MULTIMARKER STUDY OF THE EFFECTS OF CHLORINE ON AQUATIC ORGANISMS: RESULTS USING *POECILIA SPHENOPS* AS CANDIDATE SPECIES

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ALISON SHAJI JACOB

LIST OF ABBREVIATIONS

AOP	:	Adverse Outcome Pathway
CAT	:	Catalase
Ct	:	Cycle threshold
DNA	:	Deoxyribonucleic acid
GAPDH	:	Glyceraldehyde-3-phosphate dehydrogenase
GPX	:	Glutathione peroxidase
GST	:	Glutathione-S-transferase
HSP	:	Heat shock protein
NFW	:	Nuclease-free water
PAH	:	Polycyclic aromatic hydrocarbons
PCR	:	Polymerase chain reaction
qRT-PCR	:	Real-Time Quantitative Reverse Transcription PCR
RNA	:	Ribonucleic acid
ROS	:	Reactive oxygen species
SOD	:	Superoxide dismutase
UTR	:	Untranslated region

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ABSTRACT

This study investigated the differential induction of antioxidant enzymes in response to chlorine stress on ornamental fishes. The gill tissues of *Poecilia sphenops* exposed to chlorine were the subject of this study, which examined the differential activation of genes and enzymes related to oxidative stress. The upregulation of the genes was investigated. SOD (Superoxide dismutase), CAT (Catalase), GPX (Glutathione peroxidase), GST (Glutathione -S- transferase), HSP-70 (Heat shock protein-70), and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) are the genes that were investigated. The fishes were observed for 45 hours after being subjected to about 0.35g of chlorine per µl.

According to the outcomes of real-time PCR using the GAPDH gene as a reference gene, the acute exposure to chlorine toxicity has shown upregulation in two of the reactive oxygen species, such as CAT and GST. The CAT and GST genes have demonstrated a fold of induction 13414.07 and 65.809 respectively. Several aberrant physical traits, such as restlessness and irregular swimming, have been displayed by the exposed fish.

These results were obtained as a result of an acute dosage of chlorine exposed for a short period of time in ornamental fishes. When these fishes are chronically exposed, they may exhibit a differential status change in other biomarkers. The use of biomarkers facilitates the study of oxidative stress in various species and is more reliable than physical examination. As part of a risk management initiative, it can be analyzed to create adverse outcome pathways. Toxicogenomics can benefit from the use of biomarkers. This research can be expanded to look into the role of biomarkers in diseases, as well as the cellular and genetic changes caused by environmental contaminants.

AIM AND OBJECTIVES

AIM:

To study differential induction of enzymes in response to acute chlorine toxicity using multimarker in ornamental fishes.

OBJECTIVES:

- To identify potential biomarkers for chlorine stress.
- To evaluate the oxidative stress caused by chlorine poisoning in fish.
- To investigate gene regulation: compensatory mechanism in fishes.

RELEVANCE OF THE STUDY

The investigation of oxidative stress in fish as a result of chlorine toxicity allows us to identify potential biomarkers. The use of biomarkers facilitates the study of oxidative stress in various species and is more reliable than physical examination. For further investigation, these biomarkers could be used for nullifying the animal testing ethical issues. These biomarkers throw light on to study of their role as precursors in cancer detection. As a part of risk assessment, it could be used to construct the adverse outcome pathways. The use of biomarkers can benefit toxicogenomics.

INTRODUCTION

Ornamental fishes are an important part of the community's economic growth and a popular source of revenue for the residents. The development of ornamental fish farming is carried out in accordance with the mission of economic development (Cecil and T. R., 1999). The development goal is to improve the well-being of the marine and fishing communities by strengthening human resource institutions, being sustainable, increasing knowledge-based productivity and competitiveness, and expanding access to domestic and international markets. Ornamental fish are not only for entertainment but also have a multi-functional property, making them one of the most popular pets today (Nurlaili et al., 2021).

Ornamental fish production is a multibillion-dollar global industry. Originally, developed countries were the primary ornamental fish keepers as appealing hobbies, but it has recently gained traction in developing countries as well, accounting for roughly two-thirds of total export value (Portz et.al., 2006). Overall, the global aquarium fish trade has been steadily increasing due to the growing interest in aquarium fish. In the ornamental fish trade, around 2000 species and 1.0 billion ornamental fish are traded annually, involving over 100 countries. The developing world remains the primary source of ornamental fish. The United Kingdom is the largest importer of ornamental fish, followed by the United States, with Singapore being the largest exporter. The aquarium hobby has been around for about 70 years in India (Berka, 1986).

Like other captive organisms, ornamental fishes face a lot of stressors. The Ornamental Aquatic Trade Association (OATA) was established to promote high welfare standards and to establish norms of behaviour, particularly for factors like water quality, which is a crucial driver of great fish health. Stress is defined as a coordinated set of physiological and behavioural reactions to any perceived threat to homeostasis or allostasis (Braithwaite and Ebbesson, 2014). These responses are usually adaptive, but chronic, severe, or repetitive stress can be harmful to one's health and welfare, resulting in stunted growth, disease, and even death. Poor water quality is one of the most significant environmental stressors for captive fish. For up to 12 hours, most fish are transported in plastic bags with no filtering (Portz et al., 2006). Tank-based transport would make it easier to monitor water-quality variables, but it would significantly increase shipping weights and costs, making it unlikely to be adopted in the future (Berka, 1986; Lim et al., 2003).

Humanity has been using chlorine, a reactive gas, for almost two millennia. Chlorine exposure has happened in a variety of contexts, including as a chemical warfare agent, in household and industrial settings, and as a result of spills and accidents. The effects of chlorine on the respiratory system account for practically all of its toxicity. Both animals and people display similar symptomology. This includes everything from bronchospasm and sensory irritability to cellular alterations in the bronchioles and alveoli to the emergence of pulmonary diseases (Health et al., 1977).

Chlorine is being used more frequently for waste treatment and disinfection as a result of the current focus on environmental preservation and human health. The significance of chlorine toxicity has been substantially elucidated by recent investigations, including life-cycle studies with aquatic creatures. Chlorine is a chemical agent that has been discovered to be a stressor in ornamental fishes. The chlorination of municipal water to remove other contaminants endangers fish and their well-being.

The vast majority of biologists with experience keeping aquatic creatures in captivity are aware of the danger of chlorinated tap water. Numerous studies have been conducted on this harmful effect as it poses a threat to aquatic species. Chlorine or chloramine toxicity caused by adding untreated tap water to aquatic systems is a common ailment of ornamental fish. Chloramine is also formed when chlorine reacts with organic material, specifically ammonia (Winder, 2000). Both chlorine and chloramines are highly toxic to fish. Fish that have been exposed to chlorine or chloramine toxicity are found gasping for air or dead. Chronic irritant exposure causes gill epithelial hyperplasia, which is a common side effect of long-term irritant exposure (Cecil and T.R., 1999).

Fish may become poisonous to chlorine at levels lower than 0.05 mg/L. Municipal water sources typically include 0.5 to 2.0 mg/L of residual chlorine (Roberts et al., 2008). Fish exposed to tap water that hasn't been dechlorinated show clear signs of suffering, initially swimming erratically in an attempt to escape the poison, then losing colour, going dormant, and eventually succumbing to their injuries. Some species, often those that can directly breathe atmospheric air,

are less vulnerable than the rest. According to UNEP-WCMC research (2007), the following are the most traded ornamental fishes (Stevens et al., 2017):

Table 1: List of most traded ornamental fishes according to UNEP-WCMC research

HABITAT	FAMILY
Marine	Pomacentridae (damselfish, clownfish)
	Pomacanthidae (angelfish)
	Acanthuridae (surgeonfish)
	Labridae (wrasses)
	Gobiidae (gobies)
	Chaetondontidae (butterflyfish)
	Syngnathidae (seahorses, pipefish, sea dragons)
Freshwater	Cyprinidae (cyprinids)
	Poeciliidae (livebearers)
	Cichlidae (cichlids)
	Callichthyidae (armoured catfish)
	Characidae (characins)
	Gasteropelecidae (hatchetfish)
	Loricariidae (catfish)

Poecilia sphenops was previously classified as a POECILIID fish, also known as a molly, in the genus Mollienesia. It is one of the most common and numerous fishes in fresh and brackish waters of Middle America, from Mexico to Panama, and is widely used in the ornamental fish trade (Alda et al., 2013; Feltkamp et al., 1970). It was chosen as the candidate species to investigate the differential induction of antioxidant enzymes in response to chlorine stress

Scientific classification:

Poecilia sphenops

Kingdom	:	Animalia
Phylum	:	Chordata
Class	:	Actinopterygii
Order	:	Cyprinodontiformes
Family	:	Poeciliidae
Genus	:	*Poecilia*
Species	:	*sphenops*

Sole (2000) and Porte et al., (2001) proposed in two recent studies that the chemical determination of environmental contaminants be combined with the use of biomarkers of exposure and their effects to provide a more complete picture of stress situations. This approach has been shown to be an effective method of evaluating the impact of pollution in the aquatic environment. When the capacity of antioxidant defence and repair activities outpaces the rate of ROS formation, oxidative stress occurs, causing oxidative damage to biomolecules (Metcalfe et al., 2010). Medical research has been greatly influenced by the hypothesis that reactive oxygen species are mutagenic, and mutations brought on by reactive oxygen species are thought to generate and promote carcinogenesis. However, DNA damage brought on by pollution and a rise in cancer incidence is well recognised.

Biomarkers can be used to assess the risks to human health posed by chemical agent exposure. The emphasis is on the criteria for selecting and validating appropriate biomarkers of exposure, toxic effect, and susceptibility in individuals or subpopulations (World Organization, 1993). This

research looked into potential biomarkers for chlorine stress. Biomarkers can be used as indicators of contaminant exposure and measure the magnitude of the organism's response (Cajaraville et al., 2000; Dondero et al., 2006). Biomarkers are body substances that indicate the presence or host response to certain contaminants in cellular or biochemical terms (Sarkar et al., 2006).

The genes chosen for this study are SOD (Superoxide dismutase), CAT (Catalase), GPX (Glutathione peroxidase), GST (Glutathione -S- transferase), HSP-70 (Heat shock protein-70), and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase). All of the genes mentioned above are linked to oxidative stress (Afifi et al., 2017; Yi et al., 2007; Chavan et al., 2018). According to previous research, these genes have a different status when exposed to environmental contaminants. These genes were studied in order to identify potential biomarkers for chlorine stress. Oxygen species play important roles in the damage caused by neurodegenerative processes such as cell death, motor neuron diseases, and axonal injury. Antioxidant enzyme dysfunction, on the other hand, has been linked to amyotrophic lateral sclerosis, Alzheimer's disease, Huntington's disease, and Parkinson's disease (Sun and Chen, 1998; Mate´s et al., 1999).

Catalase, a key antioxidant enzyme in the body's defence against oxidative stress, is encoded by CAT, a potential biomarker. Catalase is a heme enzyme found in nearly all aerobic cells' peroxisomes (Sundaram et al., 2013). Catalase reduces the toxic effects of hydrogen peroxide by converting it to water and oxygen. Many chronic or late-onset diseases, including cancer, diabetes, asthma, Alzheimer's disease, systemic lupus erythematosus, and rheumatoid arthritis, are thought to be influenced by oxidative stress (Azad et al., 2013).

In most tissues, glutathione is the most prevalent thiol. It has two purposes: first, as an antioxidant, scavenging free radicals like O^2, and second, as a cofactor in enzymatic processes like conjugation of xenobiotics by Glutathione -S- transferase. The fact that toxicant-induced damage only manifests itself when intracellular glutathione levels are low serves as evidence of how crucial glutathione is for tissue protection (Nebert et al., 2004). A class of enzymes that functions in the detoxification of several compounds like oncogenes, environmental contaminants, and byproducts of oxidative stress is the mu class of GST. This may result in genetic abnormalities like reduced susceptibility towards oncogenes and toxic products (Bolt et al., 2006; Liu et al., 2016).

Superoxide dismutase converts free radical superoxide to peroxide, which can then be destroyed by catalase or GPX reactions. Aerobic respiration constantly generates a low level of superoxide.

Mn-SOD expression is required for aerobic life to survive and for the development of cellular resistance to oxygen radical-mediated toxicity (Mates et al., 2000). The gene Mn-SOD (SOD-2) has been proposed as a tumour suppressor. SOD-1 induction appears to be a critical enzyme for the prevention of ageing and mutation caused by oxidative stresses and hazardous environmental factors (Seo et al., 1997).

GPX is an important antioxidant enzyme that functions to remove the hydrogen peroxide free radical that forms when exposed to environmental stress. This gene is involved in detoxification in the gill tissues, as well as hepatopancreatic and intestinal functions. This antioxidant enzyme is capable of reducing hydrogen peroxide and alkyl hydroperoxides (Ren et al.,2009).

HSPs serve as molecular chaperones, assisting in the correct folding of nascent and stress-accumulated misfolded proteins and preventing aggregation. Because of their sensitivity to even mild assaults, HSPs are appropriate as an early warning bio-indicator of cellular danger (Schlesinger, 1990; Morimoto, 1993; Sikora and Grzesiuk, 2007; Saluja and Dudeja, 2008). Expression of HSP genes has been proposed as a useful biomonitor of toxicant exposure in terrestrial environments where heavy metal contamination and pesticide but also herbicide accumulation are critical problems (Pyza et al., 1997; Lee and Choi, 2006; Gao et al., 2007; Roh et al., 2007). Heavy metals, according to Stringham and Candido (1994), can induce distinct patterns of HSP expression in soil nematodes that may serve as diagnosing fingerprints for specific toxicants in soil. Toxins, including metals, have been shown to induce HSP70 in several soil invertebrates in both field and laboratory settings (Gupta et al., 2009).

Some genes appear to have been identified to nullify miRNA-mediated regulation. They use this mechanism by limiting the length of the 3′ UTR, which is the primary site of miRNA action. Housekeeping genes are well-known examples of miRNA non-target genes, also known as "anti-targets." Because housekeeping genes appear to have relatively short 3′ UTRs, they may have fewer miRNA target sites. GAPDH is a key regulatory enzyme that catalyses glyceraldehyde-3-phosphate oxidative phosphorylation during glycolysis (Sikand et al., 2012). The GAPDH gene is used as the reference gene in the experiment and control group to study the bioactivity of antioxidant enzymes.

The association of potential biomarkers, as well as their regulatory status, can be used to construct the AOP, which can then be used to fill the data gap on toxicological issues. This method avoids

the ethical issues associated with animal testing and is more technical and valid. Finding potential biomarkers for chlorine stress can provide the best solution for AOP and thus risk assessment. When it comes to species and regional differences, chlorine exposure and its compensatory response studied in histopathological examination of fish are less valid. Biomarkers are more reliable in detecting stress responses (Sachana, 2019).

There have been several microarray studies on potential biomarkers for environmental contamination. When cellular and DNA damage is considered, it does not provide spot-to-spot or duplicate results (Bartosiewicz et al., 2000). The goal of this research is to look into potential biomarkers in chlorine stress that can be extended to other organisms and regional differentiation.

REVIEW OF LITERATURE

The purpose of this study was to identify the differential induction of antioxidant enzymes in response to chlorine stress in ornamental fishes. *Poecilia sphenops* is taken as a candidate species which is a highly traded freshwater ornamental fish. The genes chosen for this study are SOD (Superoxide dismutase), CAT (Catalase), GPX (Glutathione peroxidase), GST (Glutathione -S-transferase), HSP-70 (Heat shock protein-70), and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase). All of the genes mentioned above are linked to oxidative stress (Afifi et al., 2017; Yi et al., 2007; Chavan et al., 2018).

Ornamental fishes are an important part of the community's economic development and a popular source of income for the people. The mission of economic development is carried out in the development of ornamental fish farming (Cecil and T. R., 1999). The goal of development is to improve the well-being of marine and fishing communities by strengthening human resource institutions, being sustainable, increasing knowledge-based productivity and competitiveness, and expanding access to domestic and international markets. Ornamental fish are popular as pets because they are not only entertaining but also serve multiple functions (Nurlaili et al., 2021).

Ornamental fish sales are a multibillion-dollar around the world industry. Initially, developed countries were the primary ornamental fish keepers as appealing hobbies, but it has recently gained traction in developing countries as well, accounting for approximately two-thirds of total export value (Portz et al., 2006). Because of the growing interest in aquarium fish, the global aquarium fish trade has been steadily increasing. Around 2000 species and 1.0 billion ornamental fish are traded annually in the ornamental fish trade, which involves over 100 countries. The developing world continues to be the main source of ornamental fish. The largest importer of ornamental fish is the United Kingdom, followed by the United States, with Singapore being the largest exporter. In India, the aquarium hobby has been around for about 70 years (Berka, 1986).

The vast majority of biologists who have kept aquatic creatures in captivity understand the dangers of chlorinated tap water. Numerous studies on this harmful effect have been conducted because it poses a threat to aquatic species. Chlorine or chloramine toxicity is a common ailment of ornamental fish caused by the addition of untreated tap water to aquatic systems. When chlorine reacts with organic material, specifically ammonia, chloramine is formed (Winder, 2000). Chlorine and chloramines are both extremely toxic to fish. Fish exposed to chlorine or chloramine toxicity are discovered gasping for air or dead. Chronic irritant exposure results in gill epithelial

hyperplasia, which is a common long-term irritant side effect (Cecil and T.R, 1999).

Chlorine poisoning in fish can occur at levels as low as 0.05 mg/L. Residual chlorine levels in municipal water are typically between 0.5 and 2.0 mg/L (Roberts et al., 2008). Fish exposed to untreated tap water show obvious signs of distress, swimming erratically at first in an attempt to escape the poison, then losing colour, going dormant, and eventually succumbing to their injuries (Abdelbain et al., 2016). Some species are less vulnerable than others, especially those that can directly breathe atmospheric air.

A study, which took into account the current problem of toxicity, conducted laboratory studies on various fish species. *Salmo gairdneri, Oncorhynchus kisutch, Notemigonus crysoleucas, Cyprinus carpio, and Ictalurus punctatus* fingerlings were exposed to free chlorine or monochloramine pulses in the laboratory three times daily for up to seven days. This regime simulated conditions commonly encountered in the outfall of intermittently chlorinated steam electric generating plants. Water quality characteristics included an average hardness of 45 mg/L, a conductivity of 150 MHOS, dissolved oxygen near saturation, copper of 0.05 mg/L or less, and zinc of less than 0.02 mg/L. The most sensitive to both types of chlorine were *S. gairdneri, O. kisutch, and I. punctatus C. carpio* were the most resistant species, and the most sensitive was *N. crysoleucas*. The lethality of intermittent chlorine in the experimented species was not affected by temperature. Free chlorine was three to fourteen times more toxic than monochloramine, in these types of tests (depending on the species) (Alan, 1977).

The purpose of the study by El-Bouhy et al., (2006) was to examine the stress response of swordtail (*Xiphophorus helleri*) and koi *(Cyprinus carpio*) fish to acute and sublethal chlorine poisoning. Results showed that Swordtail and Koi had 96-hour chlorine LC50s of 1.375 mg/L and 2.4425 mg/L, respectively. The exposed fish displayed aberrant swimming patterns, agitation, decreased respiration rate with anoxic symptoms, clogged gill tissues, and increased mucus secretion on the body surface of deceased fish. In addition, sublethal exposure causes reflex loss, faded body colouration, and slight destruction of the dorsal fin membrane. In fish exposed to sublethal concentrations of chlorine, there was a significant decrease in body weight, body gain, RBCs, WBCs, haemoglobin, and hematocrit (PCV%) with a significant increase in cortisol. Histopathological analysis of the gills indicated considerable lymphocyte infiltration in the lumina propria and severely clogged blood arteries and capillaries of the primary and secondary gill filaments (El-Bouhy et al., 2006).

An experiment was aimed to determine whether chlorine directly affects fish as well as how chlorine degrades in water at various temperatures. The findings demonstrated that increased chlorine concentrations in water cause chlorine to break down much more slowly. At 24–25 degrees Celsius, 0.523 ppm of chlorine in water degraded completely after 60 hours. However, after 60 hours, only 0.086 ppm of chlorine remained in the water at the original chlorine concentration of 1.733 ppm. Additionally, water with 1.081 ppm of chlorine decayed completely after 60 hours at 35 degrees Celsius, whereas water with 1.054 ppm of chlorine had 0.02 ppm of chlorine in 60 hours at 24 to 25 degrees Celsius. Chlorine's histopathological impact on the gill lamella: gill edema was generated by chlorine in the water within 8 weeks at only 0.2-0.6 ppm. There was gill hyperplasia at the base of the gill lamellae, which resulted in merged gill lamellae. The outcome of this experiment did not, however, demonstrate necrosis of hepatocytes (Thonguthai et al., 1987).

The acute toxicity of monochloramine, dichloramine, hypochlorous acid, and hypochlorite ion to emerald shiners *Notropis atherinoides*, channel catfish *Ictalurus punctatus*, and rainbow trout *Salmo gairdneri* was studied in the laboratory. Four exposure regimes were used, which are typical of chlorination schedules at operational steam electric power plants. Fish were exposed for single periods of 15 minutes, 30 minutes, 120 minutes, and four 30-minute periods. The nominal solution of hypochlorous acid was the most toxic, followed closely by the solution dominated by dichloramine; nominal monochloramine and hypochlorite ion solutions had a third to a quarter the toxicity of the other two. On average, emerald shiners were 1.8 times more sensitive to chlorine than channel catfish and 3.3 times more sensitive than rainbow trout. Short-duration chlorine exposures made the fish the most sensitive, while continuous 120-minute exposures made them the least sensitive. The differences in toxicity observed among the chlorine forms suggest that when chlorination regimes and regulations are established, careful consideration should be given not only to total residual chlorine but also to the chlorine forms, fish species present, and duration of exposure (Brooks et al., 1984).

In a dark room with a controlled temperature (27.3-28.4 °C), an experiment was carried out to determine the acute toxicity of chlorine concentration to black tiger shrimp (*Penaeus monodon fabicus*) of sizes 0.02 g, 2.75 g, 8.47 g, and 23.65 g. Toxicity tests were performed in triplicate on each of these shrimp sizes in glass jars under static conditions with no media renewal. The concentration of active chlorine that killed 50% of shrimp of each size after 24 hours was used as an indicator of acute toxicity (LC50-24 h). In the shrimp toxicity test, chlorine concentrations in

shrimp pond water ranged from 2.0 to 14.5 mg L^{-1}. Because the test water contained total suspended solids of 22.0-85.0 mg L^{-1} and total ammonia nitrogen of 0.18-0.40 mg L^{-1}, the combined residual chlorine concentrations ranged from 0.6 to 3.5 mg L^{-1}, which were the effective doses causing shrimp mortality. The test results revealed the mortality in relation to size and original dose (Husnah and Lin, 2002).

In two recent studies, Sole (2000) and Porte et al., (2001) proposed combining chemical determination of environmental contaminants with the use of biomarkers of exposure and their effects to provide a more complete picture of stress situations. This method has been demonstrated to be an effective method of assessing the impact of pollution on the aquatic environment. When the capacity of antioxidant defence and repair processes outpaces the rate of ROS generation, oxidative stress occurs, causing oxidative damage to biomolecules. The hypothesis that reactive oxygen species are mutagenic has greatly influenced medical research, and mutations caused by reactive oxygen species are thought to generate and promote carcinogenesis. However, pollution-induced DNA damage and a rise in cancer incidence are well-recognised.

CAT, a potential biomarker, encodes catalase, a key antioxidant enzyme in the body's defence against oxidative stress. Catalase is a heme enzyme found in the peroxisomes of nearly all aerobic cells. Catalase converts hydrogen peroxide to water and oxygen, reducing its toxicity. Many chronic or late-onset diseases, including cancer, diabetes, asthma, alzheimer's disease, systemic lupus erythematosus, and rheumatoid arthritis, are thought to be influenced by oxidative stress (Azad et al., 2013).

Glutathione is the most abundant thiol in most tissues. It serves two functions: first, as an antioxidant, scavenging free radicals such as O^2, and second, as a cofactor in enzymatic processes such as xenobiotic conjugation by Glutathione-S-transferase. The fact that toxicant-induced damage only appears when intracellular glutathione levels are low demonstrates how important glutathione is for tissue protection (Nebert et al., 2004). A class of enzymes that functions in the detoxification of several compounds like oncogenes, environmental contaminants, and byproducts of oxidative stress is the mu class of GST. This may result in genetic abnormalities like reduced susceptibility towards oncogenes and toxic products (Bolt et al., 2006).

Superoxide dismutase converts superoxide free radicals to peroxide, which can then be destroyed by catalase or GPX reactions. Aerobic respiration produces a low level of superoxide all the time. Mn-SOD expression is required for the survival of aerobic life as well as the development of

cellular resistance to oxygen radical-mediated toxicity (Mates et al., 2000). Mn-SOD (SOD-2) has been proposed as a tumor suppressor gene. SOD-1 plays a major role in the prevention of ageing and mutations caused by oxidative stress (Seo et al., 1997).

GPX is an important antioxidant enzyme that functions to remove the free radical hydrogen peroxide that forms when exposed to environmental stress. This gene is involved in gill detoxification, as well as hepatopancreatic and intestinal functions. This antioxidant enzyme can degrade hydrogen peroxide and alkyl hydroperoxides (Ren et al., 2009).

HSPs function as molecular chaperones, assisting in the folding of nascent and stress-accumulated misfolded proteins and preventing aggregation. Because of their sensitivity to even minor assaults, HSPs are suitable as an early warning bio-indicator of cellular hazards (Schlesinger, 1990; Morimoto, 1993; Sikora and Grzesiuk, 2007; Saluja and Dudeja, 2008). HSP gene expression has been proposed as a useful biomonitor of toxicant exposure in terrestrial environments where heavy metal contamination, pesticide accumulation, and herbicide accumulation are major issues (Pyza et al., 1997; Lee and Choi, 2006; Gao et al., 2007; Roh et al., 2007). According to Stringham and Candido (1994), heavy metals can induce distinct patterns of HSP expression in soil nematodes, which may serve as diagnostic fingerprints for specific toxicants in soil. Toxins, including metals, have been shown to induce HSP70 in several soil invertebrates in both field and laboratory settings (Gupta et al., 2009).

Some genes appear to have evolved to nullify miRNA-mediated regulation. They use this mechanism by limiting the length of the 3′ UTR, which is the primary site of miRNA action. Housekeeping genes are well-known examples of miRNA non-target genes, also known as "anti-targets." Because housekeeping genes appear to have relatively short 3′ UTRs, they may have fewer miRNA target sites. GAPDH is a key regulatory enzyme that catalyses glyceraldehyde-3-phosphate oxidative phosphorylation during glycolysis (Sikand et al., 2012).

The response of the mudskipper was tested for eight days at four different lead concentrations. Fish exposed to 2 mg/L Pb exhibited the lowest liver and gill glutathione (GSH) and glutathione peroxidase (GPX) activity. Fish exposed to 1 and 2 mg/L Pb had lower liver superoxide dismutase (SOD) levels than fish exposed to 0 and 0.5 mg/L Pb. However, the 2 mg/L Pb group had the highest levels of malondialdehyde (MDA) in the gills and liver. The 0 mg/L Pb group had decreased levels of SOD, glutathione -S- transferase (GST), heat shock proteins 70 (HSP70), and HSP90 expression in the gill and liver. This study showed that antioxidant enzymes do not

completely prevent Pb-induced ROS production. mRNA expression has an impact on the activity of functional enzymes (Jing et al., 2017).

The purpose of a previously conducted study was to look into the effects of bisphenol A (BPA) on crayfish histopathological tissue (hepatopancreas and gills), immune ability (lysozyme activity), oxidative stress, and antioxidant defence (*Pontastacus leptodactylus*). Crayfish were exposed to BPA at various concentrations (0 (control), 10, 50, and 100 g/L) in triplicate for 5 and 20 days. Histomorphological results reveal mild to extreme hyperemia and inflammatory cell infiltrations, unusual epithelial cell plans, cell degeneration, necrosis, and sloughing, hepatopancreas tubule collapse, and hyperemia, oedema, and sloughing at gill epithelial cells. In addition to gill swelling, hemocyte accumulation in the haemocoelic space of the gill lamellae was observed in relation to dosage. Antioxidant-related enzyme activities such as superoxide dismutase (SOD), Glutathione-S-transferase (GST), and glutathione reductase (GR) activity were significantly lower in the 10, 50, and 100 g/L BPA groups at 5 days ($p>0.05$). Lysozyme activity (LSZ) was not significantly increased in comparison to the control ($p<0.05$). These findings suggest that changes in antioxidant enzymes and the histological structure of crayfish hepatopancreas and gill tissue could be used as potential biomarkers for risk assessment in aquatic ecosystems (Diler et al., 2022).

The study investigated the interaction of molecular chaperons (heat shock protein-70, heat shock protein-90) with transcriptional factors (nuclear factor kappa B/nuclear factor E2-related factor 2/Kelch-like ECH-associated protein 1) in fish hepatocytes during metal-induced stress. Adult *Puntius ticto* were exposed to lead nitrate at concentrations of 0 mg/l (control), 1/50th (0.04 mg/l), and 1/20th (0.12 mg/l) of LC50 for 30 days before being sacrificed to collect liver tissues. Liver enzymes increased significantly ($p>0.05$) in both Pb-exposed groups, indicating that the liver may be damaged. Significant increases ($p>0.05$) in glutathione reductase and glutathione s-transferase activities, as well as a decrease in reduced glutathione levels in fish exposed to lower and higher Pb concentrations compared to controls, suggested metal-induced oxidative damage in fish liver. Superoxide dismutase and catalase activities increased significantly ($p<0.05$) during lower Pb concentration exposure and decreased significantly ($p>0.05$) during higher Pb concentration exposure compared to controls. Heat shock protein 70 and 90 expression levels increased significantly ($p>0.05$) during metal exposure, indicating a role in stress-induced antioxidant protein remodeling (Kumar et al., 2021).

The researchers looked at the biochemical changes in the enzymatic defence systems of common

carp (*Cyprinus carpio L.*) when they were exposed to a heavy metal-contaminated aquatic system. For 32 days, the fish were systematically exposed to heavy metal solutions containing cadmium, lead, nickel, and chromium at sub-lethal levels. The analytical results show that heavy metal toxicity in fish organs increases gradually during the exposure period and then decreases slightly on the 32nd day. The antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and Glutathione-S-transferase (GST) were activated in the fish. At $P<0.001$, this observation clearly indicates the defensive nature and adaptive mechanism of cells against free radical-induced toxicity (Vinodhini and Narayanan, 2009; Elia et al., 2006).

A study looked at the histological structure as well as the antioxidative and inflammatory responses in yellow catfish spleen and head kidney after 8 weeks of exposure to 50 and 200 g/L Cd^{2+}. It was discovered that Cd^{2+} exposure caused Cd accumulation as well as histological changes in the spleen and head kidney. In terms of antioxidative response, SOD1 expression was significantly upregulated by 50 and 200 g/L Cd^{2+} in the spleen, whereas CAT, GPX1, GST1, and NRF2 expression were upregulated by 50 but not by 200 g/L Cd^{2+}; in the head kidney, these genes were down-regulated by Cd^{2+} exposure. Cd^{2+} exposure resulted in Cd accumulation in the spleen and head kidney, as well as histological lesions, and antioxidant and inflammatory responses in both tissues (Sun et al., 2019).

The toxic effects of the antifouling biocide chlorine on marine benthic organisms were studied using transplanted green mussels (*Perna viridis*) and a panel of biomarkers. To assess the effects, biomarkers for general stress, oxidative stress (superoxide dismutase and catalase), and DNA integrity, as well as stress protein expression, were studied. Chlorine stress was indicated by a decrease in condition index and an increase in DNA strand breaks. Superoxide dismutase enzyme did not show any particular trend, but catalase activity was high during the initial days of exposure at the chlorinated site; later, it became almost equal to that at the control site. Similarly, stress protein expression (HSP60, HSP70, HSP22, GSTS1, and CYP4) followed a bell-shaped pattern throughout the study period. The multimarker approach's utility in monitoring the effects of continuous low-dose chlorination on mussels was demonstrated by a positive correlation among the endpoints (Chavan et al., 2018; De Luca-Abbott et al., 2005).

Perna viridis marine mussels were transplanted from a reference site to various polluted sites in Hong Kong. Individual mussels were tested for antioxidative responses in the gills and hepatopancreas, as well as tissue concentrations of chlorinated hydrocarbons [polychlorinated biphenyls (PCBs)] and chlorinated pesticides (CPs) after 30 days of exposure. Glutathione -S-

transferase (GST) and glutathione (GSH) levels were found to be positively related to tissue PCB levels. Only glutathione peroxidase (GPX), an enzymatic antioxidant, demonstrated a significant response to tissue PCB. There was no significant relationship found between tissue-chlorinated hydrocarbon concentrations and other enzymatic antioxidants (superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and NADPH DT-diaphorase) (DT-d). This study found a link between GST/GSH and chlorinated hydrocarbons (Cheung et al., 2002).

A study was conducted to determine the effects of disinfectant treatment on the antioxidant response of *Cyprinus carpio L.* Enzymatic activities of glutathione -S-transferases, glyoxalase I, glyoxalase II, glutathione peroxidases, glutathione reductase, catalase, and total glutathione content of carp liver exposed to three disinfectants for potabilization, sodium hypochlorite, chlorine dioxide, and peracetic acid, was investigated. Carp specimens were exposed in four experimental tanks with continuous water flow from Lake Trasimeno (Italy), three of which were treated with constant concentrations of sodium hypochlorite, chlorine dioxide, and peracetic acid for 10 and 20 days, respectively, while the control tank received untreated lake water. Differences in biochemical parameters were observed in specimens exposed to these disinfectants, with chlorine compounds inducing more pronounced biochemical variations in carp liver than peracetic acid treatment. The findings indicated that the antioxidant parameters of *Cyprinus carpio* could be used as oxidative stress biomarkers when this species is exposed to disinfectants for water potabilization (Elia et al., 2006).

Carassius auratus, a freshwater fish, was exposed to six different concentrations of 2,4-DCP (0.005-1.0 mg/l) for 40 days before liver tissues were separated for analysis. The activities of catalase (CAT) and selenium-dependent glutathione peroxidase (Se-GPX), as well as the content of oxidized glutathione (GSSG), were significantly increased 40 days later compared to the control group; superoxide dismutase (SOD) responded to 2,4-DCP exposure at only 0.005 mg/l; and the content of reduced glutathione (GSH) was continuously suppressed. SOD and Se-GPX may be used as potential early biomarkers of 2,4-DCP contamination in aquatic ecosystems (Zhang et al., 2004).

The physiological and biochemical changes in crucian carp (*Carassius auratus*) exposed to alachlor at various concentrations for 60 days were studied. The activity of hepatic antioxidant defence and detoxifying enzymes, superoxide dismutase (SOD), catalase (CAT), and glutathione -S-transferase (GST), as well as glutathione (GSH) content, were measured and compared to a control group. The activities of SOD, CAT, and GST were continuously induced ($P<0.05$),

whereas the content of reduced glutathione (GSH) was inhibited overall. These modifications indicate that the antioxidant systems of the tested fishes were impacted. As a result, the possible defence mechanistic implications of the changes were discussed. Furthermore, hepatic SOD and GST were sensitive to alachlor at low concentrations, implying that they might be potential biomarkers in alachlor early detection (Yi et al., 2007).

MATERIALS AND METHODS

1. SAMPLE COLLECTION

For this study, four *Poecilia sphenops* individuals of similar size were collected. 500ml of tap water, with a pH of 8.5 and a temperature of 27 ° C., was added to two bowls.

In 200 µl of nuclease-free water, 0.1 grams of bleaching powder (30-33% chlorine content) was weighed and combined. In order to create the experimental setup, 1 µl of the aforementioned mixture containing roughly 0.35 mg of chlorine per µl (pH = 7.1, temperature = 27 ° C) was added.

Both the experimental and control setups received feed, and they were monitored for 45 hours.

Figure 1: Experimental and control setups at zeroth hour after the addition of chlorine

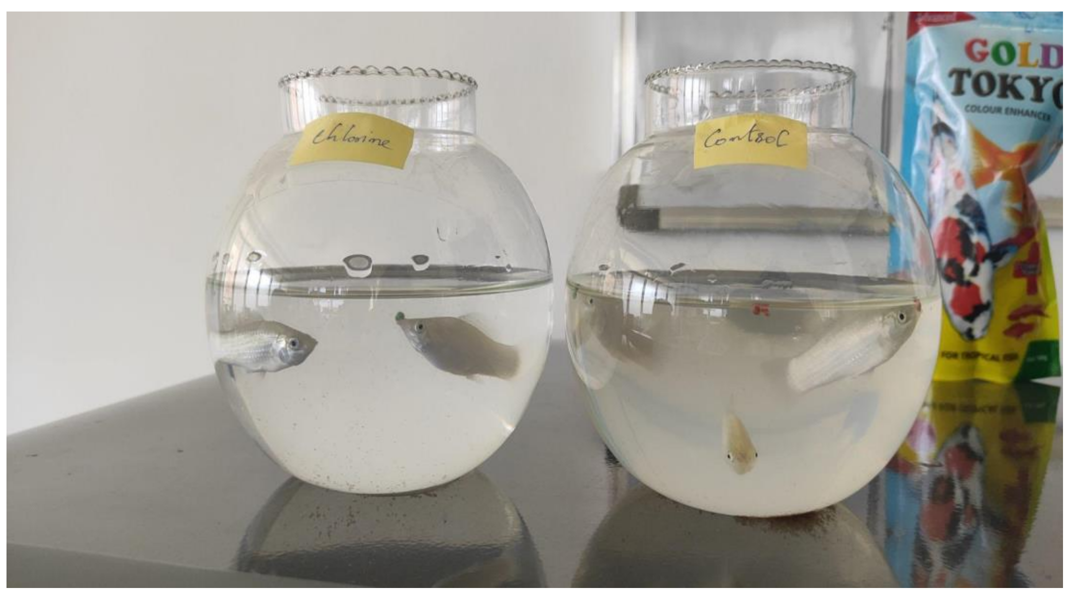

Figure 2: Experimental and control setups at the 45th hour after the addition of the chlorine

2. RNA EXTRACTION

Total RNA Extraction Kit is designed for the rapid purification of RNA from different samples using trizol reagent. This product is a mixture of guanidine thiocyanate and phenol in a mono-phase solution which effectively dissolves RNA. After addition of chloroform and centrifugation, the mixture separated into three phases: an aqueous phase containing RNA, an interphase containing DNA and an organic phase containing proteins. RNA is precipitated using isopropyl alcohol. Impurities are removed using ethanol and pure RNA is resuspended in RNase-free water. 1 ml of trizol reagent is sufficient for isolating RNA from 50-100 mg of tissue. This advanced RNA isolation procedure is an improvement to the single-step RNA isolation using phenol and guanidine isothiocyanate developed by Chomczynski and Sacchi.

❖ Precautions taken while handling RNA:

Ribonucleases (RNases) are highly active, stable enzymes that frequently do not need require cofactors to work. RNases are challenging to deactivate and even tiny quantities are enough to degrade RNA. Only glass or plastic items were used after removing any potential RNase contamination. No RNases were accidentally added to the RNA sample during or after the isolation process, extreme caution was exercised. The following measures were observed when working with RNA during pretreatment in order to establish and maintain an RNase-free environment.

- To avoid contaminating reagents and RNA samples with RNase from the surface of the skin or from dusty laboratory equipment, latex or vinyl gloves were always used when handling these substances. Tubes were closed whenever feasible and gloves were frequently changed.
- To be used in the experiment, collection tubes, tips, pipettes, electrophoresis units, etc. were exposed to UV light for 15-20 minutes.
- To avoid cross-contamination with RNases from shared equipment, sterile, disposable plastic ware and micropipettes were designated for RNA work.
- Before usage, non-disposable plastic items were treated to remove RNase.
- After carefully rinsing with 0.1M NaOH, 1mM EDTA, and RNase-free water, plastic containers were dried. RNases were inactivated by rinsing plastic containers made of chloroform-resistant material.
- Glass used for RNA processing was well cleaned with soap, and then baked in the oven for at least 4 hours at 24 °C. Glassware can also be cleaned using DEPC as an alternative (diethyl pyrocarbonate). Glassware were filled with 0.1% DEPC (0.1% in water) and let to stand at 37 °C for the next day, and then autoclaved to 100 °C for 15 minutes to remove any remaining DEPC.
- Water and other solutions were treated with 0.1% DEPC.

The total RNA extraction method is intended to quickly purify RNA from various materials.

Figure 3: Control fish for sample preparation

Figure 4: Chlorine treated test fish for sample preparation

❖ Procedure:

1. SAMPLE PREPARATION:

2 grams of cells was homogenized in 1ml of trizol reagent using autoclaved mortar and pestle and liquid nitrogen.

500µl of the mixture was transferred to a 2.0ml collection tube.

2. PHASE PREPARATION:

   A. The homogenized sample was incubated for 5 minutes at room temperature (15-25˚C) to permit the complete dissociation of nucleoprotein complexes.
   B. 200µl of chloroform added per ml trizol reagent was used. The sample was covered tightly, shaken vigorously for 15 seconds and allowed the tube to stand for 10 minutes at room temperature (15-25˚C).
   C. Centrifuged the resulting mixture at 10,000 rpm for 10 minutes at 4˚C. Following centrifugation, the mixture was separated into a lower organic phase (containing protein), an interphase (containing cell debris and DNA) and an upper aqueous phase containing RNA.

   Note: The chloroform used for phase separation should not contain isoamyl alcohol and other additives.

3. RNA PRECIPITATION:

   A. Transferred the aqueous phase-containing RNA to a fresh tube and added 500µl of isopropyl alcohol. The sample was incubated for 30 minutes at 20˚C.
   B. The sample was centrifuged at 10,000 rpm for 10 minutes at 4˚C. The RNA precipitate, often invisible before centrifugation, formed a gel-like pellet on the side and the bottom of the tube.

4. RNA WASH:

The supernatant was removed and washed the RNA pellet by adding 500µl of 75% ethanol. The sample was pipetted gently to resuspend the pellet and centrifuged at 10,000 rpm for 10 minutes at 4˚C.

5. RNA ELUTION:

Briefly dried the RNA for 10 minutes by air-drying. The pellet was resuspended with 15µl of RNase-free water.

Note: Do not let the RNA pellet dry completely, as this will greatly decrease its solubility. Do not dry the RNA pellet by centrifugation under a vacuum. Storage of the elution with purified RNA: the elute contains pure RNA, recommended to be stored at a lower temperature (-80˚C). Avoid repeated freezing and thawing of the sample which may cause denaturation of RNA. DNase treatment was also performed.

Template RNA

Total cellular RNA were isolated by standard methods suitable for use with the kit. Purified RNA was made free of salts, metal ions, ethanol and phenol to avoid inhibiting the cDNA synthesis reaction. Trace contaminants were removed by ethanol precipitation of the RNA followed by two washes of the pellet with cold 75% ethanol. For RT-PCR applications, template RNA must be free of DNA contamination. Prior to cDNA synthesis, RNA was treated with DNase I, RNase-free water to remove trace amounts of DNA. A control (TERminus) reaction which includes all components for RT PCR except for the reverse transcriptase enzyme was always performed.

RNA quantity

7000 ng of total RNA was used to generate the first strand of cDNA as the initial step of a two-step RT-PCR protocol.

3. cDNA SYNTHESIS

PrimeScript™ RT reagent Kit (Perfect Real Time):

Description

PrimeScript RT reagent Kit is designed to perform the reverse transcription optimized for real-time RT-PCR. It uses PrimeScript Reverse Transcriptase, which features excellent extendibility. The kit makes fast, efficient cDNA template synthesis for real-time PCR possible. The step of the experimental procedure in this kit is simple and suitable for high throughput analysis. This kit can be used in combination with intercalator-based real-time PCR reagents such as TB Green®Premix Ex Taq™ II (Tli RNaseH Plus) TB Green Fast qPCR Mix, or TB GreenPremix Ex Taq (Tli

RNaseH Plus), or probe-based Real-time PCR reagent such as Probe qPCR Mix, for 2 steps real-time RT-PCR. The optimized protocol for the assay can be selected in each assay condition using either an intercalator or a probe.

Protocol:

1. Prepared the following reaction mixture on ice. A slightly larger amount of master mix was prepared than required to compensate for pipetting losses. After dispensing aliquots of this mixture into the microtubes, the RNA sample was added.

Control (T1Ca)

Table 2: Contents of the reaction mixture for control

Reagent	Volume	Final concentration
5X PrimeScript Buffer (for Real-Time)	2µl	1X
PrimeScript RT Enzyme Mix	0.5 µl	
Random 6 mers (100 µM)*1	0.5 µl	50 pmol
Total RNA	5.5 µl	
RNase Free dH2O	1.5 µl	
Total		10 µl

Test (T1Ta)

Table 3: Contents of the reaction mixture for the test

Reagent	Volume	Final concentration
5X PrimeScript Buffer (for Real-Time)	2µl	1X
PrimeScript RT Enzyme Mix	0.5 µl	
Random 6 mers (100 µM)*1	0.5 µl	50 pmol
Total RNA	0.5 µl	
RNase Free dH2O	6.5 µl	
Total		10 µl

2. Incubated the reaction mixture under the following condition.

37°C 15 min (Reverse transcription)
85°C 5 sec (Inactivation of reverse transcriptase with heat treatment)
4°C at hold

A gene-specific primer was employed and reverse transcription was performed at 42°C for 15 minutes. Lowering the temperature to 50°C enhanced the findings of non-specific amplification products detected during the PCR stage.

4. REAL-TIME PCR

Real-time PCR, as its name suggests, is a method for tracking a PCR reaction's development in real-time. Additionally, a comparatively tiny amount of PCR result (DNA, cDNA, or RNA) can be quantified. Real-Time PCR works by monitoring the rise in fluorescence that a reporter

molecule emits as the reaction progresses. With each cycle of amplification, the PCR product builds up, which is why this happens. These fluorescent reporter molecules can be Sequence-specific probes such as Molecular Beacons or TaqMan® Probes, as well as dyes that bind to double-stranded DNA such as SYBR® Green. Real-time PCR makes it easier to watch the reaction develop. Starting with little amounts of nucleic acid allows for precise measurement of the final result. Additionally, there is no requirement for post-PCR processing, which saves time and costs. These benefits of the fluorescence-based real-time PCR method have radically changed how DNA and RNA measurement is done using PCR. Real-time PCR assays are now simple to use, highly sensitive, more selective, and have automation potential. Real-time PCR is also known as real-time RT PCR because it includes an additional cycle of reverse transcription, which causes an RNA molecule to turn into a DNA molecule. Because RNA is less stable than DNA, this is done.

Real-Time PCR Procedure

A fluorescent reporter molecule was employed in a real-time PCR procedure to track the PCR's development. The reporter molecule's light multiplied as the PCR product builded up after each round of amplification. Real-time PCR techniques can be broadly divided into two categories based on the molecule employed for detection:

- Non-specific Detection using DNA Binding Dyes
- Specific Detection Target Specific Probes

Non-specific Detection using DNA Binding Dyes:

The PCR reaction can be observed throughout the exponential phase by keeping track of the amount of fluorescence emission at each cycle. A linear relationship can be seen if a graph is made between the log of the initial template amount and the increase in reporter dye fluorescence that results from real-time PCR.

SYBR® Green is the most extensively used double-strand DNA-specific dye for real-time PCR. The DNA double helix's minor groove is where SYBR® Green attaches. The unbound dye fluoresces very little in the solution. When the dye is attached to double-strand DNA, the fluorescence is greatly increased. Under PCR conditions, SYBR® Green maintains its stability, and the optical filter of the thermocycler may be attached to align the excitation and emission wavelengths.

Specific Detection using Target Specific Probes:

Some oligonucleotide probes are tagged with both a reporter fluorescent dye and a quencher dye to specifically detect real-time PCR. Real-time detection probes based on several chemistries are available, such as Molecular Beacons, TaqMan® Probes, FRET Hybridization Probes, and Scorpion® Primers are also included.

Real-Time PCR setting

- **Control (T1Ca) and Test (T1Ta)**

2X Real-Time PCR smart mix	=	5µl
Forward primer + reverse primer	=	1µl
Template cDNA	=	1µl
NFW	=	3µl
Total	**=**	**10µl**

- **Non-template**

2X Real-Time PCR smart mix	=	5µl
Forward primer + reverse primer	=	1µl
NFW	=	4µl
Total	=	**10µl**

PCR Program

Polymerase activation	95℃, 10 minutes
Denaturation	60℃, 15 seconds, 40 cycles
Annealing/extension	72℃, 20 minutes

Quantitative PCR, also known as real-time PCR or qPCR, can offer a straightforward and sophisticated way for figuring out how much of a target sequence or gene is present in a sample.

Factors that can influence Ct

The point where an amplification curve and a threshold line intersect is called Ct (threshold cycle). It is a measure of the relative target concentration in the PCR reaction. Many variables influence the target's concentration in addition to Ct's absolute value. As the amount of templates decreases, the Ct value rises. The Ct value will fluctuate regardless of the template, though, if reaction mix or instrument abnormalities affect the fluorescence measurements used to calculate Ct. As a result, it is impossible to directly compare the Ct values from PCR reactions carried out under various circumstances or using various reagents.

RESULTS

This project looked into the differential induction of various antioxidant genes involved in oxidative stress. SOD (Superoxide dismutase), CAT (Catalase), GPX (Glutathione peroxidase), GST (Glutathione -S- transferase), HSP-70 (Heat shock protein-70), and GAPDH are the enzymes involved (Glyceraldehyde-3-phosphate dehydrogenase). We used qRT PCR in gills to examine antioxidant enzymes based on their cellular expression levels. At an absorbance of 260/280 fluorescence, the experimental fish exposed to 0.35 mg of chlorine per µl for 45 hours produced an RNA quantitative yield of 1285.0 ng/µl for the control and 14550.0 ng/µl for the test.

Table 4: List of primer combinations used for the Real-Time PCR

GENE	FORWARD PRIMER SEQUENCE	REVERSE PRIMER SEQUENCE
CAT	TCCTGAATGAGGAGGAGCGA	ATCTTAGATGAGGCGGTGATG
GST	TAATGGGAGAGGGAAGATGG	GAGCTTCATGCCATCCATTT
SOD 2	GGTGCCCTGGAGCCCTA	GCAACCTGTGTTGTCACGTC
HSP 70	GGGAGAGGGTTGGGCTAGAG	TTGCCTCCTGCCCAATCA
GPX	CGCCGAAGGTCTCGTTATTT	TCCCTGGACGGACATACTT
GAPDH	CGTAGCTGACTTTTCTGGATTACG	GCAGCTATGACATCATGAAATGAAG

RNA concentration

Table 5: Quantitate RNA Yield in NanoDrop 2000

SAMPLE	CONCENTRATION (ng/µl)	260/280, PURITY
Control (T1Ca)	1285.0	1.72
Test (T1Ta)	14550.0	1.83

Real Time PCR – Fold of Induction:

- The fold of induction for the data collected was calculated using the following equations:

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta CP_{\text{target}}(control - sample)}}{(E_{\text{ref}})^{\Delta CP_{\text{ref}}(control - sample)}}$$

Fold of Induction, (T1Ta)

$$\text{Ratio} = \frac{\text{Target gene}^{(\text{ct target of control - ct target of treatment})}}{\text{Reference gene}^{(\text{ct reference of control - ct reference of treatment})}}$$

Fold of Induction, (T1Ca)

$$\text{Ratio} = \frac{\text{Target gene}^{(\text{ct target of control - ct target of control})}}{\text{Reference gene}^{(\text{ct reference of treatment - ct reference of treatment})}}$$

By the application of mathematical operation, fold of induction, it was observed that the antioxidant enzyme CAT and GST genes have shown a fold of induction of 13414.07627 and 65.80914693 respectively with respect to the housekeeping gene GAPDH. CAT and GST genes have shown an upregulation which is an indication of a compensatory mechanism in response to acute chlorine toxicity. Other enzymes like SOD, HSP and GPX have not shown significant status change. So CAT and GST can be considered potential biomarkers of chlorine stress.

REFERENCE GENE: GAPDH

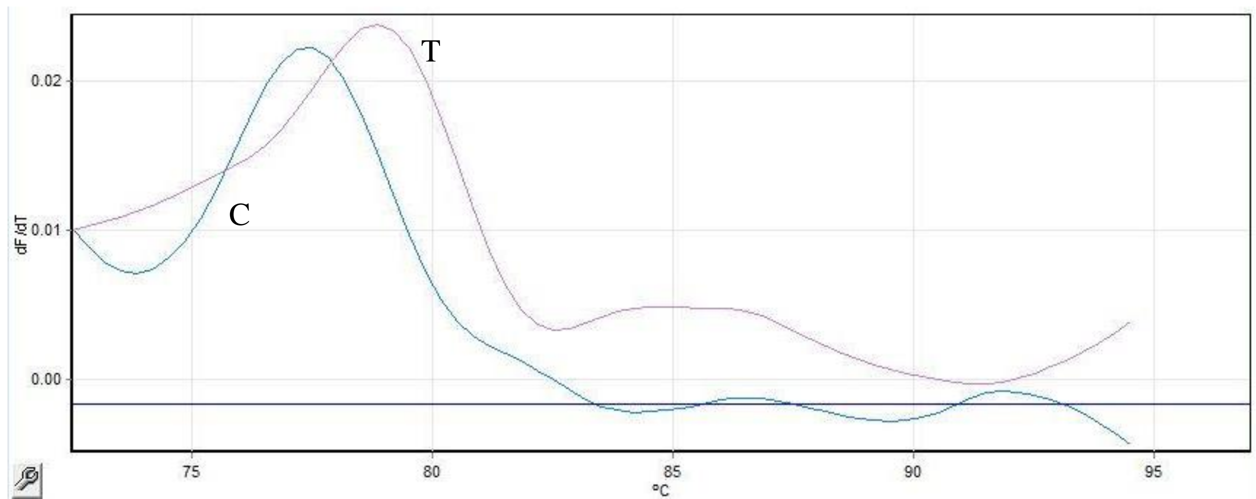

Figure 5: Melt curve analysis of the GAPDH gene

GENE 1:CAT

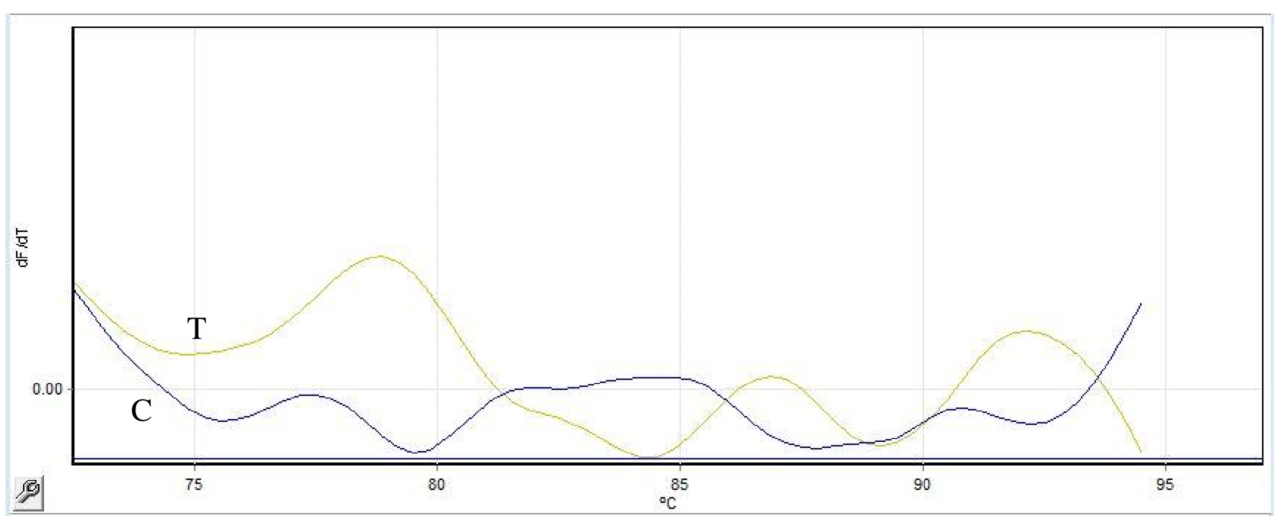

Figure 6: Melt curve analysis of the CAT gene

Table 6: Mean Ct and Fold of induction of CAT gene

Sample	Gene	Mean Ct	Fold of induction
T1Ca	CAT	26.81	1
T1Ta	CAT	6.89	13414.07627

Table 7: Ct of target gene (CAT), reference gene (GAPDH), and fold of induction

	Target Gene	Reference Gene	
PCR efficiency	**1.5720**	**1.4260**	
Ct values	**Ct target**	**Ct reference**	**fold of induction**
T1Ca	26.81	25.31	1
T1Ta	6.89	26.7	13414.07627

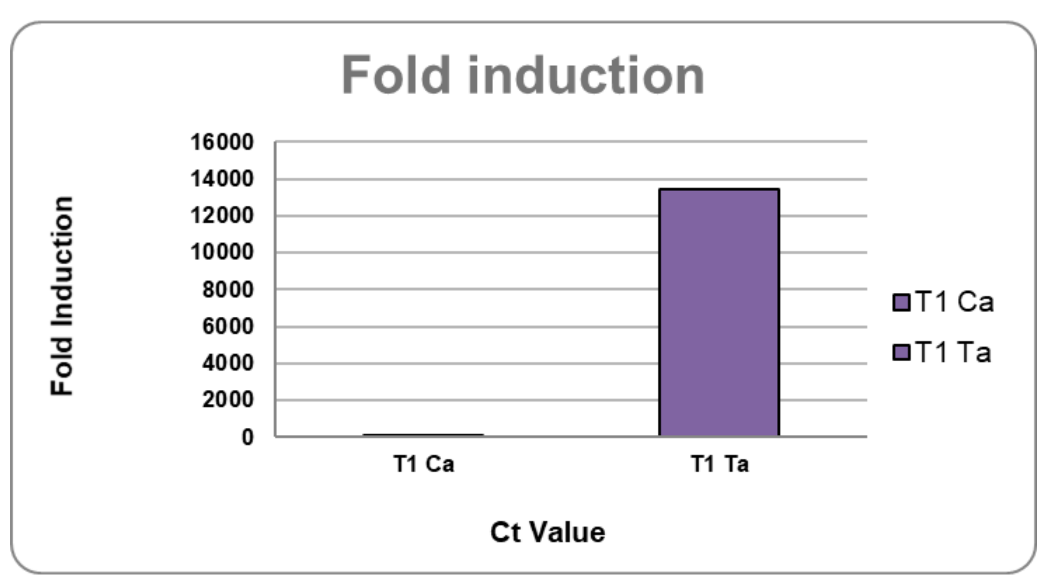

Figure 7: The fold of induction of CAT gene

GENE 2: GST

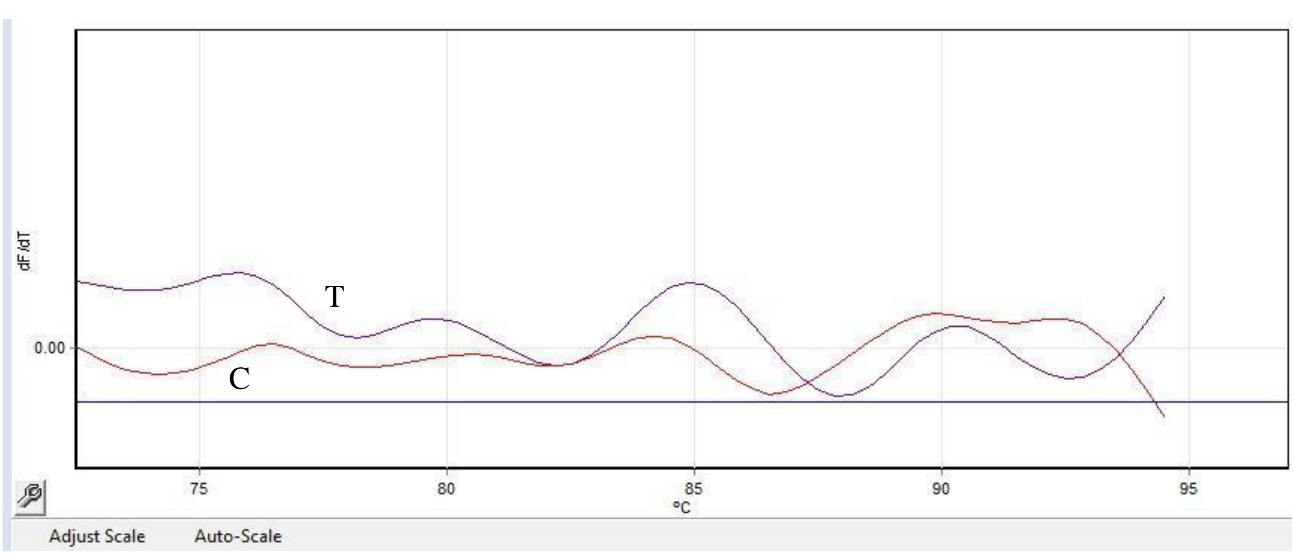

Figure 8: Melt curve analysis of the GST gene

Table 8: Mean Ct and Fold of induction of GST gene

Sample	Gene	Mean Ct	Fold of induction
T1Ca	GST	14.06	1
T1Ta	GST	6.68	65.80914693

Table 9: Ct of target gene (GST), reference gene (GAPDH), and fold of induction

	Target Gene	Reference Gene	
PCR efficiency	**1.5720**	**1.4260**	
Ct values	**Ct target**	**Ct reference**	**fold of induction**
T1Ca	26.81	25.31	1
T1Ta	6.89	26.7	13414.07627

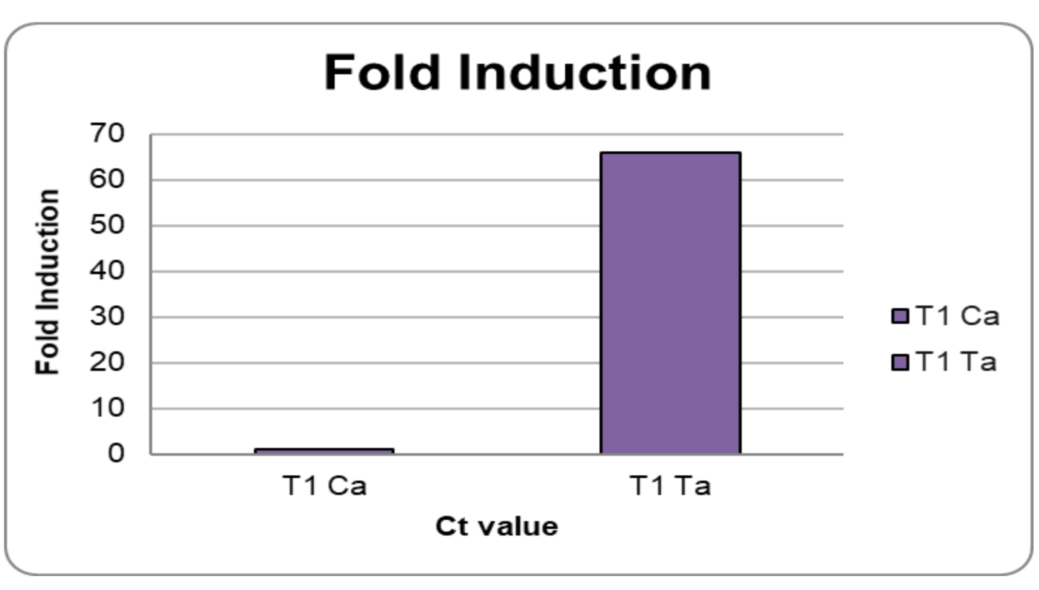

Figure 9: The fold of induction of the GST gene

DISCUSSION

Ornamental fishes are a source of economic development. It's a multibillion-dollar industry. This industry is a component of the largest economy in the developing world. Malaysia, Singapore, and China are major exporters, with the United Kingdom being the largest importer (Nurlaili et al., 2021). The ornamental fish industry in India dates back 70 years. Both developed and developing countries are contributing a major share to the export value of the ornamental fish trade in the world. Globally the fish trade has been growing due to the increased interest of people in ornamental fishes (Cecil and T. R., 1999).

The most serious threat to the ornamental industries is abiotic stress. Like other captive organisms fishes also face several stressors. One of the most significant environmental stressors for captive fish is poor water quality (Wijaya et al., 2021). The vast majority of fish are transported in plastic bags with no filtering for up to 12 hours (Sneddon et al., 2016; Braithwaite and Ebbesson, 2014). Healthy fish increase population size, provide experimentally sound data, are visually appealing and enjoyable to watch, and pose no risk to public health. Chlorinated water, high pH, hardness, temperature, and heavy metal poisoning are all examples of poor water quality (Portz et.al., 2006; Berka, 1986; Lim et al., 2003).

Many toxicological risks are associated with disinfecting drinking water with chlorine dioxide or chlorine. There have been reports of adverse health effects in animal experiments as well as human poisoning incidents. Chlorite remained the most potent chemical species in inducing hemolytic oxidative stress in animals. These effects were observed in various species with varying degrees of sensitivity. Hattersley et al., (2000) investigated the ability of disinfectants to cause haematological effects in monkeys. There was also reproductive and developmental research. Female mice were exposed to ClO_2 in their drinking water from breeding through weaning in one research, which resulted in an increase in stillbirths and fetal resorptions. This effect could be due to anoxia caused by ClO_2^-'s hemolytic effects, rather than a direct effect on prenatal development (Moore et al., 1980). The experimental results of a previous study suggest that ClO_2, but not CIO_2^-, may increase the risk of cardiovascular disease by increasing plasma cholesterol levels and the size of plaques in blood vessels of pigeons fed a high-lipid, low-calcium diet (Bull et al., 1983; Condie et al., 1986).

Chlorine exposure is mostly associated with oxidative stress in fish which is one of the fundamental events involved in physiological disorders. Heavy metals produce more reactive oxygen species (ROS) and deplete antioxidant enzymes (Huang et al., 2007). Many environmental pollutants can harm biological systems by causing oxidative stress (Aust et al., 1985; Sies, 1988; Winston and DiGiulio, 1991). In response to oxidative stress, organisms develop defence strategies such as the production of antioxidants and antioxidant enzymes, and their levels show a statistically significant positive correlation with certain tissue trace organic pollutant levels, such as PAHs (Cheung et al., 2001; Lackner and Reinhard, 1998).

Sole (2000) and Porte et al., (2001) proposed in two recent studies that the chemical determination of environmental contaminants be combined with the use of biomarkers of exposure and their effects to provide a more complete picture of stress situations. This approach has been shown to be an effective method of evaluating the impact of pollution in the aquatic environment, and the application of such a combined approach using bivalves has been successful in a number of environmental situations.

Chlorite was discovered to have the potential to act as a tumour promoter or a complete carcinogen (Condie et al., 1986). Catalase, a key antioxidant enzyme in the body's defence against oxidative stress, is encoded by CAT, a potential biomarker. Catalase is a heme enzyme found in nearly all aerobic cells' peroxisomes. Catalase reduces the toxic effects of hydrogen peroxide by converting it to water and oxygen. Oxidative stress is thought to play a role in the development of many chronic or late-onset diseases such as cancer, diabetes, asthma, Alzheimer's disease, systemic lupus erythematosus, and rheumatoid arthritis (Azad et al., 2013).

Biomarkers can be used to assess the risks to human health posed by chemical agent exposure. The emphasis is on the criteria for selecting and validating appropriate biomarkers of exposure, toxic effect, and susceptibility in individuals or subpopulations (World Health Organization, 1993). This research looked into potential biomarkers for chlorine stress. CAT and GST were discovered to be the appropriate ones (Otitoloju et al., 2011). The use of validated biomarkers to monitor vulnerable populations can serve as the foundation for early public health interventions. Toxic effects on the hepatic, renal, haematological, immune, pulmonary, reproductive, developmental, and nervous systems can be assessed using biomarkers.

In 2012, the Organization for Economic Cooperation and Development (OECD) announced an initiative to expand the notion of adverse outcome pathways (AOPs), which Ankley et al., (2010) pioneered. An AOP is not meant to be a comprehensive description of all aspects of biology and toxicology; rather, it captures only the critical steps in a toxicity pathway. To reveal and establish the important events at each major level of biological organization, a reductionist approach to biology and toxicology is used (e.g., molecular, cellular, tissue, organ, individual) (Sachana, 2019).

The association of potential biomarkers, as well as their regulatory status, can be used to construct the AOP, which can then be used to fill the data gap on toxicological issues. This method avoids the ethical issues associated with animal testing and is more technical and valid. Finding potential biomarkers for chlorine stress can provide the best solution for AOP and thus risk assessment. When it comes to species and regional differences, chlorine exposure and its compensatory response studied in histopathological examination of fishes are less valid. Biomarkers are more reliable in detecting stress responses (Sachana, 2019).

The differential expression of genes in response to various toxic substances found in the environment, in addition to the identification of potential, coincidental, and leading biomarkers, could be used for future toxicogenomics research. This database obtained can be used in the future to discover biomarkers as well (Decristofaro and Daniels, 2008). Toxicogenomics approaches have recently been used to identify key molecular pathways related to increased hepatotoxicity in a human hepatocellular carcinoma (HepG2) cell line exposed to PAHs using a whole human genome microarray (Afifi et al., 2017).

Biomarkers can be used as indicators of contaminant exposure and measure the magnitude of the organism's response (Cajaraville et al., 2000; Dondero et al., 2006). Biomarkers are body substances that indicate the presence or host response to certain contaminants in cellular or biochemical terms (Sarkar et al., 2006). In the present study, we examined the antioxidant enzymes based on their cellular expression level using qRT PCR in gills.

The findings of this study revealed an increase in antioxidant enzymes such as GST and CAT. After 45 hours of exposure, they were affected by oxidative stress. Due to a compensatory mechanism, these enzymes were induced at a low dose. However, severe oxidative stress will

disable the compensatory mechanism. Another study that investigated alachlor exposure in crucian carp liver found upregulated activities in water-soluble reductants such as GSH and enzymes such as GST, SOD, and CAT (Yi et al., 2007). Green mussel, *Perna viridis*, was used in a different study to examine the harmful effects of chlorine dose using biomarkers like CAT, SOD, HSP60, HSP70, HSP22, GSTS1, and CYP4. Similar overexpression was seen in CAT and GST in these benthic species, but other biomarkers showed downregulation (Chavan et al., 2018). Jing et al., (2017) performed a multimarker investigation on mudskippers exposed to various lead concentrations and discovered elevation in oxidative stress and stress response-related genes such as CAT, SOD, GST, GPX, and HSP. This study showed that the formation of ROS caused by Pb is not entirely stopped by antioxidant enzymes.

The current study discovered upregulation in CAT and GST genes in response to a low dose of chlorine, whereas other oxidative enzymes such as SOD, HSP-70, and GPX have shown a threshold response to chlorine content, which contradicts previous studies (Yi et al., 2007).

As suggested in a study that mentions a direct correlation between polycyclic aromatic hydrocarbons and GST levels in green sea turtles (*Chelonia mydas*), biomarkers can be used to determine the change in reproductive success when exposed to some pollutants. PAHs have been found to reduce green sea turtle egg fertilization and hatching success. The GST activity measured in this study was useful as a preliminary investigation into the biological effects of PAH pollution and in determining pollution bioavailability (Sinaei and Zare, 2019). Biotransformation enzymes are biomarkers that are responsible for xenobiotic degradation and mobilization. The phase II biotransformation enzyme glutathione transferase is a well-known example (Sole and Livingstone, 2005; Booth et al., 2006).

When a teleost *Liza aurata* was exposed to a small amount of Phenanthrene (Phe), a ubiquitous polycyclic aromatic hydrocarbon (PAH) in the aquatic environment as a result of human activities, biomarkers such as GPX, GST, and CAT varied in different organs such as the gills, kidney, and liver. Depending on the concentration of Phe, the results revealed organ-specific antioxidant defences. The liver demonstrated greater adaptive competence as evidenced by increased antioxidant defence activation, specifically GSH and GPX. The lower vulnerability of

the kidney to oxidative damage (compared to the gill and liver) seems to be related to its higher antioxidant basal levels (Oliveira et al., 2008).

A research was carried out on European eel *(Anguilla anguilla L.)*. The responses of lipid peroxidation (LPO), glutathione peroxidase (GPX), catalase (CAT), glutathione S-transferase (GST), and total reduced glutathione (GSH) to a single 24 h exposure to two copper concentrations were obtained. Cu exposure alone resulted in a significant decrease in CAT and GSH contents for both concentrations and at the highest concentration in GPX, as well as an increase in GST at the lowest concentration (Ahmad et al., 2005). The heavy metals have also indicated an upregulation in oxidant enzymes like GST and CAT, which is a common era observed in vertebrates.

This study focused on the acute toxicity of chlorine with a low dosage for a short period of exposure and resulted in the upregulation of the antioxidant enzymes like CAT and biotransformer enzymes like GST in freshwater species like *Poecilia sphenops.* Further scientific research should be done to investigate the chronic exposure and high dosage of chlorine toxicity on ornamental fishes.

CONCLUSION

The ornamental fish market is worth billions of dollars. It is vital to the economies of both developing and developed countries. People enjoy keeping fish as pets for reasons other than economic development. Fish typically live in an environment contaminated with a variety of toxic chemicals. Heavy metals such as lead, cadmium, or chlorine are examples. Water used for common activities is chlorinated, and tap water used for fish culture contains trace amounts of chlorine. Chlorine is a chemical agent that has been discovered to be a stressor in ornamental fishes. As per earlier reports, fish may become poisonous to chlorine at levels lower than 0.05 mg/L. Municipal water sources typically include 0.5 to 2.0 mg/L of residual chlorine. Fish exposed to tap water that hasn't been dechlorinated show clear signs of suffering, initially swimming erratically in an attempt to escape the poison, then losing colour, going dormant, and eventually succumbing to their injuries. Some species, often those that can directly breathe atmospheric air, are less vulnerable than the rest.

This study focused on the chlorine toxicity present in the immediate environment to which fish are exposed. The mechanisms of fish adaptation to chlorine exposure such as chemically induced changes in antioxidant enzyme systems were investigated. Numerous antioxidant parameters were measured in order to better understand the potential mechanisms of fish antioxidant defence systems. The genes chosen for this study are SOD (Superoxide dismutase), CAT (Catalase), GPX (Glutathione peroxidase), GST (Glutathione -S- transferase), HSP-70 (Heat shock protein-70), and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase). All of the genes mentioned above are linked to oxidative stress. When the gill tissues of *Poecilia sphenops* (candidate species) were examined for the differential induction of genes in oxidative stress in response to chlorine exposure, two antioxidant enzymes, CAT and GST, were found to have upregulated activities when compared to the control group. Due to a compensatory response, the enzymes CAT and GST are induced at low concentrations. These findings suggest CAT and GST genes could be used as biomarkers of chlorine toxicity for risk assessment in aquatic ecosystems especially for ornamental fishes.

This research provides an effective tool for environmental biomonitoring in order to ensure its safety. Biomarkers can be used to assess the risks to human health posed by chemical agent exposure. The emphasis is on the criteria for selecting and validating appropriate biomarkers of exposure, toxic effect, and susceptibility in individuals or subpopulations. The use of biomarkers facilitates the study of oxidative stress in various species and is more reliable than physical examination. These findings were obtained as a result of an acute dosage of chlorine to the fish exposed for a short period of time. When these fish are chronically exposed, they may exhibit a differential status change in other biomarkers. As part of a risk management initiative, such results can be analysed to create adverse outcome pathways. Toxicogenomics can benefit from the use of biomarkers. This research can be expanded to look into the role of biomarkers in diseases, as well as the cellular and genetic changes caused by environmental contaminants.

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