MATERIALS AND METHODS

1. TEST ANIMALS

Two fish species viz., *Etroplus suratensis* and *Etroplus maculatus* were chosen as the test animals. These two species inhabit the brackish-waters and estuaries of India, Sri Lanka and Pakistan. In India, the major area of distribution lie between Karnataka coast on the west and upto Chilka on the east. *E. suratensis* feeds on detritus, plankton and small aquatic insects whereas *E. maculatus* is carnivorous in nature, feeding on a wide range of food items. Both are available almost throughout the year, but the peak season is January to April and September to November. *E. suratensis* mature when they attain a size of 10-12 cm in length. They grow to a maximum length of about 25 cm. They contribute a significant percentage of the landings in Chilka lake, Pulicat lake and Kerala backwaters. It is one of the prized species in Kerala. *E. maculatus* is smaller in size. They mature at a length of about 5-6 cm. Both species are not very difficult to maintain in the laboratory as they accept artificial feeds.

Collection of test animals:

Young specimens of *E. suratensis* and *E. maculatus* were collected from the Narakkal field station of CMFRI, Malipuram and Fisheries College, Panangad. All the collection spots were not very far off from the laboratory.
Plate 1. *Etroplus maculatus* (test species)

Plate 2. *Etroplus suratensis* (test species)
Plate 3. Experimental set-up for treatments

Plate 4. U.V. irradiation system.
Specimens were transported live in oxygen bags or buckets to the laboratory, acclimatised and maintained for 20-30 days in a salinity range of 5-10 ppt. They were fed regularly with artificial pellet feed and prawns. Water was changed once or twice a week. Fibre glass tanks (40 L capacity) with outlets on the base were used for the experiments.

2. TEST CHEMICALS

a) Methyl methane sulphonate (MMS):

This known mutagen was procured from Ms John Baker Inc. of USA. It is an organic sulphate compound (liquid) easily miscible in water. This mutagen is known to directly affect the target molecule (DNA).

b) Cyclophosphamide (Endoxan):

Cyclophosphamide (CP) is also a known mutagen and is used as an anticancer drug. The chemical as such is not active but is metabolised in the body to give mutagenic metabolites while injected to any organism (Perry and Evans, 1975). Such chemicals are referred to as indirectly acting mutagens. This chemical was procured from Ms. Khandelwal, Bombay. It is soluble in water.

c) Methyl Parathion (Metacid-50)

Methyl Parathion (MP) is an organophosphorus insecticide mostly used in agricultural fields against insect pests. This compound is a contact poison and soluble in water. It was procured from Bayer (India) Ltd., Bombay. As regards the chemical nature, it is a phosphorothioate compound i.e., O, O-dimethyl O-(p-nitrophenyl) phosphorothioate.
d) **Phosphamidon (Dimecron 85% SL)**

Phosphamidon (PM) is also an organophosphorus compound acting as a systemic insecticide cum acaricide and is used in agricultural fields. The chemical is soluble in water (Manufacturers: Hindustan Ciba Geigy Limited).

The chemical is a vinyl phosphate compound, i.e., 2-chloro-2-diethylcarbonyl - 1 - methylvinyl - dimethyl phosphate.

The above two pesticides i.e., methyl parathion and phosphamidon are organophosphorous compounds inhibiting the action of the enzyme cholinesterase which normally hydrolyse acetylcholine at nerve junctions. If not hydrolysed, acetyl choline accumulate at nerve junctions as a result of the passage of the nerve impulses, and eventually blocks the transmission of these impulses to gonads and muscles. Symptoms of poisoning in animals included lacrimation, vertigo, muscular weakness, tremors and laboured respiration (Rude, 1964). Animals actually die of suffocation. There is very little residual accumulation of organophosphorous compounds in tissues although additive effects are possible if exposure is repeated before cholinesterase levels have returned to normal. The general pattern of metabolism of xenobiotics for all animals including fish is as follows:

<table>
<thead>
<tr>
<th>Phase - I</th>
<th>Phase - II</th>
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<tbody>
<tr>
<td>Xenobiotics - Oxidation, reduction or hydrolysis</td>
<td>Synthetically conjugated metabolites</td>
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</table>
The reaction of both phases is catalysed by enzymes, either through microsomal or non-microsomal mechanisms. The metabolic steps in fish include glucoronic acid conjugation, glycine conjugation, thyocyanate synthesis, hydrolysis, reduction of azo and nitrogroups, N-oxidation, hydroxylation, demethylation, and dealkylation. Other termination mechanisms in fishes include biliary excretion and gill excretion (Adamson and Sieber, 1981).

3. CHROMOSOME PREPARATION

Standardization of methods for chromosome preparation for genotoxicity studies is quite a challenging job. Most of the species have generally very small chromosomes and secondly, getting consistently good number of metaphase plates in in-vivo is not easy. Species to species variations are experienced quite often. Standardisation of procedures may vary even from laboratory to laboratory.

The various methods tried, with modifications to evolve a suitable technique were as follows:

1. Ford and Hammerton (1956)
5. Kligerman and Bloom (1977)

1. Ford & Hammerton (1956):

The method originally developed for mammalian systems was tried with modifications. Specimens injected intramuscularly with 0.1% colchicine
@ 1 ml/100 gm body weight were dissected after 3 hours, gills intestine and kidney tissues collected. Tissues were homogenised in a water bath at 37°C for 30 minutes. The material was centrifuged for 5 minutes at 1200 rpm twice at an interval of 15 minutes with addition of fixation (3:1) methanol-acetic acid in each step after decanting the supernatant. The cell suspension was dropped on alcohol chilled slides, flame dried and stained in Giemsa.


Posterior gill arches of specimen were dissected out 2-4 hours after colchicine injection (0.01%) and hypotonised in 0.4% KCl for 30 minutes at room temperature. The material was fixed in acetic-alcohol (1:3) followed by staining in 2% Giemsa for 20 minutes. The stained gill arches were shaken lightly on a clean slide until a slurry of cells were deposited. The slurry was squashed using a cover glass.


Small sized animals were allowed to swim in well aerated colchine solution (0.01%) in a container for 45 hours. Gills were dissected and hypotonised with 0.4% KCl for 20 minutes. Tissues were minced and the suspension was centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and fixative added. Slides were prepared by dropping the suspension on alcohol chilled slides, airdried and stained in Giemsa.

Kidney tissue was dissected out from fishes, 3–4 hours after injection with 0.05% colchicine (0.5 ml/100g) and hypotonised in 1% sodium citrate for 30 minutes. The material was then gently agitated in glass homogeniser and the suspension centrifuged, fixed in methanol-acetic acid (3:1). Slides were prepared on alcohol chilled slides, air dried and stained in Giemsa.

5. Kligerman and Bloom (1977):

Tissues like gill, intestine and kidney were collected from fish 3 hours after an intramuscular injection of 0.01% colchicine (1 ml/100 g), hypotonised with 0.04% KCl for 20–30 minutes followed by fixation in methanol-acetic acid (3:1). A cell suspension was prepared using 50% acetic acid and slides prepared by dropping on warm (40-50°C) slides. The drop was immediately drawn back with a pipette, forming a ring of cells on the slide.

This method gave better results in comparison to the others. However improvements were found necessary. Trials were conducted on the following aspects to evolve a suitable method.

a) **Colchicine administration**: Intramuscular injections were preferred since the animals were small. Colchicine concentrations (0.1, 0.05, 0.01, 0.005, 0.001%) @ 1 ml/100 g body weight were tried with different exposures ranging from 2 to 4 hrs. 0.01 and 0.05% colchicine gave sufficient number of contracted metaphase plates after 3 hours of treat-
ment. Colchicine (0.001%) gave good results with respect to total number of spreads. However most of the spreads were in the premetaphase stage with the same hours of treatment. 0.01 and 0.005% of colchicine (3 hours) gave moderate number of metaphase spreads. The best results were obtained with 0.005% colchicine and an exposure of 2-2½ hours.

b) **Tissues:** Gill, kidney and intestine tissues were taken for the study. Gill tissues were found to be the best since they gave the best spreads, followed by intestine and kidney. Hence gill tissue was chosen for the study.

c) **Hypotonic treatment:** Sodium chloride (1%) sodium citrate (1, 0.5%) KCl (0.4, 0.04%) and double distilled water were tried as hypotonic solutions. The duration ranged from 15 - 60 minutes both at room temperatures and in cold. Best results were obtained with 1% cold sodium citrate for 30 minutes. In most of the samples swelling was excellent in cold conditions i.e., in a refrigerator (6-8°C).

d) **Fixation:** Fixation was done in methanol acetic acid (3:1) with three changes at intervals of 15 minutes, 30 minutes and 1 hour. Good results were obtained by fixing the material at lower temperatures. Methanol storage was tried to improve chromosome morphology as shown by Bantock and Cockayne (1975). It was not very useful.

e) **Preparation of cell suspensions:** Quite often *E. maculatus* gave chromosome spreads with poor morphology i.e., fuzzy appearance of
chromosome margins. This was suspected to be caused by acetic acid used for suspension preparation. Hence acetic acid was diluted up to 20% but the problem was not entirely solved, though there were improvements.

4. METHOD DEVELOPED FOR CHROMOSOME PREPARATIONS

Based on the above trials on the various steps, the following technique was developed for chromosome preparation of the two test species in the present study:

i) An intramuscular injection of colchicine solution (0.005%) @ 1 ml/100 g body weight on the base of the fin and an exposure of 2 - 2½ hours.

ii) Hypotonic treatment in cold trisodium citrate (0.8 - 1%) for 30 minutes.

iii) Fixation by methanol acetic acid (3:1) with 2-3 changes (First after 15 minutes; second after 30 minutes; third after 1 hour). The total duration of fixation was around two hours.

iv) Cell suspension using 40 - 50% acetic acid (3-5 minutes).

v) Dropping suspension on warm slides (40-50°C) using small Pasteur pipette and withdrawing the drop with the pipette, leaving a ring
vi) Staining of slides with 4% Giemsa solution for 15-20 minutes in a phosphate buffer (pH 6.8).

This standardised method was used for all the experiments during the study.

5. DIPLOID CHROMOSOME NUMBER

For determining the diploid chromosome number, 15 animals each were studied from both species. Only well acclimatised untreated animals were used for the purpose. About 20-30 metaphases per animal were screened. The diploid number was determined on the basis of the largest number of cells showing a particular count i.e., the modal number. A total number of more than 300 metaphases were studied from each species.

6. KARYOTYPE ANALYSIS

4 good metaphase plates approximately of similar chromosome lengths from each species were photomicrographed and prints made of the same enlargement along with the scale. The individual chromosomes from each plate were cut out, visually paired and pasted on a white board paper. The arrangement was in the descending order of chromosome length in each group. In the case of E. suratensis
it was arranged according to size only since all chromosomes belonged to one group.

Chromosome lengths, relative lengths (%) and arm ratios were measured from the metaphase plates separately. Chromosomes were classified using the methodology of Levan et. al. (1964). The best among the plates considered in each species was used for karyotype display. Generalised idiogram and histograms were also made.

7. DIFFERENTIAL STAINING OF SISTER CHROMATIDS

Induction of sister-chromatid differentiation (SCD) is a prerequisite for observing sister chromatid exchanges (SCE) i.e., exchanges between two sister-chromatids in a chromosome. The chromosomes must be treated so that sister-chromatids are chemically different from one another. This is accomplished by labelling DNA during its synthesis, either by using tritiated thymidine followed by autoradiography or by using the nucleotide (thymidine) analog Bromodeoxyuridine (BrdU). Autoradiography is not required with BrdU labelling. A schematic representation of steps in DNA labelling using BrdU is given in Figs. 1 and 2.

a) Labelling with tritiated thymidine:

This was originally accomplished by Taylor et. al. (1957). Cells are allowed to replicate their DNA for one cell cycle in the presence of tritiated thymidine followed by another cycle in the presence of non-radioactive thymidine. Because of the semi-conservative replication
Fig. 1. Formation of differentially labelled chromatids

$G_1$ - Pre-synthesis

$S$ - Synthesis

$G_2$ - Growth

--- - Labelled DNA strand (BrdU incorporated)

----- - Unlabelled DNA strand.
FIG. 1. Formation of differentially labelled chromatids
Fig. 2. Formation of SCE

$G_1$ - pre-synthesis

$S$ - Synthesis

$G_2$ - Growth

--- - Labelled DNA strand (BrdU incorporated)

--- - Unlabelled DNA strand.
FIG. 2. Formation of sister-chromatid exchanges (SCE)
this treatment results in the formation of chromosomes containing one chromatid with one polynucleotide strand of its DNA labelled and its sister-chromatid not labelled. Thus the two chromatids are chemically different and this can be visualised with autoradiography techniques.

b) Labelling with bromodeoxyuridine:

This is a newer method of DNA labelling using chemical analogs of thymidine (nucleotide) that become incorporated into the chromosomes during DNA synthesis. After one round of replication with BrdU, chromosomes contain chromatids that are unifilarly substituted. After a second round of replication in the presence of BrdU the resultant chromosomes have one chromatid unifilarly substituted (one nucleotide strand labelled) while its sister is bifilarly substituted (both nucleotide strands labelled). Other analogs like bromouracil or fluorouracil can also be used.

BrdU administration is generally done through in-vivo methods like injections or administration through the medium. It is also possible to administer BrdU during in-vitro procedures (cell cultures). The labelled chromosomes are stained with fluorescent dyes like Hoechst 33258 acridine orange, DAPI and exposed to light for pre-fading. Hoechst dye provides an intense localised source of light after it is excited. (Wolf and Bodycote, 1977). Light treatment (pre-fading) results in lesser staining intensity with Giemsa in those chromatids bifilarly substituted.
This newer method is relatively better since it does not involve the use of autoradiography and good quality preparations are possible with precise treatments. Hence this basic method was adopted after modifications of the various steps.

c) Protocols evaluated:

Except for one note (Mohanty & Prasad, 1982) there is no information on the SCD/SCE on any Indian fish species. Hence it took a considerable time to evolve a suitable method for our laboratory conditions. The various protocols tried during standardisation were as follows:

i) Perry and Wolff (1974): Slides were stained in 0.5 μg/ml Hoechst 33258 in Sorensen's Buffer at pH 6.5-7.0 for at least 20 minutes. The slides were rinsed in running distilled water, dried, mounted in buffer with a coverslip and exposed to fluorescent light for 30-60 minutes. Slides were then treated with 2 X SSC (0.3 M NaCl + 0.03 M trisodium citrate) at 60°C in a waterbath. After 1 hour, slides were rinsed thoroughly in distilled water stained in Giemsa and mounted with DPX.

ii) Kligerman and Bloom (1976): Slides were treated with 0.5 μg/ml Hoechst in deionised water for about 10 minutes, rinsed in deionised water and mounted in Sorensen's buffer at pH 7.0. Treatment with light for pre-fading was done as described in the previous method.

iii) Stromberg et al. (1981) (Modified): Slides were treated with 0.5 μg/ml Hoechst in PBS solution at pH 7.0 for 5 minutes. Exposure to fluorescent
light was for 30-60 minutes. Staining was done in 4% giemsa in Sorensen’s buffer at pH 6.8.

iv) **Bloom (1982) (Modified):** Slides were treated with 0.5 \( \mu g/ml \) of Hoechst in Sorensen’s buffer at pH 7.0 for 10-15 minutes. Exposure to fluorescent light was for 30 minutes. Slides rinsed thoroughly in distilled water were airdried and stained in 4% Giemsa in phosphate buffer at pH 7.0.

v) **Kerkhoff and Gaag (1985):** Slides were treated with 5 \( \mu g/ml \) Hoechst in PBS (8 g NaCl; 0.2 g KCl; 1.44 g \( Na_2HPO_4 \cdot 2H_2O \) and 0.2 g \( KH_2PO_4 \) per litre distilled water) solution for 15 minutes. Slides were mounted in the same solution with coverslip and exposed to black light bulb (8W) for 10 minutes at room temperature. Slides rinsed in distilled water were treated with 5 N HCl solution for 15 minutes at room temperature, rinsed in distilled water, stained in 4% Giemsa in phosphate buffer at pH 6.8 for 15-20 minutes.

As described above a spectrum of treatment protocols were tried for developing a suitable method for the test species. On the basis of these, protocol variations tried with the different steps were as follows:

**BrdU administration:** Bromodeoxyuridine administrations tried were as given below. BrdU solution was prepared in double distilled water.
<table>
<thead>
<tr>
<th>Total dose (mg/g body weight)</th>
<th>Injection (Nos)</th>
<th>Duration (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mg</td>
<td>1</td>
<td>24, 48</td>
</tr>
<tr>
<td>1 mg</td>
<td>1</td>
<td>24, 48</td>
</tr>
<tr>
<td>1 mg</td>
<td>2</td>
<td>24, 48</td>
</tr>
<tr>
<td>2 mg</td>
<td>4</td>
<td>24</td>
</tr>
</tbody>
</table>

**Hoechst treatment variations:** Hoechst concentrations were 0.5, 1, 5, 50 μg/ml. Buffer used was Sorensen’s buffer (pH 6.5-7.0) and PBS. Exposure durations were 10, 15, 20 and 30 minutes.

**Light treatments and sources:**
- Sunlight: 30-60 minutes
- Fluorescent light (40W): 30-60 minutes
- U.V. germicidal tube (30w): 15-30 minutes
- U.V. black bulb (125 w): 5-15 minutes

**Post-treatment:**
- 2X SSC: 60 minutes at 60°C
- 5N HCl: 10-30 minutes

**Staining:** Giemsa 1-4% (pH 6.5-7.5)

**Mounting:**
In most cases slides faded a few days after mounting with DPX. Hence slides were screened without mounting.

**8. METHOD DEVELOPED FOR SCD/SCE IN THE PRESENT STUDY**

On the basis of the above protocols and trial experiments with the different steps described, the following fluorescence Plus giemsa (FPG) method was developed by incorporating the best results obtained in each step.
BrdU administration: 0.5 mg/g body weight (one injection) and an exposure of 24 hours.

Hoechst treatment: Hoechst (0.5 µg/ml) prepared in PBS solution (8g NaCl; 0.2g KCl; 1.44g Na₂HPo₄, 0.2 KH₂PO₄ per litre) was used. Slides were treated for 15-20 minutes.

Light treatment: Slides mounted in the above Hoechst solution and with coverslips were exposed to U.V. black bulb (125W) for 5-8 minutes at a vertical distance of about 8 cms.

Post treatment: Slides were rinsed in DDW, dried and treated with 5N HCl for 10-15 minutes.

Staining: Slides thoroughly rinsed in distilled water dried and stained in 4% Giemsa in phosphate buffer at pH ranging from 6.8-7.0.

Slides were processed on the same day the labelled chromosome spreads were prepared. Good results were obtained in 0-day old slides. All SCE experiments were conducted in subdued light.

9. CYTOGENETIC ENDPOINTS USED

The endpoints used in this study were chromosome aberrations (CA) micronuclei test (MN) and sister chromatid exchanges (SCE).

a) Chromosome aberrations

DNA damage consists of two broad categories like macrolesion and
microlesions. Macrolesions are the visible effects detectable through cytological analysis of chromosomes and microlesions refer to the invisible changes occurring at the nucleotide level.

Microlesions consist primarily of base pair substitutions or base pair addition/deletion changes. Base pair substitution mutations result from quantitative changes in nucleotide composition of a codon whereas base pair addition/deletion mutations result from the addition or deletion of one or a few nucleotide pairs from the nucleotide complement in a gene (Ames and Whitfield, 1966).

Macrolesions can be subdivided into changes in chromosome number (gain or loss of single chromosomes or sets of chromosomes) and changes in chromostructure (breaks, deletions, rearrangements...).

A schematic representation of DNA damage is given below:

 معدللا ملك

(Mutation)

(Microlesions)  (Macrolesions)

(Frameshift Mutation)  (Base-pair substitution mutation)  (Numerical changes)  (Structural changes)

- deletion
- rearrangement
- break

(after Brusick, 1978)
Classically CA involves structural changes classified according to their time of formation relative to the cell cycles. According to this the lesions induced in G phase of the cell cycle gives rise to chromosome type aberrations (in two chromatids at identical regions) and lesions induced in G₂ give rise to chromatid type aberrations (one chromatid only) when they are scored in M₁ (Brusick, 1978).

b) Micronuclei formation:

The micronuclei test is an in-vivo cytogenetic screening procedure for the detection of freshly induced structural chromosome aberrations and for revealing chromosome loss due to partial impairment of the spindle apparatus (Schmid, 1975). Micronuclei are small incomplete nuclei originated from chromatin material which lag during anaphase. In the course of subsequent division, this material is included in the cytoplasm of one of the daughter cells where it can either fuse with the main nucleus or form one or several secondary nuclei. These micronuclei represent acentric chromosome fragments or multicentrics connected by bridges. In case of spindle malformations they may consist of entire chromosomes.

c) Sister-chromatid exchanges:

Reciprocal exchange of parts between two sister-chromatids is called a sister-chromatid exchange (SCE). It is the number of exchanges between the differentially stained chromatids of a chromosome. The fluorescence plus Giemsa (FPG) technique as described earlier was
used for studying sister-chromatid exchanges. Figs. 1 and 2 show a schematic representation of BrdU incorporation and differential staining for visualising SCE.

10. BASELINE VALUE ESTIMATION

The spontaneous occurrence of CA, MN and SCE were studied using animals not deliberately exposed to any chemical. For chromosome aberrations, data was collected from 11 animals of *E. suratensis* and 8 animals of *E. maculatus*. For micronuclei studies erythrocytes from 10 specimens were screened. SCE baseline data was collected from 10 animals of *E. suratensis* and 8 animals of *E. maculatus*. The units of expression in the case of CA and SCE were chromosome aberrations or sister-chromatid exchanges per metaphase. In case of micronuclei the values were expressed as micronuclei per 1000 cells. Data from the cell populations of different animals in a particular treatment were pooled for assessing genotoxicity.

11. TREATMENTS

For treatments, sublethal doses were selected on the basis of tolerance limits of each chemical used. The maximum tolerated dose for 96 hours was considered as the highest dose in each case. Subsequent doses were fractions of the maximum dose. In each case 3 doses (lowest middle and highest) were tried. For both known mutagens viz., MMS and CP the doses were 50, 100, 150 /µg/g; 25, 50, 100 /µg/g body respectively. In the case of the two pesticides MP and PM, the doses were 0.05, 0.1,
0.2 ppm and 0.5, 0.1, 0.2 ppm respectively. The route of exposure was through intramuscular injection for the two known mutagens tested due to the risk factor involved in exposure through the medium, besides the prohibitive cost of large amounts of mutagens. The pesticides were administered through the water medium. In all cases 96 hours was the exposure duration.

The effects of the 3 different doses of the two mutagens and two pesticides in the present study were evaluated on the basis of data from different cell populations derived from each test animal in a particular treatment. 3-4 animals were used in each dose of treatment.

The exposure of the two test species and end points studied in both were as below:

**E. maculatus**

1. Methyl methane sulphonate (MMS)
   a) Chromosome aberrations (CA)
   b) Micronuclei (MN)

2. Cyclophosphamide (CP)
   a) Chromosome aberrations (CA)
   b) Micronuclei (MN)

**E. Suratensis**

1. Methyl methane sulphonate (MMS)
   a) Chromosome aberrations (CA)
   b) Micronuclei (MN)
In the case of *E. suratensis* all three cytogenetic endpoints CA, MN and SCE were studied after exposure to all the four chemicals. In the case of *E. maculatus*, SCE studies were confined only to the baseline values. Cytogenetic endpoints, CA and MN only were studied in *E. maculatus*, that too in response to MMS and CP only. Some experiments were conducted on endpoints with MP and PM also, but the chromosomes of *E. maculatus* appeared rather unsuitable for the studies. The chromosome complement of *E. maculatus* consists of even very small pairs thereby making it difficult to estimate aberrations, especially exchanges. This was noticeably so with MP and PM. Hence screening was confined to
MMS and CP only. In *E. maculatus* only the largest 4 pairs of chromosomes were considered.

The control animals in the case of the two known mutagens received intramuscular injections of the solvent (distilled water). In the case of two pesticides, animals maintained in well aerated water were used as control since the exposure was through the water medium.

a) Chromosome aberration:

For study of chromosome aberrations the animals received an intramuscular injection of 0.005% colchicine @ 1ml/100 g body weight during the last 2-2 1/2 hours of exposure. The colchicine treatment was therefore for 2-2 1/2 hours. Gills were removed, processed according to the standardised protocol and slides prepared. About 6 slides per animals were considered. A minimum of 40 well spread metaphases were evaluated. Coded slides were screened and data collected on chromosome aberrations like gaps, breaks, fragments, rings, exchanges, centromeric fusion, minute complex rearrangements, ploidy etc. The data from the cell population of each animal were pooled for each dose of the chemical and values expressed: aberrations/metaphase.

b) Micronuclei:

Blood was collected from the caudal fin region by a neat amputation and smeared on clean slides. 4-5 slides/animal were prepared and immediately fixed in absolute methanol for 10 minutes. Staining was
in May-Grunwald solution followed by Giemsa for 10 minutes. Slides were screened without mounting. 5000 cells per animal were screened for estimating the rate of MN formation which was expressed as MN per 1000 cells.

c) Sister-chromatid exchanges:

The animals were given an intramuscular injection of BrdU @ 5mg/g body weight in the last 24 hours, i.e., at 72 hours of exposure to the test chemical and colchicine injection about 2-2½ hours before their sacrifice at 96 hours. Chromosome preparations were as described earlier. All experiments involving SCD/SCE were conducted in subdued light. Only the second cells (SCD₂) were considered for screening and analysis. 10 to 30 SCE₂ plates were examined per animal wherever possible. The unit of expression was SCE per metaphase.

12. FIELD STUDY

Live specimens of *E. suratensis* were collected from known and suspected polluted spots like Thaneermukham, Thevara Ferry and Integrated Fisheries Project Jetty near Cochin. Blood samples collected from animals from Thaneermukham and Thevara Ferry were screened for micronuclei occurrence. The sensitive experiments for SCE studies could not be conducted in the field as subdued light conditions were not easily simulated. These technical problems placed restrictions on large scale field studies which were confined to one area (IFP Jetty) suspected to be polluted with oil.
13. DATA ANALYSIS:

Data from cell populations of different animals in a particular treatment were pooled for analysis. An equality of proportion test (Z-test) was used for statistical analysis of the results.