques et al., 2016). Previous studies reported that mancozeb induced chromosomal aberrations and sister chromatid exchanges in lymphocytes of occupationally exposed individuals (Jablonicka et al., 1989), DNA strand breaks in rat cells (Calviello et al., 2006) as well as chromosomal aberrations and micronuclei in human lymphocytes (Srivastava et al., 2012). Srivastava and Singh (2015) have reported increased micronuclei formation in *Calrias batrachus* erythrocyte upon exposure to 1.11 and 2.23 mg/L of propiconazole. In another study, maneb caused nucleic acid degradation testifying its genotoxicity (Ben Amara et al., 2015). Significantly increased DNA damage has been reported in the human leukocytes exposed to different concentrations (0.1-25 μg/ml) of the pyraclostrobin (Cobanoglu et al. 2019). Cytotoxicity was found to be increased in exposed human lymphocytes with increasing concentrations of fungicides. The mortality, hatching, and teratogenetic rates of eggs of zebrafish treated with azoxystrobin (144h-LC_{50}=1174.9 mg/L) and picoxystrobin (144h-LC_{50}=213.8 mg/L) demonstrated significant dose and time-dependent response, (Jia et al., 2018).

1.2.4.2 Comet assay

Initially developed by Singh et al. (1988), the Single Cell Gel Electrophoresis (SCGE) or comet assay has been accepted as a rapid, simple, and sensitive visual technique for measuring DNA damage and repair, biomonitoring, and determination of genotoxicity at the level of the individual cell (Hartmann et al., 2004). It can detect chemically or physically induced single-strand breaks and alkali-labile sites in the DNA of individual cells as well as the level and inter-cellular distribution of induced DNA damage. It is used in a wide range of studies, from regulatory safety assessment of chemicals related to the genotoxicity to DNA damage and repair mechanistic studies, and from human biomonitoring to eco-genotoxicology (Lakra and Nagpure, 2009).

Bony et al. (2008) reported enhanced score of the comets in red cells of brown trout (*Salmo trutta fario*) exposed to high levels of azoxystrobin under field conditions in a river that indicated the genotoxic nature of azoxystrobin. The adult European topminnow (*Phoxinus phoxinus*) fish treated in semi-field settings to sediments containing a combination of diuron and azoxystrobin also displayed an increase in DNA damage (Bony et al., 2008). In another study, Bony et al., (2010) using comet assay and micronucleus test, showed significant genotoxic effect of azoxystrobin on the liver and germ cells of *Danio rerio* receiving acute and chronic exposure. The comet assay used to measure DNA damage revealed a significant increase in comet length with increasing exposure time and a corresponding decrease in comet head size in Lizard (*Podarcis sicula*) treated to 1.5% Thiophanate-methyl (Capriglione et al., 2011), however, Han et al., (2016) showed an adverse genotoxic effect on the liver cells in both males and females at 10µg/L for 7 days of exposure. Huan et al. (2016) reported DNA damage in non-chordates, like earth worm, *Eisenia foetida* using comet assay and showed that the olive tail moment and percentage of DNA in the tail were significantly increased with a positive dose and duration of exposure to 0.4-3.6 mg/kg dry soil of carbendazim. DNA damage in terms of % tail DNA was increased with increasing concentration of carbendazim in *Daphnia magna* (Silva et al., 2015). A. *facetus* treated with a sub-lethal concentration (50µg/L) of azoxystrobin, demonstrated DNA damage at chronic and sub-chronic exposure (Crupkin et al., 2021). *Lynmnaea luteola* showed DNA fragmentation when exposed to azoxystrobin at 0.076mg/L for 24h.The fungicide azoxystrobin showed less, non-significant DNA damage compared to control but after 24h at

0.4mg/L, there was a significant increase in DNA damage, whereas maximum damage was seen at 0.53mg/L after 96h of exposure (Ali et al., 2021).

1.2.5 Enzyme Assay

The generation of Reactive Oxygen Species (ROS) in cells and consequent oxidative stress is an almost ubiquitous effect induced by pesticide exposure in non-target species, verified by many investigators over the years (Sayeed et al., 2003; Kavitha and Rao, 2008; Toni et al., 2013). ROS are synthesised in the cells of all animals under normal circumstances, primarily within mitochondria, chloroplasts, and peroxisomes and play a crucial role in specific cellular processes. However, production and elimination rates of ROS must be in balance to guarantee cell redox homeostasis. This balance is preserved by enzymes and molecules jointly referred to as antioxidants, given their ability to protect cells' molecules and organelles from oxidation (Droge, 2002).

1.2.5.1 Antioxidant Enzyme

Superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and glutathione peroxidase (GPx) are the primary enzymatic antioxidant defences of the cell (Apel and Hirt, 2004). These antioxidant enzymes are among the most widely used biomarkers to assess the organisms' response to the toxicants, providing essential clues to predict the impact of the chemicals in both the target and non-target organisms. Various studies have shown impacts on the activity of several enzymes in response to exposure to environmental toxicants. Fungicides are one of the major environmental toxicants that have adverse impact on the activity of the antioxidative enzymes (Liu et al., 2013; Han et al., 2016; Crupkin et al., 2021). The effect of some important fungicides on the enzyme system has been reviewed below:

Strobilurin: Strobilurin fungicides have been repeatedly reported to have effects on the antioxidant enzyme systems. Trifloxystrobin, azoxystrobin and kresoxim-methyl results in an increase in the activity of catalase and peroxidase, while a decline in the activity of superoxide dismutase in *Ctenopharyngodon idella* (Liu et al., 2013). After exposure to different concentration of azoxystrobin, the male Zebra fish showed the SOD activity was significantly decreased, while in females a notable decrease was observed after 21 days. The catalase activity was also increased compared to control (Han et al., 2016), but pyraclostrobin reduced the catalase, and SOD activities (Jiang et al., 2019). In a previous study, Jiang et al. (2018) reported that the activity of CAT, SOD and GST in the azoxystrobin and picoxystrobin-treated zebrafish larvae rose considerably with concentrations at 96 h time intervals compared to the control. Zhang et al. (2018) reported the inhibition of SOD, catalase, and GST activities in zebrafish exposed to 0.1 mg/L fluoxastrobin.

Recently, Crupkin et al. (2021) showed tissue specific inhibition of different enzymes in cichlid fish (*Australoheros facetus).* SOD was inhibited in the liver and gills of juvenile at a concentration of 0.05, 5, and 50 g/L of azoxystrobin, while the inhibition in adult was noted at concentrations of 5 and 50 g/L azoxystrobin, respectively. At all concentrations of azoxystrobin examined, GST activity was found increased in gills. CAT activity was also increased at 0.5 at 50 g/L azoxystrobin in adult fish (Crupkin et al., 2021). In non-target invertebrates, like crayfish (*Astacus leptodactylus*) exposed to azoxystrobin showed increased or decreased enzymatic activity at different sub-lethal doses (828, 414, 207mg/L). In hepatopancreas, gill and muscle tissues, SOD and GPx activity significantly increased in comparison to the control. Glutathione reductase (GR) activity significantly decreased while GST activity increased especifically in hepatopancreas (Uckun and Oz., 2021). Freshwater snail (*Lymnaea luteola*) exposed to azoxystrobin in a dose-dependent study showed induction of ROS and apoptosis in hemocytes. It was shown that glutathione and superoxide dismutase decreased, while glutathione S transferase (GST) increased in the snails exposed to different sub-lethal concentrations of azoxystrobin for 96hrs (0.079, 0.04,0.53mg/L) (Ali et al., 2020). Different biochemical parameters (ROS, SOD, CAT, POD, GST) were altered upon exposure to pyraclostrobin at concentrations of 0.25 and 0.5 mg/L in Nile tilapia *Oreochromis niloticus* (Li et al., 2021).

Triazole: Li et al. (2010) reported that antioxidant parameters (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and reduced glutathione) were altered in rainbow trout, *Oncorhynchus mykiss,* exposed to 0.2-500 µg/L of propiconazole, whereas Valadas et al. (2019) reported that exposure to 425, 850 and 1700ng/L propiconazole for 96 h could induce abnormal behavior in zebrafish and significantly increased the CAT and SOD activities. Other researchers found that CAT levels decreased significantly in the liver of zebrafish after 21 days of difenoconazole treatment, which is consistent with the downregulation of editing genes (Mu et al. 2015). The study by Tabassum et al. (2016) showed that the GST, GPX and CAT enzyme activity in *Channa punctata* was inhibited exposed to propiconazole. A significant increase in CAT, GST, and SOD activity relative to control was observed. The increase were 175 % and 207% in female and male zebrafish, respectively treated with 1.84 mg/L tebuconazole (Li et al., 2020). In a study by Yeltekin, (2022), a significant reduction of superoxide dismutase and catalase activity was noted in the gill, liver, and kidneys of *Labeo rohita* compared to the control groups, while the level of GST was higher in all vital tissues of difenoconazole-treated fish (Nataraj et al., 2023).

Others: Boscalid, a carboxamide fungicide was reported to show a dose-dependent alteration of SOD, CAT, and GST activity and led to lipid peroxidation during acute exposure in *D. magna*. SOD and GST activities notably decreased and CAT activity was induced together with the increase in the concentrations of boscalid (Aksakal, 2020). Similarly, Wang et al. (2020) reported that exposure to 5-25mg/L of boscalid decreased SOD activity and increased CAT activity and induced oxidative stress in zebrafish embryos. Exposure of *C. elegans* to 0.1 μg/L carbendazim, increased the level of SOD by 10.70% compared to the control group (Li et al., 2020). GST and LDH activities were increased at concentrations of carbendazim above 4 g/L exposed to zebrafish (Andrade et al., 2016). Freshwater fish *Jenynsia multidentata* exposed to 5 μg/L of carbendazim showed inhibition of GST activity in gills (Gotte et al., 2020). In maneb-treated mice, there was a significant decrease in the activities of catalase, glutathione peroxidase, and superoxide dismutase, as well as a reduction in glutathione levels (Ben Amara et al., 2015). Grosicka-Maciag et al. (2011) found that maneb induced high apoptotic activity and oxidative stress in cultured Chinese hamster V79 cells, which was supported by a significant increase in lipid peroxidation and a decrease in the glutathione (GSH) and glutathione disulfide (GSSG) ratio (GSH/GSSG).

1.2.6 Lipid Peroxidation and oxidative Stress

Lipid peroxidation (LPO) is another common consequence of oxidative stress. Among the different secondary products of LPO, the malondialdehyde (MDA) is one of the main product. MDA is the most frequently used biomarker for lipid peroxidation (Spirlandeli et al., 2014; Grotto et al., 2009) which represents the secondary lipid peroxidation product with the thiobarbituric acid reactive test. MDA is the final product of lipid peroxidation. The concentration of MDA is a measure of free radical induced damage to lipids. MDA is widely used as an oxidative stress biomarker due to its ease of measurement in biological samples (Draper and Hadley, 1990; Giustarini et al., 2009; Tsikas, 2017). High levels of MDA and lipid peroxidation (LPO) have been consistently observed in various organs of fish exposed to environmental pollutants, both in natural settings and controlled experiments (Dragun et al., 2017; Felício et al., 2018; Li et al., 2016; Mohanty and Samanta, 2016; Sehonova et al., 2018; Singh et al., 2019). MDA content in the azoxystrobin and picoxystrobin-treated zebrafish larvae increased considerably with increasing pesticide concentrations at 96 h intervals compared to the control. (Jia et al., 2018). MDA activity and H_2O_2 concentration at 50 g/L increased in the gills of adult fish exposed to azoxystrobin (Crupkin et al., 2021). Except for the lowest dose (207 mg/L), the amount of MDA rose in a dose-dependent way in all exposed (828, 414, 207mg/L) crayfish (*Astacus leptodactylus*) (Uckun and Oz., 2020). It was shown that LPO increased in the freshwater snails *Lymnaea luteola* exposed to different sub-lethal concentrations of azoxystrobin for 96hrs (0.079, 0.04, 0.53mg/L) (Ali et al., 2020).

In *Daphnia magna,* the MDA level was altered in a concentration-dependent manner in the boscalid exposure groups and elevated in the 5 and 10 mg/L boscalid-treated groups after 48 hour of exposure (Aksakal, 2020). Similarly, Wang et al. (2020) reported that boscalid induced oxidative stress in zebrafish embryos. Suzuki et al. (2004) showed increased lipid peroxidation in rat hepatocytes upon exposure to 25uM of captan, dichlofluanid and chlorothalonil. Freshwater fish *Jenynsia multidentata* exposed to 5 μg/L of carbendazim showed an increase of Thiobarbituric acid reactive substance (TBARs) contents in gills (Gotte et al., 2020). In another study, 43.68 μg/L of carbendazim significantly induced lipid peroxidation in the fish *Chanos chanos*. (Palanikumar et al., 2014). Difenoconazole exposure induced hepatotoxicity and lipid metabolism disorders (Jiang et al. 2020) as well as can cause the disturbances in the immune pathway apoptosis (Teng et al., 2018) in the embryonic or adult zebrafish. LPO was higher in all vital tissues of difenoconazole-treated fish (Nataraj et al., 2023). Chen et al. (2022) reported increased oxidative stress in the liver tissues of *Procypris merus* when exposed to propiconazole and difenoconazole. Similarly, when zebrafish were exposed to 5 mg/L propiconazole for 96 h, propiconazole could induce oxidative damage to fish embryos and increase ROS content (Zhao et al. 2020). Li et al. (2010) reported that oxidative stress indices (reactive oxygen species, lipid peroxidation, and carbonyl protein) were altered after exposure of 0.2-500 μg/l) of propiconazole to rainbow trout, *Oncorhynchus mykiss.* Grosicka-Maciag et al., 2011 observed high apoptotic activity and high oxidative stress in Chinese hamster V79 cells induced by exposure to maneb evidenced by a statistically significant increase in lipid peroxidation as well as a decrease of glutathione (GSH) and glutathione disulfide (GSSG) ratio (GSH/GSSG). Malondialdehyde, reactive oxygen species were elevated while reduction in mitochondrial membrane potential (MMP) was also noted by exposure to pyraclostrobin in zebrafish (Jiang et al., 2019). At 0.1 mg/L concentration of fluoxistrobin in zebrafish, lipid peroxidation and DNA damage were stimulated by ROS (Zhang et al., 2018). Kara et al. (2020) reported a concentration-dependent enhancement of reactive oxygen species in the human SH-SY5Y cell line (Kara et al., 2020). With the concentrations of 0.3 and 0.4 mM of iprodione both ROS and MDA production increased in *Oncorhynchus mykiss* (Radice et al., 2001). In *Danio rerio*, pyraclostrobin was shown to be highly toxic and induced oxidative stress and DNA damage in embryos and liver cells (Zhang et al., 2017, 2020). Han et al. (2016) reported detailed toxicological effects of strobilurins in zebrafish, such as oxidative stress, genotoxicity, and impacts on early life stages. Exposure to azoxystrobin to male and female Zebra fish at different concentrations and time intervals resulted in increased ROS accumulation in livers.

Estimation of cholinesterase activity: Fish cholinesterases, such as acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), are commonly used as biomarkers for environmental contamination due to their sensitivity to a range of toxic substances (Santana et al., 2021). Several fungicides have been reported to alter cholinesterase activities in fish. Difenoconazole has been found to inhibit the cholinesterase (CHE) activity in the brain and muscle of zebrafish (Quesada et al., 2021). Studies have shown increased AChE and LDH activities in zebra fish exposed to carbendazim (Andrade et al., 2016) and AChE increased specifically in hepatopancreas of Azoxystrobin-exposed crayfish (*Astacus leptodactylus*) (Uckun and Oz., 2020). The AChE activity in the brain and muscle tissues of *A. lacustris* and the brain of *M. nigripinnis* was reported to be significantly decreased following spraying of AZX, bifenthrin, and cyproconazole in a rice field-fish culture system (Rossi et al., 2020). *Clarias batrachus* also showed a reduction in AChE activity in response to propiconazole (Srivastava and Singh, 2015).

Tebuconazole has been shown to decrease the AChE activity in *Alburnus tarichi* (2.5 M) and *Danio rerio* (4 and 6 mg/L) after 96 hours of exposure (Altenhofen et al. 2017; Yetelkin, 2022).

1.2.7 Gene expression studies in aquatic organisms

Pollution of the aquatic environment has become an alarming in global concern due to rising level of toxins (Copat et al., 2013). Aquatic organisms, including fish, accumulate pollutants directly from contaminated water and indirectly throμgh the food chain, while the bio-concentration of potentially harmful substances in aquatic organisms poses a major threat to human health. Fish are extensively used as a bio-indicator to evaluate the health of aquatic ecosystems since they are at the top of the aquatic food chain (Camargo et al., 2007). Analyses of gene expression can reveal details about pollutant exposure and possible impacts at various biological levels (Burkand et al., 2019). In the light of above, the gene expression profile offers a sensitive, quantifiable endpoint for toxicity and can act as a precursor to a particular biological endpoint.

Antioxidant genes: A number of studies have shown the impact of pollutants/toxicants/pesticides on the expression of antioxidant/apoptic/regulatory genes (Jiang et al., 2015; Li et al., 2021; Qian et al. 2018). Study showed a significant decrease of *sod, gst, cyp4* and *nrf1*expression but increased *cat* gene expression in *Daphnia magna* after 48 hours of exposure to 5 and 10 mg/L boscalid, while, chronic exposure to boscalid showed a decreased molting frequency, the number of neonates per *Daphnia*, and the number of broods per female as compared to the control groups (Aksakal, 2020). The levels of CAT, GPX and mnSOD were up-regulated in zebrafish upon exposure to 4-500 μg/L of carbendazim after 4 days (Jiang et al., 2015). Jiang et al. (2019) reported that the activities of SOD, CAT and GPX, were altered in Zebrafish due to exposure of kresoxim-methyl. Maneb induced a dose-dependent increase in mortality, decreased hatching rate, and increased notochord deformity in zebrafish at 72 and 96 h exposure to 1.0 and 10.0 μM of maneb (Cao et al., 2019). A significant decrease in the antioxidant SOD and GPX was reported by Amara et al. (2015). Exposure to azoxystrobin led the up-regulation of catalase, MAPK1, and IGFBP1 in the liver tissue of Atlantic salmon, whereas, in the muscle tissue, transferrin, IGFBP1, and TNFR were up-regulated, while CYP1A was down-regulated (Olsvik et al., 2010). Zhang et al. (2020) reported that *Superoxide dismutase*

1 and *caspase 3* mRNA transcripts decreased in 6 dpf zebrafish larvae exposed to 2.5 µM zoxamide. After iprobenfos exposure to *Oryzias javanicus,* CAT transcription increased in the liver at all concentrations ($p<0.05$). CYP1A mRNA was induced in the intestine and liver. G6PD transcription was induced in the liver but was suppressed in muscle tissues. GPx, GR, GST and SOD expression increased in the liver and intestine or in liver only (Woo et al., 2009).

Apoptotic gene: Jiang et al. (2015) have also shown the up-regulation of p53, Apaf1, Caspase-8 and the down-regulation of Bcl2, Mdm2, and Cas3 in the apoptosis pathway in zebra fish. Both kresoxim-methyl and Pyraclostrobin altered or increased the expression of different apoptic genes, such as caspase 3 (Cas3) and caspase 9 (Cas9) and reduce intracellular calcium ion (Ca2þ) concentration and mitochondrial membrane potential (MMP) (Qian et al. 2018a, Jiang et al., 2019) and can cause mitochondrial dysfunction in zebrafish. Pyraclostrobin and trifloxystrobin were found to induce mitochondrial dysfunction in *Danio rerio*, which was linked to alterations in mitochondrial complex III activity and changes in the expression of genes related to oxidative respiration and stress (Li et al., 2021). Lipid metabolism, triacylglyceride, and cholesterol contents along with the synthesis of melanin were impaired in zebra fish embryos after exposure to 2.65 mg/L boscalid. In larval zebra fish, p53, Bax, and caspase8 was significantly upregulated in the tebuconazole treated-group relative to Control (Li et al., 2019). Recent studies showed that propiconazole up-regulated genes especially linked to reactive oxygen species (ROS) production and detoxification in Pacific oyster, *Magallana gigas* (Kuchovská et al., 2021). Difenoconazole was also reported to induce DNA double-strand breaks, intracellular generation of ROS, cleaved PARP, mitochondrial membrane potential collapse, Cyt c release, and Bax/Bcl-2 ratio increase in human SH-SY5Y cells (Wang et al., 2021).

Developmental gene: Environmental factors and chemicals/xenobiotics present in the surrounding environment of organisms greatly influence their proper development (Ashwath et al., 2023; Van der Oost et al., 2003). Studies have shown that pyraclostrobin can influence embryonic development and oxidative stress in zebra fish embryos, larvae and adults along with the alteration of mitochondrial function and immune-related transcription gene expression that impair growth and movement (Li et al., 2018; Kumar et al., 2020; Zhang et al., 2020). Azoxystrobin (200 mg/L) treated female zebrafish had lower egg production, reduced fertilization rate, decreased 17b-estradiol (E2), vitellogenin (Vtg) concentrations, gonadosmotic index (GSI) and increased Testosterone concentrations along with histoarchitechtural alterations in the ovaries and livers. Azoxystrobin was also reported to affect reproductive activities, where male zebrafish were affected more than females (Zhang et al., 2020). A significant up-regulation of steroidogenic enzyme genes (cyp17, hsd3b and hsd17b) and down regulation of gonadotropin receptor (Ihb and lhr), steroidogenic enzymes (cyp19b and cyp19a), vitellogenin gene (vtg1 and vtg2) were also seen in female zebrafish exposed to 200mg/l azoxystrobin. Whereas male zebrafish exposed to 20 and 200 mg/L azoxystrobin showed significant up-regulation of steroidogenic receptor genes (cyp19b, cyp11a, cyp17, cyp19a, hsd3b, and hsd17b), vitellogenin gene (vtg1 and vtg2), increased E2 and Vtg concentrations along with decreased testosterone concentration, and histopathological alterations in the tests (Cao et al.,2016). The results indicated that exposure to mepanipyrim and cyprodinil could elevate the expression levels of the cyp1a and ahr2 genes, as well as increase 7-ethoxy-resorufin-O-deethylase (EROD) activity, with these effects varying across different developmental stages of zebrafish (Shen et al., 2023).

Section 1.3 Objectives of the Study

1. Survey and screening of fungicide in the water collected from different sites of the river Teesta.
2. To determine the acute toxicity (LC_{50}) of relevant fungicide(s) in *Pethia conchonius* collected from river Teesta.
3. To assess the activity of biochemical markers in the selected tissue(s) of riverine and the laboratory-reared *Pethia conchonius* exposed to relevant fungicide(s) selected throµgh bioassay.
4. To assess the extent of nuclear DNA damage in the selected tissue(s) of the riverine and laboratory-reared *Pethia conchonius* exposed to relevant fungicide(s)
5. To assess the expression level of antioxidant/ apoptotic gene(s) in the selected tissue(s) of riverine and lab-reared *Pethia conchonius* exposed to relevant fungicide(s).