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Vitellogenin Induction and Histo-metabolic Changes Following Exposure of *Cyprinus carpio* to Methyl Paraben

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ABSTRACT : Methyl paraben (MP), which is used as a preservative in pharmaceutical and cosmetic (shampoo) products, foods and beverages, enters into the aquatic environment and can pose a potential fish health hazard. In this experiment, effects of MP were evaluated in adult male common carp (*Cyprinus carpio*) by exposing them to fractions (1/143rd to 1/29th) of the LC_{50} dose with every change of water for 28 days. Vitellogenin induction, metabolic enzymes, somatic indices and bioaccumulation were studied at weekly intervals. The 96th h LC_{50} of MP in fingerlings was 120 mg/L. Compared to the control, except for increases ($p<0.01$) in alkaline phosphatase (EC 3.1.3.1), alanine aminotransferase (EC 2.6.1.2) and liver size, there were decreases ($p<0.01$) in activity of acid phosphatase (EC 3.1.3.2), aspartate aminotransferase (EC 2.6.1.1), and testiculosomatic index following exposure to any dose of MP. Vitellogenin induction was significantly higher ($p<0.01$) in exposed than unexposed (control) fish. The bioaccumulation of MP in testis, liver, brain, gills and muscle tissues of fish increased significantly ($p<0.01$) with increase of dose from 0.84 ppm to 1.68 ppm. Dose and duration of exposure ($p<0.01$) indicated that an exposure period of 1 to 2 weeks was sufficient to cause changes in the quantifiable parameters studied. Fish exposed to 4.2 ppm MP became lethargic after the 26th d. Histologically, degeneration, vacuolization and focal necrotic changes in liver and fibrosis-like changes in testicular tissue were noted. (**Key Words :** Methyl Paraben, Fish, Endocrine Disruption, LC_{50}, Vitellogenin, Metabolic Enzymes, Histopathology, Bioaccumulation)

INTRODUCTION

The endocrine disrupters are a large group of chemicals which 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 of the most potent endocrine disruptors share p-substituted phenol as a molecular structure, which includes parabens. Parabens (esters of 4-hydroxybenzoic acid also known as alkyl p-hydroxybenzoates), are used as preservatives in cosmetics, and are antibacterial agents in certain toothpastes. Four main parabens are in use: methyl, ethyl, propyl and butyl parabens; many products will have 2 or more of these chemicals as part of a preservative system. In a survey of 215 cosmetic products, methyl paraben was detected in 98% of products (Rastogi et al., 1995). Methyl paraben is also used widely because of its low toxicity. These chemicals have been linked to a global health crisis, causing life-threatening and costly metabolic and neurological disorders. The EPA is concerned about parabens used as preservatives in pharmaceutical products (antimicrobial and toiletries) for many years. They preserve fats, proteins, oils, and gums in skin care products, perfumes, toothpastes, soaps, some sun-screen products, and pet foods and appear to be found in the majority of cosmetics applied to the skin. Approximately 12 parabens are used commercially as preservatives in cosmetics, food and beverage, and pharmaceutical products (Elder, 1984).

Ethyl, propyl and butyl parabens have previously been shown to possess estrogen-mimicking properties both *in vivo* in fish (Pederson et al., 2000; Bjerregaard et al., 2003) and *in vitro* (Routledge et al., 1998; Harvey and Darbre, 2004).These chemicals are important from a human health viewpoint. Estrogen is known to influence the incidence and progression of human breast cancer. In a screen with a human estrogen receptor expressed by yeast cells the

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potency of the paraben group was butylparaben> propylparaben>ethylparaben>methylparaben (Routeledge et al., 1998). When methylparaben and butylparaben were injected into immature or ovariectomized rats, butylparaben increased uterine weights (an oestrogenic effect), whereas methylparaben had no detectable effect. It is established that fish are more sensitive to toxic chemicals than rats. Thus *in vivo* studies of methyl paraben with fish, which are lacking in the literature, were conducted.

Among potential sources of methyl paraben (MP) contamination and accumulation in human beings, one is cosmetic products and the other can be fish in the diet particularly from unknown contaminated sources (Persky et al., 2001; Boseveld and vanden Berg, 2002). A recent study demonstrated that at least a proportion of parabens present in cosmetic, food and pharmaceutical products can be absorbed and retained in body tissues, based on concentration of paraben esters measured in human breast tumors. The mean concentration was 2.3 ng/g tissues.

Sewage-fed fisheries are practiced in many countries including India with waste waters being utilized for the purpose of culturing fish (Ghosh et al., 1985; Jana, 1998). Endocrine disruption and accumulation of MP are more likely to occur in sewage-fed fisheries. The major source explaining the occurrence of parabens in sewage effluent at ng/L levels is the use of these substances in shampoo and cream. Moreover, wastes from various industries and garbage containing MP-related products are released into water. Common carp, which spawns year-round (Alikunhi, 1966), has been projected as a candidate species for sewage-fed aquaculture in India (Ghosh et al., 1985; Jana, 1998; Datta and Pal, 2005). In this study with *Cyprinus carpio,* we studied effects of fractions of the LC_{50} dose of MP on metabolic enzymes, somatic indices, histopathology of liver and testis and vitellogenin induction. Except for the accumulation study where 0.84 and 1.68 mg/L was used, the dose range was 0.84 to 4.2 ppm ($1/143^{rd}$ to $1/29^{th}$ of LC_{50}).

MATERIALS AND METHODS

Test animals

Four-months-old common carp (body weight 89.25± 0.32 g) were obtained from Maritech India Pvt Ltd, Mansar (MP, India). Fish of similar weight were acclimatized to laboratory conditions for 2 weeks before initiation of experiments. Along with the control, there were three treatment groups with three replicates in each treatment. They were reared in aquaria containing 280 l dechlorinated tap water, which were provided with continuous aeration. Each tub was stocked with 20 fish. One-fourth of the water was removed every 4^{th} day and the entire water was exchanged on a weekly basis. Weekly water quality parameters were measured in each system, the means of which were: water temperature (27°C), pH (8.0), dissolved oxygen (7.25 mg/L), negligible free CO_2, hardness (290 mg/L), nitrate (0.12 mg/L) and phosphate (0.33 μg/L). Fish were fed *ad libitum* twice a day (9.30 h and 17.00 h) with pellets during the acclimation and experimental periods. The pellets were prepared with an extruder (BTPL twin screw extruder, Kolkata) at the Central Institute of Fisheries Education, Mumbai and contained 35% crude protein and 4% crude fat.

Chemicals

Methyl paraben (CAS No: 99-76-3) (Fluka Chemika, Germany), HPLC grade acetone (Merck), anti-carp vitellogenin ELISA (Cayman chemicals, MI, USA), enzymes kits for alanine transaminase, aspartate transaminase, alkaline and acid phosphatase (Siddham Chemicals, Nagpur) and clove oil containing average Eugenol concentration of 8%) (Nav Niketan Pharmaceuticals, Mumbai, India) were purchased.

LC_{50} studies

The LC_{50} studies were conducted in accordance with methods adapted from the U.S. EPA and the American Public Health Association (APHA, 1985; US EPA, 1985). An initial range finding test was undertaken to select the maximum exposure level. Stock solution was prepared in acetone. Glass aquaria were filled with water and MP was then added with constant aeration. Each aquarium was provided with 10 fingerlings (9 g) and the experiment was conducted for 96 h. Fish were fed once daily, and the excess feed was removed every 24 h.

Sublethal exposure experiments

The LC_{50} of MP determined as above was 120 mg/L and hence male fish were exposed to 0 (solvent control), 0.84 ($1/143^{rd}$ of LC_{50}), 1.68 ($1/71^{st}$ of LC_{50}) and 4.20 ($1/29^{th}$ of LC_{50}) mg/L MP concentration with every change of water. Stock solutions were prepared in methanol and the experiments were conducted in glass aquaria over a period of 28 days. This period was chosen because pseudo-equilibrium of the toxicant concentrations between exposure media and fish tissues would be expected to occur within 30 days of exposure (Veith et al., 1979). All parameters except histopathology were studied on the 7^{th}, 14^{th}, 21^{st} and 28^{th} day. For this purpose, three fish were sacrificed from each tank on that particular day.

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. (1977).

Organ isolation, weighing and somatic indices

Fish were anaesthetized with clove oil (50 µl/L) until they remained motionless. Liver and testis were dissected out and immediately taken on ice. They were weighed separately with accuracy up to 1mg for estimation of somatic indices. Organ-Somatic index was determined as a proportion of weight of organ to total body weight.

Enzymatic analysis

Enzyme activity was studied in muscle tissues of fish. After sacrificing the fish, 0.1 g of tissue was taken from the dorsal region and 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°C. The supernatant was used for estimating aspartate transaminase (AST), alanine transaminase (ALT) (Wooten, 1964), acid phosphatase (ACP) and alkaline phosphatase (ALK) (Garen and Levinthal, 1960). The end point was recorded using UV-VIS spectrometry (Spectronic Genesys 2) at 510 for ACP and ALK and 490 nm for AST and ALT.

Vitellogenin analysis

Fish were killed by decapitation and muscle tissue (0.1 g) was homogenized in ice-cold phosphate-buffered saline (PBS; pH 7.3), with a 1:2 ratio of wet mass to buffer volume, in a glass homogenizer and centrifuged at 13,000 g for 15 min at 4°C. Briefly, the ELISA protocol which was essentially that used by Denslow et al. (1999), was as follows: an amount twice the tissue weight of ice cold saline was added and the homogenate was centrifuged for 60 min at 50,000 g at 4°C. The supernatant was filtered and stored in aliquots at -20°C. All the wells were coated with capture antibody and kept overnight at 4°C and then washed with washing buffer and blocked by blocking/dilution buffer. Standard curves were prepared by dilution of 100 µl 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 µl of Vtg standard dilution was added in duplicate. After incubation overnight at 4°C, plates were washed five times with washing buffer (PBS, 0.1% Tween-20, 0.1% BSA). Horse radish peroxidase (HRP) conjugated secondary antibody (200 µl) diluted 1:500 in sample buffer was added to each well. The color 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. The standard curve was fitted using a quadratic equation.

Bioaccumulation studies

Quantitative expression of organ-wise distribution of bioaccumulated compounds was analysed using super critical fluid extraction and reverse phase high performance liquid chromatography.

Residue analysis

Muscle, liver, brain, gill, and testis samples were weighed and frozen till extraction. Prior to extraction, tissues were thawed and excess moisture was absorbed using filter paper and sodium sulphate. The tissues were thoroughly compounded in sand matrix to increase the surface area of exposure. Supercritical fluid extraction was carried out by using an SFX 220 (Model 260 D ISCO serial: 202D20283), which consisted of an SFX 220 extractor, an SFX 200 controller and a D-syringe pump with a capillary restrictor and a temperature controller. The extraction conditions were decided using existing methods. The conditions followed for extraction of MP were: temperature 40°C, pressure 12,000 Kp, 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). The samples were collected in acetonitrile, stoppered tightly and stored at 0°C till further analysis.

High performance liquid chromatography

Reverse phase high performance liquid chromatography was performed to determine MP. The chromatogram used in this study consisted of an LC-10AS pump (Waters), a SIL-9A injector, a SPD-10 A dectector and a RP18 column (300mmx3.9mm). A degassed and filtered mixture of 0.05 M potassium hydrogen phosphate pH 2.5 and acetonitritle (65:35 v/v) was used as eluent. Flow rate was maintained at 1.0 ml/min. Detection was performed at 254 nm and the runtime for each analysis was 10 min (Akhtar et al., 1996).

Histology

Internal organs, viz. liver, kidney, brain, testis and muscle were collected from all exposed fish at the end of the experimental period. Liver and testis samples were fixed in neutral buffered formalin, followed by four stepwise 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 µm were stained with hematoxylin

and eosin, and observed under a microscope (Olympus BX40). Photographs were taken by Nikon (SC 35 type 12) camera with 30 sec shutter speed.

Statistical analysis

All data were analyzed using SPSS (Version 11). Main effects of treatment, interval and their interaction were analysed by two-way analysis of variance (ANOVA). Significant differences between means were found by Duncan's Multiple Range Test (DMRT). Overall treatment (methyl paraben dose dependent) effects were emphasized for the purpose of presentation of results and their discussion.

RESULTS AND DISCUSSION

Estrogenic effects of propyl and butyl paraben in fish have been previously reported (Pedersen et al., 2000; Bjerregaard et al., 2003), however, we were unable to find any literature on methyl paraben in general and using doses relative to LC_{50} in particular. The doses employed in this study were 1/143rd to 1/29th fractions of the LC_{50}. Hence, the LC_{50} value of MP was determined first, and found to be 120 mg/L. Based on a 48 hr study, Nipa (1991) reported 50 ppm as the no observable effect concentration of MP in golden orfe fish (*Leuciscus idus*). The variation in LC_{50} is attributable to species and hardness of water. Carbonated hardness of water used in the study was 290 mg/L. The concentration of 120 mg/L is higher than that reported by Nipa et al. (1991). Pedersen et al. (2000) administered parabens by intraperitoneal injection, whereas Bjerregaard et al. (2003) dosed butyl paraben orally.

In mammals, the parabens are absorbed from the gastrointestinal tract and metabolized to p-hydroxy benzoic acid. Bjerregaard et al. (2003) reported that a fraction of propyl paraben is retained in the liver and muscle after oral administration indicating the possibility that the chemical is metabolized quickly in fish also, although the activity of esterases hydrolyzing paraben is lower. Nevertheless, where fish are continuously exposed to such chemicals, as in sewage-fed fisheries, several metabolic and histological alterations are expected.

Methyl paraben effect on enzyme activities

There was a significant ($p<0.01$) effect of MP on activities of phosphatases (acid phosphatase-ACP and alkaline phosphatase-ALK) and transaminases (alanine aminotransferase- ALT and aspartate aminotransferase-AST) (Figure 1). Whereas a dose-dependent decrease up to 1.68 mg/L MP in the activity of ACP was observed, ALK activity increased up to this level of MP exposure. Acid phosphatase (ACP) catalyzes the hydrolysis of various phosphate-containing compounds and acts as a transphosphorylase in acidic conditions. It also functions as a marker enzyme for the detection of lysosomes in cell fractions and can be altered by the presence of xenobiotics (Cajaraville et al., 2000). ALK is a non-specific phosphomonoester hydrolase found in the plasma membrane of almost all animal cells. The muscle transaminases were affected, implying an effect on gluconeogenesis in fish. It has been found that there is accumulation in lysosomes of the anionic detergent linear

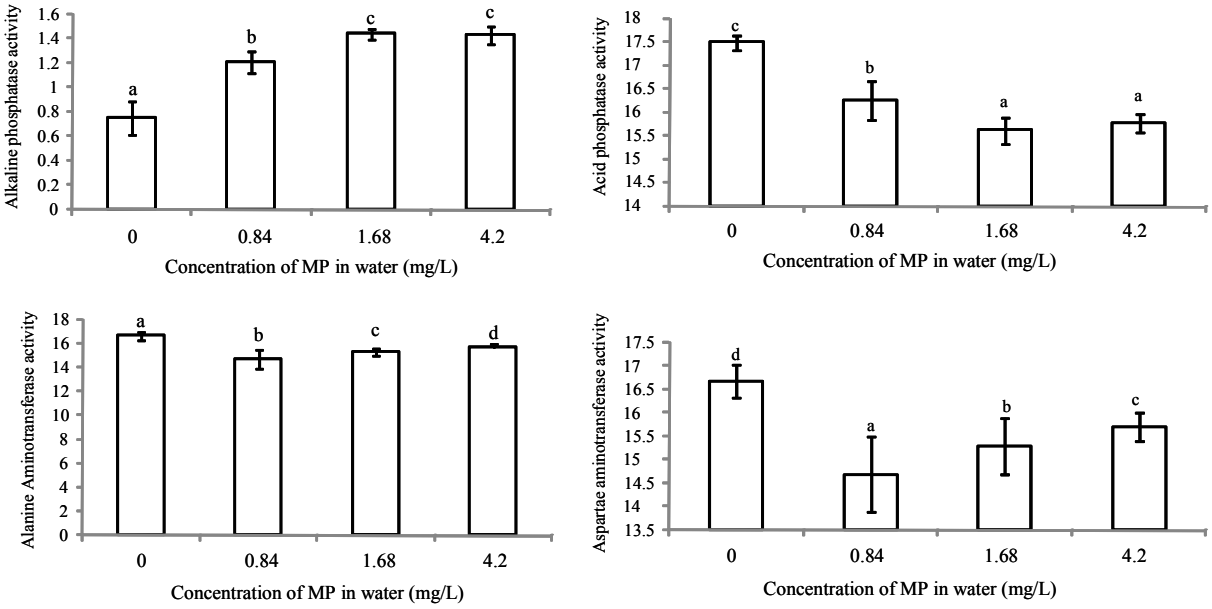

Figure 1. Effect of exposure to different concentrations of methyl paraben (MP) in water on muscle metabolic enzyme activities (μmol/g protein) in common carp, *Cyprinus carpio* (Means bearing different superscript differ significantly ($p<0.01$); Bars indicates standard error of means (n = 12 for control and each treatment).

alkylbenzene sulfonate leading to inhibition of lysosomal enzymes like ACP and ALK (Bragadin et al., 1996). Our findings agree with this report.

With regard to gluconeogenic enzymes, compared to the control AST activity was decreased in all the treatment groups. However, there were significant differences among the treatment groups with lowest activity in the group exposed to 0.84 ppm MP and highest activity in the 4.2 ppm MP group. On the other hand, ALT increased in a dose-dependent manner until 1.68 ppm. AST and ALT function as a link between carbohydrate and protein metabolism by catalyzing interconversion of strategic compounds like α-ketoglutarate and alanine to pyruvic and glutamic acids, and aspartate and α-ketoglutaric acid to oxaloacetic and glutamic acid, respectively (Watts and Watts., 1974; Martin et al., 1983). Increased ALT activity indicates increased transamination. This may mean that exposure of adult fish to MP had an effect on mitochondria during 28 days of exposure to MP. These alterations in metabolic enzymes may be due to damage to the plasma membrane resulting in leakage of enzymes. In fact, degenerative changes were noted in the liver and testis. Washburn et al. (1993) studied effects of estrogen on gluconeogenesis and related parameters in male rainbow trout. Male rainbow trout implanted with 17 β-estradiol had depressed gluconeogenesis and lower values of related attributes were seen in the plasma. Changes in the activities of transaminases and phosphatases have been reported for 4-tertiary butyl phenol (Barse et al., 2006).

There was significant ($p<0.01$) interaction of dose and duration of exposure for all the parameters. Exposure to 0.84 ppm MP for a week (ACP, ALK, and vitellogenin, testis and liver size) or two (ALT and AST) was sufficient to bring out significant ($p<0.01$) changes in these parameters (data not shown).

Methyl paraben accumulation in organs

A significant time-dependent increase of liver weight was observed in all treatments (Figure 2). Propyl paraben is known to accumulate in liver and muscle (Bjerregaard et al., 2003) causing significant effects on biological function. The liver weight of the exposed fish was significantly higher starting from the first week onwards. This corroborates the findings of Christensen et al. (1999) on increased hepatosomatic index (HSI) of juvenile rainbow trout. HSI increased at all exposure concentrations similar to a dose-dependant increase in fish injected with ethinylestradiol (Verslyke et al., 2002). The reduction in testicular size was dose dependent and maximum reduction was observed when exposed to 4.2 ppm MP. The reason for reduction of testis growth may be an effect of MP on the hypothalamic-pitutary-gonadal axis following its accumulation in testicular tissue. Similar effects were also reported in nonylphenol-treated male guppies (Billard, 1986), trout treated with alkylphenolic chemicals (Jobling et al., 1996) and carp treated with 4-tert butyl phenol (Barse et al., 2006) and diethyl phthalate (Barse et al., 2007). Reductions in gonadosomatic index have been reported in adult male trout

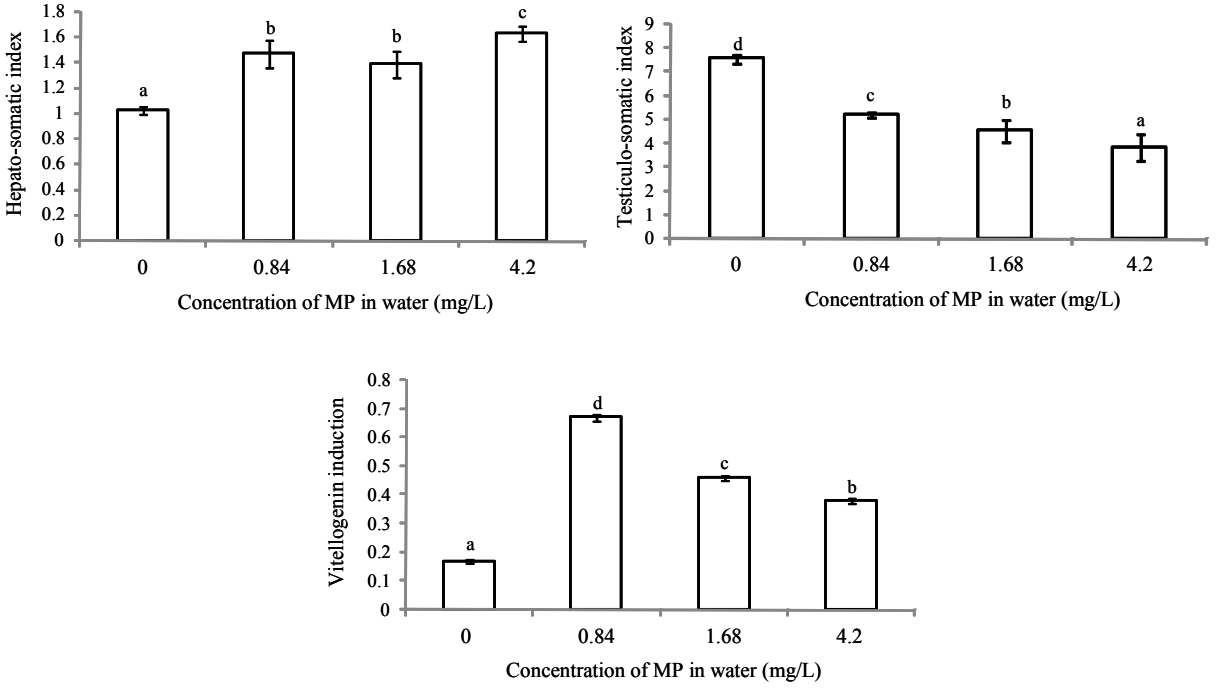

Figure 2. Effect of exposure to different concentrations of methyl paraben (MP) in water on somatic indices and muscle vitellogenin production (μg/ml extract) in common carp, *Cyprinus carpio* (Means bearing different superscript differ significantly ($p<0.01$).

and carp exposed to estrogens either in water or via food (Komen et al., 1989). Disturbance in metabolism of liver and testis and interference with the estrogen receptor causes many effects in the body, but of particular significance is induction of the protein vitellogenin, which is normally synthesized in the liver of adult female egg-laying vertebrates (Palmer et al., 1998). Vitellogenin synthesis in fish is a quite sensitive system for determining estrogenecity of a chemical compared to other test systems like the rodent uterotrophic assay (Bjerregaard et al., 2003). *C. carpio* produced significant amounts of vitellogenin when dosed with MP (Figure 2). Because it was detected in the muscle extract of male fish, response to MP was similar to xeno-estrogenic chemicals (Folmar et al., 1995; Sumpter and Jobling, 1995). Vitellogenin induction in general results in enhanced liver metabolism leading to an enlargement of the liver and consequently an increased hepatosomatic index (Korsgaard et al., 1983; Christensen et al., 1999). In rodents, 100 mg/kg/d of propyl paraben for 3 days did not induce an estrogenic response (Hossaini et al., 2000), whereas 20-30 mg/kg could induce vitellogenin in trout in 2 days. Christensen et al. (1998) found that immature rainbow trout dosed with 50 mg/kg 4-nonylphenol produced between 0.05 and 1.0 mg/ml vitellogenin after 6 days, similar to that in male *C. carpio* (0.401 and 0.425 μg/L). According to Elliot et al. (1979), vitellogenin is a fairly long-lived biomarker in male fish, and should be useful in assessing exposure of male *C. carpio* to estrogenic endocrine-disrupting chemicals in the field, especially in sewage-fed aquaculture.

One of the reasons for the metabolic changes mentioned above is the interference in metabolism due to accumulated compound in different tissues. In this study, MP residues were found in gill, liver, muscle, brain and testis (Figure 3). The bioaccumulation increased significantly ($p<0.01$) with increased dose of MP from 0.844 ppm to 1.68 ppm. Exposure to 225 μg propyl paraben^{-1} for 12 days led to concentrations of 6,700 and 870 μg propylparaben kg^{-1} liver and muscle, respectively (Bjerregaard et al., 2003). Thus methyl paraben does not behave any differently from propylparaben. Compared to the first week, bioaccumulation in liver, testis, brain, gill and muscle doubled at the end of second week of exposure (data not shown).

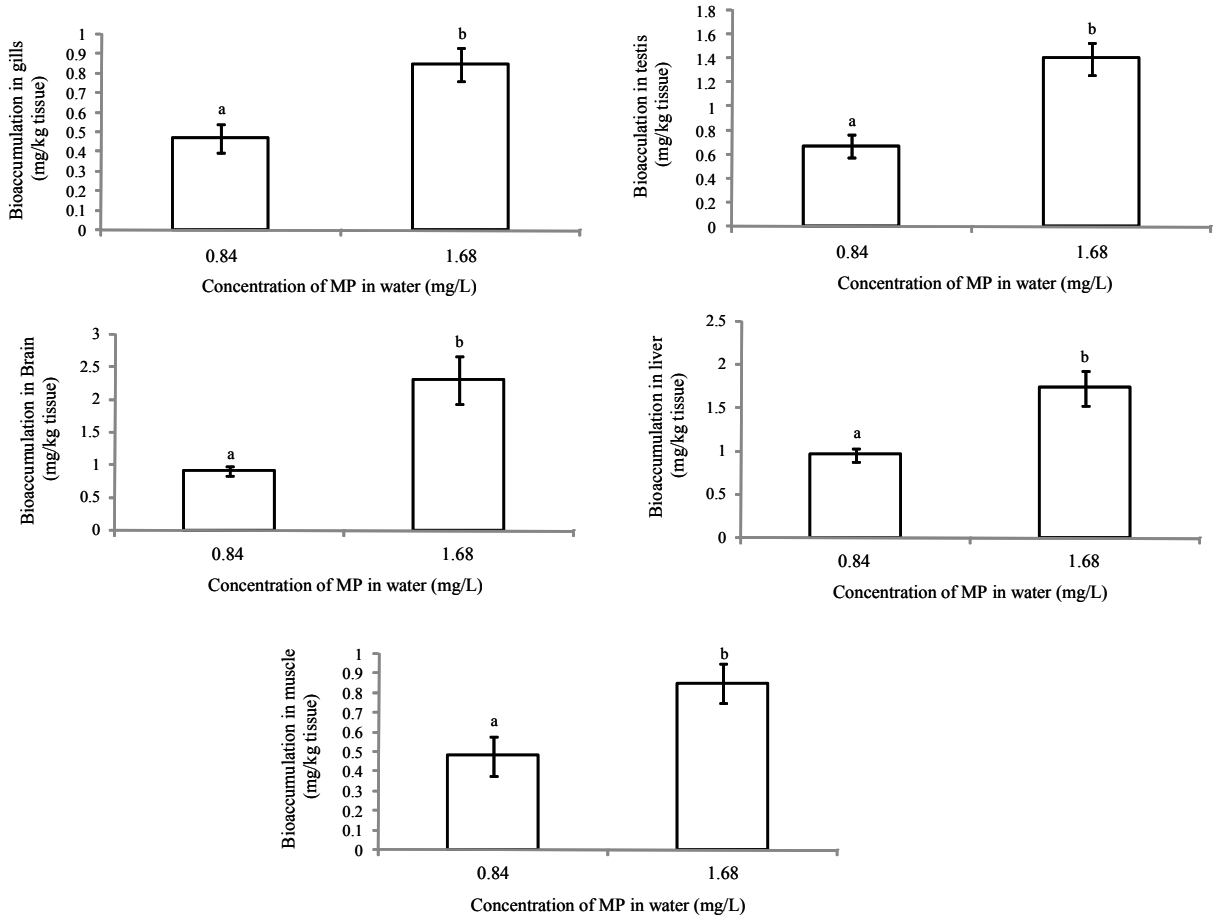

Figure 3. Effect of exposure to different concentrations of methyl paraben (MP) on accumulation in different organs in common carp, *C. carpio* (Means bearing different superscript differ significantly ($p<0.01$).

Methyl paraben causes pathological changes in organs

The other effect that accumulation may cause is pathological changes in organs. In liver from fish treated with MP, histopathological changes such as mild inflammation were noticed, with lack of clear vacuoles at lower (0.84 mg/L) exposure concentration. However, at higher exposure concentrations (1.68 and 4.2 mg/L) there was a general increase in vacuoles and appearance of focal necrosis of hepatocytes (Figure 4), but not much change in the structure. Unlike control fish testis, where the seminiferous lobule lumen was filled with spermatozoa, testis from fish exposed to MP showed altered testicular structure that included infiltration of inflammatory cells and fibrotic changes. The interstitium became narrow and there were lees spermatozoa (Figure 5). Adverse effects on testis structure of mature male carp exposed to 90-1,000/4-tert-pentyl phenol (p-alkylphenol) have been reported (Gimeno et al., 1998). These include inhibition of spermatogenesis, disappearance of spermatozoa and spermatogenic cyst and higher incidence of pathological alterations such as fibrosis, vacuolation, and atrophy of germinal epithelium.

Methyl paraben affects behaviour of fish

No behavioral changes were observed until fish were exposed to 1.68 ppm MP. While the feed was consumed within 10-15 minutes, fish swimming movements and tapping response were normal. However, with 4.2 ppm MP fish become lethargic towards the end of the experimental period (i.e. 26th day), and responded late to tapping. However, fish colour was unchanged. Changes in the

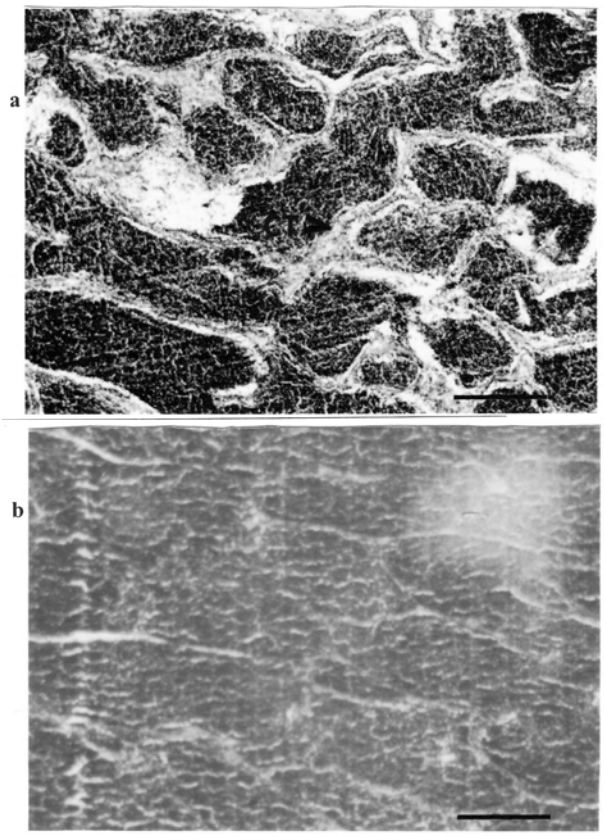

Figure 5. Histological section of testis of fish from (a) control and those exposed to (b) 0.84 ppm MP. Note: lumen of testis filled with spermatozoa in control, and fibrosis type appearance with loss of architecture in exposed fish with reduced spermatozoa (Bar is 100 μm).

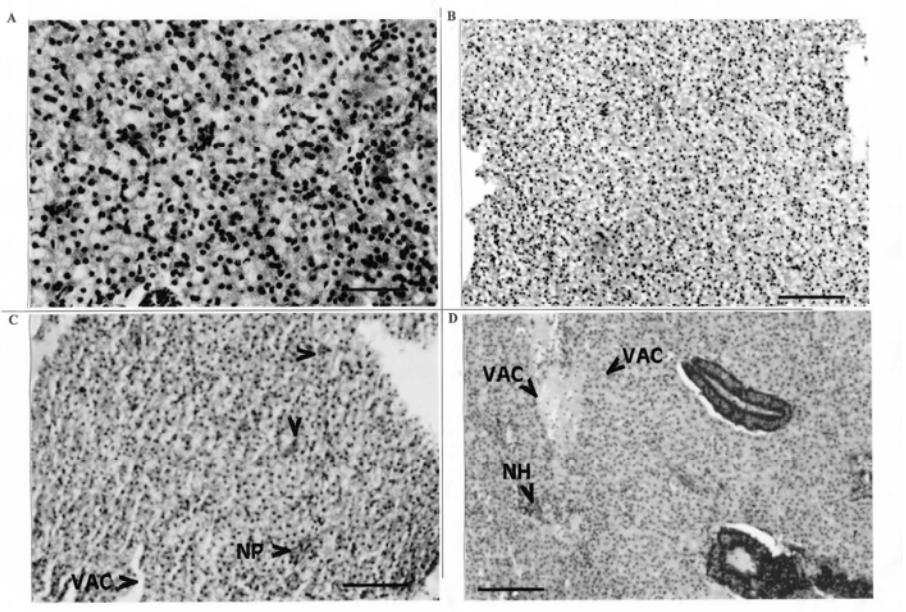

Figure 4. Histological section of liver of fish from (A) control and those exposed to (B) 0.84 ppm, (C) 1.68 ppm and (D) 4.2 ppm of methyl paraben (MP), Dose dependent changes in hepatocytes: inflammation, necrosis, vacuoles. H-Hepatocyte, VAC- Vaccuoles, NP-necrotic type patch, NH-Necrotic type hepatocyte. Changes are more prominent in 4.2 ppm MP-note large vacuole. (Bar is 100 μm for A and 40 μm for B, C, D).

activity of acetylcholine esterase (AChE) lead to changes in behaviour. Ghorpade et al. (2002) found reduced AChE activity in mrigal fish exposed to 25 ppm diethyl phthalate, a compound proved to be an endocrine disruptor (Barse et al., 2007). Altered AChE activity in fish has been found in fenvalerate toxicity (Mushigeri and David, 2005). Such a reduction in neurotransmitter activity may be due to accumulation of MP in nervous tissue, as found in the present study.

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