MATERIAL AND METHODS

III. MATERIAL AND METHODS

3.1 INTRODUCTION

Quantitative assessment of the effect of pollutants has got cardinal importance in any pollution research, both from the biological and ecological point of view. Efforts were made to evaluate the lethal and sublethal effects of pesticides and petroleum hydrocarbons individually and in combination on two selected non-target invertebrates. Both the animals used for the study were bivalves, one of them the brown mussel Perna indica is a representative of the marine environment and the other, the black clam, Villorita cyprinoides var. cochinensis has an estuarine distribution.

3.2 TEST ANIMALS

3.2.1 *PERNA INDICA*

Perna indica (Kuriakose and Nair, 1976) the common brown mussel, extensively distributed along the intertidal and subtidal rocky beaches of the South West coast of India was used for the present study. These marine bivalves attain a maximum length of 120 mm (from umbo to the posterior tip) and animals with a size range of 25 - 30 mm are abundant during October to February. Extensive beds of these animals are available on the rocks which are laid to construct a wave breaker at the mouth of the Ashtamudi Lake at Sakthikulangara, 8 km north of Quilon ($8^{o}56'$ N- $76^{o}35'$E). Using sharp chisel these animals were detached, cleaned and transported to the laboratory in large polyethylene drums of 50 l capacity with sea water from the site of collection. (See Map.)

3.2.2 *VILLORITA CYPRINOIDES VAR. COCHINENSIS*

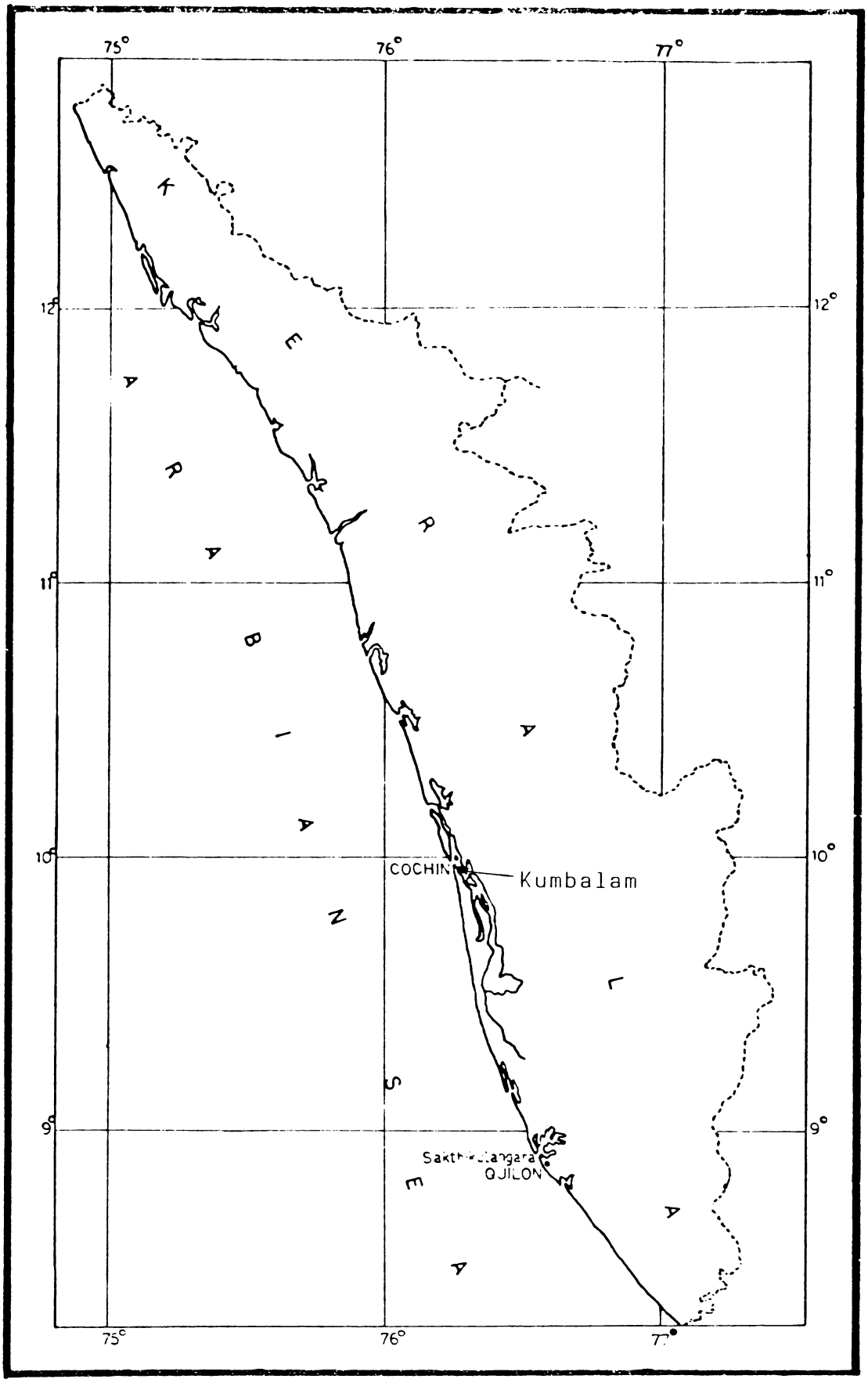
Villorita cyprinoides var. cochinensis (Hanley), popularly known as black clam, is a hardy bivalve abundantly distributed along the mesohaline and oligohaline stretches of the Cochin backwater. Though these animals are capable of tolerating wide fluctuations in salinity (Sivankutty Nair and Shynamma, 1975), the density of population had been found to decrease when the salinity exceeds ca. 20‰.

Animals were collected from the Cochin backwaters (See Map) near Kumbalam ($9^{o}53'$ W-$76^{o}17'$E) where the salinity ranges from fresh water during monsoon periods (June-August) to more or less 20‰ during high tides of peak summer (March-May). Collections for the studies were mainly made during post monsoon to pre monsoon months (September-May) when the salinity of water at the collection site ranged from 10‰ to 20‰. Individuals of V. cyprinoides var. cochinensis of the size range of 20-25 mm were collected in plastic buckets with water from the collection site and brought to the laboratory.

3.3 LABORATORY PROCEDURES

3.3.1 LABORATORY CONDITIONING OF TEST ANIMALS

The animals brought to the laboratory were maintained in polyethylene tubs of 50 l capacity with well aerated sea-water of salinity ca. 32‰ and 15‰ respectively for P. indica and V. cyprinoides var. cochinensis at room temperature ($28^{o}C \pm 1.5^{o}C$). In the case of V. cyprinoides var. cochinensis, care was taken to bring about a gradual change in salinity of water in the conditioning tank when the salinity of water in the locality of collection



Map showing the locations of collection of Perna indica (Sakthikulangara) and Villorita cyprinoides var. cochinensis (Kumbalam)

ranged widely from 15‰. The period of conditioning lasted from 36 to 48 h, during which time they were fed with the algae Synechocystis salina. The water in the holding tank was changed periodically. All organisms used for any one set of experiment belonged to one population. Only healthy and active animals of the same size were used for the experiments.

The seawater used for the experiments was collected from Arabian Sea, off Cochin. Black polyethylene carboys of 50 l capacity were used to bring the collected water. Before being employed for the experiment, the seawater was filtered through a fibre glass filter (length 32 cm, breadth 16 cm), containing glass wool and activated charcoal, using a 0.15 H.P. pump and stored in the dark in fibre glass tanks of 200 l capacity, upto 15 days. Salinity adjustments were done by diluting with de-ionised water, whenever necessary. The pH of the experimental water was 8.2 $\pm$ 0.1. All the experiments were carried out at laboratory temperature ($28.0^{o}C \pm 1.5^{o}C$)

3.3.2 TOXICANTS

The toxicants used for the study were four pesticides and petroleum hydrocarbon (PHC) derived from the Water Accommodated Fractions (WAF) of oils. The four pesticides used were Ekalux, Dimecron, Aldrex and DDT, the former two belonging to organophosphates and the latter two representing organochlorines. Both these groups are being used extensively for the control of pests in the agricultural fields. The PHCs used were derived from the Water Accommodated Fractions of Light Diesel Oil - LDO (WAF) - and Persian Gulf Crude Oil-PG Crude (WAF).

The toxicant solutions were prepared separately and were added to the test media to get respective pesticide, PHC or Pesticide-PHC concentrations.

The pesticide concentrations were prepared by mixing the commercial grade pesticide with acetone in 1:1 ratio. For getting the sublethal concentrations the initial stock solution with acetone was further diluted with distilled water.

3.3.2.1 Ekalux^{R} EC 25

Ekalux^{R} EC 25 was supplied by Sandoz India Ltd. It is a wide spectrum emulsifiable concentrate containing 25% (W/W) of the organophosphate (o.p) active ingredient 'Quinalphos' (o,o diethyl - o - quinosxalinyl (2) - thionophosphate) and 75% (W/W) of stabilizers, emulsifers and adjuvants. A suspension of this pesticide was prepared with acetone in 1:1 ratio and added to the test solution in the required concentration. Ekalux was used individually and in combination with LDO (WAF). The stock solution was prepared afresh for each experiment.

3.3.2.2 Dimecron^{R}

Dimecron^{R} is the commercial product of Hindustan Ciba Geigy Ltd. It is a systemic water soluble pesticide based on 'phosphomidon'

(C_{10} H_{19} Cl N O_5)

The procedure followed for the preparation of the test solution was similar to that followed for Ekalux. The effect of this pesticide also was studied individually and in combination with LDO (WAF).

3.3.2.3 Aldrex^{R} 30

Aldrex^{R} 30 is an organochlorine pesticide (o.c.s.) and was manufactured by National Organic Chemical Industries Ltd. It is based on Aldrin and

contain 300gms, Hexachloro Hexahydro Dimethano Naphthalene (HHDN) per Kilogram. Though it is a soil insecticide, it can also be used for foliar application in agricultural fields. Lethal as well as sublethal effects of this pesticide was studied individually and in combination with LDO (WAF).

3.3.2.4 DDT^R 25 EC

DDT^R 25 EC (Dichloro Diphenyl Trichloroethane) another representative of o.c.s. studied is an emulsion concentrate containing 25% (W/W) DDT technical. It is marketed by Premier Pesticides (P) Ltd. and is widely used in municipal drainages for the control of mosquitoes. Experiments were carried out to evaluate its individual and combined effect with LDO (WAF).

3.3.2.5 Water Accommodated Fractions of Oils

LDO and P.G. Crude supplied by the Cochin Refineries Ltd. (CRL) of Indian Oil Corporation (IOC) were brought to the laboratory in carboys of 20 l capacity and kept in the dark. The oil obtained once were used for only a month and after that fresh supplies were obtained.

The water accommodated fractions (WAFs) were prepared daily by continuously stirring a mixture of the oil and sea water of the required salinity at a ratio of 1:20 for periods upto 14 hours, using a vortex mixer. A round perspex container of 20 l capacity, fitted with an outlet at the bottom was used for this purpose. After allowing to settle the mixture for ca. 10 minutes, the aqueous fraction was drained out leaving the supernatant scum in the container. The resultant emulsion of the aqueous fraction was transferred into a thoroughly cleaned separatory funnel of 2 l capacity, for further separation. After ca. 2 h the aqueous fraction from this was collected into clean beakers of 5 l capacity and this was considered 100% WAF

of the respective oil. The dosing of the WAFs were in ppm (PHC) basis. The PHC concentration of the WAF was estimated as follows:

125 µl of the respective WAF was dissolved in 100 ml n-hexane (Spectroscopic grade) to obtain a 1000 ppm stock solution and this was filtered using a Whatman No.42 filter paper to remove the insoluble materials (Levy, 1972) especially in the case of crude oil. From this stock solution 1 ppm, 5 ppm, 10 ppm, 20 ppm, and 30 ppm of the respective oils were made by appropriately diluting with 100 ml of n-hexane. These standards were scanned over the range of 200 - 300 nm, with a spectrophotometer (Hitachi 200-20-UV-Vis)and the absorption spectra of the standards relative to n-hexane were obtained. The absorbance of the above standard solution at 225 nm (λmax), were noticed and from these readings, standard plots indicating the PHC concentrations of both the WAFs were found out.

To find out the concentration of PHC in the WAF, 50 ml of the 100% WAF prepared was taken in a hexane cleaned, dry beaker, and its pH brought down below 2 by acidifying with 1 ml of concentrated HCl. After this it is taken in a hexane cleaned separatory funnel of 100 ml capacity and extracted with about 15 ml of spectroscopic grade n-hexane, by thoroughly shaking for 2 minutes. Then the separatory funnel was allowed to remain undisturbed till the hexane fraction was completely separated. The supernatant was separated and this process of extraction was repeated twice. The combined hexane extract was passed through a column of anhydrous sodium sulphate (Gupta et al., 1980) to free it from residual water and later made up into 50 ml in a hexane cleaned, dry standard flask. The absorbance of the hexane extract was measured at 225 nm and the concentration of the PHC in the respective WAFs were computed from the standard curve.

Once the concentration of the 100% WAF's of the respective PHCs were known, required volumes to be added to the test media to obtain the desired concentrations were calculated out. The addition of WAF to the test media did not produce any significant variation in the quality of the media. The WAF to be added, was prepared daily. Variations in the PHC concentrations were observed in the 100% WAF of the P.G. Crude. PHC concentration in the 100% WAF of LDO did not vary much. However, the PHC concentration in both the WAFs were estimated every day before its application.

3.3.3 TOXICITY STUDIES

3.3.3.1 Lethal Toxicity Studies

Lethal toxicity studies give information about the relative lethality of a toxicant. This test is designed to determine the highest concentration of a pollutant that is sufficient to affect some percentage, usually 50% of a limited number of test organisms. Though this appears to be a crude method of measurement of toxic response, its importance was highlighted by many workers (Duke 1974; Buikema Jr. et al., 1982). With all its imperfections, lethal toxicity studies were considered ecologically significant, most scientifically and legally defensible, modest in predictive capacity, simple and less expensive.

3.3.3.1.1 Lethal toxicity of individual toxicants

Experiments were carried out to assess the individual lethal toxic responses to four pesticides, Ekalux, Dimecron, Aldrex and DDT and PHCs derived from LDO (WAF) and P.G. Crude (WAF) by both the bivalves, the brown

mussel, P. indica and black clam, V. cyprinoides var. cochinensis. Laboratory conditioned mussels and clams of uniform size (mussels 28-30 mm; clams 20-24 mm) were exposed to 5 l of test solution that contain graded, logarithmic series of concentrations of the toxicants. Exposure to pesticides were carried out in glass troughs (15 x 30 cm) while fibre glass troughs of the same size were used for exposing to WAFs. Ten animals were used for each test concentration of the toxicant. The experimental vessels were kept closed with a perpex sheet. The experiments were carried out at laboratory temperature ($28^{o}C \pm 1.5^{o}C$) and the animals were not fed during the duration of experiment. Duplicates and controls were run for all the experiments. The test media were replenished totally every 24 h. The animals were inspected every 12 h and were considered dead, if the valves gaped beyond 5 mm and showed no movement even under mechanical stimulation. The dead animals were removed and the cumulative percentage mortality at every 12 h recorded. The LC 50 values and their 95% confidence limits were calculated using probit analysis, (Finney, 1971).

3.3.3.1.2 Combined toxicity of toxicant mixtures

After assessing the toxic responses to the individual toxicants, efforts were made to study the combined toxic effects of a mixture of toxicants. The concentrations employed for the combination studies were derived from the respective 96 h LC50 of the individual toxicants. For studying the combined effect, the concentration of one toxicant employed (Toxicant 'A') was kept constant while that of the other (Toxicant 'B') varied. Usually 25 (5x5) combinations were tested to complete one set of experiments on combined toxicity of toxicants. All procedures observed for the study of individual toxicity were followed here.

The toxic effects of Ekalux, Dimecron, Aldrex and DDT in combination with LDO (WAF) on P. indica and in the case of V. cyprinoides var. cochinensis, toxic effects of Ekalux and Aldrex with LDO (WAF) were studied. The LC50 levels and their 95% confidence limits were found out and the additive indices were assessed following the methods of Marking and Dawson (1975).

3.3.3.2 Short-Term Sublethal Toxicity Studies

The objective of these toxicity tests were to find out the concentrations of the toxicants capable of inducing abnormal responses on the activities of the organisms. The activities studied were rate of oxygen consumption and filtration. In the case of P. indica byssogenesis was also used as an index of sublethal response.

The concentrations of different toxicants employed for the studies were derived based on the recorded 96 h LC50 values for individual toxicants. Normally 1/10 of the individual 96 h LC50 values were taken as the highest concentration along with four other concentrations fixed in the descending order. For studying the combined sublethal effect, two unvarying concentrations of one toxicant were combined with five varying concentrations of the other toxicant and concentrations that did not produce any lethality during 96 h study were selected for this study. The duration of these experiments never exceeded a maximum of 10 h. Animals used for this studies were pre-exposed to different sublethal concentrations for 2 h and 48 h to pesticides and petroleum hydrocarbons respectively. The test solutions were not aerated.

3.3.3.2.1 Rate of oxygen consumption

Mussels and clams pre-exposed to the toxicants for a period of 2 h in the case of pesticides and 48 h in the case of oil were used for this study . Experiments were conducted in conical flasks (2 l) with 2 l of test solution, each with five animals. Test solution was sealed with inert liquid paraffin (Burgoyne^{R}) to prevent gaseous exchange with the atmosphere. Duration of the experiment was 8 h and the reduction in the oxygen content was determined every 2 h by siphoning out about 30 ml of the test solution with a flexible polyethylene tube. Quantity of oxygen in the test solution was determined by Winkler's method. After the experiment, soft tissues of the animals were cleaned with distilled water, removed to a previously weighed aluminium foil, dried at 70^{o}C for 48 h and dried tissue weight taken to constancy. The results are expressed as μg oxygen h^{-1} mg^{-1} dry wt of the animal.

3.3.3.2.2 Rate of filtration

Dye clearance technique was employed to assess the rate of filtration (Abel, 1976). Animals were pre-exposed for 2 h to 2 ppm neutral red dye (A.R. Koch-Light Lab, England) along with the respective toxicants. This was done to minimize a possible initial shoot up of filtration that could be induced by the dye particle. Two animals each were introduced in glass beakers (1 litre) with 500 ml of test solution, containing 2 ppm of neutral red. Five sublethal concentrations and a control (in quadruplets) were employed. Reduction in dye concentration was recorded at 1 h interval by withdrawing 10 ml of the test solution. The experiments were terminated after 2 h. Filtration was estimated using Abel's equation (Abel, 1976). After the experiments, the soft tissues of the animals were cleaned with distilled water, removed

to a previously weighed aluminium foil and dried at $70^{o}C$ for 48 h and weighed to constancy. The results are expressed as volume of sea water (in ml) filtered h^{-1} mg^{-1} dry tissue weight of the animal.

3.3.3.2.3 Rate of byssogenesis

Five mussels each, were pre-exposed to five sublethal concentrations of pesticides and oil for 2 h and 48 h respectively. Such pre-exposed animals were allowed to produce byssus thread under the stress of pesticides and oil for a period of 10 h. Five sublethal concentration of pesticides and oil were used to assess the capacity of byssus production. After the experiment, the test solutions in the beaker was siphoned out carefully and the number of byssus threads produced by each individual were counted using a biconvex lens. Byssus threads with adhesive discs at the tip were only considered. The rate of byssus production is expressed as number of threads produced $individual^{-1}$ 10 h^{-1}.

3.3.3.3 Long-Term Sublethal Toxicity Studies

A study on the toxicity after prolonged exposure of the test organism to toxicant is a recent development in pollution experimentation.

30 mussels of uniform size were exposed to three very low concentrations (1/100 of the 96 h LC50 values were the highest) of the pesticides together with a control group. Polythene tubs of 35 l capacity containing 10 l of the toxicant solution were used for the exposure. The test media were changed daily with fresh media and the animals were fed with the algae, Synechocystis salina for 30 minutes before changing the media. The period of exposure to toxicant solution lasted for 14 days and subsequently they were

transferred to toxicant free, raw sea water for a period of 7 days. After 7 and 14 days during the period of toxicant exposure and after 7 days of exposure to toxicant free sea water 9 animals were withdrawn from each tub and used for the study of oxygen consumption and filtration.

Long-term studies involving PHCs were also conducted with mussels. For this, 100 mussels were exposed to 3 different sublethal concentrations of the respective PHCs (1/10 of the 96 h LC50 values were the highest) and a control. All the experiments were done in triplicates. Polythene tubs with 30 l of the test solution were used for this study. Test solution was replenished daily and the animals were fed with the algae Synechocystis salina for 30 minutes before replenishing the test media. After 21 days of continuous exposure the animals were transferred to toxicant free raw sea water for a period of 7 days. 20 animals were withdrawn from each tub after 7, 14 and 21 days during the period of exposure to the toxicant and at the end of the 7th day after transferring to raw sea water and these animals were pooled and used for the study of oxygen consumption, filtration and accumulation/depuration.

3.3.3.3.1 Rate of oxygen consumption during different duration of long-term exposure

The mussels removed from the toxic medium at different intervals of exposure were used for the study. Experiments were conducted in conical flasks (2 l) with 2 l of test solution each with 5 animals. 3 sublethal concentrations and a control were used in duplicate. All other procedures were the same as stated in section 3.3.3.2.1.

3.3.3.3.2 Rate of filtration during different duration of long-term exposure

Individuals of mussels removed from the toxic medium at different intervals of exposure and those transferred to raw sea water were used to asses the rate of filtration. Experiments were conducted with two animals in glass beaker (1 litre) containing 500 ml of test solution. 3 sublethal concentrations and a control were employed, in duplicate. The rest of the procedures were same as described in section 3.3.3.2.2.

3.3.4 ACCUMULATION AND DEPURATION STUDIES

Mussels have the remarkable ability to accumulate petroleum hydrocarbons (PHC) in their tissue. Bioaccumulation by an organism is one of the major factors that plays a crucial role in the biomagnification of a pollutant in the trophic system. Hence efforts were made to assess the rate of accumulation and depuration of the PHCs by the mussels.

40 mussels pre-exposed to different PHC concentrations were withdrawn at different intervals of accumulation/depuration as the case may be (0,7,14 and 21st day of accumulation and 7 days after transferring to raw sea water) and pooled into 4 sets each with 10 animals. The soft tissues of these animals were shucked out, cleaned in distilled water, drained, blotted, weighed in a preweighed aluminium foil, wrapped and preserved at $-20^{o}C$ for the analysis of tissue load.

3.3.4.1 Analysis of Petroleum Hydrocarbons

Petroleum hydrocarbons in the whole tissue of mussels were analysed by steam distillation using U.V. spectrophotometry (Donkin and Evans, 1984; Neff and Anderson, 1975).

3.3.4.1.1 Reagents

1. n-hexane (Spectroscopic grade)
2. Concentrated hydrochloric acid
3. Sodium hydroxide (G.R)
4. Silica gel (60-120 mesh, chromatographic grade)
5. Alumina
6. Sodium sulphate (Anhydrous)

3.3.4.1.2 Sample digestion and preparation

About 5 g of the soft tissues of the mussels preserved at-20^{o}C were homogenized well, with a mortar and pestle. Before use, the mortar and pestle were kept in a freezer to maintain a very low temperature preferably 0^{o}C, during homogenization.

The homogenized samples were transferred to a round bottomed distillation flask of 500 ml capacity, containing 15 ml of n-hexane and 50 ml of distilled water. The apparatus used for distillation was similar to that of Dean-Stark water estimator. About 5 ml of 4 M sodium hydroxide was poured into the flask and more distilled water was added to make the total volume ca. 250 ml. Maintaining the temperature of the homogenate sample at ca. 80^{o}C it was distilled for 2 h. Then the apparatus was allowed to cool and 20 ml of 1 M hydrochloric acid was added to the homogenate. The sides of the condenser was rinsed by pouring down 10 ml of distilled water and the process of distillation continued for 2 h at the end of which time, the distillate was collected in the condenser.

The product of steam distillation containing the accumulated oil was passed through a double layered column of anhydrous sodium sulphate and

activated alumina to free it from residual water and biogenic hydrocarbon, that might otherwise interfere with UV absorption. Finally the column was eluted with sufficient n-hexane and the extract was made upto 25 ml in a volumetric flask.

3.3.4.1.3 Sample estimation

Estimation of the hexane extracted sample was done with a spectrophotometer (Hitachi 200-20-UV-Vis.) equipped with a recorder. Using n-hexane as the blank the absorbance of the sample was measured at 225 nm and the concentration of respective PHCs was computed from the standard curve. The tissue concentration has been expressed as $\mu g\ g^{-1}$ dry wt. (for details of conversion of wet tissue wt. to dry tissue wt. see section 3.4)

3.4 COMPUTATION AND PRESENTATION OF DATA

Median lethal concentration (LC50) levels and their 95% confidence limits were calculated by probit analysis (Finney, 1971) and explained with graphs and tables. The ET50 values and toxicity curves also have been represented graphically to demonstrate the lethal effects of pollutants, following approved methods (Sprague, 1973)

Combined toxicity of toxicant mixtures on the lethal as well as sublethal responses of the animals were calculated following the methods of Marking and Dawson (1975). The data are presented with graphs and tables. The effect of mixtures of two or more chemicals was commonly referred to as additive, synergistic or antagonistic, based on the relation of the toxicity of the mixtures of that of individual toxicants. Marking and Dawson (1975) have coined a better terminology which was more clear and quantitative and there-

fore, that have been used in the present description.

The additive indices of mixtures of toxicants were derived as follows:

$$\frac{Am}{Ai} + \frac{Bm}{Bi} = S$$

Where, A and B are the toxicants; i and m are the toxicities (LC50's or EC50's) of individual and mixtures respectively for A and B and S is the sum of biological activity.

If the sum of 'S' = 1.0, then the combined action is referred to as simple additivity. If the value of 'S' is greater than or less than 1.0 it indicates that the combined toxicity is either less than additive or more than additive, respectively. Thus the value of 'S' alone could give a quantitative indication of the additive toxicity, except that values greater than 1.0 are not linear with values less than 1.0. This non-linearity can be corrected as follows and a system in which the index represents simple additivity by values '0', greater than additivity by value '+'ve and less than additivity by values '-'ve were developed (Marking and Dawson, 1975).

The linearity between values greater than and less than 1.0 are established by using the reciprocal of the values of 'S' which are less than 1.0 and by substracting 1.0 from the reciprocal ie; $\frac{(1)}{S} - 1$, to obtain a zero reference point. Index values representing less than additive toxicity were obtained by multiplying the values of 'S' which are greater than 1.0 by -1, to make them negative and a zero reference point is achieved by adding 1.0 to this negative value ie; S (-1)+1. Thus, greater than additive toxicity is represented by index values greater than zero, less than additive by negative index values and

simple additivity by values '0'. The significance of the additive indices close to zero can be assessed by substituting the lower confidence limits of the LC50 of the individual toxicants (Ai and Bi) and the upper limits of the mixtures (Am and Bm) to find out the lower limit of the index. Correspondingly, the upper limits of the individual toxicants (Ai and Bi) and the lower limits of the mixtures (Am and Bm) were substituted into the formula to determine the upper limit of the index.

Filtration rate was expressed as the quantity of sea water (in ml) filtered h^{-1} mg^{-1} dry tissue weight of the animal. This was estimated using Abel's equation (Abel, 1976) which states,

$$m = \frac{M}{nt} \quad Log_e \quad \frac{Co}{Ct}$$

Where, m = the rate of filtration in ml individual^{-1} h^{-1}

M = Volume of test solution in ml

n = number of animals in the test vessel

Co = dye concentration in the initial sample

Ct = dye concentration in the final sample

t = time between dye sampling, in hour

The value obtained in the above equation was divided by the mean dry tissue weight of the animals, to get the filtration rate in ml h^{-1} mg^{-1} (dry wt.).

Graphical representation together with tables have been used to explain the results on filtration rate, oxygen consumption and byssogenesis. These data have been analysed statistically using the student's t test to manifest the variation in response from the control at 5% level of significance.

The median effective concentration (EC50) ie; the concentration causing 50% response in the performance of the test animals was calculated, wherever possible, using regression analysis.

The tissue load of PHCs was expressed as $\mu g\ g^{-1}$ (dry wt.). For the conversion of wet weight of the mussel tissue to dry weight a ratio has been developed (Baby, 1987). Mussels used for the experiments were pooled into 10 groups of 4 animals each. The soft tissue was shucked out, cleaned in distilled water, blotted and weighed to find out the wet weight. Later these tissues were dried, and weighed to constancy. Using these data the water content of the tissues, the relationship in weight between wet and dry tissue etc. and their mean were computed, and used to express the dry weight of the tissue.

All the computations involved in the work are carried out with a personal computer (Casio, model PB700).