MATERIALS AND METHODS

Sampling sites for the hydrobiological study in Mirik Lake

Seven sampling sites were identified in Mirik Lake considering length of the lake as well as point and non-point sources of pollution. The water samples were collected every month at regular intervals from the seven sampling sites of the lake. The brief descriptions of the sites are as follows:

Site 1

Site 1 lies between $26^0 53' 08.49''$ N latitude and $88^0 11' 08.32''$ E longitude. It is 250 m away from DGHC Nursery toward North West (Fig. 11). This site is located at the point where wastes from hotels and residential area join into the lake. Car washing also takes place at this site.

Site 2

It lies between $26^0 53' 16.80''$ N latitude and $88^0 11' 14.95''$ E longitude. It is situated at the flank of the lake where tourists used to see the fishes which accumulate here to eat the food particles thrown by the visitors (Fig. 11). Wastes from hotels and residential area also join in this site.

Site 3

Site 3 lies between $26^0 53' .20''$ N latitude and $88^0 11' 01''$ E longitude. It is located at 100 m away from the concrete bridge of the lake towards South East. This site is situated near the proposed children park area. Human activity is comparatively lesser at this site (Fig. 11).

Site 4

Site 4 lies between $26^0 53' .017''$ N latitude and $88^0 10' .927''$ E longitude. It is located at the centre of concrete bridge over the lake (Fig. 11). Some amount of surface runoff joins here.

Site 5

This site lies between $26^0 53' 38.40''$ N latitude and $88^0 10' 55.11''$ E longitude. It is situated at 90 m away from the water intake point towards North East (Fig. 11). This water intake well is used for water treatment plant under PHED having a capacity of 1MGD (Million Gallon per Day) to cater the water supply for the people of Mirik Municipality area .

Site 6

Site 6 lies between $26^0 53' 27.99''$ N latitude and $88^0 10' 56.53''$ E longitude (Fig. 11). This site is located between Shibmandir and island where waste water coming mainly from Mirik Bazar area joining into the lake. Bathing and washing of clothes takes place at this site.

Site 7

Site 7 lies between $26^0 53' 34.53''$ N latitude and $88^0 10' 51.89''$ E longitude (Fig. 11). It is situated near the outlet of Mirik Lake which is joining to the Mechi River through weirs. Washing of clothes and bathing also takes place near this site.

Method of Sampling

Various physico-chemical and biological parameters of Mirik Lake were studied for two years from October, 2005 to September, 2007. The water samples were collected from seven selected sites of the lake, once in every month at regular interval. The collection of water samples from all the sites was done with the help of the paddle-boat. For the seasonal analysis, March to June were considered as summer, July to October were considered as monsoon and November to February were taken as winter.

The air and water temperature, pH and transparency were recorded in the field. DO were fixed at the field. For the other physico-chemical parameters water samples were collected in plastic bottles and transported in ice bags to the laboratory.The samples (except BOD) were stored in refrigerator. Samples for BOD tests were incubated for 5 days at 20^0 C in BOD incubator of the laboratory. The physico-chemical parameters like DO, free CO_2, total alkalinity (TA), total hardness (TH), chloride and BOD, were analyzed following standard methods (NEERI, 1984; Trivedy and Goel, 1986; APHA, 2005).

Fig. 11 Map showing the location of seven selected sampling sites of Mirik Lake, Darjeeling

Reagents for water analysis

Manganous sulphate solution

480 g of tetrahydrate manganous sulphate ($MnSO_4$, 4 H_2O) was dissolved in 1000 mL of distilled water. Then the solution was filtered through filter paper and kept in a reagent bottle.

Alkaline iodide azide reagent

500 g of NaOH and 150 g of KI was dissolved in distilled water and after cooling, it was diluted to 1000 mL with distilled water. Thus, alkaline iodide solution was prepared. Then 10 g of NaN_3 was dissolved in 40 mL of distilled water and added to the alkaline iodide solution.

Sodium thiosulphate ($Na_2S_2O_3$) solution, 0.025 N

24.82 g of $Na_2S_2O_3$, 5 H_2O was dissolved in boiled distilled water and diluted to 1000 mL which thus was 0.1 N stock solution. A pellet of NaOH was added to it as stabilizer. Then 250 mL of the stock $Na_2S_2O_3$ solution was diluted to 1000 mL with freshly boiled and cooled distilled water to prepare 0.025 N solution. 5 mL of chloroform was added to it as a preservative and was kept in a brown glass bottle.

Starch Indicator

1 g of soluble starch powder was dissolved in 100 mL of warm distilled water. It was prepared fresh each time.

Sodium hydroxide (NaOH) solution, 0.05 N

40 g of NaOH was dissolved in 1000 mL of boiled and cooled distilled water. 50 mL of the above solution was diluted to 1000 mL so that to have 0.05 N NaOH solution. The solution was standardized with HCl.

Phenolphthalein Indicator

0.5 g of phenolphthalein was dissolved in 50 mL of 95% ethanol and 50 mL of distilled water was added to it. Then 0.05 N NaOH solution was added dropwise until the solution turned to faintly pink colour.

Sulphuric acid, 0.02 N

Sulphuric acid (H_2SO_4) of 0.1 N was prepared by diluting 3 mL concentrated H_2SO_4 to 1000 mL distilled water. Then the solution was standardized against standard Na_2CO_3 0.1 N. Then appropriate volume of H_2SO_4 was diluted to 1000 mL to obtain standard 0.02 N H_2SO_4.

Methyl Orange Indicator

0.5 g methyl orange was dissolved in 100 mL of distilled water and kept in a reagent bottle.

EDTA solution, 0.01 M

3.723 g of disodium salt of EDTA was dissolved in distilled water and diluted to 1000 mL. Then the solution was stored in polythene bottle.

Ammonia buffer solution

Solution (a) 16.9 g of ammonium chloride (NH_4Cl) was dissolved in 143 mL of concentrated ammonium hydroxide (NH_4OH).

Solution (b) 1.179 g of disodium EDTA and 0.780 g of MgSO4, 7 H2O were dissolved in 50 mL of distilled water.

Then solution a) and solution b) were mixed and diluted to 250 mL with distilled water.

Erichrome Black T Indicator

0.40 g of Erichrome Black T was mixed with 100 g of NaCl and grinded.

Silver Nitrate, 0.0141 N

2.395 g of $AgNO_3$ was dissolved and diluted to 1000 mL with distilled water. Then it was standardized against NaCl, 0.014 N.

1 mL of 0.0141 N $AgNO_3$ =0.5 mg, Cl.

Then the solution was kept in dark glass bottle.

Potassium Chromate Indicator

5 g of potassium chromate (K_2CrO_4) was dissolved in 100 mL of distilled water.

Methods for water analysis

Temperature

The air and water temperature were measured by using a mercury filled Celsius thermometer (checked against a precision thermometer certified by National Institute of Standards and Technology: NIST) graduated up to 50^0 C with a precision of 0.1^0C. The water temperature was recorded by dipping the bulb in water. In each case the thermometer and the reading were recorded only after getting constant mercury in thermometer column.

Hydrogen Ion Concentration (pH)

The hydrogen ion concentration (pH) of water samples was recorded with the help of a portable pH meter (model - pHep, Hanna Instruments, Italy).

Conductivity

The conductivity of water samples was recorded with the help of a digital conductiviy meter (Systronics, model-215).

Dissolved Oxygen (DO)

The dissolved oxygen (DO) of water samples were estimated by the Azide modification of Winkler's Iodometric method. For this, the samples were collected in (100 mL.) glass stopper BOD bottles avoiding any kind of bubbling and air trapping of the air bubbles in the bottle after placing the stopper. The samples were then fixed at the site with 1 mL each of MnSO4 and alkali iodide azide reagent in 100 mL BOD bottles and later the precipitate formed was dissolved with 1 mL of Conc. H_2SO_4 in the laboratory and titrated against 0.025 N Sodium thiosulphate solution using starch as an Indicator until the blue colour disappeared. The DO was calculated by the following formula:

$$DO, mgL^{-1} = \frac{(V \times N) \times 8 \times 1000}{\text{Vol. (mL.) of sample}}$$

Where, V=Vol.(mL.) of sodium thiosulphate used

N= Normality of sodium thiosulphate

8 = Equivalent weight of the oxygen.

Free Carbon dioxide (Free CO_2)

Free CO_2 was determined by titrimetric method using phenolphthalein indicator. 100 mL of sample water was taken in a conical flask and a few drops of phenolphthalein indicator were added to it. The sample water did not turn into pink and remained clear which indicated the presence of free CO_2. Then the titration of sample water was done with 0.05 N NaOH until appearance of pink colour. The used volume of NaOH was noted and the final calculation was made as follows:

$$\text{Free } CO_2, \text{ mg } L^{-1} = \frac{(V \times N) \text{ mL. of NaOH} \times 1000 \times 44}{\text{Vol. (mL.) of sample water}}$$

Where, V=Vol. (mL) of NaOH

N= Normality of NaOH

Total alkalinity

The total alkalinity was measured by titration, method. 50 mL. of sample water was taken in a conical flask and a few drops of phenolphthalein indicator were added to it, the sample did not turn pink. Then, 2 to 3 drops of methyl orange indicator was added to the same sample and the titration was done with 0.02 N H_2SO_4 until the Yellow colour changed to pink. The volume used was recorded and the total alkalinity was calculated as:

$$\text{Total alkalinity, mg } L^{-1} = \frac{(V \times N) \text{ of titrant} \times 1000 \times 50}{\text{Vol. (mL) of sample}}$$

Where, V= mL. of H_2SO_4 used for titration

N= Normality of H_2SO_4

Total hardness

Total hardness of sample water was determined by EDTA titrimetric method. For this, 50 mL. of sample water was taken in a conical flask and then 1 mL. of buffer solution was added to it. Then, about 100 mg of Erichrome Black T indicator was added and the solution was titrated with EDTA (0.01 M) until the wine red colour changed to blue. Then the Total hardness was calculated as:

$$\text{Total hardness, } mgL^{-1} \text{ as } CaCO3 = \frac{\text{used volume of EDTA (mL.)} \times 1000}{\text{Vol. of water sample (mL.)}}$$

Chloride

The Chloride of water sample was determined by Argentometric method. 50 mL. of water sample was taken in a conical flask and 2 mL.of K_2CrO_4 solution was added to it. Then the solution was titrated against 0.0141 N $AgNO_3$ until a persistent red tinge appeared. The volume of titrant was noted and the final calculation was done as follows:

$$\text{Chloride, } mgL^{-1} = \frac{(V \times N) \text{ of } AgNO_3 \times 1000 \times 35.5}{\text{Volume of sample (mL.)}}$$

Where, V= mL. of AgNO3 used for titration

N= Normality of $AgNO_3$

Biological Oxygen Demand (BOD)

Biological Oxygen Demand (BOD) was estimated by azide modification of Winkler's Iodometric method (APHA, 2005) measuring between the initial and incubated water samples for five days at 20^0 in a BOD incubator. The calculation of BOD was done as follows:

$$BOD, mgL^{-1} = (D_0-D_5)$$

Where, D_0 = Initial dissolved Oxygen in the sample.

D_5 = Dissolved Oxygen after 5 days of incubation.

Statistical Analysis

Standard deviation, correlation coefficient were calculated by using Microsoft excel statistical function of computer software. The significance of correlation coefficient was tested by applying t- test. It was done manually. Two way ANOVA was used to test the significant and insignificant difference among sites and seasons and it was done by using Microsoft excel statistical function of computer software.

Qualitative and Quantitative Estimation of Plankton

Phytoplankton

Reagents for phytoplankton analysis

Lugol's Iodine solution

10 g of neutral potassium iodide (analytical quality) was dissolved in 20 mL of distilled water and 5 g of twice sublimed iodine was added to it. After preparation of the solution, 50 mL. of water and 5 g sodium acetate was added to it. Then the solution was stored in a 100 mL. narrow necked flask made of neutral glass with ground glass stopper which fits well.

Methods for phytoplankton analysis

Phytoplankton samples were collected without filtering the surface water. To preserve, 0.3 mL. Lugol's Iodine solution was added to 100 mL. sample. In the Laboratory, water samples were centrifuged at 1500 r.p.m. for 15 minutes and then studied under microscope (Olympus CK2, Japan). Identification of phytoplankton was done using Standard books (Desikachary, 1959; Edmondson, 1959; Ward and Whipple, 1966; Prescott, 1978 and APHA, 2005).Photographs taken by digital compact camera (Olympus SP-350).

Density

The Lacky Drop (microtransect) method (Lacky,1938) is a simple method for obtaining counts of considerable accuracy. Number of organisms per milliliter was calculated as follows:

$$\text{No. mL-I} = \frac{C \times A_t}{A_s \times S \times V}$$

Where,

C= Number of organisms counted

At= Area of cover slip, mm^2

As= Area of one strip, mm^2

S= Number of strips counted

V= Vol. of sample under the cover slips, mL.

Zoooplankton

Reagents for Zooplankton analysis

4% Formalin solution

Methods for zooplankton analysis

Zooplankton samples were collected by filtering the surface water through plankton net (conical tow net made of bolting silk) and preserved in 4% formalin solution immediately after collection of sample.

The identification was done by referring the keys (Ward and Whipple, 1966; Pennak, 1978; Battish, 1992; and APHA, 2005).

Density

In laboratory, zooplankton samples were concentrated by centrifugation at 1500 r.p.m. for 15 minutes. Sedgwick- Rafter (S-R) cell was used as a device for enumeration of zooplankton under microscope. The plankters were identified up to genera level or wherever possible upto species level, and were reported as number per cubic meter. The density was calculated as follows:

$$\text{No. } m^{-3} = \frac{C \times V^{I}}{V^{II} \times V^{III}}$$

Where,

C= Number of organisms counted

V^{I}= Volume of concentrated samples, mL

V^{II} = Volume counted, mL and

V^{III} = Volume of grab sample, m^3.

Biological indices

The indices, used on aquatic biota of Mirik Lake for drawing conclusions are delineated hereunder.

Shannon Weiner Diversity index

The most commonly accepted index in past few decades has been Shannon and Wiener equation which is based on both the number of taxon present and the relative abundance of each taxon. The Shanon Weiner Diversity index is usually used to calculate species diversity but comparisons are also made using the different taxonomic levels (Hellawell, 1978). This index is a useful measure of community diversity.

$$H = - \sum (ni/N) \log_2 (ni/N)$$

Where,

ni = number of individuals of each species in the sample.

N = total number of individual of each species in the sample

Similarity Index

Similarity index approaches to asses association based on presence, absence or binry data. When species of stations A and B are compared on the basis of their presence or absence in samples from different locations, similarity index (S) of Sorenson (1948) can be applied as:

$$S = 2c/ a+b$$

Where,

a = no. of species in community A

b = no. of species in community B

c = no. of species common to both communities

A value of 1.0 is obtained when all taxa occur at both the stations, and zero is the value, if no taxa are common amongst the two stations.

Habitat Index

Habitat is the particular environment in which a plant or animal species naturally lives or grows. It is characterized by a relative uniformity of the physical environment and fairly close interaction of all the biological species involved. Preference and availability, the key characteristics for evaluating habitat of a plant or animal in a natural ecosystem, are reflected through density and frequency of occurrence. In order to evaluate characteristics of recorded aquatic biota from an aquatic ecosystem, the data were analyzed in detail for calculating habitat index. The density measurements may over emphasize the importance of a species that consists of many small individuals; and the frequency measurements may over emphasize the importance of distribution of individuals belonging to a particular species in the biota sampled, regardless of the size or number of those individuals. The mean of these two aspects was denoted as habitat index. The steps to determine habitat index was followed as described by Ghosh, 2002.

Density = number of species A per unit area

Frequency = number of sampling points where species A occurred / number of sampling points

Relative density = (Density of species A × 100) / total density of all species

Relative frequency = (Frequency value of species A × 100) / total frequency value of all species

Habitat index = (relative density + relative frequency) / 2

Palmer Pollution Index

Palmer Pollution Index employs taxa of algae (Palmer, 1969). The most tolerant genera and species were given ratings which are used to calculate the index. An alga is recorded as 'present', if there are more than 50 individuals / mL. The sum of the ratings of the algae present was then calculated for evaluating the quality of water.

Bacteriological analysis

Reagents for bacteriological analysis

MPN of Total Coliform

To determine total coliform load in the lake water, multiple tube fermentation technique was followed.

Preparation of Media

i) 40 g of dehydrated Mac Conkey broth was dissolved in 1000 mL distilled water to obtain single- strength presumptive medium.

ii) Then 10 mL. of broth was dispensed in each culture tubes containing an inverted Durham tube and after that the culture tubes were capped.

iii) Then the culture tubes were sterilized in an autoclave at 115^0 C for 15 minutes.

iv) When the sterilized tubes came to room temperature these were stored in refrigerator.

Preparation of dilution water

A special buffered, sterilized water was used to make sample dilutions for inoculation into the culture medium. It was prepared from a concentrated stock solution of phosphate buffer. To make the stock solution , 34 g. of potassium dihydrogen phosphate (KH_2PO_4) was dissolved in 500 mL. of distilled water and pH was adjusted to 7.2 with 1 N NaOH. Then the solution was diluted with distilled water to make up to 1 litre. Then it was stored in a tightly closed bottle in refrigerator to prevent microbial growth.

At the time of use 1.25 mL. of stock phosphate solution and 5 mL. magnesium chloride solution (38 $MgCl_2$ /1 distilled water) was added to 1 litre distilled water and dispensed into bottles for sterilization in autoclave. Before sterilization, stoppers of the bottles were loosen. Then the solution was sterilized for 15 minutes at 121^0 C. After sterilization, stoppers were tightened and the dilution water was stored in a clean place until needed.

Procedure for presumptive test

1) All the water samples were shaken vigorously immediately before removing sample aliquots to inoculate the series of test tubes.

2) 0.1, 0.01 and 0.001 mL. dilutions of sample water were selected according to the expected bacterial count.

3) Samples were added using sterilized pipettes to the test tubes selected for the test and mix thoroughly. Separate pipettes were used for different samples as well as for dilutions.

4) All the tubes were placed in an incubator at 37^0 C within 30 minutes.

5) Each tube was examined for the presence of gas after 24 hours of incubation. Those tubes showing gas in Durham's vial (tube) were recorded as positive (+). Gas in any quantity even a tiny bubble was recorded as positive (+). Then the negative tubes were reincubated for a further 24 hour period. At the end of that period the tubes were again checked for gas production. Gas production at the end of either 24 or 48 hours of incubation was presumed to be due to the presence of coliforms in the sample. The tubes showing positive test were immediately subjected to confirmatory test.

Procedure for confirmatory test

1) The fermentation tubes for confirmatory test were prepared with 10 mL. BGBB (Brilliant Green Lactose Bile Broth) medium and inverted Durham's vials and then sterilized in autoclave. The number of tubes that was prepared was equal to all positive tubes of the presumptive test.

2) The fermentation tubes with positive results were shaken gently and one loopful of medium was transferred to BGBB broth.

3) Then the tubes were incubated at 37^0 C for 48 hours and the tubes with gas formation were recorded as positive.

MPN Method for fecal coliform

The presumptive test was same as for total coliforms but the confirmatory test was carried out as follows:

The confirmatory test for fecal coliforms were carried out exactly as the test for total coliforms but EC medium was taken instead of BGBB medium and then the tubes were incubated at 44.5^0 C within 30 minutes after inoculation. The tubes were removed after 24 hours and were gently observed for gas production.

Calculation of most probable numbers

The calculation of MPN of coliforms was done by combination of positive and negative results in the multiple tube tests. The values were calculated for any of the combinations given at MPN Table. (APHA, 2005; Trivedi and Goel, 1986).

Heterotrophic Plate Count (HPC)

Procedure

1) Three dilutions (0.1, 0.01 and 0.001 mL.) of sample water were selected according to expected HPC.

2) Then the undiluted and diluted samples were transferred in aseptic conditions to already marked sterilized Petri plates. After delivering the sample the tip of the pipette was touched to a dry spot in the plates.

3) After that, 12-15 mL liquefied nutrient agar medium (44^0-46^0 C) was gently poured to these petri plates by slightly opening the plates pouring the medium gently. Then the medium was thoroughly mixed with the sample in Petri plates. When the media was solidified, the plates were inverted and kept for incubation at 37^0 C for 48 hours.

Then the colonies were counted after the incubation period and the results were recorded and HPC/mL of water was calculated.

Plate 6

Explanation of figures

Fig. 12 shows the researcher recording the pH of water of Mirik Lake with the help of portable pH meter.

Fig. 13 shows the sampling of zooplankton at Mirik Lake with the help of plankton net

Fig. 14 shows the microscopic analysis of plankton at NEERI

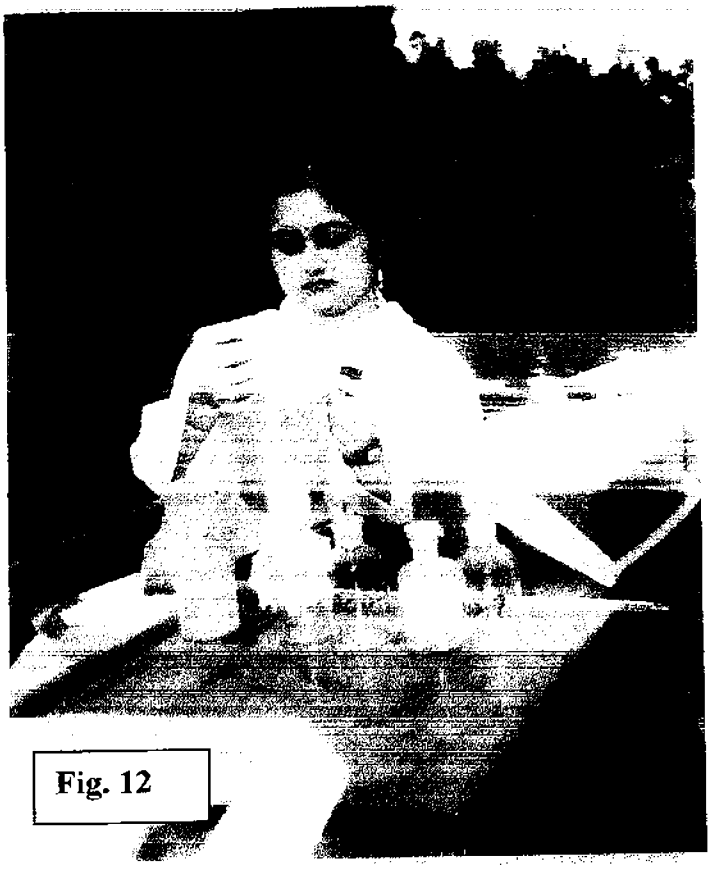

Fig. 12

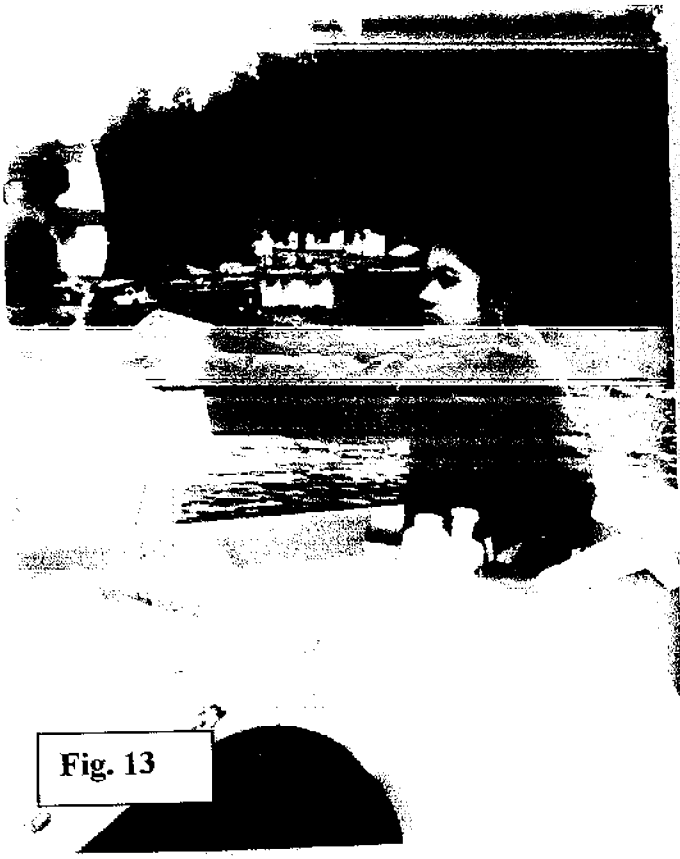

Fig. 13

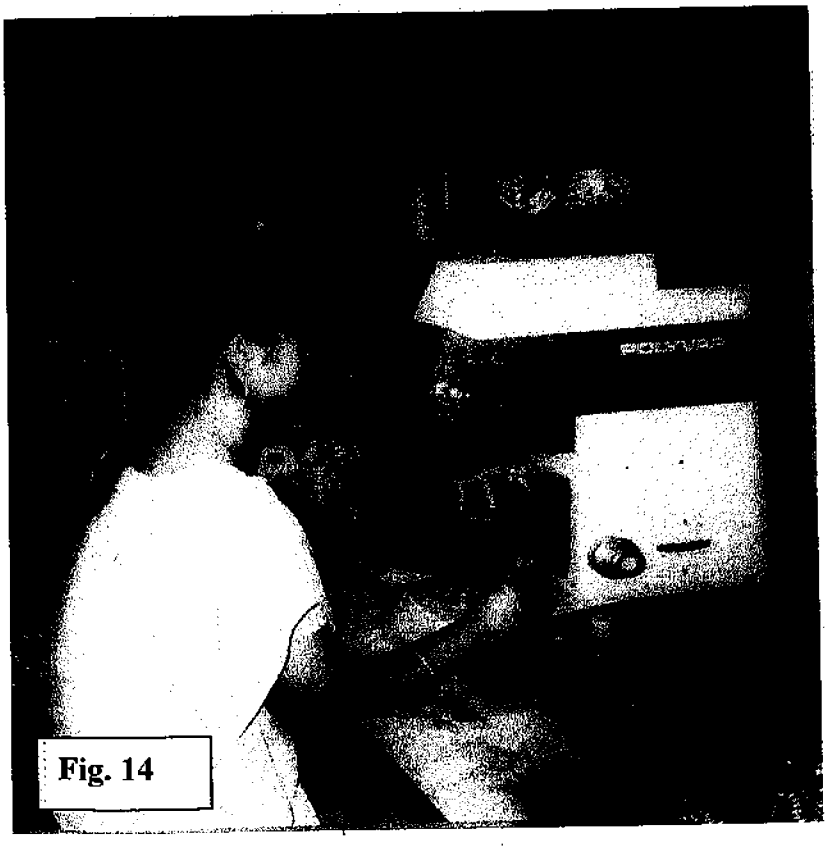

Fig. 14