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Low-Density Polyethylene (LDPE) Secondary Microplastics Feed Contamination can Affect Certain Important Biochemical Parameters (Protein & Catalase Activity) in *Anabas testudineus* (Bloch)

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Abstract

With the ever-increasing demand for the usage of plastics, plastic pollution has become an alarming concern worldwide. Disposal of plastic wastes into aquatic environments, be it freshwater or saltwater, has led to the disruption of aquatic ecosystems at different levels. In the aquatic media, discarded plastics undergo disintegration by several processes to give rise to secondary MPs. Low-density polyethylene (LDPE), one of the common forms of plastic which is widely used, can undergo the same fate to give rise to its secondary MPs in nature. Likewise, pesticides can also act as environmental pollutants as these are rampantly used to check pests and subsequently increase the crop yield. Non-target aquatic organisms like fishes may be affected by agricultural activities by means of run-off of agrochemicals from farms into aquatic ecosystems, especially freshwater ones. Monocrotophos (MCP), a popular organophosphate pesticide, finds its widespread use in agriculture. The present study reported the effects of LDPE (in the form of secondary MPs) alone and in combination with MCP on certain biochemical parameters such as tissue protein level and activity of catalase (CAT) of the climbing perch, *Anabas testudineus*, at exposure period of 24h, 48h and 72h. Fish feed was supplemented with 10% LDPE-MPs and optimum doses of MCP was administered directly to water of respective pots. When compared to the tissue protein and CAT activity in C (control), T1 (treated with MCP) and T2 (treated with LDPE MPs), the tissue protein and CAT activity in T3 (treated with both LDPE MPs and MCP) decreased and increased considerably, respectively, in a time-dependent manner. Considerable variation has been observed in the biochemical parameters of the fish from 24 h to 72 h of exposure to the pesticide MCP. After72h, the maximum decrease in tissue protein in liver (60.337±1.593 mg/g tissue) and in muscle (33.333±2.923mg/g tissue), and maximum increase in activity of CAT in liver (0.503±0.027 U/mg protein) and muscle (0.234±0.060 U/mg protein) were observed in the fish collected from T3. Statistical analyses indicated significant change between treatments. The adsorption property of LDPE MPs (adsorbent) with MCP (adsorbate) was established. The results of the study indicated that tissue protein level and CAT activity could be used as biomarkers to determine the toxicity level of MPs in water and its effect in combination with contaminants like pesticides on fishes or even other aquatic organisms.

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Keywords

Aquatic medium, plastic, polystyrene (PS), polypropylene (PP), environment.

Introduction

Plastics and Microplastics (MPs)

In the recent years, plastic pollution in the aquatic medium, be it marine or freshwater, has become a growing environmental concern worldwide. The term 'plastic' comes from the Greek word 'plastikos' which refers to the ability of being moulded or shaped into various sizes and shapes. Plastics we use nowadays are prepared from inorganic and organic raw materials, such as carbon, hydrogen, silicon, oxygen, nitrogen and chloride. Plastics are synthetic organic polymers which are derived from polymerization of monomers extracted from gas or oil. These are regarded as chief pollutants in the environment. An estimated amount of greater than 300 million tons of plastic are generated every year in the world. It consists of plastics such as polyvinyl chloride (PVC), polyethylene (PE), polystyrene (PS), polypropylene (PP), polyethylene terephthalate (PET).

These are generally non-biodegradable, however, their degradation can be hastened by UV exposure (Russell *et al.,* 2011). The process of photo-aging is slow, which releases approximately 3% of content after 2000 hours of photo-aging (Sanchez *et al.,* 2014). PS and PE are comparatively easier to get degraded in natural environmental conditions (Schirinzi *et al.,* 2017). However, these can be seen even after many decades in the form of MP and plastic litter, especially PET is mostly seen in all sorts of plastic bottles as well as promotional material. In 2014, an approximate amount of 51 million tons of PET was produced throughout the world (Schoof *et al.,* 2017).

Large plastic debris is called macroplastics. Microplastics (MPs) are fragmented pieces of large plastics which differ in size. The term 'microplastic' was coined by Richard Thompson in 2004. These are small fragments of plastics derived from the degradation of macroplastics. MPs are defined as the plastic particles having <5 mm in diameter. These arise from fragmented macroplastics due to man-made or artificial fibres for textiles released from household washing of fabric, UV exposure and mechanical abrasion, and MPs used in cosmetic and consumer products such as toothpaste, facial cleansers, etc.

Types of Microplastics (MPs)

MPs are released into the environment via two sources: primary and secondary MPs. Primary MPs are manufactured in the microscopic range. These are minute plastic particles designed for commercial purpose, such as cosmetics, clothing and other textile products including fishing nets. On the other hand, secondary MPs are minute plastic fragments or particles derived from the process of disintegration of larger plastic debris both on land and in water. The structural integrity of plastic debris can be decreased by biological, physical and chemical processes, which results in their breakdown to give rise to secondary MPs. This process of breakdown is driven by exposure to environmental factors, particularly solar radiation, water currents, tides, and waves. MPs are generally derived from fragmentation of macroplastics due to an aging and weathering phenomenon.

Environmental impact of MPs

Plastic debris produced as a result of disposal and degradation of commercial products as well as industrial waste ultimately finds its place in the estuaries, oceans, freshwater, and also even in the tap water which is now well established. Plastic litter is easily transported by currents and winds due to its light weight, and also recirculates between beach sediments and seawater. Density of polymers is a crucial factor for circulation and propagation of MPs (Eubeler *et al.,* 2010). MPs leading to entanglement and consumption by a myriad of aquatic organisms, e.g., zooplanktons, crustaceans, fishes, sea turtles, seabirds, and aquatic mammals is well documented (Free *et al.,* 2014). The smallest MPs are small enough to mimic as food for the zooplanktons (Cole *et al.,* 2013), allowing the MPs to establish and consolidate in the food chain at very low trophic levels. This leads to bioaccumulation and biomagnification. MPs of smaller size are known to induce greater toxicity in algae.

Adsorption of heavy metals, harmful polycyclic aromatic hydrocarbons and pathogens on MPs augmented the negative effects to marine biodiversity, presumably by the decrease in nutrient uptake and rise in oxidative stress. MPs pose adverse threats at the cellular and tissue level, and interfere with reproductive success, energy allocation, and signalling execution, which jeopardize environments and biodiversity. MPs are identified as an emerging environmental threat to the freshwater ecosystems and its ecological consequences. Effluents from wastewater treatment plants serve as a vital point source for MP particles for freshwater environments. Recent experimental studies suggest that MP ingestion by fishes can be detrimental to a great extent.

Polyethylene (PE) and Low-density polyethylene (LDPE)

Polyethylene (PE) is a thermoplastic polymer which comprises long chains formed as a result of the combination of monomer ethylene (CH_2=CH_2). Ethylene or ethene gets converted to ethane (CH_3–CH_3) as it takes its place in a polymer and straight sections of the polymers are the same structure as the simple chain hydrocarbons. The most problematic among synthetic plastics is PE. The resistance of PE to biodegradability (biological attack) is related to its nature of hydrophobicity and water repellency. Biodegradation of PE is a very slow process. High-density polyethylene (HDPE), linear low-density polyethylene (LLDPE) and low-density polyethylene (LDPE) are the most important grades of PE.

LDPE is defined by a density range of 0.91-0.94 g/cm^3. LDPE is one of the forms of PE which is manufactured from gaseous ethylene under extreme pressures and high temperatures (up to about 350°C) in the presence of oxide initiators. These processes yield a polymer structure having both short and long branches. LDPE has more branching (on about 2% of the carbon atoms). Its appearance varies from translucent to opaque. It is highly flexible and has low density since the branches resist the polyethylene molecules from closely packing together in hard, stiff, crystalline arrangements. It can withstand temperatures of 80°C continuously and 95°C for a short time.

Its melting point is roughly around 110°C. It is unreactive at room temperature, except by strong oxidizing agents. It possesses weaker intermolecular forces, lower tensile strength and higher resilience. It exhibits excellent resistance to concentrated and dilute acids, bases, esters and alcohols; good resistance against ketones, aldehydes and vegetable oils; limited resistance to aromatic and aliphatic hydrocarbons, oxidizing agents and mineral oils; and offers poor resistance to halogenated hydrocarbons, which is not recommended for use. It finds its uses in agricultural mulch, housewares, squeeze bottles, wire and cable insulation, toys, trash and grocery bags, packaging film, etc.

Pesticide and its environmental impact

Pesticides are substances or chemicals which are used to check pests. Target pests can comprise insects, molluscs, nematodes, fishes, birds, mammals, etc. Most pesticides are intended for the purpose of plant or crop protection. Apart from being beneficial, pesticides can pose potential toxicity to humans and other species. Administration of pesticides is usually done by sprinkling, spraying, and spreading across the agricultural lands and farms. Runoffs can carry pesticides into aquatic environments. As a result, non-target organisms such as fishes are affected by the unwanted exposure to pesticides.

Monocrotophos (MCP)

Monocrotophos ($C_7H_{14}NO_5P$), an organophosphate insecticide, is frequently used in agricultural crop fields of India. These types of pesticides are known to be neurotoxins, which affect the work of neurons in the body. MCP is commonly known as Nuvacron or Azodrin. It is a systemic as well as contact poison in nature. Despite its ban, it is still preferred because of its high efficiency against insect pests.

Uncontrolled application of MCP in farming practices has resulted in the contamination of surface and ground water, causing neurotoxicity, genotoxicity, hyperglycemic, and stressogenic effects on different organisms. Being easily soluble in water, it is placed under class I: highly toxic compounds.

Adverse effects of MCP toxicity have been studied and reported in fishes such as *Anabas testudineus* (Santhakumar *et al.,* 1999, 2000$_a$, 2000$_b$, 2001; Mercy *et al.,* 2000; Ashafali *et al.,* 2019; Yadav *et al.,* 2019; Mohapatra *et al.,* 2020$_a$), *Carassius auratus* (Tian *et al.,* 2010, 2017), *Catla catla* (Tamizhazhagan *et al.,* 2017; Tharmendira *et al.,* 2017), *Channa gachua* (Sadhu & Shafi, 1988; Koul *et al.,* 2006), *Channa punctatus* (Sadhu, 1993; Rao & Ramaneswari, 2000; Agrahari *et al.,* 2007), *Cirrhinus mrigala* (Sulekha *et al.,* 1999; Velmurugan *et al.,* 2007), *Clarias batrachus* (Narra *et al.,* 2011), *Cyprinus carpio* (Nithiyanandam *et al.,* 2007), *Danio rerio* (Zhang *et al.,* 2013; Pamanji *et al.,* 2015; D'Costa *et al.,* 2018; Kuppuswamy and Seetharaman, 2020) *Labeo rohita* (Sulekha *et al.,* 1999; Rao & Ramaneswari, 2000; Ramaneswari & Rao, 2008; Muthukumaravel *et al.,* 2013; Tamizhazhagan, 2015; Ravichandran *et al.,* 2019), *Lepidocephalichthys guntea* (Marandi and Sadhu, 2008), *Mugil cephalus* (Sathick *et al.,* 2019$_b$), *Mystus gulio* (Sathick *et al.,* 2019$_a$), *Mystus vittatus* (Rao & Ramaneswari, 2000; Sukumaran *et al.,* 2013), *Oreochromis mossambicus* (Rao, 2004, 2006), *Oreochromis niloticus* (Thangnipon *et al.,* 1995), *Puntius filamentosus* (Nair *et al.,* 2013) and *Tilapia mossambica* (Joshi *et al.,* 1988; Remia *et al.,* 2008).

Oxidative stress in animals in response to chemicals and the concept of biomarkers

The term 'oxidative stress' is often used to describe physiological conditions where there is a lack of balance between oxidants and antioxidants in the body of an organism. It can result in disrupted redox (reduction and oxidation) signalling as well as molecular damage induction in tissues and cells. Significant groups of oxidant molecules comprise free radicals and reactive oxygen species (ROS), molecules that are very reactive owing to the presence of unpaired electron(s). An uneven number of electrons allow them to cause oxidation reaction in the body by reacting with other chemical components. Antioxidants are the molecules that can donate an electron to a free radical without making themselves unstable.

This causes the free radical to stabilize and become less reactive. Animal body is constantly exposed to free radicals, ROS and other oxidants, both endogenously as by-products of normal metabolism and exogenously through the environment. Exposure to pollutants or other stress conditions increases oxidative stress in animals. Fishes like other animals have group of enzymes that are directed towards the removal of free radicals. Oxidative stress is involved in numerous pathological conditions.

The study of activity of enzymes like lactate dehydrogenase (LDH), glutathione peroxidase (GPX), catalase (CAT), acetylcholinesterase (AChE), superoxide dismutase (SOD) and process of lipid peroxidation can be widely used as biomarkers to determine the oxidative stress level in an organism. From among the various biomolecules, proteins and activity of enzyme CAT were used as biomarkers for the present study.

Proteins

Proteins are the nitrogen-containing biomolecules formed of long chain of amino acids linked through peptide linkage. The proteins are the major structural constituent of all types of cells, enzymes, hormones, blood, etc. It has been observed that the protein synthesis is affected when an organism is exposed to a stress condition as there is increase in protein catabolism.

CAT

CAT is an antioxidant enzyme which catalyses the process of breakdown of hydrogen peroxide (H_2O_2) which is toxic to the cell into water (H_2O) and molecular oxygen (O_2). The toxicity of H_2O_2 is due to the oxidation of proteins, membrane lipids and DNA by peroxide ions (O_2^{2-}).

The present experiment was based on the hypothesis that increasing the exposure duration of chemical treatment could increase the oxidative stress in fishes exposed to respective treatments.

Animal model

The effects of exposure to MPs alone and in combination with other environmental pollutants has been least studied in freshwater fishes. Hence, *Anabas testudineus* (Bloch, 1792) was selected as the model organism in the present experimental study.

Scientific classification

Kingdom – Animalia
Phylum – Chordata
Class – Actinopterygii
Order – Anabantiformes
Family – Anabantidae
Genus – *Anabas*
Species–*testudineus*

Anabas testudineus (meaning 'turtle-like') is commonly known as 'climbing perch'. It is a freshwater air-breathing fish. It is distributed widely throughout South and Southeast Asia. It is a column feeder and a larvivorous fish preying upon larvae of mosquito and hence, used to check mosquito population. It is considered as a quite hardy fish since it can survive in water containing low dissolved oxygen content, contaminated water and also water with rotting vegetation. It also draws the attention of research workers as a brilliant model for the study of stress tolerance and attenuation of physiological homeostasis with respect to environmental stress.

Geographic distribution

It is native to Asia, where it is distributed in India, Pakistan, Nepal, Bangladesh, Bhutan (most likely), Myanmar, Sri Lanka, Cambodia, Thailand, Vietnam, Laos, Malaysia, Southern China, Singapore, Brunei, Indonesia and introduced to Philippines. There has been a considerable ambiguity and debate regarding the taxonomy of the genus *Anabas*. It is often treated as

monotypic, but almost certainly represents a species complex.

Ecology

These fishes are observed mostly in lakes, ponds, canals, estuaries and swamps. Adult ones occur in medium to large rivers, streams, inundated fields and stagnant water bodies including slowly flowing canals. They are often seen in regions with dense vegetation. It can withstand highly unsuitable aquatic conditions and is associated chiefly with turbid, static waters. They remain buried in the mud during dry season. They feed on macrophytic vegetation, shrimps and fish fry. It is reported that these fishes undertake lateral migration from Mekong mainstream or other permanent water bodies to inundated lands at the time of flood season and return to the permanent water bodies at the beginning of the dry season. They stay in pools having submerged weeds and shrubs during the dry season. They have accessory air-breathing organ which assists them to survive for several days or weeks at a stretch without water provided the air-breathing organs is kept moist.

The study of the biochemical parameters of the fish can be used as biomarkers for understanding the toxicity due to LDPE secondary MPs and MCP.

Objectives

The major objectives of this study were:

To study the effects of LDPE secondary MPs on liver and muscle tissues of the experimental fish, *Anabas testudineus*, by determining the changes in certain biochemical parameters, i.e., level of tissue protein and activity of enzyme CAT.

To evaluate the probability of LDPE secondary MPs as a potential adsorbent of pesticide MCP and their combined effects on the tissue protein level and CAT activity of liver and muscle tissues *of A. testudineus*.

Materials and Methods

Experimental setup

The sample fishes (*Anabas testudineus*) used for the experiment were collected from the local resource Non-Veg World market in Bhubaneswar. The collected live specimens of fishes were disease-free, healthy and had no prior record of biochemical treatment, i.e., they were untreated. The fishes were allowed to acclimate for 3 days before using it for the experiment. The commercially formulated pesticide, Monocrotophos (36% S.L.) was purchased from the local market. LDPE secondary MPs were prepared from pristine LDPE by refrigeration of the latter at -26°C for 96 hours and then mechanical homogenization (hammering) was done with the help of industrial blender to crush and grind the plastic, which was followed by scanning electron microscope (SEM) analysis in order to ensure the size of the generated MPs. Earlier, normal fish feed (300 g) and normal fish feed supplemented with 10% LDPE secondary MPs (110 g in total) were both mechanically and manually prepared and the constituents (raw materials) of fish feed were also obtained from Central Institute of Freshwater Aquaculture(CIFA), Bhubaneswar [ref. 3.2]. For the experimental setup, 4 spherical earthen pots of optimum dimensions were used in triplicate and each of them filled was with water to required capacity and labelled as control (C), treatment 1 (T1), treatment 2 (T2) and treatment 3 (T3), respectively (ref. Table 3.1). These were purchased from the nearby local market. These pots were thoroughly cleaned and then filled with tap water almost to their brim for 1-2 days prior to experimental usage. This was done to ensure minimal loss of water by evaporation in future experimental use due to soakage or seepage of pot water, permeating through the pores to the outer surface of pots. The filled tap water was then discarded from all the pots. These pots were placed on a low raised platform of sand in order to provide a base. Pots were kept in well-ventilated laboratory conditions. The water used for the purpose of stocking fish was collected from water available in Lily Pool located in the college garden, which is free from added chemicals such as bleaching powder. The collected water was poured in requisite quantities into all the pots via a sieve in order to filter the water from macroscopic impurities. Aerators were used for continuous supply of dissolved oxygen to pot water. These were connected to all the pots. Each pot was then stocked with 5 fishes (*Anabas testudineus*). Since *Anabas*is a quite agile fish, mouth of those pots was tightly covered with nets, which were tied to the neck of the pots by elastic rubber bands and strings/threads so as to restrict the fish movement to inside of the pots only. The condition of the fishes was regularly checked from time to time with the help of flashlight and dip net. Dead fishes were eliminated by dip net. The pot water was also suitably changed as per requirement depending on the turbidity of water, mortality of fishes, etc.

The physicochemical parameters of pot water were taken well care of throughout the experiment. Measurement of

temperature, pH and conductivity of water was done using water analysis kit (Systronics Water Analyser 371). Dissolved oxygen in water and hardness of water were measured by Winkler's method and EDTA (Ethylenediaminetetraacetic acid) titration method, respectively. Mean values of water quality parameters for test water during the experimental period were as follows: pH = 7.2 ±0.05, temperature = 25°C±2.50°C, conductivity = 34.8 ± 4.22 µS, dissolved oxygen = 8.8 ± 2.0 mg/L, and total hardness ($CaCO_3$) =136.0 ± 3.4 mg/L.

Preparation of fish feed

Formulation of two types of fish feed – normal feed (100%), and 100% normal feed with 10% LDPE MPs in addition – was done as per the recommendation of ICAR-CIFA Bhubaneswar and experimental need, respectively. For the preparation of only basic normal feed (100%), Table 3.2 was followed. The composition of this type of feed contains decent amounts of fish meal (ground dried fish) since *Anabas testudineus* is mainly carnivorous. For preparing LDPE-enriched feed, LDPE MPs were added as a supplement and mixed uniformly with 100% normal feed in the ratio 1 : 10 (LDPE-MPs : normal feed).

Average weight of the fish = 26 g ± 4 g. Daily dietary requirement of fish was feeding twice a day at 5% of its body weight.

$$\text{Daily feeding amount per fish} = \frac{N \times AW \times FR}{100} = \frac{1 \times 26 \times (2 \times 5)}{100} = 2.6\ \text{g}$$

where, N = Number of fishes; AW = Average weight of fish; FR = Feeding rate

Fish feed formula

The freshly prepared fish feed was then subjected to drying in the sun as well as hot air oven at 100°C for removal of moisture or water content. After complete drying, fish feed (pellets) was packaged in labelled airtight zipper bags for storage. Fully sun-dried or oven-dried fish feed and sealed packaging are the essential criteria to ward off any fungal interference.

Fish sampling and chemical treatment

20 healthy fishes (*Anabas testudineus*) of grow-out stage belonging to age group 7± 2 weeks were procured from Non-Veg World, Patia, Bhubaneswar. The fishes were acclimatized to the laboratory conditions for 3 days in the water collected from the Lily Pool before starting the experiment. The average weight and length of fishes were found to be 26 ±4g and 15± 3 cm, respectively. During this period, normal untreated feed was provided to all the fishes belonging to all the pots by sprinkling on pot water. After 3 days, chemical treatment was started. Treatment periods of 24 h, 48 h and 72 h were carried out separately in 3 different phases. At the onset of each treatment period of the experiment, stipulated doses of formulated fish feed and 5.3 ppm sublethal concentration of MCP (36%) for respective experimental pots were given as per Table 3.3. Firstly, MCP doses were directly administered to pot water (T1 and T3) with micropipette. Secondly, fish feed was given after a short while just after MCP had mixed with pot water. As each pot had 5 fishes, 13g of fish feed was provided to respective pots for the fish to ingest at the start of each treatment period. At the end of each treatment period, pot water was changed for the next treatment period.

For *A. testudineus*, LC_{50} of MCP was calculated as per OECD guidelines (OECD, 1992) and probit analysis (Finney, 1971). 48h $LC_{50}$was found to be 106 mg/L or ppm.

For formulation of MCP,

It was already known that 48-hr LC_{50} = 106 ppm.

$\frac{1}{20^{th}}$ of LC_{50} = 106 ppm / 20 = 5.3 ppm (SL concentration), which was to be prepared.

As we had MCP solution of 36% SL conc., it can also be written as:

100 mL solution contained 36 mL MCP = 1,000,000 mL solution had 360,000 mL of MCP.

So, we had 360,000 ppm concentration of MCP.

In order to prepare 5.3 ppm SL conc. of MCP for5L water from MCP (36% SL), volume of MCP (36% SL) to be added to 5 L water was calculated from the following equation:

$C_1 \times V_1 = C_2 \times V_2 \Rightarrow 360000 \times V_1 \Rightarrow 5.3 \times (5000 + V_1) \Rightarrow V_1 = 0.07361\ \text{mL} = 73.61\ \mu\text{L}$

where, C_1 = conc. of MCP (36% SL) in ppm = 360000 ppm
V_1 = vol. of MCP (36% SL) to be added to water to yield 5.3 ppm of MCP

C_2 = conc. of MCP solution to be prepared = 5.3 ppm

V_2 = vol. of water in mL = 5 L = 5000 mL

Hence, 5.3 ppm sublethal concentration of MCP was prepared by addition of 73.6 µLof MCP (36% SL) toT1 and T3 pots containing 5 L water each.

Processing of fish for preparation of tissue sample

One healthy fish from each of the experimental pot was collected after every 24 h, 48 h and 72 h and immediately dissected to collect liver and muscle. The collected sample organs of the sacrificed animal were kept on petri dish/watch glass and stored inside ice box stuffed with ice and ice pack when not in use in order to prevent sample tissue degradation or deterioration. Then the organs were weighed on aluminium foil in a weighing balance and the readings were noted. The organs were sliced into small pieces using stainless steel scissors to facilitate thorough homogenization. 500 mL phosphate buffer of pH 7.4 was prepared before hand by adding 1.22 g KH_2PO_4 (potassium dihydrogen phosphate) and 2.8 g K_2HPO_4 (dipotassium hydrogen phosphate) to 400 mL distilled water and then the volume and pH was adjusted to 500 mL and 7.4, respectively. The prepared buffer was stored in refrigerated condition. The fish tissue was homogenized with 0.05 M Phosphate Buffer of pH 7.4 in a porcelain mortar and pestle under chilled condition to avoid tissue damage. The volume of phosphate buffer (in mL) to be added was determined as 4 times of weight of the particular tissue (in g) to be homogenized. The homogenate was centrifuged at 10,000 rpm for 10 minutes at 4°C using table top cooling centrifuge (REMI). The supernatant was collected in an Eppendorf tube and stored at -20°C in a deep freezer (Celfrost) for further use.

Sample collection for experiment

Fish samples were collected at an interval of 24 hours, 48 hours, and 72 hours from each pot respectively for biochemical analysis.

Biochemical analysis

Protein

Protein estimation was conducted according to the principle and procedure of Folin–Ciocâlteu method (Lowry *et al.,* 1951) at 700 nm (visible range) by taking bovine serum albumin (BSA) as standard protein. The first step in protein estimation involved the preparation of solution A (1 g sodium hydroxide +5 g sodium carbonate + 250 mL distilled water), solution B (0.125g copper sulphate in 25 mL distilled water), solution C (0.25 g sodium potassium tartarate in 25 mL distilled water) which were required for preparation of reaction mixture. The tissue working solution of C, T1, T2 and T3 was prepared from the supernatant obtained from fish tissue processing. Reaction mixture was prepared by adding 26 mL of solution A, 520µL solution B and 520 µL solution C in accordance with 50:1:1 ratio (as per as the experimental need). Dilution of original sample was done to make working solution of sample by adding the supernatant of the sample with distilled water in the ratio of 1: 4, respectively. This quantitative ratio was followed by adding 0.4 mL of tissue sample supernatant and 1.6 mL distilled water in Eppendorf tube. 80µL distilled water was taken in separate Eppendorf tubes in duplicate and 20µL of working solution each from C, T1, T2 & T3 was added to each of the tube and then 1 mL (1000 µL) of reaction mixture was added to each of them. Then after 20 minutes of time interval, 100 µLof Folin's reagent (400 µL Folin-Ciocâlteu phenol reagent + 800µL distilled water in accordance with 1:2 ratio as per experimental procedure) was added to each of the tube. Then after a time lap of 20 minutes the readings were noted through UV-VIS spectrophotometer (Systronics) at 700 nm. Glass cuvettes were used to take samples. The obtained O.D. values were then converted into concentration of protein of unit mg/ml by the following formula-:

Conc. of protein (mg/ml) = [(sample conc. × sample O.D.) ÷ BSA O.D.] × Dilution factor

CAT

CAT activity of the tissue sample (liver and muscle) was determined by the method as per Cohen *et al.,* (1970). It was performed preferably in dark laboratory conditions. The reaction mixture was freshly prepared by adding hydrogen peroxide (H_2O_2) and 0.05 M phosphate buffer of pH 7.4 in 1:999 ratio (H_2O_2: Phosphate buffer) as per the experimental requirement. 10 µL of H_2O_2 was added to 9990 µL of phosphate buffer in a beaker to yield reaction mixture of 10000 µL. 1975 µL of reaction mixture was taken in quartz cuvette and 25 µL of sample was added to it. Afterwards, readings were noted at 15

seconds of time interval till 1 minute (60 sec) through UV-VIS spectrophotometer (Systronics) at 242 nm which falls in the UV wavelength range. The obtained O.D. values were converted into U/mg protein by using following formula-:

CAT activity = (Diff. in CAT O.D./Protein amount required conc.) × 1000

Statistical analysis

To analyse the variation between the data those were collected from biochemical assay, two-way ANOVA (Anova: Two-Factor Without Replication) was conducted at 0.05 level of significance. $P < 0.05$ was considered statistically significant. All the graphs and statistical analyses were done using Microsoft Excel. Data was presented as mean ± SD.

Results and Discussion

Tissue protein

Time-specific significant change was observed in the total tissue protein sample collected from *Anabas testudineus* which were exposed to treatments T1, T2 and T3.

In case of liver tissue, level of protein mostly showed a decreasing trend in C, T1, T2 and T3 of each treatment period of 24 h, 48 h and 72 h. After the 24 h exposure period, level of protein in C, T1, T2 and T3 was found to be 218.987±4.778, 215.611±2.854, 217.3±8.777 and 83.544±3.798 mg/g tissue, respectively. After 48h exposure period, protein level of C, T1, T2 and T3 was found to be 246.286±6.491, 241.033±5.024, 234.704±6.962 and 75.3163±1.899 mg/g tissue, respectively. After 72 h exposure period, protein level of C, T1, T2 and T3 was found to be 277.004±2.558, 271.94±0.967, 266.244±2.035 and 60.337±1.593 mg/g tissue, respectively. T3 of 72 h showed a significant decrease.

The level of protein in C was found to be minimum (218.987±4.778 mg/g tissue) after 24 h. The amount of protein in C was maximum (277.004±2.558 mg/g tissue) after 72h. The amount of protein in T3 of 24 h (83.544±3.797 mg/g tissue), T3 of 48h (75.316±1.899 mg/g tissue) and T3 of 72 h (60.337±1.593 mg/g tissue) of exposure had shown a significant change in the trend as compared to their respective C, T1, T2 treatments of corresponding 24 h, 48 h and 72 h of exposure. The amount of protein at 72 h of exposure was found to be maximum in C (277.004±2.558 mg/g tissue), followed by T1 (271.94±0.967 mg/g tissue), T2 (266.244±2.035 mg/g tissue) and minimum in T3 (60.337±1.593 mg/g tissue). ANOVA results indicated that the variation in the tissue protein in liver between treatments were statistically significant ($p<0.05$).

In case of muscle tissue, level of protein also showed a decreasing trend in C, T1, T2 and T3 of each treatment period of 24 h, 48 h and 72 h. After the 24 h exposure period, level of protein in C, T1, T2 and T3 was found to be 95.148±5.308, 92.827±9.557, 84.81±3.294 and 44.831±2.940 mg/g tissue, respectively. After 48h exposure period, protein level of C, T1, T2 and T3 was found to be 109.493±2.9, 106.751±1.593, 103.797±2.654 and 43.776±1.198 mg/g tissue, respectively. After 72h exposure period, protein level of C, T1, T2 and T3 was found to be 121.73±2.991, 116.666±4.118, 114.979±2.759 and 33.333±2.923 mg/g tissue, respectively. T3 of 72h showed a significant increase.

As far as only readings of C are concerned, the level of protein in C was found to be minimum (95.148±5.308 mg/g tissue) after 24 h and the amount of protein in C was maximum (121.73±2.991 mg/g tissue) after 72 h.

The amount of protein in T3 of 24h (44.831±2.940 mg/g tissue), T3 of 48h (43.776±1.198 mg/g tissue) and T3 of 72h (33.333±2.923 mg/g tissue) of exposure had shown a significant change in the trend as compared to their respective C, T1, T2 treatments of corresponding 24 h, 48 h and 72 h of exposure. The amount of protein at 72h of exposure was found to be maximum in C (121.73±2.991 mg/g tissue), followed by T1 (116.666±4.118 mg/g tissue), T2 (114.979±2.759 mg/g tissue) and minimum in T3 (33.333±2.923 mg/g tissue). ANOVA results indicated a significant ($p<0.05$) change in muscle tissue protein between treatments.

CAT activity

In case of liver tissue, CAT activity showed an increasing trend in C, T1, T2 and T3 of each treatment period of 24 h, 48 h and 72 h. After 24 h exposure period, CAT activity of C, T1, T2 and T3 was found to be 0.268±0.004, 0.288±0.017, 0.296±0.295 and 0.316±0.035 U/mg protein, respectively.

After 48 h exposure period, CAT activity of C, T1, T2 and T3 was found to be 0.247±0.008, 0.266±0.010, 0.284±0.066 and 0.312±0.120 U/mg protein,

respectively. After 72 h period, CAT activity of C, T1, T2 and T3 was found to be 0.248±0.011, 0.267±0.004, 0.280±0.037 and 0.503±0.027 U/mg protein, respectively. CAT activity was found to be highest in T3 of 72 h (0.503±0.027 U/mg protein), followed by T3 of 24 h (0.316±0.035 U/mg protein) and T3 of 48 h (0.312±0.120 U/mg protein). T3 of 72 h showed the highest increase. ANOVA indicated that the variation in the CAT activity between treatments was not significant, i.e., p-value was greater than 0.05 ($p>0.05$).

In case of muscle tissue, CAT activity showed an ascending trend in C, T1, T2 and T3 for each exposure period of 24 h, 48 h and 72 h. After 24 h, CAT activity of C, T1, T2 and T3 was found to be 0.011±0.005, 0.015±0.007, 0.026±0.125 and 0.115±0.044 U/mg protein, respectively. After 48 h, CAT activity of C, T1, T2 and T3 was found to be 0.269±0.005, 0.033±0.012, 0.039±0.055 and 0.085±0.051 U/mg protein, respectively.

After 72 h, CAT activity of C, T1, T2 and T3 was found to be 0.280±0.004, 0.034±0.013, 0.040±0.026 and 0.234±0.060 U/mg protein, respectively. CAT activity was found to be highest in T3 of 72 h (0.234±0.060 U/mg protein), followed by T3 of 48 h (0.085±0.051 U/mg protein) and T3 of 24 h (0.115±0.044 U/mg protein). T3 of 72 h exhibited a sharp and significant increase. The variation in muscle CAT activity between treatments was found to be statistically significant ($p<0.05$).

From the results discussed above, it was observed that tissue protein activity proved to be the more suitable and sensitive marker in comparison to CAT.

The present experiment indicated that the LDPE MPs adversely affected the fish, *A. testudineus*, by decreasing protein level and increasing CAT activity in T2 as compared to C. It was also observed that the fishes in T3 were most affected with maximal effects on biochemical parameters. Proteins are considered as the building blocks of the animal body. The maximum decline in tissue protein was observed in T3 (72 h) due to the combined effect of pesticide MCP and LDPE secondary MPs.

Mommsen *et al.,* (1992) reported that proteins which are the main architecture of the cell and during chronic periods of stress they are broken down for energy requirement and there is impaired incorporation of amino acids in protein synthesis. Declined levels of total protein may be because of malnutrition, starvation and chronic hepatic disorders (Kirby *et al.,* 1995; Martin *et al.,* 2010). Das and Mukherjee (2000) revealed that the level of muscle protein (mg/100 mg tissue) decreased in Indian major carp, *Labeo rohita* on exposure to sublethal concentrations of quinalphos (an organophosphate pesticide) of 1.12 and 0.22 mg/L (ppm) after 15, 30 and 45 days.

A significant decrease in total protein in case of fishes exposed to MP particles and paraquat may be attributed to disorders in digestion as well as absorption process of proteins, malnutrition, and reduced synthesis of proteins in liver (Dere and Dağ, 2003; Ahmad *et al.,* 2010; Banaee, 2013). Decreased levels of total protein, albumin and globulin were reported in fish common carp (*Cyprinus carpio*) exposed to diazinon (Banaee *et al.,* 2011). The prime factors involved in reducing plasma total protein in fish exposed to treatment with environmental pollutants are as follows : starvation (deprivation of food), decreased absorption and malnutrition, disorders of nutritional behaviour caused due to reduced activity of acetylcholinesterase, decreased efficiency of dietary protein sources, peroxidation, methylation, and phosphorylation of cellular proteins with pesticides and alterations in the biochemical structure of proteins (Nwani *et al.,* 2015; Banaee *et al.,* 2016). Nematdoost Haghi and Banaee (2017) reported that content of total protein in fish, common carp (*Cyprinus carpio*) was reduced by 24-36% after co-exposure to polyethylene MPs (1-2 mg/L) and paraquat (0.2-0.4 mg/L) for an exposure time of 24 hours. Nagarjuna *et al.,* (2018) concluded that degradation of proteins was in an active stage over protein synthesis in liver, muscle and kidney of freshwater fish *Channa punctatus* at sublethal concentration of insecticide chlorantraniliprole as corroborated by the reduction in soluble protein and total protein accompanied with heightened protease activity and free amino acid levels.

Hamed *et al.,* (2019) observed that the blood biochemical parameters such as total protein increased significantly ($p<0.05$) after exposure of cichlid fish Nile tilapia (*Oreochromis niloticus*) early juvenile to 1, 10 and 100 mg/L of MPs for a period of 15 days as compared to the control group in dose-dependent manner. Total protein (g/dL) recorded in control and MP exposure (1, 10 & 100 mg/L of MPs) was found to be 4.93±0.3, 5.03±0.06, 5.30±0.1 and 6.5±0.3, respectively. Yadav *et al.,* (2019) reported that MCP has a considerable negative impact on total tissue protein content (mg of tissue/g of extract) of stomach, intestine

and gills as compared to control group of fishes (*A.testudineus*).

Table.1 Features of experimental setup

Pot no.	Treat-ment	Radius (cm)	Depth (cm)	Water qty.(L)	Fish qty.
1	C	13.2	15	5	5
2	T1	13.4	15.5	5	5
3	T2	12.9	16	5	5
4	T3	13	15.8	5	5

Table.2 Composition of normal fish feed

Constituents of fish feed	Percentage (%)
Fish meal	30%
Groundnut oil cake	20%
Soybean oil cake	20%
Rice bran	20%
Vegetable oil	5%
Vitamin and mineral	5%

Table.3 Details of chemical treatment

Treatment	Type of fish feed	Daily dietary requirement per fish	Vol. of sublethal MCP conc.
C	Normal feed	2.6 g	-
T1	Normal feed	2.6 g	73.6 µL
T2	100% normal feed mixed with supplementary 10% LDPE-MPs	2.6 g	-
T3	100% normal feed mixed with supplementary 10% LDPE-MPs	2.6 g	73.6 µL

Figure.1 Molecular and simulated structure of LDPE

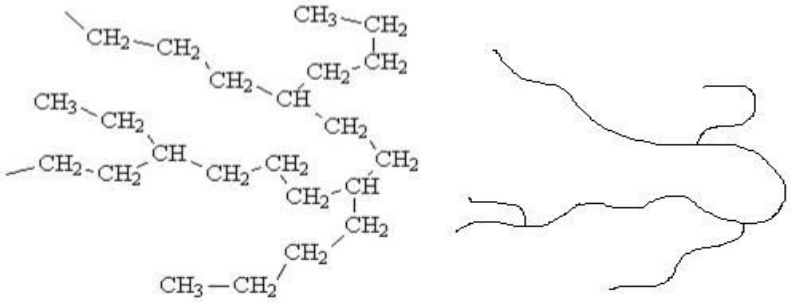


Figure.2 Chemical structure of MCP

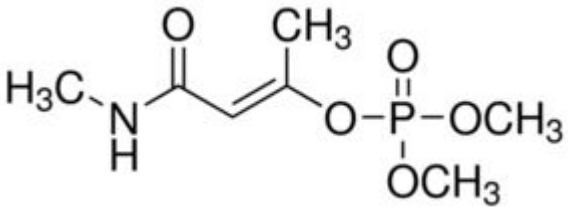


Figure.3 Simulated molecular docking between monocrotophos (MCP) and polyethylene (PE)

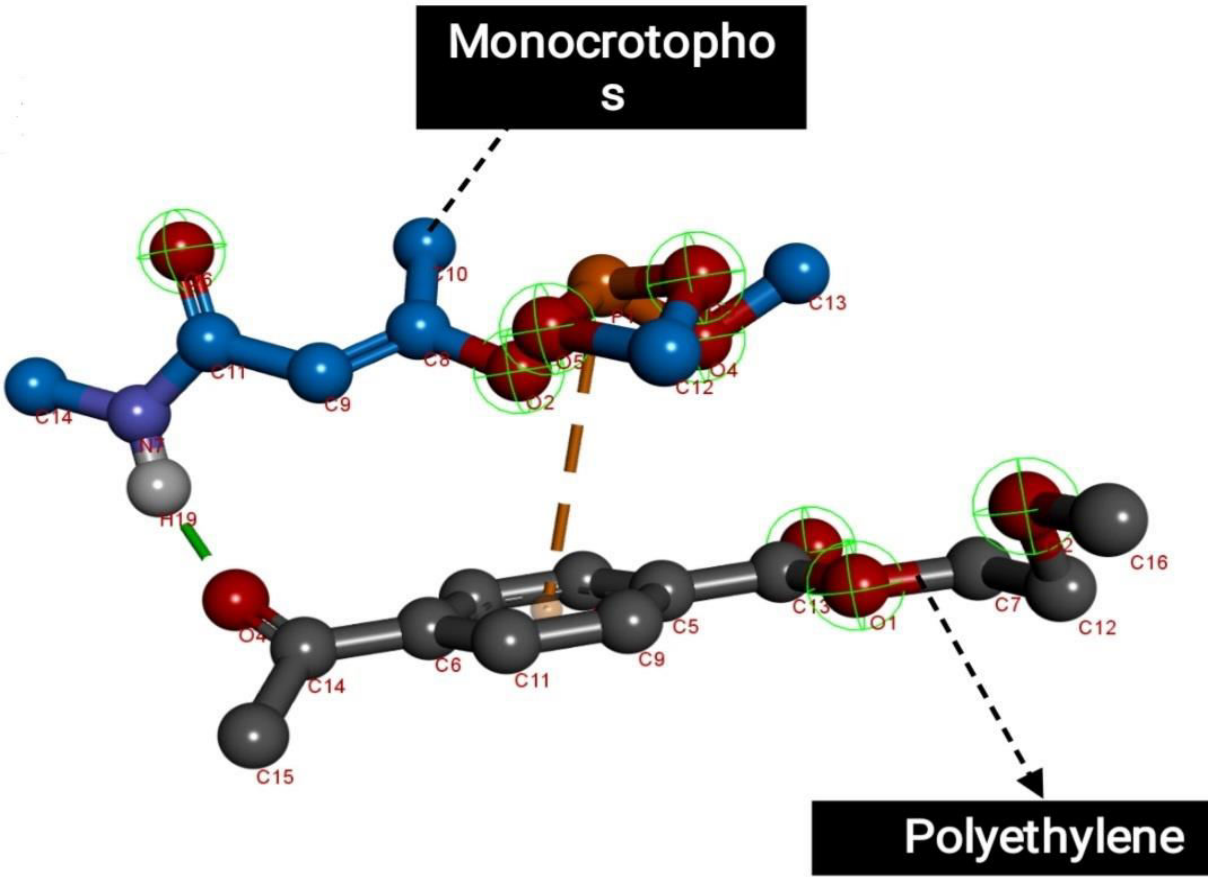


Figure.4 Schematic representation of experimental design and chemical treatment

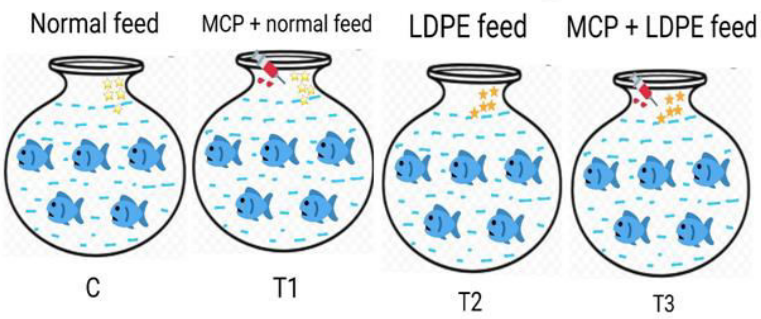


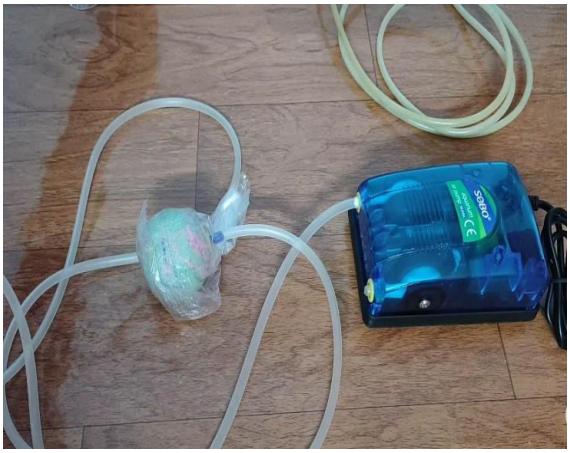
Figure.5 Experimental animal (a & b), Experimental setup (c & d) and Chemicals used for treatment (e & f)

a) Anabas testudineus

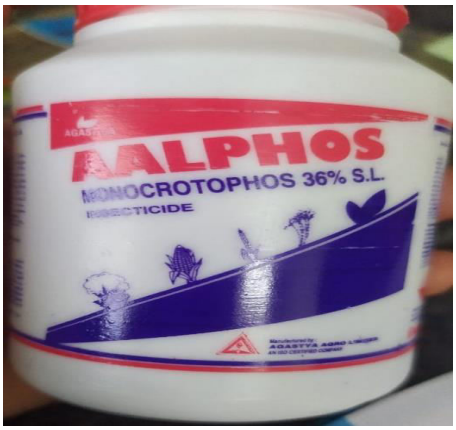
b) Measurement of length of fish

c) Experimental pots(C, T1, T2 & T3)

d) Aerator



e) Monocrotophos (36% SL)



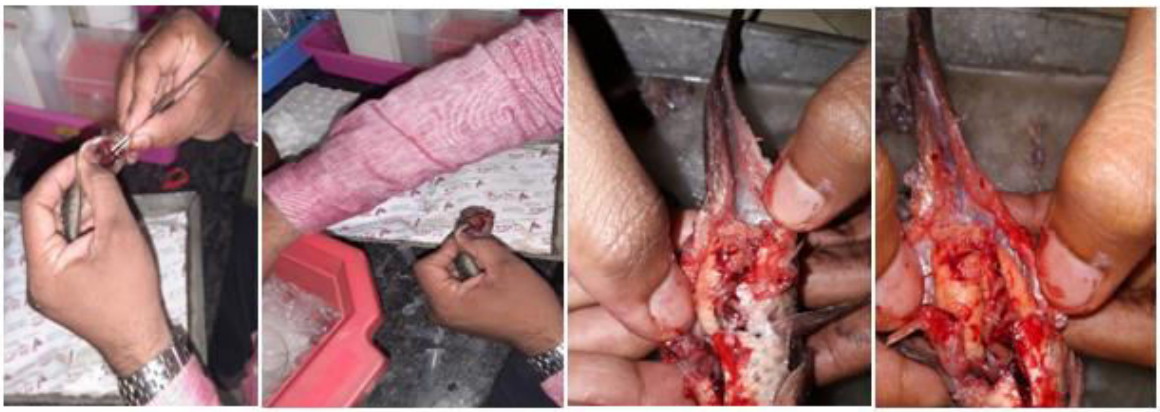
f) LDPE secondary MPs

Figure.6 Fish feed (g & h), Sacrificing of fish (i & j) and processing of tissue (k)

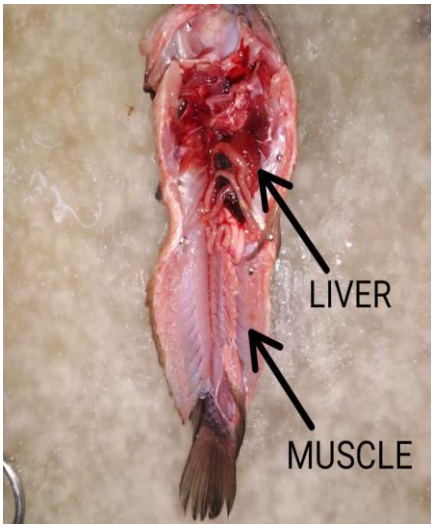
g) Fish feed (normal& LDPE-rich)

h) Fish feed in pellets

i) Dissection of fish



j) Parts of fish used in the expt.

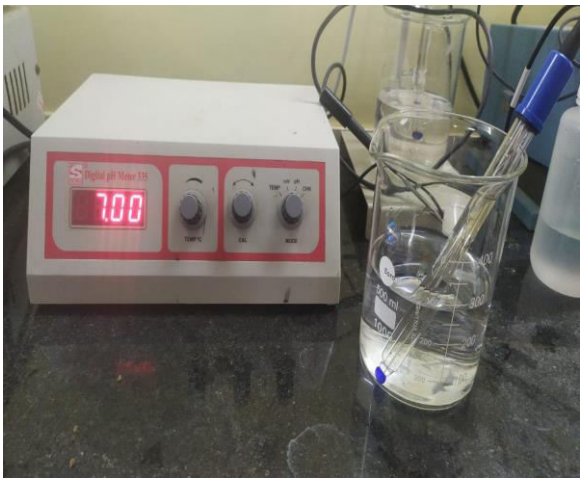


k) Tissue homogenization in ice box

Figure.7 Instruments used in the experiment (l to q)

l) Water analysis kit

m) Digital pH meter



n) Hot air oven

o) Digital weighing machine

p) Tabletop Cooling Centrifuge

q) UV-VIS Spectrophotometer

Figure.8 Process of biochemical analysis (r & s)

r) Thawing of tissue samples inside ice box

s) Protein estimation

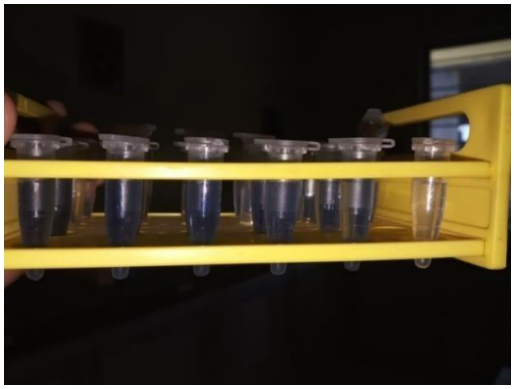


Figure.9 Biochemical changes in *A. testudineus* after exposure to T1, T2 and T3 for 24h, 48h and 72h.(a) Protein (liver) in fish,(b)Protein (muscle) in fish,(c) CAT (liver) in fish, and(d)CAT (muscle) in fish.

Figure.9(a)

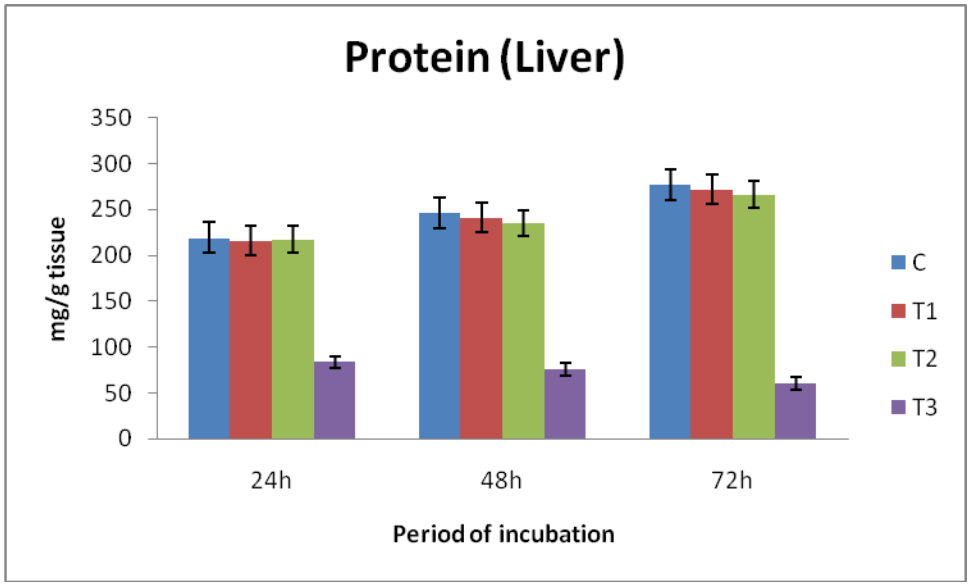


Figure.9(b)

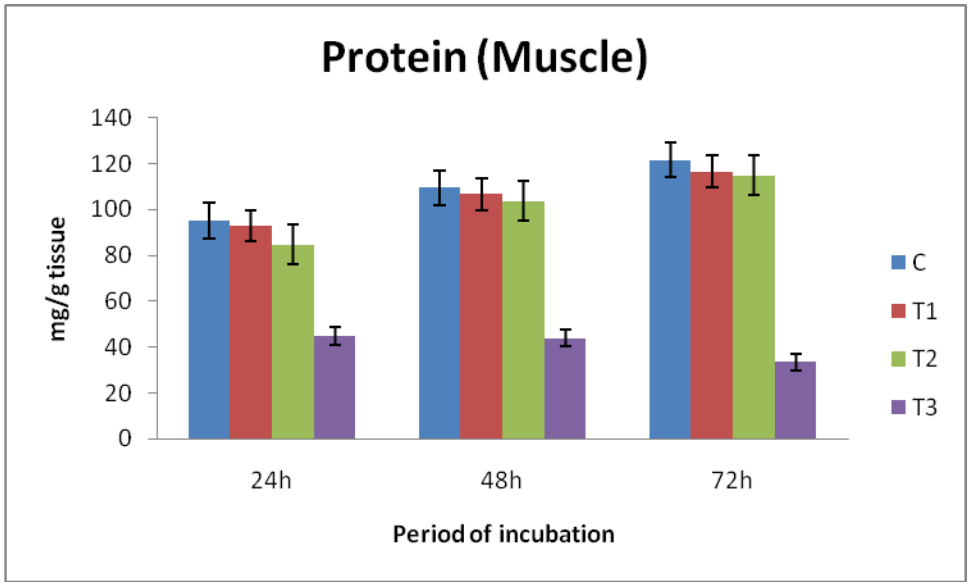


Figure.9(c)

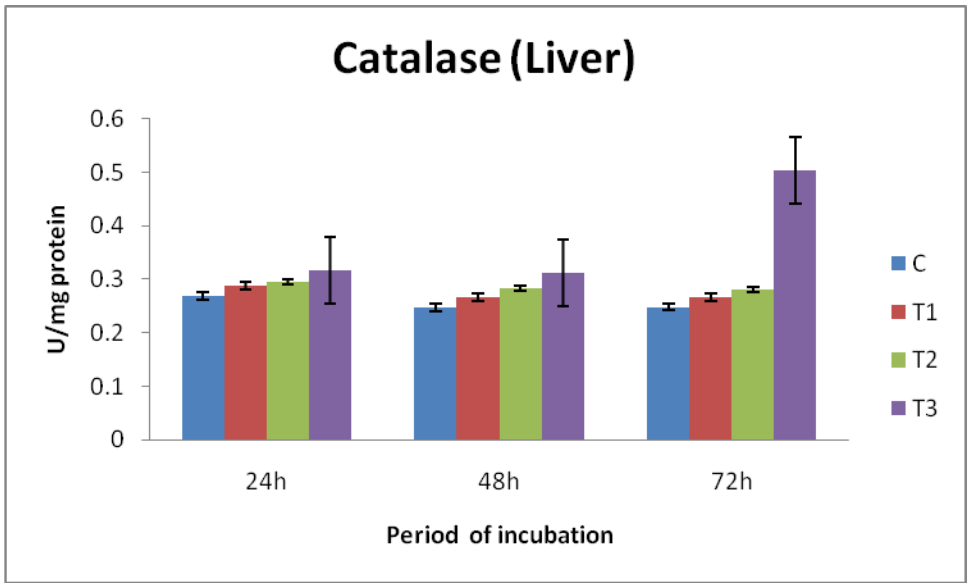
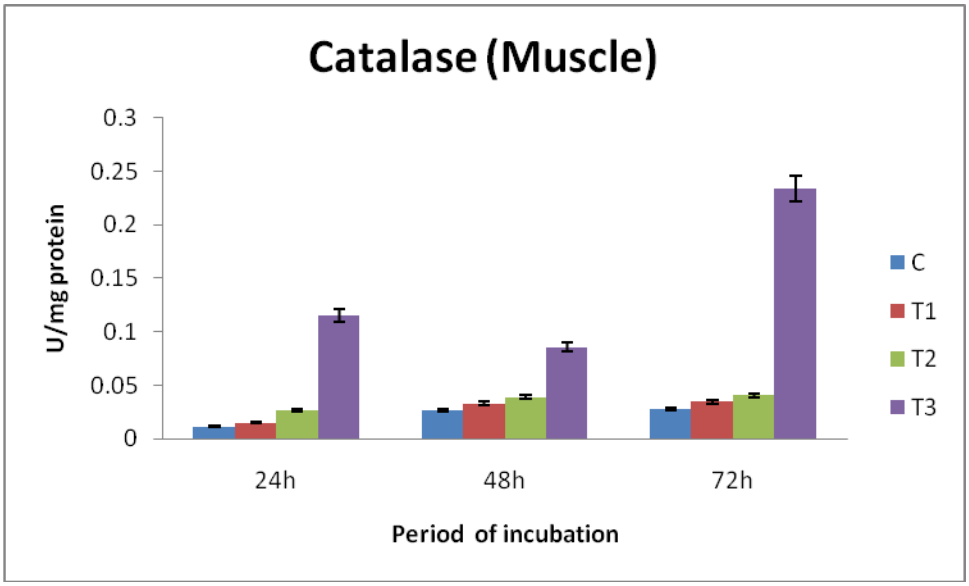


Figure.9(d)



The variation in distribution indicates differences in metabolic properties of various tissues. Fishes treated with sublethal MCP concentration (45 ppm) for 10 and 20 days exhibited significant reduction of total tissue protein content in stomach, intestine and gills. Other workers also observed that the levels of albumin and total protein reduced in the fish on exposure to various pesticides and pollutants (Vijayan *et al.,* 1997; Velisek *et al.,* 2009). Hamed *et al.,* (2020) reported that the electrophoretic pattern of muscle proteins in control group exhibited 8 bands in total. Total number of muscle protein bands declined to 7 and 5 after being exposed to 1, 10 and 100 mg/L of MPs for a period of 15 days, respectively. Protein breakdown usually dominates over protein synthesis under increased proteolytic activity.

CAT is an important indicator of oxidative stress in a cell or tissue. CAT is one of the several antioxidant enzymes that offers the first line of cellular defence to reactive oxygen species (ROS). Disequilibrium or instability between the activities of cellular antioxidant enzymes and ROS formation leads to cellular destruction and oxidative stress. The activity of CAT increases with increase in the physiological stress of the animal. It helps in scavenging the free radicals. High CAT activity is indicative of increased oxidative stress. Samanta *et al.,* (2014) reported that muscle, liver, brain and gill tissues of air-breathing teleost fishes *Anabas testudineus* and *Heteropneustes fossilis* exhibited significant enhancement in CAT activity ($p<0.05$) probably as a response to elevated levels of oxyradicals in liver such as reactive oxygen species (ROS) after exposure to glyphosate in laboratory conditions. Maximum increase in CAT activity of liver was marked in *A. testudineus*, i.e., 159.55% (85.94 – 137.12), however, minimum increase was observed in *H. fossilis*, i.e., 131.78% (30.43 – 40.10). Enhanced CAT activity of muscle tissue also showed a similar pattern. Luis *et al.,* (2015) reported that MPs led to disturbances in the levels of antioxidant enzymes in early juveniles of common goby (*Pomatoschistus microps*) after combined exposure to Cr (VI) and MPs. Karami *et al.,* (2017) emphasized that induction of antioxidant enzymes in fish treated with various pollutants can be considered as biological indicators of oxidative stress. Hatami *et al.,* (2019) reported that exposure of fish common carp (*Cyprinus carpio*) to chlorpyrifos (an organophosphate pesticide), alone or combined with polyethylene glycol (PEG), significantly enhanced the CAT level while it reduced total antioxidant level (TAN). Exposure to solely PEG rendered no effect on CAT activity and TAN level. It was established from the results that PEG can pose an antagonistic impact on chlorpyrifos toxicity which depends on the concentrations of these two materials, however, chlorpyrifos enhanced PEG toxicity. Yadav *et al.,* (2019) observed a significant rise in CAT activity in fishes (*A. testudineus*) exposed to sublethal doses of MCP. This elevation in CAT activity can be explained by stimulation of antioxidant defence system in all tissues under study (gills, stomach and intestine). Hamed *et al.,* (2020) reported that the antioxidant biomarkers such as CAT activity significantly increased ($p < 0.05$) in

case of Nile tilapia (*O. niloticus*) after being exposed to 1, 10, and 100 mg/L of MPs for 15 days in comparison to the control group. CAT activity (IU/L) readings in these groups – control, 1, 10 and 100 mg/L of MPs – were noted as 10.23 ± 0.11, 10.72 ± 0.06, 10.83 ± 0.04, and 11.03 ± 0.04, respectively. Campos *et al.,* (2021) evaluated the oxidative damage by measuring CAT activity in 20 µL of post-mitochondrial supernatant (PMS) and CAT activity was determined as per Clairborne. H_2O_2 (hydrogen peroxide) readings were taken at 240 nm wavelength for 2 minutes and its results were presented in terms of µmol/min/mg of protein. Inhibition of CAT activity occurred significantly (up to 48% and 31% in presence and absence of food, respectively) in all tested concentrations in presence of food and at 0.1 and 1 mg/L LDPE MPs in absence of food. On the contrary to the results of the present study, CAT activity decreased in zebrafish (*Danio rerio*) on exposure to polystyrene MPs (Wan *et al.,* 2019). This decrease in antioxidant enzymes such as CAT was understood by expenditure of energy of oxidative stress as a response to MP exposition. Espinosa *et al.,* (2019) reported that enzymatic activities of CAT and SOD were significantly dropped in the hepatic tissues of fish European sea bass (*Dicentrarchus labrax*) which were fed PE-MPs feeds (100 and 500 mg/kg) as compared to the fish of the control group ($p < 0.001$ in both the cases), whereas the activities of liver antioxidant enzymes were not significantly affected in fishes that were fed diets containing polyvinyl chloride MPs for 21 days.

MPs may trigger oxidative stress by various mechanisms by inducing several intracellular signal transduction pathways as reported in *Danio rerio* (Lu *et al.,* 2016), CAT activation in *Cyprinodon variegatus* (Choi *et al.,* 2018), upregulation of CAT level in the gut of *D. rerio* (Qiao *et al.,* 2019), and decline in the levels of CAT in larvae of *D. rerio* (Wan *et al.,* 2019).

The results obtained from the present study on *Anabas testudineus* are more or less in agreement with the relevant results of different workers discussed above.

Conclusion

Exposure to sublethal concentrations of MCP and LDPE MPs alone and in combination resulted in significant biochemical alterations which can be potentially disruptive for the survivability of air-breathing freshwater fish *Anabas testudineus*. In general, changes in biochemical parameters (level of protein and CAT activity) of liver and muscle tissue in *A. testudineus* exposed to MCP verify the hypothesis of this study because an increase in LDPE MP particles in water increases MCP toxicity. Therefore, MP particles present in aquatic ecosystems can increase toxicity and bioavailability of pesticides by acting as a carrier/vector or adsorbent. Polymers of plastics have various chemical additives and stabilizers because of which they absorb many toxic pollutants and contaminants from their vicinity. Fishes mistake MPs for planktons and consume them as their food, leading to bioaccumulation and bioamplification in the process. Organisms of higher trophic levels of food chain and food web of aquatic biota are affected as a result. Since humans are top-order consumers who consume fishes as food, fishes which have already ingested MPs laden with pollutants (such as pesticides) can be inimical to the health of humans. Extensive research with a large set of different combination of pollutants with microplastics/nanoplastics could bring light on this rapidly advancing plastic pollution. The results highlight the significance of carrying out more studies on the mechanisms of toxicity of MPs either alone or in combination with other environmental stressors. Since plastics have become a part and parcel of our day-to-day life, it is very difficult to completely ban or abandon its usage. It is high time to give much more emphasis on the controlled usage of plastics and proper plastic disposal at all levels. Novel and safer alternatives which can substitute the utility of plastics should be incentivized.

Authors' Contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript

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