Methodology
3.1. PLACE OF STUDY FOR SEDIMENT COLLECTION

Place of study includes seven locations from where sediment samples for lead analysis were collected which are Karaikal (10°55’47” N, 79°50’ 11” E 10.9296°N 8365°E), Nagapattinam (10°46N 79°50’E 10.77°N 79.83°), Velankanni (Lat. 10.6833, Long.79.8333, Lat. (DMS): 10°40’, 60N, Long. (DMS): 79° 49’ 60E, Time zone (est)), Kodikarai (10°17’ 16.08” N, 79°51’ 54.36”E), Muthupet (10°24’N 79°30’E 10.4°N 79.5°E), Adirampattinam and Manora (10°20’N 79°23’ E 10.34°N 79.38°E) along the East Coast of India (Fig.1; Plate 1(a-g)).

3.2. SPECIMENS FOR TOXICITY STUDIES

The specimen for toxicity studies namely the estuarine fish, Mystus gulio (Ham.) and marine shrimp Penaeus monodon (Fab.) were chosen for lead toxicity studies and collected from estuarine and backwater area of Muthupet estuary.

3.3. PERIOD OF STUDY

The study period for lead analysis and toxicity studies includes the duration from June 2004 to April 2010. The period of study not only includes study of human activity and seasonal changes. It paved a way for a detailed study of lead deposits during pre and post-tsunamic effects. The
Fig. 1. Map of the Study areas

Nagapattinam district
Karaikal
Nagpur district
Kodikarai
Karaikal (Post Tsunami)

PLATE 1a
Nagapattinam Harbour

Nagapattinam Beach (Post Tsunami)

PLATE 1b
Muthupet Back waters

PLATE 1e
Adirampattinam

Adirampattinam - Post tsunami

PLATE 1f
sampling procedure was done on monthly basis for these six years period. The research study includes toxicity studies and experimentation on the marine species of fish *Mystus gulio* and shrimp *Penaeus monodon* at sublethal concentrations of Lead nitrates at 24, 48, 72 and 96 hrs.

3.4. COLLECTION OF SPECIMEN

3.4.1. *Mystus gulio*

The brackish water fish *Mystus gulio* (length around 22 cm and weight 150 g) were collected from Muthupet - Velankanni backwaters. The collection of fishes were done with the help of local fisherman near the private fish ponds backwaters. The collected alive fishes were transferred to 50 liters plastic barrel half filled with its native water. Then the container was aerated with oxygen cylinder. Then the fishes were transferred to Thanjavur by car and the specimens were transferred to big cement tanks of approximate capacity 500 L. fed with dried acetes (test animal fed with *ad libitum* once a day). Feeding stoped 24 hrs before experimentation as per Arora *et al.* (1974) (Plate 2a).

3.4.2. *Penaeus monodon*

The marine shrimp *Penaeus monodon* size of around 10 cms length and weight around 25 grams were collected from Velankanni Private Culture Pond and transported to Thanjavur using a private car to Lab condition using oxygen cylinder and acclimatized for lab conditions for ten days dead and diseased animals were removed. The shrimps were fed with prawn feed. The same water from the pond transported to the
(a) Brackish water fish, *Mystus gulio*

(b) Marine shrimp, *Penaeus monodon*

PLATE – 2
laboratory at A.V.V.M. Sri Pushpam College, Poondi, Thanjavur district (Plate 2b).

3.5. PLACE OF ANALYSIS AND EXPERIMENTATION

The lead analysis and animal toxicity experiments were conducted at the Department of Zoology, A.V.V.M. Sri Pushpam College, Poondi, Thanjavur district, Tamil Nadu, India-613 503.

3.6. INSTRUMENTS AND EQUIPMENTS USED FOR THE RESEARCH WORK

The advanced instrument used for sediment analysis includes: Atomic Absorption Spectrophotometer (A.A.S.), Thermo Jarrell Ash (AASCAN – I USA) (A.A.S.) apart from the usual standard equipments and apparatus which include Ekman sediment grab: used for collecting surface sediment samples, Polyethylene plastic covers; to collect sediment samples, sieves size <2 mm, Mortor and Pesticle, hot water bath, Electronic balance, conical flask, measuring jars, aluminium foils, Vilas and Agitator.

The other equipments, instruments used for fish and prawn tissues analysis are 500 lit cement acclimatization tank, fish net, oxygen cylinder for transport, vehicle for transport, 20 lit capacity plastic tubs for acute bioassay test conduct, electric aerator and a battery aerator, rotary microtome (Weswox MT Chennai, India), Haemocytometer, Atomic Absorption Spectrophotometer (A.A.S.), Thermo Jarrell Ash (AASCAN-I
Collection of specimens

PLATE - 3
A thermo Jarrell Ash (AA Scan – USA) Atomic Absorption Spectrophotometer

PLATE – 4
USA), Measuring Scale, Electronic Balance, Plastic containers, pH paper or meter, Glass rods and Light microscope with Camera (Plate 3).

3.7. SAMPLING METHOD

Surface sediment samples were collected using Ekman sediment grab. Soft sediment samples were collected in polyethylene plastic covers from 7 coastal sampling stations, on monthly basis. Firstly the sediment samples were shade-dried under room temperature. After the removal of debris and large stones, the samples were sieved > 2 mm size. Then the samples were weighted and processed for lead extraction. The extraction procedure for total lead and particulate lead extraction from the sediments was adopted following the method of Loring and Rantala (1992). Detailed sample preparation extraction procedures are given in fig.2 and 3.

3.8. SAMPLE ANALYSIS

Lead concentrations in the sediment extracts were determined using Atomic Absorption spectrophotometer. The concentrate was directly aspirated into the air acetylene flame of Atomic Absorption Spectrophotometer (A.A.S), Thermo Jarrell Ash (AASCAN – I USA).

**Operation Parameters of A.A.S.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wave length</td>
<td>217.0m</td>
</tr>
<tr>
<td>Slit setting</td>
<td>1.0 nm</td>
</tr>
<tr>
<td>Light source</td>
<td>Lead hollow cathode lamp</td>
</tr>
<tr>
<td>Flame type</td>
<td>Air-acetylene flame</td>
</tr>
</tbody>
</table>
3.9. STOCK STANDARD SOLUTION

Standard stock solution was prepared by dissolving 0.1598 g of lead nitrate [Pb(NO$_3$)$_2$] in 1 per cent (V/V) nitric acid and diluted to 1 litre with 1 per cent (V/V) HNO$_3$. 1.00 ml = 1 mg. The working standard solution was prepared by making up 2 ml of standard stock solution to 100 ml using double distilled water. The sediment samples were analysed in PPM level.
3.10. SEDIMENT SAMPLE PREPARATION FOR LEAD ESTIMATION

Grab Sample

Store at 30°C

Sub sample dry at 80°C

Remove material > 2 mm

Homogenize sample

Sub sample

Extraction with HNO₃: HCl

Determination of Total Lead

Sub sample

Extraction with Acetic acid

Determination non detrital Lead

Fig. 2
3.11. THE EXTRACTION PROCEDURE FOR THE DETRITAL AND NON DETRITAL FRACTIONS OF LEAD IN THE COASTAL SEDIMENTS

Homogenized sample

Sub sample
25 ml 25% acetic acid (v/v)
Shake well 3 hrs and stand still 6 hrs
Transfer
To the above
10 ml D.W.
Stand 1 hr
Rinse and makeup 50 ml with D.W.
AAS
Non-Detrital

Sub sample
10 ml HNO₃, HCl (1:3)
Heat 3 hrs
20 ml D.W.
Shake stand 3 hrs
Transfer
To the above
10 ml D.W.
Stand 1 hr
Transfer
AAS
Detrital

Fig. 3
3.12. TOXICITY STUDIES

As earlier the heavy metal used is Pb Lead nitrate Pb(NO₃)₂ Molecular WT 331.21 used for the toxicity studies solution with different concentrations (mg/l) prepared in double distilled water. Along this a stock solutions were prepared from the stock solution for the upcoming experiment work. A stock solution was prepared using 2.5 g of the salt in 1 litre of water. From this stock solution, required concentration of test solution were prepared for the studies.

3.13. LC₅₀ DETERMINATION

24 hours before the beginning of the test the feeding of the animals were stopped. Lead nitrate with different concentrations were prepared from stock solution. In a 20 Liters capacity tank 10 lit. solutions with different concentrations were prepared. The fishes were starved to ensure evacuation of gut before the start of the experiment form this well acclimated fishes a set of 20 numbers were introduced into tanks of different concentration. A said earlier the feeding was stopped water in the container renewed for every day till 96 h with same toxicant level. The biological wastes were removed twice a day.

The determination of LC₅₀ by interpolation involved plotting the data on semilogarithmic coordinate graph morality of test animals on the arithmetic scale (X-axis). Thus a percentage of mortality was observed in each aquarium was plotted against the concentration of toxicant. A straight line was drawn between two points representing percentage
mortality at 2 successive concentrations. The concentrations at which the line crossed 50 per cent lethality line was considered as LC$_{50}$ value.

Sublethal concentration

Acute sublethal concentration of LC$_{50}$ value had been selected differentially by Rand and Petrpcelli (1985).

Heavy metal lead

Acute level-25 per cent of LC$_{50}$ value

Lead nitrate concentration

Acute level-12 per cent LC$_{50}$ value

The animal experiments on estuarine fish *M. gulio* and marine shrimp, *P. monodon* were designed to be conducted at 3 sublethal concentrations of lead nitrate 0.96, 15.50, 26.50 and 1.60, 3.33, 6.60 mg/l and the experiments were done on live specimens before histopathological studies on effect of toxicity.

3.14. HAEMATOLOGY

The count of RBC in the blood of estuarine fish *Mystus gulio* carried out with improved Neubaver ruling (Weber & Sons, England). Blood is diluted 200 times in the standard RBC pipette with Hendrick's fluid coloured light blue with methylene blue.

The WBC count studied in the *Mystus gulio* blood, in order to prevent blood from clotting, the syringe and needle were rinsed with 1% EDTA before bleeding (Mainwarning and Rowley, 1985).

Blood smear were prepared on clean slides. The smear were stained by the Panoptic method of Hoffman (1977). On the dry smear 10 drops of
May – Grunwald solution was added and after 3 minutes an equal amount of distilled water added. Without rinsing, the slides were immersed in Giemsa’s solution (1 part in 9 parts distilled water) and stained for 15-30 minutes. The slides were rinsed in running water and observed under microscope. The individual types of cells were counted and converted to percentage to get the Leukocytes differential count.

3.15. BIOCHEMICAL ASSAY

Biochemical assay was carried out for the above animal tissue using the method of Lowry et al. (1951) for protein, Folch et al. (1957) for Lipid and Roe (1955) for Carbohydrate.

3.15.1. ESTIMATION OF PROTEIN by Lowry et al. (1951) method

Principle

The carbamyl groups of protein molecules react with copper and potassium of the reagent to give a blue coloured copper potassium biuret complex. This complex together with thyroxine and phenolic components present in the protein reduce the phosphomolybdate of the Folin’s reagent.

Preparation of reagents

(i) 80 per cent ethanol

80 ml of ethanol in 20 ml of distilled water

(ii)a. 0.1 N Sodium hydroxide

0.4 of sodium hydroxide is dissolved in 100 ml of distilled water
b. Sodium potassium tartrate

500 mg sodium potassium tartrate is dissolved in 100 ml of distilled water

(iii) 1 N Sodium hydroxide

4 g of sodium hydroxide is dissolved in 100 ml of distilled water

(iv) Reagent A

12 g of sodium carbonate is dissolved in 600 ml of 0.1N sodium hydroxide

(v) Reagent B

250 mg of copper sulphate is dissolved in 50 ml of sodium potassium tartrate

(vi) Reagent C

50 ml of Reagent A is mixed with 1 ml of reagent B

(vii) Folin’s phenol reagent

Dilute the solution 1:1 ratio with distilled water

(viii) Standard solution

10 mg of bovine serum albumin is dissolved in 100 ml of sodium hydroxide

**Procedure**

To the 100 mg weighed sample, 1 ml of deproteinizing agents are added homogenized and then centrifuged at 3000 rpm of 5 minutes. Discard the supernatant and take the precipitate. Dissolve the precipitate with 1 ml of 1 N sodium hydroxide. Take separately 1 ml of 1 N sodium hydroxide and 1 ml of bovine serum albumin as blank and standard. Then,
to the sample blank and standard, 5 ml of reagent C was added and mixed rapidly, incubate it for 30 minutes. Then, the optical density of the blue colour solution was measured at 530 nm.

**Calculations**

\[
\frac{\text{Amount of protein present in the sample}}{\text{Weight of the sample}} = \frac{\text{O.D. of the sample}}{\text{O.D. of the standard}} \times \frac{\text{Conc. of the standard}}{\text{Weight of the sample}}
\]

**3.15.2. ESTIMATION OF CARBOHYDRATES by method of Roe (1955)**

The estimation of carbohydrates were done following the method of Roe (1955). One ml of the supernatant of the sample was mixed with 10 ml of anthrone reagent. The reagent blank and 0.1 per cent glucose (standard) were prepared. After heating the standard solution, sample and reagent blank placed in a boiling water bath for 15 minutes and cooled at room temperature for 30 minutes, the absorbance was measured at 620 nm and the concentration of free sugars in the sample was calculated from the known concentration of the standard.

**3.15.3. ESTIMATION OF LIPID by Folch et al. (1957) method**

**Principle**

Using chloroform methanol, the lipids are extracted from the tissue. The extracted lipids react with sulphophosphovanillin mixture to form red colour complex. The intensity of the colour depends upon the amount of the lipids present in the sample.
Preparation of reagents

1. Chloroform methanol mixture (2:1)
   50 ml chloroform was mixed with 25 ml of methanol in the ratio 2:1
2. 0.9 per cent Sodium chloride
   400 ml of Sodium chloride was dissolved in 50 ml of distilled water.
3. Concentrated sulphuric acid
4. Cholesterol standard solution
   50 mg of cholesterol was dissolved in 25 ml of chloroform methanol mixture
5. Bank Chloroform methanol mixture
6. Vanillin
   600 mg of Vanillin was dissolved in 300 ml of 80 per cent Ortho phosphoric acid

Procedure

To the 100 mg weighed sample add 1 ml of Chloroform methanol mixture and homogenize it. To that homogenate add 0.4 ml of Sodium chloride to remove non-lipid contaminants and to release the bound acids. The samples were centrifuged and the upper and middle portion were removed and lower phase were made up to 1 ml with the help of chloroform methanol mixture as standard and blank were kept in boiling water bath for 10 minutes. After cooling, 5 ml of vanillin reagent was added to all the 3 test tubes and optical density was read at 530 nm after 30 minutes.
### Calculations

\[
\text{Amount of protein present in the sample} = \frac{\text{O.D. of the sample}}{\text{O.D. of the standard}} \times \frac{\text{Conc. of the standard}}{\text{Weight of the sample}}
\]

### 3.16. BIOACCUMULATION

Accumulation test done for four days at acute level (25% of LC50 value) Lead nitrate dissolved in 25 Lit of water in 50 Lit glass tank. The water aerated through the period of study and 5 fishes were left in each glass tank. Bioaccumulation of Lead nitrate in Kidney, Muscle, gonads, liver and skin were estimated after four days.

### 3.17. DEPURATION TEST

The depuration period of this fish was carried out with 50 animals of each of same size exposed to sub lethal concentration i.e. 10 per cent of the LC50 values, fishes were transferred to metal-free water and allowed for leaching of accumulated metal from the body and 5 number of fishes were sacrificed periodically for the determination of the metal content in the respective tissues.

Similarly control animals were maintained for 21 days. Then after the prescribed period of 21 days, the fishes were sacrificed and the vital tissues were subjected to Heavy metal analysis.
The vital organs were dissected from the fishes. The major parts are 1. Gills 2. Liver, 3. Skin 4. Kidney 5. Gonad and 6. Muscle were washed in double distilled water and preserved in 10 per cent formalin. The water present over the tissues were removed using filter paper and the tissues were weighed and acid digested with Perchloric acid and concentrated nitric acid in the ratio of 1:1 (V/V) (FAO, 1975). The final acid digested extract was analysed for Lead using Atomic absorption spectrophotometer.

3.18. HISTOLOGICAL STUDIES (*Mystus gulio*)

Vital organs namely kidney, liver, gills, testis, ovary and muscle were dissected from both control and experimental animals placed at sublethal concentrations to find out the effect of Lead nitrate on the histology of the vital organs mentioned earlier. As per procedures mentioned by Pearse (1968) sections were made. The dissected tissues were fixed in Bouin’s fluid, dehydrated in alcohol, cleared in xylol, blocked in paraffin wax, sectioned at 6μ stained with Ethrilich’s haematoxylin and eosin and mounted in DPX. Photomicrographs were taken with bright field microscope (Nikon) with a zoom camera, at different magnifications from 50-1000 X.

3.19. HISTOPATHOLOGICAL STUDIES

**Tissue preparation for histological observation (*Penaeus monodon*)**

The five to eight posterior gills and Hepatopancreas were dissected and immediately fixed in Bouin’s fixative for 48 h. The preserved tissues
were processed by a routine histological method (Gurr 1962), dehydrated in an alcohol series, cleared in xylene, infiltrated with liquid paraffin at 58°C, and finally embedded in paraffin blocks were trimmed and sectioned at 5-8 μm thick cut on a rotary microtome (Weswox MT Chennai, India), were stained with Harris’ Hematoxylin and counterstained with Eosin (H and E stain). Then the slides mounted with DPX and observation under a light microscope (Woods and Ellis, 1994).

3.20. STATISTICAL ANALYSIS

Data obtained are mean ±S.E.(n=5). The controls and experimental values were analysed for correlation co-efficient to find the significant differences at 5 per cent and 1 per cent level.