This Chapter includes the findings of the various effects of Bisphenol A on *H. fossilis in vivo* and water analysis for the presence of certain EDCs and heavy metals possessing estrogenic properties. Results are presented in the form of some tables, graphs and figures.

4.1. Physicochemical parameters of experimental water

The routine examination of physicochemical properties of water revealed that the water was most favorable for fish and was useable for laboratory procedures. All the parameters were within optimal limits.

**Table 4.1.** Physicochemical parameters of the water used for experimental purposes (Drinking water specification: IS: 10500, 1992) (Reaffirmed 1993).

<table>
<thead>
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<th>Characteristics</th>
<th>Unit</th>
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</thead>
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<tr>
<td>Temperature</td>
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<tr>
<td>Dissolved oxygen</td>
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<td>30</td>
</tr>
<tr>
<td>pH</td>
<td>----</td>
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<td>6.5-8.5</td>
</tr>
<tr>
<td>FCO₂</td>
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<td>&lt; 10</td>
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<tr>
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<td>30</td>
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<tr>
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<td>75</td>
</tr>
<tr>
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<td>300</td>
</tr>
<tr>
<td>Total Alkalinity (TA)</td>
<td>mg/l</td>
<td>42</td>
<td>200</td>
</tr>
</tbody>
</table>
4.2. Evaluation of acute toxicity (L50)

The acute toxicity bioassay to determine the L50-96 h value of Bisphenol A was conducted in a semi-static system with the change of test water on every alternate day. Bisphenol-A showed signs of acute toxicity, when fish were exposed via aquarium water. Mortality was recorded at 24 h, 48 h, 72 h, and 96 h respectively. The L50 value of Bisphenol A was determined to be 7 ppm (7 mg/l) using the "probit analysis" method (Finney, 1971). The three test concentrations of Bisphenol A that was selected for present experimentation were 0.8 ppm (1/8 of L50), 1 ppm (1/6 of L50) and 5 ppm (3/4) of L50 respectively.

![Acute toxicity bioassay](image)

Fig. 4.1. Percentage mortality of *H. fossilis* after 96 h exposure to different concentrations of Bisphenol A

4.3. Solvent control group

Ethanol control at concentration 0.01 ppm was used for all the experimental purposes. It was found that solvent control animals did not reveal any significant difference with tap water control animals for all the variables used in the present study.
4.4. Effects on Liver weight & Gonad weight.

4.4.1. Effect on Relative liver weight

Fish were exposed to 0.1 ppm 17β-estradiol (positive control) and BPA at concentrations of (0.8 ppm, 1 ppm and 5 ppm) in aquarium for different duration of experimental time (24 h, 48 h, 7 days and 14 days). Liver weight was measured after sacrificing the fish as per experimental protocol. The results are shown in Fig. 4.2 and Fig. 4.3. The mean values were compared with untreated (tap water) control values. Results of comparison between the different treated groups of BPA and 17β-estradiol showed significant rise in liver weight (P<0.05) after 7 days and 14 days exposure. Interestingly, 24 h and 48 h had no effect on liver weight when compared to the untreated control group.

Bisphenol A, unlike normal control but similar to 17β-estradiol had effect on liver weight after 7 and 14 days exposure. 7 days treatment showed significant increase (p <0.05) in the medium dose of 1 ppm (1 mg/l) as well as highest dose of 5 ppm (5 mg/l). Similarly 14 days treatment showed significant effects at medium and high dose of 1 ppm and 5 ppm (p <0.05). 17β-estradiol also showed significant increase (p <0.05) in liver weight after 7 days and 14 days treatment similar to the BPA treated groups.
Fig. 4.2. Histograms showing the effect of BPA on relative liver weight after 24 h (A) and 48 h exposure (B) following 17β-estradiol (0.1 ppm) and Bisphenol A (0.8 ppm, 1 ppm, 5 ppm) treatment via aquarium water. Values are shown as mean ± SEM, (n=3). Abbreviation: Sovent c- Solvent control (ethanol), BPA – Bisphenol A, E2- 17β-estradiol
Fig. 4.3. Histograms showing the effect of BPA on relative liver weight after 7 days (C) and 14 days exposure (D) following 17β-estradiol (0.1 ppm) and Bisphenol A (0.8 ppm, 1 ppm, 5 ppm) treatment via aquarium water. Asterisks denote significant relationship with untreated control groups (* p < 0.05). Values are shown as mean ± SEM, (n=3).
4.4.2. Effect on Hepatosomatic index (HSI)

Fish were exposed to 0.1 ppm 17β-estradiol and BPA at different concentrations of 0.8 ppm, 1 ppm and 5 ppm in aquarium with continuous flow of water for different duration of experimental time (24 h, 48 h, 7 days and 14 days). Immediately after sacrificing the fish after desired exposure time the liver weight was measured with a sensitive balance. After that the hepatosomatic index was calculated with the formula

(Given in chapter 3, section 3.8.2).

The results are summarized in Fig. 4.4 and Fig. 4.5 respectively. The mean values were compared with untreated control values. Results of comparison between the different treated groups of BPA and 17β-estradiol showed significant rise in hepatosomatic index (P<0.05) after 7 days and 14 days exposure schedules.

After 7 days treatment, HSI showed significant increase at the medium dose of 1 ppm as well as at the highest dose of 5 ppm. The values were significant at P<0.05. Similar to the result of 7 days, 14 days exposure also showed significant increase of HSI at lowest dose of 0.8 ppm and the highest dose of 5 ppm. 17β-estradiol also depicted significant increase in HSI after 7 days and 14 days of treatment similar to the BPA treated groups. No significant effect on HSI of *H. fossilis* was observed after the study period of 24 h and 48 h after 17β-estradiol and BPA exposure.
Fig. 4.4. Histograms showing the effect of BPA on Hepatosomatic index (HSI) after 24 h (A) and 48 h exposure (B) following 17β-estradiol (0.1 ppm) and Bisphenol A (0.8 ppm, 1 ppm, 5 ppm) treatment via aquarium water. Values are shown as mean ± (SEM, n=3). Abbreviation given in Fig. 4.2.
4.4. Effects on Liver weight and Gonad weight

Fig. 4.5. Histograms showing the effect of BPA on Hepatosomatic index (HSI) after 7 days (C) and 14 days exposure (D) following 17β-estradiol (0.1 ppm) and Bisphenol A (0.8 ppm, 1 ppm, 5 ppm) treatment via aquarium water. Asterisks denote significant relationship with untreated control groups (* p < 0.05). Values are shown as mean ± SEM, (n=3).
4.4.3. Effect on Testicular weight

Testicular weight was measured immediately after sacrificing experimental fish following treatment with BPA (0.8 ppm, 1 ppm, 5 ppm) and (0.1 ppm) 17β-estradiol after 24 h, 48 h, 7 days and 14 days respectively. No significant effect on testicular weight was marked after 24 h and 48 h treatment. 7 days exposure also depicted no significant effect on testis weight. The values obtained were not significant in comparison to controls. Results were obtained only after 14 days treatment. 14 day exposure with BPA showed significant decrease (p<0.05) of testis weight at low (0.8 ppm), medium (1 ppm) as well as highest dose of 5 ppm. 17β-estradiol treated group depicted decrease in weight after 7 days but the value was not significant in comparison to control, whereas after 14 days of treatment significant decrease in testis weight was seen. The results are presented in Fig. 4.6 and 4.7 respectively.

![Histograms showing the effect of BPA on Testis weight after 24 h and 48 h following 17β-estradiol (0.1 ppm) and BPA (0.8 ppm, 1 ppm, 5 ppm) treatment. Values are shown as mean ± SEM, (n=4). Abbreviation given in Fig. 4.2.](image-url)

Fig. 4.6. Histograms showing the effect of BPA on Testis weight after 24 h and 48 h following 17β-estradiol (0.1 ppm) and BPA (0.8 ppm, 1 ppm, 5 ppm) treatment. Values are shown as mean ± SEM, (n=4). Abbreviation given in Fig. 4.2.
Fig. 4.7. Histograms showing the effect of BPA on Testis weight after 7 days (A) and 14 days (B) following 17β-estradiol (0.1 ppm) and BPA (0.8 ppm, 1 ppm, 5 ppm) treatment. Asterisks denote significant relationship with untreated control groups (*) p < 0.05). Values are shown as mean ± SEM, (n =3).
4.4.4. Effect on GSI

Gonadosomatic index (GSI) was analyzed following the treatment period of 24 h, 48 h, 7 days and 14 days with 17β-estradiol and BPA respectively. The mean values were compared with control values. Results of comparison between the different treated groups of BPA and 17β-estradiol showed significant reduction in gonadosomatic index (P<0.05) only after 14 days exposure schedule. GSI had no effect after the acute study of 24 and 48 h. Interestingly no change in the GSI was found in the treated groups after 7 days treatment. The result of 14 days exposure showed significant decrease of GSI only at the highest dose of 5 ppm. 17β-estradiol also depicted significant decrease in GSI only after 14 days of treatment. The results are summarized in Table 4.2.

Table 4.2. Effects of Bisphenol A on GSI of H. fossilis Mature H. fossilis. The test agents (BPA and 17β-estradiol) were dissolved in ethanol and administered. Values are given as mean ± SEM, (n=3). Asterisks denote statistically significant differences against control group. (* p < 0.05).
4.5. Effects on Histoarchitecture of Liver and Testis of *Heteropneustes fossilis*

4.5.1. Effects on Liver

Fish were exposed to 0.1 ppm 17β-estradiol and BPA at concentrations of 0.8 ppm, 1 ppm and 5 ppm in aquarium. Different duration of experimental time (24 h, 48 h, 7 days and 14 days) was selected for exposure of the chemicals. Fishes were then sacrificed and tissue was collected for histopathological analysis. The study on the liver of *H. fossilis* in the control group revealed the presence of distinct hepatocytes that surrounded the portal veins. Hepatocytes appeared as polyhedral cells with centrally located nucleus and seemed to be arranged radially in the form of distinct cords around hepatic vein. Sinusoids were distinct which separated the adjacent hepatic cords (Fig. 4.8; A). 24 h treated liver with BPA showed fatty infiltration, blood pooling and destruction of cellular structures. The borders of the hepatocytes were obscure and cell degeneration was marked (Fig. 4.8; D, E, F). 17β-estradiol and solvent control groups were similar to the control group (Fig. 4.8; B, C). 48 h 17β-estradiol and BPA treated groups elucidated swelling of hepatocytes. The compact nature and integrity of hepatic cells were lost and hepatocytes had obscure borders. Vacuolization, spotty necrosis, pycnotic nuclei and diffused hyaline necrosis was observed (Fig. 4.9; C, D, E, F). 7 days exposure to 0.1 ppm 17β-estradiol showed deposition of bile on the entire liver (Fig 4.10; C). BPA treated groups (0.8 ppm and 1 ppm) showed intense hepatocellular degeneration, destruction of cellular structures and pycnotic (condensation and reduction in size) nuclei (Fig 4.10; D, E). Fibrosis of liver and Diffused hyaline necrosis was marked in the liver at the highest dose of 5 ppm BPA. Blood pooling was also seen (Fig. 4.10; F). 14 days BPA and 17β-estradiol exposure exhibited variety of changes in the treated liver. 17β-estradiol treated group showed dissociation of hepatocytes and vacuolization (Fig. 4.11; C). BPA treated groups depicted disrupted parenchymal architecture and necrosis, pycnotic nuclei, destruction of cellular structures and extensive hepatic hemorrhage together with necrosis (Fig. 4.11; D, E, F).
Fig. 4.8. Photomicrographs of liver tissue of control and Solvent control fishes with normal features [A, B] and 24 h treated (via aquarium water) with E₂[C], 0.8 ppm BPA [D], 1 ppm BPA [E] and 5 ppm BPA [F]. 17β-estradiol showing normal features [C]. BPA treated groups showed fatty infiltration; [D], destruction of cellular structures, Blood pooling and Spotty necrosis [E], obscure hepatocyte borders and cell vacuolar degeneration [F]. White arrowheads show liver sinusoids (S). Abbreviations: FI- Fatty infiltration; SN-Spotty necrosis; BP- Blood pooling.
Fig. 4.9. Histological photomicrographs showing the changes in the liver tissue of control and solvent control fishes with normal features [A, B] and 48 h treated (via aquarium water) with 17β-estradiol [C], 0.8 ppm BPA [D], 1 ppm BPA [E] and 5 ppm BPA [F]. Control and solvent control showing normal hepatocyte structure [A], [B]. 17β-estradiol treated group showed swelling of hepatocytes, loss of integrity of compact structure and vacuolar degeneration [C]. BPA treated groups showed vacuolization and pycnotic nuclei [D], destruction of cellular structures and spotty necrosis [E], obscure hepatocyte borders and cell degeneration leading to diffused hyaline necrosis [F]. White arrowheads show liver sinusoids (S). Abbreviations: SN-Spotty necrosis; VD- Vacuolar degeneration; DHN- Diffused hyaline necrosis; PN- Pycnotic nuclei.
Fig. 4.10. Representative light photomicrographs showing the changes in the liver tissue of control and solvent control fishes with normal features [A, B] and 7 days treated (via aquarium water) with 17β-estradiol [C], 0.8 ppm BPA [D], 1 ppm BPA [E] and 5 ppm BPA [F]. Control and Solvent control showing normal features [A], [B]. 17β-estradiol treated group showed bile deposition [C], BPA treated groups depicted intense hepatocellular denaturation [D]; destruction of cellular structures and pycnotic nuclei [E]; fibrosis of liver [F]. Abbreviations: BD- Bile deposition; HD- Hepatocellular degeneration; DHN- Diffused hyaline necrosis; PN- Pycnotic nuclei; BP- Blood pooling.
Fig. 4.11. Histological photomicrographs showing the changes in the liver tissue sections of control and solvent control fishes with normal features [A, B] and 14 days treated (via aquarium water) with 17β-estradiol [C], 0.8 ppm BPA [D], 1 ppm BPA [E] and 5 ppm BPA [F]. 17β-estradiol treated group showed dissociation of hepatocytes and vacuolisation [C], BPA treated groups depicted disrupted parenchymal architecture, necrosis and pycnotic nuclei [D]; destruction of cellular structures and swollen hepatocytes, widespread hepatic degeneration [E]; extensive hepatic hemorrhage, dissolution of hepatic cords and necrosis [F]. Abbreviations: HD- Hepatocellular degeneration; DHN- Diffused hyaline necrosis; HH- Hepatic hemorrhage; v- vacuolization; BP- Blood pooling.
4.5.2. Effects on Testis

Fish were treated with BPA at concentrations of 0.8 ppm, 1 ppm and 5ppm and 0.1 ppm 17β-estradiol in aquarium for different exposure periods (24 h, 48 h, 7 days and 14 days). They were then sacrificed and tissues were collected for histopathological analysis.

The histopathological studies on the testis of *H. fossilis* in the control and solvent control group showed the presence of numerous lobules which are separated by a thin layer of connective tissue. These lobular compartments, the seminiferous consists of Sertoli cells and germ cells. The testis of *H. fossilis* is unrestricted spermatogonial type. Spermatogonia are located alongside the entire length of the tubule (Fig. 4.12; A).

24 h and 48 h treated testis showed no degenerative changes and were more or less similar to controls. Seminiferous tubules filled with spermatozoa were observed. Sertoli cells were also seen (Fig. 4.12 and Fig. 4.13).

7 days exposure to BPA and 17β-estradiol resulted in disintegration of testicular structure (Fig. 4.14; C), seminiferous tubules were shrunken and lumen was found to contain less spermatozoa (Fig. 4.14; D, E). Moreover the lobular structure of testis was lost (Fig. 4.14; F). 14 days treatment exhibited altered testicular structure with empty lobules (Fig. 4.15; C). The lobules either had scanty spermatozoa at the terminal end or were empty. Disintegrated testicular structure was also observed at low and medium doses of BPA (Fig. 4.15; D, E). Necrotic testis was visualized at the higher doses (Fig. 4.15; E, F).
Fig. 4.12. Histological photomicrographs showing the changes in the testis tissue of control and solvent control fishes with normal features [A, B] and 24 h treated (via aquarium water) with 17β-estradiol [C], 0.8 ppm BPA [D], 1 ppm BPA [E] and 5 ppm BPA [F]. Control and Solvent control group showing normal seminiferous tubules (ST); Sertoli cells (SC) and spermatozoa (SZ) [A], [B]. 17β-estradiol treated and BPA treated groups showed normal structure similar to controls [C, D, E, F].
Fig 4.13. Photomicrographs showing the changes in the testis tissue of control and solvent control fishes with normal features [A, B] and 48 h treated (via aquarium water) with 17β-estradiol [C], 0.8 ppm BPA [D], 1 ppm BPA [E] and 5 ppm BPA [F]. Control and Solvent control showing normal seminiferous tubules (ST); Sertoli cells (SC) and spermatozoa (SZ) [A], [B]. 17β-estradiol treated group and BPA treated group showed normal structure similar to controls [C, D, E, F].
Photomicrographs showing the changes in testis tissue after 7 days of exposure Roy, 2011

Fig. 4.14. Photomicrographs showing the changes in the testis tissue of control and solvent control fishes with normal features [A, B] and 7 days treated (via aquarium water) with 17β-estradiol [C], 0.8 ppm BPA [D], 1 ppm BPA [E] and 5 ppm BPA [F]. Control and Solvent control showing normal seminiferous tubules (ST); Sertoli cells (SC) and spermatozoa (SZ) [A], [B]. 17β-estradiol treated group showed slight distorted structure [C]. BPA treated group showed disintegration of testicular structure [D, E, F], seminiferous tubules were shrunken and lumen was found to contain less spermatozoa [D, E]. Lobular structure of testis was lost [F].
Fig. 4.15. Representative light photomicrographs of testis tissue of control and solvent control fishes with normal features [A, B] and 14 days treated (via aquarium water) with 17β-estradiol [C], 0.8 ppm BPA [D], 1 ppm BPA [E] and 5 ppm BPA [F]. Control and Solvent control showing normal seminiferous tubules (ST); Sertoli cells (SC) and spermatozoa (SZ) [A, B]. 17β-estradiol treated group showed empty lobules with little or no spermatozoa [C]. Disintegrated testicular structure normal lobular arrangement of the testis was lost [D, E, F]. Necrotic testis; (NT) [E, F].
4.6. Effects on Liver (SEM studies)

Fish were exposed to 0.1 ppm 17β-estradiol and BPA at concentrations of 0.8 ppm, 1 ppm and 5ppm in aquarium for different duration (24 h, 48 h, 7 days and 14 days) and were then sacrificed for collection of tissues for SEM study. Experimental conditions and treatment procedures are given in materials and methods (Chapter 3, section 3.11.1; pp 9).

Scanning electron micrographs reveals control and solvent control groups having normal hepatocytes and sinusoids (Fig. 4.16; A, B). 24 h and 48 h results were almost similar to the control group. Further study was attempted for longer duration exposure to 17β-estradiol and BPA. Deformity in the hepatocyte cell surface was observed after treatment. After 7 days, characteristic changes in the liver tissue were observed. The hepatocytes increased in size. Distinctive swelling of liver cells were visible and sinusoids were congested (Fig 4.16; D, E). In the 17β-estradiol group hepatocytes had lost their roundish contour and had increased in size (Fig. 4.16; C). At all doses of BPA the liver hepatocytes had swelled and sinusoids were not visible. Occasional blebs were also seen (Fig. 4.16; D).

After 14 days of treatment significant changes in the hepatocytes were observed. The cell boundary of hepatocytes was distorted. Characteristic swelling of cells was more evident and was accompanied with vacuoles (Fig. 4.17; D). In the 17β-estradiol group hepatocytes had lost their roundish contour and increased in size (Fig. 4.17; C). At all doses of BPA the liver hepatocytes had swelled and sinusoids were not visible. Extrusion of cellular contents was noticeable in some (Fig. 4.17; D, E, F).
Fig. 4.16. Scanning electron micrographs of liver tissue of *H. fossilis* after 7 days exposure. Control and solvent control fishes showing normal hepatocytes and sinusoids [A, B] and 7 days treated (via aquarium water) with 17β-estradiol [C], 0.8 ppm BPA [D], 1 ppm BPA [E] and 5 ppm BPA [F]. Characteristic swelling of liver cells were visible and sinusoids were congested [D, E]. 17β-estradiol group hepatocytes had lost their roundish contour and had increased in size [C]. At all doses of BPA unlike normal tap water but similar to E2 the liver hepatocytes had swelled and sinusoids were not visible. Occasional blebs (b) were also seen [D]. Abbreviations: Hepatocyte (H); Sinusoid (S); b (Bleb). The final magnification is 4000 x.
Fig. 4.17. Scanning electron micrographs of liver tissue of *H. fossilis* after 14 days exposure. Control and solvent control fishes showing normal hepatocytes and sinusoids [A, B] and 14 days treated (via aquarium water) with 17β-estradiol [C], 0.8 ppm BPA [D], 1 ppm BPA [E] and 5 ppm BPA [F]. The cell boundary of hepatocytes was distorted. Characteristic swelling of cells was more evident and was accompanied with vacuoles (v) (D). In 17β-estradiol group hepatocytes had lost the roundish contour and increased in size (C). At all doses of BPA the liver hepatocytes had swelled and sinusoids were not visible. Extrusion of cellular contents was marked (D, E, F). Abbreviations: Hepatocyte (H); Sinusoid (S). Final magnification is 4000 x.
4.7. Effects on Testis (SEM studies)

Fish were treated with BPA at concentrations of 0.8 ppm, 1 ppm and 5 ppm and 0.1 ppm 17β-estradiol in aquarium for different duration (24 h, 48 h, 7 days and 14 days). SEM study was performed thereafter. Experimental conditions and treatment procedures are given in materials and methods (Chapter 3, Section 3.11.1; pp 9).

Scanning electron micrographs reveals control and solvent control groups having well developed sperm with normal sperm tail and roundish head (Fig. 4.18 A) in the testis. In the groups treated with BPA and 17β-estradiol detachment of sperm tail from heads and shortening was marked (Fig. 4.18; C, D, E, F). 24 h and 48 h results were almost similar to the control group. Further study was initiated for longer duration exposure to the chemicals.

Breakage of tails was much more pronounced in terms of percentage in sperm population after 7 days of exposure. In the 17β-estradiol group sperm heads were de-shaped and tails shortened (Fig. 4.18; C). At lowest BPA dose of 0.8 ppm detachment of head from tail was visible (Fig. 4.18; D), while at higher doses of 1 ppm and 5 ppm higher membrane deformity in some sperm heads, intense clumping of sperm heads and tails were observed (Fig. 4.18; E, F).

Subsequent to 14 days of treatment, it was observed that majority of the sperms were affected. 17β-estradiol group showed the presence of deformed sperms with two tails and a de-shaped head (Fig. 4.19 C). In the BPA treated groups considerably large number of sperm samples with short tail and de-shaped heads were seen (Fig. 4.19 D, E, F). Moreover membrane dissolution of sperm heads and conglumeration into an irregular mass was observed at highest dose of BPA (Fig. 4.19; F).
Fig. 4.18. Scanning electron micrographs showing the changes in the testis of *H. fossilis* after 7 days exposure. Control and solvent control fishes showing well developed sperm with normal sperm head and tail; [A, B]. Shortening of tail and deformed head in 17β-estradiol group [C], breakage of tail after 0.8 ppm BPA treatment [D]; breakage of tail more pronounced after 1 ppm BPA exposure [E]; membrane deformity in some sperm head after 5 ppm BPA treatment [F]. Magnification 8000 x.
Fig. 4.19. Scanning electron micrographs showing the changes in the testis of H. fossilis after 14 days exposure. Control and solvent control fishes showing well developed sperm with normal head and tail; [A, B]. Abnormal tail and deshaped head in 17β-estradiol group [C]; intense clumping of sperms after 0.8 ppm BPA treatment [D]; membrane dissolution of sperm heads and tail breakage after 1 ppm BPA exposure [E] and conglomeration of sperm heads into an irregular mass after 5 ppm BPA treatment [F]. Differentiation between head and neck is unclear [D, E, F]. Magnification 8000 x.
4.8. Effects on Liver (TEM studies)

4.8.1. Effects on Liver cell organelles after 7 days

Fish were exposed to 17β-estradiol and BPA 0.8 ppm, 1 ppm and 5ppm in aquarium for different duration of experimental time (24 h, 48 h, 7 days and 14 days). They were then sacrificed and analyzed for TEM study. Experimental conditions and treatment procedures are given in materials and methods (Chapter 3, Section 3.11.2; pp 9, 10).

TEM study revealed the nucleus with normal shape and size in the control and solvent control group (Fig. 4.20; A, B). In the treated group with 17β-estradiol and BPA, the nuclear contour was difficult to be clearly observed, suggesting loss of the membrane integrity when compared with the control. The nucleus also appeared enlarged and de-shaped and the nucleolus showed some contour alteration characterized by projection of filamentous expansions (Fig. 4.20; C, E, F).

TEM images showed mitochondrial sets in their normal characteristics oval shape with intact membrane and cristae in the control group and the solvent control group (Fig. 4.21; A, B). Some mitochondria alterations were observed in the BPA and 17β-estradiol treated groups. After 7 days treatment almost all the mitochondria had lost their characteristic oval shape (Fig. 4.21; C, E, F). The mitochondria cristae presented degenerative alterations, fragmented in aspect or were even absent (Fig. 4.21; E, F). The outer membrane, which maintained apparent integrity of the mitochondria also, had disappeared in some (Fig. 4.21; D, E).

Rough endoplasmic reticulum (RER) depicted normal filamentous shape in the untreated control and solvent control group (Fig. 4.22; A, B). While in the treated groups RER was highly altered with some areas presenting a high concentration of expanded cisterns in concentric circles (Fig. 4.22; C D, F). It was also found that the RER volume was greatly increased (Fig. 4.22; E).
Fig. 4.20. Transmission electron micrographs showing the changes in the nucleus of control and solvent control fishes with normal features [A, B] and 7 days treated (via aquarium water) with 17β-estradiol [C], 0.8 ppm BPA [D], 1 ppm BPA [E] and 5 ppm BPA [F]. In all the treated groups' nuclear contour was de-shaped suggesting loss of the membrane integrity [C, E, F]. The nucleus appeared enlarged and de-shaped [C, E, F] and their nucleolus showed some contour alteration characterized by a "blurred" without delineated aspect, filamentous expansions were projected [C, E, F]. Abbreviations: Nu-Nucleolus; Nm- Nuclear membrane N-Nucleus, Magnification: 10 X; Scale: 1 cm = 1μ
Fig. 4.21. Transmission electron micrographs showing the changes in the mitochondria of control and solvent control fishes with normal features [A, B] and 7 days treated (via aquarium water) with 17β-estradiol [C], 0.8 ppm BPA [D], 1 ppm BPA [E] and 5 ppm BPA [F]. Almost all the mitochondria had lost their characteristic oval shape [C, E, F]. The mitochondria cristae presented degenerative alterations, fragmented in aspect or were even absent [E, F]. The outer membrane, which maintained apparent integrity of the mitochondria also, had disappeared in some [D, E]. Abbreviations: Mm- Mitochondrial membrane, *- cristae dissolution. Magnification: 40 X; Scale: 1 cm = 0.25µ
Fig. 4.22. Transmission electron micrographs showing the changes in the Rough endoplasmic reticulum (RER) of control and solvent control fishes with normal features [A, B] and 7 days treated (via aquarium water) with 17β-estradiol [C], 0.8 ppm BPA [D], 1 ppm BPA [E] and 5 ppm BPA [F]. In the treated groups RER was highly altered with some areas presenting a high concentration of expanded cisterns in concentric circles [C, D, F]. The RER volume was greatly increased [E]. Magnification: 40 X; Scale: 1 cm = 0.25μ. Abbreviations: DER- Dialated ER; CC- concentric circles.
4.8.2. Effects on Liver cell organelles after 14 days

The nucleus in the control group was prominent with normal shape and size. It was bordered with a nuclear membrane and chromatin masses. Nucleolus was present (Fig. 4.23; A). In the treated group with 17β-estradiol nucleus was de-shaped and damaged nuclear chromatin was seen (Fig. 4.23; C). In the treated groups with BPA, the nucleus also appeared de-shaped (Fig. 4.23; C, D, F). The loss of membrane integrity was marked due to the changes seen in the nuclear contour. Nucleolus also showed some contour alteration characterized by filamentous expansions. Nucleus shrinking was observed with loss of nuclear membrane in some (Fig. 4.23; D, E, F). Damaged nuclear chromatin was also seen (Fig. 4.23; D, E, F).

The mitochondria were in its normal oval structure with undisrupted membrane and cristae in the untreated group (Fig. 4.24; A, B), whereas they presented degenerative alterations in the treated groups. Cristae were fragmented in some and even absent in others (Fig. 4.24; C, D, E, F). The outer mitochondrial membrane, which maintained apparent integrity of mitochondrias, was undisrupted in the control group, while it was disrupted and broken in the treated groups. Membrane was also absent in some areas (Fig. 4.24; C, D, E, F).

Rough Endoplasmic reticulum (RER) depicted normal filamentous shape in stacks in the untreated control group. While in the treated groups RER was highly distorted. It was found that the RER volume was greatly increased (Fig. 4.25; C, D), with some areas presenting a high concentration of expanded cisterns in concentric circles (Fig. 4.25; E, F). RER could be observed all over the cytoplasm, but parallel cistern aggregates could not be identified. Most structures were altered, and it was not possible to detect the presence of two distinct membranes of the RER. At higher doses of BPA, RER aggregates were vacuolated and membrane dissolution was noticeable (Fig. 4.25; E, F).
Fig. 4.23. Transmission electron micrographs showing the changes in the nucleus of control and solvent control fishes with normal features [A, B] and 14 days treated (via aquarium water) with 17β-estradiol [C], 0.8 ppm BPA [D], 1 ppm BPA [E] and 5 ppm BPA [F]. In the treated group with 17β-estradiol nucleus was de-shaped and damaged, nuclear chromatin was seen [C]. In the treated groups with BPA, the nucleus also appeared de-shaped [C, D, F]. The loss of membrane integrity was marked due to the changes seen in the nuclear contour[C, F]. Nucleolus also showed some contour alteration characterized by filamentous expansions. Nucleus shrinking was observed with loss of nuclear membrane in some [D,E,F]. Damaged nuclear chromatin was also seen [D, E, F]. Abbreviations: Nu- Nucleolus; Nm- Nuclear membrane; N-Nucleus. Magnification: 10 X; Scale: 1 cm = 1μ
Fig. 4.24. Transmission electron micrographs showing the changes in the mitochondria of control and solvent control fishes with normal features [A, B] and 14 days treated (via aquarium water) with 17β-estradiol [C], 0.8 ppm BPA [D], 1 ppm BPA [E] and 5 ppm BPA [F]. The mitochondria were in its normal oval structure with undisrupted membrane and cristae in the untreated group [A, B] whereas they presented degenerative alterations in the treated groups. Cristae were fragmented in some and even absent in others [*C, D, E, F]. The outer mitochondrial membrane, which maintained apparent integrity of mitochondria’s, was disrupted and broken in the treated groups. Membrane was also absent in some areas [C, D, E, F]. Abbreviations: Mm-Mitochondrial membrane; c- cristae; *- cristae dissolution. Magnification: 40 X; Scale: 1 cm = 0.25μ.
Fig. 4.25. Transmission electron micrographs showing the changes in the Rough endoplasmic reticulum (RER) of control and solvent control fishes with normal features [A, B] and 14 days treated (via aquarium water) with 17β-estradiol [C], 0.8 ppm BPA [D], 1 ppm BPA [E] and 5 ppm BPA [F]. Almost all the mitochondria had lost their characteristic oval shape [C, E, F]. It was found that the RER volume was greatly increased [C, D], with some areas presenting a high concentration of expanded cisterns in concentric circles [E, F]. RER could be observed all over the cytoplasm, but parallel cistern aggregates could not be identified. Most structures were altered, and it was not possible to detect the presence of two distinct membranes of the RER. At higher doses of BPA, RER aggregates were vacuolated and membrane dissolution was noticeable [E, F]. Magnification 40 X; Scale 1 cm = 0.25μ. Abbreviations: DER- Dialated ER; CC- concentric circles; v- vacuolization.
4.9. Effects on Testis (TEM studies)

4.9.1. Effect on Sperm structure after 7 days treatment

TEM reveals well differentiated head and neck in the sperm of control and solvent control fish (Fig. 4.26; A, B), within the testis, whereas the fish groups treated with BPA and 17β-estradiol, the absence of neck and head of sperms was evident. The sperm head in controls revealed the crenate arrangement of the membrane (Fig. 4.26; A, B) which was found to be in intimate contact with the sperm head cell. In the treated groups a clear dislocation of the membrane from some parts of the head and its outward extension was evident (Fig. 4.26; E). Besides these, intense vacuolization was also observed in the sperm head cell adjacent to the dislocated and extended membrane (Fig. 4.26; D, F).

Longitudinal section of sperm tail revealed dilation of the membrane at places in the treated groups. Moreover there also appeared absence of plasma membrane in some. The sperm tail was distorted at places in the treated group. The control and solvent control samples however did not exhibit any such abnormality (Fig. 4.27; D, E, F; Round head arrow).

The transverse section of the sperm tail exhibited normal features in micro-tubular assembly and plasma membrane in control, while in the treated groups, the plasma membrane of the sperm tail showed distortion and breakage at places along with disturbances in micro-tubular assembly. (Fig. 4.27; D, E, F, Triangle arrow).
Transmission electron micrographs showing the changes in sperm heads after 7 days Roy, 2011

Fig. 4.26. Transmission electron micrographs showing the changes in the sperm heads of control and solvent control fishes with normal features [A, B] and 7 days treated (via aquarium water) with 17β-estradiol [C], 0.8 ppm BPA [D], 1 ppm BPA [E] and 5 ppm BPA [F]. Control treatment reveal the crenate arrangement of the plasma membrane and differentiation into head (H) and neck (N). 17β-estradiol and the BPA treated groups revealed clear dislocation of the membrane from some parts of the head and its outward extension[E, F]; breakage of plasma membrane at places[C] and intense vacuolization adjacent to the dislocated and extended membrane [D, E, F] and absence of differentiation between head and neck. Abbreviations: pm, plasma membrane; v, vacuole; dm, distorted mitochondria; dpm, distorted plasma membrane; H, head; N, neck. Magnification 27 X; Scale 1 cm = 0.37.
Fig. 4.27. Transmission electron micrographs showing the changes in the sperm tail (Longitudinal and transverse section) of control and solvent control fishes with normal features [A, B] and 7 days treated (via aquarium water) with 17β-estradiol [C], 0.8 ppm BPA [D], 1 ppm BPA [E] and 5 ppm BPA [F]. Control treatment showed presence of normal tubular assembly and plasma membrane (pm). 17β-estradiol and the BPA treated groups showed dilation of plasma membrane, absence of plasma membrane in some areas and distorted microtubular assembly (marked by arrow heads). Abbreviations: pm- plasma membrane; mt- microtubule assembly; TS, Transverse section (triangle head arrow); LS, Longitudinal section (round head arrow). Magnification 100 X; Scale 1 cm = 100nm
4.9.2. Effect on Sperm structure after 14 days treatment

TEM revealed well differentiated head and neck in the sperm of control and solvent control groups (Fig. 4.28; A, B), whereas the groups treated with BPA and 17β-estradiol, the absence of well differentiated neck and head was prominent (Fig. 4.28; C, D, E, F). The sperm head in controls revealed the crenate arrangement of the membrane which was found to be in intimate contact with the sperm head cell. It was present in continuation. In the 14 days treated groups a clear dislocation of the membrane from some parts of the head and its outward extension was evident (Fig. 4.28; C, D, E, F). Besides these, intense vacuolization was also observed in the sperm head cell adjacent to the dislocated and extended membrane (Fig. 4.28; C, E). Further the size of the sperm head was also found to be different from control. The neck portion showed the presence of well differentiated mitochondria in the control group but in the treated groups the mitochondria were totally distorted (Fig. 4.28; D, F). Lateral lobes were also found to be irregular in the treated groups (Fig. 4.28; C, E).

Longitudinal section of sperm tail revealed dialation of the membrane at places in the treated groups. Moreover there also appeared absence of plasma membrane in some (Fig. 4.29; C, D, E, F; Round head arrow). The sperm tail was heavily distorted at places in the treated group. The control samples however did not exhibit any such abnormality. The transverse section of the sperm tail exhibited normal features in microtubular assembly having a flagellum with the typical structure of (9 + 2) microtubules and plasma membrane. While in the treated groups, the plasma membrane of the sperm tail showed distortion and breakage at places along with heavy disturbances in micro-tubular assembly. Various degrees of degeneration of axoneme were noted. Loss of some of the peripheral and/or central microtubules was commonly observed. Some sperm tails in the treated groups did not have plasma membrane at all (Fig. 4.29; C, D, E, F; Triangle head arrow).
Fig. 4.28. Transmission electron micrographs showing the changes in the sperm heads of control and solvent control fishes with normal features [A, B] and 14 days treated (via aquarium water) with 17β-estradiol [C], 0.8 ppm BPA [D], 1 ppm BPA [E] and 5 ppm BPA [F]. Control treatment reveal the crenate arrangement of the plasma membrane and differentiation into head (H) and neck (N). Unlike untreated control but similar to the 17β-estradiol the BPA treated groups revealed clear dislocation of the membrane from some parts of the head and its outward extension, breakage of plasma membrane at places and intense vacuolization adjacent to the dislocated and extended membrane (arrow heads). Abbreviations: pm - plasma membrane; V - vacuole; dm - distorted mitochondria; dpm - distorted plasma membrane, H - head; N - neck. Magnification 27 X; Scale 1 cm = 0.37 μ
Transmission electron micrographs showing the changes in the sperm tail (Longitudinal and transverse section) of control and solvent control fishes with normal features [A, B] and 14 days treated (via aquarium water) with 17β-estradiol [C], 0.8 ppm BPA [D], 1 ppm BPA [E] and 5 ppm BPA [F]. Control treatment showed presence of normal tubular assembly and plasma membrane (pm). Unlike untreated control but similar to the 17β-estradiol the BPA treated groups showed dilation of plasma membrane, absence of plasma membrane and distorted microtubular assembly (marked by arrow heads). Abbreviations: pm- plasma membrane; mt- microtubule assembly. TS-Transverse section (triangle head arrow), LS, Longitudinal section (round head arrow) Magnification: 100 X; Scale: 1 cm = 100nm
4.10. Effects on Sperm count

Fish were subjected to 17β-estradiol (0.1 ppm) and BPA treatments (0.8 ppm, 1 ppm and 5 ppm) in aquarium for different time interval (24 h, 48 h, 7 days and 14 days) and were then sacrificed for collection of testicular milt for the analysis of sperm count. Experimental conditions and treatment procedures are given in materials and methods (Chapter 3, section 3.12.1, pp 10, 11).

Investigation of sperm count obtained from intra-testicular sperm (testis milt) after 24 h, 48 h, 7 days and 14 days of exposure to 17β-estradiol and BPA was conducted and are summarized in Table 4.3. 17β-estradiol and BPA had effects on sperms. From the experimental results, it was observed that there was significant reduction in sperm count at all doses of BPA (0.8 ppm, 1 ppm, and 5 ppm) and 0.1 ppm 17β-estradiol when compared to the untreated control (tap water) group. Sperm count was significantly reduced (p<0.001) at all doses after 7 and 14 days treatment. There was significant effects (p<0.05) only at the highest dose of 5 ppm the after 48 h study. 24 h study had no significant effect on the sperm count.

The highest decrease in sperm counts was observed in the treatment period of 7 days and 14 days. Analysis of the density of sperm count after 7 days of exposure to different doses of BPA exhibited significant decrease (p<0.001) when compared to untreated control (tap water) group. The mean values were found to be (54.78 ± 3.1, 67.73 ± 1.46, 48.12 ± 6.2, 44.52 ± 3.4) for 0.1 ppm 17β-estradiol 0.8 ppm BPA, 1 ppm BPA and 5 ppm BPA respectively when compared to control (97.25 ± 0.5). After 14 days treatment period also there was a steep decline in sperm density in the treated groups. The mean value was found as (51.05 ± 4.86, 55.1 ± 5.17, 45.85 ± 5.12, 44.26 ± 3.8) after 17β-estradiol, 0.8 ppm BPA, 1 ppm BPA and 5 ppm BPA respectively, in comparison with (97.25 ± 0.5) in control group.
Table 4.3. Effect of Bisphenol A on sperm count of *H. fossilis*. Total sperm counts in *H. fossilis* after Bisphenol A treatment. Mature *H. fossilis* was treated with 0.1 mg/l (0.1 ppm) 17β-estradiol (positive control), 0.8 ppm BPA, 1 ppm BPA and 5 ppm BPA via aquarium water. Asterisks denote significant relationship with untreated control groups (* p < 0.05; ** p < 0.01; *** p < 0.005). Values are shown as mean ± SEM, (n=3).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Route of exposure</th>
<th>Dose</th>
<th>No. of fish</th>
<th>24 h</th>
<th>48 h</th>
<th>7 days</th>
<th>14 days</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
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<td></td>
<td>3</td>
<td>97.25±0.5</td>
<td>97.25±0.5</td>
<td>97.25±0.5</td>
<td>97.25±0.5</td>
</tr>
<tr>
<td>Solvent control</td>
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<td>92.7±1±1.12</td>
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<tr>
<td>17β-estradiol</td>
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<td>3</td>
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<td>92±1.2</td>
<td>54.78±3.1***</td>
<td>51.05±4.88***</td>
</tr>
<tr>
<td>0.8 ppm BPA</td>
<td>Aquarium water</td>
<td>0.8 mg/l</td>
<td>3</td>
<td>91.7±2.1</td>
<td>91.7±2.1</td>
<td>67.7±1.46***</td>
<td>55.1±5.17***</td>
</tr>
<tr>
<td>1 ppm BPA</td>
<td>Aquarium water</td>
<td>1 mg/l</td>
<td>3</td>
<td>91.95±2</td>
<td>92.7±1.6</td>
<td>48.12±8.2***</td>
<td>45.85±5.12***</td>
</tr>
<tr>
<td>5 ppm BPA</td>
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<td>5 mg/l</td>
<td>3</td>
<td>94.65±0.57</td>
<td>80.25±1.2</td>
<td>44.52±3.4***</td>
<td>44.2±3.8***</td>
</tr>
</tbody>
</table>
4.11. Effect on Sperm Viability

Fish were subjected to 17β-estradiol (0.1 ppm) and BPA treatments (0.8 ppm, 1 ppm and 5 ppm) in aquarium for different time period (24 h, 48 h, 7 days and 14 days) and were then sacrificed for collection of testicular milt. Analysis of sperm viability was done with Nigrosin-Eosin staining. Experimental conditions and treatment procedures are given in materials and methods (Chapter 3, section 3.12.2; pp 11). In the sperm viability test with Nigrosin-Eosin stain, the dead sperms were found to take the stain while the live sperm did not take any stain and showed some glow. Analysis of sperm viability, i.e. the percentage of live and dead sperms obtained from intratesticular sperm (testis milt) after 24 h, 48 h, 7 days and 14 days of exposure to 17β-estradiol and BPA are summarized in Fig. 4.30 and 4.31. The mean values when compared with untreated control values did not exhibit any significant difference after 24 h and 48 h treatment period. Results of comparison between the different treated groups of BPA and 17β-estradiol showed significant rise in the percentage of dead sperms (P<0.001) after 7 days and 14 days exposure schedules.

Analysis of the percentage of dead sperms after 7 days of exposure to different doses of BPA exhibited significant rise (P<0.001) when compared to untreated control (tap water) group. The mean values were found to be 6 ± 3.4, 1 ± 0.57, 4 ± 2.4 and 10 ± 5.7 for 0.1 ppm 17β-estradiol 0.8 ppm BPA, 1 ppm BPA and 5 ppm BPA respectively compared to 4.72 ± 2.7 in untreated control. Similarly there was steep decline in percentage of live cells after 14 days of exposure. Percentage of live sperms were reduced significantly (p<0.001) when compared with the untreated control (tap water) group. The mean values were estimated at 7.63 ± 4.4, 6.55 ± 3.7, 4.04 ± 2.3 and 8.62 ± 4.9 for 17β-estradiol and different doses of BPA respectively.
Fig. 4.30. Histograms showing the frequency of live sperms after 24 h (A) and 48 h exposure (B) following 17β-estradiol (0.1 ppm) and Bisphenol A (0.8 ppm, 1 ppm, 5 ppm) treatment. Values are shown as mean ± SEM, (n=3).
Fig. 4.31. Histograms showing the percentage of live sperms after 7 days (C) and 14 days exposure (D) following 17β-estradiol (0.1 ppm) and Bisphenol A (0.8 ppm, 1 ppm, 5 ppm) treatment. Asterisks denote significant relationship with untreated control groups (* p < 0.05; ** p < 0.01; ***p < 0.005). Values are shown as mean ± SEM, (n=3)
4.12. Effect on frequency of Micronucleus

Fish were subjected to 17β-estradiol (0.1 ppm) and BPA (0.8 ppm, 1 ppm and 5 ppm) for different exposure period (24 h, 48 h, 7 days and 14 days) and were then sacrificed for collection of blood for Micronucleus Assay. Experimental conditions and treatment procedures are given in materials and methods (Chapter 3, Section 3.13; pp 11, 12).

The effects of different doses of BPA on induction of micronucleus (MN) via aquarium water for 24 h, 48 h, 7 days and 14 days are summarized in Fig. 4.32 and Table 4.4. From the experimental results, it was seen that micronucleus was induced by 0.8 ppm, 1 ppm and 5 ppm BPA when compared to the untreated control (tap water) group in all the exposure schedules. In 24 h and 48 h experimental schedules the % MN induction was not statistically significant, while after 7 day exposure it was found to be significant only at the lowest dose of 0.8 ppm (0.525 ± 0.04) and highest dose of 5 ppm (0.425 ± 0.1). Similar trend of results were observed in 0.1 ppm 17β-estradiol treated animals (positive control group). Amongst all, the highest number of MN was observed in the treatment period of 14 days, where 1.1 ± 0.16, 0.92 ± 0.025, 1.2 ± 0.216 and 1.15 ± 0.119 % MNT were observed in 17β-estradiol, 0.8 ppm, 1 ppm, 5 ppm BPA group respectively against 0.15 ± 0.08 % MN in control animals. These differences in % MNT were found statistically significant (p < 0.01 and 0.001).

The erythrocytes of *H. fossilis* were generally observed as elliptical with a centrally located nucleus and considerable cytoplasm around it. Distribution studies revealed one micronucleus per cell in most of the observations, though some cells had two to three MN as the concentration and exposures time increased. Random allocation of MN was generally observed in the cytoplasm. Some were even found to be attached to the cell wall which was seen quite rarely.
Fig 4.32. Photomicrographs of micronucleated erythrocytes of *H. fossilis* subjected to 17β-estradiol [A, B] and BPA [C, D, E, F]. Micronucleated cells were induced and their frequency was increased significantly after 24 h, 48 h, 7 days and 14 days exposure. Arrow heads indicate micronucleus. Stain: Giemsa. Magnification: 400 x
Table 4.4. Effects of 17β-estradiol and BPA on induction of micronucleus (MN) in adult male *H. fossilis*. Experimental conditions and treatment procedures are given in materials and methods (Chapter 3, section 3.13). Values are given as mean ± SEM, (n=4). Asterisks denote statistically significant differences against untreated control group (* p < 0.05; ** p < 0.01; *** p < 0.001).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Route of exposure</th>
<th>No of fishes/group</th>
<th>Dose</th>
<th>Total cells studied</th>
<th>% MN (MN±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Tap water</td>
<td>4</td>
<td>4000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solvent control</td>
<td></td>
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<td>0.1mL</td>
<td>4000</td>
<td>0.01±0.02</td>
</tr>
<tr>
<td>17β-estradiol</td>
<td>Aquarium water</td>
<td>4</td>
<td>0.1mg/l</td>
<td>4000</td>
<td>0.05±0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>0.8mg/l</td>
<td>4000</td>
<td>0.1±0.04</td>
</tr>
<tr>
<td>0.8 ppm BPA</td>
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<td>4</td>
<td>1mg/l</td>
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<tr>
<td>1 ppm BPA</td>
<td></td>
<td>4</td>
<td>5mg/l</td>
<td>4000</td>
<td>0.12±0.06</td>
</tr>
</tbody>
</table>

*p < 0.05; ** p < 0.01; *** p < 0.001.
4.13. Effect on frequency of morphologically altered erythrocyte

The effects of different doses of 17β-estradiol and BPA on altered erythrocyte induction via aquarium water for 24 h, 48 h, 7 days and 14 days are summarized in Fig. 4.31, 4.32, 4.33. From the experimental results, it was seen that morphologically altered erythrocytes was induced by all doses of BPA (0.8 ppm, 1 ppm and 5 ppm) compared to the untreated control (tap water) group at all the exposure programs.

In 24 h exposure schedule the percentage of altered erythrocytes was statistically significant only at the highest dose of 5 ppm (p<0.001). At 48 h experimental schedule the percentage was statistically significant at lowest dose of 0.8 ppm (p<0.05) and highest dose of 5 ppm (p<0.01). There was increase in the induction of altered erythrocytes right from 24 h which gradually increased exponentially. 7 days treated group showed statistically significant results at lowest dose of 0.8 ppm (p<0.01) and highest dose of 5 ppm (p<0.05) respectively. 17β-estradiol treated fishes showed statistically significant increase only in the treatment period of 14 days. Amongst all, the highest number of altered erythrocytes was observed in the treatment period of 14 days, where 0.27 ± 0.13, 0.63 ± 0.317, 0.46 ± 0.23 and 1.95 ± 0.97 percentage (%) altered erythrocytes were observed in 17β-estradiol, 0.8 ppm, 1 ppm, 5 ppm BPA group respectively against 0.72 ± 0.03 % altered erythrocytes in control animals (p<0.001).

Morphologically altered erythrocytes were observed in both low as well as high doses of BPA and their frequency increased when the exposure time was increased. When a closer observation in the types of erythrocyte alterations were made, spherocytes, anisocytes, echinocytes, ovalocytes and poikilocytes were predominantly noted (Fig. 4.33).
Fig. 4.33. Photomicrographs of erythrocytes of *H. fossilis* showing different types of morphological alterations. Control [A]; 17β-estradiol [B]; after treatment with BPA [C, D, E, F] under various treatment conditions (24 h, 48 h, 7 days and 14 days). Ne-Normal erythrocytes, s-Spherocyes, a-Acanthocytes, p-Poikilocytes, o-Ovalocytes, e-Echinocytes. Arrow heads indicate morphologically altered erythrocytes. Stain: Giemsa. Magnification: 400x
Fig. 4.34. Histograms showing the frequency of morphologically altered erythrocytes after 24 h (A) and 48 h exposure (B) following 17β-estradiol (0.1 ppm) and Bisphenol A (0.8 ppm, 1 ppm, 5 ppm) treatment. Values are given as mean ± SEM, (n=4). Asterisks denote statistically significant differences against untreated control group (* p < 0.05; ** p < 0.01; *** p < 0.001).

Fig. 4.35. Histograms showing the frequency of morphologically altered erythrocytes after 7 days (C) and 14 days exposure (D) following 17β-estradiol (0.1 ppm) and Bisphenol A (0.8 ppm, 1 ppm, 5 ppm) treatment. Values are given as mean ± SEM, (n=4). Asterisks denote statistically significant differences against untreated control group (* p < 0.05; ** p < 0.01; *** p < 0.001).

Estrogenic heavy metal analyses of water during the study period are presented in Table 4.5. The results showed the presence of a number of heavy metals with estrogenic activity. It was observed that the levels of these metals in the waters were higher than the permissible limits. Cd concentration was higher in Deepor beel, while Cr was high in all three sites. Pb concentration was highest in Deepor beel, followed by Bharalu River and Borsola beel. Hg was seen highest in Bharalu compared to the other two sites. Ni was seen within the permitted limit. The results demonstrated that heavy metals exceeded permissible safe levels as established by the Environmental Protection Agency and WHO. The heavy metal concentration having estrogenic activity collected from the sites was in the order Pb>Cr>Ni>Hg>Cd.

Table 4.5. Estrogenic heavy metal concentration in water (mg/l) in three major water bodies of Guwahati The Bharalu river, Deepor beel and Borsola beel, Assam.

<table>
<thead>
<tr>
<th>Metals</th>
<th>Bharalu river (mg/l)</th>
<th>Borsola beel (mg/l)</th>
<th>Deepor Beel (mg/l)</th>
<th>Permissible limits (WHO,2003)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td>0.012</td>
<td>0.012</td>
<td>0.020</td>
<td>0.003</td>
</tr>
<tr>
<td>Cr</td>
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<td>0.808</td>
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</tr>
<tr>
<td>Ni</td>
<td>0.018</td>
<td>0.024</td>
<td>0.023</td>
<td>0.05</td>
</tr>
<tr>
<td>Pb</td>
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<td>0.529</td>
<td>2.238</td>
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</tr>
<tr>
<td>Hg</td>
<td>0.0345</td>
<td>0.012</td>
<td>0.019</td>
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</tr>
</tbody>
</table>
4. 15. Gas Chromatography and Mass spectrometric (GC/MS) Analysis for Testing Estrogenic compounds

Estrogenic analyses of water with GC/MS during the study period (April 2010) are presented in Table 4.6. The results showed the presence of estrogenic chemicals Aldrin, Dialdrin in Bharalu river (µg/l). The chemicals analyzed were below the detectable limit in Deepor beel, while Borsola beel recorded the presence of Dibutyl Phthalate (µg/l). Some of the other chemicals tested were either not detected or were below the detectable limit. Permissible range of Aldrin is 0.001 (µg/l), Dieldrin 0.002 (µg/l) and DBP 0.6 µg/l, (WHO, 2003).

Table 4.6. Estrogenic compound concentration in water (µg/l) in three Major water bodies, The Bharalu, Deepor beel and Borsola beel of Guwahati, Assam. The level of these compounds were above permissible limits (WHO, 2003).

<table>
<thead>
<tr>
<th>ESTROGENIC CHEMICALS</th>
<th>BHARALU RIVER (µg/l)</th>
<th>BORSOLA BEEL (µg/l)</th>
<th>DEEPOR BEEL (µg/l)</th>
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<tbody>
<tr>
<td>Alpha HCH</td>
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<td>BDL</td>
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