3.1. Study Area

The Cochin estuary (CE) is one of the largest and productive estuaries along the south west coast of India. The average depth is 5±2 m, except in the mouth region of the estuary where the depth varies from 10 to 13 m. It serves as a repository for effluents discharged from various industries such as fertilizer, pesticide, radioactive mineral and heavy metal processing units, chemical and allied industries, petroleum refineries and fish processing units. These effluents are rich in heavy metal concentrations such as zinc, cadmium, nickel, cobalt and copper.

3.1.1. Sampling locations

Three sampling locations, viz. Vypin, Munambam and Eloor were selected for the study based on the concentration of heavy metals reported at these locations (Balachandran et al., 2005). The geographical positions of the sampling sites are shown in Figure 1. Eloor is at an intersection where the river Periyar joins the CE. It receives substantial input of untreated effluents from more than 240 industries located along the banks of the river Periyar. Vypin is situated close to the mouth of the estuary which is the major opening through which the Arabian Sea water enters into the estuary. Average depth of Vypin is 10 m and is relatively not influenced by industrial inputs. Munambam is located at the northern end of the estuary and has a fishing harbor. The average depth is 4 m and is categorized as a saline area.
Figure 1: Map showing station locations in CE.
3.1.2 Sample collection

Sediment and water samples were collected in March 2008 from the three locations. From each location, five sediment samples were collected using a van Veen grab (Mouth area - 0.05 m²). Sub-samples of the sediments were collected in plastic bag using alcohol sterilized plastic spatula to avoid metal contamination. Near-bottom watersamples were collected from the 3 sites by using a 10 L capacity Niskin water sampler. The sampler was closed with a messenger at the desired depth. For metal analysis all precautions were taken to avoid contamination from all possible sources. Nylon rope was used for operating the samplers. Samples were collected in pre-cleaned and acid washed polyethylene containers. All the samples were transported in an ice chest to the laboratory for analysis.

3.2 Analysis of Environmental Parameters

3.2.1 Water

The water samples from the 3 locations were analyzed for routine environmental parameters. Standard curves for each analysis are in the Appendix section.

3.2.1.1. Salinity and Temperature

Salinity and temperature were measured onboard with the aid of a portable Conductivity-Temperature-Depth (CTD) probe (Sea Bird Electronics, Inc., USA).

3.2.1.2. Dissolved oxygen (DO)

Dissolved oxygen was determined by the Winkler's method by Strickland and Parsons, (1972) using standard iodimetric titration. The principle of the determination and the possible sources of systematic errors are discussed by Grasshoff,(1983). Water samples were collected from the Niskin sampler in 125 ml acid washed (10% HCl) glass-stoppered bottles taking care that no air bubbles were trapped inside and fixed immediately on board with 1 ml of manganous chloride and 1 ml of alkaline iodide solution (Winkler’s reagents A & B respectively). The samples were mixed thoroughly and the precipitate was allowed to settle. The samples were transported to the laboratory for analysis. One ml of sulphuric acid (10 N) was added to each sample and the sample was shaken thoroughly for dissolving the precipitate and was titrated against 0.01 N
sodium thiosulphate using starch as the indicator. The procedure was standardized by using potassium iodate.

3.2.1.3. Nitrite

In this method, nitrite in the water sample when treated with sulphanilamide in acid solution (Appendix) results in a diazo compound, which reacts with n(1-naphthylethlenediaminedihydrochloride to form an azo dye (Grasshoff, 1983). The absorbance was measured at 543 nm. Standards were run with analytical reagent quality sodium nitrite. (precision: (0.01 µmol N-NO$_2$ T$^{-1}$).

3.2.1.4. Nitrate

Nitrate-N in the water sample was quantitatively reduced to nitrite by passing through a reduction column filled with copper coated cadmium granules and measured as nitrite. During the reduction stage, ammonium chloride buffer was added to the sample to maintain a stable pH (Grasshoff, 1983). The sample after reduction was analyzed for nitrite-N as described in section 3.2.1.3. (0.1 µmol N-NO$_3$ T$^{-1}$).

3.2.1.5. Phosphate

Phosphate-P was determined as inorganic phosphate by the formation of a reduced phosphomolybdenum blue complex in an acid solution containing molybdic acid, ascorbic acid and trivalent antimony. The method was developed by Murphy and Riley, (1962) and a variation of this method described by Grasshoff, (1983) was adopted in the present work. Instead of single solution reagent as in the Murphy and Riley procedure, two stable reagent solutions were used here. A mixed reagent of 0.5 ml containing molybdic acid and antimony tartrate followed by 0.5 ml of ascorbic acid reagent were added to 25 ml aliquots of the samples. The absorbance was measured at 882 nm within 30 minutes to reduce any possible interference from arsenite. Potassium dihydrogen orthophosphate was used as standard.

3.2.2 Metal Analysis

Concentrations of Zn, Hg, Co, Cd, Cu and Ni in the water and sediment samples were analyzed using inductively coupled plasma atomic emission spectrometer (ICP-AES - Thermo Electron IRIS INTREPID II XSP DUO). Sediment samples (5 nos.) from each
station were sub-sampled and pooled for analysis. The pooled samples were dried at 60 °C
and finely powdered using mortar and pestle. One gram of finely powdered dried
sediment was digested repeatedly with HF-HClO4-HNO3, suspended in 0.5 M HCl (25
ml) and analyzed for heavy metals following the standard protocol (Loring and Rantala,
1977). For water samples, a known volume was filtered through pre–weighed Millipore
filter paper (0.45 μm) and the filtrate was acidified using concentrated HCl. The dissolved
metals were extracted using 2 % ammonium pyrrolidinedithiocarbamate (APDC) in 10
ml of methyl Isobutyl ketone (MIBK) at pH 4.5 and brought back to aqueous layer by
back-extraction with concentrated HNO3 and made upto 20 ml with sterile de-ionized
water (Smith and Windom, 1972). The extracts were analyzed in the flame for trace
metals. The analyses were done in triplicate. The concentration of metal in water and
sediment are expressed as mg L⁻¹ and mg kg⁻¹, respectively.

3.3 Bacterial Variables

3.3.1 Abundance

Four fractions of the bacteria viz. total counts, total direct viable, total culturable
counts (colony forming units) and metal resistant bacterial counts were enumerated. The
details of the media composition and reagents used are given in the Appendix section.

3.3.1.1 Total Counts (TC)

Water samples were fixed in hexamine buffered formalin (2%) and diluted ten
times in sterile saline. For sediment samples, one gram of the pooled sediment sample
in hexamine buffered formalin (2%) was dispersed in sterile saline and sonicated in a
water bath for 10 min to dislodge the cells. Enumeration was done following the
method of Hobbie et al., (1977). Preserved samples (1 ml) were stained (in duplicate)
with 100 μl of acridine orange stain (Hi-Media, Mumbai) (final concentration 0.01%
w/v) and incubated in dark for 2 minutes before filtering through 0.22 μm black
stained Nuclepore polycarbonate membrane filter (Whatman Asia Pacific, Singapore).
The slide was then viewed under oil immersion objective (100x) of an epifluorescence
microscope (Olympus Corporation, Japan), equipped with HβO lamp and U-MWB2
mirror unit having excitation filter of 460–490 nm and emission filter of 520 nm.
About 10-12 fields of >30 bacteria per field were counted per filter using 100X oil
objective lens and the average field count was used to calculate the total bacterial abundance using the equation given below. The bacterial abundance in sediment and water are expressed as cells g⁻¹ or cells L⁻¹ respectively.

\[
\text{Total Bacterial Abundance} = \frac{\text{Filter area} \times \text{Average count} \times 1}{\text{Volume of sample} \times \text{Dilution}}
\]

### 3.3.1.2 Total Viable Count (TVC)

Total viable count was enumerated following the method of Kogure et al., (1987). The samples were incubated under dark at 28 ± 2°C for 8 hours (hr) with pre-sterilized yeast extract solution to a final concentration of 0.01 % and 50 µl of antibiotic cocktail. The samples were fixed in hexamine buffered formalin (2%). Counting procedure was the same as mentioned in section 3.3.1.1 Swollen and elongated cells were enumerated as viable bacterial cells and expressed as cells g⁻¹ and cells L⁻¹, respectively for sediment and water.

### 3.3.1.3 Culturable Aerobic Bacteria

Culturable bacterial abundance was enumerated on Nutrient agar (NA) and Peptone Yeast extract Tryptone (PYT80) agar plates (Appendix). The samples were serially diluted and 500 µl of the samples were spread plated on NA and PYT 80 agar plates. Colony forming units were counted after incubating the plates at 28 ± 1°C for 48 hr. The numbers are expressed as cfu L⁻¹ for water and cfu g⁻¹ for sediment.

### 3.3.1.4 Metal Resistant Bacteria (MRB)

The metal resistant bacteria were enumerated using the method of Hassen et al., (1998). Briefly, PYT 80 agar plates supplemented with 0.005 mM concentration of each metal (ZnSO₄·7H₂O, HgCl₂, CoCl₂·6H₂O, CdCl₂, CuSO₄·5H₂O and NiCl₂) were prepared. The samples after serial dilution were spread plated and incubated at 28 ± 2°C for 7 days. The colonies appeared on these plates were considered as MRB. The colonies were counted and expressed as cfu L⁻¹ for water and cfu g⁻¹ for sediment.

### 3.4. Studies on MRB

Morphologically different metal resistant bacteria (250 nos) were isolated from different metal plates and purified on NA plate following streak plate method. The
purity of isolates was confirmed by Gram staining. The purified isolates (Vypin 83 nos., Munambam 83 nos. and Eloor 84 nos.) were stored in NA slants amended with metal (0.005mM) at 4°C for further analysis.

3.4.1 Identification

MRB were identified based on the fatty acid profile of the bacterium developed for aerobic bacteria by MIDI Inc. USA)(Sasser, 1990). The details of reagents used for fatty acid extraction are given in the Appendix section. Briefly, the bacterial isolates were grown on Trypticase soya (TS) agar plates for 24 hr at room temperature (28 ± 2°C). Wet bacterial biomass of 40 mg was saponified at 95-100°C for 5 min in 1 ml saponification reagent. After 5 min., the reaction mixture was vortexed and continued the saponification for 25 min. Subsequently, the tube was supplemented with 2 ml methylation reagent and kept at 80 ± 1°C in water bath for 10 min. The mixture was extracted with methanol: methyl tert-butyl ether (1:1) in a laboratory rotator and the aqueous phase was discarded. The remaining fraction was subjected for a base wash with NaOH (0.25 M) in distilled water (900 ml) and the extract was transferred in to a GC sample vial. Fatty acid was analyzed in Agilent GC 6950 by injecting 2 µl of sample through a 25 m silica capillary. The oven temperature was increased from 170°C to 310°C during each run. By increasing the temperature, the fatty acids get volatilized at specific temperature and detected in a Flame Ionization Detector (FID). CHEMSTATION software converted the signal from FID into a chromatogram, which was transferred to SHERLOCK software for comparing with fatty acid profile library of known organisms. SHERLOCK software has an inbuilt library of fatty acid profiles for around 3000 bacterial species (Pendergrass and Jensen, 1997). The organisms were identified based on the similarity index. The chromatogram and composition report of a representative bacterium is given in Appendix . Previous studies have shown that more than 90 % identification of bacteria by fatty acid profile is in accordance with 16S rRNA gene sequencing method at the genus level and more than 70 % at the species level (Osterhout et al., 1991; Tang et al., 1998).

3.4.2 Multiple Metal Resistances

Bacterial resistance to heavy metals was examined by the plate diffusion method (Hassen et al., 1998). The glassware used in the experiments were leached in 2 N HNO₃.
and rinsed several times with sterile de-ionized water before using to avoid metal contamination. In the central well of PYT plates, 500 µl of 5 mM of metal solution (ZnSO₄·7H₂O, HgCl₂, CoCl₂·6H₂O, CdCl₂, CuSO₄·5H₂O, NiCl₂) was added and incubated at 28±2°C for 24 hr to allow diffusion of the metal into the agar. Six strains of bacteria were streaked in a radial fashion on each metal plate (in triplicates) and incubated for 7 days at 28 ± 2°C. Bacteria which showed visible growth were counted as metal resistant and scored for multiple resistances. Microorganisms were also screened for AgNO₃ resistance in LauriaBertani (LB) agar plates supplemented with different concentrations of AgNO₃, ranging from 0.5 mM to 1000 mM. Filter sterilized AgNO₃ solution was added to the LB agar medium just before preparing plates. Spot inoculation of 10 bacteria were done on a single plate and incubated in the dark at room temperature. The isolates showing growth in 72 hr was considered as resistant strains.

### 3.4.3 Antibiotic Resistances

Sensitivity of MRB to 12 commercially available antibiotic discs (Hi- media, Mumbai) was tested. The mode of action, chemical class and concentration of antibiotics are given in Table 1.

<table>
<thead>
<tr>
<th>Mode of action</th>
<th>Chemical Class</th>
<th>Name</th>
<th>Abbreviation</th>
<th>Concentration per Disc (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellwall synthesis</td>
<td>β-Lactams</td>
<td>Ampicillin</td>
<td>A</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Glycopeptides</td>
<td>Vancomycin</td>
<td>Va</td>
<td>10</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>Macrolides</td>
<td>Azithromycin</td>
<td>At</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Tetracyclines</td>
<td>Oxytetracyclin</td>
<td>O</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tetracyclin</td>
<td>T</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Aminoglycosides</td>
<td>Gentamycin</td>
<td>G</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amikacin</td>
<td>Ak</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Chloramphenicol</td>
<td>Chloramphenicol</td>
<td>C</td>
<td>30</td>
</tr>
<tr>
<td>Nucleic acid synthesis</td>
<td>Quinolones/Fluoroquinolones</td>
<td>Ciprofloxacin</td>
<td>Cf</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nalidixic acid</td>
<td>Na</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Nitrofurantoin</td>
<td>Nitrofurantion</td>
<td>Nf</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Sulfonamides</td>
<td>Trimethoprim</td>
<td>Tr</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 1 Details of the antibiotics used in the study
Muller Hinton agar plates were swabbed with 0.5 ml overnight grown cultures and the antibiotic discs were placed aseptically. A maximum of six discs were placed at sufficient distance from each other and all cultures were tested in duplicate. The plates were incubated at 28±1°C for 18 hr and the clearing zones formed around the discs were recorded using Hi Antibiotic Zone Scale (Hi media, Mumbai). The isolates were recorded as resistant if there was no visible zone around the discs. Multiple Antibiotic Resistance (MAR) index was calculated as the number of antibiotics to which each isolate was resistant to the total number of antibiotics tested (Krumperman and Paul 1983).

3.4.4 Enzyme Expression

The presence of enzyme among MRB was tested in NA medium prepared with 50% sea water supplemented with different substrates. MRB were grown in NA overnight and the active cultures were used for different enzyme assay. Presence of enzyme was scored based on the clearing zone or change in colour. The extent of enzyme expression or production was measured as the function of zone size of individual MRB to produce clearing zone in the substrate enriched solid media. Based on the zone size (diameter in mm), production by MRB was classified as Low (0-10 mm), Medium (11-20 mm) and High (> 20 mm).

3.4.4.1 Amylase

The bacteria were spot inoculated over the surface of a NA plate supplemented with 0.2% soluble starch and incubated for 24 hr at 28 ± 2°C. The ability of bacteria to produce amylase was identified by flooding the plates with 1% iodine solution. The zones of clearance around the colonies were measured.

3.4.4.2 Gelatinase

The colonies were spot inoculated over the surface of a NA plate supplemented with 0.4% gelatin and incubated at 28 ± 2°C for 48 hr. The ability of bacteria to utilize animal protein gelatin was recorded as the clearing zone around the colonies on addition of HgCl₂ solution (15%).
3.4.4.3 Lipase

NA medium was supplemented with 0.01% CaCl₂. Tween 80 was sterilized by autoclaving at 121°C for 20 min and was added to the molten agar medium at 45-50°C to give a final concentration of 1% (vol/vol). The medium was shaken well until the Tween was thoroughly mixed and was dispensed into petri plates and solidified. The cultures were spot inoculated and incubated at 28 ± 2°C. After 72 hr, the plates were observed for formation of an opaque zone containing crystals of calcium around the colonies as positive for lipase activity.

3.4.4.4 DNase

The ability of bacteria to produce DNase was tested using readymade DNase agar (Hi media, Mumbai). The composition of the medium is given in Appendix section. The cultures were spot inoculated and incubated at 28 ± 2°C for 24 hr. The formation of blue coloured colonies due to toludine blue was considered as positive for DNAse.

3.4.4.5 Phosphatase

Phenolphthalein phosphate agar containing sodium phenolphthalein phosphate (0.01%) was prepared in 50 % sea water. The cultures were spot inoculated and incubated at 28 ± 2°C. After 48 hr of incubation, the colonies were subjected to ammonia fumes and the formation of pink color to the colonies in 3 seconds (sec) was considered as positive.

3.4.4.6 Urease

Christensen’s urease agar medium was prepared in 50 % sea water and sterilized at 115°C for 20 min. Filter sterilized urea (40%) was added to the sterilized medium and mixed well before pouring to plate. The plates were spot inoculated and incubated for 48hr at 28 ± 2°C. Development of pink color around the colony was considered as positive.

3.4.4.7 Protease

Skim milk (5%, final concentration) incorporated in nutrient agar were used as the screening medium. The cultures were spot inoculated and incubated at 28 ± 2°C. Clear zone around the bacterial colony after incubation indicated hydrolysis of casein.
3.5. Effect of Silver Nanoparticles (AgNPs) On Mutiple Drug Resistant (MDR) Pathogenic MRB

3.5.1 Selection of MDR pathogenic MRB

Five MDR pathogenic MRB were selected for this study. Out of which three were Gram negative (*Vibrio alginolyticus, Pseudomonas aeruginosa* and *Escherichia coli*), and two Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*). These bacteria showed high levels of resistance against heavy metals and antibiotics.

3.5.2 Preparation of AgNPs

AgNPs was provided by Prof. Anantharaman, Department of Physics, Cochin University of Science and Technology, Kochi, Kerala. The preparation and characterization of the nanoparticles were done based on Thomas et al., (2008). Briefly, AgNPs was prepared using Ag–SiO$_2$ solution by employing tetraethyl orthosilicate-(TEOS), ethanol, distilled water and silver nitrate as precursors. The mean particle size was 5–6 nm. The concentration of silver present in different volumes used in this study was 0.189 ppm in 20µg.ml$^{-1}$, 0.208 ppm in 40µg.ml$^{-1}$, 153 ppm in 60µg.ml$^{-1}$, 0.136 ppm in 80µg.ml$^{-1}$ and 0.126 ppm in 100µg.ml$^{-1}$.

3.5.3 Effect of AgNPs on MDR pathogenic MRB

To study the effects of nanoparticles on MDR, pathogenic MRB, log phase cells of the cultures were treated with AgNPs (in triplicate) for 3 hr. The controls maintained were cultures without AgNPs. Both the experiment and control MDR pathogens were analysed for cell wall integrity, viability, metabolically active cells, fatty acid composition of the whole cell and genetic stability.

3.5.3.1 Cell wall integrity

*Scanning Electron Microscopic analysis*

Control and treated cell pellets were subjected to dehydration by running it through a series of increasing concentrations of acetone (10, 30, 50, 70, 90 and 100%). The samples were air dried, mounted on a stub and sputter coated with Au/Pd. The cell wall integrity was observed using Scanning Electron Microscope (SEM).
3.5.3.2. Viability

The loss of viability of bacteria (death) was tested using 2 methods viz. 1) Resistant population (%) and 2) SDS assay.

1. Resistant population

Overnight grown cultures in LB broth were dispensed separately into microcentrifuge tubes to obtain a final concentration of $10^6$ cells ml$^{-1}$. The tubes were exposed to 0, 20, 40, 60, 80 and 100 µg.ml$^{-1}$ concentrations of AgNPs separately for one hour, and were spread over the surface of LB agar plates. All plates were incubated at 28±1 °C for 24 hr before enumerating the resistant population (%).

2. SDS Assay

Overnight cultures of bacterial cells were washed copiously with sterilized phosphate buffered saline (pH 7.4) and 200 µl of the washed cells were dispensed in the wells of sterile micro plate. After measuring the initial absorbance, the test solution was supplemented with, AgNPs (100 µgml$^{-1}$) and SDS (0.1%). Control wells without AgNPs and SDS were also maintained. The absorbance at 600 nm was recorded every 15 min for a period of 2 hr. The percentage decrease in absorbance compared to the initial reading was plotted against time (Lok et al., 2006)

3.5.3.3 Metabolic active cells

Bacterial cells were treated with AgNPs for one hour and stained with 20 µl of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) solution following the procedure recommended by the manufacturer (Molecular Probes; Invitrogen, Eugene, OR, USA). Experimental and control samples were incubated for 30 min in dark condition. Samples were filtered through 0.2 µm polycarbonate filters, and counter stained with 4′,6-diamidino-2-phenylindole (DAPI). Counterstaining with DAPI allowed concurrent determination of total (i.e., viable plus non-viable) and respiring (i.e., cells exhibiting CTC-formazan fluorescence) cell counts in a single preparation. The samples were washed copiously with phosphate buffered saline and the dead and metabolically active cells were differentially counted using 100 X oil objectives under an epifluorescence microscope (Olympus, USA). Metabolic active cells were
expressed as the ratio of metabolically active cells (i.e., CTC stained) to total cells (i.e., DAPI stained, cells). If all the cells were metabolically active, then the ratio of dead to live cell would be close to one.

### 3.5.3.4 Whole cell fatty acid composition

The whole cell fatty acid profiles of pathogenic bacteria before and after treatment with AgNPs were analyzed using a gas chromatography system (16890N Network GC system, Agilent Technologies Inc., Wilmington, DE). Difference in the number and the intensity of fatty acids were compared. The details of fatty acid analysis are as described in section 3.4.1.

### 3.5.3.5 Genetic stability

The effect of AgNPs on stability of genetic material of pathogens was investigated by comet assay (Singh et al., 1999). Details of reagent preparation is given in Appendix. Briefly, AgNPs treated and control cells were washed copiously with phosphate buffered saline (PBS, pH 7.4) and re-suspended in saline and subjected to comet assay. For comet assay, 50 µl of $10^6$ bacterial cells were mixed with 500 µl of 0.5% low melting point agarose prepared in Tris acetic acid EDTA buffer (TAE). Bacterial cells impregnated in agarose solution were spread over a microscopic slide, pre-coated with a thin layer of 0.5% agarose and solidified by incubating at 4°C for 30 mins. Slides were then incubated at 37°C for 30 min and subjected to cell lysis for 1 hr at 37°C, by immersing in lysis solution. Subsequently, the cells were subjected for enzyme digestion by incubating in enzyme solution for 2 hr at 37°C. After digesting the cell wall by lysis and enzyme treatment, the slides were equilibrated with 300 mM sodium acetate and subjected to electrophoresis at 50 V for 15-20 min. Following electrophoresis, slides were immersed in 1 M ammonium acetate in ethanol for 30 min and then in absolute ethanol for 1 hr. The slides were air dried at room temperature (28±1°C) and then immersed in 70% ethanol for 30 min and air dried. Slides were then stained with 1 ml freshly prepared solution of 1 µl SyBr green in 1ml Tris EDTA (TE) buffer. The comets were recorded using a fluorescence microscope (OLYMPUS BX 61) equipped with dichroic filter pairs (Excitation filter: 470-490 nm, Emission filter 520 nm, Dichroic 500 nm) and digital camera attached to fluorescence
microscope. The comet length was measured and processed using the software Image Pro. The nucleic acids were classified based on comet length as intact/low (0 -10 mm length) or heavy damaged (>10 mm) groups and are presented as histogram.

### 3.5.4 Mechanism of resistance by MDR Staphylococcus aureus

To understand the resistance mechanism of MDR strain *S. aureus* to AgNPs resistance, Verapamil assay was carried out. Verapamil is a known calcium channel blocker for ABC transporter. *S. aureus* cells were exposed to Verapamil to a final concentration of 20 µg ml⁻¹ for 1 hr prior to the treatment with AgNPs. Total death rate and nucleic acid damage of the treated cells were determined following the method given under sections 3.5.3.2. and 3.5.3.5 respectively.

### 3.6 Statistical Analysis

Metal concentrations of water and sediment, abundance, community structure of culturable and non-culturable bacteria were subjected to various statistical analysis (Snedecor and Cochran, 1967). Depending on sample size and distribution, the data were normalized. The analyses were carried out using, student’s t test, 3 ways ANOVA. Diversity index (Shannon-Wiener H’) for culturable community (Shannon and Weaver, 1963) and Cluster Analysis were done using Primer 6 software.