3. MATERIALS AND METHODS

3.1. EXPERIMENTAL DESIGN 1: *IN VIVO* STUDY OF EXPOSURE TO LEAD AND ARSENIC SINGLY ON FISH INTESTINAL AND LIVER MACROPHAGES

a) Biological material

Freshwater fish *Channa punctatus* (Bloch.), of length 12.5–15.5 cm and weight 20.0–30.0 g, were obtained from local fish market and housed in 60 l glass aquaria. Fish of a single lot were used throughout the investigation. Prior to exposure, fish were held for 15 days for acclimatization and evaluation of overall fish health under laboratory conditions.

b) Exposure to lead and arsenic

After acclimatization, fish were divided into three groups; one served as control and the other two served as lead acetate treated and sodium arsenite treated group respectively. Sub-lethal toxicity of lead acetate solution (9.4 mg/L) (Devi and Banerjee, 2007) and sodium arsenite solution (0.24 mg/L) on the intestinal macrophages of *Channa punctatus* was analysed after an exposure of 4 days (Akter *et al.*, 2008).

c) Separation of macrophages

The fish were dissected; whole gut and liver of the fish were isolated, immediately placed in Leibovitz (L-15) medium supplemented with heparin (10 IU/ml) and fetal bovine serum (FBS) (2%), and then homogenized in ice cold condition. Cell suspension is then transferred to tubes and kept in ice for cell debris to settle. The supernatant is then layered over Ficoll (45%) and subjected to density- gradient centrifugation (Chung and Secombes, 1998). The band of macrophage- enriched fraction at the interface is collected, washed and resuspended

in L-15 medium containing FBS and allowed to adhere on plastic surface. The adherent cells were collected and tested for viability as determined by Trypan Blue dye exclusion technique.

d) Bioaccumulation, Morphological Alteration and DNA damage

i) Heavy metal bioaccumulation by Atomic Absorption Spectrophotometer:

The different organs were allowed to dry at 120ºC until reaching a constant weight, concentrated nitric acid and hydrogen peroxide (1:1 v/v) (SD fine chemicals) was added. The digestion flasks were heated to 1300ºC until all the materials were dissolved and diluted with double distilled water appropriately. The elements Pb, and As were assayed using Shimadzu AA 6200 Atomic Absorption Spectrophotometer at the Sophisticated Analytical Instrument Facility (SAIF), NEHU, Shillong, Meghalaya. The results were expressed as ppm/ g tissue.

ii) Morphological alteration of macrophages:

The cell suspension fixed in an equal volume of 2.5% glutaraldehyde in Hanks Balanced Salt Solution (HBSS) and centrifuged at 2000 rpm for 5 min. The pellet was resuspended in HBSS. Smears of cells were drawn on glass slides, air-dried, fixed in methanol and stained with Giemsa. Cells were observed under oil immersion microscope. Any cell deviating from spherical outline was scored as polarised and this was expressed as a percentage of the total number of cells counted (Ou *et al.*, 2009; Mantovani *et al.*, 2005).

iii) Ultrastructural analysis of tissue by Scanning Electron Microscope:

The intestine was excised and rinsed in heparinized saline, rinsed in 0.1 M cacodylate buffer at pH 7.5, infiltered with 2.5% glutaraldehyde for 24 hr fixation at 4ºC, rinsed in buffer, trimmed into 8.0 mm squares and subjected to post-fixation in 1% OsO_4 in 0.1 M cacodylate buffer at pH 7.5 for 2 h and dehydrated through graded acetone. The mucosal surface of each

tissue was mounted on metal stubs, coated with gold using a JFC-1100 (Jeol) ion sputter. Finally, the tissues were scanned with a JSM-6360 (Jeol) Scanning Electron Microscope at the Sophisticated Analytical Instrument Facility (SAIF), NEHU, Shillong, Meghalaya.

iv) DNA fragmentation assay:

Two volumes of solution containing [0.088 M diphenylamine (DPA), 98% v/v glacial acetic acid, 1.5 v/v sulfuric acid and 0.5 v/v of 1.6 % acetaldehyde solution.] were added to one volume of extracted DNA. The samples were stored at 4°C for 48hr. The blue color quantified spectrophotometrically at 578 nm. The percentage of fragmentation was calculated as the ratio of DNA in the supernatant to the total DNA (Pernadones, 1993).

e) Study of effect of lead acetate and sodium arsenite (*in vivo*) singly on oxidative stress and antioxidant defenses in fish *Channa punctatus*

i) Respiratory Burst Activity:

Respiratory burst activity of intestinal phagocytes of control and treated fish was measured by the method of Fujiki and Yano (1997), with some modifications. The respiratory burst activity was expressed as *A*630 nm per 10^6 cells (Fujiki and Yano, 1997).

ii) Estimation of Lipid Peroxidation (LPO):

The LPO activity was determined by the procedure of Utley *et al*. (1967), with some modifications. The rate of lipid peroxidation was expressed as nanomoles of thiobarbituric acid reactive substance (TBARS) formed per hour per milligram of protein using a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1.}$ Protein content of each sample was determined using method of Lowry *et al*. (1951) (Utley *et al*., 1967).

iii) Estimation of Protein Carbonylation (PC):

The protein carbonylation activity was determined by the method of Levine *et al.*, 1994. Carbonyl contents are determined from the absorbance at 366 nm using a molar absorption coefficient of 22,000 $M^{-1}cm^{-1}$.

iv) Catalase (CAT) activity:

The pellet was dissolved in 1 ml of 0.1 M phosphate buffer. 10-30µl of Triton X-100 was added to the sample and allowed to stand for 5 min.100µl of the sample was then added to a cuvette containing 400µl of 0.1 M phosphate buffer (pH 7.4) and the reaction was initiated by addition of 10µl of 60mmol H_2O_2. The decomposition rate of H_2O_2 was measured at 240nm for 1min using a spectrophotometer. One unit (U) of Catalase activity is defined as the amount of enzyme catalyzing 1µmol of H_2O_2 per min at 25°C (Claiborne, 1985).

v) Superoxide Dismutase (SOD) activity:

A volume of 100 µl of the sample was mixed with 100 µl of Tris-HCl buffer (ph 8.5), then 50 µl of 1mmol ethylene diamine tetraacetic acid (EDTA) and 50 µl of 2.64mmol pyrogallol was added. The absorbance of the reaction mixture was recorded at 420 nm. One unit of SOD activity was determined as the amount of enzyme that inhibited the auto- oxidation of pyrogallol by 50%. The activity was expressed as U/mg protein (Marklund and Marklund, 1974).

vi) Glutathione S- Transferase (GST) Activity:

GST activity was measured by the method described by Mannervik and Gutenberg, 1981. Enzyme activity was calculated using a molar extinction coefficient of $9.6 \times 10^3 M^{-1}cm^{-1}$.

vii) Glutathione Peroxidase (GPx) Activity:

Total cellular GPx activity was determined by measuring the decrease in absorbance (340 nm) due to the decline in NADPH at 23–25ºC (Lorentzen *et al.*, 1994). The activity of GPx

was expressed as U/mg protein and 1mU was defined as 1 nmol of NADPH consumed/min/mL of sample.

viii) Glutathione Reductase (GR) Activity:

The principle of the method is the reduction of oxidized glutathione by glutathione reductase in the presence of NADPH (Carlberg and Mannervik 1975). One Unit was defined as an amount of the enzyme which will reduce 1 μM of oxidized glutathione per minute at pH 7.6 at 25 °C, using a molar extinction coefficient of 6.22×10^3 for NADPH.

ix) Reduced Glutathione (GSH) Assay:

Non-enzymatic antioxidant, reduced glutathione, was assayed by the method previously described by Ellman (1959). The amount of glutathione was calculated using a GSH standard curve and expressed as micrograms of GSH formed/mg protein (Ellman, 1959).

f) Study of effect of lead acetate and sodium arsenite (*in vivo*) singly on innate immune responses in fish *Channa punctatus*

i) Phagocytosis assay:

The cells from both control and exposed groups were allowed to adhere separately on glass slides for one hour. Phagocytosis assay were performed with heat killed *S. aureus* and phagocytic index calculated (Czuprynski *et al*, 1984).

ii) Intracellular killing assay:

Bacteria were incubated with macrophages in L-15 FBS for 20 min at 37°C. After various time intervals, samples were plated onto nutrient agar to determine the number of viable intracellular bacteria (Leigh *et al.,* 1986). Intracellular killing is expressed as the percentage decrease in the initial number of viable intracellular bacteria.

iii) *In vitro* cell adhesion assay:

Cells were seeded separately for treated and control group in 96 well microtitre plates and allowed to adhere differentially for different time intervals. The cells were stained with crystal violet and the dye extracted from the adhered macrophage in the wells by lysing with 0.1% SDS in HBSS. Cell adhesion was expressed as the increased absorbance measured spectrophotometrically at 570 nm (Lin *et al*, 1997).

iv) Chemotactic migration of macrophages:

Chemotactic index of treated and untreated groups were determined from the ratio of directed migration to random migration (Wilkinson, 1986). Chemotactic index is expressed as:

Number of cells migrated toward serum/ Number of cells migrated toward saline.

v) Nitric oxide (NO) release assay:

Cells (10^6 cells/ml) were suspended in DPBS and were stimulated with LPS (100 ng/ml). The cell free supernatants were used for nitric oxide release assay using Griess reagent. Readings were taken in a UV spectrophotometer at 550nm (Saggers and Gould, 1989).

vi) Myeloperoxidase (MPO) release assay:

Cell suspension were taken, stimulated with LPS and centrifuged. The supernatants were collected in separate microcentrifuge tubes. Supernatants and cell lysate were allowed to react with orthophenylene dihydrochloride (OPD) substrate and readings were taking at 492 nm in a spectrophotometer (Bos *et al*, 1990).

vii) Cytokine assays:

ELISA was used for detecting fish cytokines TNF-α and IL-1β in cell lysates. The 96 well microtitre plates (Iwaki Glass, Japan) were coated with 100 µl of fish serum or cell supernatant diluted 10 times in phosphate buffer saline (PBS) and incubated in a moist

chamber overnight at 4ºC. TNF- α and IL-1β rabbit polyclonal antibody was used as the primary antibody (Sigma, USA). Vectastain ABC- PO kit (Vector Lab., USA) was applied for ELISA and peroxidase ABTS substrate kit (Vector Lab., USA) was used for coloration. The plate was read at 490 nm on a microplate reader (BioRad).

g) Statistical analysis

The data are expressed as mean± S.E. Data were analyzed using Student's *t*-test (two-sample assuming unequal variances) for determining the significant change over control values. The significance level was set at $P<0.05$.

3.2. EXPERIMENTAL DESIGN 2: *IN VITRO* STUDY OF EXPOSURE TO LEAD AND ARSENIC SINGLY ON FISH INTESTINAL AND LIVER MACROPHAGES

a) Biological material

Freshwater fish *Channa punctatus* (Bloch.), of length 12.5–15.5 cm and weight 20.0–30.0 g, were obtained from local fish market and housed in 60 l glass aquaria. Fish of a single lot were used throughout the investigation. Prior to exposure, fish were held for 15 days for acclimatization and evaluation of overall fish health under laboratory conditions.

b) Separation of macrophages

The fish were dissected; the whole gut and liver of the fish were isolated, immediately placed in Leibovitz (L-15) medium supplemented with heparin (10 IU/ml) and fetal bovine serum (2%), and then homogenized in ice cold condition. Cell suspension was then transferred to tubes and kept in ice for cell debris to settle. The supernatant was then layered over Ficoll (45%) and subjected to density- gradient centrifugation (Chung and Secombes, 1998). The

band of macrophage- enriched fraction at the interface was collected, washed and resuspended in L-15 medium containing FBS and allowed to adhere on plastic surface. The adherent cells were collected and tested for viability as determined by Trypan Blue dye exclusion technique. More than 90% cells were found viable.

c) Exposure to lead and arsenic

Cells were divided into three groups; one served as control and the other two respectively as lead acetate treated and sodium arsenite treated groups. After standardization of doses by lactate dehydrogenase (LDH) assay (Lewis *et al*., 1985) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Borenfreund *et al.*, 1988), sub-lethal doses of lead acetate solution (4 ng/ml) and sodium arsenite solution (3 ng/ml) were given *in vitro* on the intestinal and liver macrophages of *Channa punctatus* and were analyzed after an exposure of 60 minutes. More than 90% of the cells were found to be viable and non-cytotoxic at the given dose.

d) Study of effect of lead acetate and sodium arsenite (*in vitro*) on oxidative stress and antioxidant defenses in fish *Channa punctatus*

i) Respiratory Burst Activity:

Respiratory burst activity of phagocytes of control and treated fish was measured by the method of Fujiki and Yano (1997), with some modifications. The respiratory burst activity was expressed as *A*630 nm per 10^6 cells (Fujiki and Yano, 1997).

ii) Estimation of Protein Carbonylation (PC):

The protein carbonylation activity was determined by the method of Levine *et al*., 1994. Carbonyl contents are determined from the absorbance at 366 nm using a molar absorption coefficient of 22,000 $M^{-1}cm^{-1}$.

iii) DNA fragmentation assay:

Two volumes of solution containing [0.088 M diphenylamine (DPA), 98% v/v glacial acetic acid, 1.5 v/v sulfuric acid and 0.5 v/v of 1.6 % acetaldehyde soln.] were added to one volume of extracted DNA. The samples were stored at 4°C for 48hr. The blue color quantified spectrophotometrically at 578 nm. The percentage of fragmentation was calculated as the ratio of DNA in the supernatant to the total DNA (Pernadones, 1993).

iv) Catalase (CAT) activity:

The pellet was dissolved in 1 ml of 0.1 M phosphate buffer. 10-30µl of Triton X-100 was added to the sample and allowed to stand for 5 min.100µl of the sample was then added to a cuvette containing 400µl of 0.1 M phosphate buffer (pH 7.4) and the reaction was initiated by addition of 10µl of 60mmol H_2O_2. The decomposition rate of H_2O_2 was measured at 240nm for 1min using a spectrophotometer. One unit (U) of Catalase activity is defined as the amount of enzyme catalyzing 1µmol of H_2O_2 per min at 25°C (Claiborne, 1985).

v) Superoxide Dismutase (SOD) activity:

A volume of 100 µl of the sample was mixed with 100 µl of Tris-HCl buffer (ph 8.5), then 50 µl of 1mmol EDTA and 50 µl of 2.64mmol pyrogallol was added. The absorbance of the reaction mixture was recorded at 420 nm. One unit of SOD activity was determined as the amount of enzymethat inhibited the auto- oxidation of pyrogallol by 50%. The activity was expressed as U/mg protein (Marklund and Marklund, 1974).

e) Study of effect of lead acetate and sodium arsenite (*in vitro*) singly on innate immune responses in fish *Channa punctatus*

i) Phagocytosis assay:

The cells from both control and exposed groups were allowed to adhere separately on glass slides for one hour. Phagocytosis assay were performed with heat killed *S. aureus* and phagocytic index calculated (Czuprynski *et al*, 1984).

ii) Nitric oxide (NO) release assay:

Cells (10^6cells/ml) were suspended in DPBS and were stimulated with LPS (100 ng/ml).The cell free supernatants were used for nitric oxide release assay using Griess reagent. Readings were taken in a UV spectrophotometer at 550nm. (Saggers and Gould, 1989).

iii) Myeloperoxidase (MPO) release assay:

Cell suspension were taken, stimulated with LPS and centrifuged. The supernatants were collected in separate microcentrifuge tubes. Supernatants and cell lysate were allowed to react with orthophenylene dihydrochloride (OPD) substrate and readings were taking at 492 nm in a spectrophotometer (Bos *et al*, 1990).

iv) Cytokine assays:

ELISA was used for detecting fish TNF-α and IL-1β like molecules. The 96 well microtitre plates (Iwaki Glass, Japan) were coated with 100 μl of fish serum or cell supernatant diluted 10 times in phosphate buffer saline (PBS) and incubated in a moist chamber overnight at 4ºC. TNF- α and IL-1β rabbit polyclonal antibody was used as the primary antibody (Sigma, USA). Vectastain ABC- PO kit (Vector Lab., USA) was applied for ELISA and peroxidase ABTS substrate kit (Vector Lab., USA) was used for coloration. The plate was read at 490 nm on a microplate reader (BioRad).

f) Statistical analysis

The data are expressed as mean± S.E. Data were analyzed using Student's *t*-test (two-sample assuming unequal variances) for determining the significant change over control values. The significance level was set at $P<0.05$.

3.3. EXPERIMENTAL DESIGN 3: *IN VIVO* STUDY OF EXPOSURE TO LEAD AND ARSENIC SIMULTANEOUSLY ON FISH INTESTINAL AND LIVER MACROPHAGES

a) Biological material

Freshwater fish *Channa punctatus* (Bloch.), of length 12.5–15.5 cm and weight 20.0–30.0 g, were obtained from local fish market and housed in 60 l glass aquaria. Fish of a single lot were used throughout the investigation. Prior to exposure, fish were held for 15 days for acclimatization and evaluation of overall fish health under laboratory conditions.

b) Exposure to lead and arsenic simultaneously (multimetal challenge)

After acclimatization, fish were divided into four groups: Group I- lead treated, Group II- arsenic treated, Group III- multi-metal, lead and arsenic simultaneously treated and Group IV- control. Sub-lethal toxicity of lead acetate solution (9.4 mg/L) (Devi and Banerjee, 2007) and sodium arsenite solution (0.24 mg/L) (Akter *et al*., 2008) on the intestinal macrophages of *Channa punctatus* was analysed after an exposure of 4 days.

c) Separation of macrophages

The fish were dissected, whole gut and liver of the fish were isolated, immediately placed in Leibovitz medium (L-15) supplemented with heparin (10 IU/ml) and fetal bovine serum (2%), and then homogenized in ice cold condition. Cell suspension is then transferred to

tubes and kept in ice for cell debris to settle. The supernatant is then layered over Ficoll (45%) and subjected to density- gradient centrifugation (Chung and Secombes, 1998).The band of macrophage- enriched fraction at the interface is collected, washed and resuspended in L-15 medium containing FBS and allowed to adhere on plastic surface. The adherent cells were collected and tested for viability as determined by Trypan Blue dye exclusion technique.

d) Study of effects of lead acetate and sodium arsenite (*in vivo*) simultaneously on bioaccumulation, morphological alteration and DNA damage

i) Heavy metal bioaccumulation by Atomic Absorption Spectrophotometer

ii) Morphological alteration of macrophages

iii) Ultrastructural analysis of tissue by Scanning Electron Microscope

iv) DNA fragmentation assay

e) Study of effects of lead acetate and sodium arsenite (*in vivo*) simultaneously on oxidative stress and antioxidant defenses in *Channa punctatus*

i) Respiratory Burst Activity

ii) Estimation of Lipid Peroxidation (LPO)

iii) Estimation of Protein Carbonylation (PC)

iv) Catalase (CAT) Activity

v) Superoxide Dismutase (SOD) Activity

vi) Glutathione S- Transferase (GST) Activity

vii) Glutathione Peroxidase (GPx) Activity

viii) Glutathione Reductase (GR) Activity

ix) Reduced Glutathione (GSH) Assay

f) Study of effects of lead acetate and sodium arsenite (*in vivo*) simultaneously on innate immune functions in *Channa punctatus*

i) Phagocytosis assay
ii) Intracellular killing assay
iii) Chemotactic migration of macrophages
iv) In vitro cell adhesion assay
v) Nitric oxide (NO) release assay
vi) Myeloperoxidase (MPO) release assay
vii) Cytokine assays

EXPERIMENTAL DESIGN 4: STATISTICAL ANALYSIS AND ISOBOLOGRAM STUDY ON THE INTERACTIVITY EFFECTS BETWEEN LEAD AND ARSENIC EXPOSURE (*IN VIVO*) IN MACROPHAGES FROM FISH *CHANNA PUNCTATUS*

A one- tailed Student's t test was performed to compare the mean values of control and heavy metal treated groups. In case of multi metal exposure, besides one- tailed Student's t test, a statistical approach applying an additivity response surface methodology (Suhnel J., 1992) was used to test the validity of the synergy concept for the multimetals lead and arsenic. A multivariate- modified ANOVA has been applied to search for evidences of interaction between the heavy metals lead and arsenic, when administered simultaneously. However, as F tests do not provide conclusive evidences for proving heavy metal synergy, an isobologram has been plotted for NO release (μg/100 ml). For this purpose an ordinary least squares regression was run between effects (NO release) and dose levels of metals (single and multi-metal) in the log- linear form. Assuming additivity of effects, NO release was fixed at 30 units and the minimum dose levels of lead plotted for each dose level of arsenic to get the isobol for 30 units NO release. An isobologram is a two- dimensional graph with the doses of

agents A and B (lead and arsenic, in the study) as coordinate axes, in which one or several lines, the isobols, are shown connecting different dose combinations which produce the same magnitude of effect (Loewe S., 1953).

Similarly, an isobologram has also been constructed for the antioxidant status for catalase activity (U/mg protein).