Published papers

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Quantification of PCP in different tissues of cat fish, *Heteropneustes fossilis*

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Introduction

Pentachlorophenol (PCP) a phenol derivative chemical, used primarily as a preservative and protectant of wood in forestry and a biocide in agriculture and slime control in pulp and paper mills. As the retention of PCP in wood fibres is poor, discharge of the substance with the waste water from pulp and paper mills can not be avoided. It was therefore of considerable interest to examine the occurrence of PCP in various ecosystems in a receiving body of water.

Principle

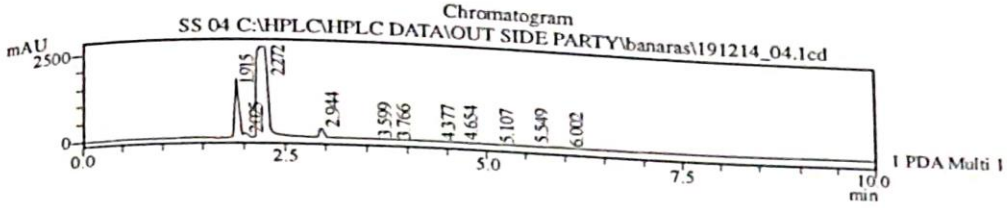
Pentachlorophenol is extracted from the acidified sample with a mixture of n-hexane and iso- propanol. The addition of iso propanol is made to minimize losses of extract due to formation of emulsions. PCP is then separated from pesticides of the chlorinated hydrocarbon type and from polychlorinated biphenyls by extracting it into a borax solution.

Material and methods

Chemicals

Chemicals were of analytical grade and purchased locally.

1. Sulfuric acid 6M- 340 ml conc.H_2So_4 was mixed carefully with water, cooled and diluted with water to 1000 ml.
2. n-hexane



1 PDA Multi 1/215nm 4nm
PDA Ch1 215nm 4nm

Peak Table

Peak#	Ret. Time	Area	Area %
1	1.919	5399396	16.640
2	2.025	550808	1.698
3	2.272	24990839	77.020
4	2.944	1368253	4.217
5	3.599	5782	0.018
6	3.766	1400	0.004
7	4.377	49708	0.153
8	4.654	58247	0.180
9	5.107	2847	0.009
10	5.549	19730	0.061
11	6.002	315	0.001
Total		32447325	100.000

Fig 4. Result of Control liver of H. fossilis.

Sample Information

Sample Name	:	SS 01
Sample ID	:	SS 01
Vail#	:	1
Injection Volume	:	50 uL
Data Filename	:	191214_01.1cd
Method Filename	:	MPD.1cmMPD.1cmMPD.lcm
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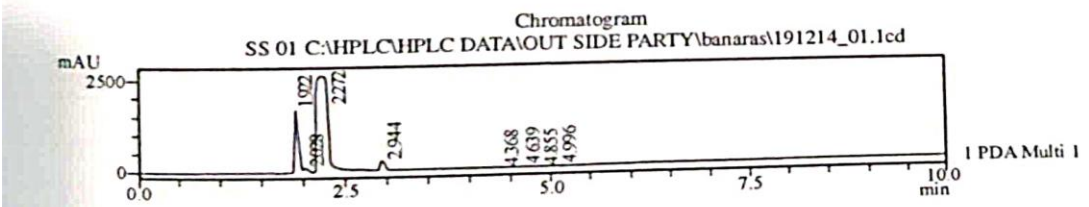

Sample Information

Sample Name	:	SS 02
Sample ID	:	SS 02
Vail#	:	2
Injection Volume	:	50 uL
Data Filename	:	191214_02.1cd
Method Filename	:	MPD.1cmMPD.1cmMPD.lcm
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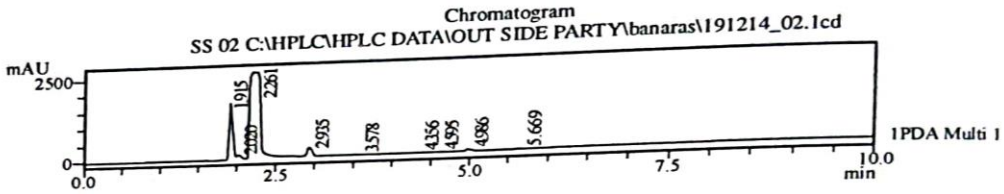

1 PDA Multi 1/215nm 4nm
PDA Ch1 215nm 4nm

Peak Table

Peak#	Ret. Time	Area	Area %
1	1.915	5486882	16.555
2	2.020	551938	1.665
3	2.261	25072350	75.647
4	2.935	1659741	5.008
5	3.578	42155	0.127
6	4.356	50161	0.151
7	4.595	2864	0.009
8	4.986	276760	0.835
9	5.669	1046	0.003
Total		33143896	100.000

Fig 3. Result of 32 ug/l treated ovary of H. Fossilis during 21 days exposure

Sample Information

Sample Name	:	SS 04
Sample ID	:	SS 04
Vail#	:	4
Injection Volume	:	50 uL
Data Filename	:	191214_04.1cd
Method Filename	:	MPD.1cmMPD.1cmMPD.lcm
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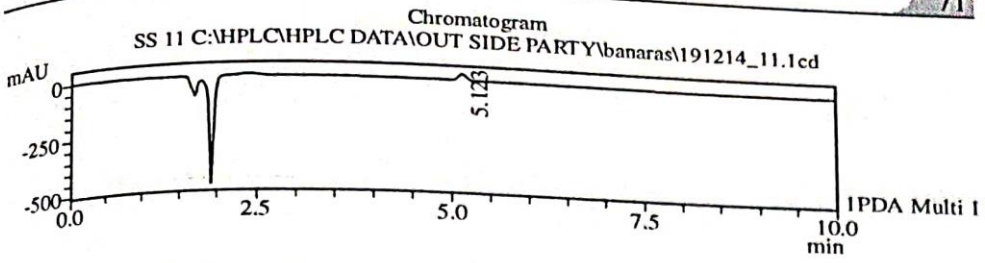
Data Processed	:	12/23/2014 3:35:17 PM



1 PDA Multi 1/215nm 4nm
PDA Ch 1 215nm 4nm

Peak Table

Peak#	Ret. Time	Area	Area %
1.	5.123	190987	100.000
Total		190987	100.000

Fig 1. Result of standard PCP Sample

Sample Information

Sample Name	:	SS 03
Sample ID	:	SS 03
Vail#	:	3
Injection Volume	:	50 uL
Data Filename	:	191214_03.1cd
Method Filename	:	MPD.1cmMPD.1cmMPD.1cm
Date Acquired	:	12/23/2014 1:44:35 PM
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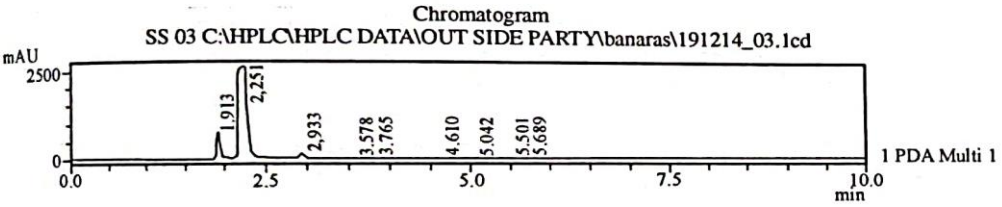

1 PDA Multi 1/215nm 4nm
PDA Ch 1 215nm 4nm

Peak Table

Peak#	Ret. Time	Area	Area %
1	1.913	2563334	9.962
2	2.016	343518	1.320
3	2.251	22423077	86.135
4	2.933	622677	2.392
5	3.578	10687	0.041
6	3.765	19103	0.073
7	4.610	4445	0.017
8	5.042	1984	0.008
9	5.501	13405	0.051
10	5.689	383	0.001
Total		26032612	100.000

Fig 2. Result of control ovary of H.Fo

3. Extraction mixture, 50 ml of isopropanol and 250 ml of n-hexane were mixed together.
4. Borax, 0.1M- Dissolved 38g 0f $Na_2B_4O_7.10\ H_2O$ in water and diluted to 1000 ml.
5. Acetylation reagent- Mixed 2ml of pyridine and 0.8 ml of acetic anhydride and a dry 5ml injection vial, cap the vial with parafilm and store it in cold.
6. Pentachlorophenol (Crystalline, 99% pure) was purchased from Acros Organics (Geel,Belgium).

Homogenized 1 g of the sample in water and transferred the homogenate to a 15 ml centrifuge tube. 5ml of water was used in total for this procedure. Added 1 ml of 6M sulfuric acid and left it for 10 min. Added 5 ml of the extraction mixture and shaked for 1min, centrifuged and chilled the tube in an ice mixture. Decanted the organic layer to a graduated centrifuge tube with conical bottom and the volume was noted. Extracted the Hexane layer with 2 ml of Borax for 1 min. Centrifuge and transferred the aqueous phase with a Pasteur pipette to a 5 ml test tube. Repeated the extraction with 2 ml of Borax. Added 0.5ml of n-hexane and 40ul of the acetylation reagent to the combined extract and shake for 1min (Rudling, 1970). Analyzed the hexane phase in HPLC (Shimadzu Prominence HPLC model Coupled with PDA detector) central salt and marine chemicals research institute, Bhavnagar.

Result

The data indicates that the fishes exposed to dose 1/10 of Lc50 for 28 days did not show detectable amount of PCP in sampled tissue. It may be suggested that accumulation of PCP in tissues needs long exposure time.

Sample Information

Sample Name	:	SS 11
Sample ID	:	SS 11
Vail#	:	11
Injection Volume	:	50 uL
Data Filename	:	191214_11.1cd
Method Filename	:	MPD.1cmMPD.1cmMPD.lcm
Date Acquired	:	12/23/2014 12:24:33 PM
Data Processed	:	12/23/2014 3:04:36 PM

1 PDA Multi 1/215nm 4nm Peak Table
PDA Ch1 215nm 4nm

Peak#	Ret. Time	Area	Area %
1	1.922	6067932	17.473
2	2.028	579200	1.668
3	2.272	25993106	74.851
4	2.944	1998247	5.754
5	4.368	52160	0.150
6	4.639	18416	0.053
7	4.855	15511	0.045
8	4.996	1920	0.006
Total		34726491	100.000

Fig 5. Result of 32 ug/1 treated Liver of H. Fossilis during 28 days exposure period

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HISTOPATHOLOGICAL CHANGES IN LIVER OF *HETEROPNEUSTES FOSSILIS* EXPOSED TO PENTACHLOROROPHENOL (PCP)

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ABSTRACT

Endocrine disrupting chemicals (EDCs) are the substances which change the course of endocrine systems in a way that adversely affects the organism itself or its progeny. EDCs call for greater attention because of their increasing utility in daily products and possible correlation with compromised health.It has been reported that PCP is the most important degradation products of phenolic compounds due of its enhanced resistance towards biodegradation.

Effect of Pentachlorophenol (PCP) on the histology of Liver of a fresh water Catfish Heteropneustes fossilis was studied by exposing the fish to 32µg/l/day (1/10 of LC_{50}) of sub lethal concentration of PCP for a period of 14 and 28 days.

Histopathological changes observed in liver was vacuolization, necrosis, rupturing of hepatocytes during different time of exposure i.e., 14th and 28th days.

In our present study PCP (32µg/l/day) showed an adverse impact on liver of fresh water Catfish H. fossilis.

Key words: *Liver, Pentachlorophenol, Histopathological, Heteropneustes fossilis.*

INTRODUCTION

The use of pesticides rises exponentially with the industrial development and agricultural growth. Side by side these pesticides create serious threat to the non-target organisms both in terrestrial as well aquatic ecosystems. Hazardous chemicals from industrial waste water and agricultural runoff are the main cause of water pollution. An aquatic organism mainly fishes accumulate many contaminants and toxicants directly through their gills and skin and indirectly via their food chain, which may causes diverse alternations in their vital organs.

Histopathology showed to be a suitable biomarker in the evaluation of the health of organism exposed to pollutants and can be used as biomonitoring tools for toxicity studies (Meyers and Hendricks, 1985). One of the great advantages of using histopathological biomarkers in environmental monitoring is that this study allows examining specific target organs, including gills, kidney and liver, that are responsible for vital functions, such as respiration, excretion and the accumulation and biotransformation of xenobiotics in the fish and other animals (Dubey et. al., 2017; Dubey and Shah 2017; Gernhofer et al., 2001). Pentachlorophenol (PCP) is used globally in the production of plastics, pesticides, wood preservatives, herbicides and is present in sewage effluents around the world (Bennie, 1999, Talmage, 1994). It has been reported that PCP is the most important degradation products of phenolic compounds because of its enhanced resistance towards biodegradation, toxicity, ability to bio accumulate in aquatic organisms, and estrogenicity (Ahelet al.,1994).The frequently occurring persistent environmental pollutant pentachlorophenol (PCP) has been prposed to be carcinogenic (WHO 2003). However, literature on toxic mechanisms of action of PCP at the cellular level is scarce. Additionally, it has been shown that several

environmental chemicals, such as lindane, pentachloronitrobenzene, and pentachlorobenzene, can be metabolized to PCP in animals and plants (Koss and Koransky 1978; Van ommen et al. 1985; Renner and Mücke 1986; WHO 2003).

The objective of the present study was to investigate toxicity effects of PCP on the liver of the catfish (*Heteropneustes fossilis*). Histological changes were monitored through the short and long term exposure of 14 and 28 days respectively.

MATERIALS AND METHODS

Pentachlorophenol (Crystalline, 99% pure,Acros organics Geel, Belgium), was dissolved in ethanol and then diluted with water. To estimate $LC_{50}$for PCP, the protocol of Secretria Estatual Do Meio Ambient (SEMA;1988) was followed. Acclimatized cat fish (30-40g) were divided in to 14 groups of 15 fish each and kept in 10 L aquaria. each group was exposed to the following nominal PCP concentration 0,5,10,20,30,40,50,60,70,80,100,200,400 and 800µg/L ,PCP was added to the water. The fish were maintained for 96 hrs. Mortality and abnormal behavioral responses were recorded every 12 hrs during 96 hr. Mortality of fish was recorded for each of the concentration during the 96 hrs exposure.

Mature male catfish *Heteropneustes fossilis* (35-45g) were purchased from local fish market in the spawning phase (June last week) of the annual reproductive cycle (Senthilkumaran and joy, 1994). They were maintained in the laboratory under normal photo period (13.0L:11.0D) and temperature (25±2°c) until use for experiments. The fish were fed egg white daily ad libitum. The adapted adult fish classified into two groups (12 fish each group), first group control, second group PCP treated (for 14 days and 28 days with 32µg/l day). The experiments were performed in accordance with guidelines for experimentation in animals and all care was taken to prevent cruelty of any kind.

Small pieces of the liver was taken and fixed in Bouin's fluid to be embedded in paraffin wax and sectioned at 7µ in thickness. They were stained with Ehrlisch hematoxylin and eosin stain (H & E) according to Bancroft and Steven, (1982) and mounted in DPX, to visualize the section using Lac Zene microscope model HL-23(Lac Zene Biosciences, India).

RESULTS AND DISCUSSION

The liver shows continuous mass of hepatic cells and cord like pattern of hepatocytes interrupted by blood vessels and sinusoids. The hepatocytes are large in size, polygonal in shape with spherical centrally located nuclei and have homogenous eosinophilic cytoplasm. The sinusoids are seen as communicating channels occupied by blood cells.

After 14 days of exposure the hepatocytes became irregular and after 28 days some cells exhibited expanded contour and loosen their polygonal shape. There were many regions in the liver of experimental fish, where cells were highly vacuolated. Many cells have exhibited necrosis. Liver also showed degeneration and disintegration in most cytoplasmic content.

Endocrine disrupting chemicals (EDCs) are the substances which change the course of endocrine systems in a way that adversely affects the organism itself or its progeny (Marcoccia et al 2017). These chemicals can be found in a variety of everyday products and goods, such as in foods, water, plastics, shampoos, clothes, toothpastes, soaps, fertilizers, paper, textiles, carpets, utensils, bedding, toy, cosmetics, deodorant, etc. (Vilela et al 2014, Zhu et al 2016). EDCs call for greater attention because of their increasing utility in daily products and possible correlation with compromised health. The endocrine system maintains homeostasis of the bodily systems through hormones that can travel long distances in the body and often have amplified effects. Differences have also been reported in urban versus rural areas showing a statistical correlation between poor semen quality and higher levels of EDCs found in pesticides,

such as alachlor, diazinon, atrazine, metolachlor, and 2,4-dichlorophenoxyacetic acid (Fisher JS 2004). Such evidence linking the increasing prevalence of EDCs to declining semen quality and male reproductive health calls attention to the detrimental effects of EDCs.It has been reported that PCP is the most important degradation products of phenolic compounds because of its enhanced resistance towards biodegradation (Ahel et al.1994). The frequently occurring persistent environmental pollutant pentachlorophenol (PCP) has been proposed to be carcinogenic (WHO 2003). However, literature on toxic mechanisms of action of PCP at the cellular level is scarce. In addition to the health risks caused by PCP itself, polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs), which are the impurities of commercial Na-PCP, released into the environment (Bao et al., 1995; Zheng et al., 2008). The problems associated with Pentachlorophenol (PCP) exposure indicate the adverse effects of PCP include immunotoxicity, carcinogenicity, oxidative stress and metabolic disorders (Yin et al.,2006; Fang et al., 2010; Pietsch et al., 2014; Chen et al., 2015).Other chlorophenol studies revealed damaging effect on the liver and gonads of fish tissues (Christtiansen et al.1998; Jobling et al.1996; Lech et al. 1996) and the corresponding metabolism.Pentachlorophenol was found to accumulate in the liver, gill, gut, fat, and kidney tissue (Ahel et al. 1993). In our present study PCP 32µg/l/ day showed adverse impact on liver of Indian catfish *H.fossilis*, however, effect on liver enzymes will be studied further.

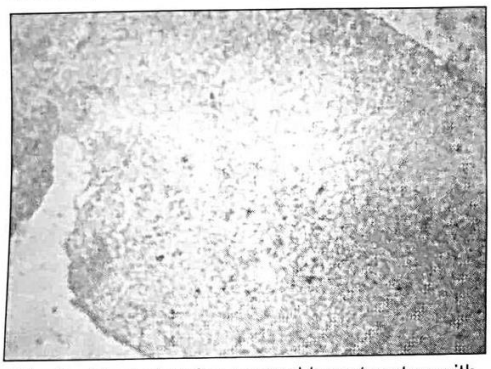

Fig A: Liver showing normal hepatocytes with visible nuclei in control groups (10X) spherical nucleus. (40X)

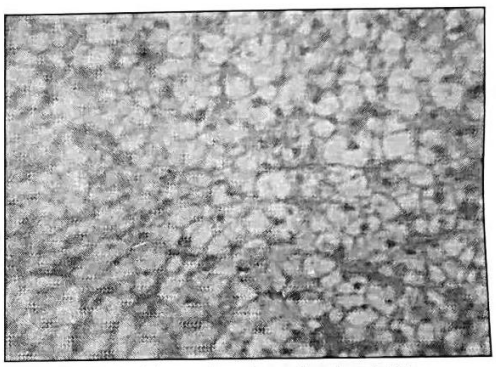

Fig B: Liver showing clearly visible Polygonal hepatocytes with central

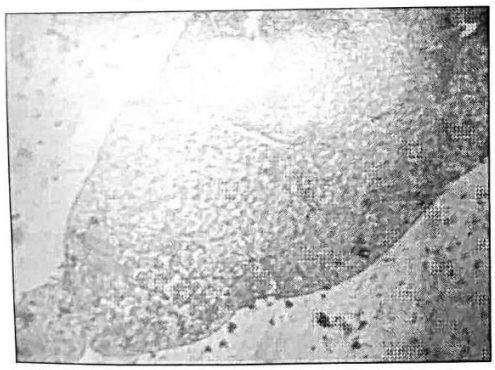

Fig C: Liver showing rupturing of hepatocytes and vacuolation of cytoplasm in 14 days PCP treated groups. (10X)

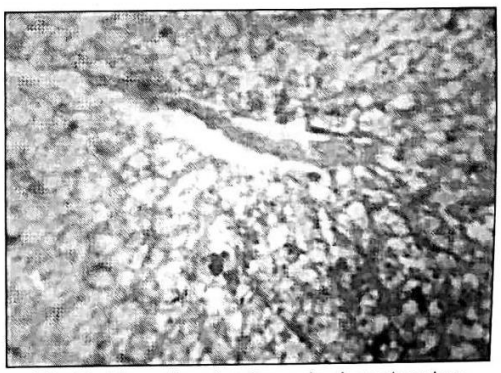

Fig D: Liver showing irregular hepatocytes with vacuolation in 14 days PCP treated groups (40X).

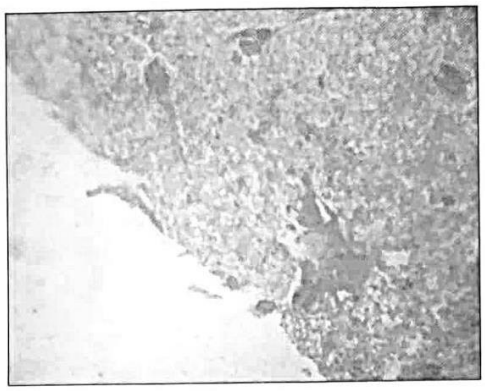

Fig E: Liver showing intensive vacuolation of cytoplasm and disintegration of hepatocytes in 28 days PCP treated groups. (10X) groups (40X).

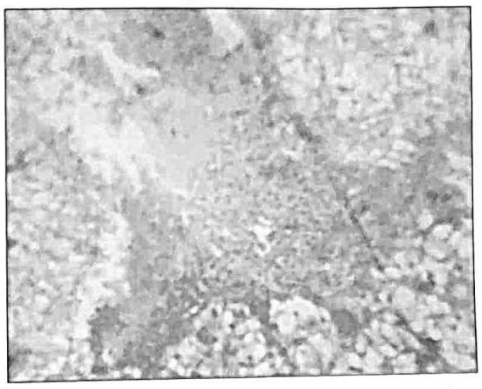

Fig F: Liver showing more irregular hepatocytes, loosing their polygonal shape, more vacuolation of hepatocytes and necrosis in 28 days PCP treated

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3

IMPACT OF PENTACHLOROPHENOL ON HISTOPATHOLOGICAL CHANGES IN THE TESTIS OF CATFISH *HETEROPNEUSTES FOSSILIS*

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ABSTRACT

The use of pesticides rises exponentially with the industrial and agricultural development. However, these pesticides create serious threat to the non-target organisms both in terrestrial as well as aquatic ecosystems. Hazardous chemicals from industrial wastes and agricultural runoff are the main causes of water pollution. An aquatic organism mainly fish accumulates many contaminants directly through their gills and skin and indirectly via their food chain, which may cause diverse alternations in their vital organs. In the present study, effect of pentachlorophenol (PCP) on the histology of testis of a freshwater catfish Heteropneustes fossilis (H. fossilis) was studied by exposing the fish to sub lethal concentration at32µg/l/day (1/10 of LC50) for a period of 14 and 28 days. Histopathological changes were observed in testis which showed shrinkage of seminiferous lobules and vacuolization in the tubular epithelium as well as in interlobular space. Histological impairment in tissues of lobules was also observed during different periods of exposure, suggesting adverse impact of PCP on testis of H. fossilis.

Key words: *Heteropneustes fossilis, Histopathology, Pentachlorophenol, Testis.*

INTRODUCTION

Endocrine disrupting chemicals draw greater attention because of their increasing utility in daily products and possible correlation of it with health. They change the course of endocrine systems in a way that adversely affects the organism itself or its progeny (Marcoccia et al., 2017).The environmental pollutant pentachlorophenol (PCP) has been proposed to be carcinogenic (WHO 2003). However, literature on mechanism of action of PCP at the cellular level is very less.It has been also reported that PCP is the most important degradation products of phenolic compounds (Ahelet al., 1994).Additionally, it has been shown that several environmental chemicals, such as lindane and pentachlorobenzene, can be metabolized to PCP. (Koss &Koransky, 1978, VanOmmenetal., 1985, Renner & Mücke, 1986 & WHO, 2003). Histopathology technique is a suitable biomarker in the evaluation of the health of organisms exposed to pollutants and can be used as bio-monitoring tools for toxicity studies (Meyers and Hendricks, 1985). One of the great advantages of using histopathological studies in environmental monitoring is that this study allows examining specific target organs, including gills, kidney and liver, that are responsible for vital functions, such as respiration, excretion and the accumulation & biotransformation of xenobiotics in the fish (Gernhofer et al., 2001, Dubey&Pandey 2015, Dubey and Pandey, 2016andVerma&Dubey,2019; Pandey and Pandey, 2013).

H. *fossilis*, a carnivorous freshwater fish is a popular delicacy relished throughout Indian subcontinent, due to its fast growth rate, high stocking-density capacities, high consumer acceptability and high resistance to poor water quality including oxygen deficiency. It is a seasonal breeder and its annual gonadal cycle comprises the preparatory (February to April), the pre spawning (May to June), the spawning

(July toAugust), and the post spawning (September to January) phases (Senthilkumaran and Joy, 1994).

The objective of the present study was to investigate toxicity effects of PCP on the testis of *H. fossilis*. Histological changes were monitored through the short and long term exposure of 14 and 28 days respectively.

MATERIALS AND METHODS

Mature male catfish H. *fossilis* (35-45g) were purchased from local fish market in the spawning phase of the annual reproductive cycle. They were maintained in the laboratory under normal photoperiod (13.0L:11.0D) and temperature (25±2°C). During the experiment, the fish were fed with boiled egg white daily. The acclimatized fish were grouped into two (12 fish each group), first group as control and the second group for PCP treated (14 days and 28 days with 32µg/l/day). The experiments were performed in accordance with ethical guidelines for experimentation in animals and care was taken to prevent cruelty of any kind.

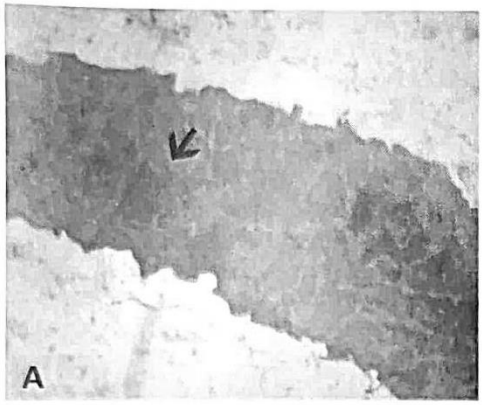
Pentachlorophenol (crystalline, 99% pure, Acros organics Geel, Belgium) was dissolved in ethanol and then diluted with water to make desired concentrations. To estimate LC_{50} for PCP, the protocol of Secretria Estatual Do Meio Ambient (SEMA) 1988 was followed. Acclimatized catfish (30-40g) were divided into 14 groups of 15 fish each and kept in 10L aquaria. Each group was exposed to the PCP concentrations as 0,5,10,20,30,40,50,60,70,80,100,200,400 and 800µg/L. The fish were maintained for 96 hrs. Mortality was recorded every 12 hrs during 96 hr.

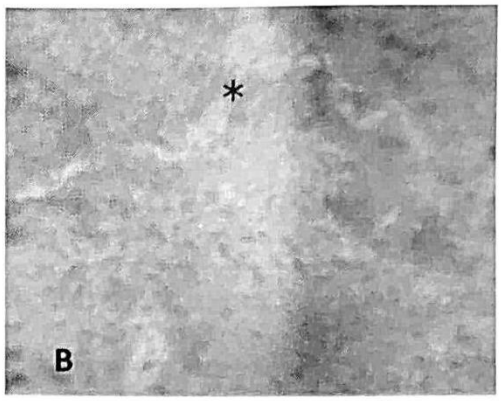
Small pieces of the testis were taken and fixed in Bouin's fluid and then embedded in paraffin wax. Tissues were sectioned at 7µm in thickness. They were stained with Ehrlich's haematoxylin and eosin stain (H&E) according to Bancroft and Steven (1982) and mounted in Dibutylphthalate Polystyrene Xylene (DPX). The sections were visualized using Lac Zene microscope (model HL-23, Lac Zene Biosciences, India).

RESULTS AND DISCUSSION

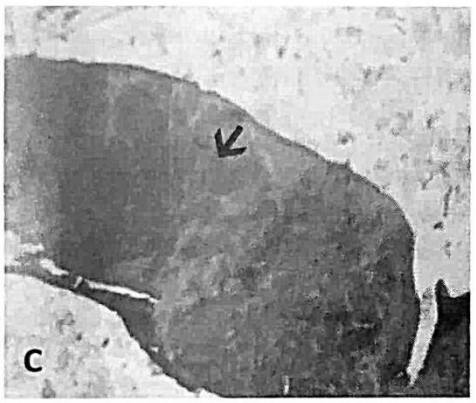
It has been reported that organochlorine compounds have effects on the immune and endocrine organs (Yokota et al., 2001,Razia et al., 2006,Chaube et al., 2016 and Choubey et al., 2015).The testis of the control *H. fossilis,* showing the seminiferous tubules lined internally with spermatogenic epithelium which produces spermatocytes (Fig A, B). In 14 daysPCP treated group, shrinkage of seminiferous lobules and vacuolization in the tubular epitheliumwere observed(Fig C, D) whereas, in 28 days PCP treated groups, both inter and intra-tubular vacuoles were observed. Shrinkage of lobules and impairment of epithelial tissues were also seen(Fig E, F).

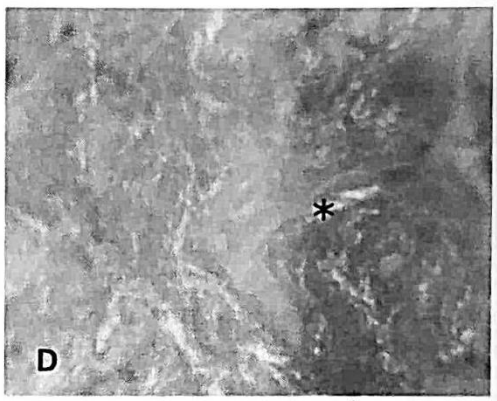
A variety of everyday products and goods, such as foods, water, plastics, toothpastes, soaps, fertilizers, paper, textiles, utensils, toy, cosmetics, deodorant, etc. contains endocrine disrupters(Joensen et al.,2009,Vilela et al., 2014&Zhu et al., 2016).The EDCs attracts the greater attention because of their increasing utility in daily products and possible correlation with health. The endocrine system maintains homeostasis of the body systems through hormones that can travel long distances in the body and often have amplified effects. Differences have also been studied in urban versus rural areas showing a correlation between poor semen quality and higher levels of EDCs found in pesticides, such as alachlor, diazinon, atrazine, metolachlor, and 2,4-dichlorophenoxyacetic acid (Fisher J.S.,2004). Such studies correlating the increasing prevalence of EDCs to decreasing semen quality and male reproductive health draws attention to the detrimental effects of EDCs.However, scarce literature is available on the mechanisms of action of PCP at the cellular level. In addition to the health risks caused by PCP itself, polychlorinated dibenzo-p-dioxins and dibenzofurans, which are the impurities of commercial Na-PCP,



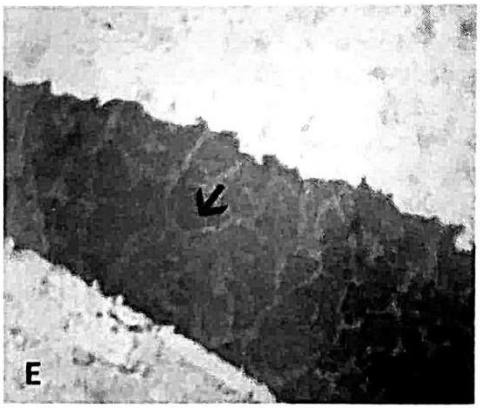


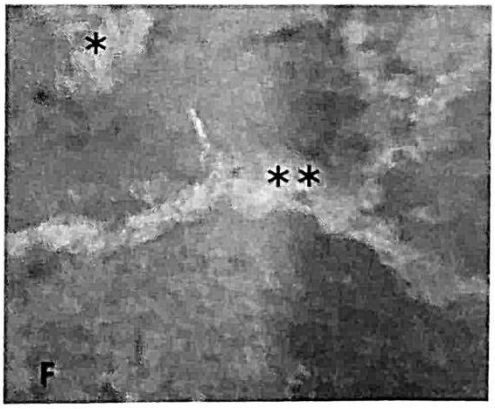
Figures showing lumen of testis filled with sperms in seminiferous tubules (↓) and interstitial cells (✳) in control group. A (10X); B (40X)





Testis showing shrinkage and vacuolization in the seminiferous lobules (↓)and vacuolization in interlobular space (✳) in 14 days PCP treated groups. C (10X); D(40X)





Testis showing shrinkage of seminiferous lobules(↓) and increase in inter(✳) & intra lobular space (✳ ✳) in 28 days PCP treated groups. E (10X); F(40X)

released into the environment causes adverse effect on health (Bao et al., 1995&Zheng et al., 2008). Studies reports that PCP exposure is associated with carcinogenicity, oxidative stress and metabolic disorders (Yin et al., 2006, Fang et al., 2010, Pietsch et al., 2014 & Chen et al., 2015).The histological study of the testis after exposure to Lead at a concentration of 1 mg/L showed that the germinal epithelium of the seminiferous tubules has been adversely affected, spermatogonial cells were not seen in treated groups, the epithelium is populated with degenerated germ cells and fibroblasts, giving an attenuated epithelial morphology (Choubey et al., 2015). Histopathological changes were also seen in the testes of *H. fossilis* under the effect of linear alkyl benzene sulphonate (Kumar et al., 2007). Apoptosis and structural abnormality in testes of *H. fossilis* exposed to PCP was reported(Ali et al., 2013). Thehistopathological impairment in tissues of liver and ovary of H. *fossilis* was studied under PCP exposure and modulation of steroid hormones were also noted in these animalsin a concentration and season dependent manner (Singh and Chaube, 2019).

In the present study, shrinkage and presence of vacuoles in seminiferous lobules indicates that, exposure to PCP can result in decreased fertility potential in male H. *fossilis*.

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