Impacts of Endocrine Disrupters on *Cyprinus carpio* (Linn.)

A thesis submitted in the partial fulfillment of the requirement for the award of degree of

Doctor of Philosophy
In
Inland Aquaculture (I.A.C.)

By
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Dedicated to
Smt. Indira J. Barse,
Smt. Kankamma W. Jacob
& Mrs. Ranajana V. Barse
Love Unlimited.....

केन्द्रीय मात्स्यिकी शिक्षा संस्थान
(समतुल्य विश्वविद्यालय) भारतीय कृषि अनुसंधान परिषद
CENTRAL INSTITUTE OF FISHERIES EDUCATION
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CERTIFICATE

Certified that the thesis entitled "Impact Of Endocrine Disrupters on *Cyprinus carpio*" is a record of bonafide research work done by Mr. Abhijeet Barse, Scholar of PhD (Inland Aquaculture), 2001-2004 batch under my guidance and supervision and that it has not previously formed the basis for the award of any other degree, diploma or other similar title to him. The thesis has now been submitted towards the partial fulfillment of the requirement for the award of Doctor of Philosophy in Inland Aquaculture.

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DECLARATION

I hereby declare that the thesis entitled "Impacts Of Endocrine Disrupters on *Cyprinus carpio*" is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associate-ship, fellowship or any other similar titles.

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वर्तमान परीक्षण में परिपक्व साइप्रिनियस कार्पियो को तीन चयनित रसायनों में 30 दिनों तक अध्ययन किया गया एवं उनके स्ट्रैस इन्जाइम, सोमाइटिक इंडिसेज, बायोएक्युमुलेशन, हिस्टोलोजिकल अल्टरेशन, व्यवहार परिवर्तन एवं विटेलोजेनिन इन्डक्शन पर प्रभाव देखा गया । सिप्रिनस कार्पियो में चुने हुए रसायनों की घातक मात्रा (एल.सी.50 एवं एल.सी.100) का जैव विश्लेषण किया गया एवं पाया गया 96 घंटे में यह मात्रा डायइथील थैलेट (डीईपी) में 48 एवं 55 मिलीग्राम/लीटर, मेथिल पेराबेन (एम.पी.) 42 एवं 45 मिलीग्राम/लीटर और टररारी ब्युटिलफीनाल (टीबीपी) में 6.9 एवं 7.2 मिलीग्राम/लीटर थी । लंबे समय के लिए कम आच्छादन सांद्रण को लिया गया । डीईपी की घातक मात्रा ज्यादा होने की वजह से विश्लेषण के लिए कम आच्छादन चुना गया । (1,5 और 20 मिलीग्राम/लीटर) मेथिल पाराबेन के घातक सांद्रण का 1/10, 1/25 एवं 1/50 भाग लिया गया । जबकि टीबीपी का 1/3, 1/5 एवं 1/10 आच्छादन के लिए चुना गया ।

एक सौ अस्सी कार्प (वजन 89.25 + 0.32g) ली गई जिन्हें 10 दिन के लिए प्रयोग हेतु अनुकूल जलवायु में रखा गया । 20 मछलियों की दर से प्रत्येक टैंक में वितरित किया गया जिसे तीन टैकों में जिसमें एक टैंक को घनात्मक नियंत्रण से रखरखाव रखा गया ।

जांच (पैरामीटर) अध्ययन हेतु नमूनों को साप्ताहिक आधार पर इकठ्ठा किया गया । प्रयोग अवधि की समाप्ति के उपरान्त यह देखा गया कि लगभग सभी प्रदर्शित सान्द्रता में इंजाइमैटिक अभिक्रिया में उल्लेखनीय वैरियसन ($P < 0.05$) दर्शा रहा है । हेपाटोसोमैटिक इनडेम्स एवं गोनाडोसोमैटिक इनडैम्स में एक चिन्हित -हास देखी गई जो सभी तीनों उच्च प्रदर्शित सान्द्रता में थी । सबसे अधिक जमाव मष्तिष्क में पाया गया जो गिल से प्रभावित था जबकि यकृत (लीवर) सबसे कम जमाव को दर्शा रहा था । हालांकि यकृत (लीवर) के संदर्भ में प्रदर्शित अवधि में जमाव में वृद्धि होता ही गया । यहां तक कि प्रदर्शित कार्प के भिन्न प्रदर्शन सान्द्रता लीवर एवं गोनाडस के हिस्टोलॉजी पर उच्च प्रभाव उत्पन्न करता है ।

Abstract

In the present investigation mature *Cyprinus carpio* were exposed to three selected chemicals individually for 30 days to study their impacts on stress enzymes, somatic indices, bioaccumulation, histological alterations, behavioral changes and vitellogenin induction. The LC_{50} was deduced by exposing the fish to a range of concentration for 96 hrs, accordingly sub-lethal concentrations were decided depending upon it. The concentration taken were 1, 5, 20 mg/L for diethyl phthalate for studies other than vitellogenin induction and for vitellogenin induction it was 100 μg/L, 1 mg/L and 5 mg/L, for methyl paraben the exposure concentrations were 0.84, 1.68 and 4.2 mg/L and for tertiary butyl phenol it was 0.69, 1.38 and 2.3 mg/L. One hundred and eighty carps weighing nearly the same (89.25 ± 0.32 g) were acclimatized for 10 days prior to experiment and distributed randomly at the rate of 20 fish per tank for each concentration in triplicate and 1 tank was maintained as positive control. Samples were taken on a weekly basis to study the parameters. At the end of the experimental period, it was observed that in almost all the exposure concentrations the enzymatic activity showed significant ($P > 0.05$) variation as compared to control suggestive of induction of stress response cause by exposure to the chemicals. There was a marked reduction in the hepatosomatic index and gonadosomatic index at the higher exposure concentrations in al the three chemicals. The highest accumulation was observed in brain followed by gills while liver showed the least accumulation. However in case of liver the accumulation increased with increase in exposure period. Even the lower exposure concentration elicited higher impacts on the histology of liver and gonads of the exposed carp. Vitellogenin induction was observed even at the lowest exposure concentration of all the chemicals. This study emphasizes the significance of *in vivo* approaches for the assessment of toxic effect of endocrine disrupting chemicals in aquatic animals.

Introduction

CHAPTER 1
INTRODUCTION

1.1 Preamble

As a result of human developmental activities, a number of environmental pollutants enter the water bodies through air, river discharge, land drainage, or direct dumping. The problem of aquatic pollution due to various types of contaminants such as organochlorine pesticides (OCP), polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs) petroleum hydrocarbons (PHCs), polychlorinated dibenzo-dioxins (PCDDs), plasticizers, heavy metals and metalloids (mercury, cadmium, arsenic, selenium, cobalt, manganese, etc.), phenolic surfactants and nutrient salts, has become a global concern because of the serious risk they pose to the fish and aquatic environment.

Water pollutants can have many different effects on aquatic organisms, always depending on the pollutant and the organism in question. There are certain groups of chemicals, which are recognized to be affecting specific organ/system of the organism. Moreover, the mechanism of toxic action of environmental chemicals is not usually thought to be restricted to one specific process as multiple toxicity sites and mechanisms are involved.

However, most of the studies have been focused on one of the aspects of pollutant impact on organism, while other accompanying impacts have been sidelined. Major effects of these are delineated here.

Genotoxicity : Many compounds that enter the body of an organism are known to cause damage to DNA. Usually when pollutants damage DNA, a natural repair system in an organism will return it to its usual state; otherwise the damaged cells undergo apoptosis. However, when repair mechanism is off for any particular reason, cells with damaged DNA can divide. Mutant cells are then produced and the defect can spread, causing the offspring of the organism in question to have serious defects that are damaging to their health and may often lead to cancer precipitation. Examples of genotoxins are PAH's, aflatoxin and vinyl chloride. Most often, it is not the original compound that reacts with DNA. Highly reactive short-lived products produced from the original compound by-enzymes usually cause the reactions.

Carcinogenity : Polycyclic aromatic hydrocarbons (PAHs) are an important class of environmental pollutants that are known to be carcinogenic and immunotoxic; ethylene dibromide has been recently added to the list. Pollutants can be inductors; which means that they introduce cancer-forming properties in the cells of an organism. They can also be promoters, which mean that they promote the growth of cells that have cancer-forming properties. Finally, they can be progressors, which mean that they stimulate unrestrained division and spreading of cancer cells. When cells are malignant, they can spread through the body rapidly through metastasis, causing defects to healthy cells and immunity mechanisms. They will destroy normal body cells and cause cancer in organs and systems.

Neurotoxicity : The nervous system of organisms is very sensitive to toxic effects of chemicals, both naturally occurring and man-made. Chemicals that cause neurological effects are called neurotoxins. Examples of dangerous neurotoxins are organochlorine pesticides, insecticides, etc. Neurotoxins disturb the normal transmission of impulses along nerves or across synapses. The consequences of neurotoxicity are varied as these may cause uncoordinated muscular tremors and convulsions, malfunction of nerves and transmissions, dizziness and depression, or even total malfunction of body parts. Neurotoxicity can be so serious that synapses are blocked. Synaptic block causes death as a result of paralysis of the diaphragm muscles and respiratory failure.

Disturbance of energy transfer: Energy transformation in organisms occurs in mitochondria through oxidative phosphorylation though substrate level phosphorylation is also in vogue. In the mitochondrion, ATP-molecules are produced which transfer energy in a variety of metabolic processes. When ATP production is disturbed, the energy transfer will cease. This will make an organism tired and lifeless and unable to function normally.

Behavioral effects: All behaviors are vulnerable to alteration by pollutants. Foraging levels can deplete, resulting in reduced production. Vulnerability to predators can increase, due to a depletion of vigilance. In these ways, effects of pollutants on behavior result in lowered production and higher mortality rates.

A common result of pollution is a loss of appetite and thus less uptake of food. Searching for preys can also be affected, due to effects of pollutants on learning, search strategy and sensory systems. These behavioral effects cause lower chances of survival of organisms, mainly animals.

Endocrine Disruption: Pollutants that cause reproductive failure due to damage to the reproductive organs are called endocrine disruptors. There are several ways in which a pollutant can act as an endocrine disruptor. The first is an estrogenic chemical; this is a chemical that can imitate an estrogen by binding to the estrogen receptor. This results in the induction of estrogenic processes, causing an organism to experience reproductive failure due to a disturbance in the reproductive system. An estrogenic chemical can also block the effects of endogenous estrogens by binding to the estrogenic receptor. This causes masculization of female organisms. It is also possible that female reproductive chemicals are found in male organisms. This causes hermaphrodites. Imposex has been widely reported in marine organisms, for instance with dog whelks by tributyltin. Another series of problems is caused when chemicals block the hormone receptor sites. In this case, the normal action of the hormone is inhibited, as it cannot bind with the receptor. This can cause infertility when it occurs over a longer period of time.

Review of Literature

CHAPTER 2

REVIEW OF LITERATURE

A number of definitions currently exist for endocrine disrupting chemicals (EDC). Kavlock *et al.* (1996) proposed a definition that includes not only the primary ways EDCs are thought to interfere with normal functioning of the endocrine system, but also some of the effects EDCs can have on an organism.

In 1996, the European Commission defined an endocrine disrupter as an exogenous substance that causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine functions (European Commission, 1996). As with Kavlock *et al.* (1996), this definition stresses that adverse health effects occur as a result of one or more changes in endocrine function. The National Research Council adopted hormonally active agents as a more neutral mechanistic descriptor, and defined them broadly as substances that possess hormone like activity, regardless of structure (NRC, 1999).

The endocrine disruptors are a large group of chemicals (over 10000) : they enter into the aquatic environment from manufacture of various industrial and consumer products, agriculture and food/drug processing, wastewater treatment plants and human wastes. This group includes certain polychlorinated biphenyls, polyaromatic hydrocarbons, dioxins furans,

pesticides, alkylphenols, synthetic steroids, phthalate esters, plant sterols and parabens. Some chemicals implicated so far are tabulated below.

Table 2.1: Chemicals Known to Cause Endocrine Disruption in Wildlife

Chemical groups	Specific chemicals	Reference
Polychlorinated compounds	Polychlorinated biphenyls Polychlorinated dioxins	Arukwe *et al.* (2001)
Organochlorine pesticides	DDT, dieldrin, lindane, atrazine, trifluralin, 2-4 D, permithrin	Fossi *et al.* (2004), Waring and Moore (1996)
Fungicide	Vinclozin	Kelce *et al.* (1997), Gray Jr. (1998), Uzumu *et al.* (2004)
Organotin	Tributyltin	Depledge and Billinghurst, (1999), Oelhmann *et al.* (1996)
Alkylphenolic surfactants	Nonylphenols	Madsen (2004), Toft and Baatrup (2003)
Phthalates	Diethyl phthalate, butyl benzyl phthalate	Rhodes *et al.* (1995), Harris *et al.* (1997), Wine *et al.* (1997), Norrgren *et al.* (1999)
Sex hormones	17 β estradiol, estrone	Gimeno *et al.* (1998), Orn *et al.* (2003), Neiminer *et al.* (2002)
Steroids	Trenbolone	Miller and Ankley (2004)

The mechanism by which these compounds have their impacts may vary, and the effects in general, can be listed as:

- Mimicking the effects of natural hormones by recognizing their binding sites

- Antagonizing the effects of these hormones by blocking their interaction with their physiological binding sites (receptors)
- Reacting directly and indirectly with hormones in question
- Altering the natural pattern of synthesis of hormones altering hormone receptor levels.

The aquatic environment is the ultimate sink for most chemicals, whether natural or man-made. However chemicals and products disposed of, whether in landfill sites or via drains to sewage - treatment works (STWs), will enter the aquatic environment either as it is or in a metabolically altered form(s). Hence, it is not coincidence that essentially all of the well-documented examples of endocrine disruption in wildlife are of animals either living in the aquatic environment e.g. alligators in Florida; (Guillette *et al.*, 1994) or fish (Bortone and Davis, 1994; Purdom *et al.*, 1994) feeding on aquatic organisms containing high levels of pollutants e.g. fish-eating birds. It is presumed that such organisms receive the highest level of exposure, and hence are the most severely affected.

In aquatic environment, the ability of an endocrine disrupter to affect an individual or population would depend on a number of factors, including the potency or efficacy of the disrupter during exposure, bioconcentration potential, presence of other endocrine disrupting chemicals, life stage, season, other environmental stressors present (e.g. temperature, salinity and other contaminant) and mobility of the individual.

The critical environmental concentrations of these compounds, which may cause endocrine effects and alter physiology are not well known yet and may be different for different species. In fish, overt endocrine disruption does not appear to be a ubiquitous environmental phenomenon, but rather more likely to occur near sewage treatment plants, pulp and paper mills, and in areas of high organic chemical contamination. However, more wide- spread endocrine disruption can occur in rivers with smaller flows and correspondingly large or numerous wastewater inputs. Some of the most severe examples of endocrine disruption in fish have been found adjacent to sewage treatment plants. Effects are thought to be caused primarily by natural and synthetic estrogens and, to a lesser extent, by the degradation products of alkylphenol polyethoxylate surfactants. Effects found in fish near pulp and paper mills include reduced levels of estrogens and androgens as well as masculinization of females, and have been linked to the presence of sitosterol, a plant sterol. Effects seen in areas of heavy industrial activity typically include depressed levels of estrogens and androgens as well as reduced gonadal growth, and may be linked to the presence of PAHs, PCBs, and possibly dioxins. Effluents released from pulp and paper mills also have a wide range of reproductive effects on fish in the receiving environment. For example, white sucker (*Catastomus commersoni*) exposed to effluent from a bleached kraft mill (BKME) on Lake Superior exhibit decreased gonad size, delayed sexual maturation, reduced expression of secondary sex characteristics, and reduced sex steroid levels (McMaster, 1995).

Fish, coming from contaminated areas, becomes a pathway of exposure for humans and animals consuming as diet (Persky *et al.*, 2001; Bosveld and Van den Berg, 2002).

2.1 Endocrine disrupters

Various effects of endocrine disrupters have been documented in wildlife species and also been demonstrated in laboratory studies. Their main effects in fish can be summarized as:

- Egg producing cells in male
- Reduced testis growth rate
- Increased liver size
- Increased levels of vitellogenin (in blood plasma) and reduced levels of endogenous hormones in both males and females
- Inhibition of spermatogenesis
- Presence of ovotestes etc.

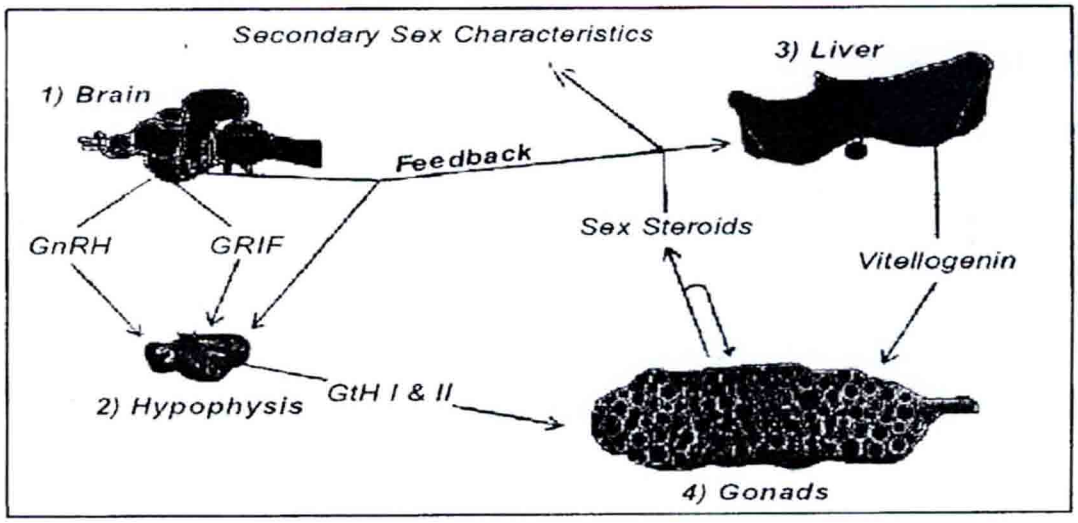

Plate no. 1 Target sites of endocrine modulators in male and female teleosts 1. Brain: CYP 19arom, neuropeptides 2. Hypophysis: Gonadotropines 3.liver: estrogen receptor, vitellogenin, and steroid catabolism. 4 Gonads: a) ovary: oocytes, steroid producing cells b) testis: spermatocytes, steroid producing cells.

2.2 Effects of Endocrine Disrupters on Biota

2.2.1 Developmental effects

Willey and Krone (2001) reported that some of the effects, observed in adult zebra fish, which were exposed to endosulphan and nonylphenols, might arise from permanent organizational changes during embryogenesis, including changes in the gonad structure and function. Delay in smolt development was observed in Atlantic salmon exposed to17 β-estradiol and 4 nonylphenol. (Madsen, 2004). According to Kime and Nash (1999), 5 ng/l nonylphenol could arrest the development of eggs produced at early blastula stage and induced vitellogenesis in exposed males.

2.2.2 Secondary sexual characters

Batty and Lim (1999) reported the loss of secondary sexual characters in male mosquito fish, which were found in a river at down stream from a sewage treatment plant. The males showed a marked reduction in the size of gonopodium (modified anal fin), which is formed under the influence of testosterone and is critical for sperm transfer.

In a study conducted by Norrgren *et al.* (1999) on the effects of potential xenoestrogens (DEHP, nonylphenol, and PCBs) on sexual differentiation in juvenile Atlantic salmon (*Salmo salar*), a significant overrepresentation of phenotypic females (64%) was recorded in a group fed 1500 mg of di-2-ethylhexyl phthalate / kg of feed, while nonylphenol at the rate of 300 mg per kg of feed did not show such effect.

2.2.3 Vitellogenin induction/ Hormonal changes

Vitellogenin (VTG) is a precursor of egg yolk that is synthesized in the liver of developing females under the control of endogenous estradiol. It circulates in high concentration in the blood of maturing female fish and is incorporated into growing oocytes where it stimulates their rapid development prior to ovulation. Vitellogenin may be determined in the blood of fish using a specific radioimmunoassay. In male, the circulating levels of endogenous estradiol are below detectable limits, and there is normally no expression of the gene responsible for VTG production. If exposed to exogenous sources of estrogens, however, male fish are able to synthesize VTG in quantities approaching those of a female fish. Hence it is used as a biomarker for male whose endocrine system has been disrupted by endocrine disrupting chemicals (Sumpter and Jobling, 1995).

Male fish exposed to estrogenic compounds showed induced production of vitellogenin. Female specific proteins were induced in the blood of male medaka that was exposed for 5 weeks to 17-β estradiol and nonylphenol at concentrations above 0.5 mg/l and 0.1 μg/l respectively (Kashiwada *et al.*, 2002). Routledge *et al.* (1998) found that alkyl hydroxy Benzoates were weakly estrogenic in nature and also had an effect on the choriogenin production in male medaka (Inui *et al.*, 2003). Recently Oshi (2002) reported that when exposed to propyl paraben, the serum testosterone concentration was decreased in a dose dependant manner. The exposure level at which this effect was observed was same as the upper limits acceptable daily intake (10 mg/kg body wt/day) of paraben in Europe and

Japan. Similar effects were also studied on daily sperm production and male reproductive system of rats, exposed to butyl paraben. Alkyl p-hydroxy benzoate has shown high binding affinity to human estrogens (Satoh *et al.*, 2000).

Exposure of adult male carp to the pseudo-estrogen TPP, or to E2 during sexual maturation, led to elevated plasma levels of vitellogenin (Gimeno *et al.*, 1998). Similar observations have also been reported in select Indian fish (Patra, 2002).

2.2.4 Sex reversal

2.2.4.1 Hermaphroditism

Hayes *et al.* (2003) showed that atrazine exposure (0.1 ppb) resulted in retarded gonadal development (gonadal dysgenesis) and testicular oogenesis (hermaphroditism) in leopard frogs (*Rana pipiens*). Slower developing males even experienced oocyte growth (vitellogenesis). Psuedohermaphroditism was observed (occurrence of male parts in addition to female genital ducts) in case of marine prosobranch *Nucella lapillus* and *Ocinebrina aciculata* in response to tributyltin (TBT) pollution (Oehlmann *et al.*, 1996).

In an experiment conducted by Tanaka and Grizzle (2002) to study the effects of nonylphenol on the gonadal differentiation of the hermaphroditic fish *Rivulus marmoratus*, it was observed that the oogenesis was significantly inhibited along with dysplasia of gonadal lumen and testicular agenesis.

2.2.4.2 Feminization

Luis *et al.* (2002) observed that in Chinook salmon alevins (103 days post-hatching) genetic males treated with 1 µg estradiol/L, BKME 100%, and SE 30% developed as physiological females, presenting ovaries identical to genetic females in the control group. The physiological female condition in XY fish was also observed in these treatments groups at 179 days post-hatch, which suggested that the effect was permanent, whereas in other groups the effect was changed between sampling periods.

The histological examination of the testes of adult male carp, exposed to tert-pentylphenol or to estradiol during sexual maturation, showed the progressive disappearance of spermatozoa and spermatogenic cysts. The occurrence of early ovo-testes in some fish with completely regressed testes suggests that oogenesis may proceed in testes, which have first been inhibited (demasculinised) following estrogenic stimulus. Gimeno *et al.*, (1998) and Yadetie & Male (2002) reported that 4-nonylphenol affected the gene expression of pituitary hormones in juvenile Atlantic salmon. Toft *et al.*, (2003) reported that sexual characteristics of relevance to male reproductive capacity were altered in the Lake Apopka mosquito fish population and the presence of chemicals with antiandrogenic effects in Lake Apopka as a possible cause of the observed alterations.

Hotchkiss *et al.* (2002) observed that the play behavior in male rats treated with flutamide (50 mg/kg), or vinclozolin (200 mg/kg) during 30-37 post-natal days was significantly reduced, resembling levels of play characteristic

of females rather than untreated males demonstrating that perinatal exposure to vinclozolin, an environmental antiandrogen, can alter androgen-dependent play behavior in the male rat.

Estradiol increased the activity of brain aromatase in sexually mature male medaka in a concentration-dependent manner at 2.5 and 25 μg/L, but the increase was lower at higher concentrations of the hormone (Melo and Ramsdell, 2001).

In vertebrates, exposure to androgens, early in development, is essential for masculinization of the male reproductive phenotype.

Desbrow *et al.* (1998) revealed that in UK estrogenic activity in some rivers was (at least in part) caused by the presence of 17-β estradiol and estrone, as well as the synthetic bied in contraceptive pills. The chemical found its way in the streams and watercourses connected to the municipal and domestic sewage. The exposure to this hormone led to feminization as well as demasculization of fish.

Sheehan *et al.* (1999) reported a complete, permanent, and functional male-to-female sex reversal in the Japanese medaka (*Oryzias latipes*, d-rR strain) after one time embryonic exposure to the xenoestrogen O'-DDT (d-rR) strain. The xenoestrogens O'-DDT were directly microinjected into the egg yolks of medaka at fertilization to parallel the maternal transfer of lipophilic contaminants to the embryo. At 10 weeks of age, microinjected medakas

were examined for mortality and sex reversal. Sex reversal of genetic males to phenotypic females (XY females) was observed in 86% (6 of 7) medakas. Histological examination of the gonads confirmed active ovaries in 100% of the XY females. In 10-day breeding trials, in which XY females were paired with normal XY males, 50% of the XY females produced fertilized embryos; representing a comparable breeding success rate to normal XX females. Fertilized eggs, produced from XY females were hatched to viable larvae.

2.2.4.3 Masculinization

Studies conducted by Orlando *et al.* (2002) showed that female mosquito fish, living downstream of a paper mill located on the Fenholloway River, Florida, had masculinized secondary sex characteristics, including altered anal fin development and reproductive behavior. Fenholloway females had masculinized anal fin development as indicated by an increase in the number of segments in the longest anal fin ray. Larsson and Forlin (2002) showed that the eelpout produced male-biased broods closed to the paper mill wastewater discharge point. They observed that when the mill was shut down for 17 days, coinciding with the period when the gonads of the eelpout embryos differentiate, the sex ratios were no longer male biased, however, after the reopening of mill, a significant male bias was reappeared.

2.2.5 Reproduction

A number of pesticides which commonly occur within the aquatic environment have been shown to have sub lethal effects upon pheromone mediated endocrine function and reproduction in the Atlantic salmon (Waring and Moore, 2004). Exposure of mature male salmon parr to environmental

levels of the pesticides atrazine, carbofuran and diazonin, inhibited the olfactory detection of the female reproductive priming pheromone, which is considered to be involved with the synchronization of spawning between the sexes of salmon (Waring and Moore, 1996).

Exposure of salmon eggs and milt to cypermethrin also reduced the level of fertilization, suggesting a further toxic impact of the pesticide on salmon reproduction. The synthetic pyrethroid inhibited the ability of male Atlantic salmon to detect and respond to the reproductive priming pheromone, PGF2α. As a result, milt and plasma levels of 17, 20-dihydroxy-4-pregnen-3-one concentrations were not significantly elevated in the male parr suggesting a significant effect on reproductive function.

In addition, low levels of the pesticide reduced fertilization rates in salmon eggs. Atrazine was also shown to have a direct impact upon the testes, modifying the release of androgens and suggesting an additional toxic mechanism to male salmon reproduction.

2.2.6 Enzymes

Many studies have suggested that compounds present in commercial effluents are known to alter the biochemical and enzymatic activities of fish in one way or the other. According to Goldemberg *et al.* (1996) the changes in phosphatase activities are regarded as indices of growth, illness and spawning.

2.2.6.1 Acid and Alkaline Phosphatase (AcP and AlP)

Gill *et al.* (1990) suggested that fish enzyme system can be used in monitoring the water quality. They studied the effect of mercury on tissue enzymes of rosy barb (*Puntius conchonius*). Rosy barb were exposed to 181 μg/l mercuric chloride for 48 h. The AcP activity was inhibited in the liver, gills, kidneys, and gut but stimulated in the gonads. With the exception of kidney, the AlP activity showed an increase in all the organs examined.

Exposure of fresh water fish *Cyprinus carpio* to fixed levels of diethyl phthalate (DEP) led to significant changes in the Acid phosphatase and Alkaline phosphatase levels. The DEP treated fish at 25 ppm inferred that DEP was capable of interfering with the metabolic process by altering enzyme activity in vital organs (Ghorpade *et al.*, 2001).

In liver homogenate of fish exposed to methyl mercuric chloride (CH_3HgCl), non-competitive inhibition of acid phosphatase activity was found by Hinton and Koenig (1975).

Banerjee *et al.* (1984) reported an elevation in acid & alkaline phosphotases in *Schizodactylus monstrossus* when the fish was exposed to pyrethrum at selected intervals. Inhibition of AcP and AlP was also reported by Verma and co-workers (1981) in response to sublethal concentrations (1/5, 1/10 and 1/15 fractions of 96-h TL_{50}) of thiotox, dichlorvos and carbofuran. Maximum (62.79%) inhibition of alkaline phosphatase in gills after thiotox

(0.00013 mg/l) treatment and minimum inhibition (6.12 %) of glucose-6-phosphatase in liver after dichlorvos (0.030 mg/l) treatment was observed.

Fish liver showed the same acid phosphatase pattern, as reported for birds and mammals in a study conducted by Panara and Pascolini (1989).

In a study conducted by Dalela *et al.* (1980) *Notopterus notopterus* was exposed to four sublethal concentrations (90, 130, 264 and 662 μg/liter) of phenol and 13·6, 20·4, 40·8 and 60·2 μg/liter of pentachlorophenol for 10, 20 and 30 days. The results showed a significant enzymic difference in the liver of control fish and those exposed to phenol- and pentachlorophenol. The activity of acid and alkaline phosphatases and succinic dehydrogenase was found to be inhibited in almost all cases. The greatest inhibition was observed in acid phosphatase after an exposure period of 30 days.

2.2.6.2 Serum glutamic oxalacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT)

Bogusz (1968) observed a decrease of acetylcholinesterase and cholinesterase activity in a group of fish exposed to organophosporous pesticides. The activities of aspartate aminotransferase, aldolase and alkaline phosphatase showed significantly higher values as compared to the control groups. There was no important change in the alanine aminotransferase activity.

Verma *et al.* (1984) concluded that mercuric intoxication at sub lethal levels (1/5, 1/10, 1/15, 1/20 or 1/25 fractions of 96 h LC_{50}) produced

alterations in the activity of serum glutamic oxalacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) of *Notopterus notopterus*. According to Pacheco and Santos (2001) fish exposed to GWSF exhibited an increase in liver alanine transaminase (ALT) activity following a short exposure, while the extended exposure revealed liver damage due to decreased ALT activity. Recently De Aguiar *et al.* (2004) concluded that increase in AST & ALT activities were directly related to the liver damage and tissue injury.

2.2.6.3 Other enzymes

Pacheco and Santos (2001) reported a time related increase in fish liver ethoxyresorufin-o-deethylase (EROD) activity as well as the appearance of erythrocyte nuclear abnormalities (ENA) after a day of exposure to diesel oil water soluble fraction (DWSF) and gasoline water soluble fraction.

Exposure studies conducted by Sastry and Rao (1982) on chronic effects of mercuric chloride on the activities of some enzymes in certain tissues of the fresh water murrel showed that there was inhibition in glucose – 6-phosphatase activities in gills, intestine, kidney and liver. Exposure to sub-lethal doses of oleandrin for 24hr and 96hr to fish caused significant alteration in the levels of total protein, total free amino acid, nucleic acid, glycogen, pyruvate, lactate and enzyme protease, phosphatases, alanine aminotransferase, aspartate aminotransferase and acetylcholinesterase activities in liver and muscle tissues. The alterations in all the above

biochemical parameters were also significantly time and dose dependent (Tiwari & Singh, 2004).

2.2.7 Behavioral Changes

Toxicant exposure often completely eliminates the performance of behaviors that are essential to fitness and survival in natural ecosystems, while frequently alter after exposures of lesser magnitude than those causing significant mortality. Unfortunately, the behavioral toxicity of many xenobiotics is still unknown, warranting their future study. Physiological effects of toxicants in the literature include disruption of sensory, hormonal, neurological, and metabolic systems, which are likely to have profound implications for many fish behaviors.

In one of the exposure studies, butyl benzyl phthalate caused fish to shoal together and also the affected fish remained at the bottom of the experimental tanks (Wibe *et al.*, 2002).

2.2.8 Bioaccumulation

Studies on rainbow trout by injecting Bisphenol-A showed accumulation of the chemical in the liver, while in muscle, levels remained below the limit of detection (Pedersen and Lindholst, 1999).

In another study, Lindholst *et al.* (2000) exposed rainbow trout to BPA for 12 days via the water phase and also under these conditions BPA was

observed mainly in the liver. They also detected BPA in the muscle, but at much lower concentrations.

2.2.9 Physiological changes

Responses of fish to cypermethrin toxicity included gill flailing, hyperactivity, loss of buoyancy and inability to remain upright in rainbow trout (Edwards *et al.,* 1986). Cypermethrin is also known to depress heart rate in goldfish (Pfuderer and Francais, 1975).

2.2.10 Somatic changes

Ram and Sathyanesan (1987) exposed a group of young and adult *Channa punctatus* to 0.20 mg/l of mercurial fungicide for a period of 6 months and showed a marked reduction in hepatosomatic index. A significantly increased liver somatic index was detected in Atlantic salmon exposed to diets contaminated with 15 and 30 17 β-estradiol, 300 mg nonylphenol or 1500 mg di-2-ethylhexyl phthalate per kg feed (Norrgren *et al.,* 1999). Increased gonadosomatic index was also observed in frogs exposed to methoxychlor (Pickford and Morris, 2003).

2.3 Recent trends

Endocrine disruption has seriously been studied for a past decade or so even though as early in the 1930s, Dodds and Lawson (1938) had explored the structural basis of oestrogenic activity, and showed that a wide range of chemicals synthesized in order to very approximately 'look like'

estradiol such as some biphenolic chemicals and alkylphenols possessed oestrogenic activity. albeit often very weakly.

It is now nearly 20 years since the report that hermaphrodite intersex fish were found in the settlement lagoons of two STWs in UK (Purdom *et al.*,1994). Initial concern was focused not on the possible adverse effects to the fishery but also on possible effects to humans supplied with treated water abstracted from the river receiving effluents from the STWs.

Research on the fisheries implications of effluent affecting sex determination began in the late 1980s after the report of caged fish downstream of sewage treatment plant being feminized (Harries *et al.*. 1996. 1997). The opposite was true in case of marine fisheries where commercially important pelagic fish such as blue fin tuna (Thunnus thynnus thynnus) and swordfish (Xiphias gladius) started showing high induction of vitellogenin males.

Very little is known presently about the behaviour of oestrogenic chemicals once they have been taken up by fish. A start has been made (Coldham et al., 1998) though much more needs to be learnt about the biotransformation metabolism, tissue distribution, bioaccumulation and persistence of estrogenic chemicals in fish before it will be possible to predict effects with any degree of confidence. Conduction of risk studies as well as quantification of these endocrine disrupters is necessary. At this time, however, there is no clear indication that large populations of fish are being seriously impacted as a result of endocrine disruption, although additional

work is needed to address this possibility. Research on the identification and effects of endocrine-active compounds has become an important area of human and environmental health research. While majority of the studies to date are directed towards reproductive impacts of endocrine disrupting chemicals on animals, there is a wide scope with reference to other impacts, which need to be taken into consideration. The challenge now is to assess how wide-ranging these adverse effects are, to determine their severity at the population level, and to gauge how serious an issue endocrine disruption is compared to other factors such as habitat loss which are also adversely impacting aquatic organisms.

2.4 Objectives

While majority of the studies to date were concentrated towards reproductive impacts of endocrine disrupting chemicals on animals, there is a wide scope with reference to other impacts, which need to be taken into consideration. The present investigation has been designed so as to take cognizance of all the important parameters in the frame of experiment.

The objectives of the study include impacts of select endocrine disrupters on Common carp with reference to the following aspects

1. Behavior and survival of fish
2. Growth performance and biochemical status
3. Bioaccumulation of the EDCs in the internal organs of fish
4. Histopathology of select organs of exposed fish
5. Vitellogenin content in exposed fish

Material and Methods

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental Setup

3.1.1 Aim of Experiment

The main objective of the present experiment was to study the effects of diethyl phthalate, methyl paraben and tert-butyl phenol on the fish *Cyprinus carpio*. Accordingly, fish were exposed separately to predetermined sublethal concentrations of these compounds.

3.1.2 Biology of the test animal

Common carp (*Cyprinus carpio communis* L) belongs to the family Cyprinidae. This exotic fish was introduced in India 1957 from Bangkok. It is now found in almost all water bodies throughout the country and constitutes a sizeable amount of fishery. It is a comparatively hardy species and can tolerate somewhat turbid waters, low oxygen and wide temperature variations. It has a silvery to slightly greenish coloration depending on the waterbody, the fins are yellowish, reddish or golden, and the anal fin becomes red in breeding season. The common carp attains maturity in 6 months and breeds throughout the year in most parts of India except for hilly regions. It can be bred artificially in confined areas by providing suitable substrate for laying eggs such as, plastic strips or aquatic plants such as, hydrilla. An omnivorous fish, feeding mainly on aquatic insects, crustaceans, molluscs, weeds, aquatic plants, algae, etc., it can be easily maintained on artificial feed. It reaches up

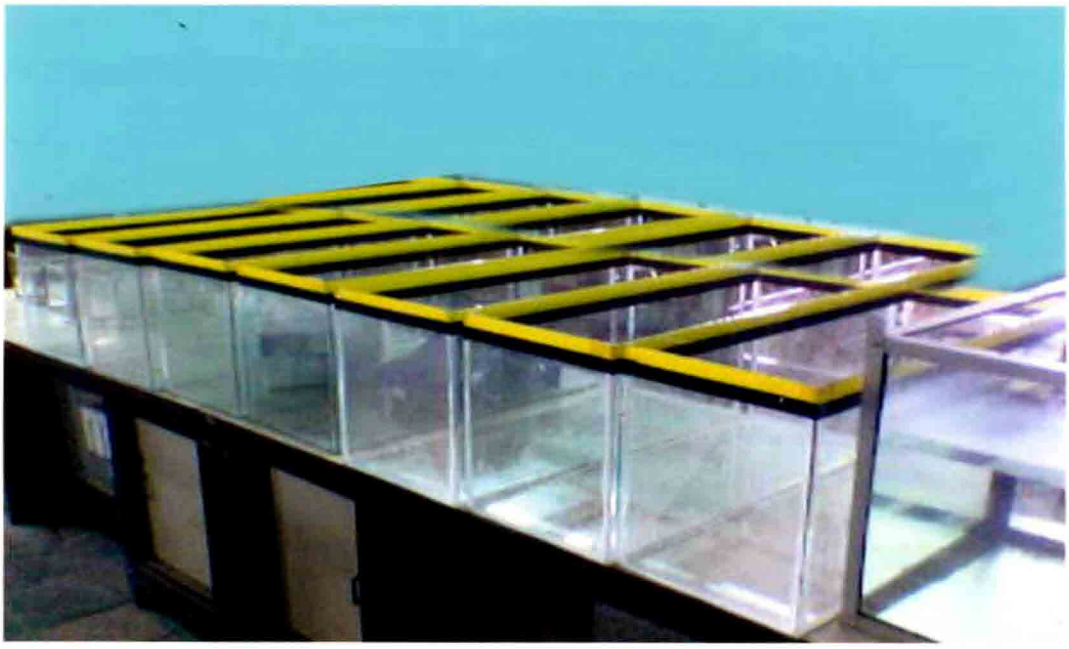

PLATE NO. 2 Experimental Setup

to 1 kg in a year; the maximum growth in confinement has been reported 10 kg in 30 months (Jhingran, 1983).

3.1.3 Site of Experiments

The experiments were conducted within a period of 2 years (August 2003 to August 2004) in the Environmental Biotechnology Division of National Environmental Engineering Research Institute (NEERI), Nagpur.

3.1.4 Toxicants selected

- **Diethyl Phthalate**: Chemically it is 1, 2-Benzenedicarboxylic acid, diethyl ester (CAS No: 84-66-2) having many industrial uses. It acts as a vehicle for fragrance and cosmetic ingredient and subsequently comes in contact with skin. Phthalate esters are key additive in many plastics and hence are important constituent of many common commercial products. It is used in pharmaceutical coatings, as a fixative in cosmetics, in the manufacture of celluloid, as a solvent for cellulose acetate in the manufacture of varnishes and ropes, in the denaturation of alcohol, in plastic for wrapping food, and as a vehicle for pesticide sprays (Okita and Okita, 1992).

- **Methyl Paraben**: Methyl paraben (CAS No. 99-76-3) is a methyl ester of p-hydroxybenzoic acid. It is a stable, non-volatile compound, used as an antimicrobial preservative in foods, drugs and cosmetics for over 50 years. Methyl paraben is readily and completely absorbed through the skin and from the gastrointestinal tract.

Parabens are widely used cosmetic preservatives, present in a large variety of products, including face, body and hand creams, lotions and moisturizers; eye makeup products; foundation and other makeup products; night creams and lotions; cleansing products; hair conditioners; bubble baths; shampoos; mud packs; underarm deodorants; skin lighteners; and sachets (Nikitakis, 1988). Methyl paraben and propyl paraben are the most commonly used preservatives in cosmetics (Berke *et al.*, 1982; Gruvberger *et al.*, 1998; Soni *et al.*, 2001).

- **4-Tert butylphenol**: 4 Tert butylphenol (CAS No: 98-54-4) comes under a group of chemicals, commonly known as Alkylphenol ethoxylates (APEs), which are widely used as surfactants in domestic and industrial products and are, commonly found in wastewater discharges and in sewage treatment plant (STP) effluents. Degradation of APEs in wastewater treatment plants or in the environment generates more persistent shorter-chain APEs and alkylphenols (APs) such as nonylphenol (NP), octylphenol (OP), butylphenols and AP, mono- to triethoxylates (NPE1, NPE2 and NPE3).

APEs can be used as detergents, wetting agents, dispersants, emulsifiers, solubilizers and foaming agents. APEs are important to a number of industrial applications, including pulp and paper, textiles,

coatings, agricultural pesticides, lube oils and fuels, metals and plastics.

3.1.5 Test organism

Four months old *Cyprinus carpio* (89.25 ± 0.32 g), used in the present investigation, were procured from the Indian Maritech Limited Fish Farm; District Mansar, Nagpur, (M.S). Prior to the experiment, the fish were held in circular plastic pools (500 l) till their use in the experimental studies. During this period they were given a practical diet at the rate of 5-10 % of body weight.

3.2 Experimental Design

The experiment was set up in 3 distinct experimental groups, each having 3 replicates in 200 l circular tanks; however, in case of diethyl phthalate all glass aquaria of 200 l capacity were used. All the tubs and aquaria were cleaned thoroughly with cleansing agent, followed by filling with dechlorinated tap water. Each tub was stocked with 20 *Cyprinus carpio*. The tubs were arranged in 3 rows following completely randomized design. At onset of experiment, 10 fish from common stock were sacrificed to study the initial parameters. Each set of experiment was conducted for 28 days with 4 distinct sampling days at an interval of 7 days, starting from the onset of experiment. During each sampling, 3 fish from each exposure concentration were sacrificed.

3.2.1 Rearing

Twenty mature *Cyprinus carpio* were stocked in each tank. The tanks were covered with iron mesh to prevent the fish from jumping out of the tanks. The fish were acclimatized for 2 weeks with aeration and feeding. No attempt was made to control the environmental conditions. A uniform volume of 200 l was maintained in each tank throughout the experimental period. Round the clock, aeration was provided using aerators of 1/8" diameter with plastic regulator to adjust the airflow uniformly in all tubs.

3.2.2 Cleaning and siphoning

The exposure water was removed completely by siphoning every alternate day. During the process, the fish were removed and released in their respective tanks after cleaning and refilling the tank with respective levels of the toxicants. Due care was taken not to stress the fish unnecessarily during water exchange. Filtration was provided in case of all glass aquariums. This exercise was carried out throughout the experimental period.

3.2.3 Feeding

Diet based on the fish protein requirement was prepared at Fish Nutrition Laboratory, CIFE, Mumbai. The fish were fed @ 5-10 % body weight once every day in the morning. The feeding rate was adjusted as per requirement. The diet contained crude protein min 32%, crude fat min 4%, crude fiber max 5%, and moisture 10%.

3.2.4 Reagents

All the chemicals and glasswares, used for the study, were of analytical grades. Diethyl phthalate, methyl paraben and 4-tert butylphenol were procured from Fluka Chemika (Prod no 348920/1896), SD Fine Chem Limited India (Product no. 39231 Batch no A04Z/0603/191/16) and Merck (S3829950 312) respectively. Vitellogenin Elisa kit was supplied by Cayman chemicals (Prod no V10002401-096, lot no 21987A-63441). All the solvents, used throughout the experiment, were of HPLC grade.

3.2.5 Sampling

As the experiment was conducted in triplicates, each group was treated separately. The fish were first anaesthetized using *Cifecalm* until they became motionless and settled at the bottom, and then rinsed. Liver, kidney, brain, gonads and gill were isolated and placed immediately on ice. Samples from all the organs were taken separately and analyzed separately in order to reduce sub-sampling error. For studying the somatic growth, all the internal organs were weighed separately with accuracy up to 0.01 mg.

3.3 Exposure

3.3.1 Dilution water

Besides being ecologically relevant, water is the most common laboratory route for exposing fish to EDCs. The delivery of a toxicant to water at different concentrations is well established. A water exposure concentration is more readily correlated with water concentrations found in the aquatic environment. The biggest challenge, however, with water exposure is

preparing aqueous solutions of high concentration relative to solubility limitations of the test substances in the water. To overcome this problem, organic solvents, such as methanol and acetone were used as carriers to assist in delivering a test substance in water. Dechlorinated tap water was filtered using an ordinary foam filter and used throughout the experimental period. The physico-chemicals characteristics of ambient water are mentioned in the **Table 4.1**.

3.3.2 LC_{50} Studies

The LC_{50} studies were conducted in accordance with guidelines provided in standard methods (APHA, 1998). Initial range finding test was undertaken to select the maximum exposure level. Stock solutions were prepared in suitable solvents. Fifteen liter capacity glass aquaria tank were filled with 10 l of dilution water and the compound was then added to the water with constant aeration. Each aquarium was provided with 10 fish and the experiment was conducted for 96 hrs. Fish were fed daily once, and excess feed was removed every 24 hour.

3.3.3 Exposure concentrations

96-hr LC_{50} tests were conducted so as to select sublethal concentrations. Ideally, the dose level used should not alter the growth of the test fish. Because the purpose of the present assay was to generate extensive dose-response data, the number of test concentrations was fixed to three-exposure concentrations.

A stock solution of each compound was prepared in suitable solvent (acetone in case of diethyl phthalate and 4-tert-butylphenol, and methanol for methyl paraben) depending on the solubility. The dosage was decided depending on the LC_{50} values of the compound. In case of higher LC_{50} values, a more realistic concentration was decided upon. The exposure concentrations were fractions of the original LC_{50} values.

3.3.4 Exposure Duration

A variety of factors were considered for the determination of the duration of the toxicant exposure. An underlying principle of toxicology is that an increase in the amount of exposure time typically results in identification of effects at lower concentrations (Ensenbach & Nagel 1997; McKim 1977; Nagel *et al.* 1998; Parrott *et al.* 2000). Thus, the longer the duration of the exposure, the more likely an effect would be observed. With respect to a reproductive screening assay, the use of a high toxicant concentration might allow for a shorter exposure period. Since the present study was directed towards an overall dose response, a much lower concentration was taken. The exposure duration was sufficiently long to allow for extensive absorption of the toxicant and potential biotransformation events (e.g., disposition) to occur to a significant extent. For practical purpose, an exposure duration lasting sufficiently long to achieve pseudo-equilibrium of the toxicant concentrations between exposure media and in fish tissues would be desirable. As for most chemicals, this equilibrium would be expected to occur within 30 days of exposure (Veith *et al.*, 1979), the period of 28 days was decided upon for present studies.

3.4 Physico-chemical Parameters

The following physico-chemical parameters of water were studied:

3.4.1 Temperature

The water temperature of each experimental tub was recorded twice a day, i.e. early in the morning and late evening with mercury thermometer having accuracy of 0.1^0 C.

3.4.2 pH

The pH of water in all experimental tanks was taken by digital pH meter (Control Dynamic pH meter Model no –175 E/C)

3.4.3 Dissolved oxygen

The dissolved oxygen (DO) level in all the experimental tanks was maintained as per the requirement by providing efficient aeration system during entire experiment. The dissolved oxygen was measured by Winkler's method (APHA 1998) twice daily, i.e. early in the morning and later in the evening and calculated as follows:

$$\text{Dissolved oxygen} = \frac{8 \times 1000 \times N \times v}{V}$$

Where,

v = volume of titrant used.
V = volume of sample.
N = Normality of titrant ($Na_2S_2O_3$).

3.4.4 Free carbon dioxide

Free carbon dioxide was measured by titrimetric method (APHA, 1998) and calculated by following formula:

$$CO_2\,(mg/L) = \frac{A \times N \times 44 \times 1000}{\text{Volume of sample (ml)}}$$

Where,

A = Volume of titrant (NaOH)
N = Normality of Titrant (N/44)

3.4.5 Hardness

Total hardness was estimated by using EDTA titrimetric method (APHA, 1998) using the following formula:

$$\text{Total hardness} = \frac{\text{Volume of EDTA used in the titration} \times 1000}{\text{Volume of sample used}}$$

3.4.6 Total alkalinity

Total alkalinity was estimated by titrimetric method (APHA, 1998) as the concentration of calcium carbonate and expressed in ppm or mg/L.

3.4.7 Nutrients

3.4.7.1 Nitrate

Nitrate content was estimated using Ultraviolet Spectrophotometric Screening Method (APHA, 1998) using the following formula:

Nitrate concentration (mg/L) = (absorbance at 220 nm) – 2 (absorbance at 275nm)/slope (derived from standard NO_3 graph)

3.4.7.2 Phosphate

Phosphate content was estimated by vanadomolybdophosphoric acid colorimetric method (APHA, 1998) using the following formula:

$$\text{Phosphate (mg/L)} = \frac{\text{mg Phosphate (in 50 ml final volume)}}{\text{ml sample}} \times 1000$$

3.5 Somatic Indices

Indices were calculated using the following formulae:

3.5.1 Craniosomatic Index (CSI)

$$\text{Craniosomatic Index (CSI)} = \frac{\text{Weight of brain}}{\text{Weight of fish}} \times 100$$

3.5.2 Renosomatic Index (RSI)

$$\text{Renosomatic Index (RSI)} = \frac{\text{Weight of kidney}}{\text{Weight of fish}} \times 100$$

3.5.3 Hepatosomatic Index (HSI)

$$\text{Hepatosomatic Index (HSI)} = \frac{\text{Weight of liver}}{\text{Weight of fish}} \times 100$$

3.5.4 Gonadosomatic Index (GSI)

$$\text{Gonadosomatic Index (GSI)} = \frac{\text{Weight of gonads}}{\text{Weight of fish}} \times 100$$

3.6 Enzymatic Analysis

Enzyme activity was studied in tissues of fish. After sacrificing the fish, 0.1 g of tissue was taken out from the dorsal region and was immediately placed on ice. It was then homogenized in 2 ml of 0.9% chilled saline using a teflon pestle tissue grinder and centrifuged for 5 mins at 4 ^{0}C. The supernatant was used for estimating serum glutamic oxalacetic transaminase (SGOT) / Aspartate amino transferase (AST) (L-aspartate: 2 oxaloglutarate aminotransferase, E.C.2.6.1.1), Serum glutamic pyruvic transaminase (SGPT) / Alanine amino transferase (ALT) (L-alanine: 2 oxaloglutarate aminotransferase, E.C.2.6.1.2), Acid phosphatase (AcP) (Orthophosphoric monoester phosphohydrolase, E.C.3.1.3.1) and alkaline phosphatase (AlP) (Orthophosphoric monoester phosphohydrolase, E.C.3.1.3.2).

3.6.1 Acid phosphatase (AcP)

(Orthophosphoric monoester phosphohydrolase, E.C.3.1.3.2)

The AcP activity was determined by the method of Garen and Levithal (1960). The assay mixture comprised of 0.2 ml acetate buffer (0.2M) pH 5.0, 0.1 ml of 0.1M $MgCl_2$, 0.1 ml tissue homogenate, 0.5 ml of distilled water and 0.1 ml of freshly prepared 0.1M paranitrophenol phosphate. The reaction mixture was incubated in water bath 37^0 C for 15 minutes and the

reaction was stopped by 1 ml of 0.1 N NaOH. The end point was recorded using UV-VIS spectrometry (Spectronic Genesys 2) at 410 nm.

$$\text{Acid phosphatase} = \frac{T}{S} \times 10$$

Where, T is optical density of test sample at 510 nm
S is the control reading

3.6.2 Alkaline phosphatase (AlP)

(Orthophosphoric monoester phosphohydrolase, E.C.3.1.3.1)

The AlP activity was determined by the method of Garen and Levithal (1960). The assay mixture comprised of 0.2 ml bicarbonate buffer (0.2M), 0.1 ml of 0.1M $MgCl_2$, 0.1 ml tissue homogenate, 0.5 ml of distilled water and 0.1 ml of freshly prepared 0.1M paranitrophenol phosphate. The reaction mixture was incubated in water bath 37^0 C for 15 minutes and the reaction was stopped by 1 ml of 0.1 N NaOH. The end point was recorded using UV-VIS spectrometry (Spectronic Genesys 2) at 410 nm.

$$\text{Alkaline phosphatase} = \frac{T - C}{S - B} \times 10$$

Where, T is optical density of test sample at 510 nm
C is the control reading
S and B are standard and blank readings respectively.

3.6.3 Serum glutamic oxalacetic transaminase/ Aspartate aminotransferase (SGOT/ AST)

(L-aspartate: 2 oxaloglutarate aminotransferase, E.C.2.6.1.1)

The AST activity was assayed in muscle tissue homogenates described by Wooton (1964). The substrate comprised 0.2M D,L-aspartic acid and 2mM α-ketoglutarate in 0.05M phosphate buffer (pH 7.4). In the

treatment and control tubes, 0.5 ml of substrate was added. The reaction was started by adding 0.1 ml of tissue homogenate in the treatment tube. The assay mixture was incubated at 37^0C for 60 minutes. The reaction was terminated by adding 0.5ml of 1mM 2, 4 dinitrophenol hydrazine (DNPH). In the control tubes the enzyme source was added after DNPH solution. The tubes were held at room temperature for 20 minutes with occasional shaking. Then 5 ml of 0.4M NaOH solution was added and the contents were thoroughly mixed. After 10 minutes, the end point was recorded using UV-VIS spectrometry (Spectronic Genesys 2) at 540 nm against blank.

$$X = \frac{Y - 0.1784}{0.0928}$$

Where, X is the SGOT value to be calculated
Y is the optical density at 490 nm.

3.6.4 Serum glutamic pyruvic transaminase/ Alanine aminotransferase (SGPT/ALT)

(L-alanine: 2 oxaloglutarate aminotransferase, E.C.2.6.1.2)

The ALT activity was assayed in muscle tissue homogenates described by Wooton (1964). The substrate comprised of 0.2M D,L-alanine acid and 2mM α-ketoglutarate in 0.05M phosphate buffer (pH 7.4). In the treatment and control tubes, 0.5 ml of substrate was added. The reaction was started by adding 0.1 ml of tissue homogenate in the treatment tube. The assay mixture was incubated at 37^0C for 60 minutes. The reaction was terminated by adding 0.5ml of 1mM 2,4 dinitrophenol hydrazine (DNPH). In the control tubes the enzyme source was added after DNPH solution. The tubes were held at room temperature for 20 minutes with occasional shaking. Then 5 ml of 0.4 M

NaOH solution was added and the contents were thoroughly mixed . After 10 minutes, the end point was recorded using UV-VIS spectrometry (Spectronic Genesys 2) at 540 nm against blank.

$$X = \frac{Y - 0.155}{0.0953}$$

Where X is the SGOT value to be calculated
Y is the optical density at 490 nm

3.7 Behavioral Monitoring

Behavioral monitoring was conducted daily throughout the 28-day exposures. The general behavior, swimming, crowding or seclusion, skin coloration, and external appearance of fish were monitored, and their startle response behavior was examined by tapping the aquarium. Fish were then fed and their feeding habits were observed until either the food had been consumed or 5 min had passed. If food had not been consumed within 5 min, the aquarium was checked periodically over the next 2 h to see if the food had been consumed (non-consumed food was then removed). Certain behavioral parameters were selected from those described by McKim *et al.* (1987).

3.8 Bioaccumulation Studies

Quantitative expression of organ-wise distribution of bio-accumulated compounds were analyzed using latest techniques such as supercritical fluid extraction, gas chromatography mass-spectrometry, reverse phase high pressure liquid chromatography and ultra violet spectroscopy.

3.8.1 Supercritical fluid extraction

Supercritical fluid extraction (SFE) is a very efficient technique to prepare samples before analysis, which saves not only time but also solvent. Extraction can be done from any biological sample without much pre-processing. The use of supercritical carbon dioxide yields a rapid and quantitative extraction. In the present study, the tissue samples were weighed and frozen till before extraction. Prior to extraction, the sample was thawed and excess of moisture was absorbed using a filter paper. The organ was then thoroughly compounded in sand matrix to increase the surface area of exposure. The extraction conditions were decided using the existing methods. The samples were collected in suitable solvents, stoppered tightly and stored at 0°C till further analysis.

3.8.1.1 Instrumental

SFE's were carried out by using an SFX 220 (Model 260 D ISCO serial: 202D20283), which consists of an SFX 220 extractor; an SFX 200 controller and a D-syringe pump with a capillary restrictor and a temperature controller. The samples were placed in stainless steel cartridges (internal volume, 2.5 ml).

3.8.1.2 Conditions

Diethyl phthalate: The condition, followed for extraction of diethyl phthalate, was at a temperature of 85 °C, dynamic extraction time 15 min, and pressure 48000 Kpa and flow rate of 2 ml/min (Marin *et al.* 1998).

PLATE NO. 3 Supercritical Fluid Extractor

PLATE NO. 4 High Pressure Liquid Chromatography

Methyl paraben: The optimum condition established for SFE was 40 ^{0}C, 12 100 kPa, CO_2 with 0.05% acetonitrile. Extracts were collected at the end of the restrictor with 7 to 8 ml of acetonitrile (Wang and Chang 1998).

3.8.2 Gas chromatography

3.8.2.1 Instrumental

A gas chromatography (Varian Cp – 3800GC) with a split / split less injection system, capillary column (30m x 0.25 mm, BP 5) and mass spectrometry (Saturn 2200 GC/MS/MS) was utilized for analysis of diethyl phthalate.

3.8.2.2 Conditions

The conditions were followed as referred in USEPA 525 method.

3.8.3 High-pressure liquid chromatography

3.8.3.1 Instrumental

A reverse phase high-pressure liquid chromatography was performed to determine methyl paraben. The chromatograph used in this study consisted of a LC-10AS Pump (waters) a SIL-9A injector, a SPD -10 A detector and a RP 18 column (300mm x 3.9mm).

3.8.3.2 Conditions

A degassed and filtered mixture of 0.05M potassium hydrogen phosphate at pH 2.5 and acetonitrile (65:35 v/v) was used as eluent. Flow rate

PLATE NO. 5 Gas Chromatography - Mass Spectrometry

was maintained at 1.0 ml per min. Detection was performed at 254 nm and the runtime for each analysis was 10 minutes (Akhtar *et al.*, 1996).

3.9 Histology

Internal organs, viz. liver, kidney, brain, gills and gonads were collected from all the exposure concentrations at the end of experimental period. Samples were fixed in neutral buffered formalin, followed by stepwise four changes of 70% alcohol, and stored in 90% alcohol until further processing. The preserved tissue was then dehydrated in a graded alcohol series and embedded in paraffin. Sections of 4 mm were stained with hematoxylin and eosin, and observed under an Olympus microscope (Olympus BX40 microscope) at 100X magnification. The photography was done using Nikon (SC 35 type 12) camera with shutter speed 30 sec.

3.10 Elisa

Prior to preparation of tissue homogenate, the sex of the fish was determined by examination of the gonads. The homogenate was prepared by crushing tissue in a mortar, filled with liquid nitrogen. An amount of twice the tissue weight ice-cold saline was added. The homogenate was centrifuged for 60 min at 50 000 X *g* at 4 ^{0}C.The supernatant was filtered and stored in aliquots at – 20 °C. All the wells were coated with capture antibody and were kept overnight at 4 °C. The coated well were washed with washing buffer and blocked by blocking/dilution buffer.

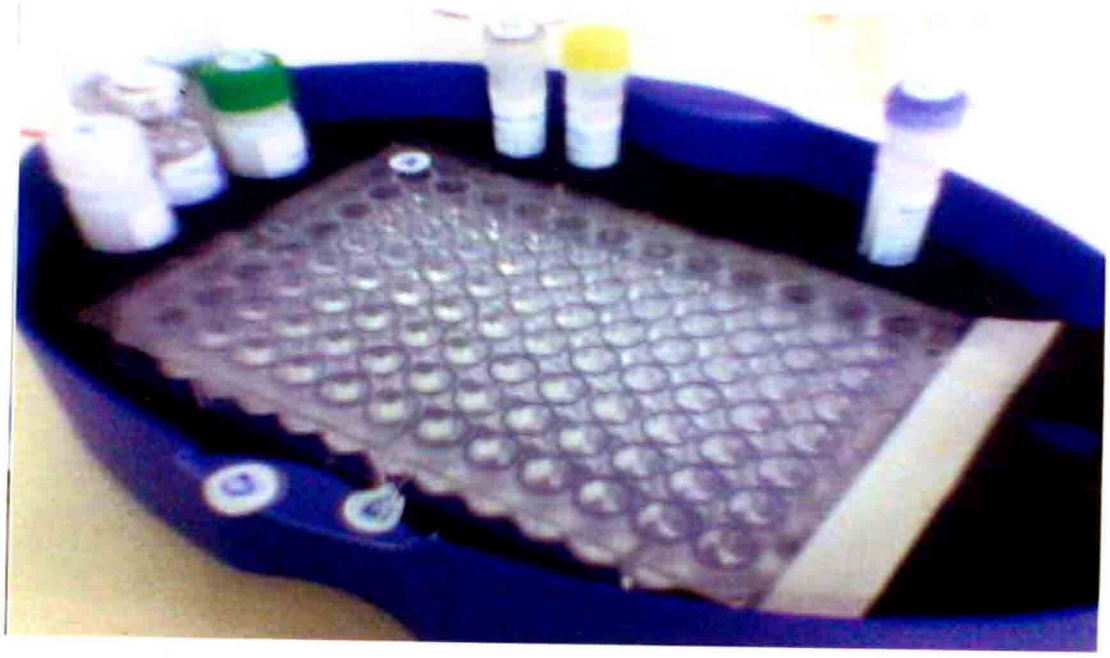

PLATE NO. 6 Vitellogenin Elisa Kit

PLATE NO. 7 Elisa Plate Reader

Standard curves were prepared by dilution of 100 ul of the stock solution in an appropriate volume of blocking buffer. Three different dilutions of each sample in cold blocking buffer were made. The tissue homogenate was diluted to 400 times. To the blocking buffer coated plate 100 ul of carp – Vtg standard dilution was added in duplicate. The colour development was stopped after 5-15 min in the dark by adding 50 μl of 0.5 M H_2SO_4 to each well. The plates were read at 405 nm on a Labsystems multiscan Elisa plate reader, and data were analyzed on the associated software Softmax.

3.11 Statistical analysis

- The difference in somatic indices, enzymatic activity, bioaccumulation and vitellogenin induction between the control group and treated groups were tested by one way analysis of variance ($P < 0.05$ and $P < 0.01$) followed by Student 't' test.
- Data was processed using Microsoft Excel in Windows 98.

Results

CHAPTER 4

RESULTS

4.1 Physico-chemical parameters of water

Data pertaining to physico-chemical parameters of water are presented in **Table no. 4.1** temperature (°C), dissolved oxygen (mg/L), pH, free carbon dioxide (mg/L), carbonate hardness (mg/L), nitrate –N (mg/L), total alkalinity (mg/L) and phosphate were recorded every alternate day.

4.1.1 Temperature

The water temperature of different experimental tubs ranged from 25.4 to 28.6 °C during the entire period of experiment.

4.1.2 Dissolved Oxygen (DO)

The dissolved oxygen levels were recorded to vary between 6.99 and 7.51 mg/L during the period of entire experiment.

4.1.3 Free Carbon Dioxide (CO_2)

Free carbon dioxide was observed to negligible in all the experimental in the experimental tubs.

4.1.4 pH

The pH of the rearing water was found to vary from 7.59 to 8.48 in all the experimental tanks.

Table 4.1 Physico-Chemical parameters of ambient water

Sr. no	Parameter	Range
1	Temperature	24-32^{0}C
2	pH	6.99 - 7.51
3	Dissolved Oxygen	7.59 - 8.48 mg/L
4	Free Carbon Dioxide	Negligible
5	Carbonate hardness	289.29 – 291.07 mg/L
6	Nitrate	0.08 to 0.16 mg/L
7	Phosphate	0.19 to 0.47 µg/L

Table 4.2 Experimental LC_{50} and Lethal Doses for the selected chemicals

Sr. no	Compound	LC_{50} (ppm)	LC_{100} (ppm)
1	Diethyl phthalate	48	75
2	Methyl paraben	42	50
3	4-tert butylphenol	6.9	7.2

4.1.5 Carbonate hardness

Carbonate hardness of the experimental water was observed within the range of 289.29 – 291.07 mg/L during all the exposure period.

4.1.6 Nitrate-N

Nitrate concentration was recorded within the range of 0.08 to 0.16 mg/L.

4.1.7 Phosphate – P

The phosphate –P was found to range between 0.19 to 0.47 µg/L in all the experimental tubs.

4.2 Acute toxicity test and LC_{50}.

Data pertaining to acute toxicity test of technical grade Diethyl phthalate, Methyl paraben and Tertiary Butylphenol to *Cyprinus carpio* in terms of treatment (concentration of compound) and cumulative response (mortality) at 96 hrs is given in **Table no. 4.2**

4.3 Diethyl phthalate

4.3.1 Acid Phosphatase (AcP)

Data pertaining to the acid phosphatase activity in *Cyprinus carpio* exposed to diethyl phthalate at 3 concentrations is given in **Tables 4.3.1** through **4.3.4** and graphically shown in **Fig. 4.1**. Starting 1st week onwards there was a significant reduction (P>0.5) in the AcP activity of all the exposed fish as compared to control. The maximum reduction observed at 1mg/L

Table 4.3.1 : Effect of DEP on Various Indices and Enzymatic Activities of *Cyprinus carpio* (Grouped by exposure concentrations) over 30 days

S.N.	Parameter	Exposure period (Week)	Mean values at exposure concentrations				Significance of 'F' Test	SE (m) Between exposure concentrations	SE (m) Within exposure concentrations	CD (5%)	CD (1%)
			Control	1 mg/L	5 mg/L	20 mg/L					
1	GSI	1	6.8494	6.7948	5.8210	5.7794	N.S.	1.0230	0.7711	--	--
		2	7.1259	6.4042	5.3441	4.8608	**	1.7724	0.5613	4.0231	6.6569
		3	7.7149	5.6269	3.0862	2.6213	**	4.1059	0.6671	9.3198	15.4213
		4	8.6078	6.4171	2.8338	1.8724	**	5.4296	0.2024	12.3244	20.3929
2	HSI	1	1.0604	1.2312	1.3959	1.5328	**	0.3541	0.1133	0.8038	1.3301
		2	0.9986	1.6195	1.5787	1.2806	*	0.5018	0.2433	1.1389	--
		3	0.8916	1.2052	1.8181	1.4990	**	0.6873	0.1863	1.5600	2.5813
		4	1.1781	1.1872	1.2027	1.2432	NS	0.0498	0.1008	--	--
3	CSI	1	1.3648	1.2889	1.4671	0.8937	N.S.	0.4344	0.2202	--	--
		2	1.0987	1.0189	1.1595	0.9768	N.S.	0.1413	0.0888	--	--
		3	0.9284	1.0952	1.1209	0.9963	N.S.	0.1544	0.1068	--	--
		4	1.5336	1.3487	1.6853	0.9963	**	0.5144	0.1779	1.1675	1.9319
4	RSI	1	0.6020	0.2958	0.5107	0.3174	*	0.2584	0.1208	0.5865	--
		2	0.5962	0.4260	0.6587	0.5952	N.S.	0.1730	0.1427	--	--
		3	0.5662	0.2760	0.4076	0.4925	*	0.1730	0.1427	1.7080	--
		4	0.4807	0.5424	0.4080	0.5620	N.S.	0.1203	0.1320	--	--
5	AcP	1	18.1060	14.2292	0.9256	1.5871	**	15.1684	0.5119	34.4300	56.9706
		2	17.6777	14.2698	3.9372	3.0392	**	12.7318	0.1871	28.8994	47.8192
		3	17.6993	13.9453	6.4397	4.4743	**	10.7897	0.8542	24.4910	40.5247
		4	16.5632	14.9501	5.5917	0.8560	**	13.0225	0.2712	29.5592	48.9110
6	AlP	1	0.1971	2.3174	3.2365	3.6216	**	2.6531	0.1457	6.0221	9.9646
		2	0.3902	0.8787	3.5586	2.6962	**	2.5896	0.2377	5.8781	9.7263
		3	0.9680	1.2363	3.6316	3.7738	**	2.6094	0.2582	5.9230	9.8006
		4	1.4587	0.9179	7.3267	8.1668	**	6.5964	0.1636	14.9728	24.7752
7	SGOT	1	15.0582	15.5463	15.4796	15.8836	**	0.5871	0.1155	1.3325	2.2049
		2	16.3753	17.0148	16.3748	17.8157	*	1.1844	0.4664	2.6884	--
		3	18.5140	11.7696	15.2739	15.3724	**	4.7729	0.1968	10.8339	17.9266
		4	16.8201	11.1121	13.1965	12.0140	**	4.3407	0.1559	9.8529	16.3033
8	SGPT	1	7.2781	7.8100	9.8360	8.7760	**	1.9513	0.1579	4.4293	7.3290
		2	8.9688	7.3457	10.7628	11.7962	**	3.4058	0.1871	7.7308	12.7919
		3	10.8757	5.7729	7.6742	7.2324	**	3.7250	0.1414	8.4552	13.9905
		4	8.8435	5.3030	5.3581	5.7037	**	2.9506	0.1558	6.6975	11.0822

*, ** indicate significance at 5 and 1% respectively (n – 6). NS = Not Significant. SE (m) = Mean Standard Error. CD = Critical Difference

AcP and AlP expressed as µmoles.mg of protein^{-1}. SGOT and SGPT expressed as nmoles oxaloacetate.mg of protein^{-1}.

Table 4.3.2 : Consistent Trends in the Variation of the effect of DEP on Important Indices and Enzymatic Activities of *Cyprinus carpio* (Grouped by exposure concentrations) during an Exposure Period of 30 days

S.N.	Parameters	Exposure period (Week)	Specific comparison among different exposure concentrations showing significant to highly significant differences
1	GSI	2	1 & 4
		3	1 & 3, 1 & 4, 2 & 3, 2 & 4
		4	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
2	HSI	1	1 & 4
		3	1 & 3, 1 & 4, 2 & 3
3	CSI	4	3 & 4
4	AcP	1	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4
		2	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		3	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4
		4	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
5	AlP	1	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		2	1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		3	1 & 3, 1 & 4, 2 & 3, 2 & 4
		4	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
6	SGOT	1	1 & 2, 1 & 3, 1 & 4, 3 & 4
		3	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4,
		4	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
7	SGPT	1	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		2	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		3	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4
		4	1 & 2, 1 & 3, 1 & 4

Exposure concentrations: 1 - Control; 2 - 1 mg/L; 3 - 5 mg/L; 4 - 20 mg/L

Table 4.3.3 : Effect of DEP on Various Indices and Enzymatic Activities of *Cyprinus carpio* during 30 days Exposure Period (Grouped by Week)

S.N.	Parameter	Exposure concentrations (mg/L)	Mean Values at Exposure Period (Week)				Significance of 'F' Test	SE (m) Between Exposure period	SE (m) Within Exposure period	CD (5%)	CD (1%)
			1	2	3	4					
1	GSI	Control	6.8494	7.1259	7.7149	8.6078	NS	1.3470	0.0000	--	--
		1	6.7948	6.4042	5.6269	6.4171	NS	0.8497	0.8198	--	--
		5	5.8210	5.3441	3.0862	2.8338	**	2.6502	0.5315	6.0155	9.9537
		20	5.7794	4.8608	2.6213	1.8724	**	3.1855	0.6641	7.2306	11.9642
2	HSI	Control	1.0604	0.9986	0.8916	1.1781	**	0.2073	0.0000	0.4706	0.7786
		1	1.2312	1.6195	1.2052	1.1872	NS	0.3578	0.2282	--	--
		5	1.3959	1.5787	1.8181	1.2027	*	0.4545	0.1820	1.0317	--
		20	1.5328	1.2806	1.4990	1.2432	NS	0.2565	0.1780	--	--
3	CSI	Control	1.3648	1.0987	0.9284	1.5336	**	0.4675	0.0000	1.0611	1.7558
		1	1.2889	1.0189	1.0952	1.3487	**	0.2706	0.0490	0.6142	1.0163
		5	1.4671	1.1595	1.1209	1.6853	NS	0.4633	0.2565	--	--
		20	0.8937	0.9768	0.9963	0.9963	NS	0.0847	0.1768	--	--
4	RSI	Control	0.6020	0.5962	0.5662	0.4807	**	0.0969	0.0000	0.2200	0.3640
		1	0.2958	0.4260	0.2760	0.5424	*	0.2152	0.0855	0.4884	--
		5	0.5107	0.6587	0.4076	0.4080	NS	0.2055	0.1700	--	--
		20	0.3174	0.5952	0.4925	0.5620	NS	0.2146	0.1652	--	--
5	AcP	Control	18.1060	17.6777	17.6993	16.5632	**	1.1470	0.0000	2.6035	4.3080
		1	14.2292	14.2698	13.9453	14.9501	NS	0.7382	1.0132	--	--
		5	0.9256	3.9372	6.4397	5.5917	**	4.2121	0.2273	9.5609	15.8201
		20	1.5871	3.0392	4.4743	0.8560	**	2.7792	0.1480	6.3085	10.4385
6	AlP	Control	0.1971	0.3902	0.9680	1.4587	**	0.9924	0.0000	2.2526	3.7274
		1	2.3174	0.8787	1.2363	0.9179	**	1.1649	0.2410	2.6441	4.3752
		5	3.2365	3.5586	3.6316	7.3267	**	3.3484	0.2269	7.6004	12.5762
		20	3.6216	2.6962	3.7738	8.1668	**	4.2404	0.2482	9.6251	15.9264
7	SGOT	Control	15.0582	16.3753	18.5140	16.8201	NS	2.4710	0.0000	--	--
		1	15.5463	17.0148	11.7696	11.1121	**	4.9716	0.4396	11.2848	18.6728
		5	15.4796	16.3748	15.2739	13.1965	**	2.3284	0.2610	5.2852	8.7453
		20	15.8836	17.8157	15.3724	12.0140	**	4.1797	0.1802	9.4872	15.6983
8	SGPT	Control	7.2781	8.9688	10.8757	8.8435	**	2.5512	0.0000	5.7908	9.5818
		1	7.8100	7.3457	5.7729	5.3030	**	2.0927	0.1688	4.7500	7.8598
		5	9.8360	10.7628	7.6742	5.3581	**	4.1743	0.1808	9.4751	15.6782
		20	8.7760	11.7962	7.2324	5.7037	**	4.5063	0.2074	10.2286	16.9251

*, ** indicate significance at 5 and 1% respectively (n = 6). NS = Not Significant. SE (m) = Mean Standard Error. CD = Critical Difference

AcP and AlP expressed as µmoles.mg of protein^{-1}. SGOT and SGPT expressed as nmoles oxaloacetate.mg of protein^{-1}.

Table 4.3.4: Consistent Trends in the Variation of the effect of DEP on Important Indices and Enzymatic Activities of *Cyprinus carpio* (Grouped by exposure period) at different Exposure Concentrations during a 30 days Exposure

S.N.	Parameter	Exposure concentrations (mg/L)	Specific Comparison among different exposure period showing significant to highly significant differences
1	GSI	5	1 & 3, 1 & 4, 2 & 3, 2 & 4
		20	1 & 3, 1 & 4, 2 & 3, 2 & 4
2	HSI	Control	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
3	CSI	Control	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		1	1 & 2, 1 & 3, 2 & 4, 3 & 4
4	RSI	Control	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
5	AcP	Control	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		5	1 & 2, 1 & 4, 1 & 3, 2 & 3, 2 & 4, 3 & 4
		20	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
6	AlP	Control	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		1	1 & 2, 1 & 3, 1 & 4
		5	1 & 4, 2 & 4, 3 & 4
		20	1 & 2, 1 & 4, 2 & 3, 2 & 4, 3 & 4
7	SGOT	1	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4
		5	1 & 2, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		20	1 & 2, 1 & 4, 2 & 3, 2 & 4, 3 & 4
8	SGPT	Control	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		1	1 & 3, 1 & 4, 2 & 3, 2 & 4
		5	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		20	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4

Exposure Period : 1 - 1st Week 2 - 2nd Week 3 - 3rd Week 4 - 4th Week

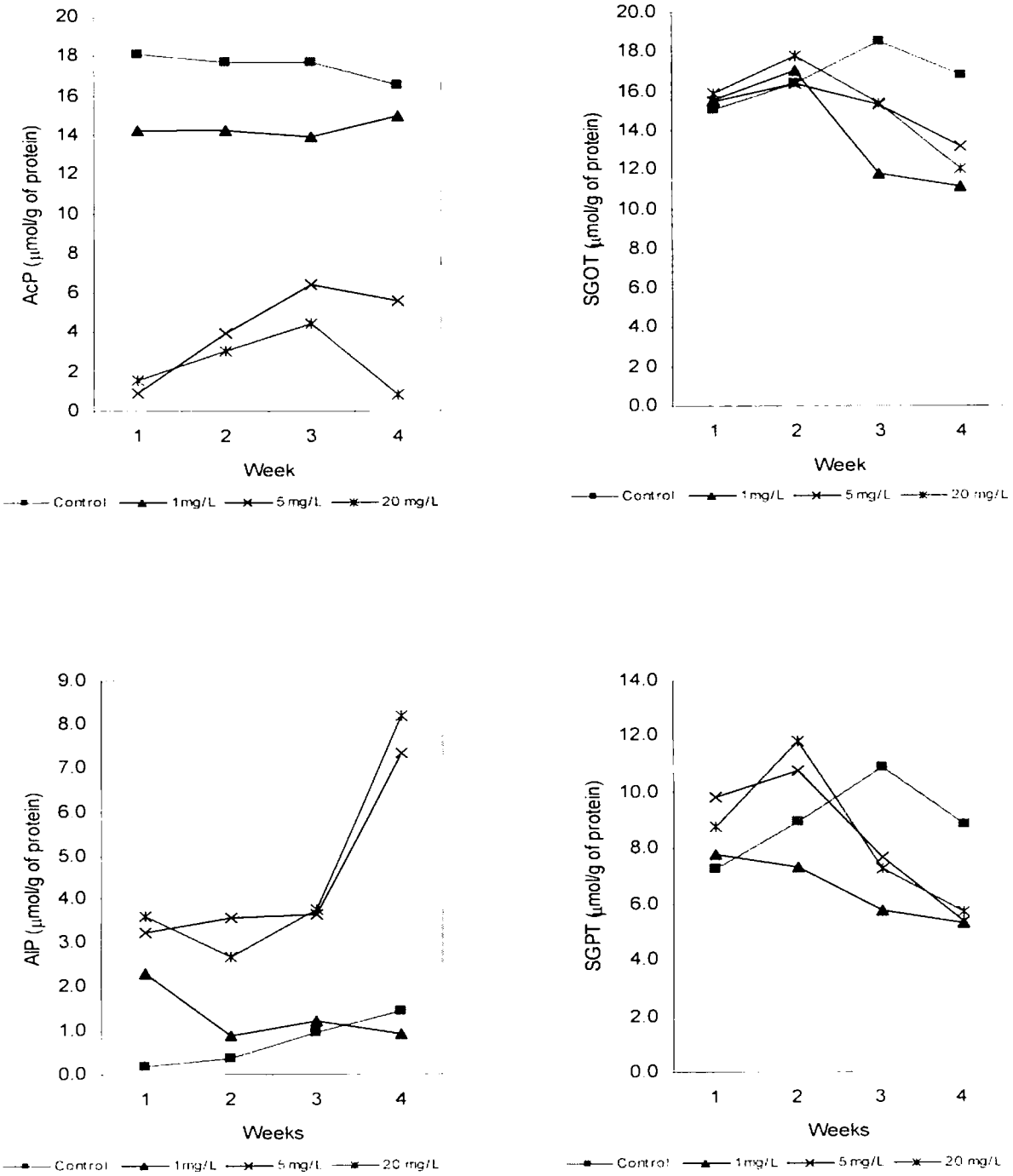

Fig. 4.1 : Mean enzymatic responses of Cyprinus carpio to Diethyl phthalate

exposure concentration was 13.945 ± 0.961 µmoles/mg of protein during the 3rd week while the highest activity 14.950 ± 0.258 µmoles/mg of protein was seen in the 4th week. At 5mg/L exposure the maximum reduction (0.925 ± 0.011 µmoles/mg of protein) of acid phosphatase activity was observed in the 1st week while the least reduction (6.439 ±0.184 µmoles/mg of protein) was observed in the 3rd week. At 20 mg/L exposure concentration the AcP activity was observed increase from 1.587 ± 0.057 µmoles/mg of protein in the 1st week to 4.473 ± 0.117 µmoles/mg of protein in the 3rd week.

4.3.2 Alkaline Phosphatase (AlP)

Impact of DEP on the alkaline phosphatase activity in *Cyprinus carpio* exposed to 3 concentrations individually is given in **Tables 4.3.1** through **4.3.4** and **Fig. 4.1**. As compared to control there was a marked increase in the alkaline phosphatase activity of all the exposed fish. The maximum activity can be seen in response to 20 mg/L exposure concentration during the fourth week of exposure, while the minimum can be seen at 1mg/L exposure during the 2nd week. At 5 mg/L exposure concentration the lowest activity (3.236 ± 0.01 µmoles/mg of protein) was seen during the 1st week while the highest activity (7.326 ± 0.121 µmoles/mg of protein) was observed for the 4th week of exposure. It can be observed that there was a time and dose dependant increase in the alkaline phosphatase activity in the fish exposed to DEP.

4.3.3 Serum Glutamic Oxalacetic Transaminase (SGOT)/ Aspartate Aminotransferase (AST)

Data pertaining to the Serum Glutamic Oxalacetic Transaminase (SGOT) in *Cyprinus carpio* exposed to DEP at 1, 5 and 20 mg/L is given in

Tables 4.3.1 through **4.3.4** and **Fig. 4.1**. The SGOT was more or less inhibited in response to DEP exposure. At the lowest exposure concentration i.e. 1mg/L the activity was higher as compared to control during the 1st and 2nd week (15.546 ± 0.088 and 17.014 ± 0.466 nmoles oxaloacetate/mg of protein respectively) which was significantly lowered ($P<0.05$) to 11.112 ± 0.105 nmoles oxaloacetate/mg of protein during the 4th week. At the intermediate concentration i.e. 5 mg/L similarly the highest activity (16.374 ± 0.24 nmoles oxaloacetate/mg of protein) was observed during the 2nd week which lowered to 13.196 ± 0.112 nmoles oxaloacetate/mg of protein during the 4th week. At the highest exposure concentration i.e. 20 mg/L the activity (15.886 ± 0.1 nmoles oxaloacetate/mg of protein) was significantly higher than the control (15.0580 ± 0.123 nmoles oxaloacetate/mg of protein) during the 1st week of exposure which reduced to 12.014 ± 0.092 nmoles oxaloacetate/mg of protein in the 4th week.

4.3.4 Serum Glutamic Pyruvic Transaminase (SGPT)/ Alanine Aminotransferase (ALT)

The Serum Glutamic Pyruvic Transaminase (SGPT) activity in *Cyprinus carpio* exposed to 3 concentrations (1, 5 and 20 mg/L) of Diethyl phthalate is given in **Tables 4.3.1** through **4.3.4** and **Fig. 4.1**. In all the exposure concentration during the 1st week the SGOT activity was higher than the control , however starting 2nd week the SGOT activity in the 1mg/L exposure lowered significantly , in case of the higher two exposure concentrations (5 and 20 mg/L) the activity remained higher than control . During the 3rd and 4th week it was reduced in all the exposures. At 1 mg/L the highest activity observed was 7.81 ± 0.112 nmoles oxaloacetate/mg of protein during the 1st

week while the lowest was 5.303 ± 0.056 nmoles oxaloacetate/mg of protein during the 4th week. In the 5 and 20 mg/L exposures the highest activities were 10.762 ± 0.120 and 11.792 ± 0.133 nmoles oxaloacetate/mg of protein respectively during the 2nd week while the lowest activities were 5.358 ± 0.12 and 5.703 ± 0.12 nmoles oxaloacetate/mg of protein during the 4th week.

4.3.5 Gonadosomatic Index (GSI)

Data pertaining to the effect of DEP on the GSI at 1, 5 and 20 mg/L exposure during a 4 week period is given in **table 4.3.1** through **4.3.4** and **Fig. 4.2**. The GSI decreased significantly ($P<0.05$) with increase in the exposure period and concentration. During the first week of exposure, there was no significant changes between the GSI of control and all the exposure concentrations. At 1 mg/L exposure concentration the highest GSI was observed to be 6.794 ± 0.133 during the 1st week while the lowest i.e. 5.626 ± 0.763 was observed during the 3rd week. In the 5 mg/L exposure concentration the GSI observed was 5.821 ± 0.556443 during the 1st week which was significantly lowered to 2.838 ± 0.038 during the last week i.e. 4th week, similar observations were recorded at 20 mg/L exposure concentration where it significantly reduced from 5.779 ± 0.682 in the first week to 1.872 ±0.085 in the 4th week.

4.3.6 Hepatosomatic Index (HSI)

Impact of DEP on Hepatosomatic Index (HSI) of *Cyprinus carpio* exposed at 1, 5 and 20 mg/L concentration individually is exhibited in **Table 4.3.1** through **4.3.4** and **Fig. 4.2**. The HSI was found to be significantly

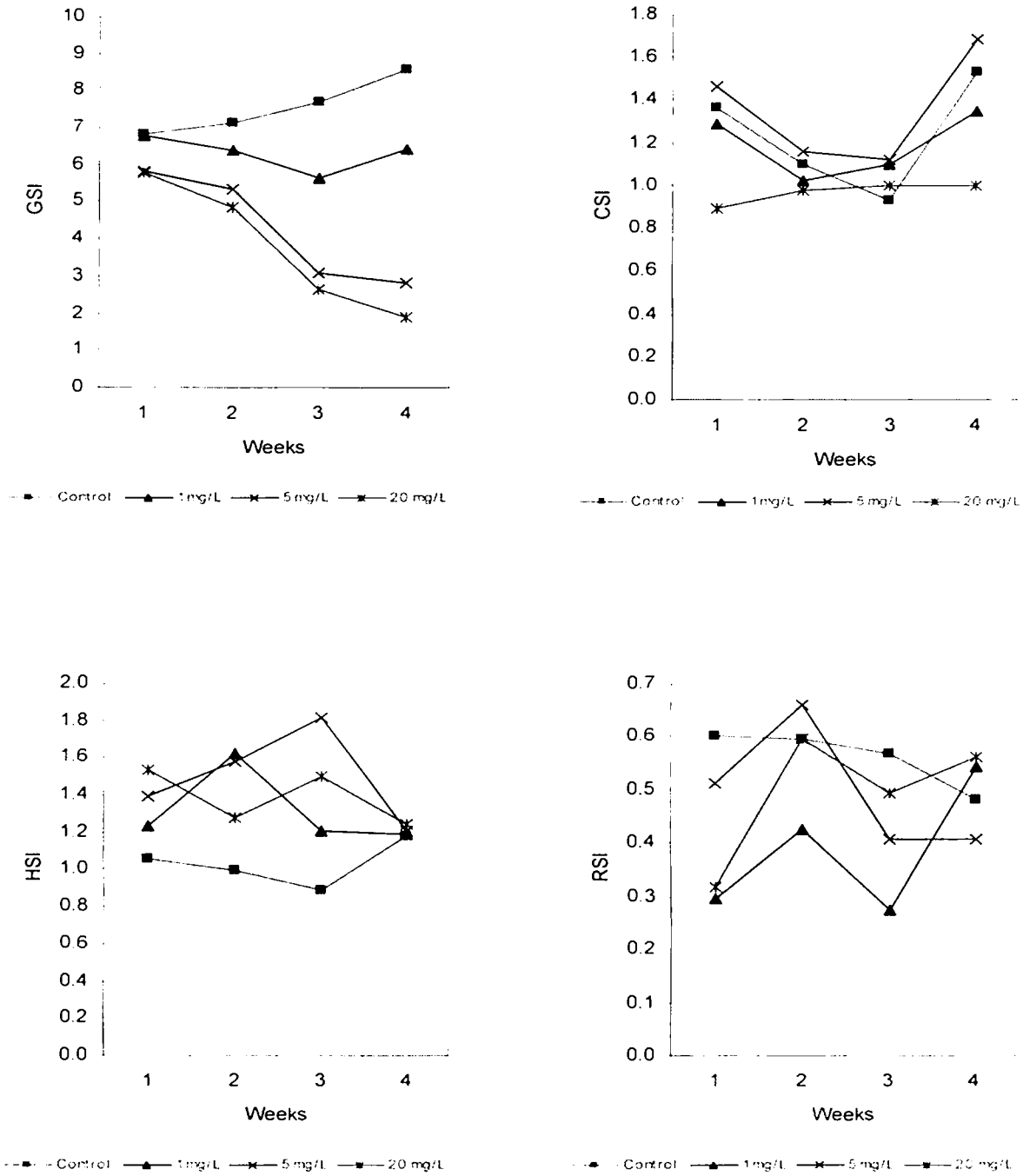

Fig. 4.2 : Somatic indices of Cyprinus carpio exposed to Diethyl phthalate

elevated ($P<0.05$) in response to all the exposure concentrations. At 1mg/L exposure the HSI was 1.231 $\pm$ 0.005 as compared to 0.998 $\pm$ 0.021 in control and was 1.187 $\pm$0.0716 during the 4th week. At 5 and 20 mg/L exposures the HSI declined over the period of time, however at all times it was higher than control. The highest HSI at 5 mg/L was 1.818 $\pm$0.092 during the 3rd week, which was reduced to 1.205 $\pm$ 0.09 during the following week (4th). At the highest exposure concentration however the HSI decreased from 1.532 $\pm$ 0.092 in the 1st week to 1.243 $\pm$ 0.014 during the 4th week, at no time however, was this value lower than the control value.

4.3.7 Renosomatic Index (RSI)

Data pertaining to the impact of DEP on the RSI of *Cyprinus carpio* is given in **Tables 4.3.1** through **4.3.4** and **Fig. 4.2**. The RSI was significantly inhibited in all the exposure concentrations throughout the experimental period. The maximum inhibition observed at 1 mg/L was 0.279 $\pm$0.02 during the 3rd week of exposure. At 5 and 20 mg/L exposures the highest values were 0.658 $\pm$ 0.055 and 0.595 $\pm$ 0.1501 respectively during the 2nd week; however it was reduced to 0.408 $\pm$ 0.1198 and 0.562 $\pm$ 0.0738 respectively during the 4th week.

4.3.8 Craniosomatic Index (CSI)

Data pertaining to the Craniosomatic index of *Cyprinus carpio* exposed to DEP at 1, 5 and 20 mg/L is given in **Tables 4.3.1** through **4.3.4** and **Fig. 4.2**. There was no significant impact of DEP on the CSI of carp at the lower exposure concentration i.e. 1 and 5 mg/L throughout the experimental period.

However at the highest exposure concentration there was marked reduction with respect to exposure period.

At 1mg/L exposure rate the CSI was 1.288 $\pm$ 0.0309 in the first week which was not significantly different from 1.348 $\pm$ 0.035 in the 4th week of exposure. In the 5 mg/L exposure rate the CSI was 1.467 $\pm$ 0.2473 in the 1st week and 1.685 $\pm$ 0.1202 in the 4th week. However at the highest exposure concentration i.e. 20 mg/L the CSI (0.897$\pm$ 0.0506) was suppressed even during the 1st week of exposure as compared to control 1.368 $\pm$ 0.1628 during the same week, which remained lower than the control throughout the remaining exposure period.

4.3.9 Fish behavior and general appearance

Data pertaining to the behavior and appearance of carp exposed to Diethyl phthalate at 1, 5 and 20 mg/L for a period of 28 days is given in **Table 4.3.5**.

4.3.10 Bioaccumulation

Data pertaining to bioaccumulation of diethyl phthalate in *Cyprinus carpio* is presented in **Tables 4.3.6** through **4.3.8** and **Fig. 4.3.** Bioaccumulation was studied in the internal organs of *Cyprinus carpio* exposed to DEP at 1 and 5 mg/L for a period of 28 days. The gills testis and brain showed a time and concentration related increased in DEP content till the 3rd week of exposure followed by a decrease, where as liver showed a steady increase in the accumulation even at the 4th week of exposure.

Table 4.3.5 : Behavioral responses of *Cyprinus carpio* exposed to Diethyl phthalate at different concentrations over 30 days

Parameter	Exposure concentrations			
	Control	**1 mg/L**	**5 mg/L**	**20 mg/L**
Behavior	non aggressive	non aggressive	non aggressive	non aggressive
Feed consumption	within 10-15 minutes	within 10-15 minutes	within 10-15 minutes	within 10-15 minutes
Movements	normal	normal	normal	lethargic towards the end of experimental period (i.e. from day 23)
Coloration	normal	normal	normal	dark coloration (day 20 onwards)
Tapping response	normal	normal	normal	late response

Table 4.3.6: Bioaccumulation of DEP in various organs of *Cyprinus carpio* exposed for a period of 30 days (Grouped by exposure concentrations)

S.N.	Organs	Exposure period (Week)	Exposure concentrations - Mean Values		Significance of 'F' Test	SE (m) Between Exposure concentrations	SE (m) Within Exposure concentrations	CD (5%)	CD (1%)
			1 mg/L	5 mg/L					
1	Gills	1	0.1053	0.1525	**	0.0578	0.0039	0.1312	0.2171
		2	0.8433	3.1325	**	2.8037	0.0459	6.3640	10.5303
		3	0.7109	1.0210	**	0.3798	0.0440	0.8621	1.4265
		4	0.1076	0.3108	**	0.2489	0.0093	0.5649	0.9347
2	Testis	1	0.0660	0.1728	**	0.1308	0.0065	0.2969	0.4913
		2	0.9564	1.2298	**	0.3348	0.0220	0.7601	1.2576
		3	1.2589	1.2875	NS	0.0350	0.0382		
		4	1.0656	1.2425	**	0.2167	0.0399	0.4918	0.8137
3	Brain	1	0.0560	0.1721	**	0.1422	0.0026	0.3228	0.5341
		2	1.8823	2.5773	**	0.8512	0.1128	1.9321	3.1970
		3	2.2409	2.8095	**	0.6964	0.1016	1.5807	2.6156
		4	1.0850	1.0522	**	0.0402	0.0321		
4	Liver	1	0.0660	0.1481	**	0.1006	0.0057	0.2282	0.3777
		2	0.7349	1.2239	**	0.5989	0.0300	1.3594	2.2494
		3	1.0351	1.4693	**	0.5318	0.0381	1.2071	1.9973
		4	0.6264	1.6824	**	1.2933	0.0477	2.9357	4.8576

*, ** indicate significance at 5 and 1% respectively (n = 6). NS = Not Significant
SE (m) = Mean Standard Error. CD = Critical Difference. DEP expressed as $mg.kg^{-1}$.

Table 4.3.7 : Bioaccumulation of DEP in Various Organs of *Cyprinus carpio* during 30 days Exposure Period (Grouped by Week)

S.N.	Organs	Exposure concentrations (mg/L)	Mean Values -Exposure period (Week)				Significance of 'F' Test	SE (m) Between Exposure period	SE (m) Within Exposure period	CD (5%)	CD (1%)
			1	2	3	4					
1	Gills	1	0.1053	0.8433	0.7109	0.1076	**	0.6772	0.0199	1.5370	2.5433
		5	0.1525	3.1325	1.0210	0.3108	**	2.3762	0.0409	5.3936	8.9246
2	Testis	1	0.0660	0.9564	1.2589	1.0656	**	0.9159	0.0251	2.0791	3.4402
		5	0.1728	1.2298	1.2875	1.2425	**	0.9367	0.0341	2.1262	3.5181
3	Brain	1	0.0560	1.8823	2.2409	1.0850	**	1.6784	0.0670	3.8098	6.3040
		5	0.1721	2.5773	2.8095	1.0522	**	2.1785	0.0870	4.9449	8.1822
4	Liver	1	0.0660	0.7349	1.0351	0.6264	**	0.7017	0.0235	1.5928	2.6355
		5	0.1481	1.2239	1.4693	1.6824	**	1.1803	0.0422	2.6792	4.4332

DEP Bioaccumulation expressed as $mg.kg^{-1}$

*, ** indicate significance at 5 and 1% respectively (n = 6) NS = Not Significant

SE (m) = Mean Standard Error. CD = Critical Difference

Table 4.3.8 : Consistent Trends in the Variation of Bioaccumulation of DEP in internal Organs *Cyprinus carpio* (Grouped by exposure period) in different Exposure Concentrations during a 30 days Exposure

S.N.	Organs	Exposure concentrations (mg/L)	Specific comparison among different exposure period showing significant to highly significant differences
1	Gills	1	1 & 2, 1 & 3, 2 & 3, 2 & 4, 3 & 4
		5	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
2	Testis	1	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		5	1 & 2, 1 & 3, 1 & 4
3	Brain	1	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		5	1 & 2, 1 & 3, 1 & 4, 2 & 4, 3 & 4
4	Liver	1	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		5	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4

Exposure Period : 1 – 1st Week; 2 – 2nd Week; 3 – 3rd Week; 4 – 4th Week

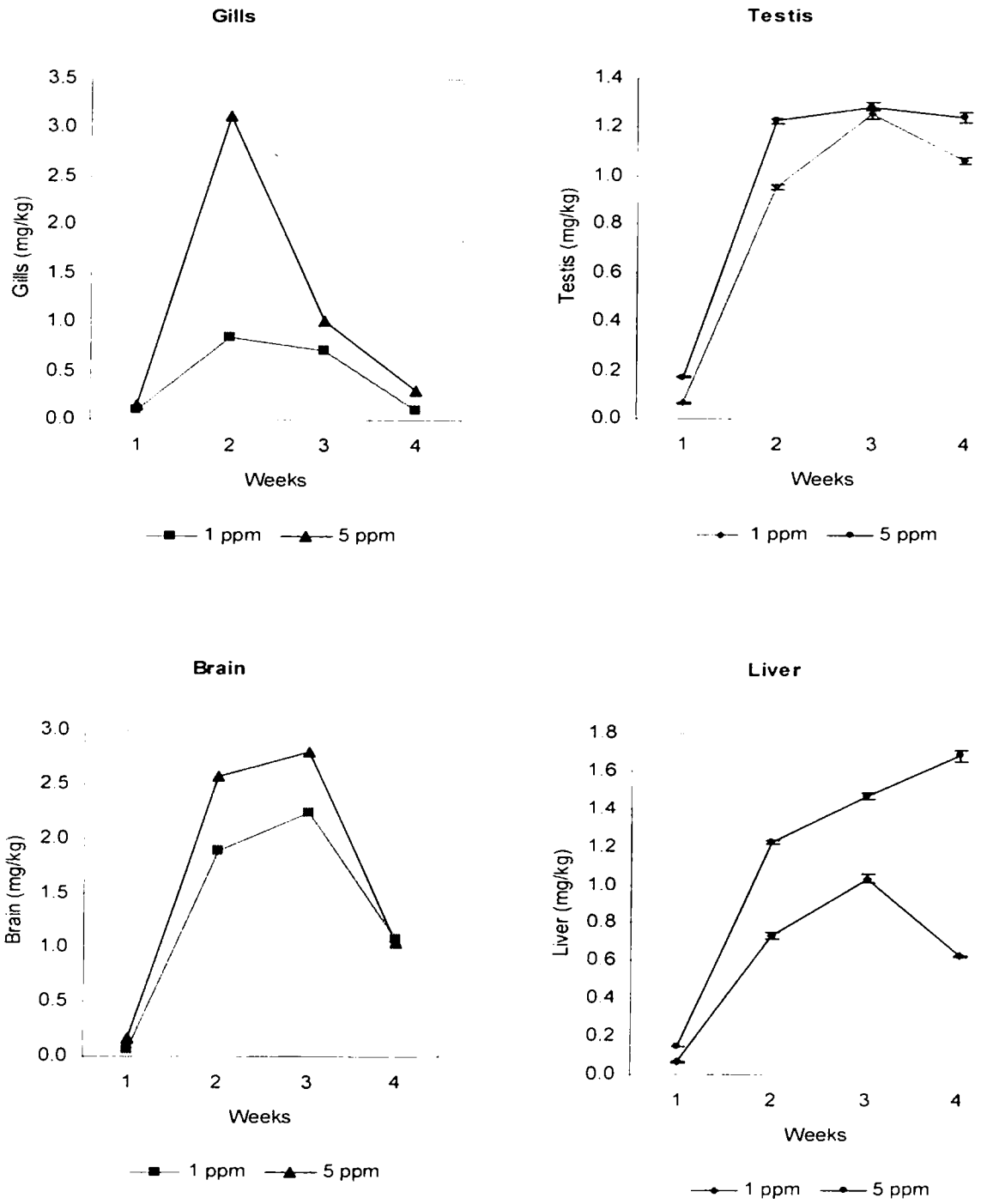

Fig. 4.3 : Bioaccumulation of Diethyl phthalate on various fish organs during different weeks

In gills at 1 and 5 mg/L exposure the highest accumulation was 0.843 ± 0.0069 and 3.132 ± 0.0256 mg/kg respectively during the second week of exposure which declined to 0.107 ± 0.0018 mg/kg and 0.31 ± 0.0051 mg/kg in the 4th .In testis the highest accumulation was 1.258 ± 0.0218 mg/kg and 1.287 ± 0.0158 mg/kg at 1and 5 mg/L exposure respectively during the 3rd week. The lowest accumulation in this case was observed during the 1st week 0.066 ± 0.0019 mg/kg and 0.172 ± 0.0035 mg/kg at 1and 5 mg/L exposure respectively. Similarly brain showed the highest accumulation of 2.24 ± 0.0366 and 2.8 ± 0.0459 mg/kg at 1 and 5 mg/L exposure respectively during the third week of exposure, while the lowest accumulation was 0.056 ± 0.0005 and 0.172 ± 0.0014 mg/kg during the 1st week of exposure.

4.3.11 Vitellogenin Induction

Data pertaining to vitellogenin induction following the 28-day exposure of male fish to DEP (100 μg/l, 1 mg/l and 5 mg/l) is given in **Tables 4.3.9** through **4.3.12** and **Fig. 4.4**. It can be observed that even at lowest exposure concentration that is 100 μg/l there is significant induction of vitellogenin starting 1st week of exposure, vitellogenin content shows a concentration and period related increase.

The highest vitellogenin content can be observed in response to 5 mg/L exposure during the 4th week of DEP exposure, while the lowest can be seen in response to 100 μg/l during the 1st week.

At 100 μg/l DEP exposure the lowest vitellogenin content was 0.285 ± 0.0082 μg/ml during the 1st week, while the highest was 0.401± 0.0093 μg/ml

Table 4.3.9 : Effect of DEP on Vitellogenin Induction in *Cyprinus carpio* (Grouped by exposure concentrations) during an Exposure of 30 days

Parameter	Exposure period (Week)	Mean Values at Exposure concentrations				Significance of 'F' Test	SE (m) Between Exposure concentrations	SE (m) Within Exposure concentrations	CD (5%)	CD (1%)
		Control	100 µg/L	1 mg/L	5 mg/L					
Vitellogenin Induction	1	0.1428	0.285	0.391	0.4485	**	0.2327	0.0109	0.5281	0.8739
	2	0.1629	0.3105	0.4001	0.4735	**	0.2316	0.0119	0.5256	0.8698
	3	0.187	0.3409	0.4575	0.5165	**	0.2517	0.0167	0.5712	0.9452
	4	0.1819	0.401	0.425	0.5100	**	0.2421	0.0147	0.5495	0.9093

Vitellogenin induction expressed as $\mu g.ml^{-1}$.
*, ** indicate significance at 5 and 1% respectively (n = 6) NS = Not Significant
SE (m) = Mean Standard Error. CD = Critical Difference

Table 4.3.10 : Consistent Trends in the Variation of the effect of DEP on Vitellogenin Induction in Cyprinus carpio (Grouped by Exposure concentrations) during 30 days Exposure

Parameter	Exposure period (Week)	Specific Comparison among different Exposure concentrations showing significant to highly significant differences
Vitellogenin Induction	1	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
	2	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
	3	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
	4	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4

Exposure concentrations : 1 – Control; 2 - 100 µg/L; 3 - 1 mg/L; 4 - 5 mg/L

Table 4.3.11 : Effect of DEP on Vitellogenin Induction in *Cyprinus carpio* during over a 30 days Exposure Period

(Grouped by Week)

Parameter	Exposure concentrations (mg/L)	Mean Values at Exposure period (week)				Significance of 'F' Test	SE (m) Between Exposure Period	SE (m) Within Exposure Period	CD (5%)	CD (1%)
		1	2	3	4					
DEP	Control	0.1428	0.1629	0.187	0.1819	**	0.0348	0.0055	0.0790	0.1308
	100 µg/L	0.285	0.3105	0.3409	0.4010	**	0.0865	0.0123	0.1964	0.3250
	1	0.391	0.4001	0.4575	0.4250	**	0.0515	0.0155	0.1170	0.1936
	5	0.4485	0.4735	0.5165	0.5100	**	0.0553	0.0167	0.1256	0.2079

Vitellogenin induction expressed as $\mu g.ml^{-1}$.
*, ** indicate significance at 5 and 1% respectively (n = 6) NS = Not Significant
SE (m) = Mean Standard Error. CD = Critical Difference

Table 4.3.12 : Consistent Trends in the Variation of the effect of DEP during a 30 days Exposure period on Vitellogenin Induction in *Cyprinus carpio* (Grouped by Exposure period) in different Exposure Concentrations

Parameter	Exposure concentrations (mg/L)	Specific Comparison among different Exposure period showing significant to highly significant differences
DEP	Control	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4
	100 µg/L	1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
	1	1 & 3, 2 & 3
	5	1 & 3, 1 & 4

Exposure Period : 1 – 1st Week; 2 – 2nd Week; 3 – 3rd Week; 4 – 4th Week

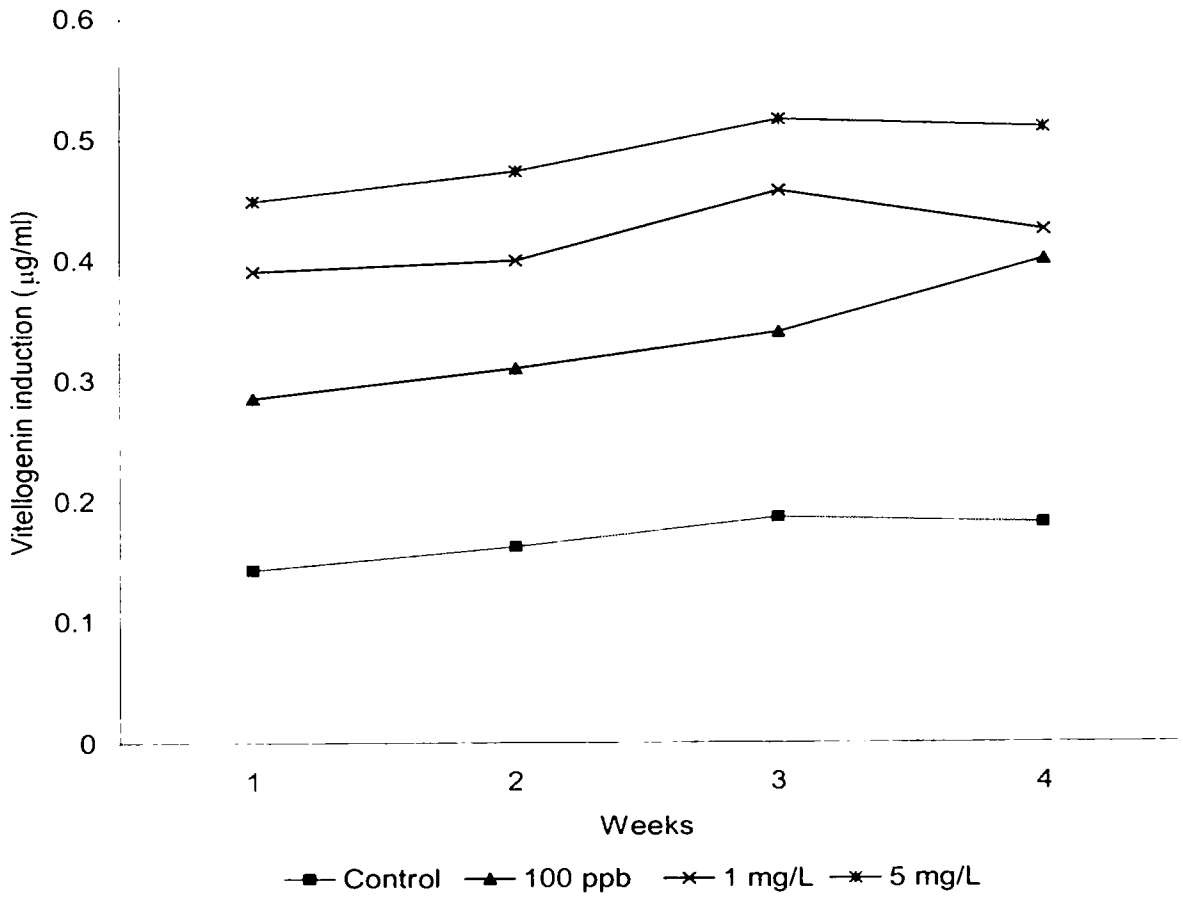

Fig. 4.4 : Effect of Diethyl phthalate on vitellogenin induction

during the 4th week. At 1 mg/L the lowest vitellogenin induction was 0.391 ± 0.0045 μg/ml in the 1st week, while the highest was 0.425 ± 0.0074 μg/ml during the 4th week of exposure. At the highest exposure concentration i.e. 5 mg/L the lowest vitellogenin induction post 1 week exposure was 0.448 ± 0.0078 μg/ml nearly double of that in 100 μg/l exposure, the highest content was observed to be 0.51 ± 0.0118 μg/ml in the 4th week of exposure.

4.4 Methyl Paraben

4.4.1 Acid Phosphatase (AcP)

Data pertaining to the acid phosphatase activity in *Cyprinus carpio* exposed to Methyl paraben at 0.84, 1.68 and 4.2 mg/L is given in **Tables 4.4.1** through **4.2.4** and graphically shown in **Fig. 4.5**. Acid phosphatase was significantly reduced in all the exposure concentrations throughout the exposure period. At the lowest exposure concentration i.e. 0.84 mg/L the highest activity was observed to be 17.668 ±0.038 μmoles/mg of protein during the 2nd week of exposure as compared to 17.677 ± 0.001 μmoles/mg of protein in control and declines to 13.997 ± 0.229 μmoles/mg of protein during the 4th week as compared to 16.532 ± 0.021 μmoles/mg of protein in control during the same period . At 1.68 mg/L exposure the highest AcP activity was observed to be 16.637±0.151 μmoles/mg of protein during the 1st week, which reduces to 14.547 ±0.259 μmoles/mg of protein during the last week of exposure. At the highest exposure concentration i.e. 4.2 mg/L the highest activity was observed to be 16.783 ±0.087 μmoles/mg of protein in the 1st week and significantly ($P<0.05$) lowered to 15.519 ± 0.27 μmoles/mg of protein .

Table 4.4.1: Effect of MP on Various Indices and Enzymatic Activities of Cyprinus carpio (Grouped by exposure concentrations) for an Exposure Period of 30 days

S.N.	Parameter	Exposure period (Week)	Mean Values at exposure concentrations				Significance of 'F' Test	SE (m) Between exposure concentrations	SE (m) Within exposure concentrations	CD (5%)	CD (1%)
			Control	0.84 mg/L	1.68 mg/L	4.2 mg/L					
1	GSI	1	6.8494	5.6295	6.5401	5.9569	**	0.9561	0.1925	2.1703	3.5912
		2	7.1259	5.3239	5.6028	5.3869	**	1.4764	0.3386	3.3513	5.5453
		3	7.7149	4.6529	2.1721	1.6496	**	4.8045	0.1913	10.9056	18.0452
		4	8.6078	5.3131	3.8347	2.3104	**	4.6589	0.1626	10.5750	17.4983
2	HSI	1	1.0604	1.7865	1.9113	1.9045	**	0.7059	0.1039	1.6024	2.6514
		2	0.9986	1.7300	1.2040	1.7420	**	0.6511	0.1958	1.4780	2.4456
		3	0.8916	1.4775	1.3381	1.4717	**	0.4787	0.1219	1.0865	1.7978
		4	1.1781	0.8825	1.1042	1.4119	**	0.3780	0.0812	0.8581	1.4198
3	CSI	1	1.3648	1.2302	1.2728	1.2745	NS	0.0981	0.0826	--	--
		2	1.0987	1.2136	1.2890	1.0415	NS	0.1932	0.1186	--	--
		3	0.9284	1.1117	1.0925	1.1037	NS	0.1515	0.1394	--	--
		4	1.5336	1.2747	1.2464	1.2320	**	0.2466	0.0424	0.5598	0.9263
4	RSI	1	0.6020	0.4337	0.4771	0.5854	*	0.1422	0.0626	0.3227	--
		2	0.5962	0.5433	0.5049	0.6127	NS	0.0856	0.1066	--	--
		3	0.5662	1.2075	0.5097	1.2063	**	0.6701	0.1169	1.5211	2.5169
		4	0.4807	1.3634	1.2334	1.3622	**	0.7342	0.1672	1.6665	2.7575
5	AcP	1	18.1060	17.1223	16.6372	16.7837	**	1.1451	0.2015	2.5992	4.3008
		2	17.6777	17.6688	16.1371	15.1906	**	2.1179	0.4361	4.8074	7.9547
		3	17.6993	16.3110	15.1794	15.6421	**	4.3145	7.1391	3.2929	4.9864
		4	16.5632	13.9976	14.5609	15.5190	**	1.9514	0.3804	4.4294	7.3291
6	AlP	1	0.1971	1.1935	1.2936	1.1760	**	0.8913	0.0811	2.0231	3.3475
		2	0.3902	1.4037	1.5001	1.7186	**	1.0223	0.1753	2.3204	3.8395
		3	0.9680	0.7483	1.4314	1.2337	**	0.5184	0.0857	1.1768	1.9472
		4	1.4587	1.5140	1.5471	1.5799	*	0.0895	0.0372	0.2032	0.3363
7	SGOT	1	15.0582	15.1412	14.9457	15.1090	NS	0.1484	0.1148	--	--
		2	16.3753	16.8975	17.4853	17.1005	**	0.8009	0.1882	1.8178	3.0079
		3	18.5140	16.6803	16.6652	16.2901	**	1.7334	0.4452	3.9346	6.5105
		4	16.8201	10.0271	12.1206	14.3794	**	5.0649	0.1598	11.4967	19.0233
8	SGPT	1	7.2781	7.3517	8.0532	8.8303	**	1.2548	0.1278	2.8482	4.7129
		2	8.9688	9.4211	9.3960	10.6857	*	1.2844	0.5897	2.9153	--
		3	10.8757	19.8675	19.8691	19.8529	**	7.7834	0.5315	17.6672	29.2334
		4	12.8435	20.8817	21.8816	22.3022	**	7.7292	0.2135	17.5442	29.0300

*, ** indicate significance at 5 and 1% respectively (n = 6). NS = Not Significant. SE (m) = Mean Standard Error. CD = Critical Difference

AcP and AlP expressed as µmoles.mg of protein^{-1}. SGOT and SGPT expressed as nmoles oxaloacetate.mg of protein^{-1}.

Table 4.4.2 : Consistent Trends in the Variation of the effect of MP on Important Indices and Enzymatic Activities of *Cyprinus carpio* (Grouped by exposure concentrations) over 30 days

S.N.	Parameters	Exposure period (Week)	Specific Comparison among different exposure concentrations showing significant to highly significant differences
1	GSI	1	1 & 2, 1 & 4, 2 & 3
		2	1 & 2, 1 & 3, 1 & 4
		3	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4
		4	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
2	HSI	1	1 & 2, 1 & 3, 1 & 4
		2	1 & 2, 1 & 3, 1 & 4
		3	1 & 2, 1 & 3, 1 & 4
		4	1 & 2, 2 & 4, 3 & 4
3	CSI	4	2 & 3, 2 & 4, 3 & 4
4	RSI	3	1 & 2, 1 & 4, 2 & 3, 3 & 4
		4	1 & 2, 1 & 3, 1 & 4
5	AcP	1	1 & 2, 1 & 3, 1 & 4
		2	1 & 3, 1 & 4, 2 & 3, 2 & 4
		3	1 & 3, 1 & 4
		4	1 & 2, 1 & 3, 2 & 4
6	AlP	1	1 & 2, 1 & 3, 1 & 4
		2	1 & 2, 1 & 3, 1 & 4
		3	1 & 3, 2 & 3, 2 & 4
		4	1 & 4
7	SGOT	2	1 & 3, 1 & 4
		3	1 & 2, 1 & 3, 1 & 4
		4	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
8	SGPT	1	1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		3	1 & 2, 1 & 3, 1 & 4
		4	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4

Exposure concentrations: 1 - Control; 2 - 0.84 mg/L; 3 - 1.68 mg/L; 4 - 4.2 mg/L

Table 4.4.3 : Effect of MP on Various Indices and Enzymatic Activities of *Cyprinus carpio* for an Exposure Period of 30 days (Grouped by Week)

S.N.	Parameter	Exposure concentrations (mg/L)	Mean Values at Exposure period (week)				Significance of 'F' Test	SE (m) Between Exposure period	SE (m) Within Exposure period	CD (5%)	CD (1%)
			1	2	3	4					
1	GSI	Control	6.8494	7.1259	7.7149	8.6078	NS	1.3470	0.0000	--	--
		0.84	5.6295	5.3239	4.6529	5.3131	**	0.7130	0.1774	1.6183	2.6778
		1.68	6.5401	5.6028	2.1721	3.8347	**	3.3517	0.2613	7.6080	12.5888
		4.2	5.9569	5.3869	1.6496	2.3104	**	3.7432	0.3392	8.4964	14.0588
2	HSI	Control	1.0604	0.9986	0.8916	1.1781	**	0.2073	0.0000	0.4706	0.7786
		0.84	1.7865	1.7300	1.4775	0.8825	**	0.7162	0.1724	1.6258	2.6901
		1.68	1.9113	1.2040	1.3381	1.1042	**	0.6251	0.1790	1.4189	2.3478
		4.2	1.9045	1.7420	1.4717	1.4119	**	0.4007	0.0939	0.9095	1.5048
3	CSI	Control	1.3648	1.0987	0.9284	1.5336	**	0.4675	0.0000	1.0611	1.7558
		0.84	1.2302	1.2136	1.1117	1.2747	NS	0.1194	0.1502	--	--
		1.68	1.2728	1.2890	1.0925	1.2464	NS	0.1562	0.0851	--	--
		4.2	1.2745	1.0415	1.1037	1.2320	NS	0.3700	0.0179	--	--
4	RSI	Control	0.6020	0.5962	0.5662	0.4807	**	0.0969	0.0000	0.2200	0.3640
		0.84	0.4337	0.5433	1.2075	1.3634	**	0.8082	0.1122	1.8346	3.0357
		1.68	0.4771	0.5049	0.5097	1.2334	**	0.6380	0.1741	1.4482	2.3963
		4.2	0.5854	0.6127	1.2063	1.3622	**	0.6943	0.1184	1.5759	2.6076
5	AcP	Control	18.1060	17.6777	17.6993	16.5632	**	1.1470	0.0000	2.6035	4.3080
		0.84	0.5854	0.4354	1.2063	1.3622	**	0.7888	0.1202	1.7906	2.9628
		1.68	16.6372	16.1371	15.1794	14.5609	*	1.6179	0.6528	3.6725	--
		4.2	16.7837	15.1906	15.6421	15.5190	**	1.2007	0.4310	2.7255	4.5098
6	AlP	Control	0.1971	0.3902	0.9680	1.4587	**	0.9924	0.0000	2.2526	3.7274
		0.84	1.1935	1.4037	0.7483	1.5140	**	0.5859	0.1170	1.3299	2.2006
		1.68	1.2936	1.5001	1.4314	1.5471	NS	0.1912	0.1428	--	--
		4.2	1.1760	1.7186	1.2337	1.5799	**	0.4569	0.1093	1.0372	1.7162
7	SGOT	Control	15.0582	16.3753	18.5140	16.8201	NS	2.4710	0.0000	--	--
		0.84	15.1412	16.8975	16.6803	10.0271	**	5.5480	0.3667	12.5933	20.8378
		1.68	14.9457	17.4853	16.6652	12.1206	**	4.1077	0.2964	9.3240	15.4282
		4.2	15.1090	17.1005	16.2901	14.3794	**	2.0979	0.2236	4.7620	7.8795
8	SGPT	Control	7.2781	8.9688	10.8757	12.8435	**	4.1622	0.0000	9.4477	15.6329
		0.84	7.3517	9.4211	19.8675	20.8817	**	12.0985	0.2412	27.4618	45.4404
		1.68	8.0532	9.3960	19.8691	21.8816	**	12.2706	0.1849	27.8526	46.0870
		4.2	8.8303	10.6857	19.8529	22.3022	**	11.5262	0.7744	26.1628	43.2909

*, ** indicate significance at 5 and 1% respectively (n = 6). NS = Not Significant. SE (m) Mean Standard Error. CD – Critical Difference

AcP and AlP expressed as µmoles.mg of protein^{-1}. SGOT and SGPT expressed as nmoles oxaloacetate.mg of protein^{-1}.

Table 4.4.4 : Consistent Trends in the Variation of the effect of MP on Important Indices and Enzymatic Activities of *Cyprinus carpio* (Grouped by exposure period) in different Exposure Concentrations during a 30 days Exposure

S.N.	Parameter	Exposure concentrations (mg/L)	Specific Comparison among different Exposure period showing significant to highly significant differences
1	GSI	0.84	1 & 3, 2 & 3, 3 & 4
		1.68	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		4.2	1 & 3, 1 & 4, 2 & 3, 2 & 4
2	HSI	Control	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		0.84	2 & 4, 3 & 4
		1.68	1 & 2, 1 & 4
		4.2	1 & 3, 1 & 4, 2 & 4
3	CSI	Control	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
4	RSI	Control	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		0.84	1 & 3, 1 & 4, 2 & 3, 2 & 4
		1.68	1 & 4, 2 & 4, 3 & 4
		4.2	1 & 2, 1 & 4, 2 & 3, 2 & 4
5	AcP	Control	1 & 2,1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		0.84	1 & 3, 1 & 4, 2 & 3, 2 & 4
		4.2	1 & 2
6	AlP	Control	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		0.84	1 & 3, 2 & 3, 3 & 4
		4.2	1 & 2, 1 & 4, 2 & 3
7	SGOT	0.84	1 & 2, 1 & 3, 1 & 4, 2 & 4, 3 & 4
		1.68	1 & 2, 1 & 3, 1 & 4, 2 & 4, 3 & 4
		4.2	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
8	SGPT	Control	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		0.84	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		1.68	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		4.2	1 & 3, 1 & 4, 2 & 3, 2 & 4

Exposure Period : 1 – 1st Week; 2 – 2nd Week; 3 – 3rd Week; 4 – 4th Week

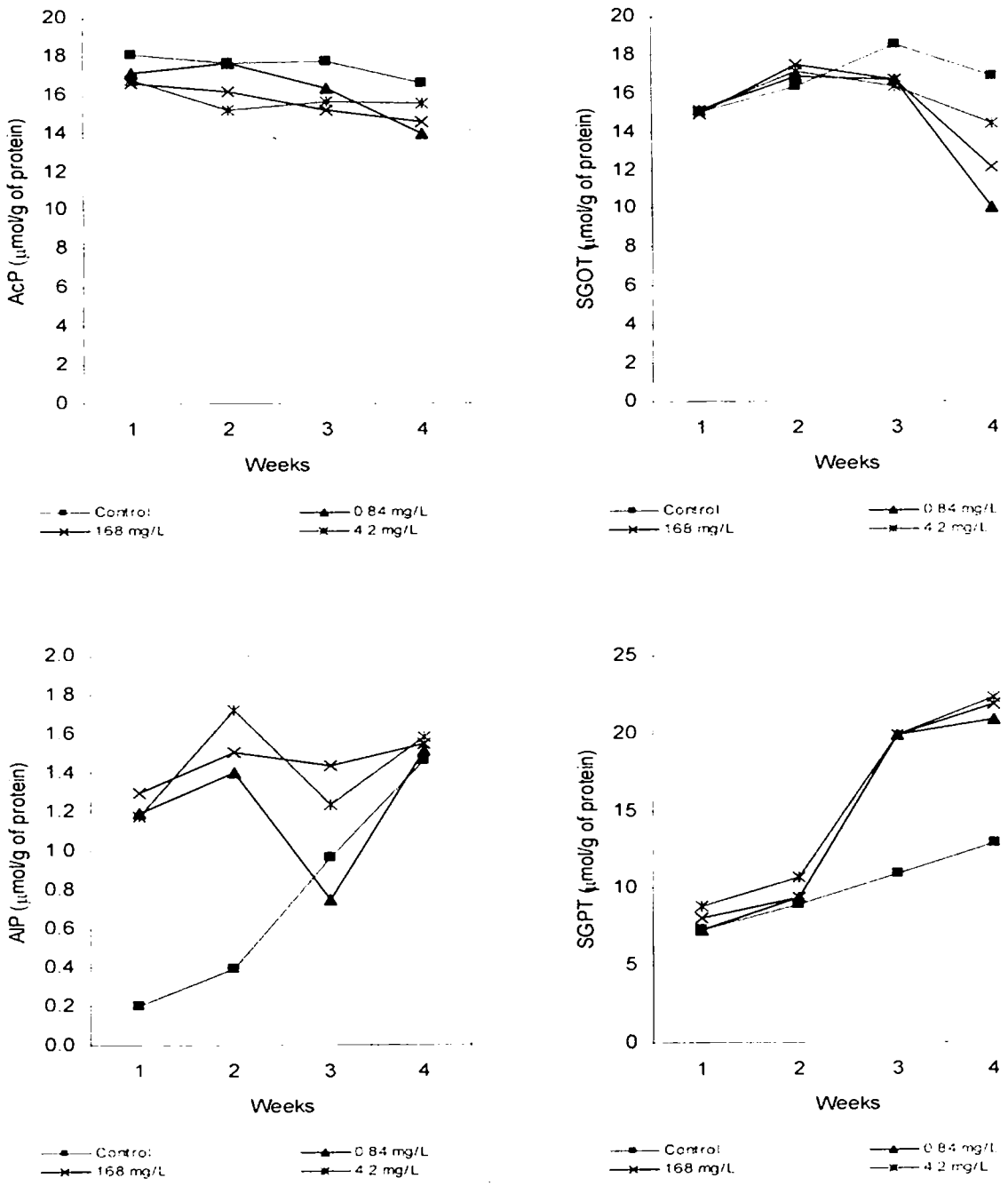

Fig. 4.5 : Mean enzymatic responses of Cyprinus carpio to Methyl paraben

4.4.2 Alkaline Phosphatase (AlP)

Impact of MP on Alkaline phosphatase activity of carp exposed at 0.84, 1.68 and 4.2 mg/L is for a period of 28 days is given in **Tables 4.4.1** through **4.4.4** and graphically shown in **Fig. 4.5**. Unlike AcP, AlP was elevated in response to MP in all the exposures, also there was a time and concentration related increase in the activity. In the control the activity increased from 0.197 ± 0.011 µmoles/mg of protein in the 1st week to 1.458 ± 0.021 µmoles/mg of protein in the 4th week. At the lowest exposure concentration i.e. 0.84 mg/L during the 1st week the AlP activity was 1.19 ±0.051 µmol/mg of protein and increased to 1.514 ± 0.031 µmol/mg of protein in the 4th week. At the highest exposure concentration i.e. 4.2 mg/L the AlP activity during the 1st week was 1.176 ±0.055 µmoles/mg of protein which was significantly ($P<0.05$) elevated to 1.579 ± 0.008 µmoles/mg of protein in the 4th week of exposure.

4.4.3 Serum Glutamic Oxalacetic Transaminase (SGOT) / Aspartate Aminotransferase (AST)

Data pertaining to effect of Methyl paraben on Serum Glutamic Oxalacetic Transaminase (SGOT) of carp is given in **Tables 4.4.1** through **4.4.4** and graphically represented in **Fig. 4.5**. The SGOT activity was significantly inhibited towards the end of experimental period i.e. 4th week. During the first week there was no significant difference ($P>0.05$) in the activity of all the exposed fish as compared to control, however towards the 4th week the activity was inhibited even in the lowest exposure concentration i.e. 0.84 mg/L. The highest activity 17.485 ± 0.006 nmoles oxaloacetate/mg of protein is exhibited at 1.68 mg/L in the second week of exposure.

4.4.4 Serum Glutamic Pyruvic Transaminase (SGPT) / Alanine Aminotransferase (ALT)

The Serum Glutamic Pyruvic Transaminase (SGPT) activity of *Cyprinus carpio* exposed to 3 concentrations (0.84, 1.68 and 4.2 mg/L) of Methyl paraben (MP) is given in **Tables 4.4.1** through **4.4.4** and graphically represented in **Fig. 4.5**. Starting 1st week onwards there was a significant increase ($P<0.05$) in a dose dependant and exposure period dependant manner. At the lowest exposure concentration there was no significant difference ($P>0.05$) during the first week however starting 2nd week onwards there is a continuous increase in the activity and is increased to more than 3 times. In the highest exposure concentration (4.2 mg/L) the activity was higher than the control right from the 1st week onwards.

4.4.5 Gonadosomatic Index (GSI)

Data pertaining to the Gonadosomatic Index (GSI) of *Cyprinus carpio* exposed to methyl paraben at 0.84, 1.68 and 4.2 mg/L for 28 days is given in **Tables 4.4.1** through **4.4.4** and graphically represented in **Fig. 4.6**. The GSI was inhibited in all the exposures starting 1st week onwards. At the highest exposure concentration i.e. 4.2 mg/L there was a significant time related inhibition in GSI of the exposed fish and is reduced to 2.31 ± 0.028 in the final week as compared to 5.956 ± 0.066 in the 1st week. At the lowest exposure concentration i.e. 0.84 mg/L the index was significantly lowered as compared to control.

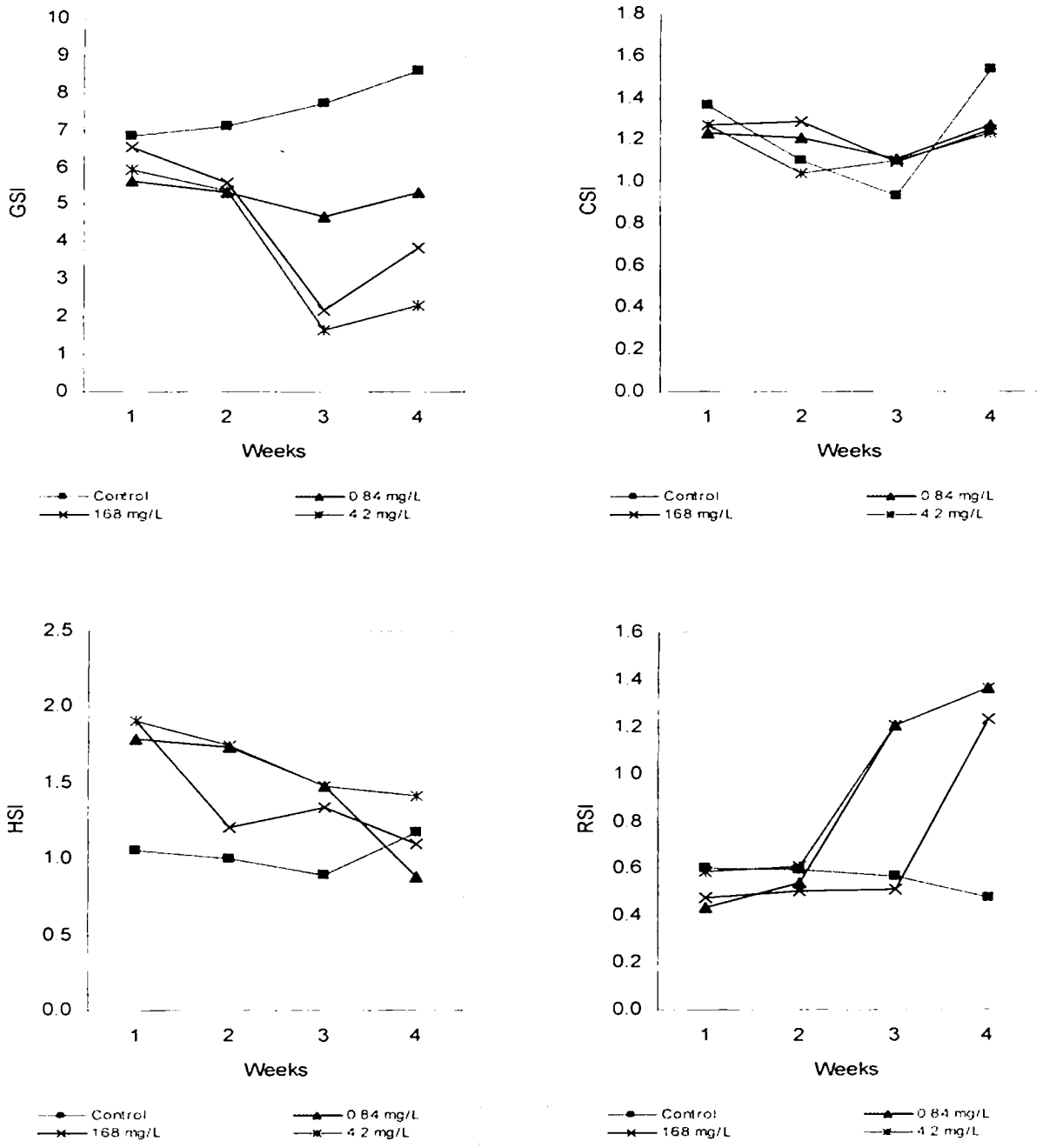

Fig. 4.6 : Somatic indices of Cyprinus carpio exposed to Methyl paraben

4.4.6 Hepatosomatic Index (HSI)

Data pertaining to the HSI of carp exposed to methyl paraben is given in **Tables 4.4.1** through **4.4.4** and graphically represented in **Fig. 4.6**. The HSI was initially elevated in all the exposures however, in the 4th week it was reduced and comparable to control in the lower exposures. The HSI at the 0.84 mg/L exposure was 1.786 $\pm$ 0.111 as compared to 1.06 $\pm$ 0.021 in control in the 1st week; however it was reduced to 0.882 $\pm$ 0.058 in the 4th week of exposure. At the highest exposure concentration it was reduced from 1.9 $\pm$ 0.039 n the 1st week to 1.411$\pm$ 0.026 in the 4th week, which was still significantly higher than control.

4.4.7 Renosomatic Index (RSI)

Data pertaining to the Renosomatic Index (RSI) of *Cyprinus carpio* exposed to Methyl paraben at 0.84, 1.68 and 4.2 mg/L for 28 days is given is **Tables 4.4.1** through **4.4.4** and graphically represented in **Fig. 4.6**. Initially the RSI was inhibited in response to all the exposure concentration but in the final week it was elevated as compared to control. During the 1st week the RSI was 0.433 $\pm$0.017, 0.471$\pm$0.069 and 0.585 $\pm$0.007 for 0.84, 1.68 and 4.2 mg/L exposures respectively which was significantly lower ($P<0.05$) than 0.602 in control. However during the 4th week it was elevated to 1.363 $\pm$ 0.073, 1.233 $\pm$0.142 and 1.362 $\pm$0. 107 for 0.84, 1.68 and 4.2 mg/L exposure concentration respectively as compared to 0.48 in the control during the same period.

4.4.8 Craniosomatic Index (CSI)

Data pertaining to impact of Methyl paraben on Craniosomatic Index (CSI) is given in **Tables 4.4.1** through **4.4.4** and graphically represented in **Fig. 4.6**. There was no significant difference (P>0.05) between any of the treatments and control CSI, during the 1st, 2nd and 3rd week, however in the final week there was a significant reduction in the CSI of all the treatments. The CSI in the 4th week was 1.274$\pm$ 0.02, 1.246 $\pm$0.042 and 1.23 $\pm$0.011 for 0.84, 1.68 and 4.2 mg/L exposures respectively which was significantly lower (P<0.05) than 1.53 $\pm$ 0.0523 in control.

4.4.9 Fish behavior and general appearance

Data pertaining to the behavior and appearance of carp exposed to Methyl paraben at 0.84, 1.68 and 4.2mg/L for a period of 28 days is given in **Table 4.4.5**.

4.4.10 Bioaccumulation

Data pertaining to bioaccumulation of methyl paraben in the internal organs (Gills, Liver, Brain and Testis) of *Cyprinus carpio* exposed at 0.84 and 1.68 mg/L is presented in **tables 4.4.6** through **4.4.8** and **Fig. 4.7**. In all the organs except for liver the accumulation increased up to the 3rd week and declined in the 4th week whereas in case of liver the accumulation showed an increasing pattern even in the 4th week.

In gill at the higher exposure rate the accumulation increased from 0.518 $\pm$ 0.022 mg/Kg in 1st week to 1.322 $\pm$ 0.032 mg/Kg in the 3rd week and finally reduced to 0.776 $\pm$ 0.003 mg/Kg in the 4th week. In testis the

Table 4.4.5 : Behavioral responses of *Cyprinus carpio* exposed to Methyl paraben at different concentrations over 30 days

Parameter	Exposure concentrations			
	Control	**0.84 mg/L**	**1.68 mg/L**	**4.2 mg/L**
Behavior	non aggressive	non aggressive	non aggressive	non aggressive
Feed consumption	within 10-15 minutes	within 10-15 minutes	within 10-15 minutes	within 10-15 minutes
Movements	normal	normal	normal	lethargic towards the end of experimental period (i.e. from day 26)
Coloration	normal	normal	normal	Normal
Tapping response	normal	normal	normal	late response

Table 4.4.6 : Bioaccumulation of MP in Various Organs of *Cyprinus carpio* over 30 days
(Grouped by exposure concentrations)

S.N.	*Organs*	Exposure period (Weeks)	Exposure concentrations - Mean Values		Significance of 'F' Test	SE (m) Between Exposure concentrations	SE (m) Within Exposure concentrations	CD (5%)	CD (1%)
			1.68 mg/L	1.84 mg/L					
1	Gills	1	0.5184	0.3061	**	0.2600	0.0298	0.5902	0.9766
		2	0.7714	0.6612	**	0.1350	0.0144	0.3064	0.5069
		3	1.3224	0.7714	**	0.6748	0.0541	1.5318	2.5346
		4	0.7761	0.1426	**	0.7759	0.0223	1.7611	2.9141
2	Testis	1	0.7714	0.1020	**	0.8198	0.0275	1.8609	3.0792
		2	1.4326	0.9367	**	0.6074	0.0242	1.3786	2.2811
		3	2.0938	0.8816	**	1.4846	0.0482	3.3699	5.5761
		4	1.3224	0.7428	**	0.7099	0.0433	1.6113	2.6662
3	Brain	1	0.4163	0.5510	**	0.1650	0.0098	0.3745	0.6196
		2	1.9836	0.8816	**	1.3497	0.0767	3.0636	5.0692
		3	3.9673	1.2122	**	3.3743	0.1173	7.6592	12.6735
		4	2.8653	1.0108	**	2.2713	0.0645	5.1555	8.5307
4	Liver	1	1.0201	0.5428	**	0.5846	0.0409	1.3269	2.1956
		2	1.2122	1.1020	**	0.1350	0.0232	0.3064	0.5069
		3	1.9836	0.9918	**	1.2147	0.0470	2.7572	4.5623
		4	2.7551	1.0254	**	2.1184	0.0831	4.8086	7.9566

*, ** indicate significance at 5 and 1% respectively (n = 6) NS = Not Significant SE (m) = Mean Standard Error. CD = Critical Difference

Bioaccumulation expressed as $mg.kg^{-1}$

Table 4.4.7 : Bioaccumulation of MP in Various Organs of *Cyprinus carpio* during 30 days Exposure Period (Grouped by Week)

S.N.	Organs	Exposure concentrations (mg/L)	Mean Values –Exposure period (Week)				Significance of 'F' Test	SE (m) Between Exposure period	SE (m) Within Exposure period	CD (5%)	CD (1%)
			1	2	3	4					
1	Gills	0.84	0.5184	0.7714	1.3224	0.7761	**	0.5871	0.0381	1.3327	2.2052
		1.68	0.3061	0.6612	0.7714	0.1426	**	0.5113	0.0284	1.1606	1.9205
2	Testis	0.84	0.7714	1.4326	2.0938	1.3224	**	0.9399	0.0476	2.1335	3.5303
		1.68	0.1020	0.9367	0.8816	0.7428	**	0.6662	0.0225	1.5121	2.5020
3	Brain	0.84	0.4163	1.9836	3.9673	2.8653	**	2.5976	0.1031	5.8962	9.7564
		1.68	0.5510	0.8816	1.2122	1.0108	**	0.4807	0.0365	1.0912	1.8056
4	Liver	0.84	1.0201	1.2122	1.9836	2.7551	**	1.3735	0.0687	3.1177	5.1588
		1.68	0.5428	1.1020	0.9918	1.0254	**	0.4377	0.0308	0.9935	1.6440

Bioaccumulation expressed as $mg.kg^{-1}$

*, ** indicate significance at 5 and 1% respectively (n = 6) NS = Not Significant

SE (m) = Mean Standard Error. CD = Critical Difference

Table 4.4.8 : Consistent Trends in the Variation of Bioaccumulation of MP in internal Organs of *Cyprinus carpio* (Grouped by exposure period) in different Exposure Concentrations during an Exposure period of 30 days

S.N.	Organs	Exposure concentrations (mg/L)	Specific comparison among different exposure period showing significant to highly significant differences
1	Gills	0.84	1 & 2, 1 & 3, 1 & 4, 2 & 3
		1.68	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
2	Testis	0.84	1 & 2, 1 & 3, 1 & 4, 2 & 3, 3 & 4,
		1.68	1 & 2, 1 & 3, 1 & 4, 2 & 4, 3 & 4
3	Brain	0.84	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		1.68	1 & 2, 1 & 3, 1 & 4, 2 & 4, 2 & 3, 3 & 4
4	Liver	0.84	1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		1.68	1 & 2, 1 & 3, 1 & 4, 2 & 3

Exposure Period : 1 – 1st Week; 2 – 2nd Week; 3 – 3rd Week; 4 – 4th Week

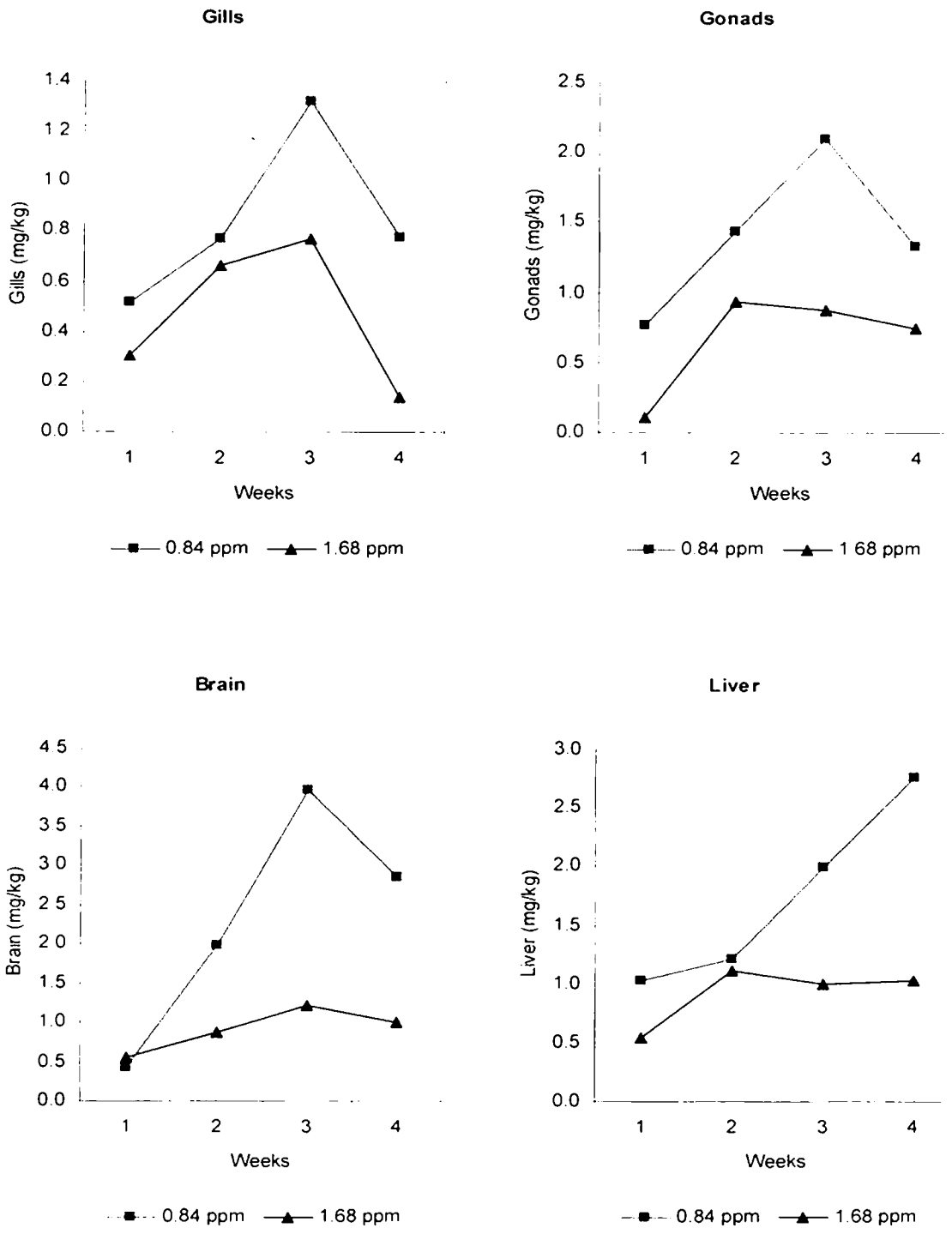

Fig. 4.7 : Bioaccumulation of Methyl paraben on various fish organs during different weeks

accumulation increased to 0.881± 0.03 mg/Kg in the 3rd from 0.102 ± 0.022 mg/Kg in the 1st week at the lower exposure concentration i.e. 0.84 mg/L and finally subdued to 0.742±0.0305 mg/Kg in the 4th week. The bioaccumulation in the brain was highest amongst all the organs studied and reached up to a level of 3.96 ± 0.091 mg/Kg in the 3rd week, finally reducing to 2.865 ± 0.0416 mg/Kg in the 4th week in 1.68 mg/L exposure . In case of liver at lower exposure rate i.e. 0.84 mg/L the accumulation increased steadily from 0.542 ± 0.002 mg/Kg in the 1st week to 1.025 ±0.017 mg/Kg in the 4th week.

4.4.11 Vitellogenin Induction

Vitellogenin induction in response to Methyl paraben exposure at 0.84, 1.68 and 4.2 mg/L is reported in **Tables 4.4.9** through **4.4.12** and **Fig. 4.8**. Vitellogenin was inducted in all the fish exposed at all the concentration starting 1st week onwards and showed a concentration and time related increase. At the lowest exposure concentration i.e. 0.84 mg/L 0.379 ± 0.018 µmol/ml vitellogenin was inducted during the 1st week and increased to 0.402 ± 0.0162 µmol/ml in the last week. At the highest exposure concentration i.e. 4.2 mg/L the initial induction was 0.625 ± 0.0066 µmol/ml and increased to 0.7 ± 0.009 µmol/ml in the 4th week.

Table 4.4.9 : Effect of MP on Vitellogenin Induction in *Cyprinus carpio* (Grouped by exposure concentrations) during 30 days Exposure

Parameter	Exposure period (Week)	Mean Values at Exposure concentrations				Significance of 'F' Test	SE (m) Between Exposure concentrations	SE (m) Within Exposure concentrations	CD (5%)	CD (1%)
		Control	0.84 mg/L	1.68 mg/L	4.2 mg/L					
Vitellogenin Induction	1	0.1428	0.379	0.421	0.6251	**	0.3427	0.0174	0.7779	1.2872
	2	0.1629	0.3235	0.4385	0.6575	**	0.3602	0.0136	0.8177	1.3531
	3	0.187	0.3999	0.5021	0.6901	**	0.3632	0.0176	0.8245	1.3642
	4	0.1819	0.4021	0.475	0.7001	**	0.3700	0.0179	0.8399	1.3898

Vitellogenin induction expressed as $\mu g.ml^{-1}$.
*, ** indicate significance at 5 and 1% respectively (n = 6) NS = Not Significant
SE (m) = Mean Standard Error. CD = Critical Difference

Table 4.4.10 : Consistent Trends in the Variation of the effect of MP on Vitellogenin Induction in *Cyprinus carpio* (Grouped by exposure concentrations) during a 30 Exposure

Parameter	Exposure period (Week)	Specific Comparison among different Exposure concentrations showing significant to highly significant differences
Vitellogenin Induction	1	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4
	2	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
	3	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
	4	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4

Exposure concentrations: 1 – Control; 2 - 0.84 mg/L; 3 - 1.68 mg/L; 4 - 4.2 mg/L

Table 4.4.11 : Effect of MP on Vitellogenin Induction in *Cyprinus carpio* during a 30 days Exposure (Grouped by Week)

Parameter	Exposure concentrations (mg/L)	Mean Values at Exposure Period (week)				Significance of 'F' Test	SE (m) Between Exposure period	SE (m) Within Exposure period	CD (5%)	CD (1%)
		1	2	3	4					
MP	Control	0.1428	0.1629	0.187	0.1819	**	0.0348	0.0055	0.0791	0.1308
	0.84	0.379	0.3235	0.3999	0.4021	**	0.0634	0.0144	0.1439	0.2381
	1.68	0.421	0.4385	0.5021	0.4750	**	0.0631	0.0170	0.1431	0.2369
	4.2	0.6251	0.6575	0.6901	0.7001	*	0.0589	0.0243	0.1337	0.2212

Vitellogenin induction expressed as $\mu g.ml^{-1}$.
*, ** indicate significance at 5 and 1% respectively (n = 6) NS = Not Significant
SE (m) = Mean Standard Error. CD = Critical Difference

Table 4.4.12 : Consistent Trends in the Variation of the effect of MP in *Cyprinus carpio* during a 30 days Exposure period on Vitellogenin Induction (Grouped by Exposure period) in different Exposure Concentrations

Parameter	Exposure concentrations (mg/L)	Specific comparison among different exposure period showing significant to highly significant differences
MP	Control	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4
	1.68	1 & 3, 2 & 3
	4.2	1 & 2, 2 & 3, 2 & 4

Exposure Period : 1 – 1st Week; 2 – 2nd Week; 3 – 3rd Week; 4 – 4th Week

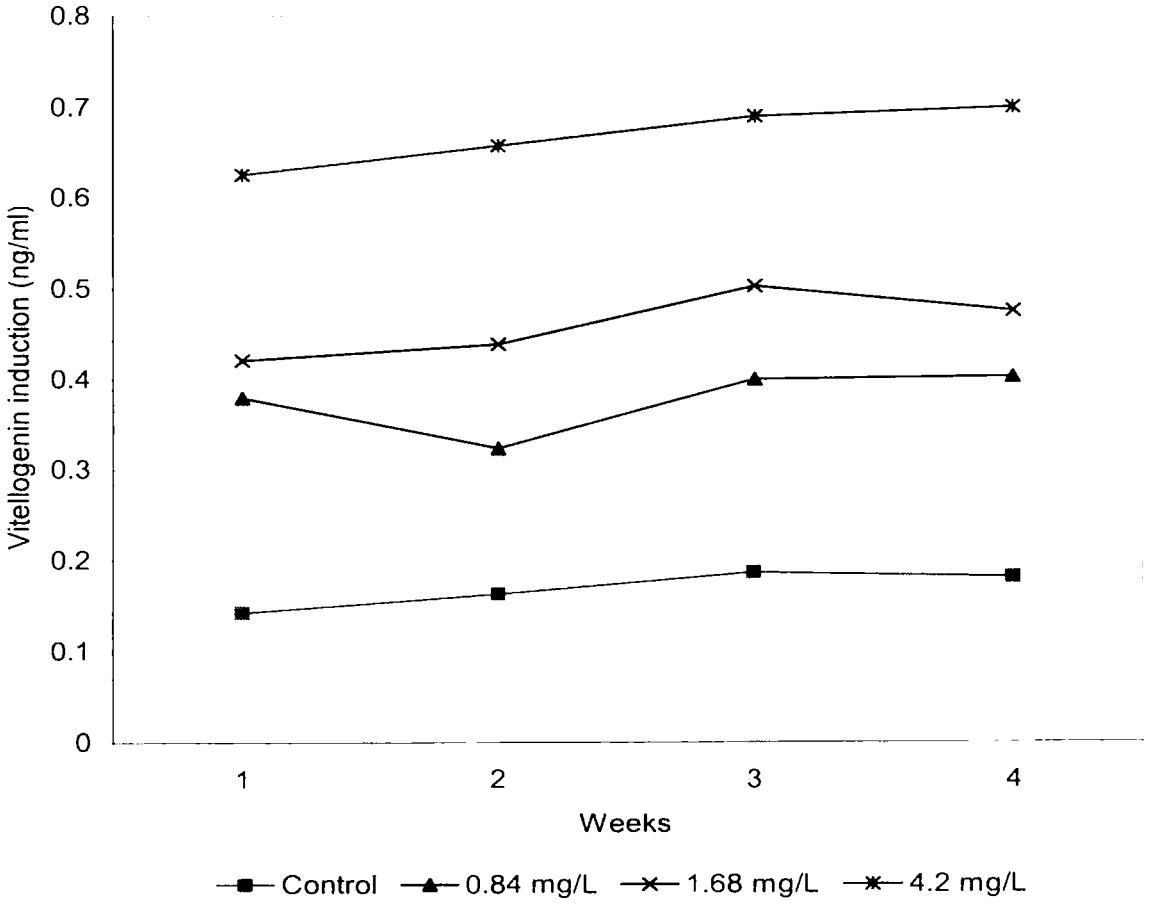

Fig. 4.8 : Effect of Methyl paraben on vitellogenin induction

4.5 Tertiary Butylphenol

4.5.1 Acid Phosphatase (AcP)

Data pertaining to Acid Phosphatase (AcP) activity in response to Tertiary Butylphenol (TBP) exposure at 0.69, 1.38 and 2.3 mg/L for 28 days is reported in **Tables 4.5.1** through **4.5.4** and **Fig. 4.9**. The AcP activity was increased in the higher two exposure concentration throughout the exposure period; however at the lowest exposure concentration it was not significantly different from control throughout the experimental period. The highest activity observed for 1.38 mg/L exposure was 25.655±0.523 µmoles/mg of protein in the 3rd week of exposure, while the lowest was 13.088 ± 1.86 µmoles/mg of protein during the 1st week. At 2.3 mg/L exposure the lowest AcP activity (17.478 ± 0.329 µmoles/mg of protein) was reported in the 1st week while the highest activity (25.655 ± 0.278 µmoles/mg of protein) was reported in the 3rd week.

4.5.2 Alkaline Phosphatase (AlP)

Data pertaining to the AlP activity of *Cyprinus carpio* in response to TBP exposure is reported in **Tables 4.5.1** through **4.5.4** and **Fig. 4.9**. Looking at the table and graph it can be deduced that TBP has an inhibitory effect on the AlP of the exposed fish, even though initially the activity was higher as compared to control but in the 4th week it was inhibited.

At the lowest exposure concentration i.e. 0.69 mg/L the initial activity was 0.493 ± 0.007 µmoles/mg of protein as compared to 0.197 ± 0.002 µmoles/mg of protein in control, however in the 4th week , it increased to 0.527 ± 0.071 µmoles/mg of protein which was still lower as compared to control (1.458 ± 0.045 µmoles/mg of protein). At the highest exposure concentration

Table 4.5.1 : Effect of TBP on Various Indices and Enzymatic Activities of *Cyprinus carpio* (Grouped by exposure concentrations) over 30 days

S.N.	Parameter	Exposure period (Week)	Mean Values at exposure concentrations				Significance of 'F' Test	SE (m) Between exposure concentrations	SE (m) Within exposure concentrations	CD (5%)	CD (1%)
			Control	0.69 mg/L	1.38 mg/L	2.3 mg/L					
1	GSI	1	6.8494	6.6772	1.7670	1.9421	**	4.9119	0.5648	11.1493	18.4485
		2	7.1259	7.1693	4.5483	4.9382	**	2.4203	0.6934	5.4938	9.0904
		3	7.7149	7.1113	4.1019	4.2516	**	3.2661	0.6308	7.4137	12.2672
		4	8.6078	7.0665	4.0080	3.9857	**	3.9920	0.7307	9.0613	14.9935
2	HSI	1	1.0604	1.0630	1.4550	0.9193	**	0.3990	0.0727	0.9058	1.4987
		2	0.9986	1.5792	1.8571	1.4320	NS	0.6208	0.4121	--	--
		3	0.8916	1.8103	1.3793	1.9591	*	0.8315	0.3416	1.8873	--
		4	1.1781	1.3422	1.1593	1.4579	NS	0.2457	0.2412	--	--
3	CSI	1	1.3648	1.2118	1.2462	1.2161	NS	0.1242	0.1630	--	--
		2	1.0987	1.2140	1.1915	1.0861	NS	0.1118	0.1917	--	--
		3	0.9284	1.3045	1.3439	0.9355	NS	0.3933	0.2750	--	--
		4	1.5336	1.0827	1.0534	1.1990	*	0.3813	0.1796	0.8654	--
4	RSI	1	0.6020	1.4937	1.3685	1.2372	**	0.6865	0.0610	1.5582	2.5783
		2	0.5962	1.3945	1.1952	1.3345	**	0.6333	0.1682	1.4374	2.3784
		3	0.5662	1.1554	1.1341	1.0838	*	0.4862	0.2138	1.1037	1.8262
		4	0.4807	1.2906	1.3042	0.8548	**	0.6830	0.1251	1.5503	2.5653
5	AcP	1	18.1060	17.2586	17.2390	17.4785	NS	0.7017	0.4040	--	--
		2	17.6777	16.6577	18.4111	19.0624	**	1.7872	0.4459	4.0568	6.7126
		3	17.6993	16.3128	25.1047	25.6557	**	8.4403	0.5237	19.1583	31.7008
		4	16.5632	16.7842	21.8210	18.0800	**	4.2142	0.9877	9.5657	15.8282
6	AlP	1	0.1971	0.4908	0.2916	0.5612	*	0.2937	0.1104	0.6668	--
		2	0.3902	0.3609	0.3972	0.3327	NS	0.0512	0.0463	--	--
		3	0.9680	0.7421	0.8218	0.4163	**	0.4043	0.1007	0.9176	1.5184
		4	1.4587	0.5271	0.7786	0.5493	**	0.7539	0.1043	1.7113	2.8316
7	SGOT	1	15.0582	11.4449	12.3713	11.4684	**	2.9511	0.1906	6.6986	11.0839
		2	16.3753	15.3398	13.9633	15.2957	**	1.7123	0.5079	3.8866	6.4310
		3	18.5140	16.1086	16.6867	16.2977	**	1.9078	0.4144	4.3304	7.1653
		4	16.8201	11.4757	11.5699	9.5935	**	5.3807	0.5618	12.2135	20.2094
8	SGPT	1	7.2781	8.3321	8.7206	8.7875	*	1.2076	0.5500	2.7410	--
		2	8.9688	20.2207	20.1229	20.2333	**	9.7202	1.5268	22.0635	36.5080
		3	10.8757	19.8143	19.9308	19.8203	**	7.7770	0.7429	17.6526	29.2094
		4	8.8435	16.5748	17.7711	18.1764	**	7.5950	0.5322	17.2396	28.5260

*, ** indicate significance at 5 and 1% respectively (n = 6). NS = Not Significant. SE (m) = Mean Standard Error. CD = Critical Difference

AcP and AlP expressed as µmoles.mg of protein^{-1}. SGOT and SGPT expressed as nmoles oxaloacetate.mg of protein^{-1}.

Table 4.5.2 : Consistent Trends in the Variation of the effect of TBP on Important Indices and Enzymatic Activities of *Cyprinus carpio* (Grouped by exposure concentrations) for an Exposure Period of 30 days

S.N.	Parameters	Exposure period (Week)	Specific Comparison among different exposure concentrations showing significant to highly significant differences
1	GSI	1	1 & 3, 1 & 4, 2 & 3, 2 & 4
		2	1 & 3, 2 & 3
		3	1 & 3, 1 & 4, 2 & 3, 2 & 4
		4	1 & 3, 1 & 4, 2 & 3, 2 & 4
2	HSI	1	1 & 3, 2 & 3, 3 & 4
3	RSI	1	1 & 2, 1 & 3, 1 & 4, 2 & 4
		2	2 & 3, 2 & 4, 3 & 4
		4	1 & 2, 1 & 3, 2 & 4, 3 & 4
4	AcP	2	2 & 3, 2 & 4
		3	1 & 3, 1 & 4, 2 & 3, 2 & 4,
		4	1 & 3, 2 & 3, 3 & 4
5	AlP	1	1 & 4
		3	1 & 4, 2 & 4, 3 & 4
		4	1 & 2, 1 & 3, 1 & 4
6	SGOT	1	1 & 2, 1 & 3, 1 & 4, 2 & 3, 3 & 4
		2	1 & 3
		3	1 & 2, 1 & 3, 1 & 4
		4	1 & 2, 1 & 3, 1 & 4, 2 & 4, 3 & 4
7	SGPT	2	1 & 2, 1 & 3, 1 & 4
		3	1 & 2, 1 & 3, 1 & 4
		4	1 & 2, 1 & 3, 1 & 4

Exposure concentrations: 1 - Control; 2 - 0.69 mg/L; 3 - 1.38 mg/L; 4 - 2.3 mg/L

Table 4.5.3 : Effect of TBP on Various Indices and Enzymatic Activities of *Cyprinus carpio* for an Exposure Period of 30 days(Grouped by Week)

S.N.	Parameter	Exposure concentrations (mg/L)	Mean Values at Exposure Period (week)				Significance of 'F' Test	SE (m) Between Exposure period	SE (m) Within Exposure period	CD (5%)	CD (1%)
			1	2	3	4					
1	GSI	Control	6.8494	7.1259	7.7149	8.6078	NS	1.3470	0.0000	--	--
		0.69	6.6772	7.1693	7.1113	7.0665	NS	0.3867	0.9062	--	--
		1.38	1.7670	4.5483	4.1019	4.0080	**	2.1628	0.6124	4.9092	8.1231
		2.3	1.9421	4.9382	4.2516	3.9857	**	2.2325	0.7317	5.0675	8.3850
2	HSI	Control	1.0604	0.9986	0.8916	1.1781	**	0.2073	0.0000	0.4706	0.7786
		0.69	1.0630	1.5792	1.8103	1.3422	NS	0.5549	0.3249	--	--
		1.38	1.4550	1.8571	1.3793	1.1593	NS	0.5046	0.4359	--	--
		2.3	0.9193	1.4320	1.9591	1.4579	**	0.7355	0.2335	1.6694	2.7624
3	CSI	Control	1.3648	1.0987	0.9284	1.5336	**	0.4675	0.0000	1.0611	1.7558
		0.69	1.2118	1.2140	1.3045	1.0827	NS	0.1580	0.2392	--	--
		1.38	1.2462	1.1915	1.3439	1.0534	NS	0.2100	0.2946	--	--
		2.3	1.2161	1.0861	0.9355	1.1990	NS	0.2241	0.1648	--	--
4	RSI	Control	0.6020	0.5962	0.5662	0.4807	**	0.0969	0.0000	0.2200	0.3640
		0.69	1.4937	1.3945	1.1554	1.2906	NS	0.2509	0.2037	--	--
		1.38	1.3685	1.1952	1.1341	1.3042	NS	0.1828	0.2128	--	--
		2.3	1.2372	1.3345	1.0838	0.8548	**	0.3622	0.0812	0.8222	1.3604
5	AcP	Control	18.1060	17.6777	17.6993	16.5632	**	1.1470	0.0000	2.6035	4.3080
		0.69	17.2586	16.6577	16.3128	16.7842	NS	0.6778	0.4755	--	--
		1.38	13.0888	16.4111	25.1047	21.8210	**	9.3180	2.0140	21.1505	34.9972
		2.3	17.4785	19.0624	25.6557	18.0800	**	6.5491	0.6878	14.8656	24.5978
6	AlP	Control	0.1971	0.3902	0.9680	1.4587	**	0.9924	0.0000	2.2526	3.7274
		0.69	0.4908	0.3609	0.7421	0.5271	**	0.2741	0.0889	0.6223	1.0296
		1.38	0.2916	0.3972	0.8218	0.7786	**	0.4629	0.0973	1.0508	1.7387
		2.3	0.5612	0.3327	0.4163	0.5493	NS	0.1904	0.1341	--	--
7	SGOT	Control	15.0582	16.3753	18.5140	16.8201	NS	2.4710	0.0000	--	--
		0.69	11.4449	15.3398	16.1086	11.4757	**	4.2984	0.4489	9.7569	16.1445
		1.38	12.3713	13.9633	16.6867	11.5699	**	3.9091	0.5496	8.8732	14.6823
		2.3	11.4684	15.2957	16.2977	9.5935	**	5.4761	0.5273	12.4300	20.5677
8	SGPT	Control	7.2781	8.9688	10.8757	8.8435	**	2.5512	0.0000	5.7908	9.5818
		0.69	8.3321	20.2207	19.8143	16.5748	**	9.5535	1.0730	21.6851	35.8818
		1.38	8.7206	20.1229	19.9308	17.7711	**	9.3250	1.1748	21.1665	35.0237
		2.3	8.7875	20.2333	19.8203	18.1764	**	9.3272	0.9682	21.1714	35.0318

*, ** indicate significance at 5 and 1% respectively (n 6). NS - Not Significant. SE (m) - Mean Standard Error. CD - Critical Difference

AcP and AlP expressed as µmoles.mg of protein^{-1}. SGOT and SGPT expressed as nmoles oxaloacetate.mg of protein^{-1}.

Table 4.5.4 : Consistent Trends in the Variation of the effect of TBP on Important Indices and Enzymatic Activities of *Cyprinus carpio* (Grouped by exposure period) at different Exposure Concentrations during a period of 30 days

S.N.	Parameter	Exposure concentrations (mg/L)	Specific Comparison among different Exposure period showing significant to highly significant differences
1	GSI	1.38	1 & 2, 1 & 3, 1 & 4
		2.3	1 & 2
2	HSI	Control	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		2.3	1 & 3
3	CSI	Control	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
4	RSI	Control	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		2.3	1 & 4, 2 & 4
5	AcP	Control	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		1.38	1 & 3, 1 & 4, 2 & 3
		2.3	1 & 3, 2 & 3, 3 & 4
6	AlP	Control	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		0.69	2 & 3
		1.38	1 & 3, 1 & 4, 2 & 3, 2 & 4
7	SGOT	0.69	1 & 2, 1 & 3, 2 & 4, 3 & 4,
		1.38	1 & 3, 2 & 3, 2 & 4, 3 & 4
		2.3	1 & 2, 1 & 3, 1 & 4, 2 & 4, 3 & 4
8	SGPT	Control	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
		0.69	1 & 2, 1 & 3, 1 & 4, 2 & 4
		1.38	1 & 2, 1 & 3, 1 & 4
		2.3	1 & 2, 1 & 3, 1 & 4

Exposure Period : 1 - 1st Week; 2 - 2nd Week; 3 - 3rd Week; 4 - 4th Week

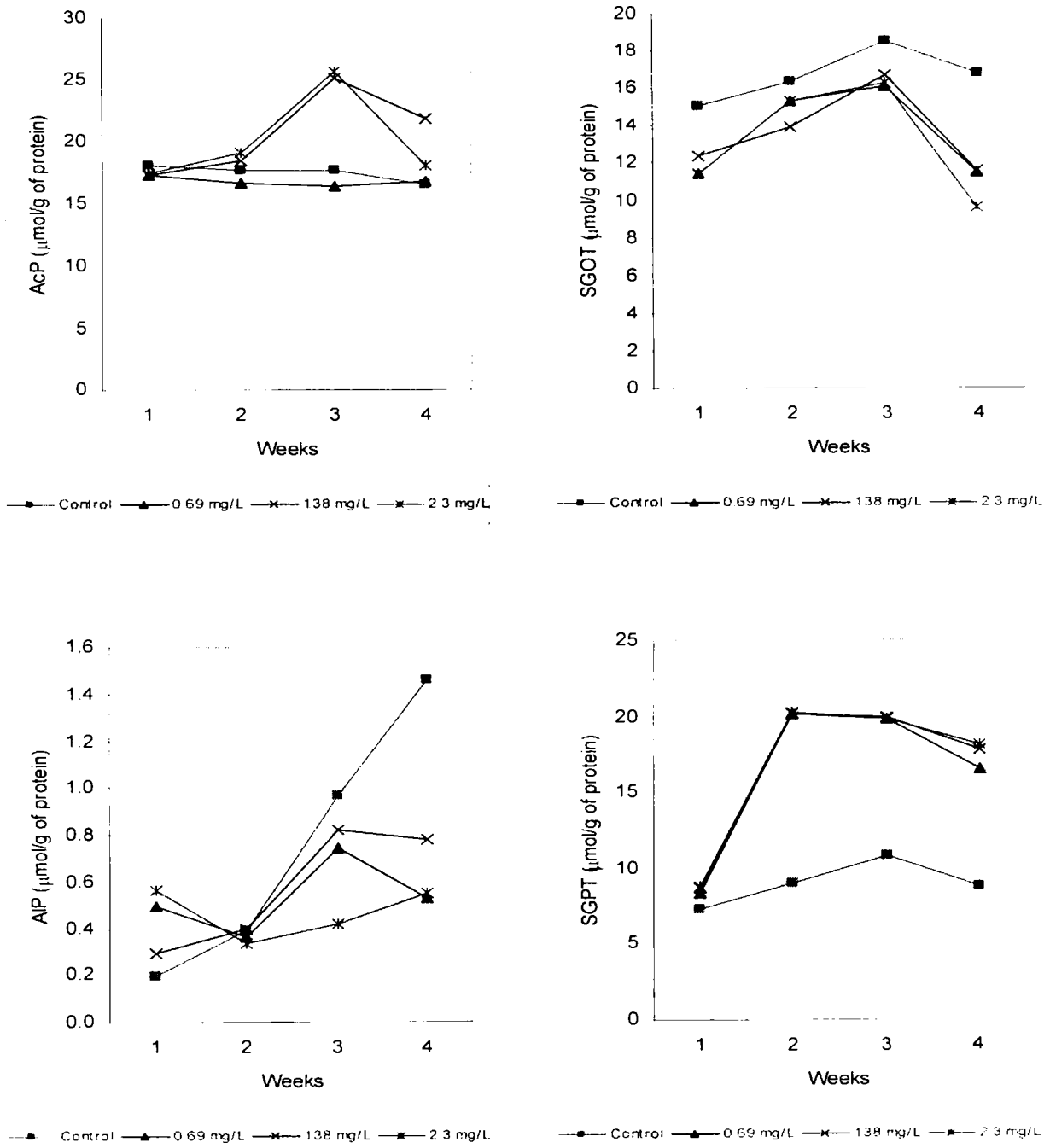

Fig. 4.9 : Mean enzymatic responses of Cyprinus carpio to 4-tert butylphenol

i.e. 2.3 mg/L the activity in the 1st week was 0.561 ± 0.0126 µmoles/mg of protein and during the final week it was not significantly different (P>0.05) from the first week.

4.5.3 Serum Glutamic Oxalacetic Transaminase (SGOT) / Aspartate Aminotransferase (AST)

Data pertaining to SGOT activity of carp exposed to TBP at 0.69, 1.38 and 2.3 mg/L is given in **Tables 4.5.1** through **4.5.4** and **Fig. 4.9**. SGOT was significantly inhibited (P<0.05) in all the exposed fish. Initially in all the exposures the activity was inhibited as compared to control irrelevant to the concentration of TBP, however the activity seemed to recover and increased up to 3rd week of exposure. Conversely in the final week of exposure the SGOT activity was again inhibited. The maximum inhibition was observed in response to 2.3 mg/L exposure in the 4th week.

4.5.4 Serum Glutamic Pyruvic Transaminase (SGPT) / Alanine Aminotransferase (ALT)

Data pertaining to Serum Glutamic Pyruvic Transaminase (SGPT) of *Cyprinus carpio* exposed to Tertiary Butylphenol at 0.69, 1.38 and 2.3 mg/L is given in **Tables 4.5.1** through **4.5.4** graphically represented in **Fig. 4.9**. SGPT activity was enhanced in response to TBP in all the exposures. Starting first week onwards there was a significant increase (P<0.05) in the activity as compared to control. At the lowest exposure concentration i.e. 0.69 mg/L the highest activity (20.22 ± 1.09 nmoles oxaloacetate/mg of protein) was observed during the 2nd week of exposure which was more than 2 times

higher than the control (8.968 ±0.034 nmoles oxaloacetate/mg of protein), in the 4th week of exposure however this activity was lowered considerably to 16.574 ± 0.28 nmoles oxaloacetate/mg of protein as compared to 8.843 ± 0.02 nmoles oxaloacetate/mg of protein in control.

4.5.5 Gonadosomatic Index (GSI)

Data pertaining the impact of TBP on the Gonadosomatic Index (GSI) of the exposed carp is given in **Tables 4.5.1** through **4.5.4** and **Fig. 4.10**. Except for the lowest exposure concentration the GSI was significantly inhibited in the higher two exposures. At the end of the experimental period i.e. 4th week the GSI for 0.69, 1.38 and 2.3 mg/L was 7.066 ± 0.74 , 4 ± 0.333 and 3.985 ± 0.23 respectively as compared to 8.607 ±0.023 in control.

4.5.6 Hepatosomatic Index (HSI)

Data pertaining to HSI of *Cyprinus carpio* exposed to TBP at 0.69, 1.38 and 2.3 mg/L, for 28 days is given in **Tables 4.5.1** through **4.5.4** and graphically represented in Fig. **4.10**. During the 1st week of exposure there the HSI was lowered at 2.3 mg/L exposure, whereas there was no significant difference in the lower two exposures. For the rest of the exposure period there was no significant impact on the HSI of all the exposed fish.

4.5.7 Renosomatic Index (RSI)

Data pertaining to RSI is represented in **Tables 4.5.1** through **4.5.4** and **Fig. 4.10**.The RSI was significantly increased in all the exposed fish starting 1st week onwards. At the lowest exposure concentration the initial RSI was 1.493 ±0.006 which did significantly differ (P>0.05) in the 4th week, same

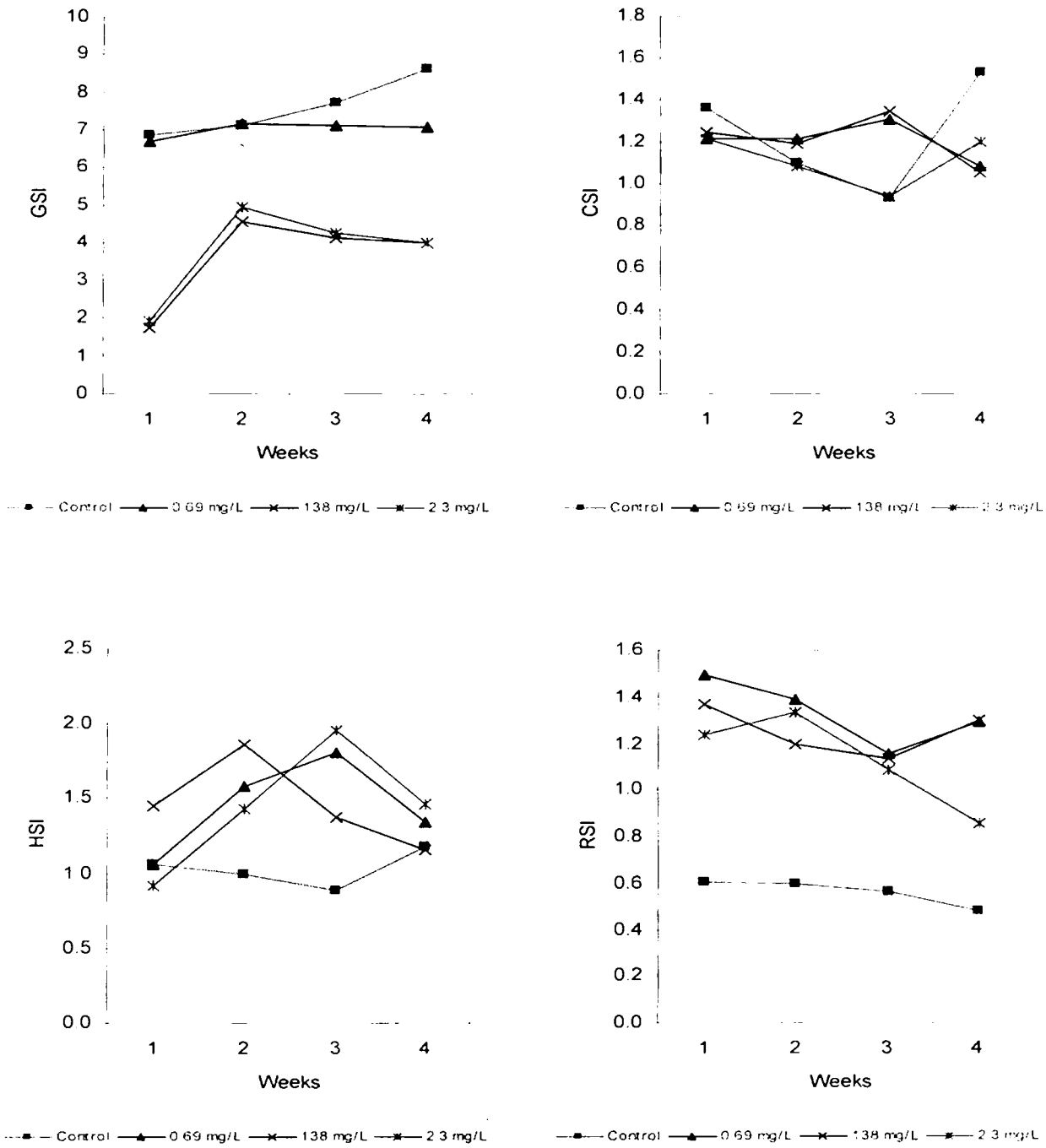

Fig. 4.10 : Somatic indices of Cyprinus carpio exposed to 4-tert butylphenol

trend was observed in the 1.38 mg/L. At the highest exposure concentration however the HSI was significantly inhibited in the 4th week as compared to the 1st week, however at no time it was lower than the control HSI.

4.5.8 Craniosomatic Index (CSI)

Impact on TBP on the Craniosomatic Index (CSI) of *Cyprinus carpio* exposed at 0.69, 1.38 and 2.3 mg/L for 28 days is given in **Tables 4.5.1** through **4.5.4** and graphically represented in **Fig. 4.10**. There was no significant difference (P>0.05) between control and 0.69, 1.38 and 2.3 mg/L during all the experimental period.

4.5.9 Fish behavior and general appearance

Data pertaining to the behavior and appearance of carp exposed to tertiary butylphenol at 0.69, 1.38 and 2.3 mg/L for a period of 28 days is given in **Table 4.5.5.**

4.5.10 Vitellogenin Induction

Data pertaining to the impact of tertiary butylphenol (TBP) on vitellogenin induction of carp exposed at 0.69, 1.38 and 2.3 mg/L for 28 days is given in **Tables 4.5.6** through **4.5.9** and **Fig. 4.11**. There was a time and concentration related increase in the vitellogenin content of the exposed carp. Even at the lowest exposure concentration i.e. 0.69 mg/L, starting 1st week onwards, there was significant increase in the vitellogenin content as compare to control, and it continued to increase till the end of experiment.

Table 4.5.5 : Behavioral responses of *Cyprinus carpio* exposed to Tertiary butylphenol at different concentrations over 30 days

Parameter	Exposure concentrations			
	control	0.69 mg/L	1.38 mg/L	2.3 mg/L
Behavior	non aggressive	non aggressive	non aggressive	non aggressive
Feed consumption	within 10-15 minutes	within 10-15 minutes	within 10-15 minutes	within 10-15 minutes
Movements	normal	normal	normal	normal
Coloration	normal	normal	normal	normal
Tapping response	normal	normal	normal	normal

Table 4.5.6 : Effect of TBP on Vitellogenin Induction of *Cyprinus carpio* (Grouped by Exposure concentrations) during 30 days Exposure

Parameter	Exposure period (Weeks)	Mean Values at Exposure concentrations				Significance of 'F' Test	SE (m) Between Exposure concentrations	SE (m) Within Exposure concentrations	CD (5%)	CD (1%)
		Control	0.69 mg/L	1.38 mg/L	2.3 mg/L					
Vitellogenin Induction	1	0.1428	0.3234	0.3131	0.32	**	0.1996	0.0123	0.4530	0.7496
	2	0.1629	0.3875	0.4968	0.565	**	0.3048	0.0146	0.6919	1.1450
	3	0.187	0.4005	0.5072	0.578	**	0.2953	0.0168	0.6704	1.1093
	4	0.1819	0.3901	0.5173	0.523	**	0.2766	0.0154	0.6279	1.0389

Vitellogenin induction expressed as $\mu g.ml^{-1}$.
*, ** indicate significance at 5 and 1% respectively (n = 6) NS = Not Significant
SE (m) = Mean Standard Error. CD = Critical Difference

Table 4.5.7 : Consistent Trends in the Variation of the effect of TBP on Vitellogenin Induction of *Cyprinus carpio* (Grouped by Exposure concentrations) during 30 days Exposure

Parameter	Exposure period (Week)	Specific comparison among different exposure concentrations showing significant to highly significant differences
Vitellogenin Induction	1	1 & 2,1 & 3, 1 & 4, 2 & 3, 2 & 4
	2	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
	3	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4, 3 & 4
	4	1 & 2, 1 & 3, 1 & 4, 2 & 4, 3 & 4

Exposure concentrations: 1 - Control; 2 - 0.69 mg/L; 3 - 1.38 mg/L; 4 - 2.3 mg/L

Table 4.5.8 : Effect of TBP on Vitellogenin Induction in *Cyprinus carpio* during a 30 days Exposure (Grouped by Week)

Parameter	Exposure concentrations (mg/L)	Mean Values at Exposure Period (week)				Significance of 'F' Test	SE (m) Between Exposure period	SE (m) Within Exposure period	CD (5%)	CD (1%)
		1	2	3	4					
TBP	Control	0.1428	0.1629	0.187	0.1819	**	0.0348	0.0055	0.0791	0.1308
	0.69	0.3234	0.3875	0.4005	0.3901	**	0.0608	0.0141	0.1380	0.2284
	1.38	0.3131	0.4968	0.5072	0.5173	**	0.1686	0.0161	0.3828	0.6334
	2.3	0.42	0.565	0.578	0.5230	**	0.1240	0.0181	0.2815	0.4659

Vitellogenin induction expressed as $\mu g.ml^{-1}$.
*, ** indicate significance at 5 and 1% respectively (n = 6) NS = Not Significant
SE (m) = Mean Standard Error. CD = Critical Difference

Table 4.5.9 : Consistent Trends in the Variation of the effect of TBP during a 30 days Exposure on Vitellogenin Induction in *Cyprinus carpio* (Grouped by Exposure period) in different Exposure Concentrations

Parameter	Exposure concentrations (mg/L)	Specific comparison among different exposure period showing significant to highly significant differences
TBP	Control	1 & 2, 1 & 3, 1 & 4, 2 & 3, 2 & 4
	0.69	1 & 2, 1 & 3, 1 & 4
	1.38	1 & 2, 1 & 3, 1 & 4
	2.3	1 & 2, 1 & 3, 1 & 4

Exposure Period : 1 – 1st Week; 2 – 2nd Week; 3 – 3rd Week; 4 – 4th Week

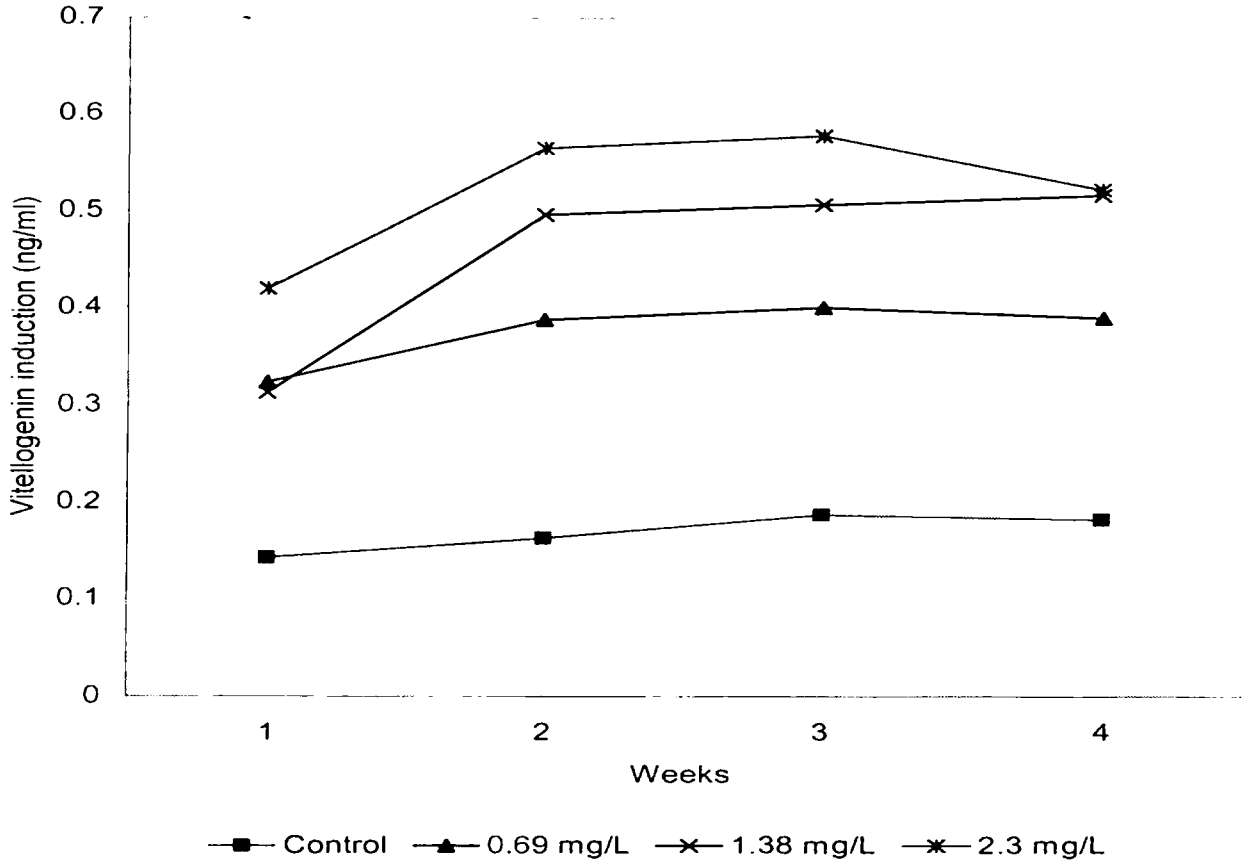

Fig. 3.11 : Effect of 4-tert butylphenol on vitellogenin induction

At 2.3 mg/L TBP exposure the vitellogenin content was 0.42 $\pm$ 0.021μg/ml during the first week as compared to 0.142 $\pm$ 0.05μg/ml in the control and increased to 0.523 $\pm$ 0.012μg/ml and 0.42 respectively during the last week.

Explanation of Photomicrographs

Explanation of Photomicrographs

Plate 8 : Liver cells of control *Cyprinus carpio* show normal architecture with regularly placed hepatocytes. Vacuolization is absent, thin cytoplasm with prominent nucleus, connective tissues.
Hematoxylin/Eosin, Bar = 100 μm

Plate 9 : Liver cells of *Cyprinus carpio* given waterborne exposure to 1 $mg.L^{-1}$ diethyl phthalate for 28 days, showing necrosis of cytoplasm. Also shows distinct increase in vacuolization and local degeneration of hepatocytes.
Hematoxylin/Eosin, Bar = 100 μm (H – Hepatocytes, Vac- Vacuoles)

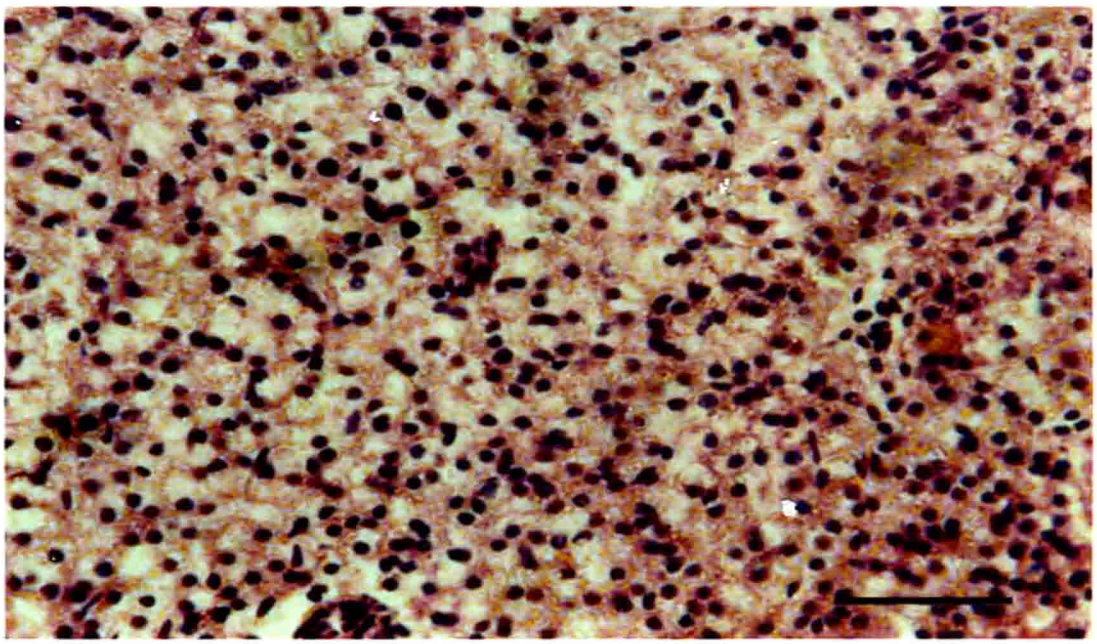

PLATE NO. 8

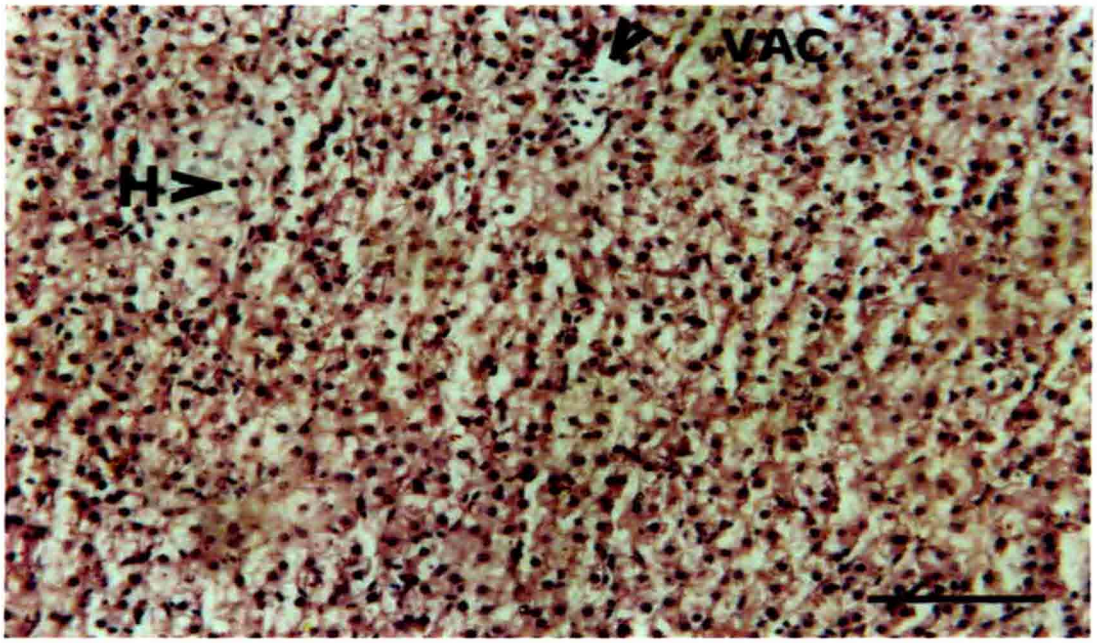

PLATE NO. 9

Explanation of Photomicrographs

Plate 10 : Liver cells of *Cyprinus carpio* exposed to 5 mg.L^{-1} diethyl phthalate for 28 days, showing an overall degeneration of connective tissue in progress. Vacuolization is also seen in abundance.
Hematoxylin/Eosin. Bar = 100 μm (NP – Necrotic patches, Vac- Vacuoles)

Plate 11 : Section of *Cyprinus carpio* liver exposed to at 20-mg.L^{-1} diethyl phthalate for 28 days, showing nuclei with irregular outlines, hyperplasia, mass degeneration of hepatocytes, large vacuoles are present, cytoplasm is totally disturbed.
Hematoxylin / Eosin Bar = 100 μm (NP – Necrotic patches, Vac- Vacuoles)

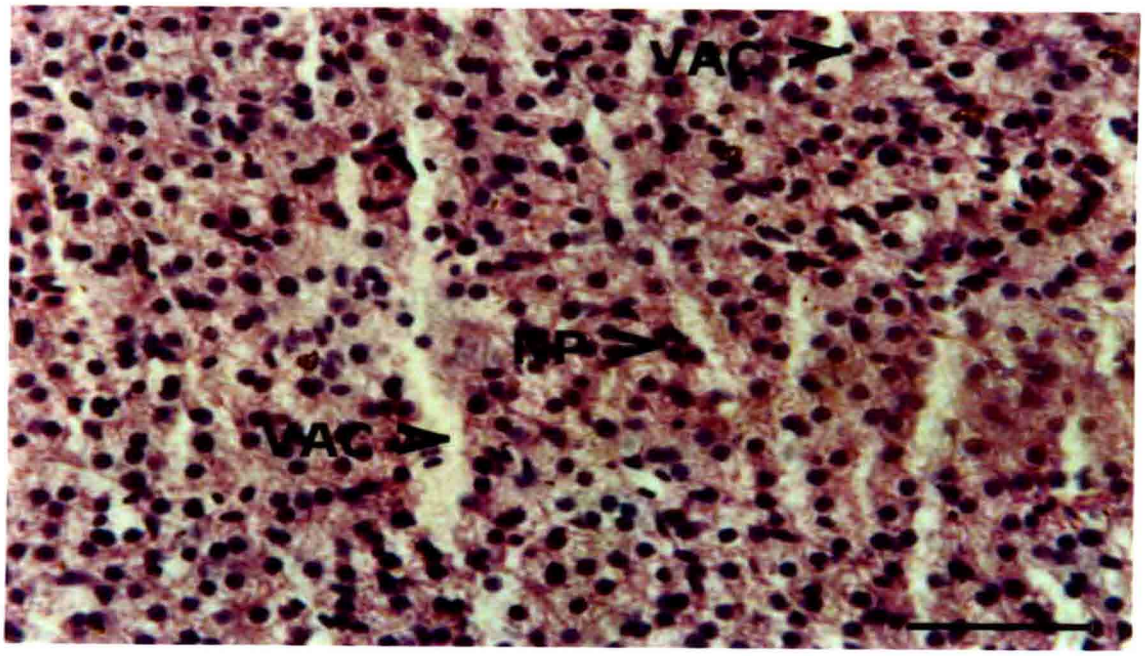

PLATE NO. 10

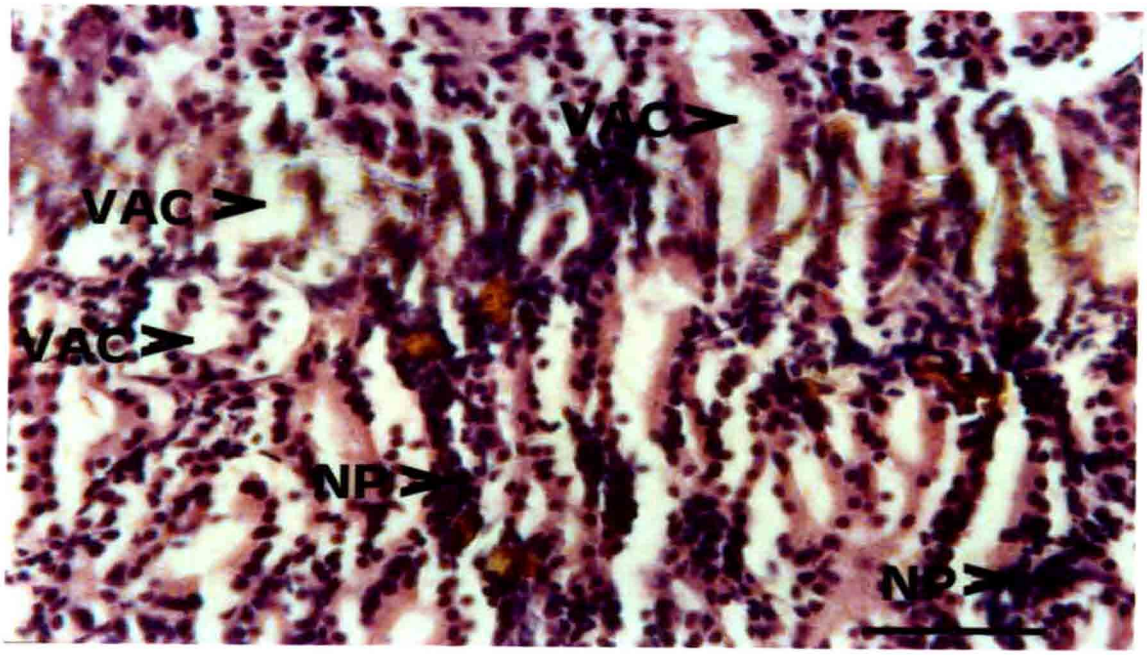

PLATE NO. 11

Explanation of Photomicrographs

Plate 12 : Section of liver of *Cyprinus carpio* exposed to Methyl paraben at 0.84 $mg.L^{-1}$ for 28 days showing mild inflammation and necrotic changes in hepatocytes.
Hematoxylin / Eosin Bar = 100 μm

Plate 13 : Liver of *Cyprinus carpio* exposed to Methyl paraben at 1.68-$mg.L^{-1}$ waterborne concentration for a period of 28 days showing increased vacuolization also necrosis of connective tissue can be observed.
Hematoxylin / Eosin Bar = 100 μm (NP – Necrotic patches, Vac- Vacuoles)

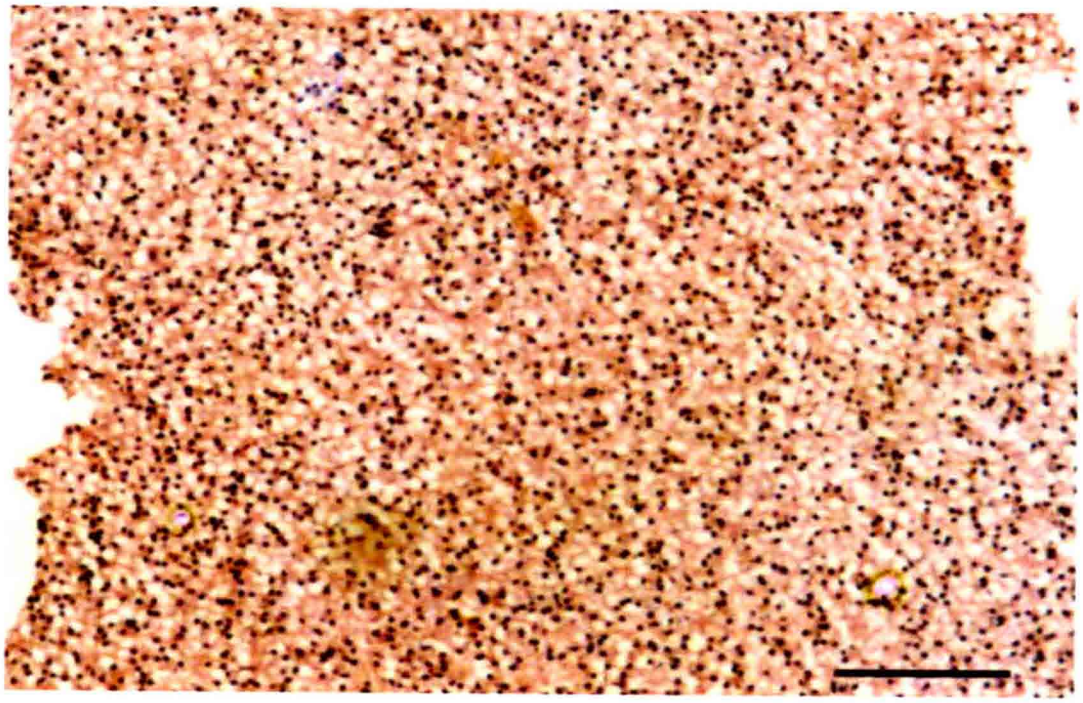

PLATE NO. 12

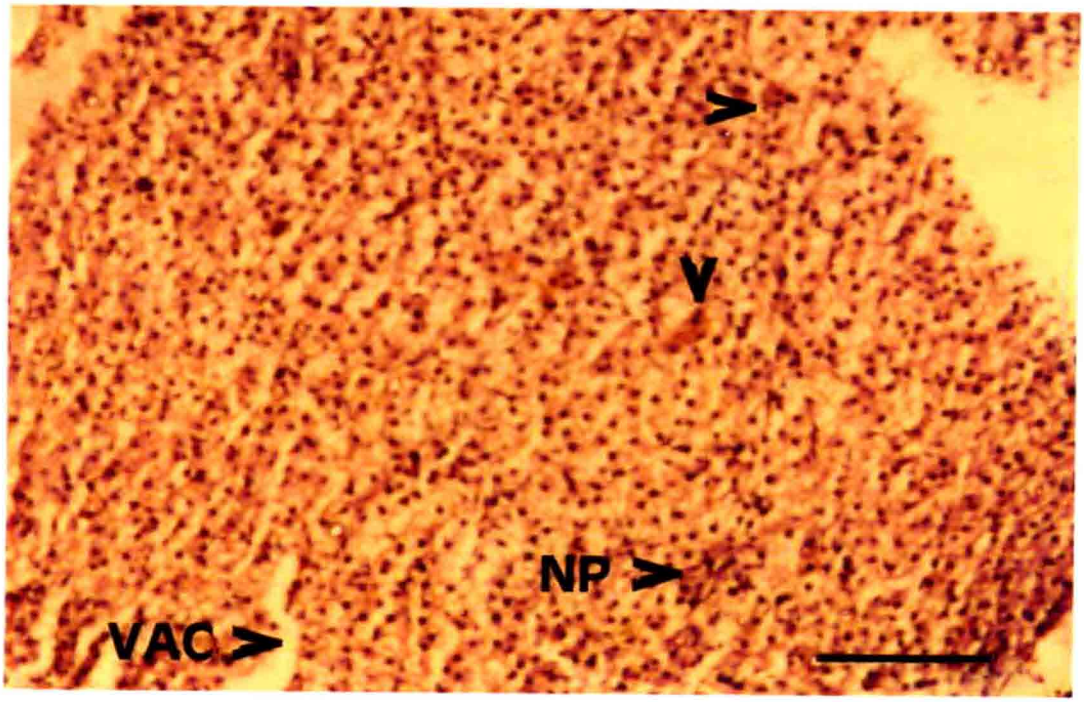

PLATE NO. 13

Explanation of Photomicrographs

Plate 14 : section of liver exposed to Methyl Paraben for 28 days at 4.2mg.L^{-1} showing necrotic hepatocytes. Also increase in size of vacuoles is seen (arrow). Hematoxylin / Eosin Bar = 100 μm (NH - Necrotic hepatocytes, Vac- Vacuoles)

Plate 15 : liver of *Cyprinus carpio* exposed to 4-tert butylphenol at 0.69 mg.L^{-1} for 28 days showing necrotic patches, also increase in size and number of vacuoles. Hematoxylin / Eosin Bar = 100 μm (Vac- Vacuoles)

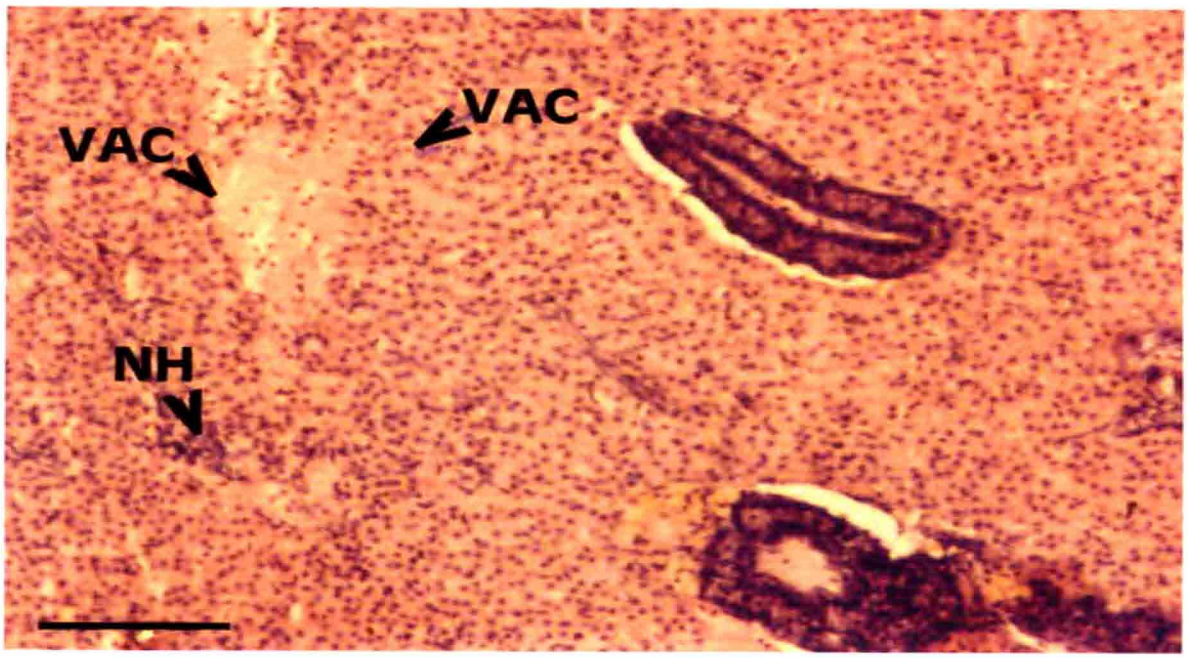

PLATE NO. 14

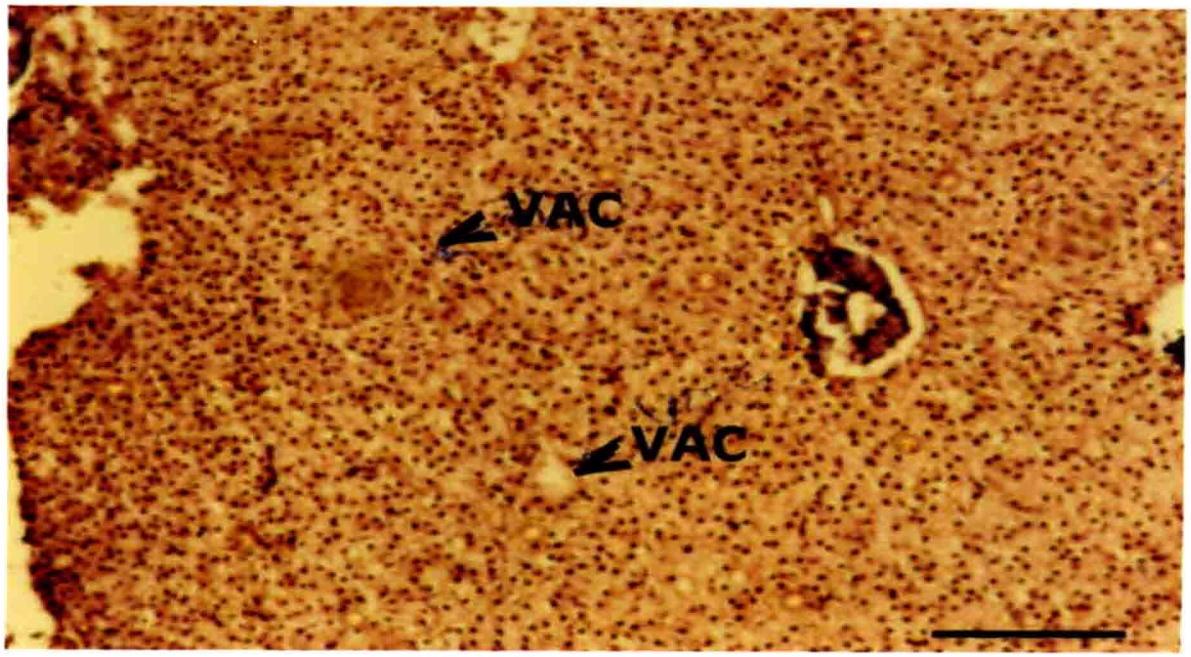

PLATE NO. 15

Explanation of Photomicrograph

Plate 16 : Section of liver exposed to 4-tert butylphenol at 1.38 $mg.L^{-1}$ for 28 days showing hyperplasia of connective tissue, extensive degeneration of hepatocytes , increased vacuolization and fibrous lesions.
Hematoxylin / Eosin Bar = 100 μm (NP- Necrotic patches, Vac- Vacuoles)

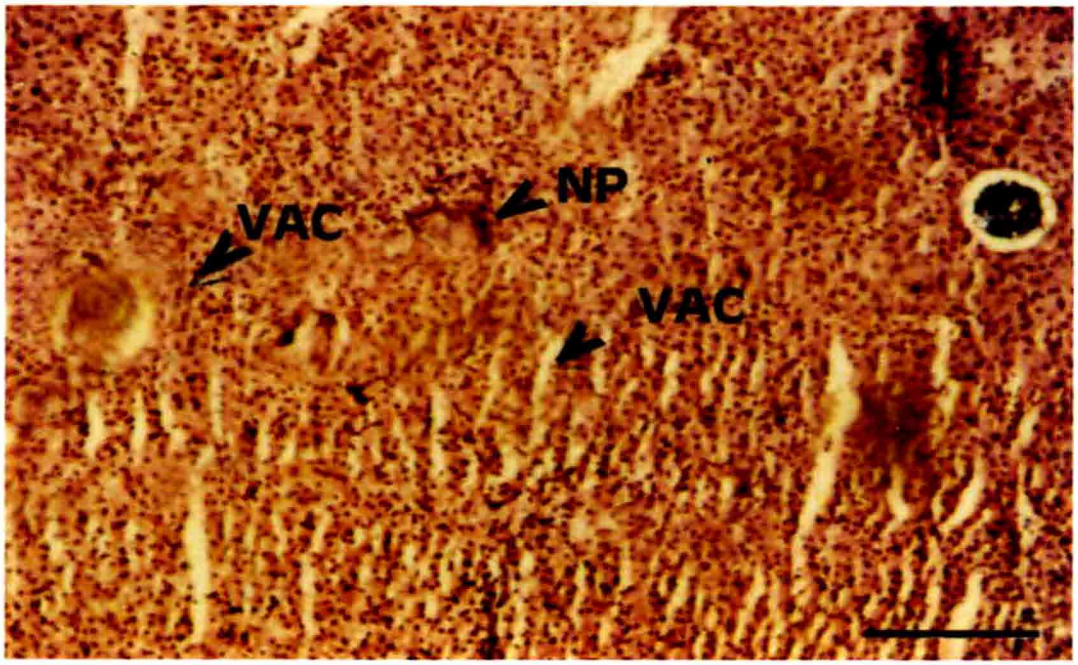

PLATE NO. 16

Explanation of Photomicrographs

Plate 17 : *Ovary of control Cyprinus carpio* showing normal architecture of oocytes.
Hematoxylin / Eosin Bar = 100 μm

Plate 18 : Ovary of *Cyprinus carpio* exposed to 4-tert butylphenol at 1.38-mg.L^{-1} showing degeneration of oocytes.
Hematoxylin / Eosin Bar = 100 μm

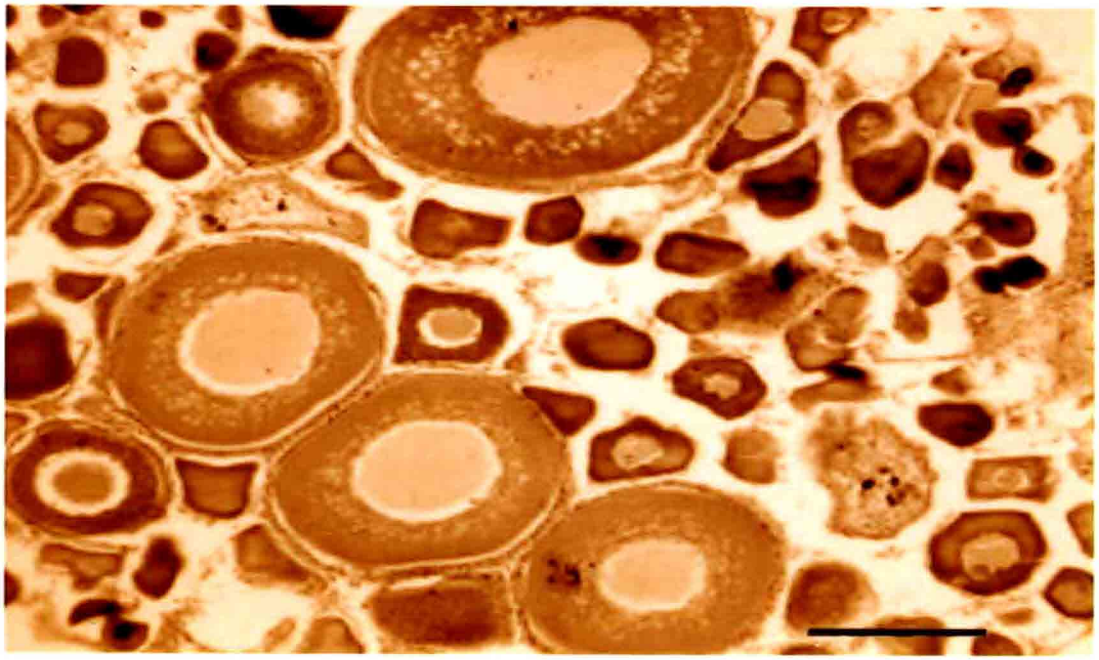

PLATE NO. 17

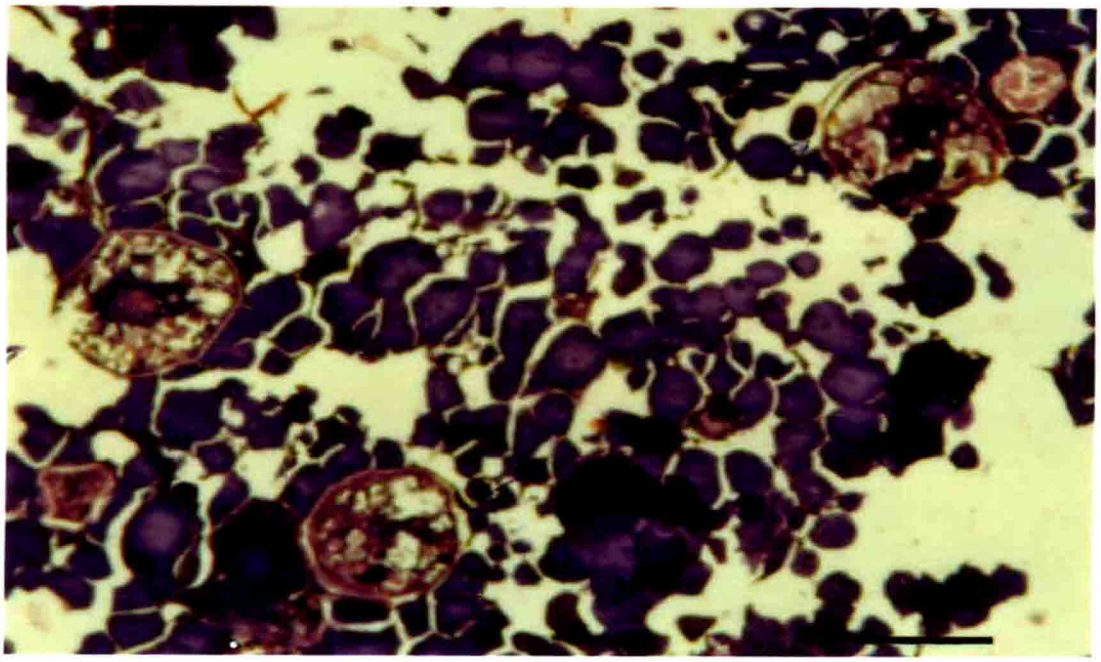

PLATE NO. 18

Explanation of Photomicrographs

Plate 19 : Section of testis of control *Cyprinus carpio* showing normal architecture. Spermatozoa can be seen in the lumen.
Hematoxylin / Eosin Bar = 100 μm

Plate 20 : Testis of *Cyprinus carpio* exposed to 1 $mg.L^{-1}$ Diethyl Phthalate for a period of 28 days. Note the disturbed architecture of lumens. The lumen is reduced as compared to control; also necrosis of spermatozoa can be seen.
Hematoxylin / Eosin Bar = 100 μm

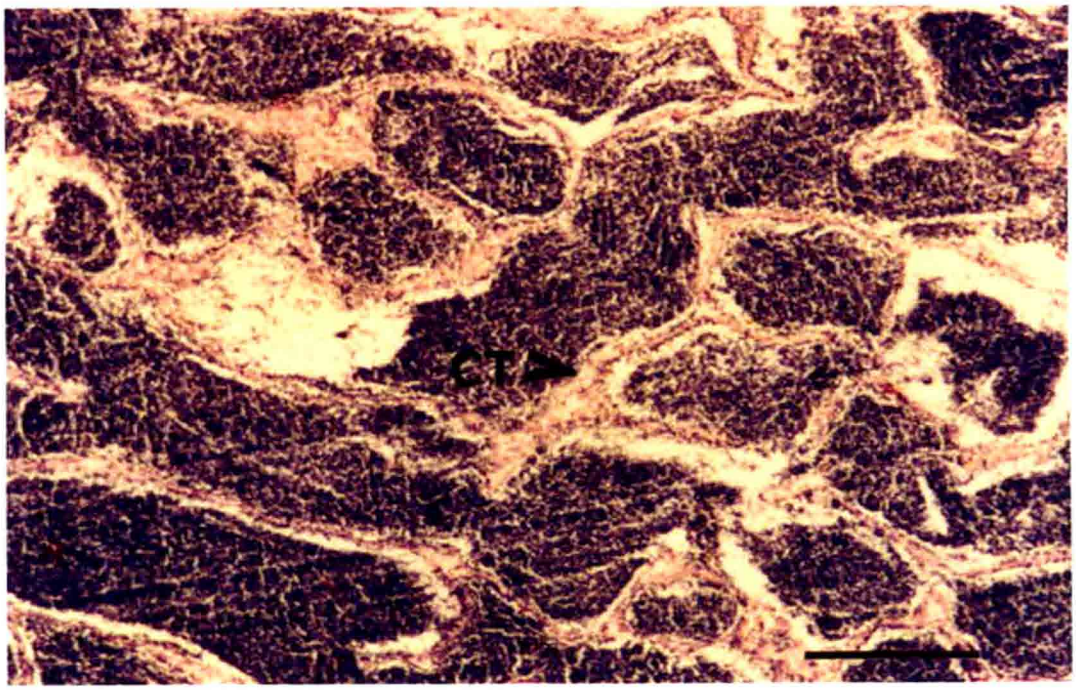

PLATE NO. 19

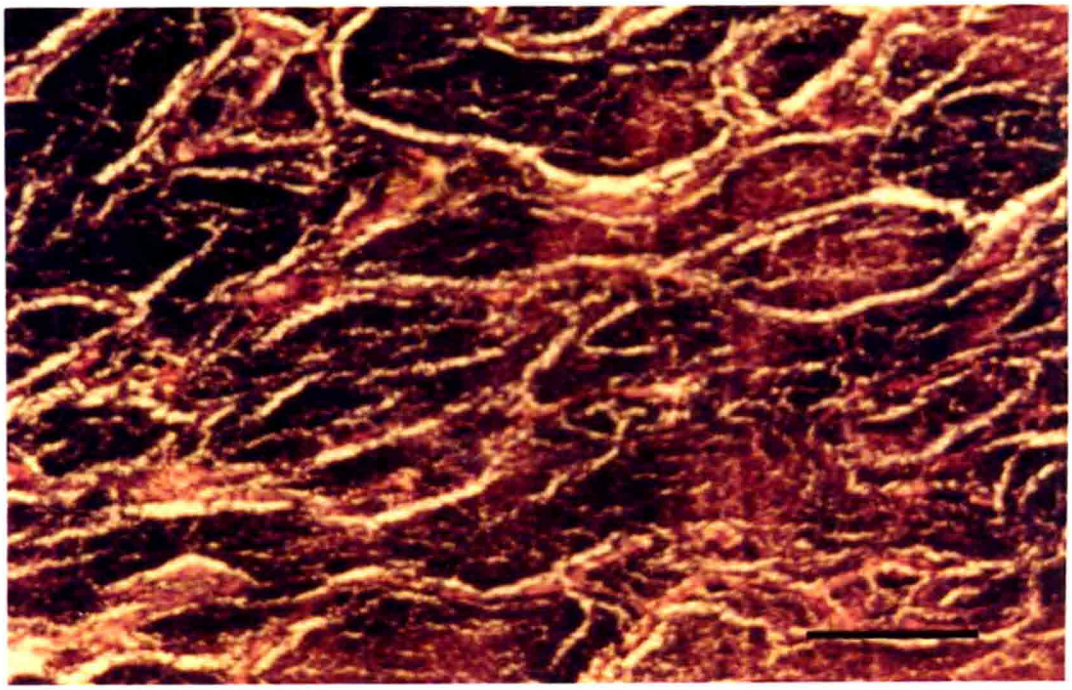

PLATE NO. 20

Explanation of Photomicrographs

Plate 21 : Section of Testis of *Cyprinus carpio* exposed to 5 $mg.l^{-1}$ Diethyl Phthalate for a period of 28 days degeneration of lumen wall can be seen. Also necrosis of spermatozoa is more pronounced as compared to control leading to spermatogenic arrest.
Hematoxylin / Eosin Bar = 100 μm

Plate 22 : Section of Testis of *Cyprinus carpio* exposed to 0.84-$mg.L^{-1}$ methyl paraben for a period of 28 days showing extensive fibrotic changes along with necrosis and inflammatory cell infiltration as well as total loss of testicular structure.
Hematoxylin / Eosin Bar = 100 μm

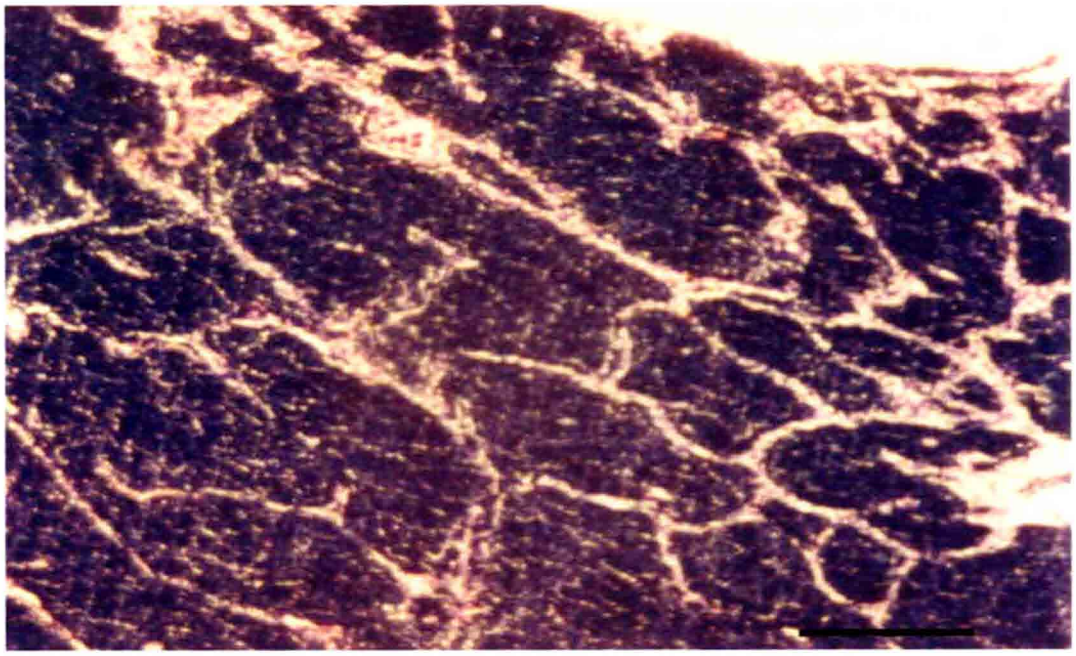

PLATE NO. 21

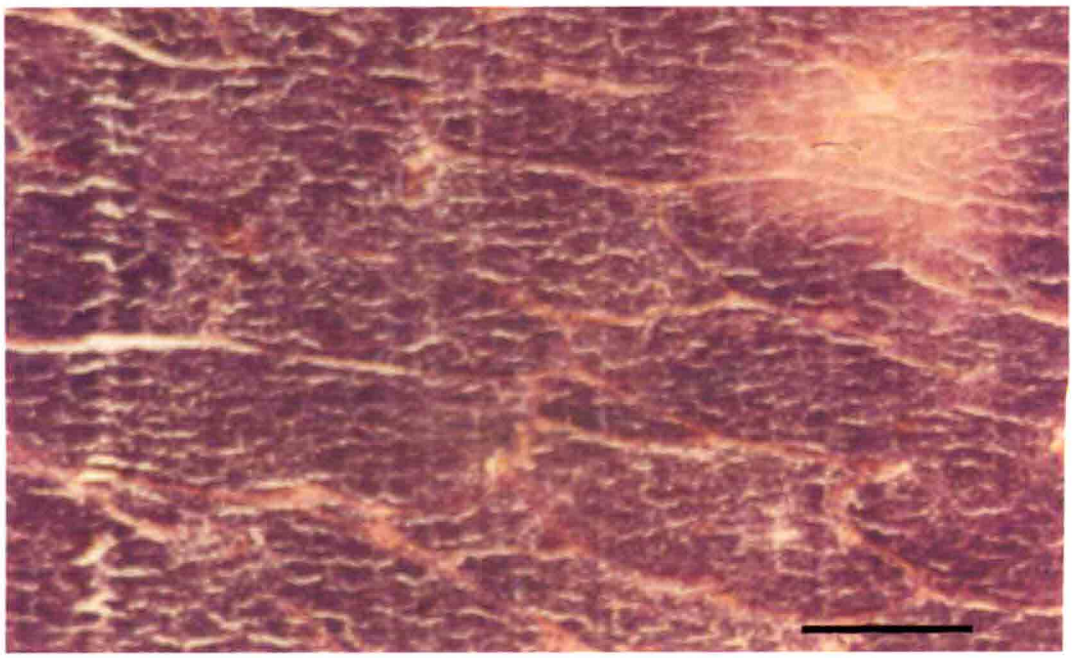

PLATE NO. 22

Explanation of Photomicrographs

Plate 23 : Section of testis of *Cyprinus carpio* exposed to 4-tert butylphenol at 0.69 $mg.l^{-1}$ for 28 days showing total loss of architecture. The lumen is disturbed along with disintegration of lumen wall and necrosis of spermatozoa.

Plate 24 : Testis of *Cyprinus carpio* exposed to 4-tert butylphenol at 1.38 $mg.L^{-1}$ for a period of 28 days showing total loss of architecture. Tissue fibrosis is abundantly seen with patch necrosis.

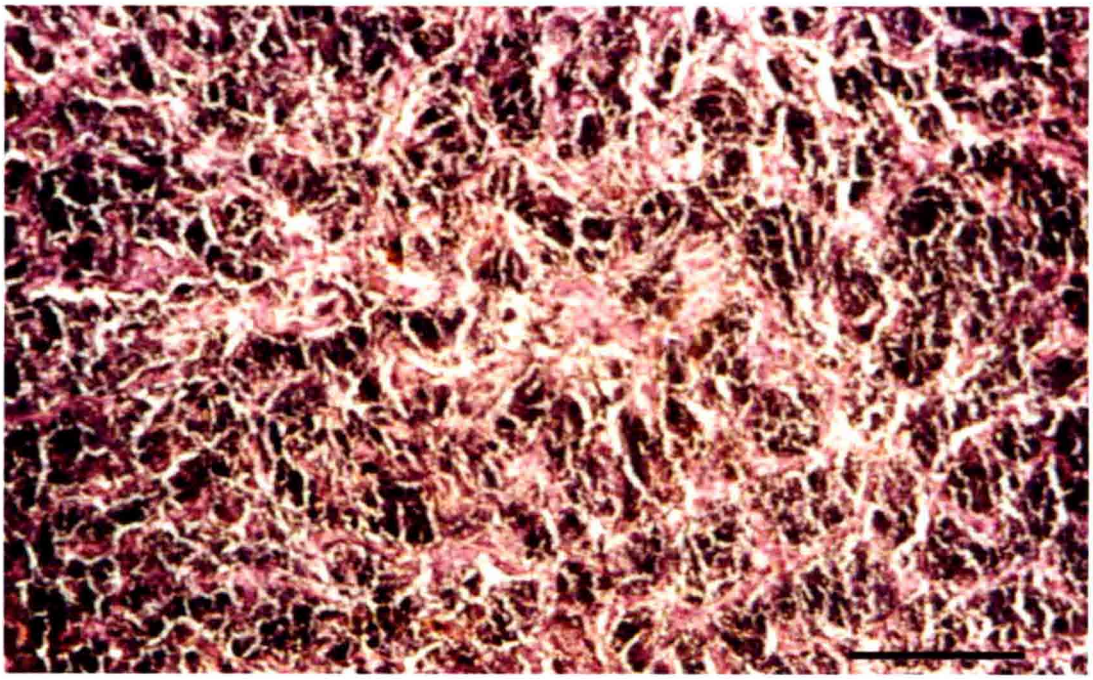

PLATE NO. 23

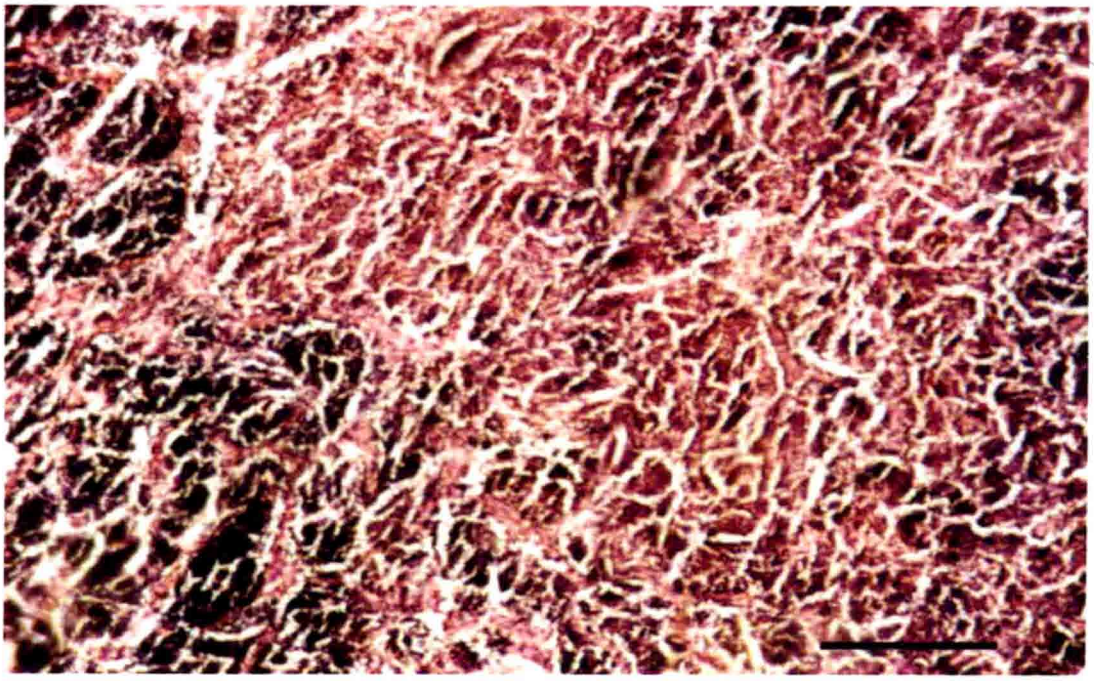

PLATE NO. 24

Discussion

CHAPTER 5

DISCUSSION

The aim of the study was to evaluate the effects of diethyl phthalate, methyl paraben and tertiary butylphenol on the fish common carp. The effect was assessed by comparing biochemical, somatic, and bioconcentration profile of control and exposed groups. The activities of selected enzymes, viz. SGOT, SGPT, AcP, AlP and growth parameters such as, GSI, HSI, CSI and RSI were measured in 20 specimens in control and 60 specimens in exposure treatments consisting of 3 selected sublethal concentrations. Induction of a female specific hormone vitellogenin was also evaluated as a response to these compounds.

5.1 Diethyl phthalate

5.1.1 Acid Phosphatase (AcP)

Acid phosphatase catalyzes the hydrolysis of various phosphate-containing compounds and acts as transphosphorylases in acidic conditions, and also functions as marker enzyme for the detection of lysosomes in cell fractions and can be altered by the presence of xenobiotics (Cajaraville *et al.*, 2000). This enzymatic activity has been studied in several organisms and the influence of heavy metals has been reported elsewhere (Del Valls *et al.*, 1998).

During the present studies, the acid phosphatase activity was estimated to be low ($P < 0.05$) in all the exposure concentrations with increase

in the exposure period. The AcP varied between 0.856 ± 0.27 and 14.950 ± 0.26 µmoles/mg of protein. The highest AcP value (14.950 ± 0.26 µmoles/mg of protein) was reported at the exposure concentration of 1 mg/l during the 4th week, while the lowest (0.856 ± 0.27 µmoles/mg of protein) was observed on 28th day of exposure at 20 mg/l, implying that AcP level was inversely related to the concentrations of DEP in water.

At 1 mg/l application, the AcP activity was recorded to vary between 13.945 ± 0.85 and 14.950 ± 0.27 µmoles/mg of protein. The lowest activity, observed at 20-mg/l exposure rate was 4.472 ± 0.85 and 0.856 ±0.27 µmoles/mg of protein. The findings of the present study are in accordance to the results recorded by Hinton and Koenig Jr. (1975) and Mohan and Verma (1981). However, a reverse trend was observed in liver AcP levels of Sprague-Dawley rats exposed to phthalate via water (Sonde *et al.*, 2000)

5.1.2 Alkaline Phosphatase (AlP)

Alkaline phosphatase (AlP) is a non-specific phosphomonoester hydrolase found in the plasma membrane of almost all animal cells. It catalyzes the hydrolysis of a wide variety of organic monophosphates. The widespread occurrence of AlP in nature suggests its involvement in fundamental biochemical processes, however, there is no positive evidence regarding its physiological function, or the nature of the natural substrates. Hydrolysis of phosphoesters, phosphate transferase activity, protein phosphatase activity, phosphate transport, modulation of organic cation transport, and involvement in cell proliferation have been suggested as

possible functions of AlP (Sarrouilhe *et al.*, 1992; Muda *et al.*, 1992). The measurement of alkaline phosphatase activity is generally carried out in clinical and ecotoxicological studies.

In response to DEP application there was an increase ($P < 0.05$) of AlP in all the exposure concentrations over the control, while the maximum increase was recorded towards the end of experimental period at 5 and 20 mg/l exposure concentrations. The lowest activity (0.878 $\pm$ 0.23 µmoles/mg of protein) was exhibited at 1 mg/l during 4th week of exposure, while the highest AlP activity was recorded as 2.317 $\pm$ 0.14 µmoles/mg of protein. At the higher exposure rate of 5 mg/l, the lowest activity was 3.236 $\pm$ 0.14 µmoles/mg of protein while at 20 mg/l application rate it was 2.696 + 0.23 µmoles/mg of protein.

Elevation of tissue AlP at 5 and 20 mg/l could be correlated with exposure time (**Tables 4.3.1** through **4.3.4** and **Fig. 4.1**) as was earlier found by Yang and Chen (2003). They reported increase in AlP activities could be attributed to pathological process such as, liver impairment, kidney function or bone disease.

5.1.3 Serum Glutamic Oxalacetic Transaminase (SGOT) and Serum Glutamic Pyruvic Transaminase (SGPT)

The serum glutamate oxaloacetic transaminase and serum glutamate pyruvate transaminase function as a link between carbohydrate and protein metabolisms by catalyzing interconversion of strategic compounds like α-ketoglutarate and alanine to pyruvic acid and glutamatic acid and aspartate

and α-ketoglutaric acid to oxaloacetic acid and glutamic acid respectively (Knox and Greengard, 1965; Watts and Watts, 1974; Martin *et al.*,1983) In the present study both aminotransferases were found to be higher in muscle tissues of exposed fish than those of the control fish during the first two weeks of exposure (**Tables 4.3.1** through **4.3.4**). This suggests that there was temporary damage to the plasma membrane and/or increase synthesis of enzymes by liver (Yang and Chen, 2003). However, during the last two weeks, there was an inhibition ($P < 0.05$, $P < 0.01$) in both the aminotransferases indicating that it (SGOT & SGPT) did not disturb the permeability and integrity of cell membranes (Masopust, 1998). The highest SGOT activity was recorded at 17.857 $\pm$ 0.46 µmoles/mg of protein at 20 mg/l exposure rate during the second week, followed by a steep reduction to 12.014 + 0.15 nmoles oxaloacetate/mg of protein in the 4th week. The same trend was shown by 1 and 5 mg/l application rates where an increase during the 2nd week followed by inhibition during final week were observed.

The SGPT followed a pattern similar to that of SGOT, where an initial increase in the second week followed by depletion in the third and fourth week were recorded. The highest activity i.e, 11.796 + 0.19 nmoles oxaloacetate/mg of protein followed by an inhibition of more than 50% a level of 5.704 + 0.15 nmoles oxaloacetate/mg of protein was exhibited at 20 mg/l exposure during the 2nd week. The activities recorded at 1 and 5 mg/l exposure also followed the same pattern. During the first week, the activity was 7.810 $\pm$0.16 nmoles oxaloacetate/mg of protein at 1 mg/l exposure

and this was steadily declined to 5.303 + 0.15 nmoles oxaloacetate/mg of protein in the last week of exposure.

The SGOT activity in all the three-exposure concentrations was greater than the SGPT. Irrespective of the trend of SGOT and SGPT, there was general enhancement in the activities of both SGOT and SGPT confirming the existence of stress to the fish. Since stress, in general, is known to elevate aminotransferase activity (Knox and Greengard, 1965), the elevation of SGOT and SGPT activities testify the existence of sublethal stress during exposure to individual. It was likely that this stress might be the result of augmentation of energy demands during the toxicity period where the possibility of depletion in energy resources was high.

5.1.4 Gonadosomatic Index (GSI)

There was reduction ($P < 0.05$) in GSI of *Cyprinus carpio* exposed to DEP in all the exposure concentrations. The GSI was drastically lowered at 20 mg/l progressively with time and it was reduced from 5.779 $\pm$ 0.77 during the first week to 1.879 $\pm$ 0.20 in the last week. However, there was a lesser impact on the GSI of carp exposed to DEP at 1 mg/l application rate which reduced from 6.795 $\pm$ 0.77 to 6.417 $\pm$ 0.20 during the four weeks of exposure (**Tables 4.3.1** through **4.3.4** and **Fig. 4.2**). Decrease of GSI was earlier reported in adult male trout, exposed to estrogenic alkylphenolic compounds (Jobling *et al.*1996).

Studying the impact of prochloraz and nonylphenol diethoxylate (NPEO) on trout, Le Gac *et al.*, (2001) similar effect in addition to inhibition of

spermatogenesis was reported. The reduction in GSI might be because of reduction in the number of germ cells (Leatherland *et al.*, 1996; Jobling, 1992) and also the exposure might have had adverse effect on the testis structure (Kinnberg *et al.*, 2000). This is again supported histological findings made in the present investigation which shows mass degeneration of spermatogonia at higher exposure concentrations.

5.1.5 Hepatosomatic Index (HSI)

Hepatosomatic index is a good indicator of liver energy content (Lambert and Dutil, 1997) as such any changes in the liver size can be correlated to imminent impact on fish health and metabolism. At 5 mg/l exposure, the HSI was increased from 1.396 $\pm$ 0.13 to 1.818 $\pm$ 0.24 during the 3rd week, and then reduced to 1.203 $\pm$ 0.10 during the last (fourth) week . At 20 mg/l exposure, the initial HSI was 1.533 $\pm$ 0.11 which was reduced to a level of 1.243 $\pm$ 0.10 in the fourth week. However, the HSI was higher ($P < 0.05$) in all the exposure concentrations as compared to control. There was a steady increase till the third week but surprisingly there was no significant difference ($P > 0.05$) between the HSI of all exposure concentration in the fourth week. The increase in HSI, observed in DEP treated carp, was reported in the same species under xenobiotic treatment by Gimeno *et al.* (1998) and may be correlated to Vtg induction (Cardinali *et al.*, 2004).

5.1.6 Renosomatic Index (RSI)

The tissue damage of the aquatic organisms, including minor lesions, ulceration and necrosis, is ascribed mainly to the non-specific disturbance of

cell membrane caused by the lipophilic xenobiotics (Wester and Canton, 1987). Such tissue aberrations facilitate invasion and growth of infectious agents (Bucke, 1993; Vethaak *et al.*, 1996).

Although the RSI was lower in the exposed fish than in control, it cannot be conclusively termed as decrease since the RSI in almost all the exposure concentrations showed a lot of variations during the 4 weeks of exposure. The highest (0.659 $\pm$ 0.14) RSI value was observed in the 5 mg/l exposure during the 2nd week of exposure, however, this rise was followed by an equally steep decline in the following week. The lowest RSI value (0.276 $\pm$ 0.14) was observed at 1 mg/l exposure during the 3rd week but it was subsequently increased to 0.542 $\pm$ 0.13 in the 4th week. At 20 mg/l exposure, the RSI increased from 0.317 $\pm$ 0.14 to 0.562 $\pm$ 0.13 during the same period.

5.1.7 Craniosomatic Index (CSI)

Literature reveals that not much work has been undertaken towards the impact of EDCs on CSI. CSI did not follow a set pattern in all the exposure concentrations except at the highest exposure level (20 mg/l), where it was inhibited through out the experimental period. In the lowest application rate i.e. 1 mg/l it was increased from 1.289 $\pm$ 0.22 in the first week to 1.349 $\pm$ 0.18 in the 4th week, however, at 5 mg/l it varied from 1.467 $\pm$ 0.22 and 1.655 $\pm$ 0.18. These variations were statistically insignificant ($P > 0.05$).

5.1.8 Histological Changes

5.1.8.1 Liver

Hepatocytes are the most common cells in fish liver (>80% of all liver cells) and are arranged as tubules of cells with their apices directed toward the central bile canaliculus and/or bile preductule (Hinton *et al.*, 1989). Bile, synthesized in the liver parenchyma, is secreted into the central bile canaliculus and ultimately flows into the gall bladder. Blood circulates through the sinusoids that separate the tubules of hepatocytes and the hepatocytes remove nutrients and xenobiotics. A number of histopathological studies were undertaken in the liver of carp exposed to diethyl phthalate. Studies identified the sites of inflammation, hepatocyte necrosis, enlarged vacuoles and fibrosis (**Plates 9, 10 and 11**). Necrotic hepatocytes with lesions along the periphery of the fibrotic zone, 'spotty necrosis' (i.e. scattered throughout) along with nuclei with irregular outlines, hyperplasia, large vacuoles and disturbed cytoplasm were seen in carp exposed to 20 mg/l DEP for 28 days (**Plate 11**). Necrotic hepatocytes were also observed in fish from the remaining treatment groups, although these lesions were not associated with fibrosis. Typically, at 1 mg/l exposure to diethyl phthalate for 28 days, liver cells of *Cyprinus carpio* showed necrosis of cytoplasm. Also, distinct increase in vacuolization and local degeneration of hepatocytes were seen (**Plate 9**) as necrotic hepatocytes.

At 5 mg/l diethyl phthalate, the liver showed an overall progressive degeneration of connective tissue. Vacuolization along with spotty necrosis was also present in numerous fish. Inflammation was occasionally associated

with necrotic lesions but also occurred in areas of normal liver parenchyma. Similar dose dependant changes were also reported in the liver of rainbow trout (*Onchorynchus mykiss*) exposed to nonylphenol (Uguz *et al.*, 2003).

5.1.8.2 Testis

Mature testis of a control male carp, are characterized by the presence of spermatogenic cysts containing dividing cells and by a lumen filled with spermatozoa (**Plate 19**).

In the majority of the males, the spermatogenic tubules contained abundant cysts of differentiating germ cells near the periphery of the gonad and spermatozoa near the central region. As compared to the normal testis (**Plate 19**) *Cyprinus carpio* exposed to 1 ppm Diethyl Phthalate for a period of 28 days showed disturbance in the architecture of lumen, and also necrosis of spermatozoa was observed. In case of 5 mg/l exposure, degeneration of lumen wall and necrosis of spermatozoa were more pronounced as compared to control leading to spermatogenic arrest.

5.1.9 Fish Behavior and General Appearance

The behavior of carp in the control group was considered 'normal' for comparisons to the behavior of carp exposed to DEP. Barring the carp exposed to 20 mg/l phthalate, carps exposed to lower levels of DEP exhibited apparently similar behavior to the control fish (**Table 4.3.5**).

Abnormal behavior was observed in carp from highest exposure concentration. The most common modifications in behavior were delayed or

absent startle response and development of dark skin coloration towards the end of experimental period. However, there was no impact on the feeding behavior of the fish, and also no changes were seen in the social behavior and aggressiveness towards other fish.

5.1.10 Bioaccumulation

Bioaccumulation of diethyl phthalate was studied in *Cyprinus carpio* during a period of 28 days, during which samples were collected every 7 day from internal organs of the exposed fish.

Data presented in **Tables 4.3.6** through **4.3.8** and **Fig. 4.3** indicated that the chemical was assimilated and rapidly accumulated in different organs including brain during exposure period. In water, attempt was made to maintain the initial concentration by replacing it with freshly exposed water every alternate day. The amount of DEP residue was varied considerably in each organ.

During the first week, rate of accumulation of phthalate was very low as compared to the remaining exposure period. The rate increased ($P < 0.05$) in all organs but mostly in gills, which might be attributed to continuous contact with treated water. The highest accumulation (3.132 ± 0.05 mg/kg) in gill was observed during the second week at 5 mg/l level, which was followed by a steep reduction ($P < 0.05$) to 0.311 ± 0.41 mg/kg in the third week. A sizable concentration of DEP was observed to be accumulated in the brain of the fish. Also another notable phenomenon was the obvious reduction in the accumulated compound after second week in almost all the organs except in

the liver at 5 mg/l exposure level, where it was steadily increased. At 1 mg/l, it was reduced to a level of 0.626 ± 0.05 mg/kg after reaching its peak value at 1.035 ± 0.04 mg/kg in the third week.

The bioaccumulation was measured to be lowest in testis as compared to all the organs. Highest accumulation in the testis was 1.287 ± 0.04 mg/kg during the 3rd week of exposure at 5 mg/l rate which did not alter significantly ($P > 0.05$) for rest of the exposure period. At 1 mg/l, however, after reaching 2.241 ± 0.10 mg/kg in 3rd week it reduced to 1.066 ± 0.04 mg/kg in the last week.

While studying changes in dietary bioaccumulation of other EDC namely, tributyltin chloride (TBTCL) and PCBs in red sea bream (*Pagrus major*) through diet, Ikeda and Yamada (2003) reported that PCBs concentration also declined gradually with time.

5.1.11 Vitellogenin Induction

Vitellogenin (VTG) is normally synthesized in the liver of adult female egg-laying vertebrates (Palmer *et al.*, 1998). Therefore, when detected in the serum of male fish, VTG can be used as a biomarker of exposure to estrogenic chemicals (Folmar *et al.*, 1995; Heppel *et al.*, 1995). Several researchers have demonstrated that exposure of male fish to xenoestrogenic chemicals results in the induction of VTG synthesis (Sumpter and Jobling, 1995) .

Following the 28-day exposure of male fish to DEP (100 μg/l, 1 mg/l and 5 mg/l), enzyme-linked immunosorbent assay (ELISA) of tissue homogenate samples from all exposed male fish revealed increasingly

intense VTG induction as DEP concentrations increased. Tissue samples were analyzed on a weekly basis to study the induction trend.

Even at the lowest exposure concentration of 100 μg/l there was a significant increase ($P < 0.05$) in the vitellogenin content (0.285 ± 0.05 μg/ml) from 7th day onwards, and it reached the peak value of 0.401 ± 0.97 μg/ml during final week (**Tables 4.3.9** through **4.3.12** and **Fig. 4.4**). At 1 and 5 mg/l levels the vitellogenin contents were higher ($P < 0.05$) than the level at 100 μg/l. The vitellogenin contents were increased ($P < 0.05$) with the increase of sublethal exposure concentrations and period of exposure. The highest vitellogenin content of 0.516 + 0.02 μg/ml was observed on 21st day of exposure at 5 mg/l application rate. Histological analysis of the testes of exposed fish revealed that the structure was disturbed indicating inhibition of production of spermatocytes and spermatozoa due to DEP exposure. This was in direct conjunction with the studies conducted by Okoumassoun *et al.* (2002), who reported a similar increase in the vitellogenin content in Tilapia (*Sarotherodon melanotheron*) exposed to organochlorine pesticide. However, some *in vitro* studies have indicated that DEP did not cause estrogenic activity; EPA (1996) opined that there was insufficient evidence to demonstrate hormonal disruption due to DEP exposure.

5.2 Methyl Paraben

5.2.1 Acid Phosphatase (AcP)

The acid and alkaline phosphatase activities are involved in a variety of metabolic processes, such as, molecule permeability, growth and cell differentiation and steroidogenesis (Ram and Sathayanesan, 1985). Their

activities have been used to evaluate the effects of crude oil (Ozretic and Krajnovic-Ozretic, 1985) and methylparathion.

Acid phosphatase catalyzes the hydrolysis of various phosphate-containing compounds and act as transphosphorylases at acid pH. AcP was significantly lowered ($P< 0.05$ and $P< 0.01$) in all the exposure concentrations starting from first week onwards. Even at the lowest exposure level, i.e. 0.84 mg/l, there was significant reduction ($P < 0.05$) in AcP activity during the first week (17.122 + 0.21 µmoles/mg of protein), while in the 2nd week it was increased to 17.669 $\pm$ 0.44 µmoles/mg of protein and subsequently reduced to 13.998 $\pm$ 0.38 µmoles/mg of protein during the 4th week. At higher two exposures, i.e. 1.68 and 4.2 mg/l the AcP activity was inhibited ($P < 0.05$) throughout the experimental period, the highest (16.677 $\pm$ 0.21 µmoles/mg of protein) being at 1.68 mg/l and the highest for at 4.2 mg/l was 14.561 $\pm$ 0.38 µmoles/mg of protein (**Tables 4.4.1** through **4.4.4** and **Fig. 4.5**).

Mohan and Verma (1981) reported similar inhibition in *Mystus vittatus* as a response of exposure to synthetic detergent (Swascofix E45), while Swascofix ED38 exhibited a reverse effect on the acid phosphatase of *Clarias batrachus* and *Cirrhina mrigala* (Gupta and Dhillon, 1983).

5.2.2 Alkaline Phosphatase (AlP)

Alkaline phosphatase is composed of several isoenzymes that are present practically in all tissues of the body, especially in cell membranes.

These enzymes catalyze the hydrolysis of monophosphate esters and have a wide substrate specificity (Duncan and Prasse, 1986).

AIP was significantly increased ($P < 0.05$) in all the exposure concentrations throughout the exposure period. Even at lowest exposure dosage, i.e. 0.84 mg/l, it showed elevation ($P < 0.05$) in the activity, the highest being 1.514 $\pm$ 0.89 µmoles/mg of protein and the lowest 0.748 $\pm$ 0.08 µ mol/mg of protein. While at the highest dosage, i.e. 4.2 ppm, it was 1.176 $\pm$ 0.08 µmoles/mg of protein during the first week and remained significantly high (1.579 $\pm$ 0.04 µmoles/mg of protein) till the 4th week. Increased serum activities of AIP could be attributed to pathological processes such as, liver impairment, kidney dysfunction, and bone disease, although its precise biochemical functions, which act in the organism, are yet to be known (Bogin *et al.*, 1994; Atroshi *et al.*, 2000),

Banerjee *et al.* (1984) reported similar increase in AIP activities in brain and nerve cord of *Schizodactylus monstrossus* after exposure to an insecticide pyrethrum.

5.2.3 Serum Glutamic Oxalacetic Transaminase (SGOT) and Serum Glutamic Pyruvic Transaminase (SGPT)

Liver is the metabolic center for detoxicification of chemicals; hence any damage to the liver could affect the metabolism of the organism. Enzyme leakage from the liver was investigated by measurement of serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) activities in the tissue.

There was no difference ($P < 0.05$) between any SGOT activities of any of the exposure concentrations during the first week (15.546 $\pm$ 0.11 μmol/g of protein at 0.84 mg/l, 15.480 $\pm$ 0.11 nmoles oxaloacetate/mg of protein at 1.68 mg/l and 5.884 $\pm$ 0.12 nmoles oxaloacetate/mg of protein at 4.2 mg/l) while during the second week of exposure there was an increase ($P < 0.05$), followed by inhibition of SGOT activities during the 3rd (16.680 $\pm$ 0.31 nmoles oxaloacetate/mg of protein at 0.84 mg/l, 16.665 + 0.61 nmoles oxaloacetate/mg of protein at 1.68 mg/l and 16.290 + 0. 42 nmoles oxaloacetate/mg of protein at 4.2 mg/l) and 4th week (10.027 + 0.15 nmoles oxaloacetate/mg of protein at 0.84 mg/l, 12.120 + 0.15 nmoles oxaloacetate/mg of protein at 1.68 mg/l, 14.379 + 0.45 nmoles oxaloacetate/mg of protein at 4.2 mg/l). Similar kind of inhibition was reported by Gill *et al.* (1990), in which *Puntius conchonius* was exposed to mercuric chloride.

However, SGPT activity was significantly higher ($P < 0.05$) in highest exposure concentration, i.e. 4.2 ppm, throughout the exposure period starting with 8.303 $\pm$ 0.55 nmoles oxaloacetate/mg of protein in the 1st week to 22.302 $\pm$ 0.53 nmoles oxaloacetate/mg of protein, while at the lowest exposure concentration, i.e. 0.84 mg/l, the activity was increased from 7.352 $\pm$ 0.55 nmoles oxaloacetate/mg of protein to 20.882 + 0.53 nmoles oxaloacetate/mg of protein in the fourth week, increasing ($P < 0.05$) throughout the experimental period. Similar findings were reported by Verma *et al.* (1984) while studying effect of mercuric chloride stress on serum transaminase

activity in *Notopterus notopterus*. As regards to changes in SGOT and SGPT levels, it is a clear indication that liver damage due to toxic injury led to the escape of these two crucial enzymes of liver function into the serum of animals. The increased activities of SGPT in fish process have also been observed in fish after exposure to pesticides (Natarajan, 1985).

5.2.4 Gonadosomatic Index (GSI)

Kumar and Mukherjee (1988) suggested that accumulation of ovarian cholesterol, coupled with reduction of GSI values in sexually maturing cyprinus carpio treated with phenol and sulfide, had result from reduced steroidogenesis and caused a gradual and significant increase ($P < 0.05$) of hepatosomatic index values. In the present study too, GSI was significantly lowered ($P < 0.05$) in all the exposure concentration starting from 2nd week to 4th week while hepatosomatic index had increase. The highest reduction (1.650 ± 0.19) of GSI was observed in the highest exposure concentration, i.e. 4.2 ppm during the third week from 5.956 ± 0.33 during the first week. At the lowest exposure concentration, i.e. 0.84 mg/l during the first week the GSI was 5.629 ± 0.19, which reduced significantly ($P < 0.05$, $P<0.01$) to 5.313 ± 0.16 in the 4th week (**Tables 4.4.1** through **4.4.4** and **Fig. 4.6**). In another study by Noaksson *et al.* (2001) it was found that there was 36% reduction in GSI of *Rutilis rutilis* exposed to public refuse dump.

5.2.5 Hepatosomatic Index (HSI)

The liver is an important organ involved in metabolic processes and in detoxification of xenobiotics. In some situations, materials may accumulate in

the liver to toxic levels and cause pathological alterations (Meyers and Hendricks 1984). During the first week of exposure the HSI was significantly ($P < 0.05$) increased in all the exposure concentrations the highest was 1.911 $\pm$ 0.10 at 1.6 mg/l, while at lowest exposure concentration, i.e. 0.84 mg/l, the HSI during this period was 1.786 $\pm$ 0.21. The liver enlargement possibly expressed the increased metabolism of the exposure compound; similar findings were reported by Andersson *et al.* (1988).

5.2.6 Renosomatic Index (RSI)

Many polar water-soluble foreign compounds (xenobiotics) are eliminated, unchanged from the organism. Fat-soluble (hydrophobic) compounds, on the other hand, have to be transformed into more polar metabolites before the organism is capable of eliminating them. Fat-soluble substances will either simply remain in the fat or be resorbed by the kidney tubules or equivalent organs and will continue to recirculate for a very long time, causing an increase in the weight of the organ of deposition. Hence, it becomes important to study the RSI along with other impacts.

The RSI was suppressed in all the exposure concentrations during the 1st week. During the 2nd week there was a significant increase ($P < 0.05$) in the RSI (0.612 $\pm$ 0.11) of the carp exposed to MP at 4.2 mg/l however at 0.84 mg/l and 1.68 mg/l exposure rates, it was lower than the control value. In the 3rd week increase ($P < 0.05$) was seen in the RSI at 0.84 mg/l (1.207 $\pm$ 0.11) which increased to 1.363 $\pm$ 0.11 during the 4th week.

5.2.7 Craniosomatic Index (CSI)

As per the literature survey done not much study has been done on the direct impact of xenobiotic on size of brain, however, these studies might be of additionally used in conjunction with other impacts. In the present study CSI was not significantly different ($P > 0.05$) from control in lower exposure concentrations (0.84 and 1.68 mg/l) while at highest exposure (4.2 mg/l) rate the CSI was significantly reduced ($P < 0.05$) throughout exposure period. At the lowest exposure concentration (0.84 mg/l) it was recorded 1.230 $\pm$ 0.15 during the 1st week and 1.245 $\pm$ 0.12 during the final week, which was similar to control the CSI during the same periods. At the highest application rate (4.2 mg/l), it however decreases from 1.274 $\pm$ 0.85 in first week to 1.230 $\pm$ 0.25 in the 4th week.

5.2.8 Histological Changes

5.2.8.1 Liver

In livers of all the fish exposed to methyl paraben at varying concentrations, the only notable feature was the presence of vacuolization. Pathological studies in Boston flounder (*Pseudopleuronectes americanus*) was linked to hepatic tubular vacuolation with xenobiotic contamination (Moore *et al*, 1989).

Liver of *Cyprinus carpio* exposed to Methyl paraben at 0.84 mg/l for 28 days, showed mild inflammation and necrotic changes in hepatocytes. Lack of clear vacuoles also observed (**Plate 13**). At the higher exposures (1.68 and

4.2 mg/l) there were not much changes in the structure except a general increase in vacuolization and necrosis of hepatocytes (**Plates 14** and **15**).

5.2.8.2 Testis

As compared with the control (**Plate 19**), testis of *Cyprinus carpio* exposed to 0.84 ppm methyl paraben for a period of 28 days, showed extensive fibrotic changes along with necrosis and inflammatory cell infiltration, total loss of testicular structure. The lumen wall was restricted to a large extent (**Plate 22**).

5.2.9 Fish Behavior and General Appearance

Behavior of an organism represents the final integrated result of a diversity of biochemical and physiological processes and is known to be highly sensitive to changes in the steady state of on organism (Warner *et al.*, 1966). Scheren (1975) expressed that behavioral tests may sometimes ascertain lower thresholds than physiological function because the response results from an integrated, functional system. There were no behavioral changes observed even in the highest level of the chemical. While the feed was consumed within 10- 15 minutes, the fish were observed to be lethargic towards the end of experimental period, there were no observed tendency to cluster in any corners of the aquarium nor aggressiveness and discoloration of the fish (**Table 4.4.5**).

5.2.10 Bioaccumulation

There have been no studies linking bioaccumulation and biochemical effects of methyl paraben. To address this problem we exposed *Cyprinus*

carpio to 3 concentrations of methyl paraben to assess the bioaccumulation parameter in liver, brain, gill and testes.

Cyprinus carp rapidly accumulated methyl paraben and contained measurable concentrations by day 7 of uptake in all the organs in both the exposure concentrations (**Tables 4.4.6** through **4.4.8** and **Fig. 4.7**). There was no methyl paraben detected in any of the control fish. Estimated uptake and assimilation patterns were similar for gills, gonads and brains between treatments except for liver. Highest overall accumulation was observed in the brain during the 3rd week of exposure (3.973 $\pm$ 0.08). In almost all the organ a deposition trend can be seen a gradual increase followed by reduction. Gill accumulated, 0.306 $\pm$ 0.01 and 0.518 $\pm$ 0.02 mg/kg respectively for 0.84 and 1.68 mg/l exposure during the first week, and steadily increased to 0.771 and 1.322 mg/kg respectively for 0.84 and 1.68 mg/l exposure during the third week of methyl paraben exposure. However, the level came down to 0.143 and 0.776 mg/kg respectively for 0.84 and 1.68 mg/l exposure during the fourth week.

The gonads more or less followed the same deposition pattern. Initial accumulation was seen at 0.102 and 0.771 mg/kg for 0.84 and 1.68 mg/l, respectively. It increased to 0.882 and 2.094 mg/kg for 0.84 and 1.68 mg/l respectively during the third week, then declined to 0.743 and 1.322 mg/kg for the 4th week in the respective exposure concentrations.

The brain initially accumulated 0.551 mg/kg at 0.84 mg/l exposure level during the first week and the assimilation was 1.011 mg/kg in the final week, while at the higher exposure concentration, i.e. 1.68 mg/l, it accumulated

0.416 mg/kg in the first week and towards the end it had accumulated 2.865 mg/kg.

Liver showed a steady but definite increase in both the exposure concentrations through out the period of experiment. At 0.84 mg/l exposure, accumulation for 1st week was 0.543 mg/kg which increased to 1.025 mg/kg during the final week, where as in case of 1.68 mg/l, it increased from 1.020 mg/kg to 2.755 mg/kg.

Monosson *et al.* (2003) found tissue differences in PCB accumulation, with PCB levels in gonad> liver > muscle on a wet weight basis for fish from PCB-contaminated sites.

5.2.11 Vitellogenin Induction

Vitellogenin (VTG) is well known as an estrogen-responsive phosphoprotein, and can also be used as a biomarker of contaminant exposure in fish such as common carp. In oviparous vertebrates VTG is produced in the liver (Ng and Idler, 1983). Recently, it has become possible to conveniently measure carp VTG concentration, since enzyme-linked immunosorbent assay (ELISA) has been developed for measurement of carp VTG.

In the present study the Vitellogenin content in *Cyprinus carpio* males exposed to methyl paraben at 0.84, 1.68 and 4.2 mg/l showed an increase ($P < 0.05$) in comparison to male in control, starting first week there was a significant ($P < 0.05$, $P < 0.01$) increase (0.379 + 0.01 μg/mg at 0.84 mg/l, 0.421 + 0.1 μg/mg at 1.68 and 0.6251 ± 0.01 μg/mg at 4.2 mg/l) in the

vitellogenin content, also the increase was directly dose and exposure dependant (**Tables 4.4.9** through **4.4.12** and **Fig. 4.8**).

At the lowest exposure rate, the induction was 0.379 + 0.02 μg/mg in the first week as compared to 0.143 + 0.01 μg/mg in control and 0.625 + 0.03 μg/mg in 4.2 mg/l. The vitellogenin in carp exposed for 28 days to 0.84 mg/l was 0.402 + 0.18 μg/mg and 0.700 + 0.02 μg/mg for 4.2 ppm.

Bjerregaard (2003) stated increases in average plasma vitellogenin levels to oral exposure to 33 mg propyl paraben/ kg/ 2 d; the most sensitive fish responded to 7 mg/kg. The increases in vitellogenin synthesis were 35, 31 and 22 mg/kg/ 2 d at day 3, 6 and 11, respectively. Exposure to 225 mg propyl paraben increased vitellogenin synthesis, but exposure to 50 mg/l did not.

Corresponding results were given by Inui *et al.* (2003) who reported an increase of plasma VTG concentration and mRNA expression of VTG-1, VTG-2, CHG-L, CHG-H, and ER in the livers of male medaka (*Oryzias latipes*) exposed to propyl paraben.

However, Routledge *et al.* (1998) reported that parabens are weekly estrogenic. In an *in vitro* yeast based estrogen assay, methyl paraben produced the weakest response in comparison to 17-Bestradiol.

In mouse eutrotrophic assay a significant increase in mouse uterus weight on day 4 was considered as estrogenic effect (Afossaint *et al.* 2000).

5.3 Tertiary butylphenol

5.3.1 Acid Phosphatase (AcP)

AcP is known to be localized in the lysosomes and is surrounded by a lipoprotein membrane. The increase in the activities of AcP and AlP enzymes in the serum may result only consequent to impairment of the function of tissues with subsequent liberation of the enzymes into the circulation from the damaged tissue. Ram and Singh (1988) found an elevation in AlP and AcP activity levels in the liver of carbofuran-treated *Channa punctatus*, however, Sharma (1990) reported a reduction in the activities of these enzymes in fish, (*Channa gachua*), exposed to endosulfan. Conversely, in our observation there was no significant difference ($P > 0.05$) between AcP activity in any of the exposure concentrations and control during the first week of exposure. The AcP activity in the lowest exposure concentration was similar to the control during all the exposure periods with the highest activity (17.25 $\pm$0.40 µmoles/mg of protein) during the first week and the lowest (16.78 + 0.99 µmoles/mg of protein) during the last week, however there were increases ($P < 0.05$) in the enzyme activity at 1.38 and 2.3 mg/l concentrations in the 3rd and 4th week of the exposure. At the highest exposure concentration, i.e. 2.3 mg/l there were significant variations in the AcP activities in all the sampling days. The activity was increased from 17.47 + 0.40 µmoles/mg of protein in the first week to 25.55 $\pm$ 0.52 µmoles/mg of protein in the third week and finally was reduced to 18.08 + 0.99 µmoles/mg of protein during the fourth week (**Tables 4.5.1** through **4.5.4** and **Fig. 4.9**). The 1.3 mg/l exposure concentration also exhibited a similar effect on the AcP. Similar kinds of observations were earlier reported by Gill *et al.* (1990). Our results suggest that both the higher concentrations of 4 tert butylphenol do not have any strong effect on the activities of AcP initially and but prolonged exposure could cause destruction of cell membranes and lysosomes which might be

responsible to liberate these hydrolyses into the cell system of tissues to facilitate autolysis.

5.3.2 Alkaline Phosphatase (AlP)

AlP activity is mainly localized in the cell membrane. As such any damage in hepatic cells may result in alteration in AlP activity. During the present investigation, inspite of an initial increase there was a statistically significant inhibition ($P < 0.05$, $P < 0.01$) of AlP activities in the fish, exposed in all the concentrations. Even at the lowest exposure concentration, i.e. 0.69 mg/l, there was inhibition ($P < 0.05$) in the AlP activity through out the period. Starting second week, the AlP activity was lesser (0.36 + 0.05 µmoles/mg of protein) than the control value, however, it was increased significantly ($P < 0.05$) in the next week but this increase of 0.74 + 0.11 µmoles/mg of protein was lower than control value (0.96 + 0.1 µmoles/mg of protein). At the highest application rate i.e. 2.3 mg/l the maximum inhibition 0.33 + 0.05 µmoles/mg of protein was in the second week and was increase 0.54 + 0.10 µmoles/mg of protein in the fourth week (highest), but at no point the activity was higher than the control.

Similar findings were reported in a study conducted by Das and Mukherjee (2003) where the depletion of AlP and elevations of AcP activity were attributed to alterations of DNA and RNA in muscle tissue.

5.3.3 Serum Glutamic Oxalacetic Transaminase (SGOT) and Serum Glutamic Pyruvic Transaminase (SGPT)

Transaminases are intracellular enzymes that exist in only a small amount in the serum. Any damage to the liver cell may result in leakage of these enzymes into the plasma due to a large concentration gradient (Wroblewski and La Due, 1955). Asztalos *et al.* (1988) have reported elevation in SGOT activity in *Cyprinus carpio* which had hepatic cellular damage caused by methidation and paraquat. Conversely, in this study, the SGOT enzyme activity was observed to be significantly ($P < 0.05$) inhibited on 7, 14, 21 and 28 days of exposure to 4-tert butylphenol, (**Tables 4.5.1** through **4.5.4**). Similarly, Sadhu *et al.* (1985) have demonstrated decreases SGOT and SGPT activities in *Channa striatus* following exposure to 0.1 mg/l Malathion for 10 days. In the present experiment at the lowest exposure concentration i.e. 0.69 mg/l the SGOT activity 11.44 + 0.09 µmoles/mg of protein was decreased during the first week then ascended to 16.1 + 0.414 nmoles oxaloacetate/mg of protein in the third week and finally lowered to 11.476 $\pm$ 0.56 nmoles oxaloacetate/mg of protein in the 4th week. At the highest exposure rate (2.3 mg/l) the activity was noted as 11.46 $\pm$ 0.19 nmoles oxaloacetate/mg of protein which was not significantly ($P > 0.05$) different from that at 0.69 mg/l and followed the same pattern as that at the lowest exposure concentration. However, the inhibition (9.59 $\pm$ 0.56 nmoles oxaloacetate/mg of protein) was more than that at 0.69 mg/l application rate.

Starting first week onwards there was a significant increase ($P < 0.05$, $P < 0.01$) in SGPT activity in all the exposure concentrations throughout the exposure period. The lowest activity was recorded for 0.69 mg/l for 1st week of

exposure, followed by steep rise that was followed in all the exposure concentration. The highest activity 20.23 + 0.53 nmoles oxaloacetate/mg of protein was exhibited by 2.3 mg/l in the second week, for the 3rd week it remains more or less constant followed by a non significant ($P > 0.05$) reduction (18.17 ± 0.53 nmoles oxaloacetate/mg of protein) in the activity during the 4th week. At the lowest exposure concentration (0.69 mg/l) the SGPT activity was recorded (8.332 ± 0.25 nmoles oxaloacetate/mg of protein), in the 1st week followed by a steep increase (20.22 ± 0.53 nmoles oxaloacetate/mg of protein), in the second week and finally recedes to 16.57 + 0.19 nmoles oxaloacetate/mg of protein.

Poleksic and Karan (1999) also reported comparable activity in carp in response to trifluralin application.

5.3.4 Gonadosomatic Index (GSI)

GSI was decreased in adult male carp exposed to 17α-methyltestosterone and 17β-estradiol, either through water or via food (Komen *et al.* 1989). In a study by Le Gac *et al.* (2001) when fish in the initial stage of spermatogenesis were exposed for 21–27 days to 580 nmol/l NP2EO, a 20–40% reduction of the gonadosomatic index was observed 41/2 weeks post-exposure, and the spermatogenetic process was partly inhibited. In the present study, except for the lowest exposure concentration, i.e. 0.69 mg/l the GSI was inhibited in all the exposure concentrations throughout the experimental period. At exposure concentrations of 1.38 and 2.3 mg/l the GSI was increased from 1.76 ± 0.56 and 1.94 ± 0.49 to 4.54 ± 0.65 and 4.93 ±

0.69 respectively during the period of 1st to 2nd week, but in the preceding week it was lowered ($P < 0.05$). A significant ($P < 0.05$) overall reduction was observed after 28 days of exposure to most of the treatments (**Tables 4.5.1** through **4.5.4** and **Fig. 4.10**). However, due to small sample size, the variability was high and might have been prevented any noticeable effect.

In another study, conducted by Hecker (2001), a similar variation was found in GSI of bream (*Abramis brama*), exposed to municipal waste.

5.3.5 Hepatosomatic Index (HSI)

When fish are normally exposed to toxic substances, these toxicants would have a tendency to be removed from circulation so as to reduce cell damage (Klaasen *et al.1993*). The liver is the main site of detoxification, and the presence of certain contaminants in the blood stimulates the synthesis of hepatic detoxification enzymes. In hepatocytes, enzymes are produced that conjugate the toxin with a glucuronide or a sulfate group. Livers that are stimulated and actively producing these enzymes are known to increase in size (Munkittrick *et al.*, 1992; McMaster *et al.*, 1991). The findings of the present study are very much in line of the above observation. HSI was increased ($P < 0.05$) in all the exposure concentrations during the first three weeks of exposure, followed by a reduction, which might because the liver acquire an equilibrium stage. At the lowest exposure concentration, i.e. 0.69 mg/l, the HSI (1.06 ± 0.07) was significantly different from control during the first week of exposure, however, it was increase to 1.81 ± 0.34 during the third week and was subsequently reduced to 1.34 ± 0.241 during the 4th week of exposure. There was no significant difference ($P > 0.05$) between HSI of all

the fishes exposed to different concentrations. The HSI at the highest exposure concentration, i.e. 2.3 mg/l, was 0.91 ± 0.07 during the 1st week, in the 3rd week it was increased to 1.95 ± 0.34 (nearly double to that in control), however, in the subsequent week, i.e. 4th, it declined to 1.45 ± 0.24.

5.3.6 Renosomatic Index (RSI)

The RSI had shown an increase in all the exposure concentrations throughout the experimental period. Even at the lowest exposure concentration, i.e. 0.69 mg/l, the RSI showed about 100% increase. It started at 1.49 ± 0.02 in the first week followed by reduction upto 1.29 ± 0.18 during the termination of the experiment. At the highest exposure concentration i.e. 2.3 mg/l, the RSI was lower as compared to the values recorded at 0.69 mg/l exposure during all the respective weeks. Looking at the **Tables 4.5.1** through **4.5.4** and **Fig. 4.10** it can be assumed that prolonged treatment led to deleterious effects on body organs, since similar kind of results were reported by Simone (1990) as a consequence of exposure of channel catfish (*Ictalurus punctatus*) to synthetic steroid 17-alpha-methyltestosterone which led to a marked edema in the renal tubules and many of the renal corpuscles of the kidney, as also hepatotrophic response causing increase of weight of the liver. However, conversely the renosomatic indices of dimethazine fed rainbow trout were significantly greater than those of the controls (Matty and Cheema, 1978).

5.3.7 Craniosomatic Index (CSI)

It is evident from literature survey that no study has been directed on this aspect of pollution. Direct impact of compound on the size of brain can be considered as an additional information. In the present study no significant ($P > 0.05$) changes in CSI of the carp exposed to tert-butylphenol, was observed at all exposure concentrations. At the lowest exposure concentration, the CSI was 1.21 ± 0.24 during the first week which was not significantly different ($P > 0.05$) from 1.08 ± 0.32 as observed during the last week of exposure. Even at the highest exposure concentration similar trend of CSI during the first week was 1.22 ± 0.18 during the last week of exposure was recorded (**Tables 4.5.1** through **4.5.4** and **Fig. 4.10**).

5.3.8 Histological Changes

5.3.8.1 Liver

In the control group, there were no pathological abnormalities. The hepatocytes were organized (**Plate 8**). Vacuoles started to appear, in addition, cell borders disappeared, nuclei became smaller, and there was a dramatic decrease in the number of hepatocytes in fish treated with 0.69 and 1.38 mg/l TBP for 28 days (**Plates 15 and 16**). Hepatocytes in the liver of fish, treated with 0.69 mg/l TBP, appeared to be floating in an empty space (**Plate 15**), also there was hemorrhage, lymphocyte infiltration and vacuoles were observed in extracellular space.

Carp, exposed to 4-tert butylphenol at 1.38 mg/l for 28 days, showed hyperplasia of connective tissue, extensive degeneration of hepatocytes, increased vacuolization and fibrous lesions.

According to a report by Hibiya (1982), areas reminiscent of vitellogenin production in the liver of sexually mature females were marked with the nuclei, as well as nucleoli were hypertrophy.

5.3.8.2 Testis

As compared to control (**Plate 19**) testis of *Cyprinus carpio,* exposed to 4-tert butylphenol at 0.69 and 1.38 mg/l, showed total loss of architecture. The lumen was disturbed along with disintegration of lumen wall and necrosis of spermatozoa, also tissue fibrosis was observed along with patchy necrosis. There was a total loss testicular architecture in both the exposure concentrations.

5.3.8.3 Ovaries

As compared with the "normal" ovary (**Plate 17**) ovary of *Cyprinus carpio* exposed to 4-tert butylphenol at 1.68 mg/l, showed degeneration of oocytes. Also a total loss of architecture was observed in the exposed ovary.

5.3.9 Fish Behavior and General Appearance

There were no changes observed in the behavior of fish treated with tertiary butylphenol at 0.69, 1.38 and 2.3 mg/l (**Table 4.5.5**). As compared to the control there was no erratic swimming, jerking, movements, crowding in

were no changes in coloration observed in any of the exposure concentrations. Feed was consumed with 10-15 minutes.

5.3.10 Vitellogenin Induction

Vitellogenin, the precursor of yolk, is normally produced only in females in response to estrogens from the ovary. Male fish do not normally synthesize vitellogenin (Copeland *et al.*, 1986), because they have very low (often undetectable) concentrations of circulating estrogens. However, male fish respond to exogenous estrogens, administered by injection or via the water, by synthesizing vitellogenin (Purdom *et al.*, 1994; Jobling *et al.*, 1995) in the same way that females do. Thus, the presence of vitellogenin in the plasma of male fish can be used as a biomarker of exposure to estrogenic chemicals (Sumpter and Jobling, 1995).

There was a significant ($P < 0.05$, $P < 0.01$) and dose dependent increase of vitellogenin content of male *Cyprinus carpio* exposed to 0.69, 1.38 and 2.3 mg/l during all the exposure periods. The highest exposure rate, i.e. 2.3 mg/l, exhibited highest vitellogenin content. Even at the lowest application rate there was significant ($P < 0.05$) induction of vitellogenin. Starting with the first week there was induction of vitellogenin that is 0.32 $\pm$ 0.01 µg/ml, 0.31 $\pm$ 0.01 µg/ml, and 0.42 + 0.01 µg/ml at all the exposure concentration i.e. 0.69 mg/l, 1.38 mg/l and 2.3 mg/l respectively (**Tables 4.5.6** through **4.5.9** and **Fig. 4.11**). There was significant difference ($P < 0.05$) between induction at 0.69 mg/l and 1.38 mg/l exposure; however, even at the lowest concentration the vitellogenin increased upto 0.3909 mg/ml during the last

Summary and Conclusion

CHAPTER 6

SUMMARY AND CONCLUSION

Anthropogenic compounds can cause many unwanted changes not only in the environment but also on the organisms inhabiting it. A variety of effects on both embryos and adults including changes in somatic indices and enzymes, impairment of reproductive system, egg shell thinning, impaired incubation in animals, and also changes in behavior have been reported. Although it is still uncertain exactly how many or what type of chemicals cause these effects, nor are the mechanisms of the actions of many of the chemicals established, it is very likely that many of the effects observed are a consequence of endocrine disruption. The exact manner in which these chemicals react after entering the organism is still a subject of discussion.

Often this disruption occurs at the larval stage, which is exquisitely sensitive to hormones and their mimics, but it is not until the organism matures that the consequences become noticeable; likewise adult exposure also causes irreversible changes. However, reasonable progress has made in identifying the chemicals likely to be causing the effects noted in caged and wild fish.

More than 70,000 chemicals have been implicated so far, and these include a wide variety of compounds such as, pesticides, alkylphenolic surfactants, PAHs, polychlorinated biphenyls, natural and synthetic hormones,

phthalates and parabens. Despite the fact that substantial advancement has been made in the last decade in understanding endocrine disruption in the aquatic environment, many questions remain unresolved.

Fish provide a very useful model for determining the effects of environmental pollutants on vertebrate reproductive function. They are the species, which are most at risk from pollution; and are readily exposed in the laboratory to environmentally realistic doses.

Wild fish from habitats, subjected to pollution, can also easily be compared with specimens exposed in the laboratory under more controlled conditions. Although the endocrine system of fish differs in the detail of its structure and hormones, reflecting the enormous variety of reproductive strategies, effects found in fish would be expected to have parallels in mammals.

It is alarming to note that the rivers Ganga and Yamuna get polluted due to many industries and urban sewage besides agriculture runoff. It is also difficult to say authentically whether these concentrations are high enough to affect wild fish and as these rivers enter the sea even more fish population falls at risk. Not much attention has been given to the aspect of endocrine disruption in India, where the load of pollution is increasing day by day without any effective inspection methods in practice.

In this investigation, a suite of biochemical variables, somatic indices, bioconcentration and vitellogenin inductions were measured simultaneously from the same organism to determine the integrated (multivariate) response of fish to anthropogenic compounds (diethyl phthalate, methyl paraben and tertiary butylphenol). The chemicals targeted for the present investigation were selected because these were known to affect the endocrine system; but a thorough study was lacking.

- Bioassay test were performed to determine the lethal concentrations of the selected chemicals to the fish *Cyprinus carpio*. The 96 hr LC_{50} and LC_{100} values were 48 and 55 mg/l respectively for diethyl phthalate, 42 and 45 mg/l respectively for methyl paraben and 6.9 and 7.2 mg/l respectively for 4-tert butylphenol.

- The sub-lethal doses for chronic exposure were selected as fractions of the LC_{50}. Since the LC_{50} value was too high it was decided upon 1, 5 and 20 mg/l of diethyl phthalate for biochemical and somatic parameters and bioconcentration profile, and for the vitellogenin induction 100 μg/l, 1 and 5 mg/l were selected. For methyl paraben 1/10 , 1/25 and 1/50 of LC_{50} were taken where as for tertiary butylphenol 1/3, 1/5 and 1/10 of LC_{50} were selected.

- Aspects of organ damage such as acid phosphatase (AcP), alkaline phosphatase (AlP), serum glutamate pyruvic transaminase (SGPT) and serum glutamate oxalacetic transaminase (SGOT) were examined as biochemical indicators. Bioaccumulation, as a consequence of continuous exposure and also formation of vitellogenin, were taken in

consideration. The somatic indices measured were hepatosomatic index, craniosomatic index, renosomatic index and gonadosomatic index. A canonical discriminant analysis procedure was used to compare and evaluate the multivariate response of fish exposed to the given set of chemicals. The results of this study would demonstrate whether the response of fish to chronic stress could differ depending on individual or multiple response variables.

Experimental Design

- Matured *Cyprinus carpio* were exposed individually to diethyl phthalate (1,5 and 20 mg/l), methyl paraben (0.84, 1.68 and 4.2 mg/l) and 4-tertiary butylphenol (0.69, 1.38 and 2.3 mg/l) for 28 days, and compared with control to study the following : (i) biochemical parameters: AcP, AlP, SGOT SGPT; (ii) somatic indices: GSI, HSI, RSI and CSI; (iii) bioconcentration in liver, testis, gills and brain; (iv) Vitellogenin induction; (v) Histopathology of liver, testis and ovary .

- To assess the effect on the pre-decided parameters; by exposing to the selected chemicals, 3 exposure concentrations for each chemical were trialed (in triplicates) for 28 days. A control was maintained for the same period. Stocking density was maintained @ 500 litre water for 20 fishes per tank (capacity 100L). Fishes were fed @ 5% of their body weight once a day. However, feed was adjusted fortnightly according to the body weight.

- Complete water was exchanged every alternate day and continuous aeration was provided through aerators. Electrical sponge filters were provided to maintain the water quality.

- Samples were taken on a weekly basis to measure the changes in parameters; and utmost care was taken during handling and sacrificing the fish.

Biochemical Parameters

- Diethyl phthalate (DEP) and methyl paraben (MP) had a more or less inhibitory effect on the Acid phosphatase (AcP) activity of the fish, while tertiary butylphenol (TBP) caused an increase. The highest activity (25.655 $\pm$ 0.53 μmol/g of protein) shown in response to TBP at 2.3 mg/l for 3rd week as compared to 17.69 $\pm$ 0.40 μmol/g of protein in the control during the same period. The maximum inhibition (0.85 $\pm$ 0.27 μmol/g of protein) was observed in response to DEP exposure at 20 mg/l, which was nearly 16 times less as compared to 16.5 $\pm$ 0.212 μmol/g of protein during the 4th week. Studies revealed that apparently 0.84 mg/l MP did not cause adverse effect on AcP activity but prolonged exposure might cause inhibition even at a very low level. However, even at lowest exposure concentration of DEP, i.e. 1 mg/l, AcP was inhibited.

- The alkaline phosphatase (AlP) activity was elevated by DEP and MP exposure, while TBP exposure inhibited the AcP activity. The highest activity of AlP (8.16 $\pm$ 0.16 μmol/g of protein) was observed at 20 mg/l

DEP exposure in 4th week, which was nearly 5.5 times more as compared to the control (1.45 ± 1.55 μmol/g of protein). The lowest activity of AIP (0.54 ± 0.10 μ mol/g of protein) in response to TBP was recorded at 2.3 mg/l exposure in 4th week. Considering AIP activity 0.84 mg/l MP exposure, could be considered to the test fish.

- The SGOT activity was more or less inhibited by all exposure levels of the 3 chemicals. The maximum inhibition was observed at 2.3 mg/l TBP exposure during the 4th week. Comparatively less differences of SGOT activities between control and exposure levels of MP were recorded.

- The SGPT activity in DEP exposure showed inhibitory trend with respect to exposure period and concentrations. The reverse phenomenon was seen in case of MP and TBP exposure, where all the exposure levels for 4 weeks led to increase of SGPT from 7.35 ± 0.29 μ mol/g of protein in 1st week to 20.88 ± 0.001 μ mol/g of protein in the 4th week at 0.84 mg/l MP exposure which was the lowest for this chemical and similarly increasing trend was also seen for the lowest exposure rate of TBP (0.69 mg/l) which was enhanced from 8.96 ± 0.29 μ mol/g of protein to 20.23 ± 1.55 μ mol/g of protein from 1st to 4th week. The maximum inhibition of 5.30 ± 0.02, 5.35 ± 0.17 and 5.70 ± 0.20 μ mol/g of protein at 1, 5 and 20 mg/l DEP respectively was observed during the 4th week of exposure .The inhibition rates were less as compared to 8.84 ± 0.10 μ mol/g of protein in control during the

same period. None of the exposure concentrations can be termed safe as far as SGPT is concerned.

Growth and somatic indices

- GSI was highly reduced in all the 3 chemicals with respect to time and concentration. The highest reduction (1.64 $\pm$ 0.19) was about 4.5 times that of control in response to MP exposure at 4.2 mg/l in the 3rd week, while the least 7.06 $\pm$ 0.73 was in response to TBP exposure at 0.69 mg/l in the 4th week. The least impact on GSI was observed in TBP at 0.69 mg/l and could apparently be said safe to the test fish.

- Likewise there was marked increase in RSI of fish exposed to MP and TBP, while in response to DEP exposure, observed RSI could not be conclusively termed as increasing or decreasing since it varied widely with time and concentration. The maximum increase in RSI was seen at 4.2 mg/l exposure to MP during the 4th week, whereas in case of TBP exposure the maximum increase (1.49 $\pm$ 0.06) was recorded at 1st week, i.e. in the beginning of the experiment, which was steadily decreased to 1.29 $\pm$ 0.12 at 0.69 mg/l level.

- The HSI was more or less increased in exposure levels of all the three chemicals while maximum increase (1.95 $\pm$ 0.12) was seen in response to TBP exposure for 3rd week. The HSI was observed to be closer to control at all the lower exposure concentrations during the 4th week.

- The CSI was not affected in all the chemicals except for DEP exposure at 20 mg/l, where it was reduced to 0.99 $\pm$ 0.176 as compared to

control (1.53 ± 0.001) during the 4th week of exposure. The rest of the concentrations of all the three chemicals did not significantly have impact on CSI.

Histological Impacts

- The histological impacts were studied for the lowest exposure concentrations of all the three chemicals. The organs (liver, testis, ovary) studied showed various degrees of damage at the end of the exposure period.

- The maximum liver damage was sustained by DEP at 1 and 5 mg/l and TBP at 0.69 and 1.38 mg/l after 28 days of exposure. A mass degeneration of tissues followed by a complete loss of architecture was observed. Partial damage was observed in the case of MP exposure at 0.84 and 1.68 mg/l. These changes might be temporary and may be reverted in case the exposure was stopped.

- The testis structure showed maximum damage after being exposed to DEP at 1 and 5 mg/l, which was comparable to the same cause due to TBP exposure at 0.69 and 1.38 mg/l. However, MP exposure did cause much damage to the testis internal structure.

- Histological changes in ovary were studied only in TBP exposure at 1.68 mg/l. Studies indicated that the ovarian structure was drastically altered.

Bioaccumulation

- DEP accumulated rapidly in the internal organs of the fish exposed to 1 and 5 mg/l, the highest being in the brain, while in liver it showed an increase with the increase in exposure period. Accumulation was also sustained in the gonads and gills. In case of MP exposure also the highest accumulation was observed in brain while liver showed a steady increase in deposition over the experimental period. The gills and gonads were reached equilibrium after accumulating the MP to a certain level.

Behavioral Changes

- Barring color changes at the highest DEP (20 mg/l) exposure, there was no visible alteration of external appearances due to exposure to all the chemicals. Changes in behavior such as delayed startle response were observed at the highest exposure concentrations of the toxicants (DEP and MP).

Vitellogenin Induction

- The lowest exposure concentrations (100 μg/l DEP, 0.84 mg/l MP and 0.69 mg/l TBP) of all the chemicals induced to produce significant amount of vitellogenin on the first day of sampling, i.e. 7th day of exposure. The highest induction was occurred at 4.2 mg/l MP exposure at the end of 4th week, while the lowest was observed at 100 μg/l exposure after 1st week. Also there was increase in the induction of hormone with the increase in concentration and period of exposure in all the chemicals.

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