

MATERIALS & METHODS

ANIMAL SELECTED

The Indian major carp *Cyprinus carpio* (Linnaeus, 1758), is an economically important edible fish, having great commercial value. It is abundantly available in the fresh water tanks and ponds in and around Anantapur. Besides its wide availability and commercial importance, this carp fish is known for its adaptability to laboratory conditions and appear to be suitable test animal to toxic studies (Sreenivasan and Swaminathan, 1967). Hence this fish is selected as the ideal experimental model for the present investigation. Since this investigation is an ecotoxic physiological nature, brief account of the biology of the fish involved may constitute a suitable preamble to this study.

Systematic position of *Cyprinus carpio*



Kingdom	:	Animalia
Phylum	:	Chordata
Subphylum	:	Vertebrata
Division	:	Ganthostomata
Super class	:	Pisces
Class	:	Osteichthyes
Sub class	:	Actinopterygii
Super order	:	Teleostei
Order	:	Cypriniformes
Family	:	Cyprinidae
Genus	:	<i>Cyprinus</i>
Species	:	<i>carpio</i>

Biology of *Cyprinus carpio*

Cyprinus carpio is a widespread freshwater fish of eutrophic waters in lakes and large rivers in Europe and Asia. It inhabit warm, deep, slow-flowing and still waters such as lowland rivers and large, well vegetated lakes (Kottelat and Freyhof, 2007). Carp exploit large

and small manmade and natural reservoirs, and pools in slow or fast moving streams. They prefer larger, slower-moving bodies of water with soft sediments but they are tolerant and hardy fish that thrive in a wide variety of aquatic habitats. (Froese and Pauly, 2002; Page and Burr, 1991). It is popularly known as common carp, widely present in temperate regions of the world, which is extensively cultivated in freshwater ecosystems. It is an exotic species and native of the temperate region of Asia, especially in China (Gunther, 1968). It is introduced in Indian waters in the year 1939 from Ceylon (Alikunchi, 1957). It is a very divergent species. At present the common carp enjoy global distribution occurring in tropical as well as temperate regions acclimatized to variety of habitats and extremes of environmental conditions (Alikunchi, 1957; Bashamohideen and Subba Rao, 1982; Parvathi, 1982).

It is a typical warm water fish. It can tolerate a wide range of temperatures between 20°C to 30°C. It is a non predatory and has a non-elongated body with an abdomen conspicuously larger than the rest of the body and there are a pair of barbels at the side of the mouth. The fins are yellowish to red, but become red during breeding season. *Cyprinus carpio* is an omnivorous fish feeding on vegetable debris, insects, worms and planktonic algae. This fish is known for its rapid growth and attains sexual maturity at the end of first year. The maximum length of the fish to which it grows is about 76 cm (approximately 6.8 kg by weight) which has been recorded in Yarcqud lake, Ootacamund (Wealth of India, 1962). As the smaller fish are considered to be more sensitive to toxic pollutants rather than larger ones (Anderson and Weber, 1975), small sized fish weighing around 10 ± 2 grams were selected for the present investigation.

Procurement and maintenance of fish

Cyprinus carpio were collected from the department of fisheries, Anantapur, Andhra Pradesh and were immediately transported in big 20 Lts fish containers each with 50 fish to the laboratory. Then they were released into large cement tanks with sufficient dechlorinated tap water. The fish were fed with commercial fish pellets *ad libitum* having around 50% protein content daily and allowed to acclimatize for 15 days. Then the fish were separated into the batch of having the size 10 ± 2 gm and were maintained in static water without any flow (Doudoroff et al., 1951). Water was renewed every day to provide freshwater, rich in oxygen. During experimentation water was aerated once a day to prevent hypoxic conditions, if any (Khorram and Knight, 1977). As the level of toxicity is reported to vary with the interference of various extrinsic and intrinsic factors like temperature, salinity, pH, hardness of water,

exposure period, density of animals, size, sex etc., (Sivaramakrishna et al., 1991), precautions were taken throughout this investigation to control all these factors as far as possible. As a part of it water from the same source has been used for maintenance of fish. The animals were starved for 24 hours prior to each estimation, to avoid any influence of differential feeding. The size of the animals selected was also maintained strictly throughout the investigation.

Physico, chemical factors of water used for the experiment

pH	7.5 to 7.7
Salinity	0.193 gm/liter
Dissolved O ₂	6-8 ml/ liter
Alkalinity	88 ppm (as Ca CO ₃)
Chlorinity	0.112 gm/liter
Sodium	1.23 m.moles/liter
Potassium	30.6 m.moles/liter
Calcium	4.32 m.moles/liter
CO ₂	2.09 mg/liter
O ₂ % saturation	8
Hardness of water	160 ppm (as Ca CO ₃)

Pesticide selected for the study

Commercial grade of phorate , an organophosphorus insecticide was selected as representative of organophosphate pesticides group for the study of the effects on fish *Cyprinus carpio* . The pesticide was obtained from the local market. Commercial formulation of this pesticide is used because only commercial preparation is used in agriculture.

Phorate

It is a soil and systemic insecticide and miticide, used for the control of sucking and chewing insects, mites and soil dwelling pests in sugarcane, rice, beetroot, carrots, maize, sorghum, potatoes, tomatoes, soyabeans, wheat, chillies, onion, sunflower, cotton, groundnut, coffee, some ornamental plants, herbaceous plants and bulbs. Phorate (*O,O*-diethyl *S*-ethylthiomethyl phosphorodithioate) is widely used throughout the world as a broad-spectrum insecticide for numerous crops and acaricide in pine forest and field crops. It is highly toxic to fish, with the 96-hour LC₅₀ ranging from 2 to 280 µg/L in several fish species (Johnson and Finley, 1980).

Physico - chemical properties of phorate

Common name	:	Phorate
Chemical name	:	O,O-diethyl S-ethylthiomethyl phosphorodithioate
Commercial Names	:	Thimet, Rampart, Granutox, Agrimet etc.
Pesticide Class	:	Organophosphate, cholinesterase inhibitor
Pesticide Type	:	Insecticide, acaricide, nematocide
Molecular Formula	:	C ₇ H ₁₇ O ₂ PS ₃
Molecular Weight	:	260.38
Physical form	:	Granules S
Chemical Structure	:	CH ₃ -CH ₂ -S-CH ₂ -S -P (O CH ₂ CH ₃) ₂
Melting Point	:	Less than -15 ⁰ C
Boiling point	:	118-120 ⁰ C at 0.8 mm of Hg
Stability	:	Stable in normal storage conditions for at least 2 years. Decomposes in damp conditions and in alkaline media.
Regulatory Status	:	Phorate is a highly toxic compound in EPA toxicity class I. Labels for products containing it must bear the Signal Words DANGER - POISON.
Manufacturers	:	Cyanamid, Crystal, M/S. pesticides
Water Solubility	:	50 mg/L at 25 ⁰ C.

In this investigation stock solutions were prepared in tap water. The quantity of tap water used to be non-toxic to non-target animals and it was biologically safe in preparation of stock solution of pesticides (Jagannatha Rao, 1981).

Solubility in other solvents : Miscible with carbon tetrachloride, dioxane, vegetable oils, xylene, alcohols, ethers, esters and other organic solvents.

Toxicological Effects**Acute toxicity**

Phorate is highly toxic via the oral route with reported oral LD₅₀ values of 1.1 to 3.7 mg/kg in rats (Gallo and Lawryk, 1991; Kidd and James, 1991), and 2.25 to 6.59 mg/kg in mice (Gallo and Lawryk, 1991; Wayne, 1992). It is highly toxic via the dermal route as well,

with reported dermal LD₅₀ values of 2.5 to 6.2 mg/kg in rats (Gallo and Lawryk, 1991; Kidd and James, 1991) and 5.2 mg/kg in rabbits (Gallo and Lawryk, 1991; Wayne, 1992). Guinea pigs reportedly have a dermal LD₅₀ of 20 to 30 mg/kg during a 24-hour exposure (Kidd and James, 1991; Wayne, 1992). The acute 1-hour inhalation LC₅₀ for rats is reported as 0.06 mg/L (Kidd and James, 1991). Symptoms of acute exposure to phorate are similar to those caused by exposure to other organophosphate pesticides, except that they may occur at lower doses. Symptoms of acute exposure to organophosphate or cholinesterase-inhibiting compounds may include the following: numbness, tingling sensations, incoordination, headache, dizziness, tremor, nausea, abdominal cramps, sweating, blurred vision, difficulty breathing or respiratory depression, and slow heartbeat. Very high doses may result in unconsciousness, incontinence, and convulsions or fatality. Toxicity appears to vary with age, with the young being more susceptible (U.S. Public Health Service, 1995). Several poisoning cases involved workers from 16 to 18 years old, wearing inadequate protection while applying phorate to crops, or working around machines used to apply phorate (Gallo and Lawryk, 1991; U.S. Public Health Service, 1995). Studies indicate that direct eye exposure may cause blurring, tearing, and ocular pain (Gallo and Lawryk, 1991).

Chronic toxicity

Repeated low-level exposures may result in cholinesterase inhibition and the associated neurological and neuromuscular effects (Gallo and Lawryk, 1991). A survey of workers exposed to phorate revealed toxic effects in 60% of the males tested (after a 2-week exposure). Symptoms included a lowering of the heart rate. Effects on cholinesterase in the blood of the workers were also noted in this study (Gallo and Lawryk, 1991; U.S. Public Health Service, 1995). In a study on dogs, moderate to high doses of phorate 6 days each week for 13 to 15 weeks lowered cholinesterase activity, but produced no tissue damage (Wayne, 1992).

Reproductive effects

Long-term studies of mice fed high doses of 98.7% pure phorate showed no effects on fertility, gestation, or viability (Wayne, 1992). Maternal and embryo toxicity occurred at dietary doses of 0.5 mg/kg/day fed to rats (U.S. Environmental Protection Agency, 1985). Available data suggest that phorate is unlikely to cause reproductive effects.

Teratogenic effects

No birth defects were found in two studies on the rat (Wayne, 1992; U.S. Environmental Protection Agency, 1985). Available data suggest that phorate does not cause birth defects.

Mutagenic effects

Studies of phorate in both bacterial systems or in mice indicate that it is nonmutagenic effects (Gallo and Lawryk, 1991).

Carcinogenic effects

Studies in both rats and mice produced no evidence of carcinogenicity (U.S. Environmental Protection Agency, 1985).

Organ toxicity

Phorate's main target organ, as determined by animal testing and human use experience, is the nervous system.

Fate in humans and animals

Phorate is readily absorbed by the skin and the gastrointestinal tract. The major breakdown products of phorate in mammals are more toxic and have greater anticholinesterase activity than phorate (Gallo and Lawryk, 1991). Phorate may have a long residence time in mammalian systems; for example, rats given a high oral dose excreted less than 40% in 6 days. The liver, kidney, lung, brain, and glandular tissue held the remaining residues (Vettorazzi, G, 1979).

Ecological Effects**Effects on aquatic organisms**

Phorate is very highly toxic to fish. Reported 96-hour LC₅₀ values range from 2 to 13 ug/L in cutthroat trout, bluegill sunfish and largemouth bass. Other 96-hour LC₅₀ values are 110 ug/L in northern pike and 280 ug/L in channel catfish (Johnson and Finley, 1980). Reported 96-hour LC₅₀ values for the compound in freshwater invertebrates such as stoneflies and scuds are 4 ug/L, also indicating very high toxicity. Other LC₅₀ values are 0.006 ug/L for amphipods and 0.11 to 1.9 ug/L in other freshwater invertebrates (U.S. Environmental Protection Agency, 1985). The acute oral LD₅₀ of phorate is 85 mg/kg in bullfrogs (Smith, 1993).

Effects on birds

Phorate is very highly toxic to birds. The reported acute oral LD₅₀ values are 12.8 mg/kg in chukar, 7.5 mg/kg in starlings, 0.6 to 2.5 mg/kg in mallards, 7 to 21 mg/kg in northern bobwhite quail, 1 mg/kg in red-winged blackbirds, and 7 mg/kg in ring-neck pheasants (Smith, 1993). The 5- to 8-day dietary LC₅₀ values are reported as 370 to 580 ppm in Japanese quail, mallard, northern bobwhite quail, and ring-neck pheasant (Smith, 1993).

Effects on other organisms

Phorate is toxic to bees, with a reported topical application LD₅₀ of 10 ug per bee (Kidd and James, 1991).

Environmental Fate**Breakdown in soil and groundwater**

Phorate is of moderate persistence in the soil environment, with reported field half-lives of 2 to 173 days. A representative value may be approximately 60 days (Shepard, 1986). Actual residence times may be influenced by soil clay and organic matter content, rainfall, and soil pH (U.S. Public Health Service, 1995). Soil treatments often leave more residues in plants than foliar treatments, because the compound persists in the soil and is readily taken up by plant roots (Kidd and James, 1991; U.S. Public Health Service, 1995). Phorate binds moderately well to most soils and is slightly soluble in water (Wauchope et al., 1992). It should therefore not be highly mobile in most soils, and should mainly be transported with runoff via sediment and water. Phorate has minimal potential to leach through the soil and contaminate groundwater. This is most likely, where soils are sandy and aquifers are shallow. Field studies indicate that leaching is very low in soils high in clay and organic matter content, and lower in sandy soils (U.S. Public Health Service, 1995).

Breakdown in water

The half-life of phorate in acidic water solutions is between a few days and a few weeks, depending on temperature; the half-life in alkaline (basic) water may be much shorter (U.S. Public Health Service, 1995, U.S. Environmental Protection Agency, 1985). Phorate is degraded by waterborne microorganisms and hydrolysis (U.S. Public Health Service, 1995, U.S. Environmental Protection Agency, 1985). As it breaks down in water, nontoxic, water-soluble products are formed.

Breakdown in vegetation

Phorate itself is not persistent in plants, but plants metabolize phorate to very potent anticholinesterase agents such as the sulfoxide and sulfone derivatives of the compound (Gallo and Lawryk, 1991). This activity will usually peak several days following application before decreasing (U.S. Public Health Service, 1995). Phorate and its soil metabolites are absorbed from the soil by plant roots and are translocated to above-ground portions of the plant. Following treatment with a 10% granular formulation at 1 pound a.i./acre, phorate residues persisted at very low levels for 28 days in the kernels, cobs, or husks. After 83 days, there were no detectable residues of phorate or breakdown products (U.S. Public Health Service, 1995).

METHODOLOGY

Evaluation of toxicity

Lethal concentration (LC_{50}) of phorate to *Cyprinus carpio* was determined by “Probit method” of Finney (1971).

The percent mortality of the fish in different concentrations of phorate was determined immediately after 96 hours of exposure. For this the experimental fish were divided into batches of thirty each and were exposed to different concentrations of phorate and each batch to one concentration, ranging from 0.4 ppm to 1.1 ppm. This range was obtained on trial and error basis. Toxicity evaluation was carried out in static water and mortality rate was observed in all concentrations of phorate immediately after 96 hours of exposure period. A batch of fish separately maintained along side in fresh water medium without phorate served as control. The experiment was repeated thrice for accuracy. The mortality rate at each concentration, derived from the mean of three replications was converted as percent mortality value. From this, the probit mortality value was obtained (Finney, 1971).

As the evaluation of toxicity of pesticides to an aquatic organism is by the determination of its LC_{50} , the percent mortality values as well as probitt mortality values were plotted separately against pesticide concentration and LC_{50} s at 96 hours were derived from these two curves. For subsequent verification the LC_{50} s obtained by graphical methods, Dragstedt and Beheren’s method as given by Carpenter (1975) was employed. As per this method the animals were exposed to log 2 concentrations of pesticides for the same exposure period. The percent mortality values were calculated from the cumulative mortalities, with

them LC_{50} was derived by adopting the following formula

$$\text{Log } LC_{50} = \text{Log } A + \frac{50 - a}{b - a} \times \text{Log } 2$$

Where

A = Concentration of the pesticide which has a percent mortality immediately below 50%

a = Percent mortality observed immediately below 50%

b = Percent mortality observed immediately above 50%

Finally the LC_{50} at 96 hours of phorate was obtained by taking the mean of LC_{50} s derived from percent and probit mortality curves and Dragstedt and Beheren's method.

Fixation of lethal and sublethal concentration

Taking into consideration the fact that the effect of a pesticide on fish become consistent within 96 hours of exposure, $LC_{50}/96$ hours (0.71 ppm/l) of phorate was taken as lethal concentration to study acute toxicity in the physiological, biochemical responses and histopathological changes in the fish, *Cyprinus carpio*. However, knowledge on the concentration of toxicant that kills 50% of the test animals in fixed period of time could become insufficient to assess various responses of the animal to toxicant (Nobbs and Pearu, 1976; Hoppenheit, 1977)). Further studies on acute toxicity have significant limitations like the possibility of ignoring the occurrence of adaptation of test animal to the imposed toxicity (Stockner and Anitha, 1976; Hoppenheit, 1977). Hence, Perkin (1979) viewed the need for sublethal studies because distinct changes involving a sequence of events in the responses of test animal could occur in sublethal concentrations. So about one-tenth of the $LC_{50}/96$ hours (0.07 ppm/l) concentration of phorate was taken as the sublethal concentration for further (chronic toxicity) studies (Reed and Muenchi, 1938; Konar, 1969).

Fixation of exposure periods

Since the duration of exposure is having a great influence on the toxicity of a compound (Radhakrishnaiah and Busappa, 1986), the effect of lethal and sublethal concentration of pesticide in acute and chronic toxicity studies respectively on *Cyprinus carpio* was studied at different periods of exposure in order to understand the influence of time over toxicity. So from shorter duration of exposure to longer duration, 1, 4 days for acute

toxicity study and 1, 7, 15, 30 days for chronic toxicity study were selected to study the effects of lethal and sublethal concentrations respectively. The result obtained at these exposure periods may give an insight on some specific events of responses of the fish to acute and chronic pesticide stress at short term and long term exposures.

Experimental Design

160 fishes divided into two batches, again batch I divided into 3 groups and batch II divided into 5 groups comprising of 20 fishes each. Batch – I was exposed for acute toxicity of Phorate (exposed to lethal concentration = LC_{50} - 0.7168 ppm) and Batch – II was exposed for Chronic toxicity of Phorate (exposed to sub lethal concentration = 1/10 of LC_{50} - 0.07168 ppm)

Batch - 1

Group I	:	Normal control
Group II	:	exposed for 1 day
Group III	:	exposed for 4 days

Batch - 2

Group I	:	Normal control
Group II	:	exposed for 1 day
Group III	:	exposed for 7 days
Group IV	:	exposed for 15 days
Group V	:	exposed for 30 days

After the completion of stipulated exposure period, blood was collected from the caudal region of the fish for hematological studies and then the fish were sacrificed and tissues of gills, liver, muscle, kidney and brain were isolated under laboratory conditions for biochemical analysis and histopathological studies. DMR (Duncan's Multiple Range) test had been employed for the statistical analysis of the data. P value (level of significance) is significant at < 0.05 .

General Experimental Procedure for further Studies:

Further studies in this investigation were carried out in the gills, liver, muscle, kidney and brain of the fish *Cyprinus carpio* at 1, 4 days on exposure to lethal concentration of phorate in acute toxicity study and 1, 7, 15, 30 days on exposure to sublethal concentration of phorate in chronic toxicity study. Selection of the tissues such as the gills, liver, muscle,

kidney and brain were to understand the difference in the effects of phorate on osmoregulatory organs (gills and kidney) and non-osmoregulatory organs (liver muscle and brain) of the fish. Prior to estimation, groups of fish were exposed to the respective lethal and sublethal concentration of phorate and were maintained upto stipulated period of exposure.

At the end of exposure period, the healthy fishes were taken out and blood was collected from incision at the caudal vein region of the fish into the heparinized capillary tubes for hematological studies, and then fish were stunned to death and the target organs like gills, liver, muscle, kidney and brain were dissected out from each animal using sterilized instruments. The organs were weighed accurately on a Sartorius electrical semi microbalance and transferred into ice jacketed micro beakers containing fish ringer solution. The fish solution was prepared as per the composition given by Ekberg (1958). An equilibrium time of 15 minutes was allowed to the organs to regain normalcy from a state of shock, if any due to handling and dissecting procedures. The biochemical analysis of each experiment was carried out in the organs from six animals at each exposure period and the mean of six is taken into consideration. Similar studies were made in the animals from normal medium served as controls.

HAEMATOLOGY

Cyprinus carpio exposed to lethal concentration of phorate in acute toxicity study for 1 and 4 days and sublethal concentration of phorate in chronic toxicity study for 1, 7, 15 and 30 days, at the end of exposure period, the healthy fishes were taken out and the blood from the control and treated fingerlings was collected from incision at the caudal vein region of the fish into the heparinized capillary tubes for hematological studies. The treated and control blood samples were used to estimate hematological parameters.

Red blood corpuscle (RBC) count

RBC count was made with a Neubauer crystalline counting chamber as described by Davidson and Henry (1969).

The RBC count was determined in *Cyprinus carpio* at different lethal and sublethal exposure periods including control. The RBC count was determined in six individual fishes at each exposure period including control and the mean of six was taken into account.

The blood collected was diluted with Hayem's fluid (5gm of sodium sulphate, 1gm of

sodium chloride and 0.5gm of mercuric chloride were dissolved in 200 ml of distilled water) and the RBC number was represented in millions per cubic millimeter (mm^3). The blood was drawn up to 0.5 marks in the RBC pipette by caudal incision of the fish and immediately the Hayem's fluid was taken upto 0 mark. The blood was mixed thoroughly by rotating the pipette and the mixture was allowed to stand about 2-3 minutes for uniform mixing. The counting chamber and coverslip were cleaned and the coverslip was placed over the portified area. Again the solution was mixed gently and stemful of solution was expelled and a drop of fluid was allowed to flow under the coverslip by holding the pipette at an angle of 40° , it was allowed to stand for 2 to 3 minutes to allow RBC to settle. Afterwards the portified area of the counting chamber was focused under the microscope and the number of RBC were counted in five small squares of the RBC column (the RBC were counted in the outer four corner squares and the central square) under high power and the number of RBC cubic millimeter (mm^3) were calculated using the following formula.

$$\frac{\text{Number of cells} \times \text{dilution factor} \times \text{depth factor}}{\text{Area counted}}$$

White blood corpuscles (WBC) count

Blood is drawn from the vial into WBC pipette up to 0.5 marks and immediately the diluting fluid is drawn up to 11 mark. The solution is mixed thoroughly by shaking gently. The rest of the procedure is the same as described by Davidson and Henry (1969) for RBC count. In case of WBC, count was made in bigger squares of the chamber. The WBC count was expressed in cu. mm.

Some aspects of Carbohydrate metabolism

The levels of blood glucose and total carbohydrate content in gills, liver, muscle, kidney and brain were estimated under this study.

Estimation of Blood glucose

The level of blood glucose was estimated by colorimetric method as described by Nelson and Somogyi (1952), in the fish *Cyprinus carpio* at 1, 4 days on exposure to lethal concentration of phorate in acute toxicity study and 1, 7, 15 and 30 days on exposure to

sublethal concentration of phorate in chronic toxicity study. 0.1 ml of blood was collected and to it 3.9 ml of deprotenizing solution (5% zinc sulphate and 0.3N sodium hydroxide in 1:1 ratio) was added and the mixture was centrifuged at 3000 rpm, for 10 minutes. The supernatant of 1 ml from this mixture was added with 1 ml of alkaline copper reagent, was shaken vigorously and heated in a boiling water bath for exactly 20 minutes. Then it was cooled and 1 ml of arsenomolybdate colour reagent was added. The entire volume of the solution was raised to 10 ml by adding distilled water and the optical density of the colour developed was measured in a spectrophotometer at wavelength of 540 nm. A blank and glucose standards were also run simultaneously. Glucose content was expressed as mg of glucose/100 ml of blood.

Estimation of total carbohydrates

The total carbohydrate content was estimated by the method of Carroll et al., (1956). A 10% homogenate (w/v) of the tissues, gills, liver, muscle, kidney and brain was prepared in 10% TCA. The protein precipitate was removed by centrifuging the homogenates for 15 minutes at 3000 rpm. The clear supernatant was taken for the estimation of total carbohydrates. To 0.5 ml of TCA filtrate, 5 ml of anthrone reagent was added and kept in boiling water bath for 15 minutes. Then the contents were cooled and read at 620 nm against a reagent blank in a spectrophotometer. The total carbohydrate levels were expressed as mg/gram wet weight of the tissue.

Protein metabolism

The levels of total proteins were estimated separately in different tissues such as gills, liver, muscle, kidney and brain of the fish *Cyprinus carpio*, under this study.

Estimation of total proteins

The total proteins were estimated using Folin phenol reagent method as described by Lowry et al., (1951).

A 1% homogenate (w/v) of the tissues was prepared in 0.25M ice cold sucrose solution. 1.0 ml of homogenate was taken, to it 1.0 ml of 10% trichloro acetic acid (TCA) was added and centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded and residue was taken for the estimation of total proteins. The residue was dissolved in 1.0 ml of 1N NaOH and to this 4.0 ml of CuSO₄ reagent and 0.4 ml of Folin-ciocalteau reagent were

added. The contents were stirred well and kept aside for 30 minutes at room temperature. Then the optical density of the solutions was read at 600 nm in spectrophotometer. A mixture of 1.0 ml of 1N NaOH, 4.0 ml of CuSO₄ reagent and 0.4 ml of Folin-ciocalteau reagent was used as blank. Bovine serum albumin was used for the preparation of protein standards. The total protein content was expressed as mg/gram wet weight of the tissue.

Study of Enzymatic activities

Estimation of Acetylcholinesterase (Acetylcholine hydrolase, EC: 3.1.1.7) activity (AChE):

Acetylcholinesterase activity in the organs was estimated by the method of Metcalf (Glick, 1957).

1% homogenate of brain and 5% homogenate of other tissues were separately prepared in 0.25M ice-cold sucrose solution. The reaction mixture consisted of 1.0 ml of buffer acetylcholine chloride solution (4.0 vols of 0.1M phosphate buffer (pH 7.2) + 1.0 vol of 0.04M acetylcholine chloride solution for the brain and 9.0 vols of 0.1M phosphate buffer (pH 7.2) + 1.0 vol of 0.04M acetylcholine chloride solution for other organs) was added to 0.5 ml of each organ homogenate. The mixture was incubated at 37°C for 30 minutes and the reaction was stopped by adding 2.0 ml of alkaline hydroxylamine hydrochloride (1M hydroxylamine hydrochloride + 35M sodium hydroxide solution in 1:1 ratio) and 1 ml of HCl (1:1 HCl:H₂O). The contents were thoroughly mixed and centrifuged at 1000 rpm for 15 minutes. To the supernatant 0.5 ml 10% ferric chloride solution was added to develop colour. The colour was measured against the blank at 540 nm in spectrophotometer. The blank contained 1 ml of buffer solution instead of buffer substrate mixture. The enzyme activity was expressed as μ moles of Ach hydrolysed /mg protein/hr.

Estimation of Succinate dehydrogenase (Succinate:Acceptor) oxidoreductase, EC: 1.3.99.1) activity (SDH):

Succinate dehydrogenase activity in the organs was estimated using the colorimetric method of Nachlas et al., (1960).

A 5% homogenate (w/v) of the tissues was prepared in 0.25M ice cold sucrose solution, centrifuged at 3000 rpm for 10 minutes and the supernatant was taken as the source of the enzyme. The incubation mixture consisted of 0.2 ml of 0.4M phosphate buffer (pH 7.7), 0.2 ml of 0.2M sodium succinate, 1.0 ml of 0.004M 2-(p-indophenol)-3-p-nitrophenyl-5-

phenyl tetrazolium chloride (INT), 0.1 ml of 0.005M phenazine methosulphate and 0.5 ml of enzyme preparation. The mixture was incubated at 37⁰ C for 30 minutes and the reaction was stopped by adding 6.0 ml of glacial acetic acid. The formazone formed was extracted into 6.0 ml of toluene overnight at 0⁰ C and the optical density of the colour developed was read at 495 nm in a spectrophotometer. A blank taking 0.5 ml of distilled water and control by taking 0.5 ml of boiled enzyme were also run similarly. INT standards were prepared alongside for comparison. The enzyme activity was expressed as μ M of formazone formed/mg protein/hr.

Estimation of Malate dehydrogenase (L-Malate NAD-oxidoreductase, EC: 1.1.1.37) activity (MDH):

Malate dehydrogenase activity in the organs was estimated by the method of Nachlas et al., (1960) with slight modification as suggested by Prameelamma and Swami (1975).

A 10% tissues homogenate were prepared in 0.25M ice-cold sucrose solution and centrifuged at 2500 rpm for 15 minutes. The supernatant was taken as the source of the enzyme. The reaction mixture in a final volume of 2.0 ml contained 100 μ moles of phosphate buffer (pH 7.4) + 4 μ moles of INT + 50 μ moles of sodium malate + 0.1 μ moles of NAD + enzyme. This mixture was incubated at 37⁰ C for 30 minutes and the reaction was stopped by adding 5.0 ml of glacial acetic acid. Then the colour was extracted into 5.0 ml of toluene by keeping overnight at 5⁰C and the optical density of the colour developed was read in a spectrophotometer at a wavelength of 495 nm. A blank taking 0.5 ml of distilled water and control by taking 0.5 ml of boiled enzyme were also run similarly. INT standards were prepared alongside for comparison. The enzyme activity was expressed as μ M of formazone formed/mg protein/hr.

Estimation of Lactate dehydrogenase (L-Lactate NAD oxidoreductase, EC: 1.1.27) activity (LDH):

Lactate dehydrogenase activity in the organs was estimated using the method of Srikanthan and Krishnamoorthi (1955) as modified by Govindappa and Swami (1965).

A 5% homogenate (w/v) of the tissues was prepared in 0.25M ice-cold sucrose solution, centrifuged at 2500 rpm for 15 minutes and the supernatant was taken as the source of the enzyme. The incubation mixture consisted of 0.2 ml of 0.4M phosphate buffer (pH 7.4), 0.5 ml of 0.1M lithium lactate, 1.0 ml of 0.0001M nicotinamide adenine dinucleotide (NAD), 1.0 ml of 0.004M 2-(p-indophenol)-3-p-nitrophenyl-5-phenyl tetrazolium chloride (INT) and

0.5 ml of enzyme preparation. The mixture was incubated at 37⁰ C for 30 minutes and the reaction was stopped by adding 6.0 ml of glacial acetic acid. The formazone formed was extracted into 6.0 ml of toluene overnight at 0⁰ C and the optical density of the colour developed was read at 495 nm in a spectrophotometer. A blank taking 0.5 ml of distilled water and control by taking 0.5 ml of boiled enzyme were also run similarly. INT standards were prepared alongside for comparison. The enzyme activity was expressed as μ M of formazone formed/mg protein/hr.

Histopathology

The histological sections of the gills, liver, muscle, kidney and brain of acute and chronic toxicity exposed fish were taken by adopting the procedure as described by Humason (1972).

The tissues were isolated from control and phorate treated fish and gently rinsed with physiological saline solution (0.9% NaCl) to remove mucus and other debris adhering them. They were fixed in Bouin's fluid (75 ml saturated aqueous picric acid, 25 ml 40% formaldehyde and glacial acetic acid) for 24 hours. The fixative was removed by washing through running tap water for overnight. Then the tissues were processed for dehydration. Ethyl alcohol was used as the dehydrating agent, as it is the most suitable and economical besides its hardening effect. The alcoholic transfer schedules were so arranged as to utilize both dehydration and hardening effect. The tissues were passed through successive series containing 30%, 50%, 70%, 80%, 90%, 95% and absolute alcohols. Then the tissues were cleaned in methyl benzoate and embedded in paraffin wax. Sections of 5 μ thickness were cut using "SIPCON" rotatory microtome. The sections were stained with Harris hematoxylin (Harris, 1900) and counter stained with eosin, dissolved in 95% alcohol. After dehydration and cleaning, the sections were mounted in Canada balsam. Photomicrographs of the section preparations were taken using Magnus photomicrographing equipment.

Statistical analysis

All the results obtained in this investigation were subjected to statistical analysis. For this, the data were fed to the computer, the standard deviation to each mean and percent change over the means of controls and experimental were derived. The significance of the data among controls and experimental were derived at 5% level using the DMR test, and is represented in the respective tables.