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M.Sc. in Biochemistry Science and Technology

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BIOCHEMICAL RESPONSES OF *ONCORHYNCHUS MYKISS* TO THE STRESS INDUCED BY PHOSMET

M.Sc. THESIS

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Firas MUHAMMED

ABSTRACT

BIOCHEMICAL RESPONSES OF *ONCORHYNCHUS MYKISS* TO THE STRESS INDUCED BY PHOSMET

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M.Sc. in Biochemistry Science and Technology
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Organophosphorus pesticide phosmet is an anticholinesterase showing its toxicity following contact, ingestion and inhalation. It finds a use in agriculture for apple, peach and olive trees against pests in our country and there are limited numbers of study related to its sublethal effects of on non-target organisms. Therefore this investigation was performed to evaluate oxidative stress inducing potential and anticholinesterase activity of phosmet together with biochemical blood parameters on liver and brain tissues of juvenile *O. mykiss* following 24h, 48h, 72h and 96h of exposure to 5 µg/L, 25 µg/L and 50 µg/L concentrations. Phosmet application resulted in important decrease in the levels of serum glucose, protein and cholesterol while a significant increase in the activities of ALT, AST and ALP were recorded. Its anticholinesterase activity was confirmed in both liver and brain tissues being more pronounced in brain possibly due to abundant tissue concentration. In both tissues, the activities of SOD, CAT and GPx and level of GSH were increased. Similarly, TBARS level exerted ascending tendency. Obtained findings reveal the inhibitory effect of phosmet on AChE activity and its oxidative stress potency. The observed changes in ALT, AST and ALP activities present the hepatotoxic potential of phosmet meanwhile determined hypoproteinemia and hypoglycaemia were evaluated as adaptive responses to cope with the applied stress.

Key Words: Organophosphorus Pesticide, Fish, Liver, Brain, Oxidative Stress.

ÖZET

ONCORHYNCHUS MYKISS'İN PHOSMET İNDÜKLÜ STRESE BİYOKİMYASAL YANITLARI

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Yüksek Lisans Tezi, Biyokimya Bilimi ve Teknolojisi
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Organofosforlu pestisit phosmet temas, oral yolla alınım ve soluma sonrası toksik etkilerini gösteren bir antikolinesterazdır. Ülkemizde başlıca elma, şeftali ve zeytin ağaçlarında zararlılara karşı kullanılmakta ve hedef olmayan canlılar üzerindeki subletal etkileri ile ilgili sınırlı sayıda çalışma bulunmaktadır. Bu nedenle, bu araştırma 24, 48, 72 and 96 saat 5 µg/L, 25 µg/L ve 50 µg/L derişimlerde phosmet etkisinde bırakılan juvenil *O. mykiss* karaciğer ve beyin dokularında insektisitin oksidatif stresi indükleme potansiyelini, antikolinesteraz aktivitesini ve biyokimyasal kan parametrelerine olan etkilerini araştırmak üzere yapılmıştır. Phosmet uygulaması serum glukoz, protein ve kolesterol düzeylerinde önemli bir azalmaya neden olurken ALT, AST ve ALP aktivitelerinde önemli düzeyde artış belirlenmiştir. Pestisitin antikolinesteraz aktivitesi her iki dokuda da gözlenmiş ancak beyin dokusunda enzim derişiminin daha yüksek olması sebebiyle etkinin daha belirgin olduğu kaydedilmiştir. Her iki dokuda da SOD, CAT ve GPx aktiviteleri ile GSH düzeyinde artış meydana gelmiştir. Benzer şekilde, TBARS düzeyleri de artan bir eğilim sergilemiştir. Elde edilen sonuçlar, phosmetin AChE aktivitesini inhibe edici etkisini ve oksidatif stresi indükleme kuvvetini ortaya koymaktadır. ALT, AST ve ALP aktivitelerinde gözlenen değişimler phosmetin hepatotoksik etkisini kanıtlarken, hipoproteinemi ve hipoglisemi durumları uygulanan stres ile mücadele etmek üzere oluşturulan adaptif yanıtlar olarak değerlendirilmektedir.

Anahtar Kelimeler: Organoforforlu Pestisit, Balık, Karaciğer, Beyin, Oksidatif Stres.

"Dedicated to my family"

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CHAPTER I

INTRODUCTION

Toxicology is an interdisciplinary science that investigates the negative effects of chemicals on the biological systems. All living things that live on the planet's surface are increasingly exposed to an enormous variety of natural and synthetic chemicals. Currently, these chemicals differ in their nature and source according to the site they affect. In agricultural environments, these chemicals include herbicides and pesticides. Whereas in the chemical industries they include solvents, metals, and feedstocks for manufacturing of chemical substances or manufacturing of components, like nanoscale engineering materials.

Toxicology was discovered by Paracelsus (1493-1541) in the 16th century. Paracelsus found that chemicals often have both curative and toxic properties, and he also found that the distinction between being curative or toxic can only be done through dosage. His observations were the first step to conceptualizing the relationship between dose and response. “All substances are poisons; there is none which is not. Only the dose differentiates a poison from a remedy” Four centuries ago, Paracelsus said that. In toxicology, the relationship between compound dose and response is a key concept. Responses of organisms regardless of animal venoms that are naturally toxic to the source, pesticides, industrial chemicals, or therapeutic drugs give a relationship between dose and response. It is evident in this relationship that the size of the effect increases with increasing dose (Eaton et al., 2010).

1.1 Pesticides

Although the frequent use of chemical products in many areas of life brought many benefits to humans, but in return it also brought a large number of threats to the environment and thus to the health of people. These transformations have had a major role in the faster progress of civilization. This is because a great progress has been made in the agricultural fields, especially with regard to various methods through the

use of technology and agrochemicals. The quality of agricultural products depended not only on the climate and soil, but also on fertilization and agricultural methods. Thus, to ensure abundant yields and high quality, the cultivation of crop plants requires more procedures and precautions, such as the introduction of fertilizers and the addition of genetic improvements that return the desired gains and yields, not just adding pesticides. In addition to the above, herbicides certainly help reduce competition between cultivated plants and unwanted weeds, and also have a major role in helping to increase yields. Pesticides are natural or synthetic materials that are frequently used to combat harmful or unwanted organisms, whether in agriculture or in homes. They are commonly used in protection, forests and water bodies. Pesticides are represented by many groups of chemical compounds and by a large number of procedures and classifications that exist on the following basis:

First: the target organism for control (insecticides, herbicides, fungicides or biocides).

Second: The mechanism of physiological action within each of these groups, which is usually related to specific chemical structures.

In fact, the effect of pesticide residues on the environment is one of the biggest problems in their use. In general, the main reason for the exposure of living organisms to toxic properties is the persistence of foreign substances resulting from residues of these pesticides in many elements of the environment (Buszewski et al., 2019).

1.1.1 Insecticide Groups

Insecticides are classified into four main groups: organophosphorus compounds, organochlorine compounds, carbamates and pyrethroids. Insecticide is a type of pesticide and is used in many fields, including: agriculture, environment, human and animal health. While most pesticides include different types of chemicals that have many mechanisms of action, disrupting the functions of the nervous system is one of its greatest effects when it comes to using pesticides in agriculture in general. The purpose of using insecticides worldwide is to prevent harm from the presence of insects in agricultural areas. As pesticides consist of different chemical formulas, they may be dangerous to the environment due to their persistence, bioaccumulation and toxicity (Kayhan et al., 2013).

Also, these insecticides can be mainly distributed into two groups depending on the penetration of the active substance into the pest, through two forms: the first: the direct form through the exoskeleton or the respiratory system, and the second indirectly through the poisoned plant. Other classifications of pesticides and plant protection products include classification according to use. Plant protection chemical products in gardens and fields have a wide range of forms of use. It is therefore important to know the threats that these pesticides can pose (for example, disrupting plant growth) and the special ways in which individual formulations work as well as mixtures of pesticides (Buszewski et al., 2019).

Producers were not concerned about the privacy of agricultural products when the development of pesticides began on an industrial scale, as the huge general harm of using these pesticides was overlooked. They do not harm people, animals and agricultural crops, but include enormous damage to the surrounding environment and everything related to it. In 1948, PH Müller was awarded the Nobel Prize in Physiology or Medicine "for discovering the high efficacy of Dichloride-Diphenil-Trichlorethane [DDT] as a contact poison against many arthropods". Global agricultural production is mainly based on the use of pesticides, and as a result, abandoning or reducing their use will cause lower production, higher costs, and high prices for consumers, and in some areas, it will also lead to hunger and malnutrition in many countries of the world (De Castilhos Ghisi, 2012). All over the world, the freshwater systems in the agricultural regions are contaminated by use of mixtures of numerous pesticides input aquatic ecosystems through diffuse access pathways consisting of spray drift, unintended overspray, runoff and drainage. The pesticide contamination of floor waters is most often associated with several kinds of substances rather than a single compound because several lively components are often applied concurrently to crops and many kinds of crops are usually intermingled in agricultural basins. Despite the fact that the mixtures of pesticides are generally illustrative of genuine exposures, the measurement and portrayal of their harmfulness and collaborations has got restricted consideration recently. So, past researches have resulted in a spectrum of interactions relying at the mode of poisonous movement and chemical properties of the insecticides that are examined (Brodeur et al., 2016). Pesticides, as herbicides and insecticides, may collect in aquatic environments and exert toxic outcomes on aquatic organisms (Soares et al., 2016).

1.1.2 Organophosphates (OP)

The term “organophosphates” has been used generically in order to include all organophosphorus esters. These compounds include approximately 40% of the pesticides registered worldwide. The chemistry of organic phosphorous appeared for the first time in the mid-nineteenth century, while the development of organic phosphorous compounds for use as insecticides was in 1936 (Salem and Olajos, 1988).

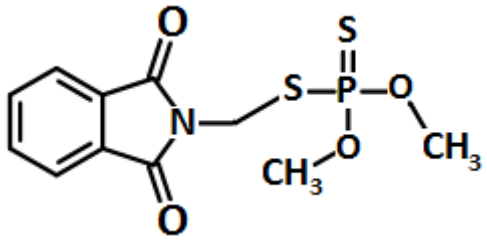
Organophosphates are complex organic materials that contain phosphorous. They were manufactured as insecticides in the 1950s. It is also one of the most prevalent pesticides. And depending on the roots attached to the phosphorous molecules, these compounds are classified as phosphoric acid esters. According to the molecule, the pharmaceutical form, and the types of affected animals, this toxicity differs. Animals such as bees, water birds, cats, and fish are some of the species that are relatively most sensitive to these compounds. It is important to know that organophosphorus compounds are cholinesterase inhibitors. Where organophosphates contain similar proportions and levels of toxic effect on vertebrate and invertebrate organisms alike, and they cause special toxicity in fish even at low concentrations (ng-µg / mL). These main effects are also shown by inhibiting the effect of some enzymes in invertebrate organisms and aquatic vertebrates. For example, organophosphates cause a toxic effect by inhibiting acetylcholinesterase enzyme (AChE) responsible for terminating nerve impulse transmission in fish. Acetylcholine (ACh) is considered to be one of the most important neurotransmitters circulating in the body. Organophosphates cause excessive accumulation of ACh and activation of ACh receptors by inhibiting the hydrolysis of ACh in the peripheral and central synapses. Organophosphate compounds are widely used in agriculture and domestic purposes to control insect pests. Because of their rapid solubility in water and low environmental stability, the use of organophosphate compounds has largely replaced organochlorine in recent years. Depending on the compound, water quality and exposure time, reactions by aquatic organisms to the use of these insecticides have been widely reported. Especially by fish, as they were clearly sensitive to the particles of these pesticides. Also, organophosphates can promote the oxidation of lipids with cell membrane by direct reaction. Acetylcholinesterase is used frequently as an early warning marker to detect pesticide toxicity. Inhibition of AChE is one of the most common use of

organophosphate insecticides, and although insecticides in natural water inhibit Acetylcholinesterase in fish, they also affect recombination and hormonal regulation of the enzyme. This has been shown to inhibit Acetylcholinesterase in the brain will reduce the fish's skills to survive the early stages of development and will cause physiological and behavioral modifications as well. It should be noted that the inhibitory effects of OP depend on their ability to bind to the active enzyme site and the rate of phosphorylation with respect to behavior and age (Kayhan et al., 2013). Organophosphate pesticides are actually synthetic chemicals and are widely used in household pest control as well as in agricultural applications; Since OPs cause acute and chronic toxic effects by inhibiting AChE or by affecting target organs directly in living organisms, their effect is apparent on the peripheral and central nervous system by reducing oxidation and reduction processes, activating oxidative stress, and disrupting the growth process of neurons (Chang et al., 2018). Organic phosphates are one of the many groups of pesticides used in agriculture on a large scale. These compounds have high neurotoxicity affecting non-target organisms such as fish and aquatic invertebrates. The near-lethal doses of OPs may be the cause of physiological impairment of vital functions such as feeding, avoidance of predators and reproduction in fish due to their short persistence in days as OPs are usually found at low levels in aquatic systems. Environmental toxicity assessments should use low doses of exposure to better understand the effects of OPs in an environmentally realistic context, similar to the levels found in the environment (Herrera et al., 2019). Organophosphorus pesticides and carbamate are considered to be the most important pesticides in regard to usage. These types of pesticides are highly toxic to non-target organisms such as birds and aquatic organisms, and at the same time they are more biodegradable than others and less stable than organic chlorine, and they can last for several days in the environment, but their effect on tissues is greater as it can last for several weeks This makes them more useful than the chemical residues of environmental monitoring programs in place to assess biomarkers, such as cholinesterase. Monitoring programs in biochemistry and physiology allow us to discover the presence of pollutants faster in the environment and more specifically to find the pesticide compounds used before their harmful effects appear in the upper levels of biological systems, which is an early warning. Initial studies and analyzes are necessary to know the biochemical behavior of organisms and their living molecules under normal conditions, to see the changes

that toxic substances may cause to these organisms and their molecules (Assis et al., 2012).

Jian-Ke et al. (2009) reported frequent cases of poisoning due to exposure to residues of organophosphates and carbamates, as the excessive use of them in agricultural products is a cause of acute toxicity to humans and animals. Organophosphorus compounds have been used widely among many different groups of pesticides, due to their relatively low stability and high efficacy in eliminating insects and pests in general under normal conditions (Nataraj et al., 2017). Given the important role of organophosphates in controlling parasitic infections, which is a major constraint in aquaculture, it has become necessary to use them in fish farms in order to improve productivity (Baldissera et al., 2019). According to Patel et al. (2018), as a result of the increase in the global population and the increasing demand for food, the appropriate use of organophosphates is essential in protecting fish from infection with parasites to maintain or improve fish meat productivity, as fish is an essential source of protein and essential nutrients for human health. As a result of human greed to increase the meat yield of these fish, organic phosphates have been used excessively (Baldissera et al., 2019). In spite of the selective toxic effects of pesticides and poisonings with organophosphates, we cannot deny the beneficial effect of these pesticides on agriculture and on human health. Several anticholinesterase compounds, like tetraethyl pyrophosphate, diisopropyl fluorophosphate, echothiopate, and paraoxon, are used in the treatment of humans, in particular in the treatment of many common diseases, including intestinal and bladder smooth muscle spasms, glaucoma, and myasthenia gravis (Salem and Olajos, 1988).

1.1.3 Phosmet



Formula: $C_{11}H_{12}NO_4PS_2$
Molecular weight: 317.31 g/mol
Density: 1.03 g/cm^3 (20°C)
Solubility in water: 25 mg/L (25°C)
Melting Point: 72 °C

Figure 1.1 Structure of phosmet and its properties.

Phosmet (Figure 1.1) is a very effective and widely used insecticide, which has been used extensively all over the world to control some types of insects that are found on horticultural crops and ornamental plants. But improper applications of phosmet can cause environmental and food pollution, and phosmet residues in agricultural products and environmental water systems also affect the lives of humans, wildlife, and pets. In fact, one of the most important toxic effects associated with exposure to phosmet is permanent inhibition of the enzyme acetylcholinesterase, which leads to minor symptoms such as dizziness and stomach upset in addition to the possibility of serious problems such as loss of consciousness, convulsions, and even death if excessive doses are applied (Fan et al., 2014). In order to ensure food safety around the world, the Codex Alimentarius Commission for the Food and Agriculture Organization, the World Health Organization (FAO/WHO, 1984), China (GB2763-2012), and the European Union (EU, 2005) have set the tolerance for pesticide residues in meat, fruits and vegetables. In general, the remaining phosmet residues in fruits and meat are 5-10 mg/g and 0.1 mg/g respectively, but in vegetables they differ according to species (FAO/WHO, 1984; GPO, 2013; The Japan Food Chemical Research Foundation, 2006). Monitoring phosmet levels in fruits and vegetables is an essential but laborious task, as is the case with many other pesticides. It should be noted that one of the most important areas of research in the field of food research is the research in developing rapid analytical methods (such as high performance liquid chromatography and gas chromatography–mass spectrometry) to replace the traditional methods used and often expensive (Fan et al., 2014).

1.2 Neurotoxicity

Inhibition of AChE and/or butyrylcholinesterase (BChE) is a primary biomarker for detecting neurotoxicity and disturbance in the metabolism of other neurotransmitters such as-aminobutyrate (GABA) as a result of exposure to pesticides such as organophosphate and carbamate, as GABA receptors block the transmission of nerve impulses. And this leads to disturbances in this receptor that may cause nerves to be overstimulated. The assessment of fish exposure to these pesticides is usually by identifying changes in AChE in the brain, plasma, muscle, liver, and other tissues. AChE is an enzyme that disrupts the neurotransmitter acetylcholine. In the event that AChE is inhibited, this leads to the accumulation of the neurotransmitter acetylcholine

in the cholinergic synapses space, which leads to the blockage of these synapses and thus disrupting the transmission of signals, resulting in a change in the swimming behavior of fish, vibration paralysis and convulsions, in addition to impeding feeding, recognition, avoidance and escape from predators. It also affects reproductive behavior and many other unwanted effects. As is well known, nerve tissue has a much weaker antioxidant defense system than the antioxidant defense systems found in other tissues of living organisms. Since the brain is the center of the nervous system in fish, it contains lower levels of enzymatic and non-enzymatic antioxidants and higher oxidative levels of unsaturated fats and catecholamines. As a result, nerve tissues are among the tissues present in living organisms most sensitive to oxidative stress damage due to pesticide toxicity in general when compared to other tissues. In fact, similar results have also been reported by other scientists (Banaee, 2012).

1.2.1 Acetylcholine and Acetylcholinesterase

ACh is considered an important neurotransmitter in the peripheral and central nervous systems. It is secreted at the postganglionic parasympathetic nerve endings and at some sympathetic nerve endings, at sympathetic and parasympathetic ganglia, at the adrenal medulla, at motor endplates at the neuromuscular junction of skeletal muscles, and at particular synapses in the central nervous system. ACh is rapidly catabolized by AChE, an enzyme found in the central nervous system, the neuromuscular junction, and red blood cells. This enzyme mainly hydrolyzes ACh and has very little affinity towards butyrylcholine (BCh). Serum BChE is found mainly in serum and liver and hydrolyzes BCh at a way faster rate than ACh. AChE, in contrast to BChE, is a membrane- bound enzyme, and is localized in close proximity to the action sites of ACh (Alvares, 2017). Within the central nervous system there are some subcortical regions, such as the nucleus caudatus and globus pallidus, in which AChE is abundantly present in the form of multiple molecular shapes, including a spherical part of the brain that is soluble. The difference reflects probably the mode of membrane attachment rather than a different catalytic activity. Therefore, selective access to the inhibitor rather than differential sensitivity to AChE is evidence of many differences in AChE inhibition in the brain after receiving systemic doses of organophosphate compounds (Lotti, 1992). AChE and BChE exist in various biological fluids and tissues of birds, mammals and fish. AChE is mainly found in red blood cell and nervous tissue, while BChE is in

blood plasma and serum with brain and peripheral nervous tissue also contributing to the pool of plasma AChE (St Omer and Rottinghaus, 1992). In fact, AChE determination has been shown to be a very sensitive and effective method for detecting the presence of herbicides, especially carbamate-based insecticides and organophosphorus pesticides, in various tissues of fish tissues (Modesto et al., 2010). Also, we cannot overlook the main and important role of AChE in the cholinergic system, especially the process of impulse transmission in synapses, as it splits acetylcholine into acetate and choline. Whereas, toxic chemicals can target the AChE enzyme present in the brain leading to its inhibition, thus disrupting neurological functions as a result of the excessive accumulation of ACh. This enables the evaluation of neurotoxic changes by AChE being a biomarker. The brain has become the target organ in many neurotoxicity studies and in particular with regard to the nervous system for its physiological and regulatory role in fish exposed to pesticides (Topal et al., 2017; Assis et al., 2012). The hydrolysis of the neurotransmitter acetylcholine is carried out by the enzyme acetylcholinesterase, which is responsible for this process. If this enzyme is inhibited, the various nerve processes are disrupted, resulting in an overstimulation of the nerve due to the continuous stimulation of neurotransmitters in the nerve cells, in addition to muscle contraction and paralysis and disruption of swimming performance in fish. We cannot conclude that a significant impact on the individual performance of fish has occurred as a result of exposure to pesticides and organophosphates as a result of inhibition of acetylcholinesterase (Herrera et al., 2019). It should be noted that the prevention of the hydrolysis of the neurotransmitter ACh is a consequence of exposure to OPs, which leads to activation of ACh receptors and thus the excessive accumulation of ACh in the synapses (Peña-Llopis et al., 2003).

1.3 Oxidative Stress and Antioxidant

In recent years, studies of antioxidant defense systems have increased due to the expected benefit of oxidative responses in providing biochemical data and indicators. These studies focused on the enzymes that contribute to providing the first line of defense against the toxicity of various pesticides. Among the most important of these studied are: glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT), and lipid peroxidation (Nataraj et al., 2017). There may be a close correlation between increased production of reactive oxygen species (ROS) that lead to oxidative damage

and exposure to pesticides in fish. Typically, in fish and vertebrates, reactive oxygen species are rapidly eliminated by antioxidant systems that are actually composed of antioxidant enzymes such as SOD and CAT and other non-enzymatic substances such as glutathione (GSH), vitamin C and vitamin E. After different types of fish were exposed to a number of pesticides such as cypermethrin and deltamethrin, significant changes were reported in the antioxidants that were the cause of oxidative stress, and many studies reported a close association between increased production of reactive oxygen species and poisonings caused by exposure to pesticides (Buszewski et al., 2019). Typically, the effects of oxidative stress are mitigated under normal conditions by first line of defense antioxidant enzymes such as CAT, SOD, and glutathione peroxidase (GPx), and many previous studies have indicated the predictive importance of membrane lipid damage as a vital predictor of oxidative stress (Dogan et al., 2011). Antioxidants in animals and plants consist of a variety and complex systems of elements, compounds, and enzymes that have the ability to prevent or slow down the oxidation process with the goal of protecting other molecules by neutralizing reactive oxygen species. Thus, preventing the production of free radicals that lead to cell damage or death, and these systems and enzymes are represented by catalase, superoxide dismutase and various peroxides, and if these systems fail, they cause oxidative stress in the cell.

CAT is present in high concentrations in mitochondria and peroxisomes, while many forms of SOD have been recognized:

- Mn-SOD is present in all mitochondrial air tissues and acts on their protectors.
- Cu-Zn SOD is present in cytosol.

The mechanism of action of antioxidant enzymes is as follows:

First: By converting reactive oxygen species to less active hydrogen peroxide (H_2O_2) and oxygen (O_2) by SOD and preventing them from converting into harmful forms.

Second: GPx catalyzes H_2O_2 to H_2O. CAT also breaks down H_2O_2 and turns it into H_2O and O_2.

Glutathione is a short polypeptide made of three amino acids: glycine, cysteine, and glutamate. It is found in tissues and plays an important role in metabolism and as an

antioxidant as it protects the cell from oxidative damage by neutralizing reactive oxygen species such as lipid peroxides and inhibiting the formation of free radicals within the cell. GSH is oxidized by GPx to form oxidized glutathione (GSSG), and GSH is reconstituted from GSSG by stimulating the enzyme glutathione reductase, which is dependent on the presence of NADPH (Varjovi et al., 2015). CAT is recruited when high concentrations of H_2O_2 are found.

Oxygen is an essential and important element in the production of energy by oxidizing food, however, the reduction of this element is not complete in natural conditions, so that the mitochondria are an important source of reactive oxygen species inside the cell. Therefore free radicals are formed, and they work to attack and destroy the components of the cell. Extreme damage to its genetic material and its various cellular functions. With the increasing accumulation of these free radicals, many diseases appear (Jaeschke, 2011).

1.3.1 The Role of Oxidative Stress and Antioxidants in Liver

The increase in free radicals or the decrease in the antioxidant systems in most liver cells leads to oxidative stress, and the latter can cause damage, and the cells lose their functional integrity, which leads to their cell death, so there must be defense systems for all cells, enzymes and antioxidants, to neutralize and eliminate reactive oxygen species (Jaeschke, 2011).

Fish have enzyme defense mechanisms to overcome the oxidative stress caused by exposure to toxic pesticides in aquatic environment. These enzymes form an antioxidant cellular defense system. It also has non-enzymatic antioxidant systems, including:

- Amino Acids
- Vitamins (vitamin A - vitamin E - vitamin C)
- Carotenoids
- Polypeptides

Both these enzymatic and non-enzymatic systems together constitute the overall antioxidant capacity that acts to inhibit the production of free radicals in aquatic organisms (Mirvaghefi et al., 2016). Catalases are a common enzyme found in nearly all organisms that are exposed to oxygen such as bacteria, plants and animals, as it catalyses the hydrogen peroxide breakdown into H_2O and O_2, an enzyme that is very important in protecting the cell from oxidation caused by reactive oxygen species. The highest turnover number of all enzymes as one catalase molecule can convert millions of hydrogen peroxide molecules into H_2O and O_2 every second. Catalase also contains four groups of haem that contain iron and allow the enzyme to interact with hydrogen peroxide. In fact, catalase is found in peroxisomes inside cells in aerobic organisms to help get rid of toxic oxides such as peroxide, a mineral enzyme that contains iron in the form of haem. Lipid oxidation is used as an effective method for measuring oxidative stress in organisms exposed to various types of chemicals such as deltamethrin and glyphosate as well as toxic metals such as iron, mercury and lead (De Castilhos Ghisi, 2012). In the event of an imbalance and disturbance in the balance between the generation of reactive oxygen species and the action of antioxidant defenses, it results in our oxidative stress, and if it is not controlled, it will cause the damage and death of all components of the cell, including proteins, fats and DNA, and therefore it is important to maintain this state of balance within the living cells. The damage caused by the lipid oxidation caused by herbicides is one of the main causes of cell damage and death.

SOD is an important enzyme in reducing the speed of cell destruction and restoration of vitality. Zinc, copper and manganese availability helps in its work, and is mainly found in peroxisomes. GPx is mainly concentrated in mitochondria and cytosol, and it is considered one of the most important antioxidant enzyme systems due to its ability to displace many free radicals and hydroperoxides resulting from oxidation, and it plays an important role in regulating various peroxides by accelerating the transformation of GSH into GSSG after removal of various peroxides (such as hydrogen peroxide, lipid peroxides, or organic peroxides). GPx is part of the system that protects cells against oxidative stress and its products, thereby reducing cell damage caused by excess free radicals (Modesto and Martinez, 2010).

1.4 Biomarkers

Biomarkers are widely used to assess the effects of near-fatal and fatal toxicity as a result of exposure to various pesticides in fish (Herrera et al., 2019).

1.4.1 Biochemical Blood Parameters

Biochemical tests are used as a basic tool in studies of chronic and acute neurotoxicity as a result of exposure to various pesticides as well as to know the physiological and health status of fish, especially the blood biochemistry test, as this test is widely used to detect target organs and tissues, as these damaged cells in this tissue release specific enzymes in the blood plasma. When deficiencies or malfunctions occur in the functioning of these tissues, especially liver and brain tissues, this will reduce a number of biochemical factors in the blood, and thus the extent of the damage caused by exposure to pesticides will be determined. Changes in tissue function have been reported in the following fish: (*Cirrhinus mrigala*, *Cyprinus carpio*, *Heteropneustes fossilis*, *Colisa fasciatus*, *O. mykiss* and *Clarias batrachus*) as a result of exposure to various pesticides (Banaee, 2012).

1.4.2 Biomarkers of Oxidative Stress

Although several studies have been conducted in the past years, no single biomarker has been identified specifically for detection of oxidative stress due to exposure to pesticides and other toxic substances (Slaninova et al., 2009). However, these field studies indicated several of the most sensitive biomarkers that can be used in combination to detect oxidative stress, including:

- GSH: GSSG ratios
- lipid peroxidation, especially thiobarbituric acid reactive substances (TBARS)
- activities of GR, GST and GPX

Thus, were the field studies reported by Dorval et al. (2005), Eufemia et al. (1997) and Machala et al. (2001) is widely credited with documenting several biomarkers of oxidative stress induced by the use of insecticides.

These biomarkers of oxidative stress have also been divided into two main groups:

- Biomarkers of free radical damage
- Antioxidant defenses

1.5 Fish as Bioindicator

In aquatic toxicology, fish are classified as the most sensitive organisms to pollutants and toxic substances in the aquatic environment (Moura et al., 2017). They are also used as biological indicators in evaluating the expected risks resulting from the leakage of chemicals into the aquatic environment (Nataraj et al., 2017). These pollutants cause significant damage to tissues and cells in fish and thus disrupt vital physiological processes (Durmaz et al., 2006). As a result of the above, conducting toxicological tests became a necessity to assess the potential effects on water quality in water bodies as a result of direct and indirect exposure to insecticides and various toxic chemicals (Wang et al., 2020). In recent years, many studies have focused on the use of fish as biomarkers in detecting various pollutants in the environment (De Castilhos Ghisi, 2012). As a result of the accumulation of toxic chemicals inside fish by contact with water or sediments or through food, they have been recognized as important biological indicators and an early warning sign against environmental hazards (Rossi et al., 2020). Fish provide early information about environmental hazards as a result of the leakage of pollutants to the aquatic environment. They also play a major role in the biotransformation, accumulation and excretion of pollutants, so the brain and liver are among the most important target organs in fish (Dogan et al., 2011). The use of fish as biological indicators has become very important as a result of their concentration at the top of the aquatic food web and their direct contact with the surrounding environment, which provides an integrated perception of environmental problems. In the event that the toxic agent reaches fish, because it is used as a main source of food for many organisms, it will threaten the health of these organisms, especially humans. Many studies have reported the negative consequences of the excessive use of pesticides, as they cause toxic effects on the lives of fish as well as humans (Soares et al., 2016).

CHAPTER II

LITERATURE REVIEW

The organophosphate pesticides have important area of usage mainly in agriculture, therapeutic medicine and as insecticides in domestic and public health applications. They may be used safely when they are handled correctly and with appropriate protective, precautionary and guidance measures. However, extensive usage, potential risk of misuse and possible adverse effects, however, pose a risk for aquatic ecosystems as the final destination of pollutants. Therefore, they have been subjected to many aquatic toxicological studies.

Mozhdeganloo et al. (2016) studied the effect of permethrin on *O. mykiss*. They measured some biochemical parameters reflecting oxidative stress in liver. They observed decreasing in antioxidant levels. Authors suggest that using vitamin C increases the efficiency of the antioxidant defense system and reduces oxidative stress in the liver.

The biochemical effects of dimethoate on liver and brain tissues of *O. mykiss* were studied in a 30 days study (Dogan et al., 2011). They observed both increasing and decreasing trend in antioxidant levels. Alteration recorded in antioxidant enzymes were interpreted as dimethoate induced oxidative stress. Authors suggest brain tissue as a sensitive organ when compared to liver due to low defense ability as a result of the increase in lipid peroxidation in the brain more than in the liver.

Rossi et al. (2020) studied the effect of glyphosate, azoxystrobin, cyproconazole and bifenthrin on *Astyanax lacustris* and *Markiana nigripinnis* before and after fumigation. They measured neurotoxicity biomarkers which reflecting oxidative stress in liver, muscle, gills and brain at both periods. They observed inhibition and failed antioxidant mechanisms. Authors concluded that application of pesticide mixture exposure

resulted oxidative stress in both fish and decreased acetylcholinesterase activity in muscle and brain were significantly of *A. lacustris*.

The oxidative stress effects of Roundup on muscle, liver and brain tissues of juvenile *P. lineatus* was studied in a 6, 24 and 96 h study (Modesto et al., 2010). The authors noted that the decrease in acetylcholinesterase activity, as a result of exposure to Roundup, was evident in the muscles after 24 and 96 hours and in the brain after 24 hours. Alteration recorded in AChE activity was interpreted as oxidative stress. Besides authors concluded Roundup has a toxic and pollutant effect.

Banaee et al. (2011) studied the effect of diazinon on juvenile *O. mykiss*. They measured some biochemical parameters of blood reflecting the toxicity. They observed decreasing in AChE activity in plasma. Authors suggest that Alteration recorded in AChE activity, albumin, protein and globulin provides tools for assessing toxicity from exposure to diazinon.

Assis et al. (2012) studied changes in AChE activities on brain tissues of *O. niloticus*, *Arapaiama gigas* and *Rachycentron canadum*. Authors suggest that changes recorded between nonpurified and purified enzymes could be used as a biomarker for detecting exposure to various insecticides.

Wang et al. (2020) They investigated in the effect of triazophos on *Danio rerio* was studied in a 96 h study. They measured some biochemical parameters reflecting oxidative stress in liver and brain. They observed both increasing and decreasing trend in antioxidant levels. They concluded that triazophos exposure resulted to oxidative stress in *D. rerio* tissue.

Disruption of the blood-brain barrier as a result of trichlorfon exposure on brain tissues of *Rhamda quelen* was studied in a 24 and 48 h study (Baldissera et al., 2019). They observed decreasing in AChE activity in brain both exposure periods. They concluded the rupture of blood-brain barrier was a cause of neurotoxicity and oxidative damage in the brain.

Durmaz et al. (2006) studied the effect of diazinon on *O. niloticus* in a 30 days study. They measured some biochemical parameters reflecting oxidative stress in muscle, gill and kidney. They observed both increasing and decreasing trend in antioxidant levels.

Authors concluded that diazinon exposure resulted oxidative stress in fish and kidney is more vulnerable than muscle and gill.

Herrera et al. (2019) studied the behavior of fish upon exposure to organophosphate pesticides. They measured changes in AChE activity when exposed to low doses of the pesticide, especially when accompanied by studying the behavior of fish as a biomarker. Authors concluded of the utility of using AChE as a biomarker in neurotoxicity studies.

Topal et al. (2017) investigated in the effect of imidacloprid on *O. mykiss* was studied in a 21 days study. They reported that extensive use of imidacloprid promoted oxidative stress, demonstrated with increased in some biochemical parameters such as (MDA, SOD, CAT and GPx). They concluded that imidacloprid could induce neurotoxicity to *O. mykiss* by enhancing AChE inhibition.

The biochemical effects of profenofos on *Labeo rohita* was studied in 21days study (Nataraj et al., 2017). They observed both and decreasing increasing trend in antioxidant levels. Authors suggest that profenofos exposure resulted pathological changes in the studied tissues in addition to genotoxicity in *L. rohita*.

Baldissera et al. (2021) studied the effect of trichlorfon on *R. quelen*. They evaluate the behavioral effects and measured some biochemical parameters reflecting oxidative stress. They observed both increasing and decreasing trend in antioxidant levels and a decreasing trend in AChE activity during the exposure period.

The biochemical effects of permethrin on brain of *N. notopterus* were investigated in a 15 days study (Moniruzzaman et al., 2020). Brain dysfunction due to loss of oxidative balance has been interpreted as oxidative stress with permethrin. Besides authors suggest that melatonin could be used to reduce oxidative stress caused by pesticides.

Zhao et al. (2021) studied the effect of cypermethrin/sulfamethoxazole on juvenile *grass carp*. The authors found that acetylcholinesterase was inhibited during the exposure period. They concluded that exposure to the mixture of the two pesticides caused a much greater toxicity than exposure to each individual.

Faria et al. (2021) studied the effect of glyphosate on zebrafish. They measured some biochemical parameters reflecting oxidative stress and neurotoxicity in brain of fish. They concluded that glyphosate affects the survival of fish in aquatic ecosystems.

Yang et al. (2020) investigated the biochemical effects of pyrethroid on liver, muscles and gills of fish such as (trout, tilapia and carp). The changes reported in antioxidant enzymes after exposure to pyrethroid pesticides were explained to make fish more vulnerable to oxidative stress in the aquatic environment.

Farag et al. (2021) studied the effect of bifenthrin on *O. niloticus*. They measured some biochemical parameters reflecting oxidative stress, inflammatory and neurobehavioral in brain of fish. They observed decreasing trend in antioxidant levels in brain. The authors concluded that bifenthrin increased oxidative damage and affected fish behavior and viability.

CHAPTER III

MATERIALS AND METHODS

3.1 Material

3.1.1 Test Organism

Juvenile rainbow trout (*O. mykiss*), (72.80±4.85g, 16.24±1.03cm), were taken from the breeding ponds of from Firniz Alabalik (Kahramanmaras) and adapted to laboratory conditions for 2 weeks. During the adaptation period, the fish were kept in 200 L aquariums containing tap water and ventilation was provided with a central system. Fish were fed daily with commercial trout food (Optima trout-Skretting) throughout acclimatization. The feeding were stopped 24 h before the experiments The 12:12 photoperiod was applied.

3.1.2 Water

The characteristics of water were: temperature temperature 9.6 ± 0.55, pH 7.8 ± 0.28, dissolved oxygen 8.7 ± 0.71 mg/L and conductivity 745.5±10.11 µS cm^{-1}.

3.1.3 Test Compound

A commercial grade formulation of Phosmet (Barco 50 WP, Gowan Crop Protection Limited), [O,O-dimethyl S-phthalimidomethyl phosphorodithioate] was used as test compound.

3.2 Method

3.2.1 Experimental Design

Sublethal concentrations were selected based on 96 hour LC_{50} value of phosmet for *O. mykiss* (500µg/L) (Johnson and Finley, 1980). Selected concentrations were 5 µg/L, 25 µg/L and 50 µg/L which corresponds to 1%, 5% and 10% of the 96h LC_{50}.

Insecticide was dissolved in test water to prepare stock solution and further dilutions were performed to achieve test concentrations in aquariums. After adaptation period, fish were divided into 4 groups. Group 1 treated as control and maintained in pesticide-free water. Groups 2, 3 and 4 were exposed to 5 µg/L, 25 µg/L and 50 µg/L phosmet for 24, 48, 72 and 96 hours. There were 24 fish in each treatment and experiments were performed three times. Fish were fasted during the experiment and the aquarium water was renewed at 24 hour intervals (APHA, 1980). Fish were monitored regularly and no death was observed. The experimental protocols were authorized by the Local Ethics Committee of Animal Experiments of Gaziantep University.

3.2.2 Biochemical Analysis

At the end of durations, 6 fish selected randomly and they were anesthetized with MS-222 (50 mg/L, buffered with $NaHCO_3$ (Ross and Ross, 2008). Then they were euthanized through spinal cord section. Blood samples taken from caudal vein were taken into anticoagulant-free tubes and centrifuged at 5.000 rpm for 10 min (Hettich Universal 320R) to obtain serum samples. The levels of glucose, cholesterol, triglyceride, protein and the activities of ALT, AST and ALP were analyzed using a biochemical analyzer (Siemens ADVIA 2400 Chemistry System) by using kits provided by Siemens Healthcare Diagnostics Inc. (Munich, Germany).

Liver and brain samples were dissected, rinsed with ice-cold NaCl (0.59%). They were homogenized (1:5 w/v) in 0.1 M pH 7.4 PBS (phosphate buffer saline) via Ultraturrax homogenizator (Isolab). To obtain supernatant, homogenates were centrifuged at 13.000 rpm for 30 min at 4 ^{o}C (Hettich Universal 320R). Spectrophotometric methods were used for analysis of biochemical parameters (Shimadzu UV Mini-1240).

3.2.2.1 Organosomatic Index

The weights of liver tissue were recorded and used in the calculation of liver somatic indice with the following formula:

$$\text{Organosomatic index} = (\text{Tissue weight} / \text{Body weight}) \times 100$$

3.2.2.2 Acetylcholinesterase Activity

AChE activity was measured by using the method described by Ellman et al. (1961). The method is based on the ability of cholinesterase to catalyze the breakdown of acetylcholine to thiocoline and acetate. The intensity of color formed during the reaction of Ellman reagent (DTNB; 5,5-dithio-bis-(2-nitrobenzoic acid) with thiocoline was read at a wavelength of 412 nm.

3.2.2.3 Superoxide Dismutase Activity

SOD catalyzes the dismutation of superoxide radical ($O_2^{\cdot -}$) to H_2O and molecular oxygen. Superoxide radicals form a colored complex with 2- [4-iodophenyl] -3 [4-nitrophenol]-5-phenyltetrazolium chloride in the presence of xanthine. The formation of formazone is inhibited by SOD to remove $O_2^{\cdot -}$ from the environment. SOD enzyme activity was measured by percent (%) inhibition of formation of formazone at 505 nm (McCord and Fridovich, 1969).

3.2.2.4 Glutathione Peroxidase Activity

GPx catalyzes the oxidation of GSH to GSSG by H_2O_2. In the reaction mixture containing t-butyl hydroperoxide as hydrogen peroxide source, GSH is oxidized to GSSG with the activity of GPx. GPx activity was measured by reading the dismutation of t-butyl hydroperoxide at 340 nm (Beutler, 1984).

3.2.2.5 Catalase Activity

CAT catalyzes the conversion of H_2O_2 to water and molecular oxygen. CAT activity was measured by reading the destruction rate of H_2O_2 at 230 nm wavelength (Beutler, 1984).

3.2.2.6 Protein

Amino acids reduce the phosphotungaustic acid-molybdic acid separator (Folin-Ciocalteu) and form blue complex. Protein amount was measured by using the method of Lowry et al (1951). In the reaction mixture, proteins react with Cu^+ to form copper-peptide bond-protein complex. Copper-protein complexes combine with tyrosine and

tryptophan residues of Folin-Ciocalteu reagent in the reduction process. The absorbance of the colored complex was measured at 750 nm.

3.2.2.7 Thiobarbituric Acid Reactive Substances (TBARS)

Thiobarbituric acid reactive substances are by products of lipid peroxidation. TBARS assay was performed by using the method of Ohkawa et al (1979). The method based on measurement of colored complex formed during reaction of thiobarbituric acid with TBARS at 532 nm.

3.2.2.7 Glutathione

The amount of GSH in the tissues was determined by method described by Ellman et al (1959). The method includes the oxidation of GSH by DTNB. The absorbance of resulting yellow complex of 5'-thio-2-nitrobenzoic acid was read at 412 nm.

3.2.3 Statistical Analysis

Two-way ANOVA (analysis of variance) followed by Tukey's test were applied to (SPSS 22.0 computer program; SPSS Inc. Chicago, Illinois, USA) considering $p<0.05$ as statistically significant. The strength of association between variables was examined by Pearson's correlation analysis.

CHAPTER IV

RESULTS

4.1 Behaviour and Organosomatic Index

Control group exerted normal behaviour during experimental period. Control like behaviour was observed in group treated with 5 µg/L. In group exposed to 25 µg/L phosmet frequent surfacing and faster swimming on 72 h and 96 h were recorded. Behavioural abnormalities like positioning at the corner/bottom of aquarium, loss of equilibrium and erratic swimming were observed throughout the experimental period. Phosmet treatment did not cause any significant change in liver somatic index ($p>0.05$; Figure 4.1).

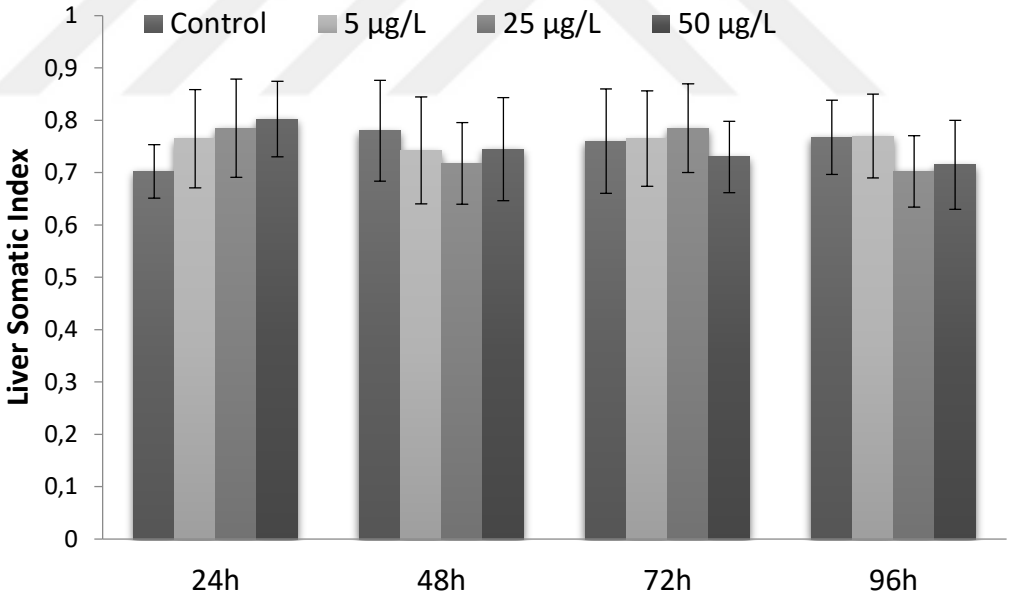


Figure 4.1 The effect of phosmet on the liver somatic index in *O. mykiss*.

4.2 Serum Biochemistry

4.2.1 Glucose

Effect of phosmet application on serum glucose level is given in Figure 4.2. Significant decrease in serum glucose level being more pronounced at high concentration with extended period of exposure was determined. The estimated increase in the levels were 44%, 43% and 25% for 5 µg/L, 25 µg/L, and 50 µg/L concentrations on 96 h, respectively ($p<0.01$).

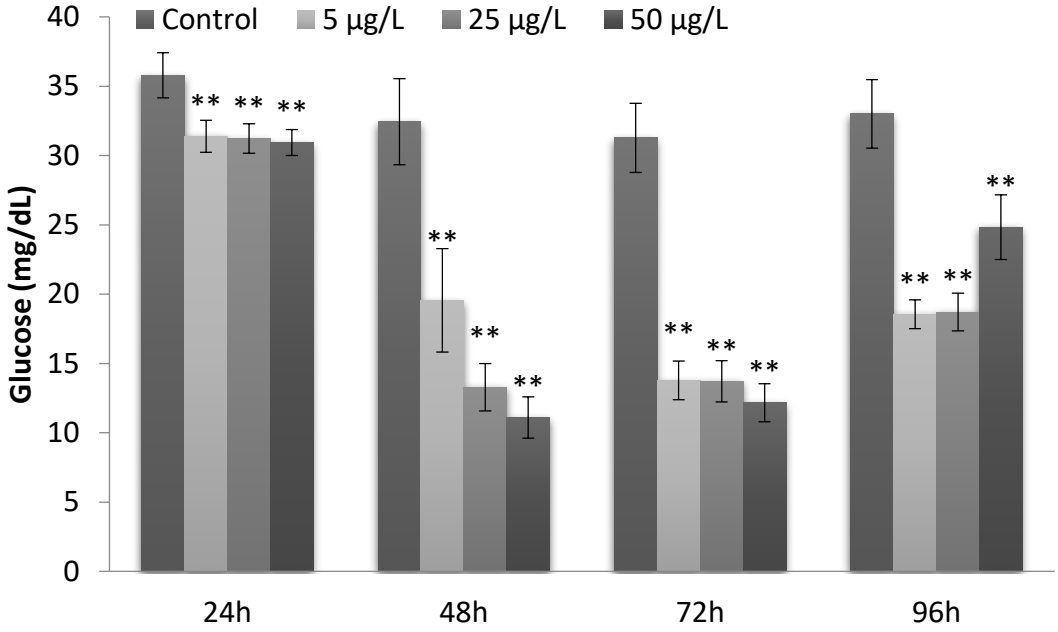


Figure 4.2 Effects of sublethal phosmet exposure on serum glucose (mg/dL) level of *O. mykiss*. **p < 0.01.

4.2.2 Cholesterol

Effect of phosmet treatment on serum cholesterol level is given on Figure 4.3. Application resulted in significant decrease in cholesterol level reaching maximum of 32% following 50 µg/L phosmet exposure for 72h ($p<0.01$).

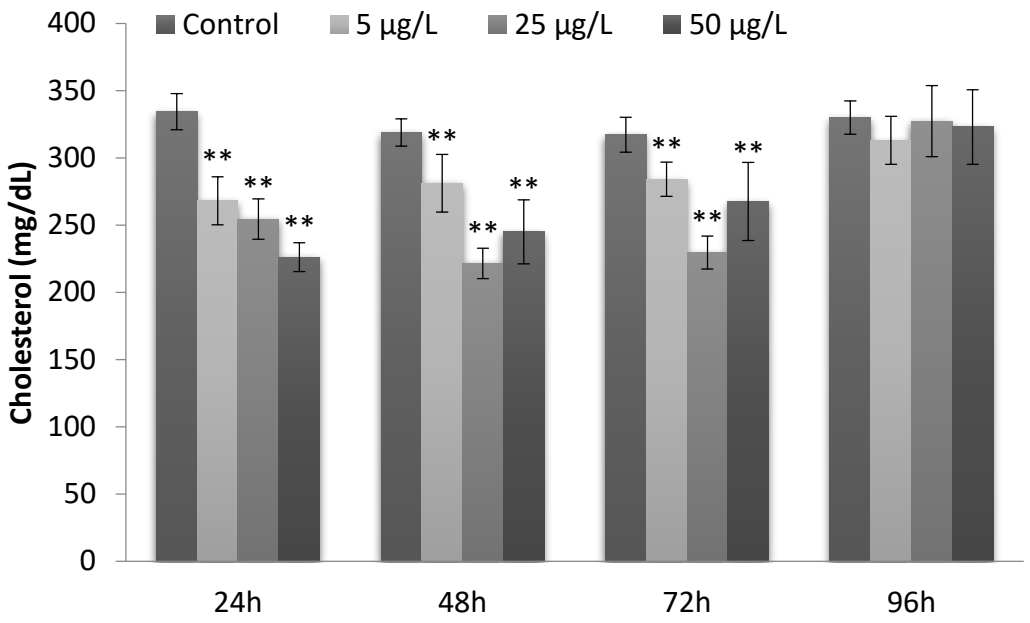


Figure 4.3 Effects of sublethal phosmet exposure on serum cholesterol (mg/dL) level of *O. mykiss*. **p < 0.01.

4.2.3 Protein

An important decline in serum protein level after 24h and 48h phosmet exposure reaching 13% was determined (Figure 4.4, p<0.01).

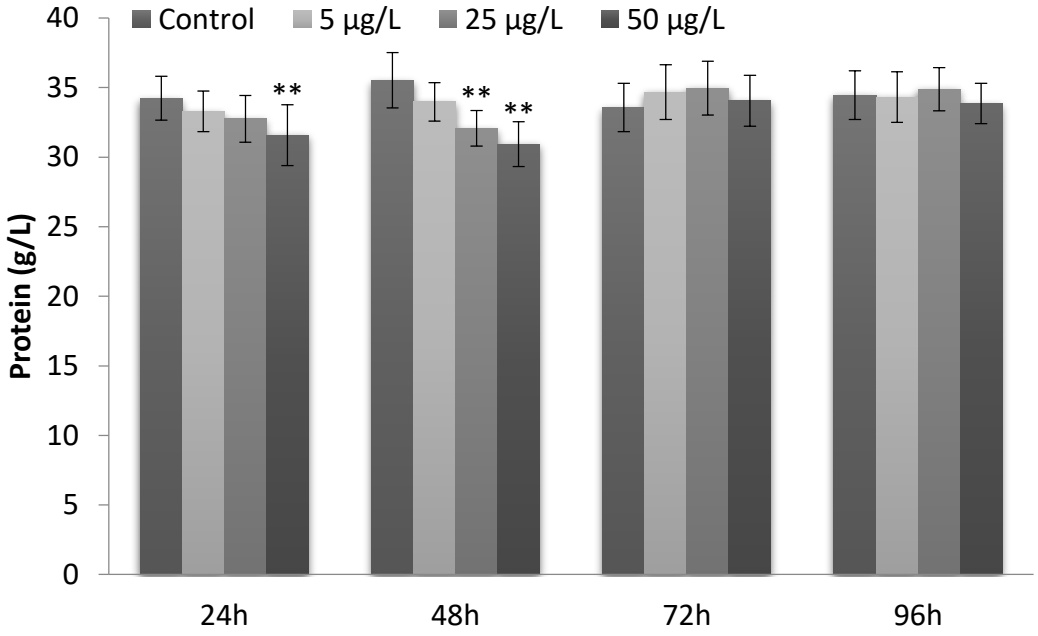


Figure 4.4 Effects of sublethal phosmet exposure on serum protein (g/L) level of *O. mykiss*. **p < 0.01.

4.2.4 ALT

The activity of ALT showed an ascending trend following phosmet treatment. The maximum percentage change was calculated as 172% at 50 µg/L concentration for following 96h (Figure 4.5, $p<0.01$).

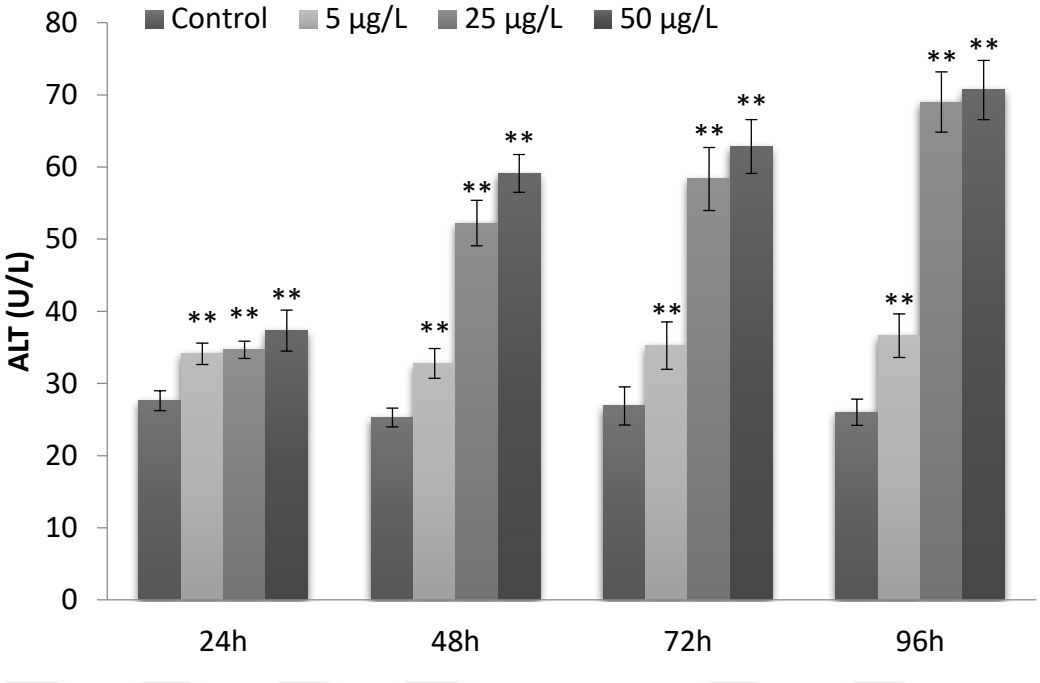


Figure 4.5 Effects of sublethal phosmet exposure on serum ALT activity (U/L) of *O. mykiss*. **p < 0.01.

4.2.5 AST

Phosmet application caused significant increase in serum AST activity. The maximum percentage changes was calculated as 123% at 50 µg/L concentration following 96 h (Figure 4.6, $p<0.01$).

4.2.6 ALP

ALP also showed increasing trend following phosmet exposure reaching maximum of 29% on 96h at 50 µg/L concentration (Figure 4.7, $p<0.01$).

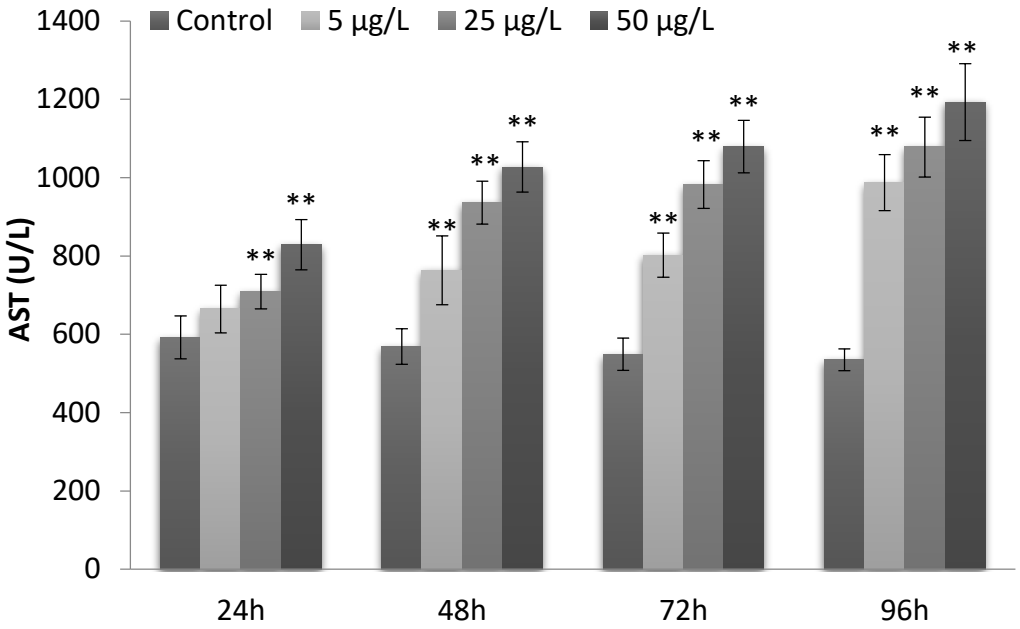


Figure 4.6 Effects of sublethal phosmet exposure on serum AST activity (U/L) of *O. mykiss*. **p < 0.01.

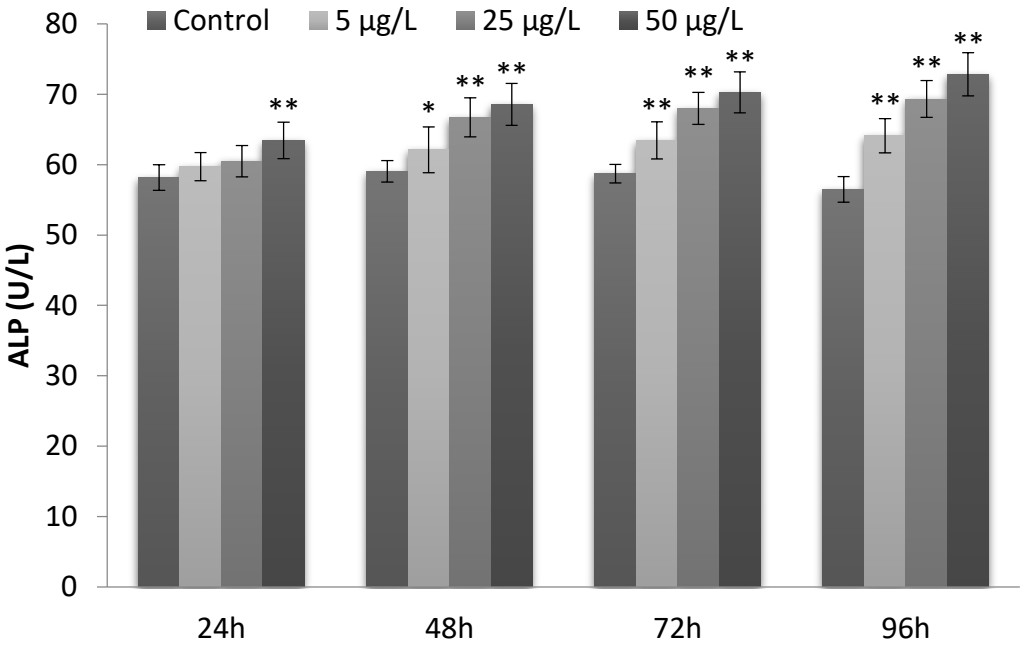


Figure 4.7 Effects of sublethal phosmet exposure on serum ALP activity (U/L) of *O. mykiss*. *p<0.05 and **p < 0.01.

4.3 AChE Activity

Percentage changes in specific activity of AChE in liver and brain tissues are given in Figure 4.8 and Figure 4.9, respectively. A decline reaching maximum of 14% in liver tissue was statistically insignificant (p>0.05). Phosmet treatment resulted in inhibition

of brain AChE activity ($p<0.01$). The estimated maximum inhibition rates were as 34%, 44% and 46% for 5 µg/L, 25 µg/L, and 50 µg/L concentrations following 96 h exposure, respectively.

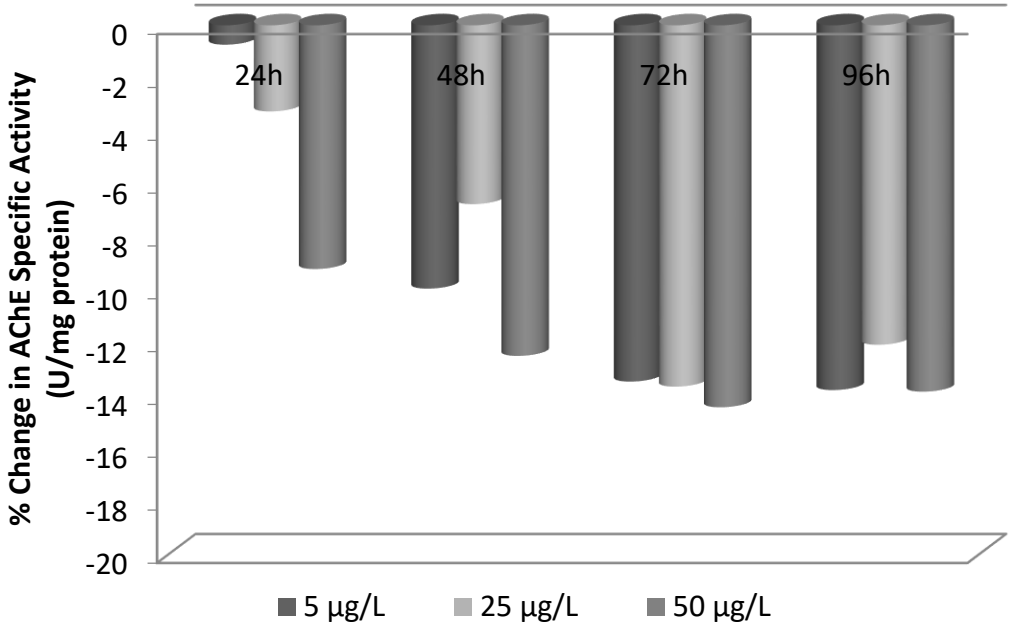


Figure 4.8 Effects of sublethal phosmet exposure on liver AChE activity (U/mg protein) of *O. mykiss*.

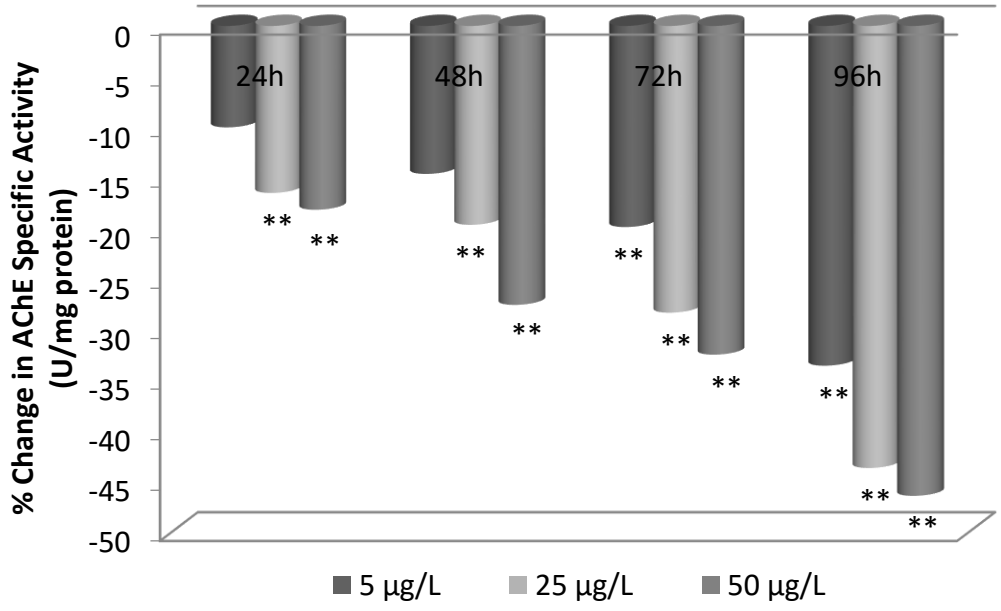


Figure 4.9 Effects of sublethal phosmet exposure on brain AChE activity (U/mg protein) of *O. mykiss*. **$p < 0.01$.

4.4 Antioxidant Enzyme Activities

4.4.1 SOD Activity

Percentage changes in specific activity of SOD in liver and brain tissues are presented in Figures 4.10 and 4.11. In liver tissue, phosmet treatment caused a significant increase in SOD activity reaching maximum of 124% on 96h at 50µg/L ($p<0.01$). Similarly, rise in SOD activity in brain tissue was observed and determined induction was 46% at 50 µg/L concentration on 96h ($p<0.01$).

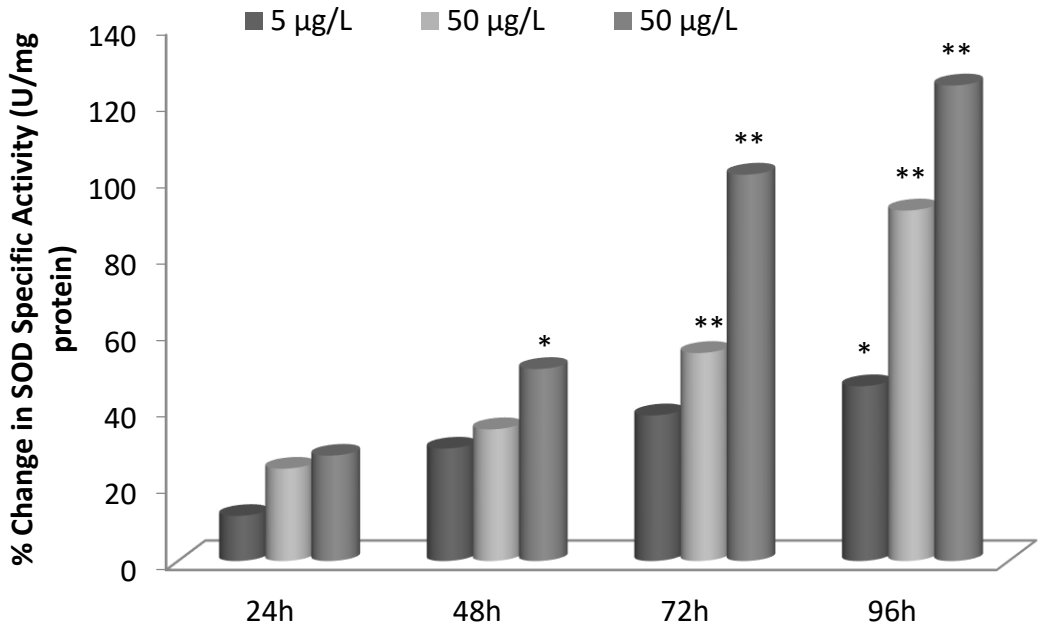


Figure 4.10 Effects of sublethal phosmet exposure on liver SOD activity (U/mg protein) of *O. mykiss*. *$p<0.05$ and **$p < 0.01$.

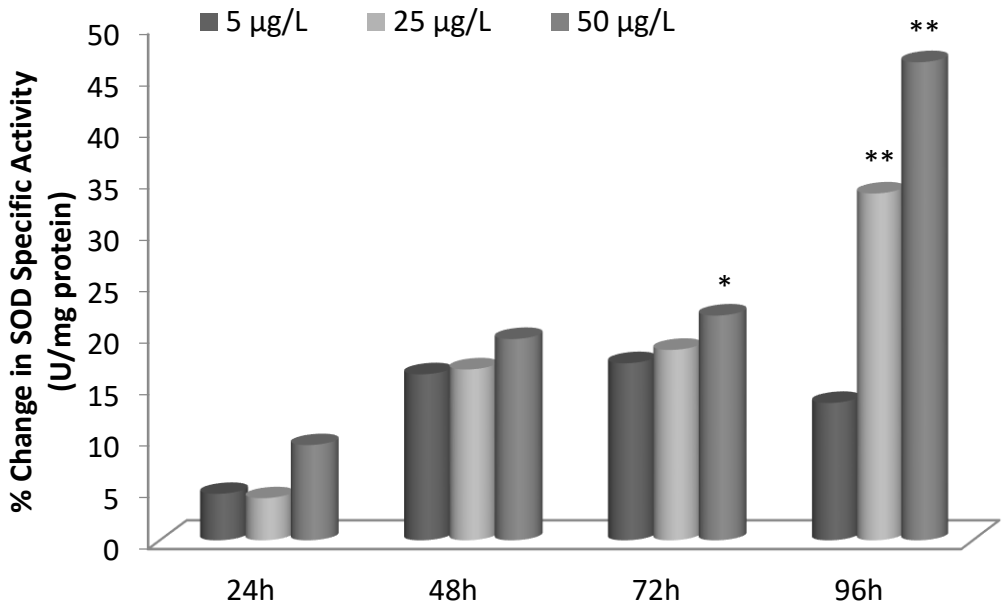


Figure 4.11 Effects of sublethal phosmet exposure on brain SOD activity (U/mg protein) of *O. mykiss*. *p<0.05 and **p < 0.01.

4.4.2 CAT Activity

Percentage changes in specific activity of CAT in liver and brain tissues were given in Figure 4.12 and Figure 4.13. In liver tissue, no alteration was recorded in CAT on 24h and 48h (p>0.05). Following 72h and 96h, 12% and 21% increase were calculated on at the highest phosmet concentration, respectively (p<0.01). In brain tissue, phosmet treatment resulted in induction of CAT activity. On 96h, 22% and 23% increase were calculated at 25 µg/L and 50 µg/L concentrations (p<0.01).

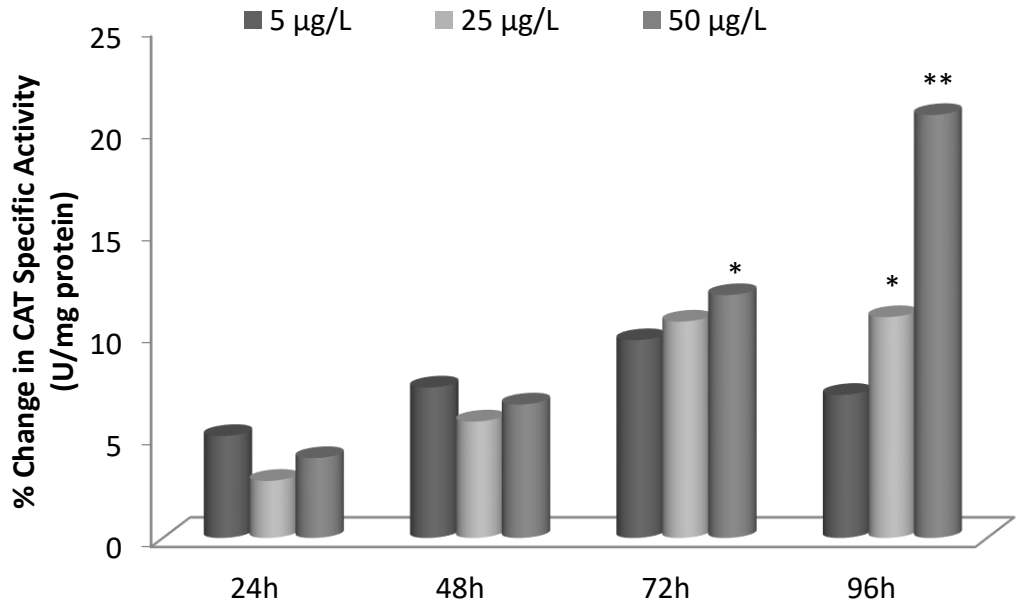


Figure 4.12 Effects of sublethal phosmet exposure on liver CAT activity (U/mg protein) of *O. mykiss*. *p<0.05 and **p < 0.01.

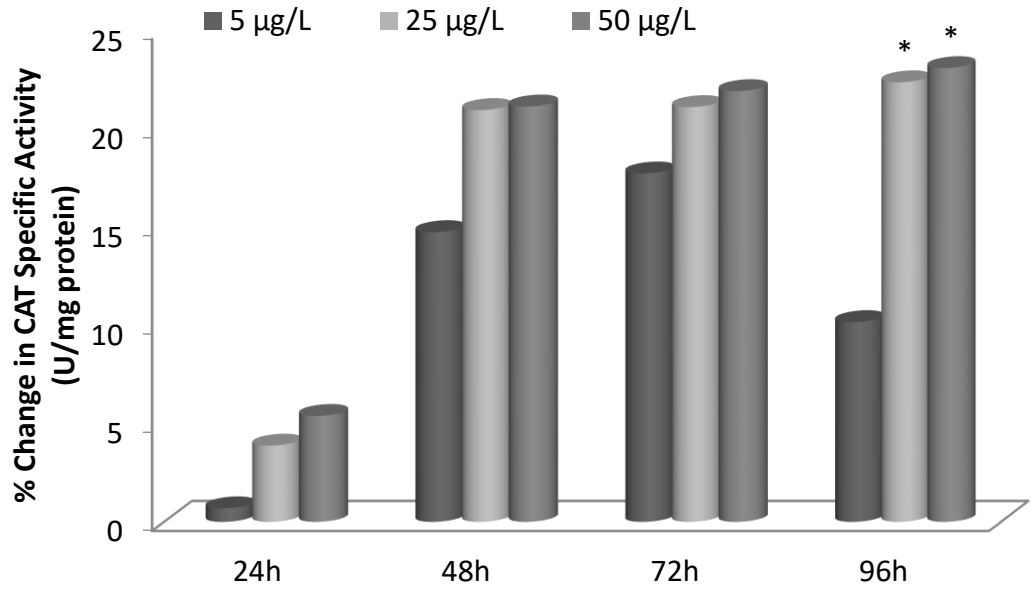


Figure 4.13 Effects of sublethal phosmet exposure on brain CAT activity (U/mg protein) of *O. mykiss*. *p<0.05 and **p < 0.01.

4.4.3 GPx Activity

Percentage changes in specific activity of GPx in liver and brain tissues were given in Figures 4.14 and 4.15. Phosmet application caused increase in GPx activity in both

tissues. The maximum of percent changes were calculated as 17% and 18% for liver and brain tissues at 50 µg/L phosmet concentration on 96h, respectively ($p<0.01$).

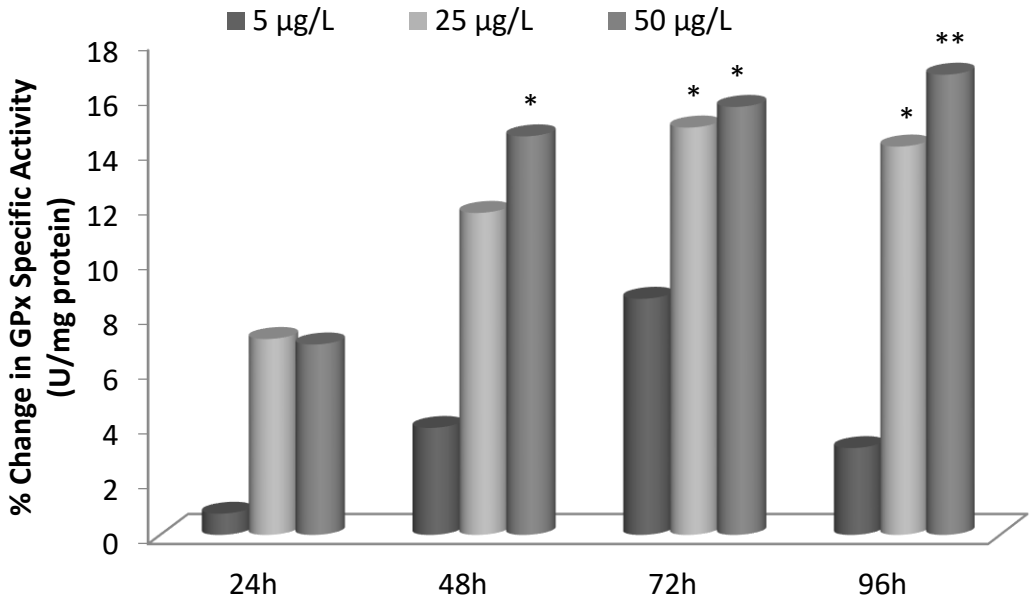


Figure 4.14 Effects of sublethal phosmet exposure on liver GPx activity (U/mg protein) of *O. mykiss*. *p<0.05 and **p < 0.01.

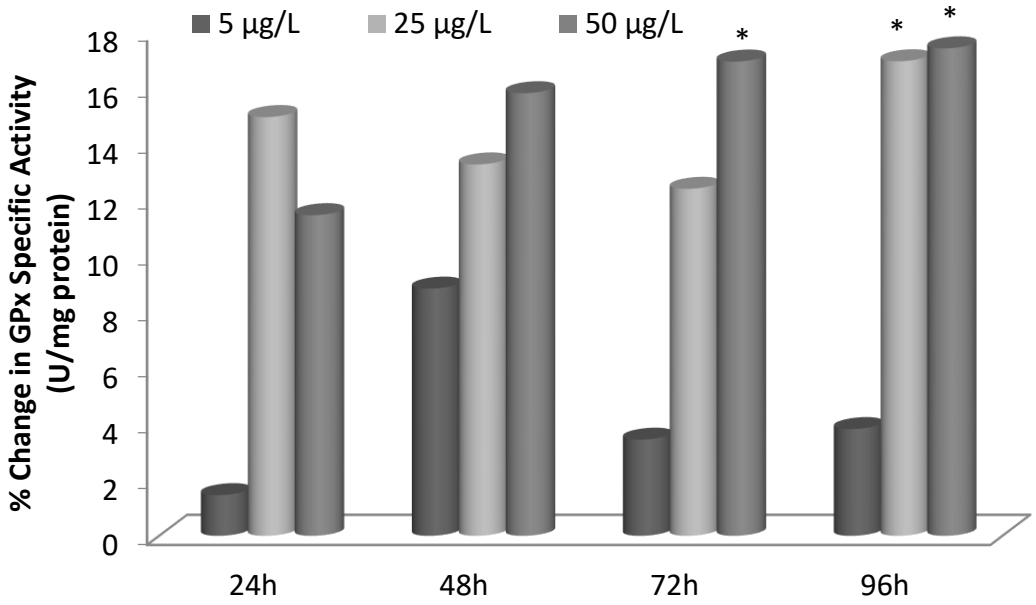


Figure 4.15 Effects of sublethal phosmet exposure on brain GPx activity (U/mg protein) of *O. mykiss*. *p<0.05 and **p < 0.01.

4.5 TBARS Level

The effect of phosmet application on TBARS levels of liver and brain tissues of *O. mykiss* were given in Figure 4.16 and Figure 4.17. In liver tissue, phosmet exposure caused concentration and duration dependant increase TBARS contents ($p<0.01$). On 96 h, 56%, 55% and 78% increase were determined for 5 µg/L, 25 µg/L, and 50 µg/L concentrations ($p<0.01$). Increasing tendency was also observed for TBARS content of brain tissue. 62%, 84% and 97% rise were estimated for 5 µg/L, 25 µg/L, and 50 µg/L concentrations, respectively, following 96h phosmet exposure ($p<0.01$).

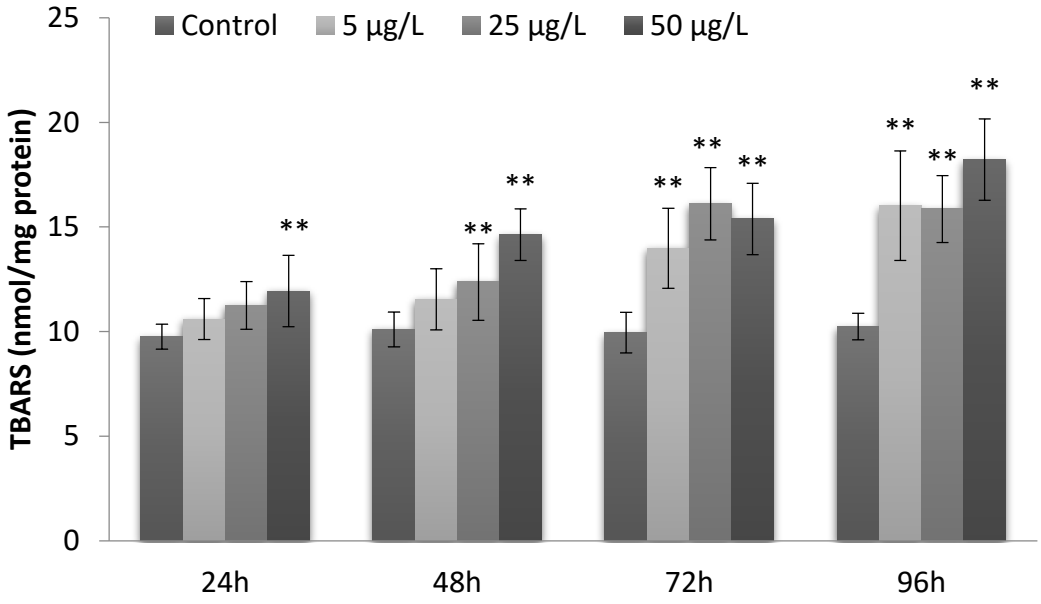


Figure 4.16 Effects of sublethal phosmet exposure on liver TBARS content (nmol/mg protein) of *O. mykiss*. **p < 0.01.

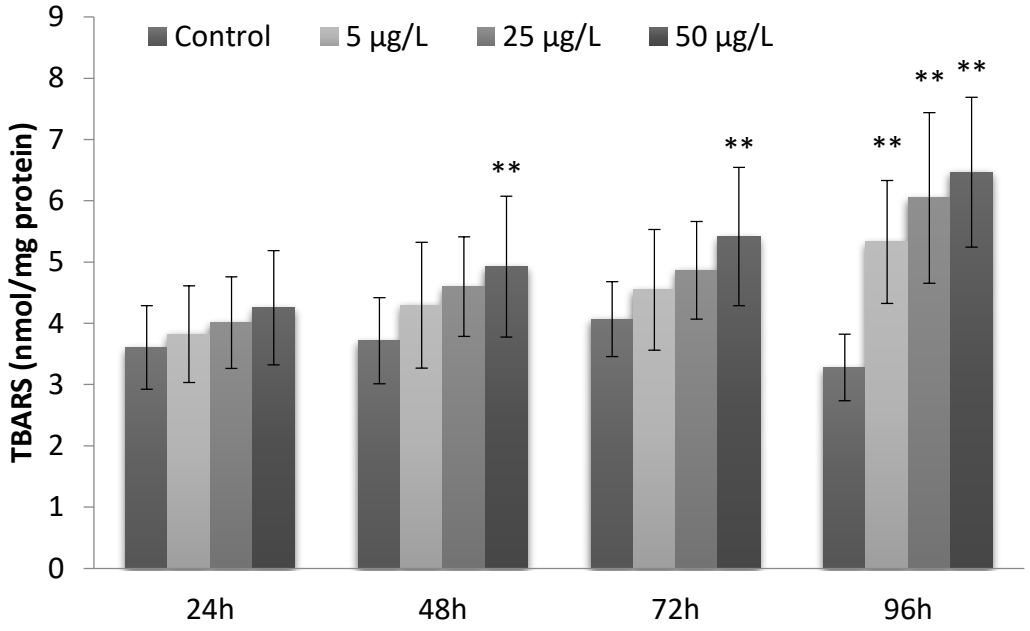


Figure 4.17 Effects of sublethal phosmet exposure on brain TBARS content (nmol/mg protein) of *O. mykiss*. **p < 0.01.

4.6 GSH Level

The effect of phosmet application on GSH content of liver and brain tissues of *O. mykiss* was given in Figures 4.18 and 4.19. GSH content did not exert any significant change following phosmet treatment for 24h and 48h in liver tissue ($p>0.05$). On the other hand, 21% increase was detected at 50 µg/L concentration on 72h and 42% and 49% rise were observed in groups exposed to 25 µg/L and 50 µg/L for 96h ($p<0.01$). In brain tissue, 26% and 35% increase in GSH content were estimated following 25 µg/L and 50 µg/L phosmet application for 96h ($p<0.01$).

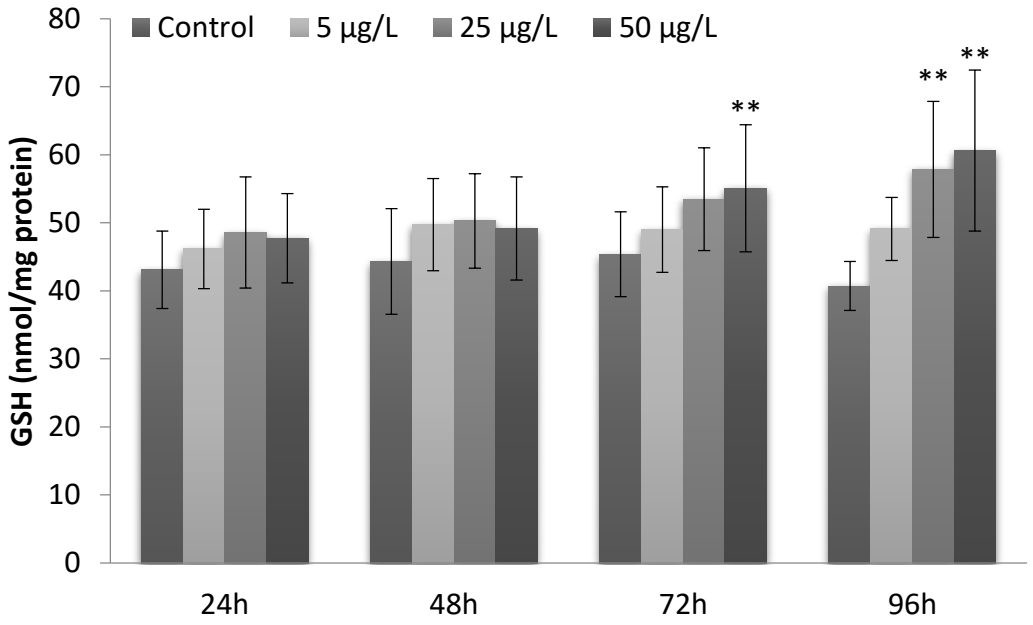


Figure 4.18 Effects of sublethal phosmet exposure on liver GSH content (nmol/mg protein) of *O. mykiss*. **p < 0.01.

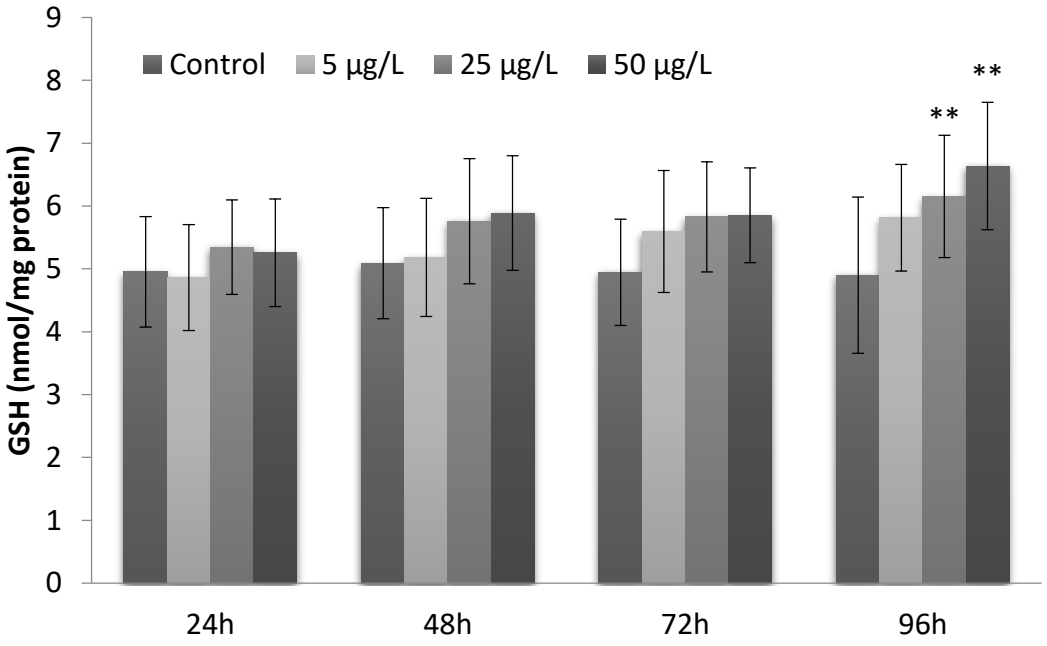


Figure 4.19 Effects of sublethal phosmet exposure on brain GSH content (nmol/mg protein) of *O. mykiss*. **p < 0.01.

4.7 Protein Level

The effects of phosmet application on protein content of liver and brain tissues of *O. mykiss* were given in Figures 4.20 and 4.21. The liver protein content did not show any alteration on 24h and 48h of phosmet treatment while a concentration and duration dependant decrease was determined on 72h and 96h ($p<0.01$). In brain tissue, only significant change observed was 19% decrease in protein content at 50 µg/L concentration on 96h ($p<0.05$).

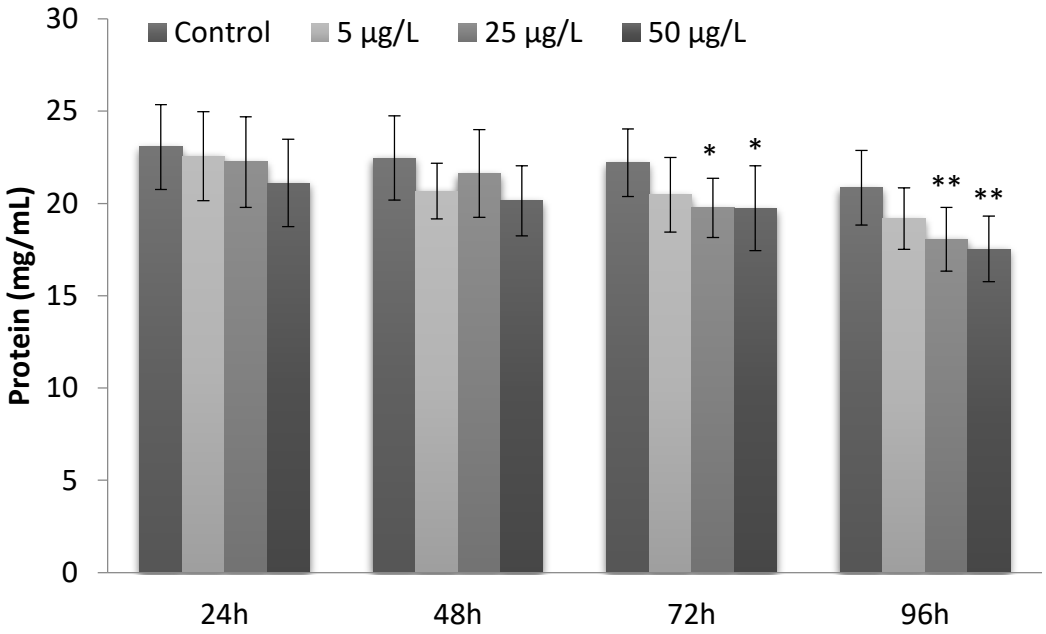


Figure 4.20 Effects of sublethal phosmet exposure on liver protein content (mg/mL) of *O. mykiss*. *$p<0.05$ and **$p < 0.01$.

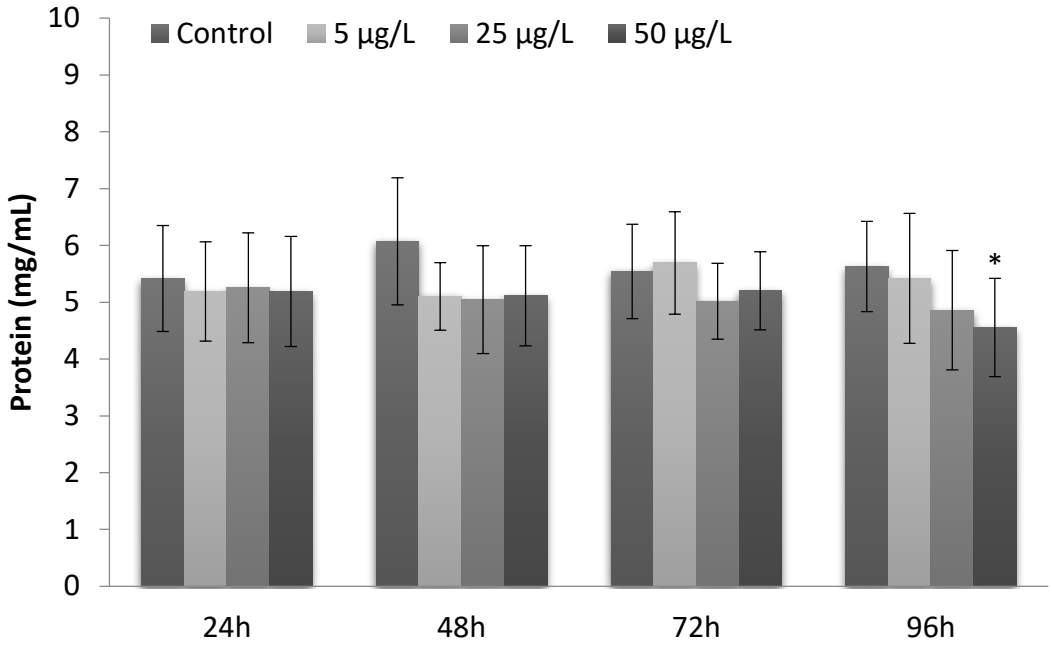


Figure 4.21 Effects of sublethal phosmet exposure on brain protein content (mg/mL) of *O. mykiss*. *p<0.05 and **p < 0.01.

The correlation coefficients calculated for the relationship among ALT, AST and ALP were significant and positive (p<0.01). The correlation analyses revealed a significant and negative relationship between AChE activity and TBARS content in liver and brain tissues (r=-0,228 and r=-0,214, p<0.01). The antioxidant enzymes and GSH levels were found to be positively correlated to TBARS content for both tissues (p<0.01).

CHAPTER V

DISCUSSION

There is growing concern around the world about environmental pollution problems caused by pesticides that are already affecting non-target organisms, such as fish. Actually, fish are very important as they can be used as biological indicators because they accumulate chemicals through contact with water or sediments or by eating their food. Another thing is that biological responses in fish are considered an early warning sign of potential environmental problems due to exposure to various pollutants, including pesticides in particular. As a result of the widespread spread of pesticides, they directly enter the bodies in the water and cause a general risk to the aquatic ecosystem (Malaj et al., 2020; Chatterjee et al., 2021; Ghasemzadeh et al., 2015).

As we know, pesticides have been used for more than half a century increasing crop production and pesticide pollution has thus become a major concern in regards to health. Acetylcholinesterase is a frequent target due to exposure to various pesticides and toxins (Moniruzzaman et al., 2020; Lee et al., 2020). Authors report a decrease in acetylcholinesterase activity as a result of exposure to organophosphate pesticides of more than 20 percent (Santana et al., 2021). Actually, even exposure to very small concentrations of pesticides or their fragments causes inhibition of acetylcholine esterase activity and thus affects the survival and behavior of fish in the aquatic environment. For example, authors have reported changes in speed and irregular swimming patterns (Bretaud et al., 2000; Huang et al., 2016; Dogan and Can, 2011; Laetz et al., 2013; Marigoudar et al., 2009; Baldissera et al., 2021; Qiu et al., 2017), cognitive abilities, defensive behaviours (Zala and Penn, 2004), decreases growth rate in addition to other metabolic processes that occur in fish (Moniruzzaman et al., 2020) and loss of ability to avoid predators (Sandoval Herrera et al., 2019), as one of the consequences of exposure to pesticides.

Alkaline phosphatase, alanine transaminase, and aspartate aminotransferase have been used as biochemical parameters recently in toxicology studies to evaluate tissue damage, liver and muscle, induced by various pollutants in the environment (El-Sayed, 2007). The current study showed exposure of *O. mykiss* to phosmet increased levels of ALP, ALT and AST throughout the duration of the exposure. Changes in these parameters indicates the involvement of liver in the toxic mechanism of action of phosmet (Nwani et al., 2014; Bhattacharya et al., 2008; Banaee et al., 2011).

The changes in the levels of protein and glucose were interpreted as indicators of general health status and energy metabolism in fish (Abhijith et al., 2012; Dogan and Can, 2011). The authors reported that the inhibition of protein biosynthesis or its use by repairing damaged cells as a source of energy was the result of exposure to the herbicides in *O. mykiss* (Pereira et al., 2013). The breakdown of proteins into amino acids (protein catabolism), and increasing ATP synthesis where they are utilized as an energy source to provide cells with energy levels (Geneshwande et al., 2012). Likewise, other authors have reported decreased protein in fish tissues exposed to various environmental pollutants (Samanta et al., 2014; Dogan and Can, 2011; da Fonseca, 2008).

AChE is a vital marker for determining neurotoxicity, particularly as a result of exposure to organophosphorous compounds and carbamates (Tompson et al., 1988; Olsvik et al., 2019; Pereira et al., 2019; Liu et al., 2020). Thus, biomarkers are used to understand the extent of pollution caused by the use of various pesticides in the aquatic environment. Inhibition of AChE activity is common in fish exposed to various toxins (Kim and Kang, 2015; dos Santos Carvalho et al., 2020). Phosmet exposure resulted in reduction in AChE in both tissues being statistically signidicant and pronounced in the brain. Inhibition of the enzyme activity in brain causes accumulation of ACh at the synapses of the nerves and disrupts the function of the nerve which can result in behavioral abnormalities in exposed fish. Similar results were reported for different fish species following pesticide exposure (Baldissera et al., 2019; Dutta and Arends, 2003; Woo and Chung, 2020). As indicated by the results, it is demonstrated that the inhibition of AChE is specifically caused by organophosphorus insecticide phosmet (Woo and Chung, 2019; Wang et al.,2015; Hai et al.,1997; Varga et al.,1997; Monserrat et al., 2002). The results of the present study are consistent with previous

reports of pesticide-induced AChE decrease in fish tissue (Almeida et al., 2010; Xing et al., 2010; Dogan and Can, 2011; Topal et al., 2017).

In the current study, our data indicated that the exposure of phosmet significantly increased TBARS contents in the tissues of the liver and brain of *O. mykiss* reflecting increased oxidative stress and lipoperoxidation. We also found an important and negative relationship between the activity of AChE and TBARS content in the tissues of the liver and brain, there can be a significant correlation between the increase in lipid peroxidation and the inhibition of the activity of AChE in both of the tissues of *O. mykiss*. That is why it can be considered to be of lipid peroxidation caused by phosmet and is related to its anticholinergic activity. Similar results have been reported in *O. niloticus* that is exposed to etoxazole (Sevgiler et al. 2004). The excessive generation of reactive oxygen species explains the disruption of the balance between oxidants and antioxidants, which causes cellular oxidative stress (Nwani et al., 2014). The production of reactive oxygen species is a by-product in normal biochemical functions, but the increased production of reactive species may lead to oxidative stress and lipid oxidation. (Awasthi et al., 2018; Maharajan et al., 2018; Wang et al., 2020). The elevated TBARS values obtained in this study are consistent with previous reports in the fish that are exposed to different pesticides (Nwani et al., 2010; Blahová et al., 2013; Guilherme et al., 2012; Modesto et al., 2010; Nwani et al., 2014).

The antioxidant defense system works by altering antioxidant enzyme activity and antioxidant content. Changes in enzymatic activity provide information and an early warning of the abiotic stress to which organisms are exposed in the aquatic environment (Wang et al., 2020). In compatible with our study, pesticide elicited oxidative stress status in different fish species were reported as a response to handle the stress (Pandey et al., 2003; Farombi et al. 2008; Zheng et al., 2016; Cheng et al., 2018; Wang et al., 2020). Therefore, the occurrence of oxidative stress in *O. mykiss* tissues induced by exposure to phosmet has been demonstrated while further studies are required in order to investigate the damage from oxidative stress.

In conclusion, this study shows phosmet can adversely affect juvenile rainbow trout by providing insight into its toxicity mechanisms. Endpoints of this investigation, also presents the threat at population level due to consequences of adverse effects on growth, survivability and reproduction. The results of the current study not only reflect

the potential threat to wild fish populations but also provide an understanding of the potential impacts on human health from exposure to phosmet. Further studies may focus on investigating potential adverse effects under the environmentally relevant concentration considering co-exposure to different chemicals together in nature for risk assessment studies.

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