Acute Toxicity Test:

Toxicity of a substance depends on its chemical and physical properties, which may be harmful to animals or plants. The toxicity of any compound is related to its dose. A highly toxic substance causes severe symptoms even in small doses as toxicity can either be acute or chronic. Acute toxicity is the ability of a substance to cause harmful effects within few hours to few days. Usually, the acute toxicity test is conducted for 96 hours exposure time period. In the bioassay test, the experimental organisms are subjected to a series of concentrations of known toxicant, under adequately controlled conditions. The LC_{50} is the concentration of a toxicant or stressor, which is lethal to 50% of the population of test animals for a specific time period.

For the present investigations following parameters were taken into account.

1. Physico-chemical analysis of effluents.
2. Calculation of 96h LC_{50} values of effluents for the preparation of sublethal concentrations.
3. Behavioural responses and morphological changes exposed to two effluents.
4. Chromosome Aberration Test (CAT).
5. Micronucleus Test (MNT) and Erythrocytes Aberrations Test (EAT).
6. Histopathological alterations in gills, kidney and liver

1. Physico-chemical analysis of effluents:

The physico-chemical analysis of both the effluents was done by Punjab Pollution Control Board (PPCB), Patiala.

Tannery and Paint effluents:

Physico-chemical characteristics of the tannery effluent, collected on 20/05/2013, 18/06/2013 and 16/07/2013 were shown in Table I and of the paint effluent, collected on 12/04/2014, 10/05/2014 and 07/06/2014 were summarized in Table II.

1. Tannery effluent was grey in color because of sub-processes of tanning like pickling, neutralization, dyeing and fat liquoring. The pungent smell can also be due to decomposition of hides. On the other hand, colour of

the paint effluent was black due to the pigments used in paint, while unpleasant odour was because of reaction/decomposition of heavy metals and phenolic compounds.

2. The tannery effluent was acidic (pH 4.5,5.7, 3.9), which deplete the water quality and dissolved oxygen, while paint effluent was alkaline (pH 9.6, 9.8, 9.5), which was unfit for aquatic organisms because it striped the slime coat.
3. The electrical conductivity was high in both the effluents, due to addition of salts during processing of hides/skins in tannery effluent and inorganic salts added by the processing of paint.
4. Total suspended solids (TSS) of tannery effluent were 648, 740, 786, while of paint effluent were 186, 194, 190, which were found to be beyond permissible limit 100mg/L. They caused turbidity, resulting in poor penetration of light in aquatic system, reduced photosynthetic activity, increased algal bloom and decreased oxygen concentration.
5. Total dissolved solids (TDS) of tannery effluent were 2400, 2872, 2547, while of paint effluent were 2750, 2715, 2735, which exceeded the permissible limit of 2100 mg/L. The increase of TDS in tannery effluent resulted due to soaking, liming, deharing, defleshing and deliming of hides, whereas in paint due to the presence of different binders, pigments and resins.
6. The BOD level of tannery effluent was 520, 524, 528 mg/L, while of paint was 507, 515, 525, which exceeded the limit of 50mg/L. The increase in BOD was resulted in the high organic load, which depletes the dissolved oxygen and caused hypoxic conditions responsible for adverse affects on the aquatic biota. Further, the presence of organic matter promoted anaerobic conditions, which lead to accumulation of toxic compounds in water bodies.
7. The COD level of tannery effluent was 295, 284, 298 mg/L, while of paint effluent was 815, 809, 825 mg/L, which exceeded the permissible

limits of 250mg/L because the effluents contain high concentration of inorganic compounds.

8. The oil and grease in tannery effluent was 25, 27, 24 mg/L, while of paint effluent was 32, 28, 30 mg/L, which exceeded the limit of 10 mg/L. These conditions occurred due to presence of fatty substances, which create very high oxygen demand, causes reduction of light penetration and photosynthesis.
9. In tannery effluent, ions especially hexavalent chromium, total chromium, sulphides, boron and chlorides imparted hardness to water. Chloride level in the effluent was 1524, 1421, 1540 mg/l, which exceeded the permissible level (1000 mg/L) and caused salinity. Presence of chlorides in the tannery effluent might be due to soaking and pickling processes. Sulfide level was 6.4, 6.8, 7.4 mg/L, which exceeded the permissible limit of 2 mg/L, resulted due to liming and unhairing. Total chromium were (4.1, 4.8, 4.5) exceeded the permissible limit of 2.0 mg/L and hexavalent chromium (0.3, 0.5, 0.7 mg/L) exceeded the permissible limit of 0.1 mg/L. Chromium is mainly found in the effluent due to chrome tanning process.

In paint effluent, toxic metals, such as lead, chromium (hexavalent and total chromium), copper, nickel, zinc and phenolic compounds were present. The values for various metals were lead 4.2, 4.5, 4.7 mg/L (limit 2.0 mg/L), hexavalent chromium 0.6, 0.3, 0.4 mg/L (limit 0.1 mg/L), total chromium 2.9, 2.8, 2.4 mg/L (limit 2.0 mg/L), copper 3.7, 3.9, 3.7 mg/L (limit 2.0 mg/L), nickel 2.6, 2.6, 2.8 mg/L (limit 2.0 mg/L) and zinc 7.0, 6.7, 6.8 mg/L (limit 5.0 mg/L). Phenolic compounds were 4.8, 5.0, 5.1 mg/L, which exceeded the limit of 1.0 mg/L. All these substances are highly toxic and may cause endocrine dysfunction, liver dysfunction, genotoxic effect, reduced growth rate and accumulate in a significant amount.

2. Calculation of 96h LC_{50} values of effluents for the preparation of sublethal concentrations.

The 96h LC_{50} value of tannery and paint industrial effluents against *Labeo rohita* was determined by using the method given by Finney (1971). The 96h LC_{50} value of tannery effluent was 15.48% (Graph 1, Table III) and of paint was 31.62 % (Table IV, Graph 2). Two sublethal concentrations 1/2 and 1/8 of 96h LC_{50} of both the effluents were selected for the experiments. The sublethal concentrations of tannery effluent were 1/2 (7.74%), 1/8 (1.93%) and of paint were 1/2 (15.81%), 1/8 (3.95%). The fishes were subjected to sublethal concentrations of both the effluents for 24h, 48h, 72h, 96h and 120h time intervals.

3. Behavioural responses and morphological changes:

Behavioural responses and morphological changes are considered to be a promising tool in ecotoxicology. Behaviour allows organisms to adjust to external and internal stimuli in the environment for the survival. Behaviour is sequence of quantifiable actions and selective responses operating through the central and peripheral nervous system. Morphological responses are external changes occur in the body of organism due to the effect of toxicants present in the environment. Therefore, behavioural responses and morphological changes are necessary for the survival and are also used to detect the impact of toxicants on fishes. In the present study, controls as well as treated fishes were daily monitored for 30 minutes to record the behavioural responses and morphological changes under the influence of both the effluents. The data related to behavioural responses and morphological changes of control and treated fishes is summarized for tannery effluent (Table V) and for paint effluent (Table VI).

A) **Behavioural responses:**

Control:

The control fishes were maintained in well aerated water with sufficient amount of feed. They were acclimatized in laboratory for 20 days prior to the experiment. Control fishes showed active feeding, normal schooling behaviour,

well-synchronized body movements and attentive to slight disturbance near the tank. Normally, the fishes settled at base of experimental tub, sometimes came on the surface of water. No mortality was recorded in the control group. The behaviour of the control groups did not show any change upto 120h.

Treated fishes:

Treated fishes exhibited various altered behavioural responses due to the exposure of two industrial effluents under various time intervals. Following changes were observed during the study:

i) Erratic and rapid swimming: Initially, when fishes subjected to sublethal concentrations of both the effluents showed erratic and rapid swimming. In tannery effluent, at 7.74% concentration, fishes exhibited fast swimming upto 48h then slow down with passage of time. The time durations were 27 min (24h), 20 min (48h), 18 min (72h), 12 min (96h) and 7 min (120h). On the other hand, in 1.93% concentration, fast swimming was observed at 24h then slows down with time. The time intervals were 17min (24h), 10min (48h), 8min (72h) and 5 min (96h and 120h). However, in paint effluent, fishes in 15.81% concentration, showed erratic swimming for 15 min (24h) then only for 5 min in each durations (48h, 72h, 96h and 120h), while at 3.59% concentration, fishes exhibited slight erratic swimming for less than 5 minutes then settled at base during all the exposure periods.

ii) Gulping air at surface or jumping out of water: This was the second interesting behavioural response observed in the fishes. In tannery effluent, the effect was prominent in 7.74% concentration from 24h to 120h, whereas in 1.93% concentration, it was present only at 96h and 120h. In paint effluent, in 15.81% concentration, gulping of air was less upto 48h then increased from 72h to 120h, whereas in 3.58% concentration, it was less upto 72h then increased at 96h and 120h.

iii) Opercular movements: In fishes opercular movements possessed different patterns at different time durations. In tannery effluent, at 7.74% concentration, fishes showed fast opercular movements at 24h and 72h, while it was slow at 96h and 120h. At 1.93% concentration, opercular movements were normal at

24h and 48h then it was fast at 72h, while slow down at 96h and 120h. In paint effluent, at 15.81% and 3.59% concentrations, the opercular movements were normal upto 72h then slow at 96h and 120h.

iv) **Loss of equilibrium**: In fishes loss of equilibrium was rarely seen. In tannery effluent, at 7.74% concentration, it was not seen upto 48h and observed at 72h, 96h and 120h, whereas in 1.93% concentration, it was present only at 96h and 120h. In paint effluent, in 15.81% concentration, it was seen at 96h and 120h, while in 3.59% concentration, it was absent in all durations.

v) **Hitting against the wall:** Fishes were hitting against the wall in 7.74% concentration of tannery effluent, at all time intervals, while it was seen at 96h and 120h in 1.93% concentration. In paint effluent, at 15.81% concentration, fishes were showing this behaviour after 72h upto 120h, whereas at 3.59% concentration, it was present at 96h and 120h.

vi) **Restlessness:** In tannery effluent, fishes were restless upto 48h in 7.74% concentration, while 1.93% concentration, it was seen upto 72h. In paint effluent, at 15.81% and 3.59% concentrations, it was present upto 72h.

vii) **Sluggishness:** In tannery effluent, fishes became sluggish after 72h upto 120h in 7.74% concentration, whereas they remained sluggish at 96h and 120h in 1.93% concentration. In paint effluent, at 15.81% concentration, fishes showed same behaviour at 96h and 120h, while in 3.59% concentration, fishes were moderately sluggish at 120h.

viii) **Death:** At 120h, two fishes were died in 7.74% concentration of tannery effluent and one fish in 15.81% concentration of paint effluent. However, no mortality was recorded in 1.93% concentration of tannery effluent and 3.59% concentration of paint effluent. Fishes, before death, jumped out of water, gulped air and lied at surface of water with jerky movements. Lastly, dead fishes lied at the surface of water with ventral side upward and mouth open.

B) Morphological changes:

Control:

Morphologically, *Labeo rohita* is laterally compressed, fusiform, blackish grey coloured fish with large bulging eyes, prominent snout and fringed lips. Body is covered with overlapping cycloids scales (Fig. 1).

Treated fishes:

Treated fishes were subjected to two sublethal concentrations of tannery and paint effluents at various time intervals and they exhibited following morphological changes:

i) Loosening of scales: In tannery effluent, at 7.74% concentration, loosening of scales was observed from 72h to 120h, while in 1.93% concentration, it was present at 96h and 120h. In paint effluent, at 15.81% concentration, it was seen at 96h and 120h, whereas in 3.59% concentration, it was noted only at 120h (Fig. 2).

ii) Sinking of eyeball: In tannery effluent, sinking of eyeball was examined from 48h to 120h, in 7.74% concentration, whereas it was present at 96h and 120h, in 1.93% concentration. In paint effluent, at 15.81% concentration, it was seen from 72h to 120h, while in 3.59% concentration, it was recorded only at 120h (Fig. 3).

iii) Redness of eye: In tannery effluent, redness of eyes was recorded from 48h to 120h in 7.74% concentration, while it was present at 96h and 120h, in 1.93% concentration. In paint effluent, at 15.81% concentration, this behaviour was shown from 72h to 120h, whereas it was observed at 96h and 120h, at 3.59% concentration (Fig. 4).

iv) Profuse mucous secretions: In tannery effluent, profuse mucous secretions were seen at all the exposure periods, in 7.74% concentration, while it was noted from 72h to 120h, in 1.93% concentration. In paint effluent, profuse mucous secretions were shown from 48h to 120h, in 15.81% concentration, whereas it was recorded at 96h and 120h, in 3.59% concentration (Fig. 5).

v) **Bleeding from gills:** At 120h, bleeding from gills was recorded, at 7.74% concentrations of tannery effluent and at 15.81% concentration of paint effluent, while it was absent in all durations in 1.93% concentration of tannery effluent and 3.59% concentration of paint effluent (Fig. 6).

vi) **Haemorrhages:** In tannery effluent, haemorrhages near lips were recorded, in 7.74% concentration from 72h to 120h, while it was present at 120h, in 1.93% concentration. In paint effluent, it was observed in 15.81% concentrations at 96h and 120h, whereas it was absent in 3.59% concentration (Fig. 7).

4. Chromosomal analysis:

Control:

Slides for chromosome analyses were prepared from the kidney tissue. Well spread somatic metaphase plates were selected to study the chromosome number and morphological characteristics of chromosomes. Morphometric data of chromosomal analysis is summarized (Table VII).

1. Somatic metaphase plate:

Diploid chromosome number, 2n = 50 (Fig. 8):

2. Karyotype prepared from somatic metaphase (Fig. 9): Chromosomes are differentiated on the basis of centromeric position:

a. Number of metacentrics = 5 (biarmed)

b. Number of submetacentrics = 9 (biarmed)

c. Number of subtelocentrics = nil

d. Number of telocentrics = 11 (uniarmed)

3. Chromosomal formula = 10m + 18sm + 22t

4. Sex chromosomes = Not distinguishable

5. Morphometric data of somatic metaphase karyotype:

a. Actual mean length of the largest chromosome = 6.09 µ

b. Actual mean length of the smallest chromosome = 2.62 µ

c. Relative length percentage of the largest chromosome pair = 100.00

d. Ratio of the largest and smallest chromosome = 2.32 µ

e. Mean total haploid length = 47.65 µ

f. Mean total diploid length = 95.31 µ

g. Fundamental arm number = 78

Treated fishes:

Slides were prepared from the kidney tissue to study the chromosomal aberrations in fishes exposed to two sublethal concentrations of tannery and paint effluents for 24h, 48h, 72h, 96h and 120h. 300 somatic metaphase plates, in each concentration and time interval of treated fishes were examined. Eleven types of chromosomal aberrations were observed.

a) Chromosomal fragmentation (Cf):

A chromosomal fragment is originated by the break at telomeric region of chromosome or it lacks centromere. These acentric fragments unable to attach the spindle and are not equally distributed to the daughter cells during cell division (mitosis and meiosis). One (Fig.10) and two (Fig.11) chromosome fragments were observed in the metaphase plates of treated fishes.

b) Ring chromosome (Rc):

Ring chromosomes are formed when telomere ends of both arms of chromosomes are eroded or deleted, which resulted in fusion due to stickiness and having a shape of ring. One (Fig. 12) and two (Fig.13) ring chromosomes were present in the metaphase plates.

c) Terminal chromatid deletion (Tcd):

A terminal end of a chromosome is deleted due to break, which resulted in unequal size of chromosome arms of submetacetric and metacentric chromosomes. Terminal chromatid deletion in one (Fig. 14) and two (Fig.15) chromosomes were observed in metaphase plates.

d) Minutes (M):

Minutes are small, spherical fragments, which formed due to chromosomal fragmentations and these are smaller than chromosome fragments. Presences of one (Fig. 16) and two (Fig.17) minutes in metaphase plates were seen.

e) Centromeric gaps (Cg):

Centromeric region of chromosomes is stretched or elongated and becomes narrow/thin or lightly stained/unstained, resulted in centromeric gap. Presence of one (Fig. 18) and two (Fig. 19) centromeric gaps in metaphase plates were noticed.

f) Terminal association with chromosome (TAC):

The ends of two chromosomes joined due to stickiness resulted by deletion of telomeric regions (Fig. 20).

g) Stickiness (Stk):

The terminal ends of two or more chromosomes stick together and remained intact due to DNA depolymerisation, while the individual chromosomes can also be easily distinguishable (Fig. 21).

h) Clumping (C):

It is advance form of stickiness, in which all the chromosomes of the complement clump together and individual chromosomes cannot be differentiated (Fig. 22).

i) Pyknosis (Py):

Pyknosis, or karyopyknosis is the irreversible condensation of chromatin in the nucleus of a cell, which undergo necrosis. Differential staining of chromosomes occurs, in which some parts of chromosomes are darkly stained and other parts are lightly stained. The darkly stained regions show positive pyknosis, whereas lightly stained regions possess negative pyknosis (Fig. 23).

j) Stretching (Stch):

When normal chromosome complement is showing unusual lengthening and thinning of chromosomes known as stretching (Fig. 24).

k) Pulverization (P):

When whole of the chromosome complement undergoes fragmentation and ultimately, resulted in cell death is called as pulverization (Fig. 25).

The chromosome aberrations are categorized as clastogenic and physiological type. The clastogenic types of aberrations are chromosomal fragmentation, ring chromosome, terminal chromatid deletion, minutes and

centromeric gaps, whereas physiological types of aberrations are terminal association of chromosomes, stickiness, clumping, pyknosis and stretching.

Frequencies of all the eleven types of chromosomal aberrations and their mitotic indices in fishes treated with tannery and paint industrial effluent were summarized (Table VIII and IX), respectively. Comparative data of chromosomal aberrations on the basis of Mean±S.E. of both the effluents is shown in Graph 3. $p<0.05$ was considered to be the level of significance.

Tannery effluent:

In 1.93% concentration, there was decrease in all types of chromosomal aberrations with increase of exposure periods, particularly after 96h and 120h. Overall, the frequency of Cf was highest, followed by Tcd, Cg, whereas the frequency of Stch and Stk were lowest. The decreasing order of the aberrations was Cf>Tcd>Cg>Rc>M>P>Tac>C>Py>Stk>Stch. The mean frequencies of total aberrations were at 24h (45.66 ± 0.88^{a}), 48h (40.00 ± 0.57^{b}), 72h (35.00 ± 1.52^{c}), 96h (27.00 ± 1.73^{d}) and 120h (22.00 ± 0.57^{e}), which indicated that overall decrease in aberrations from 24h to 120h. However, in 7.74% concentration, there was increase in chromosomal aberrations from 24h to 120h. Overall, the frequency of Rc was highest, followed by Cf and Cg, while frequency of Stch, Stk and C were lowest. The decreasing order of the aberrations was Rc>Cf>Cg>M>Tcd>P>Py> Tac>C>Stk>Stch. The mean frequencies of total aberrations were at 24h (47.00 ± 1.52^{a}), 48h (53.66 ± 0.88^{b}), 72h (60.66 ± 0.33^{c}), 96h (67.33 ± 0.33^{d}) and 120h (72.66 ± 0.88^{e}), which showed the increasing trend of chromosomal aberrations from 24h to 120h. In both the concentrations, clastogenic effects were more as compared to physiological effects.

Mitotic indices:

Mitotic indices of control were at 24h (15.90±0.33), 48h (15.60±0.66), 72h (15.55±0.66), 96h (15.20±0.57) and 120h (15.20±0.57), which depicted that number of dividing cells were approximately same in all the durations. At 1.93% concentration, mitotic indices were at 24h (13.58±0.57), 48h (13.86±0.33), 72h (14.58±0.57), 96h (14.72±0.66) and 120h (14.86±0.66), which showed that increasing trend with respect to exposure period, while after

48h there was prominent increase from 72h to120h. At 7.74% concentration, mitotic indices were at 24h (12.36±0.88), 48h (12.19±0.88), 72h (11.52±1.45), 96h (11.16±1.00) and 120h (11.08±0.57), which indicated the decreasing order with respect to exposure periods, whereas after 48h there was drastic decrease in mitotic indices, followed upto 120h (Table VIII).

Paint industry:

In 3.95% concentration, there was decrease in chromosomal aberrations from 24h to 120h. Overall, frequencies of M, Tcd, Cf were highest, whereas P and Stch were lowest and Stk, Stch and P were not observed at 120h. The decreasing order of the aberrations was M>Tcd>Cf>Rc>Tac>C>Cg>Stk> Py> Stch > P. The mean frequencies of total aberrations were at 24h (38.66 ± 0.88^{a}), 48h (31.33 ± 1.15^{b}), 72h (26.00 ± 0.66^{c}), 96h (19.33 ± 1.72^{d}) and 120h (12.00 ± 1.45^{e}), which showed decreasing trend for aberrations from 24h to 120h. In 15.81% concentration, there was increase in chromosomal aberration from 24h to 120h. Overall, frequencies of Tcd, M, Cf were highest, while Stch and Tac were lowest and Stch was absent at 120h. The decreasing order of the aberration wasTcd>M>Cf>Rc>Cg>Py>C>P>Stk>Tac>Stch. The mean frequencies of total aberrations were at 24h (40.33 ± 0.66^{a}), 48h (46.00 ± 0.88^{b}), 72h (50.66 ± 1.15^{c}), 96h (57.00 ± 0.66^{d}) and 120h (65.33 ± 1.15^{e}), which indicated that increasing trend of in aberrations from 24h to 120h.

Mitotic indices:

Mitotic indices of control were at 24h (18.75±1.73), 48h (18.63±1.45), 72h (18.69±2.02), 96h (18.61±0.88) and 120h (18.75±2.30), which were almost same in all exposure periods. At 3.95% concentration, mitotic indices were at 24h (15.00±2.30), 48h (15.47±1.85), 72h (15.58±1.73), 96h (15.94±1.33) and 120h (16.19±1.76), which indicated increasing order with respect to exposure periods. At 15.81% concentration, mitotic indices were at 24h (14.19±1.15), 48h (14.52±2.33), 72h (14.25±0.57), 96h (14.11±2.33) and 120h (13.69±2.90), which showed decreasing order from 24h to 120h, while prominent decrease was observed at 120h (Table IX).

5. Micronucleus test:

Micronucleus test was performed on blood from kidney of *Labeo rohita*. Micronuclei formation can occurs in dividing cells of all the organisms including fishes having nucleated erythrocytes. Micronuclei are chromosome fragments, which lag behind during the cell division because of their acentric nature and their size is 1/3rd of the main nucleus. MN test is proved to be a simple, reliable, sensitive and inexpensive procedure to assess the biological impact of water pollution on aquatic organisms. It can also be used to assess the genotoxicity of physical and chemical agents after direct or indirect exposure.

In *Labeo rohita* (Control) mature erythrocytes were fairly large in size with centrally placed elliptical or rounded nucleus. They had well defined boundary and clear cytoplasm (Figs. 26, 27). Position of micronuclei was varied, in some cells they located very near to nucleus and in other cells they were present at the periphery of the cell. In erythrocytes (treated fishes), single micronucleus (Figs. 28, 29) was commonly occurred, whereas two (Figs. 30, 31) and three micronuclei (Figs. 32, 33) in a cell were also seen due to the clastogenic effects.

Fishes treated with two sublethal concentrations of both the effluents for 24h, 48h, 72h, 96h, 120h were found to be significantly different ($p<0.05$) from the control. 6000 cells in control as well as in treated fishes were examined to find out the occurrence of micronuclei. Frequencies of micronuclei in fishes treated with tannery and paint industrial effluent were summarized in (Tables X and XI), respectively. Comparative data of micronuclei in control and treated fishes of both the effluents is shown in Graph 4. The graph indicated that frequency of one micronucleus is higher than two and three micronuclei in all sublethal concentrations of both the effluents.

Tannery effluent:

Control:

In control, there was negligible number of micronuclei. There was no micronuclei upto 48h, while one cell each of one micronucleus, two micronuclei and three micronuclei after 72h were seen. After 96h, two cells of one

micronucleus, one cell each of two micronuclei and three micronuclei were observed, whereas one cell each of one micronucleus and two micronuclei and two cells with three micronuclei were present at 120h.

Treated fishes:

In 1.93% concentration, mean frequency of micronuclei was decreased at 24h (26.33 ± 0.66^{a}) then steady decreased at 48h (23.00 ± 1.15^{b}), at 72h (22.50 ± 0.57^{c}) and 96h (17.83 ± 0.33^{d}), while sharp decrease was detected at 120h (13.16 ± 0.88^{e}). In 7.74% concentration, mean frequency of micronuclei was induced at 24h (32.16 ± 1.20^{a}) then steady increased at 48h (37.66 ± 0.33^{b}), at 72h (42.33 ± 0.88^{c}) and at 96h (49.66 ± 1.45^{d}), whereas sharp increase was observed at 120h (56.00 ± 1.15^{e}).

Paint effluent:

Control:

In control, there was negligible number of micronuclei. After 24h, four cells with one micronucleus and two cells with two micronuclei were seen. After 48h, two cells each of one micronucleus and two micronuclei were observed, while after 96h, one cell each of one micronucleus, two micronuclei and three micronuclei were recorded.

Treated fishes:

In 3.95% concentration, mean frequency was decreased steadily from 24h (15.66 ± 2.02^{a}), 48h (14.66 ± 0.66^{b}), 72h (12.33 ± 1.76^{c}), 96h (11.00 ± 2.30^{d}) and 120h (9.16 ± 1.45^{e}). However, in 15.81% concentration, mean frequency was steadily increased from 24h (18.66 ± 0.88^{a}), 48h (23.33 ± 1.20^{b}), 72h (25.33 ± 0.66^{c}) and 96h (28.33 ± 2.02^{d}) and sharp increase was seen at 120h (35.83 ± 2.78^{e}).

6. Erythrocyte Aberrations (EA):

Erythrocyte aberrations are genotoxic analogues of micronuclei that may also be the originated due to the impact of genotoxic agents present in aquatic system. Fish erythrocytes are distinct because they possess a nucleus and any morphological changes in the cells are considered as important bio-indicator. There are two types of nuclear aberrations and cellular aberrations.

1) Nuclear Aberrations (NA): Five types of nuclear aberrations were examined during the present investigations.

Nuclear extrusion (NE):

When nucleus starts moving towards periphery and changes the shape of the cell (Figs. 34, 35).

a) Blebbed (B):

A small bulge containing chromatin material on the nuclear membrane is present (Figs. 36, 37).

b) Binucleate (BN):

A cell with two nuclei sharing common cytoplasm (Figs. 38, 39).

c) Lobed (L):

Nuclear membrane showing several lobes (Figs. 40, 41).

d) Notched (N):

Nucleus having sharp depression, which appears like a notch (Figs. 42, 43).

2) Cellular Aberrations: Six types of cellular aberrations were recorded during the present investigations.

a) Enucleated cell (EnC):

When nucleus is absent in erythrocyte cell (Figs. 44, 45).

b) Vacuolated cell (VC):

Nucleus is surrounded by number of vacuoles (Figs. 46, 47).

c) Deformed cell (DC):

The shape of erythrocytes altered due to the unequal distribution of cytoplasm (Figs. 48, 49).

d) Echinocytic cell (EC):

Cells are showing spine like projections on the membrane (Figs. 50, 51).

e) Spindle shaped cells (SC):

When erythrocytes are having a shape of spindle, that is, thick in middle and tapering at both sides (Figs. 52, 53).

f) Apoptotic cell (AC):

Cell is programmed to the cell death due to the toxic effect of pollutants (Figs. 54, 55).

6000 cells were examined in control as well as in treated fishes of both the effluents after 24h, 48h, 72h, 96h and 120h to study erythrocyte aberrations. These aberrations were found to be significantly different ($p<0.05$) with respect to their control. Frequencies of erythrocytes in fishes treated with tannery and paint industrial effluents were summarized in (Tables XII and XIII), respectively. Comparative Mean($\%_0$)±S.E. frequencies of cells with erythrocyte aberrations in control and treated fishes are shown in Graph 5.

Tannery effluent:

Control

In control, minor aberrations were observed. After 72h, cells with nuclear extrusion (1), enucleated (1), deformed (3), apoptotic (1) were seen, while after 96h, nuclear extrusion (1), enucleated (2), apoptotic (2) cells were recorded and after 120h, nuclear extrusion (1), enucleated (2), spindle shaped (1), apoptotic (1) cells were seen.

Treated fishes:

In 1.93% concentration, deformed, echinocytic and enucleated cells were predominant, while binucleated were lowest. The mean frequency of erythrocytes aberrations were decreased from 24h (72.83 ± 1.20^{a}) to 48h (70.33 ± 1.76^{b}), 72h (63.83 ± 1.20^{c}) upto 96h (60.33 ± 1.76^{d}), while there was sharp decrease at 120h (46.50 ± 1.15^{e}). The overall decreasing trend for aberrations was DC>EC>Enc>L>SC>VC>B>AC>NE>N>BN. In 7.74% concentration, deformed, spindle shaped and echinocytic cells were predominant, while binucleated were lowest. The mean frequency was increased from 24h (94.16 ± 2.08^{a}) to 48h (108.83 ± 1.20^{b}) then sharp increase was seen, after 72h (163.33 ± 0.66^{c}), 96h (177.50 ± 1.73^{d}) and 120h (195.83 ± 2.60^{e}). The overall decreasing trend was DC>SC>EC>VC>L>Enc>NE>N>AC>B>BN.

Paint effluent:

Control:

In control after 24h, deformed (3), echinocytic (1) and apoptotic (1) cells were seen. After 48h, nuclear extrusion (1), deformed (1) and spindle shaped (1) cells were observed, whereas after 96h, nuclear extrusion (1) was recorded.

Treated:

In 3.95% concentration, apoptotic, echinocytic and spindle shaped cells were predominant, while binucleated were very rarely seen. The mean frequency was decreased steadily from 24h (47.66 ± 1.20^{a}), 48h (40.66 ± 1.15^{b}), 72h (34.66 ± 2.60^{c}), 96h (29.66 ± 3.18^{d}) and sharp decrease was noted at 120h (19.33 ± 2.90^{e}). Overall, the decreasing trend for aberrations was AC>EC>SC>NE>DC>N>L>Enc>VC>BN. In 15.81% concentration, echinocytic, nuclear extrusion and spindle cells were prominent, while binucleated were rare. The mean frequency was increased at 24h (69.16 ± 1.45^{a}), 48h (81.33 ± 0.66^{b}) and sharp increase was seen after 72h (107.00 ± 2.30^{c}), 96h (137.00 ± 1.15^{d}) and 120h (154.66 ± 2.16^{e}). Overall, the decreasing trend for aberrations was AC>EC>SC>NE>DC>N>L>B> Enc>VC> BN.

7. Histopathology:

Semi-Quantitative histological assessment:

Takashima and Hibiya (1995) and Sindermann (1979) proposed the classification for the histological assessment of organs for experimental animals. Later, Bernet *et al*. (1999) published the standard protocol for semi-quantitative histological assessment of each target organ. The pathological changes are also called as alterations and divided into five reaction patterns. Each reaction pattern (rp) includes several alterations (alt) related to either functional unit of the organ or to the whole organ. These reaction patterns are enumerated as follows:

Reaction pattern 1 (rp_1): Circulatory Disturbances (CD)

Circulatory disturbances result due to changes in the blood flow and movement of tissue fluid. These are:

a**)** Haemorrhages: Blood leaking from blood vessels.

b**)** Aneurysm: Dilation of arterial blood vessels in form of balloon.

c**)** Intracellular oedema: Tissue fluid leaked from capillaries into interstitial tissue.

Reaction pattern 2 (rp_2): Regressive Changes (RC)

Regressive changes are processes, which suppress the functions of an organ and ultimately, result in loss of an organ. They are as follows:

a) Architectural and structural alterations (change in structure, shape and arrangement of cells)

Gills:

1) Broken cartilage: Break in the cartilage lamellae.

2) Damaged lamellae: Destruction in lamellae.

3) Loss of lamellae: Lamellae are lost due to erosion.

4) Lamellar telangiectasia: Dilatation of the capillaries in form of bulges at the tips of lamellae.

Kidney:

1) Occlusion of tubular lumen: Closing of lumen of tubules.

2) Narrowing of tubular lumen: The partial closing of lumen.

3) Widen lumen: Area of tubular lumen increased due to necrosis.

4) Increased Bowman's space: Decrease in size of glomerulus.

5) Shrunken glomerulus: Reduction in size of glomerulus.

6) Decreased hematopoietic tissue: Necrosis of hematopoietic tissue.

7) Dilation of Bowman's capsule: Dilation or erosion in the wall of Bowman's capsule.

Liver

1) Disintegration of central vein: Necrosis in central vein.

2) Fatty acid degradation: Fats degraded into metabolites.

3) Dilation of sinusoids: Thinning of blood vessels.

4) Melano-macrophage centers: Aggregation of macrophages with melanin pigment.

5) Necrosis: Irreversible loss of functions of cell/tissue.

Reaction pattern 3 (rp_3): Progressive Changes (PC)

Progressive changes are processes, which lead to an increased activity of cells or tissues. These are as follows:

a) Hypertrophy: Enlargement of cell volume or tissue without increase in cell number.

b) Hyperplasia: Enlargement of tissues or organs by the increase in number of cells without any change in the volume of the cell. However, hyperplasia in gills leads to fusion of primary and secondary lamellae (Gluing of lamellae) and Curling/Curving of lamellae.

Reaction pattern 4 (rp_4): Inflammation (I)

Inflammatory changes mostly related to infections, which result in:

Lymphatic infiltration: Leucocytes infiltrating the surrounding tissue *via* blood vessels.

Structure of Gills:

Gills are characteristic feature of fishes, which used for vital functions like respiration, osmoregulation, nitrogen excretion, acid base regulation and detoxification. Fish gills are located on both sides of pharynx covered by operculum. *Labeo rohita* has five pairs of gill arches, however, last pair of arches formed the pharyngeal bone, which helps in respiration. Gill arche possesses cartilaginous skeleton associated with striated muscles, which helps in movement of gills. Each gill arch has number of gill filaments, called as holobranchs, which again divided into two halves, known as hemibranchs. Numerous lamellae are present in theses hemibranchs. The lamellae are again divided into primary and secondary lamellae. The primary lamellae consist of cartilaginous support, vascular system, multi-layered epithelium and mucous cells on afferent and efferent edges. The secondary lamellae originated along the entire length of primary lamellae and consist of blood arterioles supported by pillar cells. Chloride cells surrounded by flattened pavement cells are also present at the primary and secondary lamellae (Fig. 56).

Control:

Gills of control fishes of tannery effluent showed normal morphology with some alterations like loss of lamellae, necrosis, fusion of primary and secondary lamellae and curling of lamellae at very few regions, whereas in paint control fishes possessed minor alterations like aneurysm, loss of lamellae, necrosis, hypertrophy and fusion of primary and secondary lamellae.

Treated fishes:

Treated fishes of tannery and paint effluent showed histopathological changes under various reaction patterns were summarized (Table XIV and XV), respectively.

Major histopathological alterations in gills of treated fishes were Haemorrhages (Fig. 57), intracellular oedema (Fig. 58), aneurysm (Fig.59), broken cartilage (Fig. 60), damaged lamellae (Fig. 61), loss of lamellae (Fig. 62), lamellar telangiectasia (Fig. 63), necrosis (Fig. 64), hypertrophy (Fig. 65), hyperplasia (Fig. 66) fusion of primary and secondary lamellae (Fig.67), curling of lamellae (Fig. 68) and lymphatic infiltration (Fig. 69).

In tannery effluent, at 1.93% concentration, haemorrhages, intracellular oedema, lamellar telangiectasia, hyperplasia and lymphatic infiltration were significant alterations in the gills, while necrosis, broken cartilage, aneurysm, damaged lamellae loss of lamellae were seen at few regions. Hypertrophy, increased number of cells lead to fusion of primary and secondary lamellae and curling of lamellae were less observed. On the other hand, in 7.74% concentration, gill damage was more severe than above concentration. Haemorrhages, intracellular oedema, broken cartilage, damaged lamellae, necrosis, hyperplasia, hypertrophy, lamellar telangiectasia, aneurysm and lymphatic infiltrations were significant changes, while loss of lamellae, curling of lamellae and fusion of primary and secondary lamellae were least significant changes.

In paint effluent, at 3.95% concentration, damaged lamellae, loss of lamellae, lamellar telangiectasia and curling of lamellae were significant changes, while intracellular oedema, haemorrhages, aneurysm, broken

cartilage, hypertrophy, hyperplasia and lymphatic infiltration were less pronounced changes. Necrosis and fusion of primary and secondary lamellae were rare. Moreover, at 15.81% concentration, marked changes were intracellular oedema, aneurysm, haemorrhage, damaged lamellae, loss of lamellae, necrosis, telangiectasia and lymphatic infiltration of cells, whereas fusion of primary and secondary lamellae, curling of lamellae, broken cartilage and hyperplasia were rare.

Structure of Kidney:

Kidneys are long paired reddish brown structures running dorsally in the body cavity above the gas bladder and beneath the vertebrate column. They are one of the primary organs involved in excretion, homeostasis and osmoregulation. In *Labeo rohita,* mesonephric kidney is divided into two portions, anterior head composed of hematopoietic, lymphoid and endocrine tissue, while posterior trunk consists of numerous nephrons surrounded by interstitial lymphoid tissue. The basic functional unit of the kidney is nephron, which includes Bowman's capsule containing a well vascularized glomerulus and renal tubules. Renal tubules include proximal segment, distal segment and collecting duct system (Fig.70).

Control:

In tannery effluent, control fishes showed minor histological alterations in kidney like increased Bowman's space, shrunken glomerulus, occlusion and narrowing of tubular lumen, while in paint effluent, narrowing of tubular lumen, increased Bowman's space and shrunken glomerulus at few regions of the tissue were seen.

Treated fishes:

Fishes treated with tannery and paint effluent showed histopathological changes and various reaction patterns were summarized (Table XVI and XVII), respectively.

Major histopathological alterations resulted to the impact of effluents were haemorrhages (Fig. 71), intracellular oedema (Fig. 72), occlusion of tubular lumen (Fig. 73), narrowing of tubular lumen (Fig. 74), widen lumen of

tubules (Fig. 75), increase Bowman's space (Fig. 76), shrunken glomerulus (Fig. 77), decreased hematopoietic tissue (Fig. 78), dilation of Bowman's capsule (Fig. 79), necrosis (Fig. 80), hypertrophy (Fig. 81), hyperplasia (Fig. 82) and lymphatic infiltration (Fig. 83).

In tannery effluent, at 1.93% concentration, presence of hypertrophy, hyperplasia and widen lumen of tubules were significant alterations, whereas haemorrhages, intracellular oedema, occlusion of tubular lumen, narrowing of tubular lumen, increase Bowman's space, shrunken glomerulus, decreased hematopoietic tissue, dilation of Bowman's capsule, necrosis and lymphatic infiltration were less significant changes. On the other hand at 7.74% concentration, there was marked cellular and structural damage of the tissue, which included mass intracellular oedema, haemorrhages, occlusion of tubules, necrosis, increased Bowman's space, shrunken glomerulus, dilation of Bowman's capsule, decrease in hematopoietic tissue, widen lumen, hyperplasia, while lymphatic infiltrations, narrowing of tubular lumen and hypertrophy were less significant changes.

In paint effluent, at 3.95% concentration, lymphatic infiltrations, hypertrophy and dilation of Bowman's capsule were more pronounced changes, while haemorrhages, intracellular oedema, necrosis, decreased hematopoietic tissue, narrowing of tubular lumen, occlusion of tubular lumen, widen lumen, increase Bowman's space, hyperplasia, shrunken glomerulus were less significant alterations. However, at 15.81% concentration, intracellular oedema, hypertrophy, lymphatic infiltrations, occlusion of tubular lumen, narrowing of tubular lumen, necrosis, shrunken glomeruls, increased Bowman's capsule, decreased hematopoietic tissue as marked alterations, while haemorrhages, widen lumen, dilation of Bowman's capsule were less significant. However, hyperplasia was rarely seen.

Structure of Liver:

Fish liver is a key organ, which control many functions and play an important role in fish physiology. It is a bilobed organ and consists of right large lobe and small left lobe. The left lobe is further divided into two lobes,

anterior and posterior. The gall bladder is embedded in the right lobe. The normal liver is formed of hepatocytes, which are polygonal in shape. Hepatocytes possess a centrally located nucleus, which contains nucleolus and homogenous cytoplasm. Hepatocytes are arranged in the form of thick cord (double cell) between two neighboring sinusoids (Fig. 84).

Control:

In control of tannery effluent, showed minor changes in the liver like disintegration of central vein, dilation of sinusoids and necrosis, whereas control fishes of paint effluent possessed disintegration of central vein and dilation of sinusoids at few regions of the tissue.

Treated fishes:

Fishes exposed to tannery and paint effluents showed histopathological changes and various reaction patterns were summarized (Table XVIII and XIX), respectively.

Major histopathological alterations resulted due to the effluent were haemorrhages (Fig. 85), aneurysm (Fig. 86), intracellular oedema (Fig. 87), disintegration of central vein (Fig. 88), fatty acid degradation (Fig. 89), dilation of sinusoids (Fig. 90), melano-macrophage centers (Fig. 91), necrosis (Fig. 92), hypertrophy (Fig. 93), hyperplasia (Fig. 94) and lymphatic infiltration (Fig. 95).

In tannery effluent, at 1.93% concentration, marked histological alterations were fatty acid degradation, dilation of sinusoids and presence of melano-macrophage centers, while disintegration of central vein, haemorrhages, intracellular oedema, aneurysm, hyperplasia, necrosis, hypertrophy and lymphatic infiltration were least observed. However, at 7.74% concentration, haemorrhages, disintegration of central vein, necrosis, dilation of sinusoids, melano-macrophage centres and fatty acid degradation were significant changes, while cellular damages like intracellular oedema, aneursym, hypertrophy, hyperplasia and lymphatic infiltration were also seen in many regions of the tissue.

In paint effluent, at 3.95% concentration, significant alterations were hypertrophy, disintegration of central vein, fatty acid degradation and dilation of sinusoids, while haemorrhages, intracellular oedema, melan-macrophage centers and necrosis were less marked changes. Aneurysm, hyperplasia and lymphatic infiltration were rarely recorded. On the other hand, 15.48% concentration, melano-macrophage centers, hyperplasia, dilation of sinusoids, fatty acid degradation, intracellular oedema and aneurysm were significantly present, whereas haemorrhages, disintegration of central vein, necrosis, hypertrophy and lymphatic infiltration were least seen.

Mean index values: These values indicate the extent and intensity of histological alterations in the respective tissues. On basis of these values, qualitative results are converted into a semi-quantitative value. The mean index values for organs of fishes treated with both the effluents were summarized (Tables XIV to XIX).

At 1.93% concentration, of tannery effluent, the mean index value were ($I_{G,}$ 23.33), ($I_{K,}$ 19.33) and ($I_{L,}$ 15.66), whereas at 7.74% concentration, these values were ($I_{G,}$ 33.33), ($I_{K,}$ 32.66) and ($I_{L,}$ 30.33) in comparison to control ($I_{G,}$ 3.66), ($I_{K,}$ 2.30) and ($I_{L,}$ 2.60). However, at 3.97% concentration of paint effluent, these values were ($I_{G,}$ 20.66), ($I_{K,}$ 17.66) and ($I_{L,}$ 14.33) and at 15.81 % concentration, these values were ($I_{G,}$ 29.66), ($I_{K,}$ 28.66) and ($I_{L,}$ 26.66) in comparison to control ($I_{G,}$ 3.60), ($I_{K,}$ 2.00) and ($I_{L,}$ 1.33). These values clearly indicated that at low concentrations of both the effluents, the mean index values of gills, kidney and liver were ranged in class II (index <10-25), which represented moderate alterations, while at higher concentrations of both effluents, these values were placed in class III (index <26-35), which showed pronounced alterations in the organs as compared to control.

I_{rp} index (Reactive index): The I_{rp} values for tannery and paint effluents were summarized and compared in Table XX. These values clearly depicted that there were more regressive changes in the fishes treated with tannery effluent as compared to paint effluent.

Moreover, the semi-quantitative results of both the effluents at low and high concentrations also indicated that regressive changes were more prevalent in all the selected organs (gills, kidney and liver). In both the effluents, there were more circulatory disturbances than progressive changes at high concentration, while progressive changes were more than circulatory disturbances at low concentrations. Inflammatory changes were present in the form of blood infiltrations but there was no tumor formation in all the tissues treated with effluents.

Total reaction index values ($TotI_{rp}$), Organ indices (I_{org}) and Organ indices (I_{org})

The Total reaction index values ($TotI_{rp}$), Organ indices (I_{org}) and Organ indices (I_{org}) of both the effluents were summarized and compared (Tables XXI and XXII).

Total reaction index values ($TotI_{rp}$): The total reaction index values ($TotI_{rp}$) of all target organs represent the sum of various reaction patterns observed under different concentrations of effluents. These values indicate that which of the reaction pattern is more prevalent within the sample with highest index value. TotIrp values were 308 (7.74% concentration), 162 (1.93% concentrations) and 46 (control) of tannery effluent, whereas these value were 256 (15.81% concentration), 158 (3.95% concentration) and 32 (control) of paint effluent. All these values indicated the overall condition of the target organs under the influence of various concentrations of both the effluents with respect to their controls. In tannery and paint industrial effluents, regressive changes were more prevalent.

Organ indices (I_{org}): The organ indices (I_{org}) depict overall condition of the individual target organ in the fishes treated with both the effluents. In tannery, I_{org} values were 364 (gills), 326 (kidney) and 292 (liver), while in paint effluent, these were 324 (gills), 290 (kidney) and 254 (liver). In both the effluent, gills were highly affected than kidney and liver.

Total organ indices ($\mathbf{TotI_{org}}$): The total organ indices ($TotI_{org}$) indicate the entire health of the fishes based on selected target organs. In tannery effluent, $TotI_{org}$ value came out to be 982, whereas for paint effluent was 868, which represented that organs were more damaged in tannery effluent as compared to paint effluent. Comparative mean organ indices of gill, liver and kidney of *Cirrhinus mrigala* exposed to both the effluents are shown in Graph 6 indicating gill is more affected than kidney and liver.