In the present study, genotoxic potential and histopathological changes in fishes exposed to two sublethal concentrations of both the effluents have been done by chromosomal aberration test (CAT), micronucleus test (MNT), erythrocyte aberration test (EAT) and Histopathology. Behavioural responses and morphological changes have also been studied to determine the early warning signs shown by fishes. *In vivo* toxicity tests were conducted in the laboratory and following steps were undertaken:

1. Selection of the test animal:

Rand and Petrocelli (1985) and Wepener (1987) gave following points to assess the 'health status' of an aquatic body.

- It should be the representative of that ecosystem.
- It should be commercially and ecologically important.
- It should have a broad range of sensitivity.
- It should easily be maintainable in the laboratory.
- It must be able to accumulate pollutants rapidly, therefore, reflecting environment levels of pollutants.

The selection of fish as a model in eco-genotoxicological studies is necessary because fish is a very sensitive biomarker to indicate the water quality. It also highlights the potential danger of new chemicals introduced in the aquatic environment and respond to toxicants in a similar manner to higher vertebrates (Al-Sabti and Metcalfe, 1995).

Major Indian carps are abundant in Satluj river of Punjab and about 14 species of family Cyprindinae are present (Dua and Parkash, 2009). For the present study fish, *Labeo rohita* has been selected as model because of its fast growth rate, easily availability and also highly consumed by the people of Punjab. It can sustain stress, remains alive for a longer period and can be easily acclimatized in laboratory conditions.

Systematic position of the fish

Kingdom : Animalia

Phylum : Chordata

Class : Actinopterygii

Order : Cypriniformes

Family : Cyprinidae

Genus : *Labeo*

Species : *rohita* (Hamilton-Buchanan, 1822)

2. Collection and maintenance of the fish specimens:

Healthy live specimens of *Labeo rohita,* about 6-8 cm in length and 32–60 gms in weight were collected in wide mouthed plastic collected bags containing freshwater and oxygen from government fish seed farm, Patiala. They were acclimatized in laboratory for 20 days and were treated with 0.1% of $KMnO_4$ solution for 30 minutes to remove any external infections. They were fed with pelted feed and feeding was stopped 24h prior to commencement of genotoxicity tests. They were not fed during experimental periods.

3. Test chemical used:

Tannery and paint industrial effluents were used to study genotoxicity and histopathological changes in a freshwater fish, *Labeo rohita*. Effluents were directly taken from the waste outlets of tannery industrial units in Jalandhar and paint industrial units in Ludhiana.

4. Physico-chemical analysis of effluents:

Tannery and paint industrial effluents were characterized from laboratory of Punjab Pollution Control Board (PPCB), Patiala for the assessment of various constituents present in the effluents.

5. Experimental Design:

5.1 Estimation of median-lethal concentration (LC_{50}) of test chemical

LC_{50} is a concentration that can be expected to cause 50% death of the organisms under a defined set of experimental conditions. The 96h LC_{50} of tannery and paint industrial effluent was calculated by following the method given by Finney (1971). In each group, 10 fishes were exposed to 50L of

normal water (control) and five concentrations (0%, 10%, 15%, 20%, 25% and 30%) of tannery effluent and five concentrations (0%, 15%, 30%, 45%, 60% and 75%) of paint effluent. Mortality of fishes was recorded in each concentration for 96h. The same set of experiment was repeated three times. Mortality was converted into probits and concentrations were converted into log values. The graph was plotted and 96h LC_{50} was obtained. 96h LC_{50} values came out to be 15.48% and 31.62% for tannery and paint industrial effluents, respectively.

5.2 Exposure to sublethal concentrations of the effluents

Experiments were conducted in plastic tubs of 70L capacity. Apparently healthy, uninjured and uninfected fish specimens were taken and acclimatized. Fishes of control group were maintained in well aerated water, while fishes for treatment were exposed to 50L each of two sublethal concentrations of tannery (7.74% and 1.93%) and paint (15.81% and 3.95%) effluents based on 1/2 and 1/8 of 96h LC_{50} values. The experiments were repeated three times. Fishes were not fed during experiment. Effluent reached to the target organs *via* long route along blood circulation and absorption through operculum, gill epithelium and body surface as observed in nature (Rishi and Grewal, 1995b; Chandra and Khuda-Bukhsh, 2001; Rishi *et al*., 2001). Behavioural responses and morphological changes were recorded after 24h, 48h, 72h, 96h and 120h durations of exposure.

6. Chromosomal preparations:

Anterior kidney cells were selected for chromosomal preparations because rate of cell division is fast. For control and treated groups, slides were made after 24h, 48h, 72h, 96h and 120h by following the method given by Manna and Sadhukhan (1986) with minor modifications. Fishes of control and treated groups were given an intraperitoneal injection of colchicine (0.05%, 1 ml/100 g body weight). After one hour, fishes were dissected from the ventral side to remove the kidney tissue. Kidney tissue was cut into pieces and tissue was kept in hypotonic solution (0.56% KCl) for 30 minutes and fixed in freshly prepared Carnoy's fixative. Three changes of fixative each of 15 minutes were

given. After this, tissue was teased on clean slides with the help of forceps and slides were air dried. Slides were stained in Giemsa working solution, differentiated in distilled water and air dried as suggested by Tijo and Whang (1965).

6.1 Karyotype preparations

Karyotype was prepared from well spread somatic metaphase plate of the control fish. Individual chromosomes were cut from the mitotic metaphase plate and homologous chromosomes were paired on the basis of their length, arm ratio and morphology then arranged in a descending order according to their lengths. After morphometric measurement, karyogram was prepared and various parameters were studied, which include i) Chromosome length ii) Long arm length (q) iii) Short arm length (p) iv) Arm ratio (q/p). Morphological classification of chromosomes proposed by Levan *et al*. (1964) was followed to differentiate a metacentric, submetacentric, subtelocentric and telocentric chromosome on basis of following chart:

Centromeric positions	**Arm ratio (q/p)**	**Chromosome type**
Median	1.00 - 1.70	Metacentric (M)
Submedian	1.71– 3.00	Submetacentric (SM)
Subterminal	3.01- 7.00	Subtelocentric (ST)
Terminal	7.00 - onwards	Telocentric (T)

6.2 Morphometric analysis of chromosomes

Chromosomes were classified as uniarmed and biarmed as suggested by Chen and Ebelling (1971) and fundamental arm number (NF) was calculated. Mean total length (MTL), total complement length (TCL) and its percentage, centromeric index percentage (CI%), relative length percentage (RL%) and arm ratio (AR) were calculated by the following formulae:

1. MTL = Mean total length of both arms

2. TCL = Adding mean total length (μ) of all chromosomes

3. TCL % = $\frac{\text{Mean total length of a chromosome}}{\text{Total complement length}} \times 100$

4. CI% = $\frac{\text{Short arm of chromosome}}{\text{Mean total length of a chromosome}} \times 100$

5. RL% = $\frac{\text{Absolute length of a chromosome}}{\text{Absolute length of a largest chromosome}} \times 100$

6. AR = $\frac{\text{Length of long arm of a chromosome (q)}}{\text{Length of short arm of a chromosome (p)}}$

7. Genotoxicity assays:

7.1 Chromosome aberration test (CAT)

Well spread metaphase chromosome plates of control and treated fishes were selected to study the chromosomal aberrations. Total 300 plates, each of control and treated groups for each concentration and duration of exposure were examined.

Mitotic Index (MI): Mitotic index is the ratio of number of dividing cells and total number of cells.

7.2 Micronucleus test (MNT) and Erythrocyte Aberrations Test (EAT)

Micronucleus test for control and treated groups after 24h, 48h, 72h, 96h and 120h were performed by using the method given by Ayllon *et al.* (2000). Blood was collected from anterior kidney of control and treated fishes after sacrificing and immediately spread on the pre-cleaned slides to obtain a thin smear. Slides were kept for night at room temperature then fixed in absolute methanol for 14-20 minutes and air dried for half an hour. Slides were stained with Giemsa solution for 15 minutes then washed with distilled water to remove excess stain and air dried. Total 6000 cells, each of control and treated groups for each concentration and duration of time were studied.

8. Scanning and photomicrography:

Slides of normal chromosomes, chromosomal aberrations, micronuclei and erythrocytes aberrations were scanned at the magnification of 1000X by using Nikon microscope and photomicrographed by using Nikon camera.

9. Statistical anaylsis:

Data of chromosomal aberrations, micronuclei and erythrocytes aberrations tests were subjected to statistical analysis by applying Annova and

Tukey test. Statistical analysis was done by using computer software 'Graph pad prism'. $p<0.05\%$ was considered to be the level of significance. Statistical significance of frequencies of chromosome aberrations, micronuclei, erythrocytes aberrations of control and treated groups of each concentrations and durations were evaluated.

10. Histopathology:

Histopathological studies were undertaken to study pathological changes in tissue structure and morphology of cells.

10.1 Fixation of the tissue

Fishes of control and treated groups were sacrificed to take out gills, liver and kidney tissues after 120h. All the tissues were fixed in Bouin's solution for 24h.

10.2 Tissue processing (washing, dehydration, clearing, infiltrations and embedding)

Tissues samples were washed with distilled water to remove extra Bouin's solutions and dehydration was done in (30%, 50% and 70% of ethanol grades by giving changes of 15 minutes each). After that, tissues were preserved and these were proceeded for embedding by dehydrating in 90% ethanol (15 min), absolute alcohol (10min), absolute alcohol: xylene (1:1 for 10 min), xylene (3 min), xylene : wax (1:1 for 12 h). Before embedding, the tissues were given two changes of pure wax for 2h. Glycerine was evenly applied to the selected mould and molten wax was added in it. When wax began to solidify then tissue was placed in the center of mould. Tissues were extracted from mould and were fixed in the wooden blocks for microtomy.

10.3 Sectioning

The tissues in the wooden blocks were fixed on microtome with the help of clips. The ribbon of sections was prepared and fixed on clean slides coated with thin film of Mayer's fixative. The sections were stretched on hot plate at 30^{o}C and slides were kept for overnight.

10.4 Staining

The stretched slides were dipped in xylene for overnight then dip in xylene : absolute alcohol (1:1 for 10 min) and dehydration was done by the various grades of ethanol, 100% (15min), 90% (17 min), 70% (20 min), 50% (23 min), 30% (25 min). The slides were differentiated in distilled water to remove extra alcohol and stained with Haematoxylin stain for 5 minutes. The slides were dip for 1 minute in HCL: water followed by dip in water then dip in ammonical water. After that, slides were dipped in various grades of ethanol, 30% (2 min), 50% (4 min), 70% (6 min), 90% (8 min). The slides were stained with Eosin for (2 min), dipped in 90% ethanol (2 min), absolute alcohol (2 min), absolute: xylene (1:1 ratio for 2 min) and xylene (2 min).

10.5 Mounting

The slides were mounted with DPX and dried.

10.6 Scanning and photomicrography

Slides of histology of gills, kidney and liver were scanned at the magnification of 400X by using Nikon microscope and photomicrographed by using Nikon camera.

11. Semi-quantitative histological assessment

Semi-quantitative histological assessment was done to quantify the histopathological alterations observed in the gills, liver and kidney tissues by following the protocol of Bernet *et al.* (1999) and Van Dyk *et al.* (2009). They gave five reaction patterns to assess histopathological damage in organs/tissues. During the present study, four reaction patterns were followed:

i) **Reaction pattern 1 (rp1): Circulatory disturbances (CD)**

ii) **Reaction pattern 2(rp2): regressive changes (RC)**

iii) **Reaction pattern 3(rp3) : progressive change (PC)**

iv) **Reaction pattern4 (rp4): inflammation (I)**

11.1 Importance factor

Bernet *et al.* (1999) assigned 1, 2 and 3 as importance factor for tissue alterations. These are (1) for minimal pathological importance, (2) for moderate pathological importance and (3) for marked pathological importance.

11.2 Score value (a)

Bernet *et al.* (1999) assigned a score values 0 to 6 for the assessment, which depend on the degree and extent of the alterations. These are (0) unchanged (2) mild occurrence or focal alteration (4) moderate occurrence (6) severe occurrence or alterations.

11.3 Reaction index value (Irp)

This index value determines the degree of damage within a single reaction pattern (Circulatory disturbances, regressive change, progressive change and inflammation) for organ. The value is calculated by the sum of the multiplied score values (a) and importance values (w) of all the alterations (alt) identified in a single reaction pattern (rp) of a specific organ. It is calculated for the five reaction patterns.

$$\mathbf{(I_{rp})} = \sum \mathbf{alt}\ (\mathbf{a_1}\ \text{org}\ \mathbf{_1}\ \text{rp}\ \mathbf{all}\ \text{alt} \times \mathbf{W}\ \mathbf{_1}\ \text{org}\ \mathbf{_1}\ \text{rp}\ \mathbf{all}\ \text{alt}).$$

11.4 Total reaction index values ($\mathbf{TotI_{rp}}$)

These values detect the degree of damage by single reaction pattern in all the organs. The index value is calculated by the sum of the multiplied score values (a) and importance factors (w) of all alterations (alt) identified in a single reaction pattern (rp) of all organs (org). This index represents the quality of the histological alterations in all examined organs of an individual. These values indicate the overall condition of the target organs under each concentration of the effluent.

$$\mathbf{(TotI_{rp})} = \sum \text{rp} \sum \mathbf{alt}\ (\mathbf{a}\ \mathbf{all}\ \text{org}\ \mathbf{1}\ \text{rp}\ \mathbf{all}\ \text{alt} \times \mathbf{W}\ \mathbf{all}\ \text{org}\ \mathbf{1}\ \text{rp}\ \mathbf{all}\ \text{alt})$$

11.5 Organ indices ($\mathbf{I_{org}}$)

These indices evaluate the overall damage of the target organ in the entire sample. The index value is calculated by the sum (∑) of the multiplied score values (a) and importance factors (w) of all alterations (alt) identified in all the reaction patterns (rp) of a specific organ (org). A high index value indicates a high degree of damage.

$$\mathbf{(I_{org})} = \sum \mathbf{rp} \sum \mathbf{alt}\ (\mathbf{a_1}\ \text{org}\ \mathbf{all}\ \text{rp}\ \mathbf{all}\ \text{alt} \times W_1\ \text{org}\ \mathbf{all}\ \text{rp}\ \mathbf{all}\ \text{alt})$$

11.6 The total organ indices (Tot I_{org})

These indices determine the cumulative damage to all the selected target organs. The index value is calculated by the sum of the multiplied score values (a) and importance factors (w) of all alterations (alt) indentified in the reaction patterns (rp) of all organs (org). This index represents the overall health status of an individual.

$$\textbf{(Tot } I_{\textbf{org}}\textbf{)} = \sum_{org} \sum_{rp} \sum_{alt} \textbf{(a all} \text{ org } \textbf{all} \text{ rp } \textbf{all} \text{ alt} \times \textbf{W all} \text{ org } \textbf{all} \text{ rp } \textbf{all} \text{ alt)}$$

11.7 Mean Organ Index

The mean organ index protocol was given by Zimmerli *et al.* (2007) and modified by Van Dyk *et al.* (2009). According to them, mean organ indices are divided into four classes:

Class 1 (index <10) - tissue with slight histological alterations.

Class 2 (index <10-25) - tissue with moderate histological alterations.

Class 3 (index <26-35) - tissue with pronounced histological alterations.

Class 4 (index <35) - tissue with severe histological alterations

12. Preparation of reagents:

12.1 Colchicine (0.05%): Colchicine -50mg

Distilled water-100ml

12.2 Hypotonic solution (0.56% KCl): KCl -560mg

Distilled water-100ml

12.3 Carnoy's Fixative: Methanol -75 ml

Glacial acetic acid - 25ml

12.4 Preparation of Giemsa stain: Stock solution: Giemsa -1gm

Glycerin- 66ml

Incubate for 2 hours at 60^0C.

Methanol - 66ml

Working solution

Distilled water-40 ml

Stock solution- 20 ml

12.5 Bouin's Solution: 70% alcohol -500 ml

Picric acid powder- 35 gm

Solution is allowed for one week to ripe.

Formaldehyde - 100 ml

Glacial acetic acid - 20 ml

12.6 Delafield's Hematoxylin: Hematoxylin crystals -1 gm

Absolute alcohol - 10 ml

Saturated ammonium sulphate -100 ml

Leave it exposed to light and air for one month. Filter the solution

Glycerine - 25 ml

Methy alcohol -25 ml

12.7 Eosin stain: Eosin -2.5 gm

Distilled water -100 ml

Glacial acetic acid -5 ml

70% alcohol - 500 ml

12.8 Mayer's fixative: Egg albumin-10ml

Glycerol-10ml

12.9 Ammonical water: Ammonia -2-3 drops

Distilled water -100 ml

12.10 HCL: HCL -2-3 drops

Distilled water -100 ml