# Chapter II

# *Experimental*

# II. EXPERIMENTAL

## II.1. Hydrochemical analysis

Complete chemical analysis (major and minor ions) of the collected water samples were conducted according to the Standard Methods. The results of the chemical analysis were expressed in milligrams per liter (ppm), milliequivalents per liter (epm) and epm percentage (epm %).

***The following parameters and constituents were included:***

### II.1.1. Hydrogen Ion Concentration (pH)

The pH was determined using the electrometric method according to the ASTM D1293, The pH of the water samples were measured at 25°C using a digital pH-meter, Hanna model pH 213, equipped with a combination pH electrode, HI 1230B, consisting of glass electrode and silver-silver chloride reference electrode.

The pH-meter with the combination electrode was standardized under controlled parameters using three buffer solutions of pH 4.01, 6.86 and 9.18 at 25°C before measurements of the pH of the water samples were carried out.

### II.1.2.Electrical Conductivity

The electrical conductivity was determined using the instrumental method according to the ASTM D1125, The conductivity of the water samples was determined at 25°C using a digital conductivity meter, WTW, model cond 330i, equipped with a conductivity cell. The cell was rinsed thoroughly several times with deionized water and then with the sample to be measured. The temperature of the sample was stabilized at 25°C in a thermostat.

### II.1.3. Total dissolved solids (TDS):

TDS were determined experimentally according to ASTM D-1888. Awell-mixed sample is filtered through a weighed standard glass fiber filter and the filtrate is evaporated to dryness in a weighed dish and dried to a constant weight at 180°C. The increase in dish weight represents the Total dissolved solids.

### II.1.4. Alkalinity:

The bicarbonate alkalinity was determined by potentiometric titration method according to the ASTM D1067, A suitable aliquot of the water sample was titrated potentiometrically to pH 4.5 with a standard hydrochloric acid solution. The acid solution was added automatically, while the sample was gently mixed with a magnetic stirrer. The bicarbonate alkalinity, as calcium carbonate, was calculated.

### II.1.5. Ion Chromatography (IC)

Cations and Anions were determined experimentally according to ASTM D 4327 using Dionex IC model DX 600 equipped with high capacity columns. Anions concentrations (Fluoride, Chloride, Nitrite, Bromide, nitrate, Phosphate, and Sulfate) were determined using the following system:

| | |
|---|---|
| Column | : Ion Pac AS9-HC (4X 250nm) |
| Eluent | : 9.0 mM Na2CO3 |
| Flow rate | : 1.0 ml/min |
| Detector | : Electrochemical detector (Dionex, ED50) |
| Injection volume | : 10 µl |

Cation concentrations (Lithium, Sodium, Potassium, Magnesium, and Calcium) were determined using the following system:

| | |
|---|---|
| Column | : Ion Pac CS12A (4X 250nm) |
| Eluent | : 20 mM Methanesulfonic acid |
| Flow rate | : 1.0 ml/min |

Detector : Electrochemical detector (Dionex, ED50)
Injection volume : 10 µl

Each water sample was filtered using Whatman No.42 filter paper and diluted with deionized water prior analysis. Total of five and six mixed anion and cation standard solutions were used for instrument calibration.

Aliquots of the water samples were diluted with measured volume of deionized water to a suitable volume to be within the measuring range, and 10µl of the solution was injected into the column.

## II.2. Oil content study:

### II.2.1.Surface water samples

***Apparatus***

- Separating funnel.
- Round bottomed flask with a ground joint.
- 100 ml volumetric flask.

***Reagents***

- Carbon tetrachloride (pure)
- Anhydrous sodium sulphate (A.R)

***Procedure***

100 ml of the brine was shaken with 100ml of carbon-tetrachloride in a separating funnel for 15minutes. After 30 seconds agitation and 3min settling period, the aqueous layer was discarded. The process was repeated till all of the brine sample has been extracted. The obtained extract was dried using anhydrous sodium sulphate (≈ 30 g). The dried extract was then transferred to a weighed beaker and finally evaporated by electric furnace at 60°C till constant weight **(Moustafa et al., 1997)**.

## II.2.2. Sediment samples:

***Apparatus***

- Round bottomed flask.
- Reflux condenser.
- Separating funnel.

***Reagents***

- Methanol (pure)
- n-Hexane (pure)
- Potasium hydroxide (A.R)
- Anhydrous sodium sulphate (A.R)

***Procedure***

To 100 g of wet sediment sample in a round bottomed flask, 100 ml redistilled methanol and 3 g Potassium hydroxide were added. The mixture was refluxed for 1.5 hr. The saponified extract was cooled to room temperature transferred to a separating funnel and then extracted with n-hexane (≈ 25 ml). The extract obtained was then dried through a column packed with anhydrous sodium sulphate. The excess solvent in a weighed beaker was evaporated to constant weight and finally the oil content was then calculated **(Viguri et al., 2002).**

## II.2.3. Fish samples

***Apparatus***

- Reflux condenser
- Round bottomed flask
- Separating funnel

***Reagents***

- Methanol
- Potassium hydroxide
- Sodium sulphate anhydrous
- Hexane
- dichloromethane

***Procedure***

Fish samples were thawed before gills, muscles and skins were cut down into small pieces, each sub-sample was ground in a pestle and mortar with 2 g anhydrous sodium sulphate. The cake was then extracted with 60 ml mixture hexane/dichloromethane (1:1) v/v. The mixture was filtered and the tissue was extracted twice more. Organic solvent fractions were combined and filtered through filter paper with 2g anhydrous sodium sulphate. The extract was then reduced to about 2 ml using a rotary evaporator.

Saponification; The concentrated fish extract (2 ml) was transferred to a round-bottom flask and 100 ml aqueous methanolic KOH was added. The mixture was refluxed for 3h.

Saponified material was transferred to a separating funnel. The round-bottom flask was quantitatively rinsed with 150-200 ml mixture of methanol-distilled water 4:1 v/v. The mixture was transferred to a separating funnel and 100ml of n-hexane was added and then funnel shaken for 3min. Layers were allowed to separate and then organic layer was collected in another beaker. The extraction of the aqueous layer was repeated with 50ml n-hexane and layers were allowed to separate and the aqueous layer discarded. Organic layers were collected and carefully evaporated in a rotary evaporator to about 1ml. **(Meddleditch et al., 1977).**

## II.3. Gas Chromatographic (GC) Analysis

All the oil extracted from studied (water, sediment and Fish) were analyzed using capillary column, according to IP 318/75(82) **(IP, 1995)**.

***Apparatus***

- All extracted samples were analyzed using Aglient model 6890 plus gas chromatograph. The condition of analysis was as follows **(IP, 1995)**:
- Column: HP-1 (100% methyl silicon siloxane) 30m length and 0.25 ID mm,
- Oven temperature: 80-300oC (3°C /min)
- Detector: flame Ionization Detector (FID) 325°C
- Injector: splitter injector 300°C using N2 (2 ml/min) as carrier gas.

***Reagent***

- Methylene chloride (A.R).

***Procedures***

The column was left overnight with carrier gas flowing through it, and at maximum working temperature used, disconnected from the detector. The extracted hydrocarbons were dissolved in the least amount of methylene chloride and about 1µl was injected into the apparatus. The temperature of the oven was programed (before injection) from 100-300°C at fixed rate (5 degree / min.). The column was then left at its maximum temperature until the sample has been completely eluted i.e. no more peaks appear. The identification of the n-paraffin peaks was established by chromatographing a reference mixture of n-paraffins of known composition.

## II.4. Ultraviolet spectroscopic analysis:

Scanning of the collected samples was performed using a double beam UV/VIS/NIR spectrophotometer model Jasco V 570 between 400nm-190nm.

## II.5.High performance liquid chromatographic analysis (HPLC)

PAHs identification and quantification in the extracted oil of all samples was performed using HPLC model Waters 600E equipped with auto sampler Waters 717 plus and dual wavelength absorbance detector Waters 2487 set at 254 nm. PAHs standards were obtained from Supelco. The condition of operation is as follows **(Lal and Khanna, 1996)**:

- ***Column:*** Supelcosil LC-PAH, 15 cm x 4.6mm ID, 5 μm particles size.
- ***Mobile phase:*** Acetonitrile/water HPLC grades, gradient from 50:50 to 100% acetonitrile.
- ***Flow rate:*** Gradient program; 0-2min, 0.2 ml/min then 2-45min, 1.0 ml/min.

## II.6. Biotreatment of oil contaminated sediments

### II.6.1. Luria-Bertani medium (LB)

According to **Kirimura et al., (2001)**; a defined LB medium consists of;

- Tryptone 10g
- Yeast extract 5g
- NaCl 10g

  - All the above constituents were dissolved in 1liter distilled water
  - pH was adjusted to 7.0 with 10% NaOH before sterilization.

For maintenance of isolates; LB solidified with 2% agar was used to prepare LB slants.

### II.6.2. Basal Salts medium (BSM)

According to **Piddington et al., (1995)**; a defined BSM consists of;

- $Na_2HPO_4$ 5.57g

- $KH_2PO_4$ 2.44g
- $NH_4Cl$ 2.0g
- $MgCl_2.6H_2O$ 0.2g
- $FeCl_3.6H_2O$ 0.001g
- $CaCl_2.2H_2O$ 0.001g

- All the above constituents were dissolved in 1liter distilled water.
- pH was adjusted to 7.0 using 10% NaOH before sterilization.

## II.6.3. Bacterial culture

The bacterial strain used in bioaugmentation experiment was previously isolated from hydrocarbon polluted soil and identified as *Staphylococcus gallinarum* NSh37 **(El-Gendy, 2004)**. Pure culture of the isolate was stored on LB-agar slants at 4°C as working stock culture and preserved in 80% (v/v) glycerol -20°C.

## II.6.4. Inoculum preparation

Inoculum of NSh37 was grown for 24hours in LB supplemented with molasses (2%) as carbon source at 30°C and 200rpm. Centrifuged at 5000 rpm for 5min, the biomass was harvested and washed with BSM twice then respended in BSM.

## II.6.5. Sediment slurry biodegradation

- Each sediment sample was divided into sterile and non-sterile portions.
- For sterile sediment slurry, 5g of each fresh sediment samples were added into a 100ml conical flask containing 50ml BSM and autoclaved at 121°C for 30min.
- The autoclaving step was omitted for the non-sterile sediment slurry.

***To study the effect of Staphylococcus gallinarum* NSh37**

5ml of enriched bacterial culture suspended in BSM was inoculated into each flask containing sterile sediment slurry to give an initial inoculum

size of ≈ $10^7$cell $ml^{-1}$ at the beginning of each degradation experiment (zero time).

***To study effect of bioaugmentation***

5ml of enriched bacterial culture of NSh37 suspended in BSM was inoculated into each flask containing non-sterile sediment slurry and the total viable count (TCFU) was determined at the beginning of each degradation experiment (zero time).

***To study the effect of biostimulation***

The non-sterile flasks indicate the degradation capacity of native bacteria naturally present in sediments.

***Incubation***

- All the flasks were incubated in a shaking incubator 200rpm at 30°C for six weeks.
- Total viable count (TCFU) and biodegradation percentage of TPH was determined at time intervals of zero, 1, 2, 4 and 6 weeks.
- GC analysis was done to determine the effect of different biotreatment methods on TRP of oil contaminating the sediment samples.
- HPLC analysis was done to determine the effect of different biotreatment methods on PAHs of oil contaminating the sediment samples.