Chapter 3

Experimental

CHAPTER III: EXPERIMENTAL

3.1 Introduction

Petroleum oil is a complex mixture of compounds consisting predominantly of hydrocarbons along with a small amount of oxygen, nitrogen, sulphur and metal compounds. The hydrocarbon portion contains straight-chain and branched alkanes, cyclic alkanes (naphthenes), alkenes and aromatic compounds. The relative content of these compounds varies with type of oil and from sample to sample of the same oil, and therefore, because of the wide variety of physical and chemical properties exhibited by constituent compounds, there is no single technique available which can determine all component of petroleum oil unambiguously, and yield an entirely accurate quantification of the sample to satisfy all the requirements of research and monitoring programme. However, the growing awareness of hydrocarbon pollution has led to refined methods of analysis and application of modern sophisticated analytical techniques for detailed environmental studies. New methods and equipment are developed and revised from time to time according to research requirements and problems encountered.

Various techniques have been applied for hydrocarbon pollution studies either from catastrophic oil spills or monitoring of long term involving small but continuous sources (such as discharges of industrial effluents) of hydrocarbon pollution input, encompassing the analysis of the pollutants in water, sediments as well as biological tissues. The major analytical methods used for determining the concentration of petroleum hydrocarbons in environmental samples include gravimetry, gas-liquid chromatography, spectrophotometric techniques (infra-red, ultra-violet and fluorescence) and gas chromatography. Most of the techniques require prior extraction of hydrocarbons from the sample media into an organic solvent.

In the present study, two techniques were adopted to measure petrogenic hydrocarbons in water, sediment and cockle tissue. In the first approach, the relatively simple technique

of fluorescence spectrometry was employed to measure the concentration of petrogenic hydrocarbons in the environmental samples. A particular compound is said to be fluoresce when it absorbs light at a particular wavelength and become excited. In the process of returning to the ground state, it emits light at a longer wavelength which is called fluorescent light. Fluorescence is not a property shared by all types of hydrocarbons, but only occurs with aromatic compound which are comparatively stable and capable of carrying considerable energy without destruction of the molecule (Ehrhardt, 1983). When irradiated by light of particular wavelength they can be excited to an elevated level of electronic energy and emitting electromagnetic radiation when return to the ground state. The frequency distribution reflects the energy difference between the two levels and characteristic of certain molecule. The wavelength of both excitation and emission can be varied, but the most commonly used procedure involves excitation of sample at 310 nm and emission at 360 nm (Law, 1981).

The determination of petrogenic hydrocarbons by fluorescence spectroscopy is a widely accepted analytical procedure based on the analysis of aromatic and PAHs, and indicative of the concentration of crude oils and oil products in the marine environment. Also included in this measurement are PAH arising from incomplete combustion of oil and other carbonaceous materials such as in internal combustion engines and industrial processes. While PAHs are important components of petrogenic hydrocarbons, they have never been demonstrated to be derived or present in biogenic hydrocarbons. Therefore, due to this selectivity, spectrofluorometry has found widespread use in the determination of petrogenic hydrocarbons in the marine environment. Hence, the spectrofluorometric technique of analysis does not allow for determination of non-aromatic hydrocarbons.

In the second approach, to allow for the determination of aliphatic hydrocarbons, gas chromatography was employed. In the present study, straight-chain hydrocarbons were identified and quantified by Gas Chromatography - Mass Spectrometry (GCMS). GCMS is a technique in which gas chromatography is coupled with mass spectrometry as the detector system providing mass spectra of the eluted components. Combined with

computerised data system, it is the most powerful instrument available to identify and quantify all components of petroleum hydrocarbons. It has been used extensively in recent years for unambiguous identification of petroleum and combustion-derived aromatic hydrocarbons in water, sediments and biota (NAS, 1975). GCMS can be operated to acquire either a full range of mass spectra data which allows for total ion chromatogram (TIC), or in selected ion monitoring (SIM) which allows the mass spectrometer output to be scanned at a preselected mass and the resultant mass chromatogram is recorded. Quantification can be done by computer-assisted integration of an ion current plot for a compound derived from the mass chromatogram. The area is then compared to an internal standard ion current and corrected based on relative mass spectral response factors.

The present study also included the determination of the extent of uptake and depuration of hydrocarbons by the cockles. Experiments were carried out under controlled conditions in which cockles were exposed to water-soluble fractions of a crude oil and a refined oil.

3.2 Chemicals and Glassware

All chemicals used for the present study are given in Table 3.1. Only borosilicate glassware was used in all the procedures. Solvents were used without further purification. Other materials such as glass wool, boiling chips, anhydrous sodium sulphate, extraction thimbles, silica and alumina were also pre-cleaned by Soxhlet extraction and the appropriate blanks determined (see Section 3.4).

Table 3.1 **Solvents and chemicals used in the present study.**

	Chemicals	Supplier	Purity
1.	Acetone	BDH Ltd. England	Analytical grade
2.	Dichloromethane (DCM)	BDH Ltd. England	Analytical grade
3.	Hexane	BDH Ltd. England Fisher Chemical	Analytical grade Optima grade
4.	Hydrocarbon Standards	Alltech	Analytical grade
5.	Methanol	J.T. Baker, London	Analar
6.	Seligi Crude Oil	ESSO	
7.	Aluminium Oxide	E.Merck, Germany	Neutral, 90 % active with 70-230 mesh, ASTM
8.	Hydrochloric acid	BDH Ltd. England	Analytical grade
9.	Mercury	COMAK Ltd. London.	
10.	Potassium Hydroxide	E.Merck, Germany	Analar
11.	Silica Gel	E.Merck, Germany	70-230 mesh, ASTM

3.3 Sample Collection and Storage

Two sampling sites were chosen in the West Coast of Peninsular Malaysia; Kuala Selangor in Selangor and Kuala Sepetang in Perak. These two areas are widely known as the most important cockle farming areas in the country. Sampling was carried out at five stations chosen along an imaginary transect (about one km from the estuarine area) drawn on predetermined latitude and longitude as indicated in the map in Figure 3.1 and 3.2 for Kuala Selangor and Kuala Sepetang respectively. The exact latitude and longitude of each station which were about 500 - 1000 metres from each other were recorded on site using Geographical Positioning System. Sampling was carried out in March 1998.

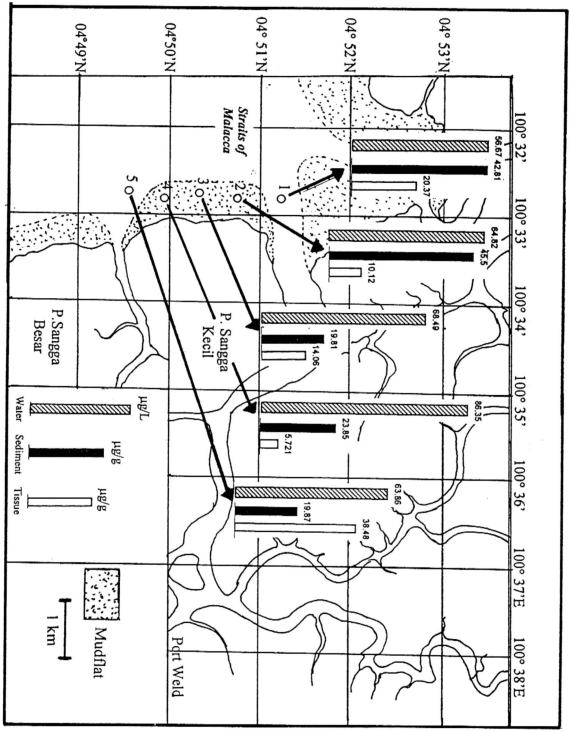

Figure 3.2: Sampling stations at Kuala Sepetang

At each station, water, sediments and cockles samples were collected. Water was collected from a depth of one metre using a Van Dorn sampler. The water samples were then stored in pre-cleaned Winchester bottles and acidified with hydrochloric acid to pH 5. Samples of surficial sediment along with the cockles were collected by scooping the culture bed with a long-handle rake normally used by the cockle farmers to collect cockles. The cockles were then separated from the sediments and both were wrapped in hexane-cleaned aluminium foil. In the laboratory, water samples were extracted within a period of 48 hours after sampling, while sediments and cockles samples were kept at -20 °C prior to freeze-drying and extraction.

3.4 Cleaning of Glassware and Other Materials

Analysis of environmental samples usually involve trace (1 – 100μg/g) and ultra-trace (<1μg/g) levels. Hence, quality control and accurate measures were essential to produce meaningful results within a stated defined level of confidence. Hence, it was essential that various steps were taken to prevent the introduction of contaminants during all the procedures involved in the study including any material that the samples come into contact with such as glassware, solvents and adsorbents and reagents.

Cleaning of glassware

All glassware were cleaned by soaking in laboratory detergent (Decon-90) for 24 hours, rinsed thoroughly with water followed by distilled water and acetone before drying in an oven at 400 °C overnight. The glassware were capped with aluminium foil to prevent any contamination from dust and other airborne contaminants. Glassware was rinsed once with hexane prior to use.

Cleaning of extraction thimbles.

Extraction thimbles were cleaned by carrying out Soxhlet-extraction with 250 ml hexane: DCM (3:2) for 8 hours. Thimbles used were of the disposable type and hence were only used once.

Cleaning of silica and alumina

Silica and alumina were Soxhlet-extracted successively with hexane and methanol for 8 hrs. The procedure was deemed sufficient as observed in the procedural blank based on both spectrofluorometric and GCMS determinations. The adsorbents were activated at 120° C for 4 hrs. Deactivation was carried out by adding water (5 % w/w) to the fully active sorbent.

Blank determinations

Both solvent blanks and procedural blanks were determined. The former was determined for both the fluorescence and gas chromatographic measurements using identical total volumes of all the solvents used in the procedure followed by solvent concentration. The procedural blanks were determined by following the procedure for the extraction of seawater using artificial seawater, while for the sediment and tissue the appropriate procedure was followed in the absence of the matrices.

3.5 Sample Treatment and Extraction

The hydrocarbons extraction from seawater and sediment samples were in accordance with the methods suggested by Parsons et. al. (1984) and was also carried out by Abdullah et. al (1994) and Tahir et. al. (1997). For tissue samples, the extraction was carried out according to the method proposed by IAEA (1995) for the extraction of petroleum hydrocarbons from fishes and bivalves.

Seawater samples

Seawater samples (1.0L) were extracted with hexane (3 x 30 ml). The combined extracts were then dried over anhydrous sodium sulphate and then concentrated in a rotary evaporator (temperature : < 40 °C). The residue was then transferred quantitatively into a 5 ml pre-cleaned sample bottle and reconstituted to 1 ml with the aid of a stream of purified nitrogen placed above the surface of the solution. The extract was now ready for fractionation.

Sediment samples

A sample of freeze-dried sediments (10.00g) were placed in a pre-cleaned cellulose extraction thimble and Soxhlet extracted for 12 hours with hexane-dichloromethane (3:2). The extracts were then evaporated under reduced pressure to about 5 ml, transferred into capped test tubes and shaken vigorously with mercury to remove sulphur or sulphurous compounds which might present quenching effects on the fluorometric measurement. The procedure was repeated several times with fresh mercury added each time until the formation of black mercuric sulphide was no longer observed. The extracts were then reconstituted to 1 ml prior to fractionation.

Cockles samples

Freeze-dried cockle tissue (10.00g) were cut and minced and then transferred into pre-cleaned cellulose extraction thimble. The tissue was Soxhlet-extracted with 300 ml of hexane-methanol (3:2) for a period of 12 hours. Upon extraction with hexane (3 x 30 ml), the extract was refluxed with 0.5M methanolic KOH (100 ml). The extract was then dried over anhydrous sodium sulphate, concentrated under reduced pressure and finally reconstituted to 1ml prior to fractionation.

3.6 Fractionation

The fractionation was done in accordance with the method suggest by IAEA (1995). The elution volume for every fraction (using the above stated mesh size for silica and alumina) were predetermined. The fractionating column was prepared by slurry-packing pre-cleaned 5% deactivated silica (5.0g) into a 50ml burette followed by pre-cleaned 5% deactivated alumina (10.0g) and topped with anhydrous sodium sulphate. The concentrated extracts, as prepared above from seawater sediment and tissue, were then introduced onto the column and eluted successively with the solvents shown in Table 3.2. Each fraction collected was then evaporated to about 1 ml and transferred quantitatively into a pre-cleaned sample vial and reconstituted to 10ml.

Table 3.2 **Eluents used for fractionation**

Fraction	Eluent	Class of Compounds
F1	20 ml hexane	n-alkanes
F2	30 ml of 10% DCM in hexane	Alkenes and aromatics
F3	20 ml of 50% DCM in hexane	PAHs

3.7 Analysis

Fluorescence Spectrometry

The concentration of petrogenic hydrocarbons in each fraction was determined using a Hitachi F-2000 Fluorescence Spectrophotometer. The fluorescence of the samples were measured in 1 cm quartz cell at 310 nm excitation and at 374 nm emission wavelength. Seligi crude oil and chrysene were used as calibration standards. Solvent blanks were run for each batch of samples and one or more of the standard solutions of Seligi Crude Oil measured on each working day to verify the calibration program prior to the analysis of the samples. A representative curve for the measurement is given in Appendix A.

Gas chromatography-mass spectrometry

A Hewlett-Packard GCMS was used to identify and quantify aliphatic hydrocarbons. The following operating conditions were employed:

Apparatus	:	GCMS HP6890
Detector	:	Mass spectrometer
Injection technique	:	Manual
Injector temperature	:	290 C
Injection volume	:	1 μL
Injector mode	:	Splitless
Carrier gas	:	Helium
Carrier gas flow	:	15 mL/min

Column type	:	Capillary column: HP 19091S-433
Length	:	30.0 m
Internal diameter	:	250.00 μm
Stationary phase	:	5 % Phenyl methyl Siloxane
Film thickness	:	0.25 μm
Column pressure	:	10 psi
Column initial flow	:	1.2 mL/min
Temperature programming	:	
Initial temperature	:	60 C for 1 minute
Temperature rate	:	8.00 C/min
Final temperature	:	280 C
Final hold time	:	30 minutes
Total running time	:	54 minutes

Identification of components peaks was based on comparison with the GC retention time and mass fragmentation patterns of standard materials, and confirmed with the aid of the National Institute of Standards and Technology library incorporated in the data system of the GCMS.

3.8 Uptake and Depuration of Petroleum Hydrocarbons in Cockles

The experimental design for uptake and depuration was obtained from Sophia and Balasubramaniam (1991) which was assumed applicable for cockle culture.

Tests animals

Cockles collected from Kuala Selangor were acclimatised in the laboratory for a period of 1 month before exposure commenced. The seawater used in the holding tank was renewed frequently and continuously aerated. Salinity was checked daily and maintained at 30 ppt. Cockles of approximately equal size were selected for the uptake and depuration study.

Exposure oils

The hydrocarbons used in the study included *Seligi* crude oil and a refined oil commonly used in marine vessels. Water-soluble fractions of the hydrocarbons were prepared as the exposure media.

Preparation of water-soluble fractions

Water-soluble fractions (WSF) of the test oils were prepared in accordance with the method of Anderson *et al.* (1974). Hence, stock solutions were prepared by stirring (with a magnetic stirrer) a mixture of 1 part oil and 9 parts artificial seawater placed in a 10L conical flask for a period of 24 hr at room temperature. The flask was covered with aluminium foil to minimize evaporation of volatile hydrocarbons. At the end of 24 hr the oil and water phases were allowed to separate before the water phase was siphoned out. This process was carefully carried out to exclude oil residues in the water phase. A portion (100 ml) was removed for extraction and analysis of hydrocarbons, while the remainder was used immediately in the exposure experiments.

Exposure regime

In a preliminary experiment, various concentrations of the WSF were prepared by diluting the initial water phase (25%, 50%, 100% and 200% dilutions). The hydrocarbons in these solutions were then extracted into hexane in accordance to the method of extraction for seawater analysis (Section 3.5), but without carrying out the fractionation. Cockles (10 animals per beaker) were also exposed to the solutions and observed for mortality. Based on the results of this preliminary study it was decided that the initial WSF was most suitable for the exposure study as it had the highest concentration of dissolved hydrocarbons, while at the same time the exposure did not result in mortality of the cockles over a 48hr period.

Cockles were exposed to the newly-prepared WSF in triplicate. Each tank contained approximately equal size animals and were continuously subjected to gentle aeration. The animals (5 from each tank) were sampled at specific time intervals for 7 days. The

exposure media were renewed every 24hrs. The sampled cockles were extracted using the procedure presented in Section 3.5. Water samples were also extracted immediately after renewal and again 24hrs later. At the end of the 7th day the remaining cockles were transferred into clean artificial seawater. Animals were again sampled at specific time intervals to assess the extent of depuration.